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PH.D. THESIS

QUANTITATIVE AND QUALITATIVE ASPECTS OF SANDY SEDIMENT MICROBIOTA ON THE ROMANIAN BLACK SEA LITTORAL: THEORETICAL AND APPLICATIVE SIGNIFICANCE

SUMMARY

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INTRODUCTION

Prokaryotes are distributed in all living environments. Obviously, marine sediments are not an exception. Bacteriobenthos is much richer, regarding microbial density, than plankton.

Bacteria on sediment surfaces and in sediment layers play very important roles in marine ecosystems. They perticipate in biogeochemical cycles, ensuring the recycling of different bioelements By decomposing organic compounds and releasing nutrients, they increase the productivity of oceanic basins. Furthermore, bacteria take part in physico-chemical transformation essential to the genesis of different types of marine sediments.

Unfortunetely, even though numerous studies on bacteriobenthos were published, few of them concern arenicolous bacteria and even fewer tried to find correlations between the density, biomass and structures of microbial communities, on one hand, and various environmental factors (sediment grain size, temperature etc.), on the other hand.

Keywords: bacteria, sandy sediments, density, biomass, morpho-structural diversity, seasonal variations, physiological groups, bioremediation.

PART I. CURRENT STATE OF KNOWLEDGE

Chapter 1. Marine sandy sediments – generalities

Marine sediments can be classified in two categories: shallow sea sediments and deep sea sediments. Shallow sea sediments have several subcategories, important for the current paper being littoral sediments. These consist mostly of different grain sized-sands, descending up to 15-40 below sea level (Bondar et al., 1973, p. 103).

Sands can be defined as mobile sediments with distinct grains and few colloidal particles. They are classified mostly according to their granulometry: very fine sands (with a grain diameter of 0.016-0.12 mm), fine (0.1-0.25 mm), medium (up to 0.5 mm) and coarse sands (0.5-2 mm). Above 2 mm sediments are considered gravel (Gomoiu, 1969).

Sandy sediments are not uniform on all the Romanian coast, grain size progressively increasing from fine, alluvial sands in the north, to medium and coarse sands, resulting from the disintegration of mollusk shells and calcareous rocks, to the south of Constanţa. There is also a vertical variation of grains size, decreasing in deeper waters. It also varies in time, being influenced, for example, by the mollusk species present in that region.

Chemical composition also varies. Sands in the north are mostly made of silica, while in the south, calcium carbonate predominates.

The amount of organic matter in the sediment may vary between 0.4% and 14.2%, being more abundant around the waterline (Gomoiu, 1969).

The dissolved oxygen amount in the interstitial water and light penetrating depth are two important factors for microorganisms and they depend on grain size, water depth and, for oxygen, water temperature (Gomoiu, 1969).

Chapter 2. Marine bacteriobenthos

2.1. Quantitative aspects regarding bacteriobenthos

The distribution of benthic bacteria is determined by several factors. Sediment depth influences the access to nutrients and oxygen. The maximum densities are found at the water-sediment interface (Pora & Oros, 1974).

Geographical location is also important. In near-shore areas, bacterial densities increase due to a higher amount of organic compounds resulting from plant and animal waste deposition. Other favouring factors are high oxygenation and higher temperatures. Unfavourable factors are temperature and salinity variations and water dynamism (Novitsky

& MacSween, 1989, Pusceddu et al., 2005, Mudryk & Podgórska, 2006b, Podgórska et al., 2008)

The type of sediment also influences bacterial abundance. Mud, clayey mud, detritic sediments are very rich in nutrients and also microorganisms, unlike sands.

By direct counting methods, littoral benthic microbial densities determined by various authors in different parts of the world fall within the range of around 10^6-10^9 cells per cm^3 (Montagna, 1982, Novitsky & MacSween, 1989, Danovaro et al., 1994, Epstein et al., 1997, Hymel & Plante, 1998, Kuwae & Hosokawa, 1999, Dietrich & Arndt, 2000, Proctor & Souza, 2001, Luna et al., 2002, Lunau et al., 2005, Pusceddu et al., 2005, Šestanović et al., 2005, Ferrara-Guerrero et al., 2007, Røberg et al., 2007, Podgórska et al., 2008, Šestanović et al., 2009), with circadian and seasonal variations (Šestanović et al., 2005, Ferrara-Guerrero et al., 2007, Šestanović et al., 2009).

It should be noted that of the bacterial cells counted by direct count methods, rarely more than 60% are alive (Luna et al., 2002), and those being culturable may constitute even less than 1‰ (Khiyama & Makemson, 1973).

Bacteria colonize only a small part of the sediment grains' surface (below 1%), preferring locations protected from abrasion, water movement and consumers (fissures, cracks). Detritus deposits obviously favour microbial colonization (Weise & Rheinheimer, 1977, DeFlaun & Mayer, 1983, Novitsky & MacSween, 1989, Mudryk & Podgórska, 2006b).

2.2. Prokaryote physiological groups and their metabolic activity in marine sediments

Regarding the ecology of sediment microbiota, it should be noted that the parameters of various environmental factors (light, oxygen, organic and inorganic substrates) show strong vertical variations, with large differences over milimeter-sized distances. Oxygen gradients are the most important. Thus, sediments have an upper, aerobic zone, an oxic-anoxic transition zone and an anoxic zone. Their thickness varies according to sediment granulometry, the organic matter amount, the presence or absence of cyanobacteria or microalgae, or to surface microtopography (which can be modified by water dynamism or animals; Brune et al., 2000, Kogure & Wada, 2005).

The highest bacterial densities are found at the water-sediment interface, due to the accumulation of large organic matter amounts and to high oxygenation. This is where most degradative activities take place. Complex polysaccharides (cellulose, lignin, chitin),

oligosaccharides, lipids and, especially, aminoacids are trophic resources for the heterotrophic microbiota (Pora & Oros, 1974, Mudryk et al., 2005, Mudryk & Podgórska, 2006a). Most benthic bacteria show a limited trophic versatility, being specialized on a small number of organic substrates (Khiyama & Makemson, 1973).

The anaerobic zone of the sediments can be further subdivided in the upper layers, with positive redox potential, and the lower ones, with negative redox potential. In the upper layers, nitrates, the manganic (Mn^{4+}) and ferric (Fe^{3+}) ions are the main final electron acceptors used by anaerobic bacteria in their respiratory processes. Their reduced compounds reach then the oxic-anoxic interface, where other bacteria reoxidize them.

In the lower, reduced, layers sulfates and carbon dioxide are the main final electron acceptors used in anaerobic respiration. Hydrogen sulfide and methane produced by sulfate-reducing and methanogenic bacteria are oxidized in the layers with positive redox potential (Hansen & Blackburn, 1991, Brune et al., 2000).

Sediment microbiota is of great ecological importance. First of all, most of these bacteria take part in the decomposition of organic materials resulted from plant and animal waste. They release nutrients that form the basis of benthic and pelagic trophic chains, thus playing an important role in the productivity oceanic and marine basins (Pora & Oros, 1974).

