**ROMANIAN ACADEMY**

**THE BIOLOGY INSTITUTE OF BUCHAREST**

# SUMMARY

# PHD THESIS

## *„DYNAMICS OF PLANKTONIC MICROBIOTA IN MICROCOSMS SYSTEMS SUPLEMENTED WITH: HIDROCARBONS, DISPERSANT AND NUTRIENTS- FUNDAMENTAL AND APPLIED ASPECTS„*

**Scientific coordinator,**
**Prof. univ. dr. Ioan ARDELEAN**

**PhD,**

**Manea MIHAELA**

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**PURPOSE OF THE THESIS**

***The study of the dynamics in marine endogenous planktonic prokaryotes able to degrade / tolerate hydrocarbons (oil) in microcosms systems in the absence of bacteriovorus (filtered seawater – with pors 0.45 µm) or in the presence of bacteriovorus (unfiltered seawater).***

## PERSONAL RESEARCH OBJECTIVES

1. ***Selecting optimal concentrations of nutrients used to stimulate growth, multiplication and metabolic activity of endogenous marine prokaryotes in microcosms systems in laboratory with or without bacteriovorus.***
2. ***Selecting optimal concentrations of dispersants used to stimulate growth, multiplication and metabolic activity of endogenous marine prokaryotes in microcosms systems in laboratory with or without bacteriovorus.***
3. ***Selecting optimal concentrations of gasoline (oil) used to stimulate growth, multiplication and metabolic activity of endogenous marine prokaryotes in microcosms systems in laboratory with or without bacteriovorus.***
4. ***Quantifycation of pollutant consumption (diesel) in microcosms systems in laboratory with or without bacteriovorus., under optimum dispersant and nutrients concentrations.***
5. ***Dinamics of endogenous marine prokaryotes (living, death and hydrocarbon oxidizing bacteria) in microcosms systems in laboratory under addition of dispersant, poluant and nutrients***
6. ***Isolation, purification and identification of hydrocarbon oxidizing bacteria of endogenous microbiota, for bioaugmentation subsequent experiments.***
7. ***Testing susceptibility of hydrocarbon oxidizing microorganisms to antibiotic .***

## THE STRUCTURE OF PHD THESIS

The thesis is structured in two different parts, first part is containing two chapters and Part II with seven chapters, get a total of 124 pages, 45 figures and 9 tables and 162 references.

**The thesis is organized as follows:**

**Partea I** – is structured in 2 chapters with information regarding:
Stage of knowledge of eviromental pollution and bioremediation solutions, hydrocarbon pollution with 6 subchapters regarding to: generality, brief history of environmental pollution

by hydrocarbons , the effects of oil pollution on the environment, bioremediation, environmental factors influence the biodegradation of petroleum hydrocarbons, hydrocarbon-tolerant microorganisms resistance to antibiotics and toxic compounds, importance of the subject.

**Partea II** – contains „ **PERSONAL CONTRIBUTIONS**" and is divided into seven chapters, the first four chapters are dedicated to each of the 4 experiments performed DURING the 5-year study, Chapters 1-6 are structured into chapters MATERIALS AND METHODS, RESULTS AND DISCUSSION, CONCLUSIONS:

CHAPTER I - Density dynamics in microbial cells from marine microcosms supplemented with diesel and dispersant nacol c .

CHAPTER II - CHAPTER III - Selecting the optimal concentration of nutrients, dispersant and diesel oil to enhance metabolic activity and diesel oil consumption by marine endogenous microbiota

quantified by resazurine reduction in microplates experiments.

CHAPTER III - Selecting the optimal concentration of nutrients to enhance metabolic activity and diesel oil consumption by marine endogenous microbiota quantified by resazurine reduction in microcosms experiments at 15 ° C .

CHAPTER IV- Diesel fuel consumption in long term (15 months) microcosms experiments with continuous addition of inorganic nutrients .

CHAPTER V - Isolation, selection and identification of bacteria oxidizing hidrcarbon.

CHAPTER VI - Testing antibiotic susceptibility of hidrcarbon oxidizing bacteria isolated .

The thesis is finalizing with CHAPTER VII –GENERAL CONCLUSIONS AND PERSPECTIVES - In setting out the main conclusions of the research conducted, presenting original contributions and propose some future research prospects.

**I.2.6. IMPORTANCE OF THE SUBJECT.**

Petroleum hydrocarbons clean environment is a real problem. A better understanding of the mechanisms of biodegradation has high ecological significance, which depends on native capacity of organisms to convert organic contaminants or mineralise them. The biodegradation process helps to eliminate microbial oil leaked into the environment after the discharge of large quantities, they have been removed after the critical spil by various physical and chemical methods.

This is possible because microorganisms enzymatic systems gives them the ability to degrade and to use various hydrocarbons as a source of carbon and energy.

It is unanimously endorsed the idea (Bull. 1992, Crook., 1996) that bioremediation compared with other methods of physico-chemical treatment, are more effective and economical, and does not disrupt the ecological balance of the environment, preserving biodiversity of the ecosystem. Physical remediation methods (adsorption, filtration, extraction), in some cases they are more efficient compared to bioremediation, but has the disadvantage that it does not convert waste into less toxic constituents. Chemical treatments, in turn, can lead in the end to products with some risk to the environment, in that it can provide new sources of pollution being more refractory than the original products.

Generally in polluted areas, is an increase in the number of micro-organisms that metabolize the polluting substrate and a descreasing of the taxonomic diversity. From this aspect, it must be used in bioremediation applications the bacterial strains from polluted sites, which has a flexibility and a metabolic enzyme suitable equipment, enabling the integration into the polluted area without affecting the ecological balance of the area. The most effective microbial communities utilised to remedy the contaminated environments are those that are acting sinergic on the organic pollutants.

All these arguments supporting the implementation of remediation biotechnologies, justified the interest in addressing issues of environmental protection and decontamination of areas affected by the presence of pollutants. At the same time, deepening studies of molecular biology or by

applying techniques of taxonomic identification or for making genetic changes in order to increase the efficiency of metabolising contaminants or for characterization of metabolism products (enzymes, biosurfactants), puts the microbiological reserch of bioremediation at the bordering to the actual current of nanotechnologies.
Bioremediation process has a higher yield when certain conditions are met, related to: the type of pollutant and polluted environments, microorganisms involved in remediation technology and physical-chemical parameters.

This topic presents an theoretical and methodological importance, which will be the basis for future studies regarding the involvement of microorganisms from the seawater in bioremediation processes. Their number, their metabolic activity, their capacity to use as a source of carbon hydrocarbons and so their degradation, helped by certain concentrations of organic / inorganic nutrients and dispersants, are issues we consider important in knowing their role in bioremediation and therefore taken into account in studies from this thesis.

## PART II- PERSONAL CONTRIBUTIONS

Study of marine planktonic prokaryotes able to tolerate/ degrade hydrocarbons (oil) under the conditions set during experiments was conducted using the following techniques work:

### MATERIALS AND METHODS UTILISED:

**Installation of microcosms experiments** (from seawater filtered and unfiltered) polluted with diesel fuel, supplemented by organic compounds and / or inorganic in the presence of dispersants, substances were added in various concentrations (in the 4 series of microcosms developed in this thesis for determining optimal concentrations and characteristics of the systems for maximizing cellular growth and multiplication and the intensity of energy metabolism of natural populations / of marine microbiota.

**Fluorescent microscopy-** It is widely used in microbial ecology. There are several advantages in using it. It's quick and fairly easy to use, allows the visualization of the spatial distribution of cells in the sample with an appropriate mix of fluorescent stains, it is possible to distinguish

between viable and dead cells. However, the direct identification of microorganism is not possible by conventional fluorescent staining. Therefore, distinguish cells based on morphology it is important as fluorescent stains are not specific to bacterial species or genera (Kepner, 1994).

Image analysis systems allow rapid quantification of several parameters, for example: fluorescence intensity, different sizes of micro-organisms and the percentage of area covered by the biofilm. (Johanna, 2003, Bloem, 1995, Keevil, 1992, Møller, 1995.)

They are also widely used in microbial ecology and extremely important in ecological research. There are many advantages to using them. They are fast and quite easy to use, allows the visualization of the spatial distribution of cells in the sample and, with an appropriate mix of fluorescent stains, it is possible to differentiate between viable and dead cells. (Lecoeur, 2002). The number of bacteria present in the original sample is calculated from the average cell counted / grid area used sample volume filtered and effective filtration area following Jones. 1979 formula. (Jones, 1979).

**Sizing and quantifying the number of bacterial cells by manual, semiautomatic and automatic method.**

Manual method (classical) counting of bacteria on the filter. Photographic methods are often used to measure the size and number of bacteria in natural environments (Fry. 1990) .

