THE BIOREMEDIATION POTENTIAL OF MARINE SANDY SEDIMENT MICROBIOTA

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Abstract. The natural microbiota from marine sandy sediments on the Romanian sea coast was tested for resilience in case of hydrocarbon contamination, for estimating the number of (culturable) hydrocarbon and lipid oil-degrading microorganisms and for determining the influence of inorganic nitrate and phosphate nutrients on hydrocarbon spill bioremediation process, by microcosm experiments.
Results show that hydrocarbon contamination affects the bacteriobenthos both in terms of cell numbers and composition. Bacterial numbers showed a rapid decrease (28% in four days), followed by a relatively fast recovery (two weeks). The pollution favoured the increase of Gram-positive bacterial proportion (from around 25% to 33%)
Sandy sediment microbiota in both sites studied contained microorganisms able to use mineral or lipid oils as sole carbon sources, usually around 10^3-10^4/cm^3, with variations according to the sediment grain size and substrate used.
The biostimulation experiments showed that, in absence of water dynamism (and, implicitly, an efficient oxygenation), the addition of nitrogen and phosphorus can be ineffective and even inhibit the remediation process, probably due to eutrophication.

Keywords: bacteria, sandy sediment, bioremediation, hydrocarbons, lipid oils, nutrients

INTRODUCTION

Bioremediation is an ecological reconstruction process, by which natural microbiota is used to neutralize or lower the concentration of pollutants [26]. One of the most frequent and dangerous forms of pollution in the marine environment is the one involving petroleum hydrocarbons [6]. Similar to oil pollution is the contamination with biological (mostly vegetable) oils. Their increasing use for biofuel production and other industrial purposes makes them a more and more frequent source of pollution, especially in inland waters, but also in the marine environment [3, 4].

There are many factors influencing the bioremediation process: the physical state of the pollutant, solubility, external surface, concentration, environmental temperature, nutrients (especially nitrogen and phosphorus), oxygen availability, salinity, hydrostatic pressure etc. [3, 6, 10, 13, 20, 28, 44].

Knowledge of these factors and their influence is useful in designing biostimulation strategies (i.e. supplying the right amount of oxygen and nutrients, or even chemical surfactants in order to enhance the process [3, 6, 13, 20, 26, 44]).

Littoral marine sediments are one of the environments most affected by hydrocarbon contamination. Its effects vary between different grain-sized sediments. Larger grained sands have a higher self-cleaning potential, but also a higher permeability, allowing sometimes heavier hydrocarbons to settle in deep, compact layers. Finer sediments are more affected because they are usually found in areas with low water dynamism, causing petroleum products to accumulate, sometimes in compact surface layers [24].

The current paper has three main goals: evaluating the resilience of sandy sediment microbiota in the case of hydrocarbon spills, quantifying culturable microorganisms able to degrade several hydrocarbon and lipidic substrates and determining the influence of nitrate and phosphate nutrients on hydrocarbon spill bioremediation in coastal sandy sediments.

MATERIALS AND METHODS

Effect of hydrocarbon contamination on the sediment microbiota. In order to assess the impact of hydrocarbon contamination on the sandy sediment microbiota, microcosm experiments were carried. Sediment taken from several beaches in Constanța was mixed and distributed in two 1 L transparent plastic vials (around 500 cm^3 in each) and covered with a thin layer (around 100 cm^3) of filter-sterilized (0.2 μm) seawater. One microcosm served as control (labeled „M"), the other one („C") was contaminated with diesel oil (20 g/L), mixed directly into the sediment. Microcosms were kept at room temperature and natural light for 14 days.

5 cm^3 sediment cores were taken, using improvised piston corers, every two days, beginning just before the spill, from microcosm C and in days 1, 8 and 14, for the control microcosm. The cores were fixed with formalin (4% final) and stored at +4°C [18, 33, 34].

Bacteria were dislodged from sand grains using chemical (Tween 80) and mechanical procedures and observed by epifluorescence microscopy using SYBR Green I (1:10,000) and hexidium iodide (HI; 10 mg/L) according to a procedure described elsewhere [17, 29, 33, 34]. Microorganisms were counted and classified according to their Gram character (HI-positive, i.e. orange-red fluorescing, being Gram-positive, and those with green fluorescence being counted as Gram-negative) and morphology: cocci, rods, filamentous cells [42].

Estimation of potential mineral and natural oil degrader density. The second experiment consisted in quantifying culturable microorganisms from coastal sands able to use hydrocarbon and vegetable oils as the sole carbon source. Samples were taken from two beaches in Constanța (September, 2011), one with large-grained sands and one with fine sands (labeled as sites A and, respectively, B). Microorgansims were tested for seven substrates: four hydrocarbon mixtures – petroleum ether (mostly C_5-C_6 hydrocarbons), gasoline (C_4-C_{12}), diesel oil (C_{10}-C_{15}) and paraffin wax

$(C_{20}-C_{40})$ – and three vegetable oils – sunflower, olive and linseed oil.

