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SELECTING THE OPTIMAL CONCENTRATION OF NUTRIENTS, DISPERSANT AND DIESEL OIL TO ENHANCE METABOLIC ACTIVITY AND DIESEL OIL CONSUMPTION BY MARINE ENDOGENOUS MICROBIOTA AT 15 ^{0}C

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Abstract

In this paper we present our results concerning further screening for optimal concentration of dispersant (Nacol C), nutrients and diesel oil to sustain metabolic activity of marine autochthonous microbiotain order to enhance the biological consumption of diesel oil. The results show that the overall metabolic activity (reported as ng reduced resazurine/hour/wheel), is decreased by diesel addition (10% final "concentration") either in the absence or in the presence of dispersant (Nacol C 1/10.000 dilution); inorganic nutrients additions (both ammonium acetate and ammonium phosphate 0.5%) greatly (4 to 10 times) increase the rate of resazurine reduction whereas further organic nutrient addition sustains an even higher rate of resazurine reduction. Diesel oil consumption is 1.5 times enhanced by dispersant addition but organic and/or inorganic nutrients additions do not have a further effect on diesel consumption, the values obtained being within the level of standard deviations.

Keywords*: diesel oil consumption, dispersant (Nacol C), resazurine, microcosms, Black Sea.*

INTRODUCTION

Extensive oil exploration activities sometimes lead to (severe) environmental pollution (Atlas, 1981; Venkateswaran et al., 1995; Habe and Omori, 2003; Zhang et al., 2010). In the last decades, there is an increasing interest in the use of dispersants to enhance hydrocarbon degradation by microorganisms (Atlas, 1981; Lewis, 2001; Cohen, 2002; Van Hamme et al., 2003; Molina-Barahona et al., 2004; Head et al., 2006; Kempf, 2010; Shata, 2011; Uzoigwe et al., 2012; Manea and Ardelean, 2013; Manea et al., 2013). 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. In the absence of dispersants the oil slick remains practically compact at the sea surface, the natural dispersion being the only way to break the oil silk. 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 (Lewis, 2001). However, the use of dispersants in near shore areas is expected to increase the exposure of aquatic organisms to petroleum (Milinkovitch et al., 2011). 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 (Venosa and Xueqing, 2003).

Based on literature (Atlas, 1981; Lewis , 2001; Cohen, 2002;Van Hamme et al., 2003; Molina-Barahona et al., 2004; Head et al., 2006; Kempf, 2010; Shata, 2011; Enon et al., 2011; Uzoigwe et al., 2012) and on our previous experiments concerning the screening for optimal concentrations of nutrients, dispersant and diesel oil to enhance microbial growth and metabolic activity (Ardelean et al., 2009; Ghita and Ardelean, 2010, 2011, 2012; Popoviciu and Ardelean, 2011; Manea and Ardelean, 2013; Manea et al., 2013), in this paper we focus on the relationships between the rate of metabolic activities and the consumption of diesel oil by endogenous marine microbiota incubated at 15 C in protist-free bacterial communities (filtered sea water).

MATERIALS AND METHODS

Sampling

The sea water used in this experiment was collected in sterile bottles from the Black Sea at 0.5m depth (Constanta; 44°21"14'N; 28°64"43' E), then was filtrated with 0.45 µm Millipore filter to avoid the inclusion of bacteriovores microorganisms (e.g. heterotrophic nanoflagellates) in the filtrate. However, it has to remember that 0.45 µm filtration causes the exclusion from the microbial community of larger bacteria as well, which are, in general, in good metabolic status; however, the elimination of bacteriovores is needed in order to overcome sub-evaluation of microbial cell densities (Cynar et al., 1985; Jurggens et al., 1999; Vazquez-Dominguez et al., 2005; Sherr and Sherr, 2002).