The measurement can be done automatically with any device attached to the microscope able to achieve images at low light levels. Bacteria are enumerated directly on the image using a micrometer or a ruler, or indirectly by using a highlighting marker on the picture of the bacterial cell.

The automated method for counting bacteria in the filter. For automatic sizing and quantification of bacterial cells we used two programs: Image J and CellC. For the semi-automatic method DotCount was used. Image J was the main software to measure the length of the cell and software CellC is the second software used in automatic image analysis for our microscopy cells enumeration and measurements of cells (size, shape, intensity) (Selinummi., 2008) as shown above (Ardelean., 2009, Ghita., 2010b).

Using software CellC we conted the bright cells on a dark background (epifluorescence). CellC default option is to present the parameters measured in pixels.

Semi-automatic method for counting bacteria in the filter. Quantification of cells was also achieved by a semi-automated method using software DotCount. DotCount is a program that count the number of points in an image. Points are considered to be regions connected with approximately the same intensity. Its original purpose was to count the pictures of skin pigmentation spots for Cancer Research (Dr. Martin Reuter, http://reuter.mit.edu/software/.) Thus we scored, like the classical method, the bacteria in pictures using a digital marker and then processed with Dot Count.

**Highlighting the bacterial metabolic activity using microplate resazurine type experiments. Quantitative determination of the resazurine reduction.**

Resazurine (10-oxide 7-hydroxy-3H-phenoxazine-3-one) is a blue dye, nonfluorescent, it is reduced to resorufin (pink and very fluorescent), which is further reduced to hydroresorufin (colorless nonfluorescent ) with the aid of the oxidoreductase from the viable cells present. Resazurine is mainly used as an indicator of oxidation-reduction in cell viability assays. (Fig.1) (O'Brien et al, 2000; www.promega.ro).

Resazurine reduction test is used for about 50 years to monitor bacterial and yeast contamination of milk, also assessing the quality of semen. It is not yet known how this reduction occurs or intracellular activity of the enzyme . Recently, resazurine has become very popular as a very simple and versatile method for measuring cell proliferation and cytotoxicity. (O'Brien et al, 2000).

Resazurine conversion to fluorescenct resorufin is proportional to the number of metabolically active cells, present a viable population.

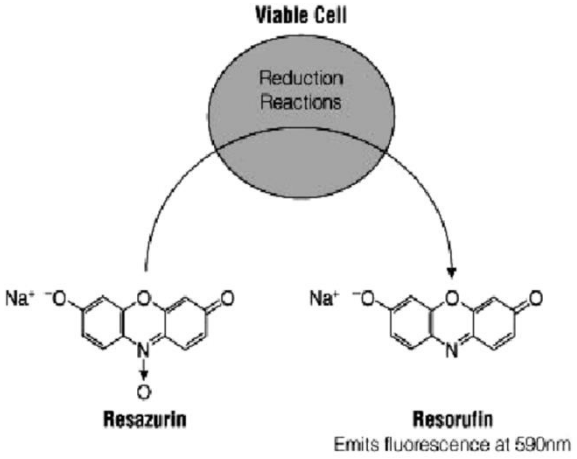


Fig.1.8. Reducing resazurine (O'Brien et al, 2000; /www.promega.ro)

**Using dispersants in microcosms experiments .**

Because of the uneven distribution of bacterial populations and immiscible structure of hydrocarbon used in the experiments, were used dispersants (NACOL C) to facilitate their distribution in the microscopic field so that they can achieve counts microscope much easier and eliminating the emergence of errors such as not taking into account the clustering of bacterial populations. (Buesing, 2002 Lunau et al. 2005).

**Identification of hidrcarbon oxidant bacteria** can be achieved by studying: characters of the culture, how they growth, form of the colonies, their growth on selective media and morfoctintorial characters by Gram stain and depending on the result will be identified biochemicaly of the isolated species.

**Specific antibiotic susceptibility testing of** hidrcarbon oxidizing bacteria. Is performed by Kirby-Bauer disc diffusion method and / or by microdilution method (MIC). (CLSI 2017).

## CHAPTER I - Density dynamics in microbial cells from marine microcosms supplemented with diesel and dispersant NACOL C.

Given the data in the literature on the dynamics of bacterial microcosms contaminated with diesel, protists -free (Ardelean et al., 2009a; Ghita, 2010; Va'zquez et al., 2005; Sherr., 2002, Shat. , 2011), the purpose of a first experiment was to measure the temporal evolution of the number of bacterial cells in microcosms lacking protists, including bacteriovores-obtained by filtering seawater in filter with pores of 0,45 mm, supplemented with diesel and dispersion agent (NacolC) compared with non-supplemented control microcosms.

**Water samples** were collected in sterile bottles from the Black Sea (the Tomis sea port at a depth of 0.5 m, 44 ° 10 '42' 'N, 28$^{o}$ 39' 36 " E), which was used to configure the microcosms in transparent polyethylene bottles. Microcosms were kept at room temperature in the dark.
To monitor changes in the bacterial cell density micrococsmosms free from bacteriovors, the bacterial communities were obtained by filtering seawater through a sterile filter 0,45 mm (Millipore) using vacuum filtration device, to avoid including nanoflagellates / heterotrophs protists in the filtrate. (Jürgens et al., 2000; Sherr et al., 1999, 2002, Vasques- Domninques et al., 2005, Sherr., 2002).

Excluding heterotrophic nanoflagellates (and bacteriovors or eukaryotes) from microcosms, alows to measure total cell number when prokaryotes are not consumed by microorganisms such as bacteriovors. This is unusual for bacterioplankton populations in the wild, but it simplifies the subject, to better understand the interaction between a smaller number of factors. However, it should be noted that the filtration by 0.45 mm filter exclude larger community microbial bacteria that are generally in good metabolic state. (Vasques- Domninques et al., 2005).

**Mounting microcosms.** For the microcosms mounting was used dispersant NACOL C, a mixture of organic and inorganic nutients, nonionic surfactants, etc., was diluted 10,000-fold in the filtrate - seawater.

Experimental variants were: filtered seawater M1 - control without any addition and M2- control supplemented with dispersant (1 / 10,000) and diesel (1% w / v) (15 years old).

The advantages of using microcosms as experimental models in laboratory, allows to control the experimental parameters, such as: temperature, the presence or absence of bacteriovors, the concentration of pollutant and / or nutrients. This control allows an easier interpretation of the results obtained in microcosms compared to the natural environment and provide a more easier understanding of the natural interaction of various factors. On the other hand, there are some disadvantages: compared with the natural environment, the microcosm is a simplified system and the results thus obtained can not be extrapolated per se. In addition microcosm does not remain the same throughout the experiment and the time evolution of the microbiota is also different from that which occurs in the natural environment.

In Figure 1.1. we present representative images of the 20 fields inspected of the marine microbiota from samples taken at different times during the experiment, the M1 (control) and M2 at T0, T1 (3 days), T2 (7 days), T3 (17 days), T4 (23 days), T5 (38 days), and T6 (52 days).

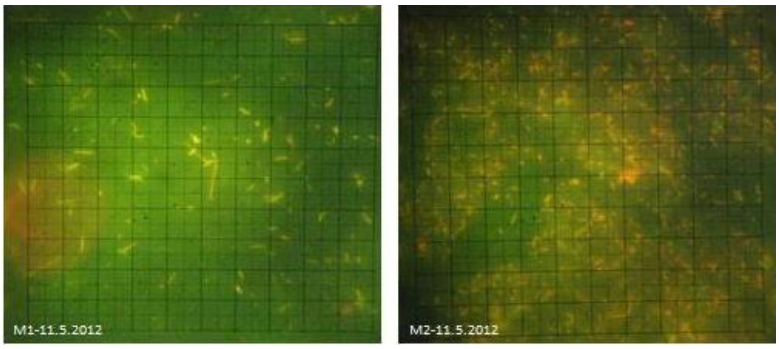


Figure 1.1. Marine microbiota from samples taken at: T0, T1, T2, T3, T4, T5, T6 from M1 (control) and M2 (microcosm supplemented with diesel 1% and dispersant Nacol C. (It may be noted that in the presence of dispersant and diesel (M2), the cell densities are higher than in the control (M1) and the control cells are larger than those grown in the presence of a dispersant and the gas oil (M2).

Were calculated cell densities from each sampling time (see Materials and Methods). In Figures 1.2 and 1.3 we can see the dynamics in the cells densities in M1 and M2. Cell enumeration was performed: manually, automatic using image analysis (CellC software) and semi automated using image analysis (DotCount).