Microorganisms were cultivated on Bushnell-Haas medium (liquid, for liquid substrates, and agarized, for petroleum ether and paraffin wax; [23, 27, 35, 49]), supplemented with the substrate (0.5% hydrocarbons; [1, 22, 43, 47], respectively, 1% lipidic oils; [4]) and triphenyl tetrazolium chloride (TTC, 0.01%) as an indicator of microorganism growth [27]. Serial dilutions were inoculated on mictotiter plates, respectively Petri dishes containing the Bushnell-Haas medium, incubated for two weeks at room temperature and natural light. TTC-positive (red-coloured) wells (microcolonies for solid medium) were counted and microbial density was estimated by a Most Probable Number (MPN) method [35, 49], using MPN Calculator free software.

Nutrient influence on bioremediation. The third experiment concerned the influence of inorganic nutrients on bioremediation. Six microcosms similar to the first ones were set up. Each of them was contaminated with diesel oil (20 g/L) and supplemented with ammonium nitrate and monopotassium phosphate as follows: microcosm 1 – control; microcosm 2 – 5 mg/L NH_4NO_3, 0.5 mg/L KH_2PO_4 [21, 39]; microcosm 3 – 25 mg/L NH_4NO_3, 2.5 mg/L KH_2PO_4; microcosm 4 – 50 mg/L NH_4NO_3, 5 mg/L KH_2PO_4; microcosm 5 – 100 mg/L NH_4NO_3, 10 mg/L KH_2PO_4; microcosm 6 – 200 mg/L NH_4NO_3, 20 mg/L KH_2PO_4. Microcosms were kept at room temperature and natural sunlight.

3 cm^3 cores were taken weekly, and total (extractable) petroleum hydrocarbons (TPH) were determined by the gravimetric method of Nwaogu et al.

[30], but using chloroform as a solvent [15, 25]. Cores were dried at at 50°C, for water evaporation, hydrocarbons were extracted with chloroform and the solvent was evaporated at 50°C in preweighed recipients, and weighed using a three-decimal semi-analytical balance.

After 35 days since the beginning of the experiment, the density of hydrocarbon-degrading microorganisms in each microcosm was estimated using the method above (culturing was done on liquid Bushnell-Haas medium, with 0.5% diesel oil). Bacteria isolated were also investigated by epifluorescence microscopy, with SYBR Green and HI.

RESULTS

Effect of hydrocarbon contamination on the sediment microbiota. The evolution of microbial cell density in both microcosms is shown in Fig. 1, and that of the distribution of bacterial morpho-structural groups in Fig. 2.

In the control microcosm, the density and structure of benthic microbiota remained the same throughout the experiment, diesel oil addition in the other microcosm had significant effects. A major drop in bacterial numbers was observed during the first four days (a 28% decrease). The microbiobenthos recovered, after two weeks, bacterial numbers being close to the initial ones.

The structure of the microbiota also suffered modifications after the contamination: there was a significant increase of Gram-positive rod-shaped bacteria (from around 25% to 33%).
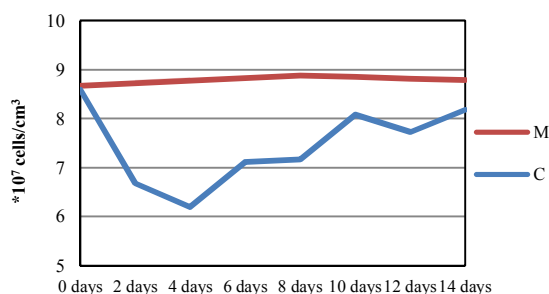

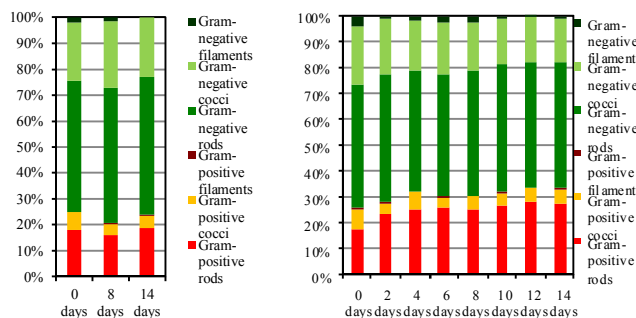**Figure 1.** Evolution of microbial density in the two microcosms (M = control, C = diesel oil-contaminated)

Figure 2. Evolution of percentual proportion of different bacterial morpho-structural groups (control, respectively contaminated sediment)

Estimation of potential mineral and natural oil degrader density. After two weeks of incubation, and counting the positive TTC reactions, the microbial densities estimated for each substrate were those in Table 1.

Nutrient influence on bioremediation. Fig. 3 shows the evolution of TPH (expressed as parts-per-million – ppm) in each of the six microcosms.