Benthic microorganisms produce chemical alterations that are essential to the formation and evolution of different types of marine sediments, especially by calcium carbonate precipitation and humus formation. Some of them are involved in the genesis of hydrocarbon and ferromanganese deposits

Overall, it can be said that benthic microbiota maintains the dynamic chemical balance of marine sediments and neighbouring water masses (Pora & Oros, 1974).

2.3. Morpho-structural and taxonomical features of littoral sandy sediment microbiota

From a morphological point of view, littoral sediments contain various types of prokaryotic cells: cocci, coccobacilli, rods, vibrios, filamentous cells, disk-shaped cells etc. There are both solitary and colonial forms.

Various factors influence the distribution of bacterial morphotypes: hydrodynamism, seasonal variations, sediment grain size, amount of nutrients, selective grazing by consumers (Novitsky & MacSween, 1989, Šestanović et al., 2005, Šestanović et al., 2009). Colonial (aggregate or filamentous) forms are usually rare, found mostly among cyanobacteria and

some sulfate-reducers. In sediments with low dynamism, biofilms can occur (DeFlaun & Mayer, 1983, Novitsky & MacSween, 1989, Mudryk & Podgórska, 2006b).

Concerning dimensions, small size prokaryotes, with an average biovolume around 0.1 μm^3 are dominant in littoral sands (Kuwae & Hosokawa, 1999, Mudryk & Podgórska, 2006b, Podgórska et al., 2008).

The large majority of benthic bacteria live attached to sediment grains, only few of them living in the interstitial water (Khiyama & Makemson, 1973, Mudryk & Podgórska, 2006b). That is why they have specific structures for substrate adhesion – glycocalyx, polysaccharidic filaments or other, still little known, mechanisms (Weise & Rheinheimer, 1977, Moriarty & Hayward, 1982, Novitsky & MacSween, 1989, Mudryk & Podgórska, 2006b).

Regarding the repartition of marine bacteria according to the structure of their cell walls, it is well established that Gram-negative bacteria are dominant (70-90%), with Gram-positives as a minority (Khiyama & Makemson, 1973, Moriarty & Hayward, 1982).

Genetical methods of investigation allowed the determination of taxonomic diversity in marine sediments and even the discovery of previously unknown taxa.

Concerninc the distribution of different taxonomical groups in coastal sediments, researchers obtained various results, generally proteobacteria (Alpha-, Beta-, Gamma-, Deltaproteobacteria), and the *Cytophaga-Flavobacterium-Bacteroides* group being dominant, followed by Planctomycetes and Gram-positives (Llobet-Brossa et al., 1998, Gillan et al., 2005, Muşat et al., 2006, Tănase, 2009, Kunihiro et al., 2012). Archaea are much less numerous (Tănase, 2009).

Chapter 3. Bioremediation potential of marine sediments in case of mineral or vegetable oil pollution

Bioremediation is an ecological reconstruction process by which natural microbiota is used to lower the concentration, in a certain environment, or the toxicity of pollutants, by biodegradation (Korda et al., 1997). Although it has certain limitations, bioremediation is an extremely attractive method of ecological reconstruction, due to its low costs (Doboș & Puia, 2010).

One of the most frequent and dangerous forms of pollution in marine ecosystems is petroleum oil spills. Petroleum and its derived products are complex mixtures containing different types of hydrocarbons: alkans, cycloalkans, alkenes, aromatics and polyaromatics, asphaltenes, sulfurated hydrocarbons. Some microorganisms possess metabolic pathways to

oxidize such compounds (usually aerobic oxidation), converting them mostly to fatty acids Generally, each of the hydrocarbon-oxidizing species or strainis strictly specialized on a narrow range of hydrocarbons (Atlas, 1981, Korda et al., 1997, Harayama et al., 1999, Okoh, 2006, Das & Chandran, 2011).

Various environmental factors can affect the way bioremediation occurs: the physical state of the pollutant, its solubility, the surface available for pollutant-microorganisms interactions, concentration, previous spills (which select hydrocarbon-degrading microbes), temperature, nutrients (nitrogen, phosphorus), oxygen, salinity and hydrostatic pressure (Atlas, 1981, Leahy & Colwell, 1990, Van Hamme et al., 2003, Okoh, 2006, Doboș & Puia, 2010, Hazen, 2010, Das & Chandran, 2011).

Knowing the principles of the process and its favouring and inhibiting factors, natural bioremediation can be artificially enhanced through biostimulation (nutrient addition in the environment) or bioaugumentation (addition of degrading organisms). It was shown that biostimulation can have good results in various environments. Stimulation techniques include supplementing nitrogen and phosphorus sources, artificial oxygenation (if required) and surfactant addition (Atlas, 1981, Korda et al., 1997, Van Hamme et al., 2003, Doboș & Puia, 2010, Hazen, 2010).

Marine coastal sediments are one of the enviroments most oftenly affected by hydrocarbon contamination, its effects varying according to the sediment type (IPIECA, 2000).

Similar to this kind of pollution, regarding the effects, are the contaminations with lipid mixtures of biological origin (mostly vegetable), more and more frequent due to the increasing use of such oils in biofuel production and other industrial activities. Vegetable oils (especially sunflower, canola, linseed and palm oils) are one of the most frequent source of pollution in many countries (Al-Darbi et al., 2005, Aluyor et al., 2009).

Vegetable oils are very different in regard to their composition, especially the proportion of saturated and unsaturated fatty acids and the length of their chains. Composition, together with various environmental factors, determine their biodegradation rate (Aluyor et al., 2009).

PART II. PERSONAL CONTRIBUTIONS TO THE QUANTITATIVE AND QUALITATIVE STUDY OF LITTORAL BENTHIC MICROBIOTA

The purpose and objectives of the thesis

The purpose of the current paper is to study, in quantitative and qualitative terms, the microbiota in sandy sediments on the Romanian Black Sea littoral, with the following objectives:

1. Determining the characteristics (abundance, biomass, morpho-structural diversity) of benthic microbiota in sandy sediments taken from different locations along the Romanian coast.
2. Determining the relation between sediment grain size and the characteristics of its microbiota.
3. Studying the seasonal variations of benthic bacterial abundance, biomass and morpho-structural diversity, in relation to several environmental factors (chlorophyll concentration – expressing primary producers' density, water temperature).
4. Determining the estimative numbers of several microbial functional groups taking part in some important biogeochemical cycles in the sediment.
5. Studying the impact of hydrocarbon pollution on the quantitative characteristics and morpho-structural diversity of benthic microbiota, by microcosm experiments.
6. Assessing the bioremediation potential of benthic microbiota in case of hydrocarbon or vegetable oil contamination, by destimating the density of potential degraders.
7. Determining the influence of nitrate and phosphate nutrients addition on bioremediation efficiency, by laboratory microcosm experiments.