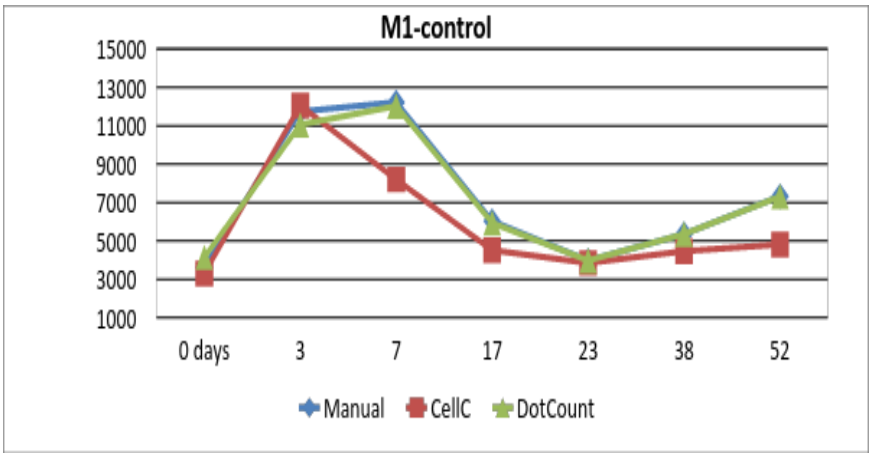


Fig. 1.2. Dynamics of microbial cell density in M1 enumerated by: manual , automatic or semi automatic image analysis using (CellC and DotCount) -values submitted on ordinate represents the number of bacterial cells quantified.

In connection with the time course of the microbiota in the control microcosm (M1) can be observed (Figure 1.2), there is a very good correlation between the number of cells counted manually and the number of cells counted semi automatic ( with software DotCount), the same it applies to the calculation made by automated image analysis (software CellC), except the third sample and to a lesser extent in the last sample. These differences are due to poor quality images and high cell densities.

In connection with the time evolution of microbiota in microcosm supplemented with dispersant and diesel (M 2) can be observed (Figure 1.3) that there is a very good correlation between the number of cells counted manually and the number of cells counted semi –utomated (with DotCount) as in the case of the control (Figure 1.2). However, except time zero, there are large differences between the number of cells counted manually or semi-automatically (with DotCount) and the results obtained using the automated image analysis. These differences may be determined / due to higher cell densities found in M2, as shown in Figure 1.1, making it difficult to differentiate between bacterial organisms and the background.

Considering these results, it is assumed that counting manual and semi-automatic provides a better measurement of cell densities than automatic image analysis in these experiments.

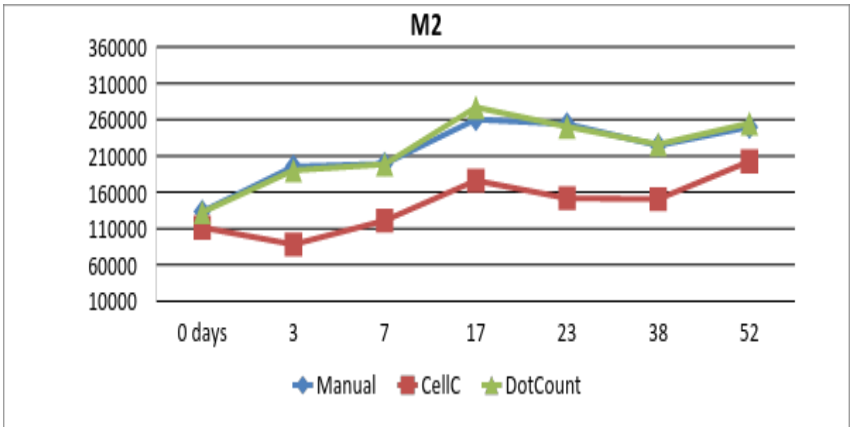


Fig. 1.3 Dynamics of microbial cell density in M2 enumerated by: manual , automatic or semi automatic image analysis using (CellC and DotCount) –values submitted on ordinate represents the number of bacterial cells quantified.

To get a better view of the dynamics of microorganisms in both microcosms in Figure 1.6 it shows the results of the length of cells with standard deviation values (Table 1.2).

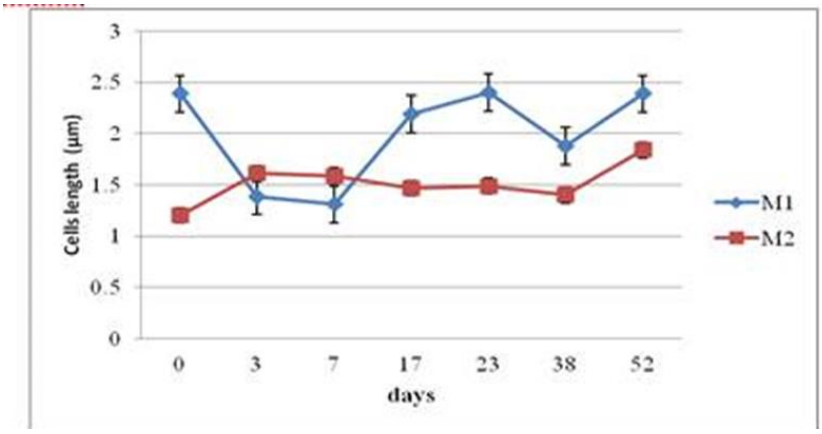


Fig. 1.6. Evolution of cells lengths in control (M1) and in the presence of both diesel and dispersant (M2). As you can see the length control cells is higher than in M2.

These differences in cell size could be related to the presence of dispersant (C Nacol, a biodegradable product) and diesel, which could be used as carbon source by endogenous microflora thereby supporting cell growth and multiplication.

**CONCLUSION CHAPTER I.**

***The results show that in the presence of dispersant and diesel cell densities are higher than the control group, whose cells are larger than those grown in the presence of dispersant and diesel.***

## CHAPTER II - Selecting the optimal concentration of nutrients, dispersant and diesel oil to enhance metabolic activity and diesel oil consumption by marine endogenous microbiota quantified by resazurine reduction in microplates experiments.

Subsequently, a second screening was performed using microplates. Was used several variables: the concentration of dispersant, the amount of diesel and nutrient organic / inorganic. To follow the cellular multiplication and intensity of energy metabolism of marine microbiota, it was followed the **resazurine rate reduction.**

**Highlighting the bacterial metabolic activity using resazurine. Quantitative determination of resazurine reduction.** The data obtained for each well ( reduced resazurine ng /well) are represented in Figure 2.3.

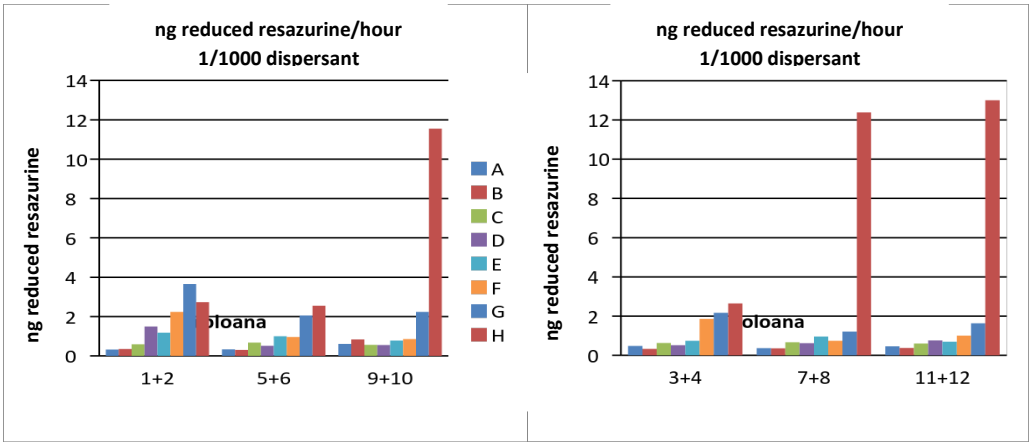


Fig.2.3. The amount of reduced resazurine 1-12 per hour wells.

**Growth and multiplication of microorganisms in the microplate.** Growth and cell proliferation was much slower for screening experiments number 2.