Microcosms

Five microcosms were constructed in glass transparent bottles with: 200 mL of filtered seawater (0.4 µm pores, in order to eliminate bacteriovorus microorganisms and to overcome the sub-evaluation of microbial cell densities), different quantities of organic nutrients (1/10 yeast- peptone medium), and inorganic nutrients (both ammonium acetate and ammonium phosphate 0.5%), diesel oil (10%) and dispersant (Nacol C-1/10.000) were added as is shown in Figure 1.

The five microcosms were then incubated at 15°C in the dark.Periodically samples were collectedfrom eachmicrocosmto determine: metabolic activity (reduction ofresazurine) and consumptionof diesel oil.

Figure 1A. Microcosms bottles

	200 seawater filred 0,45 µm filter	Filtred diesel 1/10	Dispersant 1/1000	Ammonium acetate si ammonium phosphate 0,5 %	YP 150µl
M1	x				
M2	x	x			
M3	x	x	x		
M4	x	x	x	x	
M5	x	x	x	x	x

Figure 1B. Composition of the five types of microcosms

Metabolic activity of the samples was carried outas previously shown (Manea and Ardelean, 2013). Resazurin (10-oxide 7-hydroxy-3H-phenoxazin-3-one) is a blue non-fluorescent dye; it can be reduced by metabolically active cells to resorfine (pink and highly fluorescent), which can be further reduced to hydroresorufin (colorless and non-fluorescent). Resazurine was used primarily as an indicator of oxidation-reduction reactions in cell viability tests (Figure 2). The test is used from about 50 years for monitoring bacterial contamination of milk also for assessing the quality of the semen. Recently resazurine became very popular as a simple and versatile method for measuring cell proliferation and cytotoxicity (O'Brien et al., 2000).

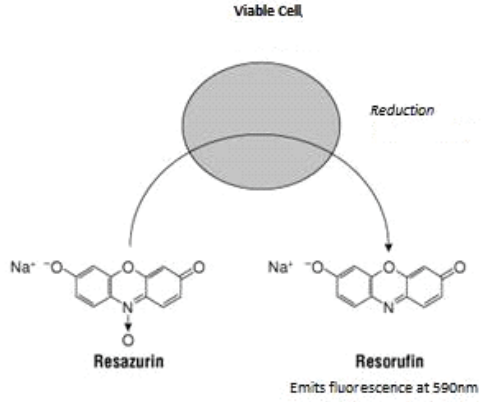

Figure 2. Resazurine reduction

Conversion of resazurin to resorufin is proportional to the number and metabolism intensity of cells present in a population. In each wheel 5ng resazurine was added and the optical densities were readed at 570 and 600 nm at appripriate intervals for a period of 450 hours. The microplate was incubated at 15 °C during the experiment. The amount of reduced resazurin was calculated according to the formula (1) (Al-Nasiry et al., 2007).

$$\%resazurin = \frac{(Eoxi600xA570) - (Eoxi570xA600)}{(Ered570xA"600) - (Ered600xA"570)} \quad (1)$$

where:

Eoxi600-= molar extinction coefficient of resazirin in the oxidized form at 600 nm=117,216
Eoxi570-= molar extinction coefficient of resazirin in the oxidized form at 570 nm=80,586;
Ered570- molar extinction coefficient of resazirin n the reduced form at 570 nm=155,677;
Ered600- molar extinction coefficient of resazirin in the oxidized form at 570 nm=14,652;
A570 -absorbance of test wells at 570 nm;
A600-absorbance of test wells at 600 nm;
A"600- absorbance of negative control.

Determination of diesel oil consumption in the microcosms was performed by extraction with toluene (Odu, 1972), as follows: 5 ml of samples taken from microcosms were mixed with 2 ml of toluene, the mixture was strongly mixed and then centrifuged 10 minutes at 8000 rpm. Then the supernatant was collected and read absorbance at 420 nm. Based on the standard curve the amount of diesel consumed in microcosms was calculated.