Chapter 4. Material and methods

4.1. Sample collection and fixation

4.1.1. Determination of microbial characteristics in different grain-sized sandy sediments from the Romanian Black Sea coast

Samples, consisting of sediment cores, were collected from eight sites along the Romanian coast, on September 24, 2010, at around 0.5 m depth. Site I is located in Vama Veche, site II in Mangalia, site III in Neptun, site IV in Costineşti, site V in Eforie-Nord, site VI in Constanţa, site VII in Mamaia and site VIII on the Vadu beach (fig. 1). Sediment samples were also taken for granulometry studies.

Samples were collected using improvised piston corers, the top 5 cm^3 (corresponding to a depth of 17.5 mm) of each core being taken to analysis, suspended in buffered formalin (4% final concentration) and stored by refrigeration at +4°C (Fry, 1990, Epstein et al., 1997, Luna et al., 2002).

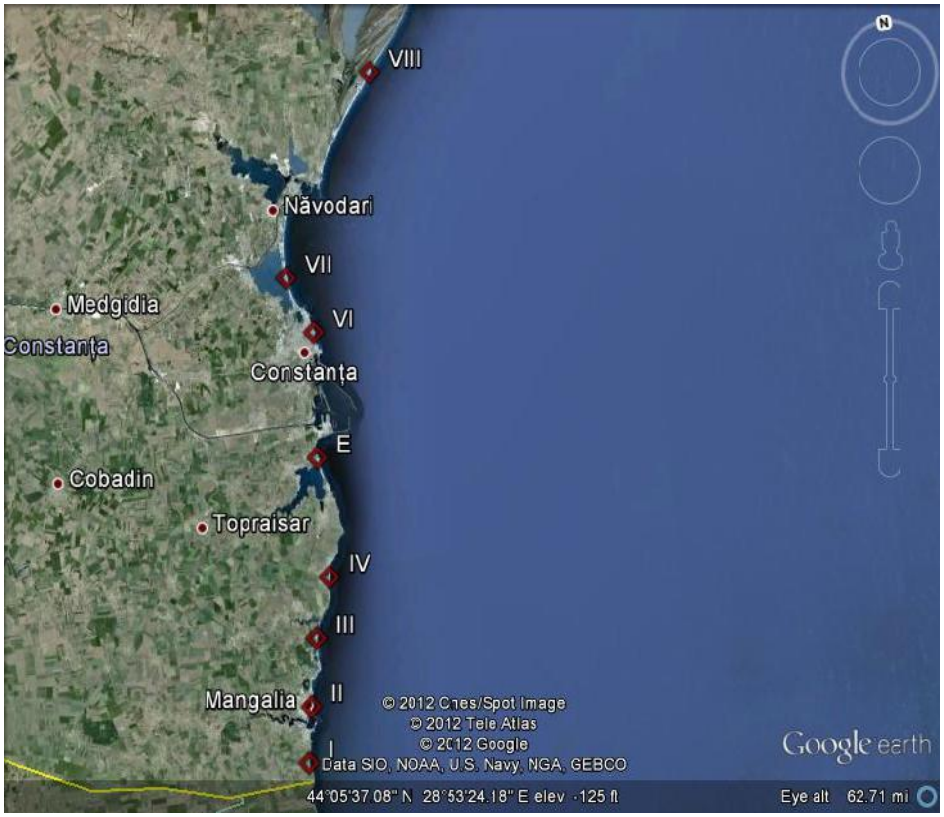


Fig.1. Location of sample collection sites (September 2010; Google Earth image - http://www.google.com/earth/index.html).

4.1.2. Evolution of benthic microbiota in different grain-sized marine sediment microcosms

Marine sand for the microcosms was collected from several beaches in Constanţa, washed with Tween 80 (1 mg/L final), sterilized at la 105°C, sorted according to the grain size, using an Endecotts Minor granulometry device and distributed in four 1 L plastic recipients as follows: microcosm A – 0-200 µm diameter grains, microcosm B – 200-400 µm, C – 400-800 µm and D – 800-1 000 µm. Seawater was added to a total volume around 700 cm^3, and the microcosms were covered with transparent foil and kept at room temperature and natural illumination.

Samples were collected every two weeks (for ten weeks), collection, fixation and storage being effectuated according to the previous section.

4.1.3. Determination of seasonal variations in littoral benthic microbiota characteristics

Fig. 2. Location of seasonal sample collection sites (Google Earth image).

Sediment cores were collected monthly from three beaches (mediolittoral zone) in Constanţa (labeled as Sites A, B and C; fig. 2), starting in April 2011. Sites A and B both have coarse sands but different exposures and site C is located in a protected bay, with gentle slope and fine sediment. Collection, fixation and storage were done according to the previous sections.

4.1.4. Determination of seasonal variations in sediment chlorophylls' concentration

Monthly, besides the samples for microbiological analysis, separate cores were collected from sites A and C (see section 4.1.3) for determining photosynthetic pigment concentrations

(starting in August 2011). The top 3 cm^3 of each core were taken and stored at -20°C prior to analysis (Roelfsema, 1999).

4.1.5. Density estimation of several microbial physiological groups in littoral sediments

For the experiments involving culturing and density estimation of several physiological groups, including hydrocarbon and lipid degraders (see sections 4.6 and 4.7.2), 5 cm^3 unfixed sediment cores, taken as shown in section 4.1.1 were used as inocula. The cores were taken from sites A and C mentioned in section 4.1.3.

4.2. Sediment granulometric analysis

Sediment samples were dried and analyzed using an granulometry Endecotts Minor device, with 1,000, 800, 400, 200, 180, 125, 90 and 53 µm sieves. The mass of each fraction and its percentual proportion were determined.

4.3. Microscopic analysis

4.3.1. Cell dislodgement from sediment grains

For dislodging substrate-attached microorganisms, prior to microscopic analysis, sediment suspensions were diluted, incubated with Tween 80 (1 mg/L final) for 15 minutes and vortexed at 2,400 r.p.m. for 5 minutes (protocol adapted from Epstein & Rossel, 1995, Kuwae & Hosokawa, 1999, Bennett et al., 2006).

4.3.2. Direct microbial count

Microorganisms were observed by epifluorescence microscopy, using SYBR Green I, a strongly green-fluorescing compound when bound to nucleic acids, staining both living and dead cells (Noble & Fuhrman 1998).

The protocol employed was adapted from Noble & Fuhrman (1998). Sediment suspensions were diluted to an optimal cell density, incubated for 15 minutes with SYBR Green I (1:10,000 final), vacuum-filtered trough 0.2 µm Millipore filtering membranes and examined by microscopy (1,000× enlargement), employing an eyepiece grid micrometer.

Fluorescing cells were manually counted, excluding anorganic fluorescing particles and obviously eukaryotic structures (by size and shape) and the mean cell density was calculated for each sample.