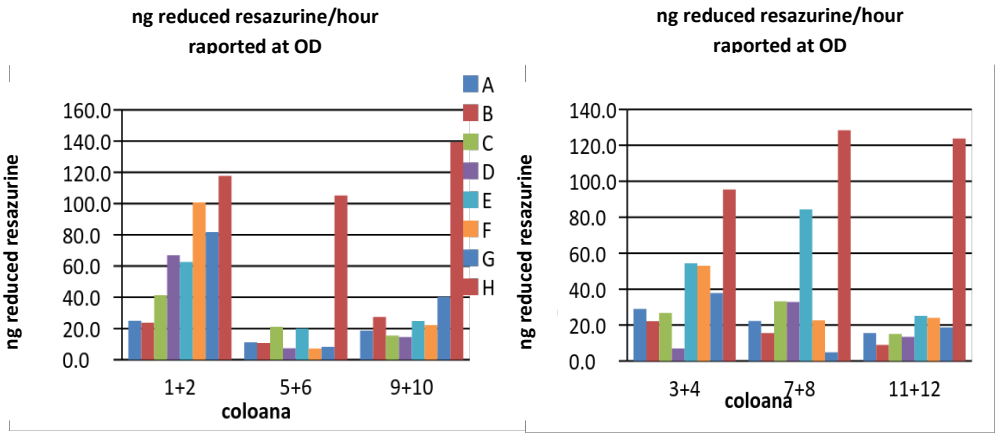


Fig.2.4. Intensity of resazurine reduction ng reduced resazurine / hour / DO.

It can be seen a greater amount of reduced resazurine in columns with an amount of 20µL of diesel fuel. (1-2, 3-4) High activity in the control array that does not contain diesel (H -1-12) it has dispersant and ammonium phosphate + ammonium acetate + yeast peptone medium.

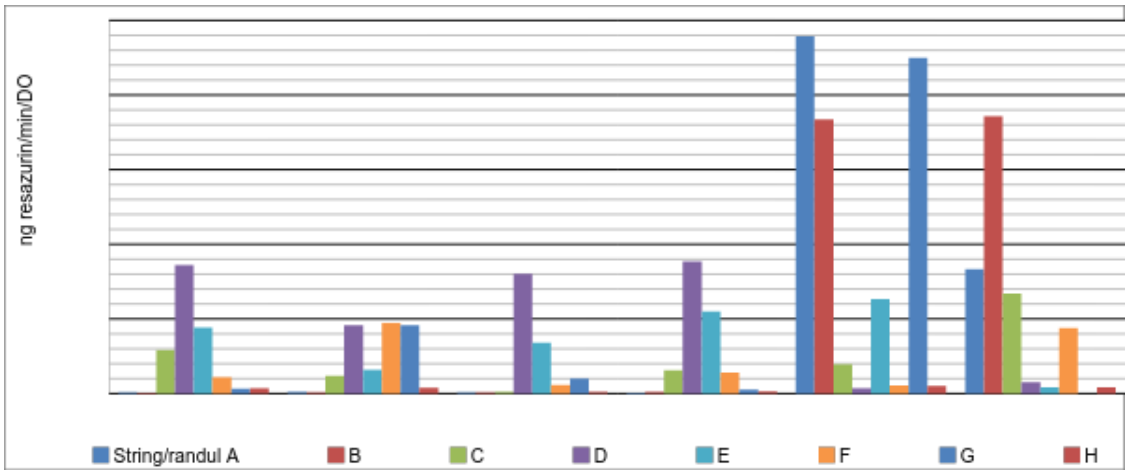


Fig.2.5. The intensity of resazurine reduction ng reduced resazurine / hour / DO.

## CONCLUSIONS CHAPTER II.

**The results of this chapter suggests following optimum in terms of the three variables:**

**Variable diesel: ● optimum amount of diesel is 20µL (10%).**

**Variable dispersant: ● optimum amount of dispersant (1/1000).**

## CHAPTER III - Selecting the optimal concentration of nutrients to enhance metabolic activity and diesel oil consumption by marine endogenous microbiota quantified by resazurine reduction in microcosms experiments at 15 ° C.

This screening was conducted in order to select the best solutions for: the optimal concentration of nutrients, dispersant and diesel to stimulate microbial growth and metabolic activity (by resazurine reducing). (Ardelean et al, 2009 ;. Ghita, 2010, 2011, 2012; Popoviciu, 2011; Manea Ardelean, 2013; Manea et al., 2013) and the relationship between the rate of metabolic activity and diesel consumption by marine endogenous microbiota incubated at 15ºC in bacteria communities - without protists (filtered seawater through the filter with pores of 0,45 mm).

Were mounted five microcosms in glass bottles with 200 ml of filtered seawater with different amounts of organic nutrients (1/10 yeast- peptone medium) and inorganic nutrients (ammonium acetate and ammonium phosphate 0.5% ), diesel oil (10%) and added dispersant (Nacol C-1 / 1.000), as shown in table 3.1. The five microcosms were then incubated at 15 ° C in the dark. Samples were collected periodically to determine in each microcosm: metabolic activity (by resazurine reduction) and diesel consumption.

Tabelul 3.1. Working protocol for experiment 3 – the content of each microcosm.

| | 200 µl sea water filtred by 0,45 µm pores | Filtred diesel | Dispersant 1/1000 | Ammonium acetate and ammonium phosphate 0.5% | Yeast petone 150 µl |
|---|---|---|---|---|---|
| M1 | X | | | | |
| M2 | X | X | | | |
| M3 | X | X | X | | |
| M4 | X | X | X | X | |
| M5 | X | X | X | X | X |

## III.2. RESULTS AND DISCUSSIONS

Figure 3.3 presents the results in terms of reducing the rate of resazurin (Resazurin expressed as ng / hour / well) during the experiment by native microbiota of the five types of microcosms.

As you can see, the rate is lower in control (M1) compared with microcosms with added inorganic nutrients (M3-M5), suggesting that inorganic nutrients support the growth of endogenous metabolic activity of marine microbiota. This increase is acording with the data literature (Fuhrman, 1980, Atlas, 1981, Lewis 2001, Cohen, 2002; Van Hamme et al, 2003 ;.- Barahona Molina et al, 2004, Munn, 2004 Cap et al 2006 ;. Ducklow, 2008, Gasol 2008 Kirchman 2008 Kempf, 2010; Shat 2011; Enon et al, 2011 ;. and Popoviciu, 2011; Uzoigwe et al, 2012.; Ardelean et al.,. 2009 Ghita 2010, 2011, 2012, Manea 2013 ;. Manea et al, 2013).

When it comes to adding diesel fuel, the situation deserves further attention.

In M2, which was added only diesel with seawater, the rate of resazurine reduction is lower compared to control (M1), this suggests that the pollutant (diesel) determines the reduction in the intensity of metabolic activity of marine endogenous microbiota, this data are in agreement with previous results reported (Atlas, 1981; Venkateswaran et al., 1995; Habe, 2003; Zhang et al., 2010; Manea, 2013; Manea et al., 2013).

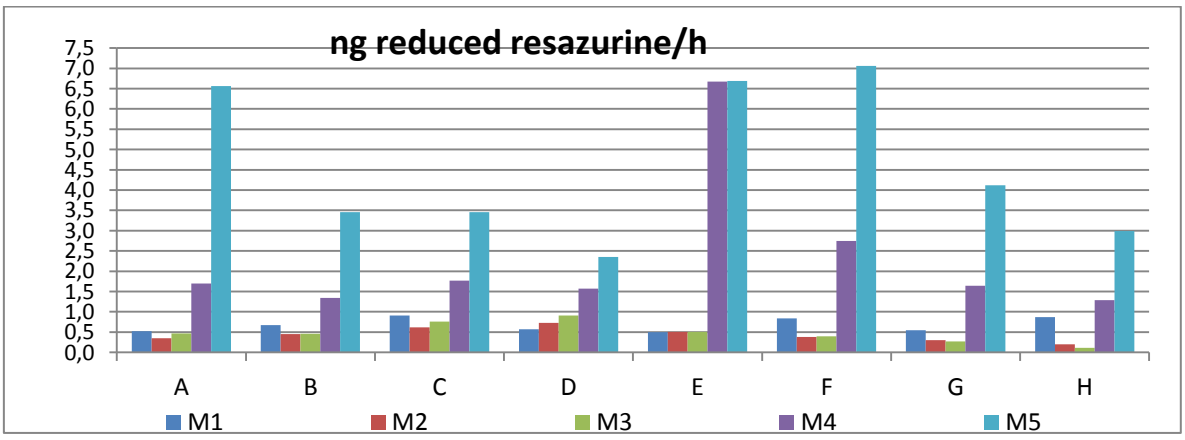


Fig. 3.3. Evolution of metabolic activity over time (measured in ng / resazurine / hour / well) of endogenous microbiota in microcosms with filtered water supplemented with dispersant (Nacol C), oil, inorganic and organic nutrients.