RESULTS AND DISCUSSIONS

In Table 1 there are presented the results concerning the rate of resazurine reduction, (expressed as ng resazurine/hour/wheel) during the experiment by native microbiota living in 5 types of microcosms. As one can see, the rate is lower in control (M1) as compared with the microcosms where inorganic nutrients have been added (M3-M5), suggesting that inorganic nutrients addition sustain an increase in metabolic activity of marine endogenous microbiota. This increase is in agreement with reports in the literature (Fuhrman and Azam, 1980; Atlas, 1981; Lewis, 2001; Cohen, 2002; Van Hamme et al., 2003; Molina-Barahona et al., 2004; Munn, 2004; Head et al., 2006; Ducklow, 2008; Gasol ,2008; Kirchman, 2008; Kemp, 2010; Shata, 2011; Enon et al., 2011; Popoviciu and Ardelean, 2011; Uzoigwe et al., 2012.; Ardelean et al., 2009; Ghita and Ardelean, 2010, 2011; 2012; Manea and Ardelean, 2013; Manea et al., 2013).
When it comes to diesel addition, the situation deserves further attention. In M2, where only diesel is supplemented to sea water, the rate of resazurine reduction, all over the experiment, is lower as compared to control (M1) with the exception of one sampling time. These results suggest that pollutant (diesel oil) addition causes a decrease in the intensity of metabolic activity of endogenous marine microbiota which is in agreement with previous reportedresults (Atlas, 1981; Venkateswaran et al., 1995; Habe and Omori, 2003; Zhang et al., 2010; Manea and Ardelean, 2013; Manea et al., 2013).

Table 1. The time evolution of metabolic activity (measured as ng/resazurine/hour/wheel) of endogenous microbiota in the filtered sea water microcosms, supplemented with dispersant (Nacol C), diesel oil, inorganic ans organic nutrients (sea materials and methods for details). The standard deviations are within 10%

Days	0	7	14	27	35	42	48
M1	0.52	0.67	0.91	0.57	0.5	0.84	0.55
M2	0.35	0.45	0.62	0.73	0.51	0.38	0.3
M3	0.47	0.46	0.76	0.91	0.51	0.4	0.27
M4	1.7	1.34	1.77	1.57	6.67	2.75	1.64
M5	6.56	3.46	3.46	2.35	6.69	7.06	4.12

Similar results have been obtained in the presence of both diesel and dispersant (M3), in agreement with our previous results concerning the absence of toxicity of this dispersant at this low concentration (Manea and Ardelean, 2013; Manea et al., 2013). Inorganic nutrient addition (both ammonium acetate and ammonium phosphate 0.5%) to M4 greatly increase the rate of resazurine reduction as compared with the rate measured in their absence (M1-M3); further organic nutrient addition to M5 (see materials and methods) sustains 2-3 times higher rate of resazurine reduction in M5 as compared with the rates measured with no organic supplements (M4). All these results are in agreement with reports in the literature (Atlas, 1981; Venkateswaran et al., 1995; Habe

and Omori, 2003; Zhang et al., 2010) suggesting that the concentrations of inorganic and organic nutrients in unpolluted marine environments are in limiting concentrations (De Long and Karl, 2005; Costello et al., 2010; Liu et al., 2010).
In the next figure there are presented the results concerning the time evolution of diesel consumption in the four type of microcosms (M2-M5).

Table 2. The time evolution of diesel consumption by endogenous microbiota in the filtered sea water microcosms, supplemented with dispersant (Nacol C), diesel oil, inorganic and organic nutrients (sea materials and methods for details). The standard deviations are within 10%

Days	7 days	14 days	35 days
M1	0	0	0
M2	0.7	0.19	2.1
M3	1.3	2.7	3.2
M4	1.5	2.5	3.4
M5	1.6	2.9	3.3