4.3.3. Microbial biomass quantification

The size of all microorganisms observed was measured using the micrometer and each cell's biovolume was calculated, a cell shapes being approximated to that of a straight-sided cylinder, with hemispherical ends (Fry, 1990).

For converting biovolume values to dry biomass, the conversion formula of Loferer-Krößbacher et al. (1998) was used.

4.3.4. Discrimination between Gram-positive and Gram-negative bacteria by epifluorescence microscopy

Hexidium iodide is an orange-red-fluorescing compound, that cannot pass through the cell walls of Gram-negative bacteria. Simultaneously using SYBR Green I and hexidium iodide (10 mg/L, 15 minutes incubation), bacterial cells appear differently stained (Mason et al., 1998, Forster et al., 2002).

Cells were classified according to morpho-structural criteria, considering both the Gram character and shape (cocci – spherical cells, rods and filamentous forms – those with a length at least five times larger than the width; Šestanović et al., 2005).

4.3.5. Discrimination of bacteria according to their membrane integrity

Propidium iodide is a strongly orange-red-fluorescing stain, that can only enter cells with compromised membrane integrity (dead cells). It can be used together with SYBR Green, to distinguish cells with intact membrane (most probably, viable cells) from dead ones (20 mg/L, 15 minute incubation; Grégori et al., 2001, Falcioni et al., 2006).

4.4. Sediment chlorophylls quantification

The protocol employed was taken from Roelfsema (1999). Pigment extraction was done using 90% acetone solution, and the mixture was centrifuged at 3,000 r.p.m. for 25 minutes.

The supernatant was analysed through spectrophotometry and the concentrations of the main chlorophyll pigments (A, B, C) was determined using the trichromatic equations of Jeffrey & Humphrey (1975, cit. by Namsaraev, 2009).

4.5. Correlations between various parameters

In order to determine the existence of correlations between various characteristics of analyzed microbiota and between them and environmental factors, **Pearson's correlation**

coefficient was calculated (Dale, 1974, Paulson, 2008, Šestanović et al., 2009). Values close to 1 or -1 indicate a significant, positive, respectively, negative, correlation. Values close to 0 indicate a weak correlation between the two parameters.

To investigate correlation between a microbial feature (density, biomass) and a set of independent variables, multiple regression was used (Dale, 1974, Paulson, 2008, Šestanović et al., 2009).

4.6. Density estimation of several microbial physiological groups in littoral sediments

In order to determine the density and distribution of some of the main microbial physiological groups, sediment cores collected from two beaches in Constanța, one with coarse and one with fine sand (sites A and C from section 4.1.3; collection was done in April-May 2012) were used as inocula.

Nitrogen-fixing, ammonifying, nitrifying and denitrifying microorganisms were taken into consideration with regard to the nitrogen cycle, and also sulfate-reducers.

For aerobic diazotrophs, a liquid nitrogen-free culture medium, with sucrose (Hanson & Gundersen, 1976, Zuberer & Silver, 1978, Tejera et al., 2005, Torpee, 2009) and triphenyltetrazolium chloride (TTC; 0.01%), as an indicator of microbial metabolic activity (Kutty, 2009). Incubation was aerobic, at room temperature and natural light.

Ammonifiers were cultured on peptone broth (aerobically), using Nessler's reagent as an indicator of ammonium presence (Gołaś et al., 2008).

For nitrifiers (more precisely, nitrosifyers – ammonia-oxidizing bacteria) a medium containing ammonium sulfate was used (aerobic incubation), negative reactions with Nessler's reagent being taken into consideration (Burton & Prosser, 2001, Gołaś et al., 2008).

For denitrifiers, a medium with beef extract, peptone and KNO_3 was used (anerobic incubation; Britton & Greeson, 1987). Bromothymol blue was used as an indicator (10 g/L; Saitoh et al., 2003)..

Sulfate reducers were cultured on a medium containing sodium thioglycolate (anaerobic incubation), observing sulfide precipitation (Hines & Buck, 1982, Chandrika et al., 1990).

In all cases, estimation of these microorganisms' numbers was done through a Most Probable Number (MPN) method, using the free software MPN Calculator (http://www.i2workout.com/mcuriale/mpn/ index.html).

4.7. Assessment of the bioremediation potential of littoral benthic microbiota

4.7.1. The effect of hydrocarbon pollution on littoral benthic microbiota

In order to evaluate the impact of hydrocarbon pollution on marine sandy sediment microbiota, experiments were carried on microcosms similar to those in section 4.1.2. (around 500 cm^3 sediment in each, covered by a thin sterile seawater layer). The first one served as control, while diesel oil (20 g/L) was added in the other one and mixed into the sediment. Microcosms were kept at room temperaturea and natural light.

Sediment cores were collected and fixed as shown in section 4.1.1., as follows: from the control microcosm – in days 1, 8 and 14, and from the contaminated microcosm – immediately before contamination and every two days, for two weeks.

Microorganisms were observed by epifluorescence microscopy, using SYBR Green and hexidium iodide, counted and classified into morpho-structural classes.

4.7.2. Density estimation of hydrocarbon and vegetable oil-degrading microorganisms

In this study, benthic microorganisms able to use as sole carbon source hydrocarbons from the following mixtures were counted: petroleum ether, (containing mostly compounds with five or six carbon atoms), gasoline (C_4-C_{12} hydrocarbons), diesel oil (C_{10}-C_{15}) and paraffin wax (C_{20}-C_{40}). Regarding vegetable oils, sunflower, olive and linseed oils were used.

Density estimation was done through a MPN method, using Bushnell-Haas culture medium (Horowitz & Atlas, 1977, Wrenn & Venosa, 1996, Ramsay et al., 2000, Kutty, 2009), supplemented with TTC (Kutty, 2009) and the hydrocarbon mixtures (0.5%, Walker & Colwell, 1976, Horowitz & Atlas, 1977, Adoki, 2007) , respectively, vegetable oils (1%, Al-Darbi et al., 2005). For volatile (petroleum ether) or solid hydrocarbons (paraffin wax), the Bushnell-Haas medium was used in its solid form (with 2% agarose).

Sediment cores taken, in September 2011, from sites A and C (see section 4.1.3) served as inocula.

4.7.3. Nutrient influence on hydrocarbon-polluted sediment bioremediation – microcosm experiments

An important factor favouring natural remediation of hydrocarbon-polluted ecosystems is the amount of nutrients. Thus, nitrate and phosphate addition is a frequently used stimulation method.

In this study, microcosms similar to thse in section 4.7.1 were assembled (500 cm^3 sand, covered with a 100 cm^3 layer of sterile seawater). In each of them, diesel oil was mixed into the sediment (20 g/L). Microcosms were kept at room temperature and natural lught.