Similar results were obtained in the presence of both diesel and dispersant (M3), in agreement with our previous results regarding the absence of toxicity of the dispersant at this low concentration (Manea et al, 2013). Adding the inorganic nutrient (both ammonium acetate and ammonium phosphate, 0.5%) to M4 greatly increase the rate of resazurine reduction compared to the rate measured in the absence of them (M1-M3); plus, the nutrient addition in M5 support a higher rate by 2-3 times of resazurine reduction compared to the rates measured in M5 without organic supplement (M4). All these results are consistent with reports in the literature (Atlas 1981 ;. Zhang et al, 2010), suggesting that concentrations of inorganic and organic nutrients in unpolluted marine environments are limited concentrations (Karl, 2005; Costello et al, 2010).

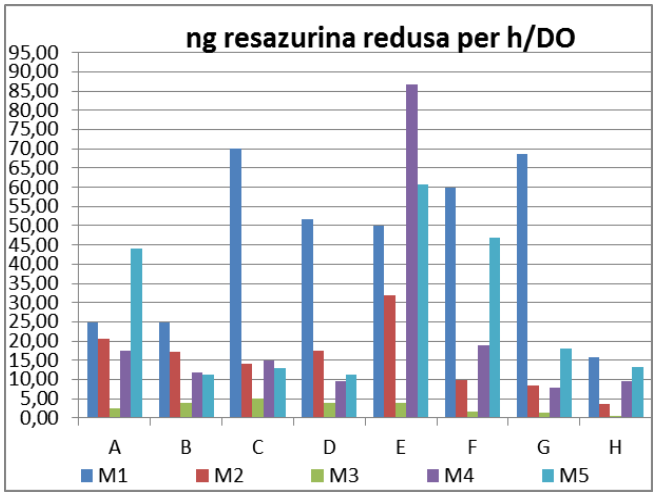


Fig. 3.4. Estimating the consumption of diesel by endogenous microbiota in microcosms with filtered water supplemented with dispersant (Nacol C), diesel, inorganic and organic nutrients by resazurine reducing.

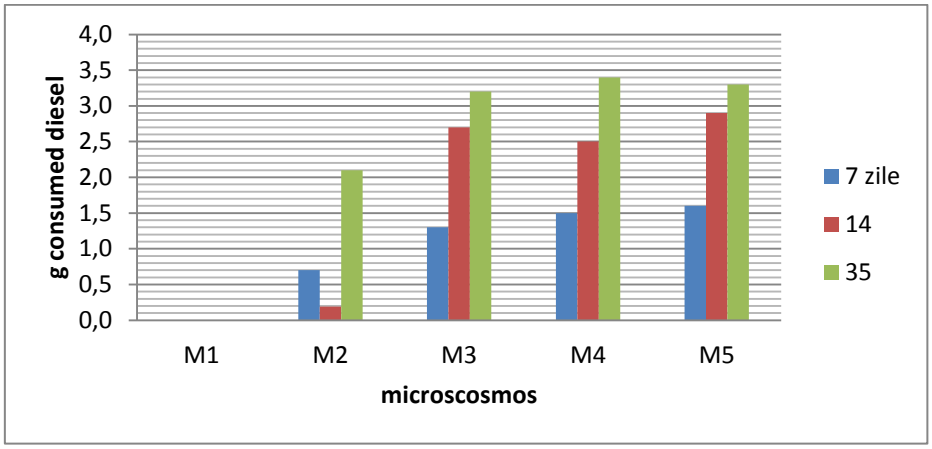


Fig. 3.5. In time evolution of diesel consumption -it can see the amount of diesel consumed from the initial 10 g (10%).

The dispersant addition (M3), increase diesel oil consumption by 1.5 fold or more compared to M1, where only diesel oil was added to the filtered seawater. These results are consistent with the use of different types of dispersants (non toxic at the work concentration) to enhance the complex interactions between microbial cells and petroleum hydrocarbons, thereby supporting an increased pollutant rate consumption (Atlas, 1981; Lewis, 2001; Cohen, 2002; Van Hamme et al, 2003, Molina-Barahona et al, 2004 ;. Cap et al, 2006 ;. Kempf, 2010; Shat 2011; Uzoigwe et al, 2012 ;. Manea, 2013; Manea et al. 2013).

As shown in Figure 3.6., in the absence of diesel oil (M1) it can see prokaryotes isolated, stained with acridine orange (Ghita and Ardelean, 2010), while in the presence of diesel, the cells start to aggregate (M2), in addition in the presence of dispersant (M3-M5) can see the micro-vesicles of different sizes, with the bacteria to the surface, like true micro bioreactors, containing within them dense bacterial population; This spatial arrangement of the bacteria in the micro-vesicles enhances physical contact between the cells and diesel oil, and bacterial consumption (Lewis, 2001; Cohen, 2002; Van Hamme et al, 2003 ;.-Barahona Molina et al, 2004; Head et al, ;. Kempf 2006, 2010, 2011 ;. Uzoigwe Shati et al, 2012).

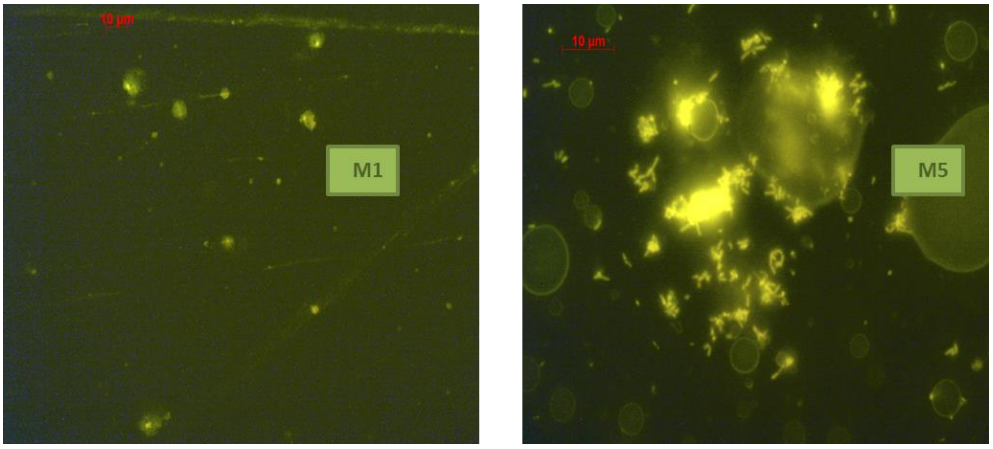


Fig 3.6. The spatial distribution of bacterial cells in the presence and absence dispersant, diesel and nutrients (M1) and in the presence of dispersant, diesel and nutrients (M5). -it can observe the distribution of bacteria that adhere to droplets of diesel, bacterial density was higher in the presence of dispersant (M5) and defending diesel droplets dispersed in water mass in the presence of dispersant- Acridine Orange Staining(AO).

As shown, the diesel fuel consumption is slightly higher (1.5-fold) by adding dispersant (M3, M4, M5) as compared with M2 and M1, but nutrient organic and / or inorganic add does not have an effect on fuel consumption, the values obtained being the standard deviation. However, the increase in fuel consumption (1.5 fold) is much lower compared to the growth rate of resazurin reduction (10-fold or more), suggesting that in our experiments, the additions of inorganic and organic nutrients have a limited positive effect on consumption of diesel.

**CONCLUSIONS CHAPTER III.**

**The rate of resazurine reduction is descresed in microcosms without nutrients compared to microcosms with added inorganic nutrients.**

**In microscosmos were it has been added only diesel resazurine reduction rate is lower compared to the control.**

**Adding inorganic nutrient greatly increase the speed reduction of resazurine, plus the addition of nutrient claims 2-3 times higher rate of resazurine reduction than the rates measured without organic supplement.**

**Diesel fuel consumption is improved (by 1.5 times) by adding dispersant. The addition of nutrients of organic and / or inorganic do not have an effect on fuel consumption.**

## CHAPTER IV- Diesel fuel consumption in long term (15 months) microcosms experiments with continuous addition of inorganic nutrients.

In further experiments presented in the previous chapters, the experiments in this chapter provide new elements: long-term monitoring of fuel consumption and metabolic activity of edogenous marine microbiota, and with periodic supplementation with inorganic nutrients based on nitrogenous and phosphorous in small quantities to prevent inhibition of endogenous microbiota metabolism . (Atlas, 1981 floodgate, 1984. Choi, 2002. Kim, 2005, Bruss, 1998, Atlas 1981 Chaillan, 2006).