Table 1. Most probable number of microorganisms ($\times 10^3$) degrading hydrocarbon and vegetable oils per cm^3 of sediment for each site studied

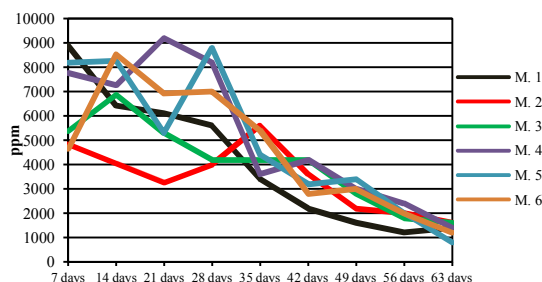| Substrate	Site A	Site C
Petroleum ether (0,5%)	0	0.6
Gasoline (0,5%)	6.5	3.3
Diesel oil (0,5%)	1.2	2.4
Paraffin wax (0,5%)	0.6	4
Sunflower oil (1%)	1.8	16
Olive oil (1%)	8.1	23
Linseed oil (1%)	1.7	4.5

Figure 3. Evolution of TPH in microcosm sediments

In all microcosms, the initial amount of diesel oil added was 20,000 ppm. Preliminary tests showed that, using this gravimetric method, volatile fractions representing up to 34% of the oil's mass are lost by evaporation.

As for the density of culturable hydrocarbon degraders in each microcosm (after 35 days from contamination), it is shown in Table 2.

Table 2. MPN of hydrocarbon degraders ($\times 10^3$) per cm^3 in microcosm sediments (35 days from spill)

Microcosm 1	1
Microcosm 2	0.9
Microcosm 3	2.6
Microcosm 4	2.1
Microcosm 5	8.8
Microcosm 6	11

DISCUSSIONS

Various researchers, studying the short-term impact of hydrocarbon pollution on the natural microbiota in various environments (marine bacterioplankton, beach sediments, soils), noticed massive drops in cell density during the first days, due to the toxic effect of the pollutant, followed by a recovery to normal densities,

caused by the selection of hydrocarbon-tolerant bacteria, usually after 2-3 week-periods [2, 11, 12]. Contamination with petroleum products also affects the taxonomic composition of the microbiota [37] and its metabolic activity [12].

The MPN estimation showed that up to 0.01% of the total benthic microorganisms (compared to microbial total counts in those sediments; [33, 34]) were able to use hydrocarbons as sole carbon source, and 0.002-0.025% could do this with vegetable oils (only culturable microorganisms). Site B, with finer sediments, has significantly higher numbers of degraders (except for gasoline). In site A, microorganisms that oxidize light hydrocarbons (gasoline, C_4-C_{12}) are dominant, while in site B, most are able to consume heavier hydrocarbons (C_{10}-C_{40}).

These densities are similar to those determined by other researchers in uncontaminated marine littoral sediments in various parts of the world, with variations due to the sediment type, location and substrate used for culturing: 10^1-10^5 MPN/CFU/cm^3 [8, 9, 11, 22, 35, 36, 38, 40, 45, 47].

Regarding vegetable oils, olive oil had most potential degraders, while linseed oil had the least, although some researches showed it would be easier degradable than, for example, sunflower oil [4].

As for the influence of nutrients, results show that a moderate addition of nutrients (5-25 mg/L NH_4NO_3, respectively 0.5-2.5 mg/L KH_2PO_4 – microcosms 2 and 3) causes a fast and effective biodegradation in the water column. Overall, the fastest decrease in TPH was observed in the control microcosm.

It is well established that nitrogen and phosphorus are often limiting factors of hydrocarbon degradation, especially in a confined environment, such as sediments [6]. Biostimulation experiments in various environments showed the positive role that the addition of inorganic nitrogen and phosphorus can have in accelerating natural bioremediation of oil spills [1, 3, 6, 7, 10, 14, 19, 20, 23, 28, 31, 32, 39, 40].

There are, however, situations when this type of biostimulation has little or no efficiency [3, 21, 28, 39, 41].

In the current experiment, the addition of nitrogen and phosphorus led to a significant numerical increase of microorganisms capable of using diesel oil hydrocarbons as sole carbon source, but inhibited the biodegradation process. Microcosms where nitrate and phosphate nutrients were added had a lower, or almost equal rate of TPH decrease with the control microcosm. Such negative effects were also encountered by several researchers [16, 41, 48]. The main cause of bioremediation inhibition in aquatic environments is eutrophication [26, 39], which leads to an excessive growth of primary producers. In absence of any significant water dynamism, this may cause anoxia and, furthermore, potential hydrocarbon degraders can shift to a more easily assimilable source of food.

So, at least in sediments less subjected to hydrodynamism, using phosphates and nitrates as

biostimulants can decrease the biodegradation rate. In such cases, oxygenation, by bioventilation or other means, should be the main priority [41].

Epifluorescence microscopic analysis showed that, among culturable diesel oil degraders, Gram-positive rod-shaped bacteria were dominant (45.8%), followed closely by Gram-negative rods (42%). Of the most frequent aquatic hydrocarbon-consuming bacteria, species of the genera *Nocardia*, *Arthrobacter*, *Brevibacterium* and *Bacillus* would fit in the first category, while *Alcanivorax*, *Pseudomonas*, some *Enterobacteria*, *Achromobacter*, *Alcaligenes*, *Flavobacterium* and some *Cyanobacteria* would fall in the latter [6, 19, 28, 46].

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