Microcosm number 1 served as control, in all others, different amounts of inorganic nutrients were added (with a constant nitrate : phosphate ratio; Heitkamp & Cerniglia, 1989, Röling et al., 2002), as follows: microcosm 2 – 5 mg/L NH_4NO_3 and 0.5 mg/L KH_2PO_4; microcosm 3 – 25 mg/L NH_4NO_3 and 2.5 mg/L KH_2PO_4; microcosm 4: 50 mg/L NH_4NO_3 and 5 mg/L KH_2PO_4; microcosm 5 – 100 mg/L NH_4NO_3 and 10 mg/L KH_2PO_4; microcosm 6 – 200 mg/L NH_4NO_3 and 20 mg/L KH_2PO_4.

The evolution of the bioremediation process was monitored by collecting, every seven days, surficial sediment cores (3 cm^3) and determining total petroleum hydrocarbon (TPH) concentration, according to the method of Nwaogu et al., 2008 (drying at 50°C, extraction, solvent evaporation at 50°C in preweighed recipients, weighing the residue), using instead chloroform, as a solvent (Kalėdienė et al., 2003, Ezekiel et al., 2011). The hydrocarbon amount per gram of dry sediment was calculated and expressed as parts-per-million (ppm).

Furthermore, hydrocarbon-oxidizing microorganisms in each microcosm were quantified (in the 35^{th} day of the începerea experiment), according to the method shown in section 4.7.2. Microorganisms thus islolated were also investigated by epifluorescence microscopy (with SYBR Green and hexidium iodide).

Chapter 5. Results and discussion

5.1. Microbial characteristics in different grain-sized sandy sediments from the Romanian Black Sea coast

5.1.1. Sediment granulometry

Sediments collected from sites I, III and IV were the coarsest, at the opposite end being sites VIII and, especially, II (fig. 3).

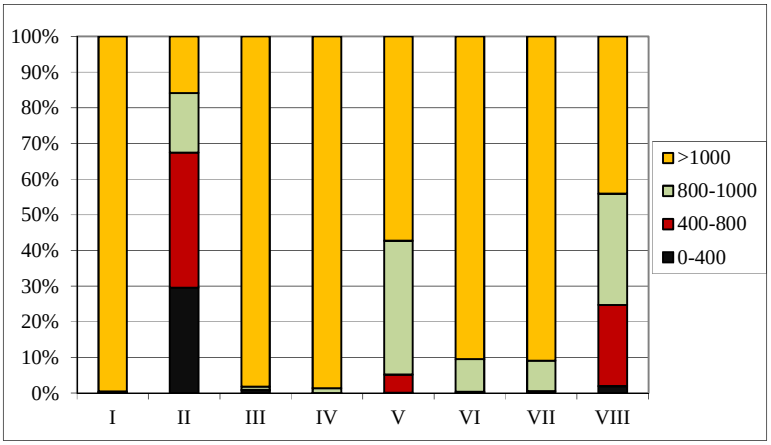


Fig. 3. Proportion of different sized sediment fractions (µm).

5.1.2. Quantitative aspects: cell density and biomass

Among the eight sites, microbial density varied between 4.62-9.64 × 10^7 cells/cm^3 sediment, with an average of 7.15 × 10^7 cells/cm^3 (fig. 4).

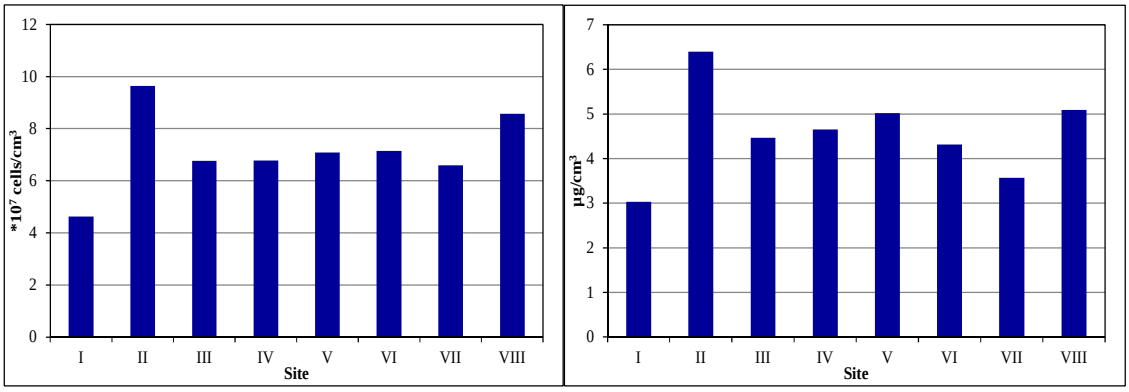


Fig. 4. Abundance and dry microbial biomass per cm^3 sediment in each site.

These values fall within the variation limits of coastal sandy sediment microbiota (around 10^6-10^9 cells per cm^3), comparable to those found by various authors in littoral sediments from different parts of the world (Montagna, 1982, Novitsky & MacSween, 1989, Danovaro et al., 1994, Epstein et al., 1997, Torréton et al., 1997, Hymel & Plante, 1998, Kuwae & Hosokawa, 1999, Dietrich & Arndt, 2000, Proctor & Souza, 2001, Lucas et al., 2003, Lunau et al., 2005, Pusceddu et al., 2005, Šestanović et al., 2005, Ferrara-Guerrero et al., 2007, Hewson et al., 2007, Røberg et al., 2007, Podgórska et al., 2008, Šestanović et al., 2009).

There is a noticeable tendency towards a negative correlation of bacterial density and sediment grain size, a fact observed by many researchers (Novitsky & MacSween, 1989,

Kuwae & Hosokawa, 1999). This would be due to a larger available surface and higher amounts of organic matter in fine sands. However, this relation is neither absolute nor proportional, suggesting other environmental factors determine as well the distribution of microorganisms.

Biomass showed significant variations among the eight sites (fig. 4), between 3.03 and 6.39 $\mu g/cm^3$ sediment, generally following the variations in microbial abundance. Considering the cell density, these results are similar to those found by Danovaro et al. (1994), Luna et al. (2002), Pusceddu et al. (2005), Šestanović et al. (2005) or Ferrara-Guerrero et al. (2007).

Average cell biovolume was 0.12 μm^3 (0.10-0.13 μm^3), value similar to those determined by Kuwae & Hosokawa (1999) or Mudryk & Podgórska (2006b).

5.1.3. Distribution of main microbial morphological groups

Rod-shaped bacteria (including coccobacilli and few vibrios) formed most of the cells observed (58-65%; fig. 5). Their proportion was different among the eight sites, with a tendency to increase their numbers with decreasing grain size. Filamentous forms were rare, and the same for colonial ones (in concordance with the observations made by Novitsky & MacSween, 1989). It should be noted that electron microscopy studies in littoral sediments have shown the existence of disk-shaped bacteria, that cannot be correctly identified by epifluorescence microscopy (Mudryk & Podgórska, 2006b).