Were mounted 10 microcosms- 5 clear glass vials with GREEN stoppers (180 mL of filtered seawater) and 5 with BLACK stoppers (180 ml of seawater unfiltered) with different amounts of organic nutrients (ureea) and nutrients inorganic (potassium dihydrogen phosphate), diesel fuel (1/10) and dispersant (Nacol C-1/1000).
Unfiltered water (group I):

1. were added 200 ml unfiltered seawater in all the 5 bottles.
2. were removed with 20 ml of seawater M1-------M3.
3. were added to 10 ml of filtered diesel M1-------M3.
4. was added to 10 ml of the stock solution 1/50 of the dispersant in M3 a, b, c.
5. and 20ml of buffered formalin 10X stock solution was added in -180 ml microcosm M2.

Filtered water (group II):

1. were added 200 ml filtered seawater in all the 5 bottles.
2. were removed with 20 ml of seawater M1-------M3.
3. were added to 10 ml of filtered diesel M1-------M3.
4. was added to 10 ml of the stock solution 1/50 of the dispersant in M3 a, b, c.
5. and 20ml of buffered formalin 10X stock solution was added in -180 ml microcosm M2.

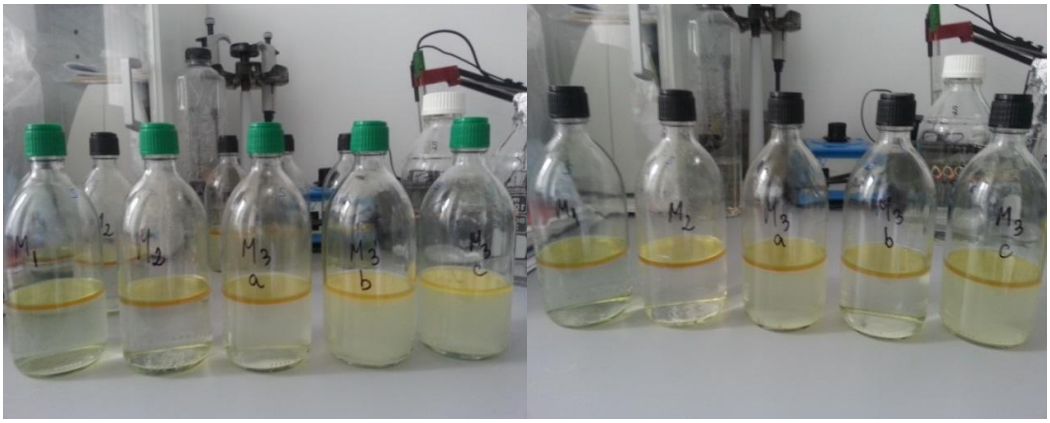


Fig. 4.1. Microcosms of GROUP I (left) and Group II (right) - experiment mounted in. 5 .5 2015.

The ten microcosms were incubated at 15 ° C in the dark. Samples were collected periodically to determine in each microcosm: metabolic activity (resazurine reduction) and diesel consumption.

## IV.2. RESULTS AND DISCUSSIONS.

Following the centralization of the data obtained during the seven months in which measurements were made of the activity of resazurine reduction as a marker of metabolic activity of the microorganisms in the microcosms. (Figures 4.5 and 4.4), could see the rate of resazurine reduction lower in control (M1 and M2), compared with microcosm with organic nutrients in different proportions (M3, b and c), suggesting that nutrients support the increasing the metabolic activity of the marine endogenous microbiota. This increase is consistent with the literature data (Fuhrman, 1980, Atlas, 1981, Lewis 2001, Cohen, 2002; Van Hamme et al, 2003 ;.-Barahona Molina et al, 2004, Munn, 2004 Cap et al 2006 ;. Ducklow, 2008, Gasol 2008 Kirchman 2008 Kempf, 2010; Shat 2011; Enon et al, 2011 ;. and Popoviciu Ardelean, 2011; Uzoigwe et al, 2012.; Ardelean et al., 2009, Ghita, 2010, 2011, 2012, Manea 2013 ;. Manea et al, 2013).

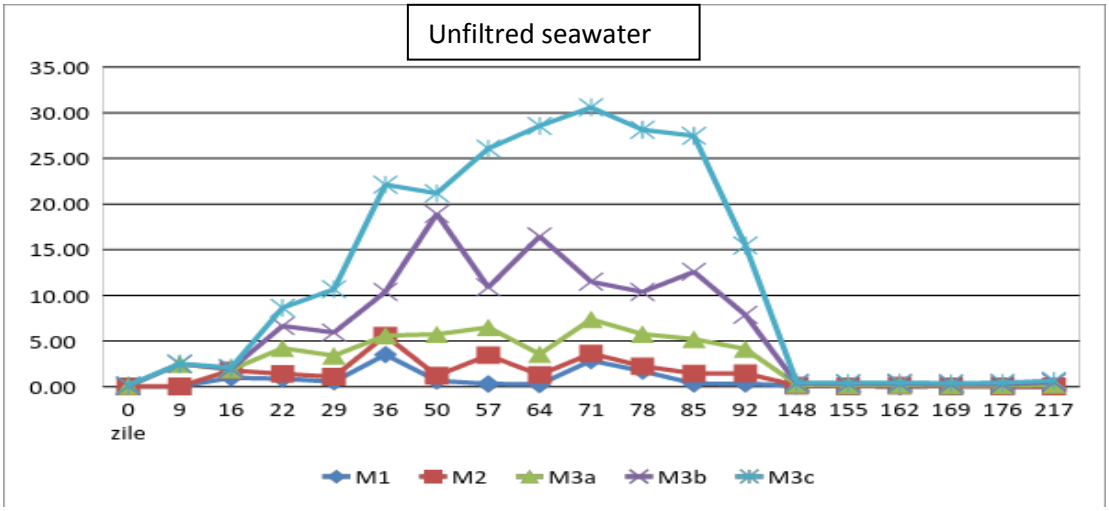


Fig. 4.4. The evolution of metabolic activity for 7 months of the endogenous microbiota in microcosms with unfiltered water, supplemented with dispersant (Nacol C), diesel, inorganic and organic nutrients.

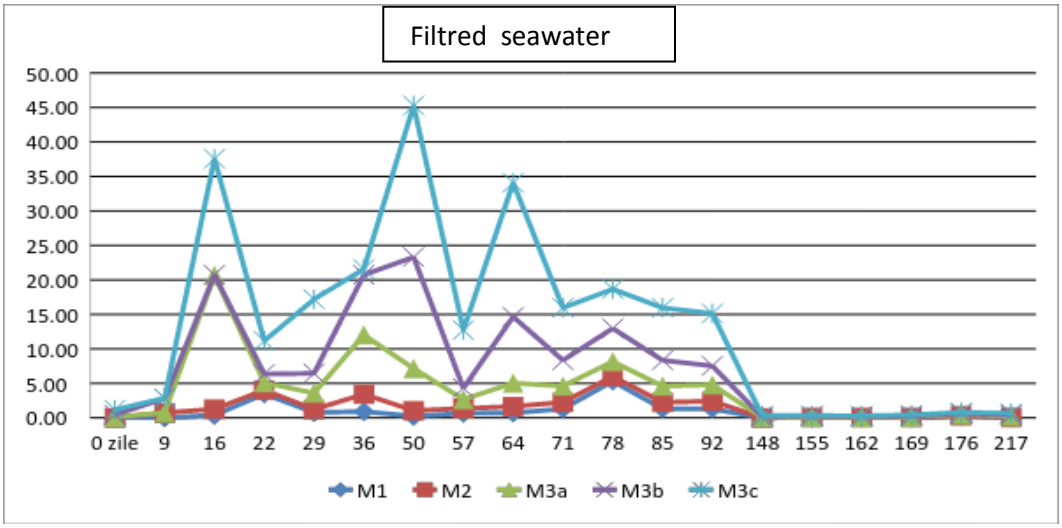


Fig. 4.5. Evolution in metabolic activity for 7 months of endogenous microbiota in microcosms with filtered water supplemented with dispersant (Nacol C), diesel, inorganic and organic nutrients.

But it can be seen and inhibition in the rate of resazurine reduction in microcosms with a greater amount of organic nutrients (M 3a, M3b) unlike the quantity is less (M3c). This increase is consistent with data from the literature (Atlas, 1985) confirming that the biodegradation process nutrients are needed, but in some concentrations it can become limiting factors.

It can be seen an increase in metabolic activity of marine endogenous microbiota microcosm M3c with a smaller amount of organic nutrient 1.08g / 200ml, compared to 2.17g / 200ml in M3a and 3.25g / 200ml in M3b. All these results are consistent with the literature (Atlas, 1985; Venkateswaran et al 1995 ;. Habe 2003 ;. Zhang et al, 2010), which confirms the fact that nutrients are needed biodegradation process but can become in certain concentrations limiting factors.