Dispersant addition (M3) 1.5 times increases diesel consumption by endogenous microbiota, as compared with M1, where only diesel has been added to filtered sea water. These results are in agreement with the use of different types of dispersants (non toxic at the working concentration) to enhance the complex interactions between microbial cells and petroleum hydrocarbons, thus sustaining an increased rate of pollutant consumption (Atlas, 1981; Lewis, 2001; Cohen, 2002; Van Hamme et al., 2003; Molina-Barahona et al., 2004; Head et al., 2006; Kempf, 2010; Shata, 2011; Uzoigwe et al., 2012; Manea and Ardelean, 2013; Manea et al., 2013). As shown in Figure 3, in the absence of diesel oil (M1), one typically can see only isolated prokaryotes, labeled with acridine orange (Ghita and Ardelean, 2010), whereas in the presence of diesel, cells starts to aggregate (M2) (Figure 3); furthermore, in the presence of dispersant (M3-M5) one can see micro-vesicles with different size, having bacteria at the surface and, the larger ones ,true micro- bioreactors, containing inside them dense populations of bacteria (Figure 3); This spatial arrangement of bacteria at the level of micro-vesicles enhances the physical contact between cells and diesel oil, and its consumption by bacteria (Lewis, 2001; Cohen, 2002;Van Hamme et al., 2003; Molina-Barahona et al., 2004; Head et al., 2006; Kempf, 2010; Shata, 2011; Uzoigwe et al., 2012).

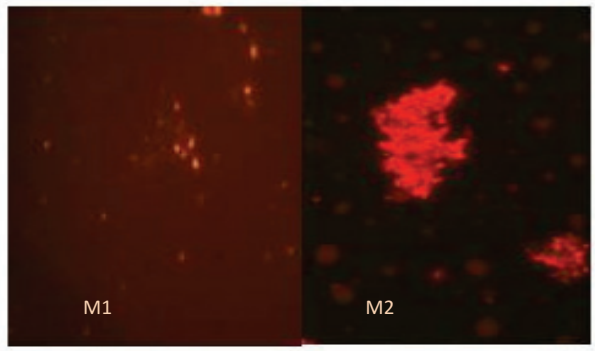

Figure 3a. Microbial cells spatial distribution in the absence of dispersant: (M1 and M2) but in the presence of diesel oil (M2)

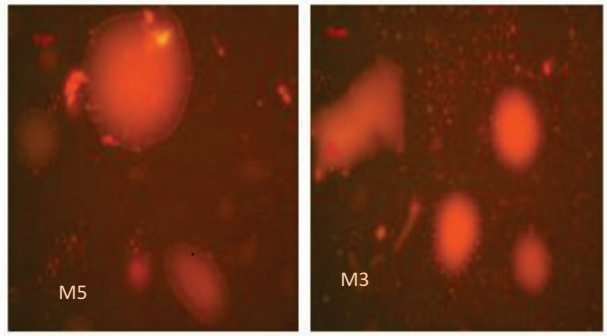

Figure 3b. Microbial cells spatial distribution in the presence of both dispersant and diesel oil (M3 and M5). Similar results in M4

As shown, diesel oil consumption is slightly (1.5 times) enhanced by dispersant addition (M3 as compared with M2) but organic and/or inorganic nutrients additions do not have an effect on diesel consumption, the values obtained being within the level of standard deviation.. However, the increase in diesel consumption (1.5 times) is far much lower as compared with the increase in resazurine reduction rates (10 times or more), suggesting that, in our experiments, inorganic and organic nutrients additions has a limited positive effect on diesel oil consumption, but a strong positive effect on general metabolic activity of the endogenous microbiota, as measured by the rate of resazurine reduction.

CONCLUSIONS

Metabolic activity(reported as ng reduced resazurine/hour/wheel), as compared with the control (M1) is decreased by diesel addition (10% final “concentration”) either in the absence (M2) or in the presence of dispersant

(Nacol C 1/10.000 dilution). Inorganic nutrients additions (both ammonium acetate and ammonium phosphate 0.5%) greatly (4 to 10 times) increase the rate of resazurine reduction in M4, as compared with the rate measured in their absence whereas further organic nutrient addition to M5 sustains a 2-3 times higher rate of resazurine reduction as compared with M4. Diesel oil consumption is slightly (1.5 times) enhanced by dispersant addition (M3 as compared with M2) but organic and/or inorganic nutrients additions do not have an effect on diesel consumption, the values obtained being within the level of standard deviation.

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