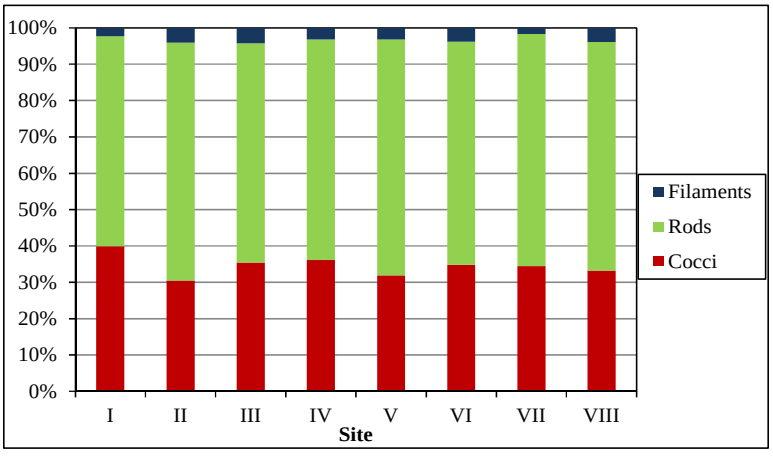


Fig. 5. Percentual proportion of main bacterial morphological groups.

5.1.4. Bacterial membrane integrity

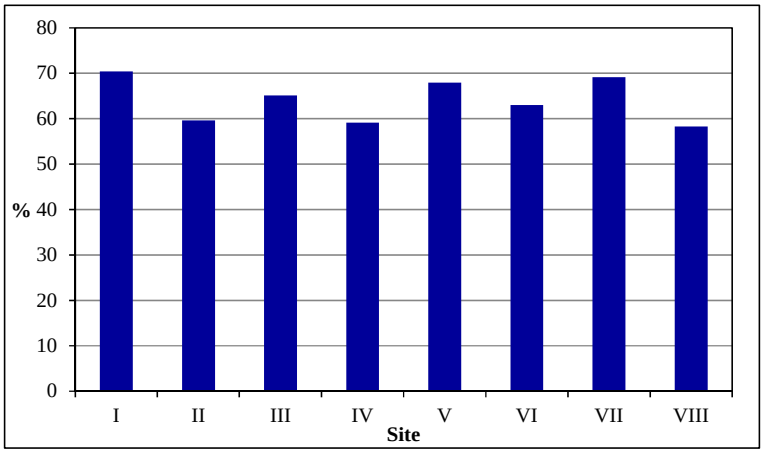


Fig. 6. Percentual proportion of viable (PI-negative) cells.

58-70% of the microorganisms counted had a stained negatively with propidium iodide (PI), having, thus, intact cell membranes. Generally, finer sediments had a lower percentage of viable cells, while the highest values were reported in site I (with the largest grain size; fig. 6).

These percentages are much higher than those determined, for instance, by Luna et al. (2002), in muds and fine sands, supporting the idea that there is a negative correlation between sediment grain size and the proportion of viable bacteria.

5.2. Evolution of benthic microbiota in different grain-sized marine sediment microcosms

After the first two weeks, bacterial cell density reached values as high as 7.7-22.5 × 10^7 cells/cm^3 sediment (fig. 7). These values are similar to those determined in natural sediment samples (see sections 5.1.2., 5.2.1.). Thus, colonization of sterile sediments can be very rapid (under a lack of water dynamism and thermal variations). Growth rate decreases afterwards, with a tendency towards microbial density stabilization.

Mean cell biovolume ranged between 0.069-0.1 μm^3. Most cells observed were rods and coccobacilli, cocci accounting for less than one third in all analyzed samples, and filamentous forms being very rare.

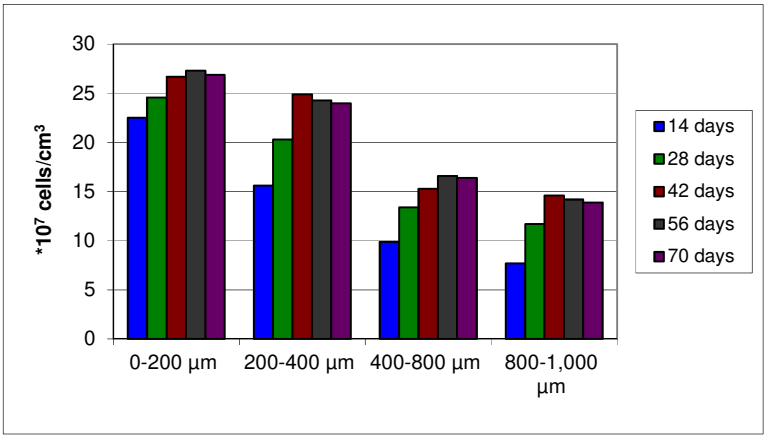


Fig. 7. Evolution of microbial density in each microcosm.

The existence of a correlation between microbial density and sediment's granulometric characteristics is obvious. There are diverging opinions among specialists regarding thic correlation and the way it operates, ranging form an almost proportional relation between bacterial abundance and grain diameter or surface area (Dale, 1974, DeFlaun & Mayer, 1983) to a total absence of any predictable relation, other factors (temperature, grazers) being determinant (Cammen, 1982).

In this experiment, under controlled environmental conditions (constant temperature, lack of water dynamism), there is an obvious negative correlation (an average Pearson's coefficient of -0.97), but not a proportional one (one reason could be the grain shape; this is variable, and the area is not directly poportional to the diameter). The closest regression equation (with a determination coefficient of only 76%) was the following:

$A = 26{,}75 - 0{,}017 \times \phi$

where A = microbial abundance ($\times 10^7$ cells/cm^3) and ϕ = mean grain diameter (µm)

Regarding mean cell dimensions, they vary independently from granulometry (Pearson's coefficient of -0.18).

5.3. Seasonal variations in the characteristics of littoral benthic microbiota

5.3.1. Quantitative aspects: cell density and biomass

Microbial densities ranged between 4.32-12.64 × 10^7 cells/cm^3 (fig. 8), also comparable to those in literature.

The highest densities were found in warmer months (May-October) and the lowest during winter, especially February 2012 (with a seawater freezing).

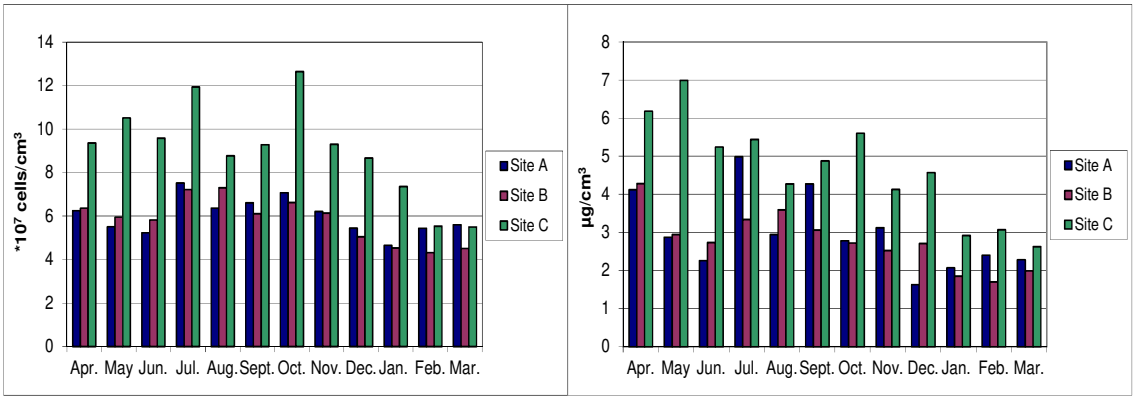


Fig. 8. Evolution of microbial density and dry biomass in each site (April 2011-March 2012).