But concentrations of inorganic and organic nutrients in unpolluted marine environments are in limited concentrations (De Long 2005; Costello et al, 2010; Liu et al .., 2010) suggesting that nutrient augmentation can help accelerating the process of bioremediation .

**Diesel consumption in the 10 microcosms illustrated in Figure 4.6, as the difference between the initial concentration and the final concentration (gravimetric method).**

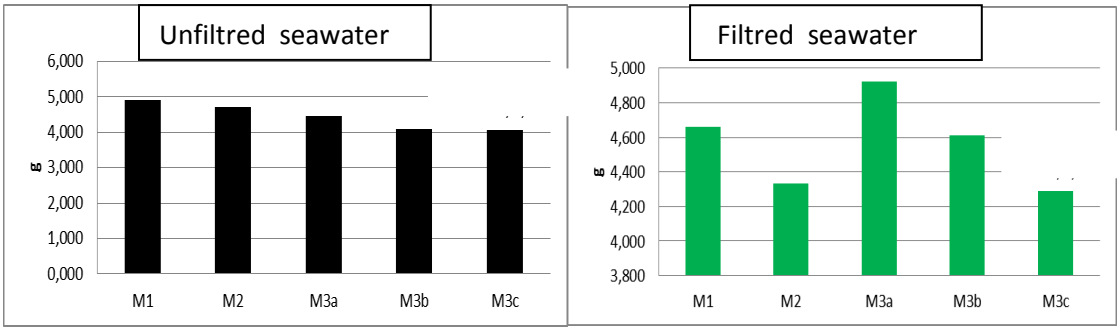


Fig 4.6. Microcosms remaining quantities of diesel fuel at the end of the experiment, the difference between the initial concentration and the final (July 2016). It can be seen in microcosm M3c higher consumption with nutrient less than those with higher concentrations (M 3, M3b).

Following the dosing fuel consumption by gravimetric method it was possible to highlight a decrease in the amount of hydrocarbon in microcosm supplemented with quantities of nutrients in moderate amounts (M3c) than those with higher concentrations (M 3, M3b).

**Quantification of hydro-carbon- oxidizing bacteria in microcosms supplemented with nutrients.** The number of colonies on the plates inoculated , counted on 9/30/2016 at 148 days from the beginning of the experiment are presented in Table 4.6.

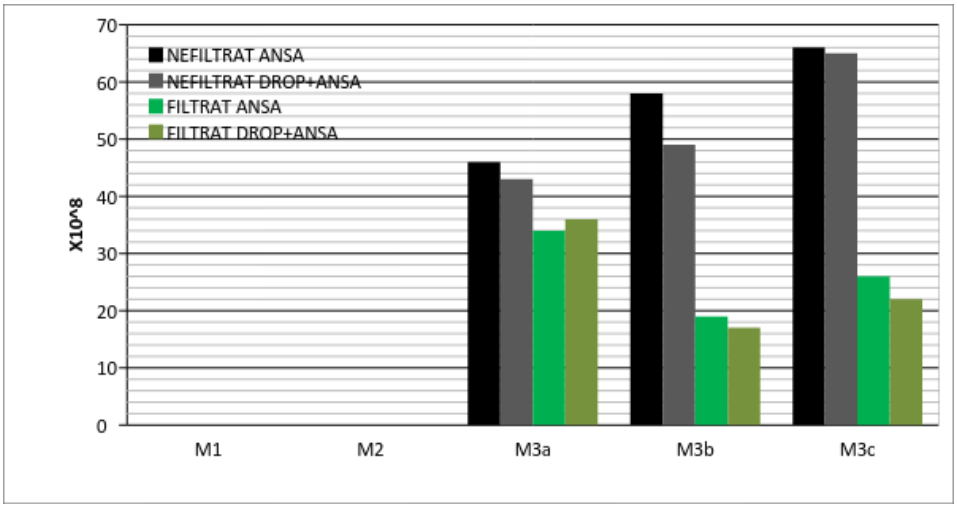


Fig.4.7. Number of hydrocarbon oxidizing UFC 148 counted in days. from the start of the experiment .

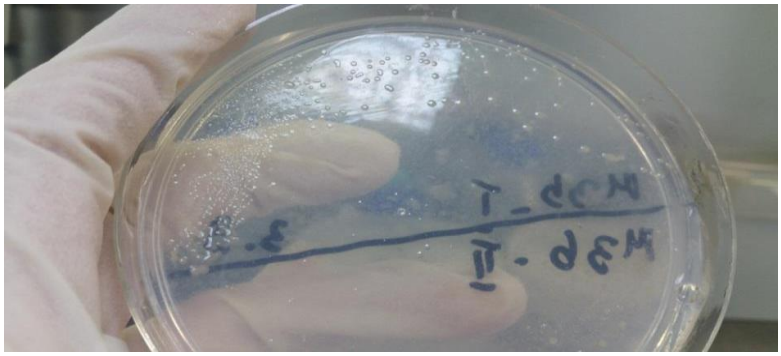

Fig.4.8. Colonies isolated from microcosms M3b filtered (below) and unfiltered (above).

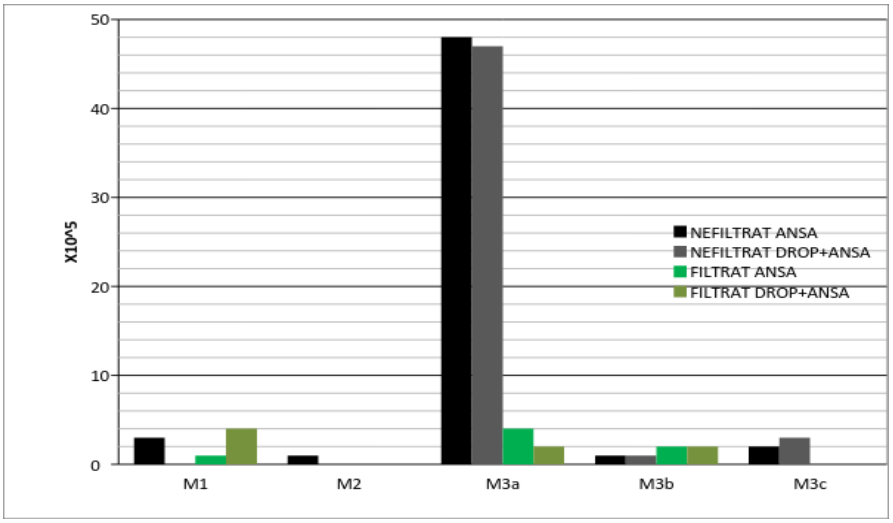


Fig.4.10. Number of hydrocarbon oxidizing UFC counted in day 305 from the start of the experiment .

Following the selection of hidrocarbon -oxidizing bacteria in microcosms supplemented with nutrients. It could notice a decrease in the viable colony density in phase II. Following the colony counts was able to observe a variety colony for more than microcosms unfiltered to the filtered.

**CONCLUSIONS CHAPTER IV.**

**In long term microcosm experiments (15 months) intermittent addition of nutrients nitrogen and phosphorus resulted in consumption of a larger amount of diesel compared with microcosms control both the microcosms with filtered and unfiltered seawater.**

**Hydro-carbon oxidizing cell density is greater in microcosms with the addition of nutrients compared with control microcosm, both in microcosms with filtered seawater as well as those with unfiltered seawater;**

## CHAPTER V - Isolation, selection and identification of bacteria oxidizing hidrcarbon.

After analyzing the characteristics of: cultures, GRAM smears, were identified two morphological types:

**Type 1-**on Columbia agar medium with 5% sheep blood – circular colonies , convex type S, with entire edges.

Colony diameter = 1-1.5 mm after 24 hours at $37^{o}$C and 3.0-3.5 mm to 48 hours at $28^{o}$C.

Without hemolysis, Non-motile. (Fig.5.1)

On MacConkey agar there is good growth after 24h at $37^{o}$C. and 3.0-3.5 mm at $28^{o}$C. lactic-negative colonies. (Fig.5.1)

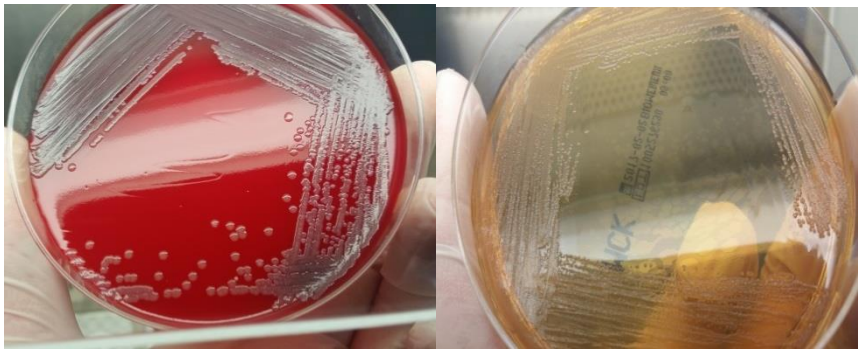

Fig.5.1. Morphological type 1 isolated colonies from microcosms. (COS left, right MC).