While sites A and B were similar from this point of view, site C showed much higher microbial densities (due to differences in granulometry and water dynamism; Novitsky & MacSween, 1989).

Biomass also showed significant variations between months and collection sites (fig. 8), ranging from 1.63 to 6.99 $\mu g/cm^3$.

Mean cell biovolume was 0.096 μm^3, its variation limits being 0.07-0.14 μm^3, for each sample. Its variations did not follow a pattern and did not correlate to other variables determined in the current study.

Generally, there are two factors regulating mean bacterial biovolume: available organic nutrients (favouring size growth of microorganisms) and bacteriovorous protists (which select either very small either large sized bacteria, less accesible to consumers). The effects of these factors vary with depending on the environment. While, studying plankton, some authors found a seasonal cyclicity of mean biovolume, with the highest values in warm months, Šestanović et al. (2005, 2009) found, in fine infralittoral sands, a reverse cycle, with the highest values in winter.

5.3.2. Distribution of main microbial morpho-structural groups

All bacteria observed were classified according to their Gram character (with taxonomical value) and morphology (fig. 9).

Most cells counted were Gram-negative (70-86%), in accordance to scientific literature (Khiyama & Makemson, 1973, Moriarty & Hayward, 1982). The proportion of Gram-positives was significantly higher in coarser sediments (sites A and B) compared to fine ones, and in the colder months of the year.

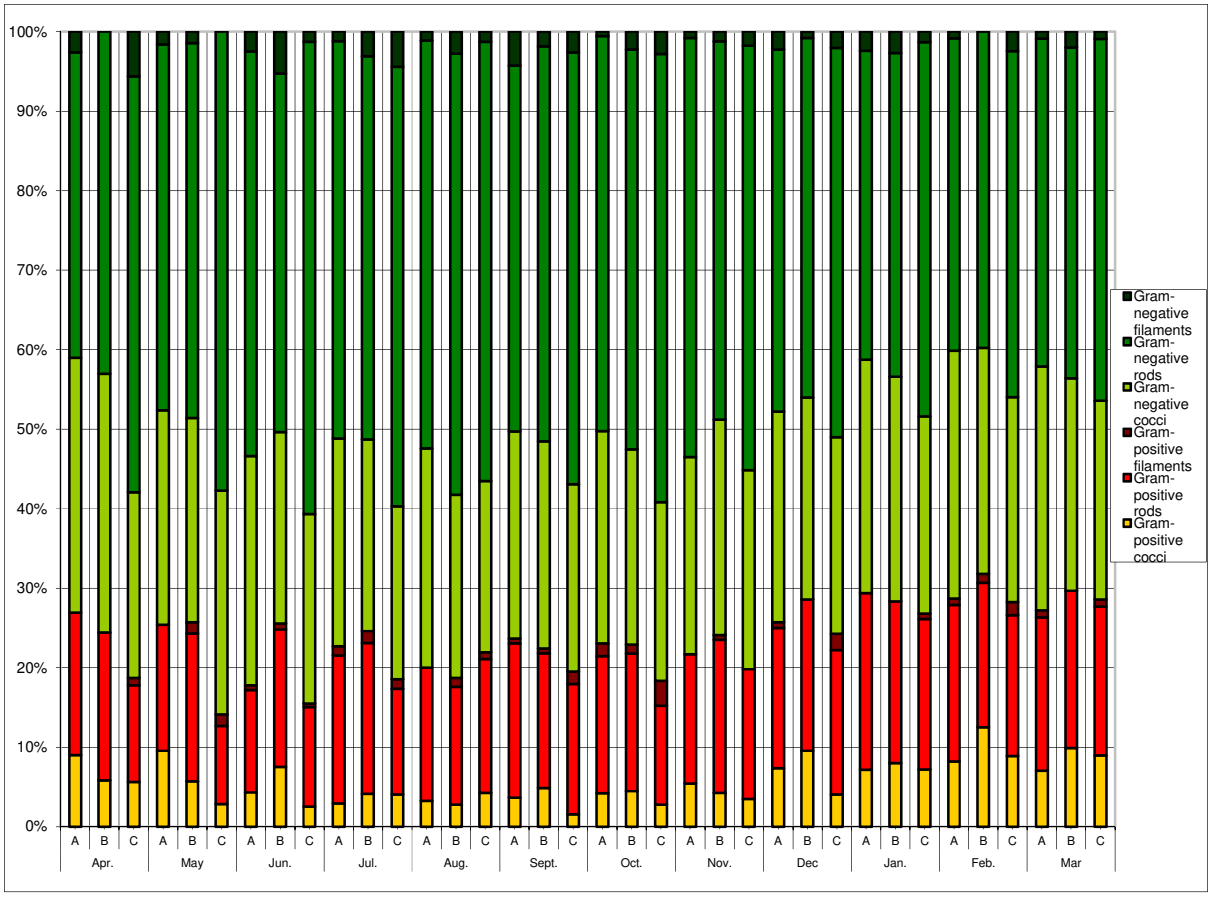


Fig. 9. Percentual proportion of main bacterial morpho-structural groups in each site (April 2011-March 2012).

Regarding morphology, rods were dominant (56-72%). Cocci had a higher occurrence in coarser sediments and colder months, contrary to the observations made by other researchers (Novitsky & MacSween, 1989, Šestanović et al., 2005, Šestanović et al., 2009). Filamentous forms were rather rare in all studied samples.

5.3.3. Seasonal evolution of sediment chlorophylls' concentration

Very large differences were found between sites A and C regarding chlorophylls' concentration. During the study (August 2011-July 2012) total chlorophylls varied between 0.11-0.41 µg/cm^3 in site A and 0.15-2.64 µg/cm^3 in site C, with maximum values in autumn and a minimum following the freezing period (February-March 2012).

The distribution of various types of chlorophylls is shown in fig. 10. Chlorophyll A is common to all oxygenic photosynthetic organisms, Chlorophyll B is found in green algae and land plants and chlorophyll C is common to several groups of algae, important in this case

being diatoms. The amount of photosynthetic pigments is a clue of sediment primary productivity and available organic matter; it derives from microphytobenthos, but also plankton deposition or decaying macroalgae. In this study, chlorophylls A and C were dominant and their concentrations were correlated, confirming the main role diatoms play in littoral sediment primary production (García-Robledo et al., 2010).