**Identification with Remel -** According to the identification performed using the kit Remel in a proportion of 99% type one species are belonging to the genus *Acinetobacter* .

**Identification by polytropic media.** As identified using biochemical tests performed and interpreted individual with ABIS software online at a rate of approx. 92% species is identified is *Acinetobacter lwoffii.*

The genus *Acinetobacter*, isolated in our experiments represents a group of Gram-negative bacteria, imobile and oxidase negative, nonfermentative belonging to the family *Moraxellaceae*. *Acinetobacter* species are able to survive on various surfaces (both wet and dry). **These bacteria are intrinsically resistant to many classes of antibiotics.** According Gerischer *Acinetobacter* bacteria is a suitable biotechnological exploitation **purposes**. (Gerischer., 2008).

*Acinetobacter* genus belongs alongside *Pseudomonas* genus to the Order of *Pseudomonadales*. Both species include nonfermentative genders involved in bioremediation ubiquitous. *Pseudomonas aeruginosa* is a bacteria that can tolerate organic solvents due to metabolic mechanisms that allow their development in various ecosystems, including polluted ones. (Lazaroaie, 2010. 2009 Stancu. MM, 2011).

**CONCLUSIONS CHAPTER V.**

**They were isolated, purified and identified from the level of genera in the forth experiment a strain of *Acinetobacter sp*. and a strain of *Bacillus sp*.**

**The strain identified at gender could be identified at a rate of approx. 92% level as species named *Acinetobacter lwoffii*.**

## CHAPTER VI - Testing antibiotic susceptibility of hidrcarbon oxidizing bacteria isolated.

After incubation, the plates were examined visually for the presence or absence of bacterial growth, the lack of growth was recorded as resistance , while the bacterial growth being registered as susceptibility .

It could highlight the resistance of the isolated strain for the class of: cephalosporins, carbapenems and beta-lactamases. (Table 6.1). according to the data from the literature, such as Gupta., antibiotic susceptibility tests for *Acinetobacter sp.* Isolate showed resistance to piperacillin (55%), ceftriaxone (46%), ceftazidime (46%) of cefepime (44%), cefotaxime (43%), amikacin (42%) and imipenem (22%). (Gupta, 2015).

At the same time it was able to observe the lack of resistance to class: Aminoglycosides: Trimethoprim + sulfamethoxazole, Quinolones and Tetracyclines. The data obtained are consistent with the literature, according to Sharma, 2016. the bacterial isolates from oil spills are resistant to ampicillin in large number 12/28, to penicillins 10/28 and only 1/28 resistant tentracicline, suggesting that this antimicrobial agent would be quite efective. (Sharma, 2016).

Because of the resistance to cephalosporins and carbapenems, the strain was tested for the production of ESBL and Carbapemenase. The tests were negative. (Fig. 6.1), which may explain the existence of other mechanisms that can cause resistance of the strain to certain classes of antibiotics and susceptibility to others; These mechanisms could be acording to the literature: activation and / or inactivation of efflux pumps in the cell wall. (Stancu. MM, Grifoll. M, 2011, Lazaroaie, 2010, .2009., Poole, 2001, 2005).

Also species of *Acinetobacter spp*. isolated from medical samples under. Abbott presents in a large number resistance, thus 39.63% of isolates were multidrug resistant (at least 2 or 3 classes: penicillins, cephalosporins, aminoglycosides, fluoroquinolones and carbapenems). (Abbott .I, 2013, .N Gupta, 2015).

Tabel 6.1. Antibiogram of hydrocarbon- oxidizing izolate strain of *Acinetobacter lwoffii*. (Can be seen the resistance to cephalosporins and carbapenems, resistance to amoxicillin-clavulanate intrinsic confirmed)

| ***Antibiotic*** | ***Antibiotic contained for disc*** | ***Antibiotic class*** | ***Diameter read:*** | ***Incadration Recording CLSI*** | ***Diameter read in the presence of hidrocarbon:*** | ***Incadration Recording CLSI*** |
|---|---|---|---|---|---|---|
| Tobramycin | (10 μg), | Aminoglycosides | 27 mm | Susceptible | 26 mm | Susceptible |
| Trimethoprim + sulfamethoxazole | (1.25+23.75 μg) | Inhibitor al căii folatului | 26 mm | Susceptible | 34 mm | Susceptible |
| Levofloxacin | (5 μg) | Quinolones | 24 mm | Susceptible | 25 mm | Susceptible |
| Ciprofloxacin | (5 μg) | Quinolones | 26 mm | Susceptible | 25 mm | Susceptible |
| Doxycicline | (30 μg) | Tetracyclines | 20 mm | Susceptible | 25 mm | Susceptible |
| Tetracycline | (30 μg) | Tetracyclines | 16 mm | Susceptible | 26 mm | Susceptible |
| Gentamicin | (10 μg) | Aminoglycosides | 15 mm | Susceptible | 15 mm | Susceptible |
| Amikacin | (30 μg) | Aminoglycosides | 12 mm | Resistant | 15 mm | Resistant |
| Piperacillin + Tazobactam | (100+10 μg) | beta-Lactams | 16 mm | Resistant | 24 mm | Susceptible |
| Ampicillin + Sulbactam | (10+10 μg) | beta-Lactams | 0 mm | Resistant | 0 mm | Resistant |
| Ceftazidime | (30 μg) | Cefalosporine | 0 mm | Resistant | 0 mm | Resistant |
| Cefepime | (30 μg) | Cefalosporine | 0 mm | Resistant | 0 mm | Resistant |
| Ceftriaxone | (30 μg) | Cefalosporine | 0 mm | Resistant | 0 mm | Resistant |
| Imipenem | (10 μg) | Carbapenems | 0 mm | Resistant | 0 mm | Resistant |
| Meropenem | (10 μg) | Carbapenems | 0 mm | Resistant | 0 mm | Resistant |
| Piperacillin | (100 μg) | Penicillins | 0 mm | Resistant | 0 mm | Resistant |

**CONCLUSIONS CHAPTER VI.**

**The *Acinetobacter lwoffii* strain isolated shows resistance to: cephalosporins, beta-lactams, carbapenems, penicillins and sussceptibility to: tetracyclines, qinolone, aminoglycosides, false metabolites. in the presence and absence of diesel.**

**Exceptions in the presence of diesel: the strain became sussceptibile to Piperacillin-tazobactam and for Doxycicline, Tetracycline, Trimethoprim + sulfamethoxazole strain has greater susceptibility expressed by increasing the diameter read.**

## CHAPTER VII –GENERAL CONCLUSIONS AND PERSPECTIVES .

1. The results show that the presence of dispersant and diesel are higher cell densities than the control group, whose cells are larger than those grown in the presence of dispersant and diesel.
2. From the view point of reducing resazurine, the optimal concentration is 10% diesel and dispersant 1/1000.
3. The diesel fuel consumption is stimulated by 1.5-fold by addition of dispersant, but the addition of organic and / or inorganic nutrients has no effect on fuel consumption .
4. In microcosm experiments of long duration (15 months), the addition of nitrogen and phosphorus nutrients resulted in consumption of a larger quantity of diesel compared to both the control microcosms with filtered seawater and unfiltered.
5. Hydro-carbon oxidizing cell density is greater in microcosms with the addition of inorganic nutrients compared with witness microcosms, both in microcosms with filtered seawater as well as those with unfiltered seawater .
6. Were isolated, purified and identified from the level of genera in the forth experiment a strain of *Acinetobacter sp*. and a strain of *Bacillus sp.*
7. The strain identified at gender could be identified at a rate of approx. 92% level as *Acinetobacter lwoffii* species.
8. The strain of *Acinetobacter lwoffii*. .isolated show resistance to cephalosporins, beta-lactams, carbapenems, penicillins and susceptibility to tetracyclines, qinolone, aminoglycosides, false metabolites. in the presence and absence of diesel them.
9. Exceptions in the presence of diesel: the strain became sussceptibile to Piperacillin-tazobactam and for Doxycicline, Tetracycline, Trimethoprim + sulfamethoxazole strain has greater susceptibility expressed by increasing the diameter read.

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