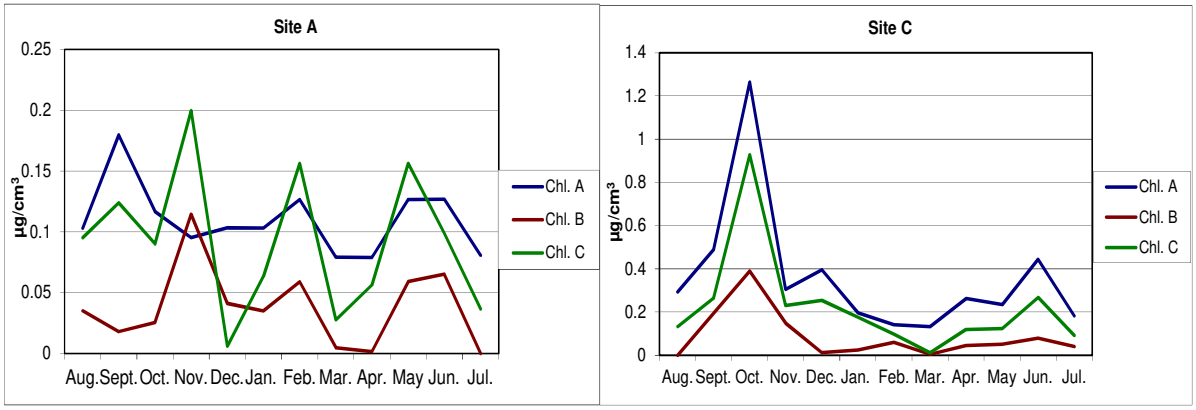


Fig. 10. Chlorophyll concentrations (sites A and C, August 2011-July 2012).

5.3.4. Correlations between microbial characteristics and environmental factors

Table 1 shows the values of Pearson's coefficient for different pairs of analyzed variables.

Microbial abundance and biomass showed significant correlations to water temperature and chlorophyll concentration. Thus, microorganism growth is favoured during warmer periods of the year and during microphytobenthos (especially diatom) booms. Average cell sizes are weakly correlated to temperature and, practically, have no correlations to other microbial or environmental features.

Unfavourable environmental conditions (low temperatures, low primary production) seem to favour Gram-positive and sphere-shaped bacteria (considering both seasonal and granulomerty-linked variations). Regarding Gram-positives, the phenomenon is probably due to their capacity to generate resistance forms such as endospores. For cocci, the explanation is probably connected to their sizes. Most of the cocci counted had diameters below 0.25 micrometers. Apparently, the lack of nutrients can cause bacteria to change to very small cryptobiotic forms (Velimirov, 2001).

Close relations between sediment microbiota and microphytobenthos, in terms of quantity and diversity, were also found by other researchers, such as Šestanović et al. (2009) or Kunihiro et al. (2012), with permanent organic and inorganic nutrient exchanges among

them. Gonzalez-Acosta et al. (2006) found a positive correlation (R = 0.6) between water temperature and culturable heterotrophs in tropical sediments.

Table 1. Pearson correlation matrix for studied parameters: A = microbial abundance, V cel. = mean cell biovolume, B = microbial biomass, % coci = percentage of sphere-shaped bacteria, % Gram+ = percentage of Gram-positives, Chl A/B/C = chlorophyll A/B/C concentration, Total Chl = total chlorophyll concentration, Temp. = seawater temperature at sample collection time (cf. http://www.meteoconstanta.ro.).

	A	**V cel.**	**B**	**% coci**	**% Gram+**	**Chl A**	**Chl B**	**Chl C**	**Total Chl**	**Temp.**
A	1,00	0,25	0,79	-0,74	-0,70	0,64	0,39	0,61	0,61	0,71
V cel.		1,00	0,76	0,06	-0,22	0,29	-0,09	0,09	0,15	0,35
B			1,00	-0,42	-0,61	0,77	0,38	0,69	0,70	0,76
% coci				1,00	0,73	-0,50	-0,37	-0,44	-0,49	-0,86
% Gram+					1,00	-0,40	-0,49	-0,50	-0,52	-0,84
Chl A						1,00	0,54	0,72	0,80	0,27
Chl B							1,00	0,83	0,88	0,05
Chl C								1,00	0,98	0,13
Total Chl									1,00	0,16
Temp.										1,00

The evolution of chlorophylls' concentrations during the studied period had no correlations to water temperatures, following its own seasonal cycle, with the highest values in autumn.

These results show that benthic microbial abundance and biomass variations can be linked to both temperature variations and changes in primary producers' density.

5.4. Density estimation of several microbial physiological groups in littoral sediment

Table 2 shows the MPN of each group of microorganisms studied, per cm^3 of sediment.

Regarding nitrogen fixers, the method employed only allows culturing and counting aerobic or oxygen-tolerating ones. The most common are species belonging to *Azotobacter* and *Azospirillum* genera, and sometimes *Campylobacter*, *Beggiatoa*, some cyanobacteria etc.

(Capone, 1988, Herbert, 1999), all being Gram-negative and, usually, rod-shaped, a morpho-structural group that was by far dominant in analyzed samples (with an average value over 67%).

Table 2. MPN of culturable microorganisms in each physiological group ($\times 10^3$) per cm^3 of sediment in the two sites.

Physiological group	Site A	Site C
Nitrogen-fixers	3.7	11
Ammonifiers	5.3	16
Nitrifiers	3.7	8
Denitrifiers	4.4	8.8
Sulfate-reducers	3.5	170

In various types of marine sediments, several authors counted from below 10^2 to over 10^4 diazotrophs/g (both aerobic and anaerobic; Hanson & Gundersen, 1976, Zuberer & Silver, 1978, Kannan, 2004).

Culturable ammonifiers accounted for below 0.02% of the total directly quantifiable bacteria. Generally, their abundance varies among different sediment types, depending on the available amount of organic nitrogen (Herbert, 1999): from below 10^3 to over 10^6 bacteria/g (Podgórska & Mudryk, 2007, Kannan, 2004, Brambilla, 2006).

Ammonifying microorganisms play an important role in nitrogen recycling in marine ecosystems, converting organic nitrogen into ammonium salts, more accessibile to primary producers. Among the most common such bacterial taxa are species of *Pseudomonas*, *Vibrio*, *Serratia* (Gram-negative), *Bacillus*, *Clostridium*, many Actinomycetes (Gram-positive), but also some fungi (Herbert, 1999). Epifluorescence analysis of microorganisms grown on the medium showed a celar dominance of Gram-positive bacteria (rod-shaped and filamentous.

Nitrifying bacteria (precisely nitrosifying, ammonia-oxidizing bacteria) had densities around $10^3/cm^3$ and a similar proportion within the total microbiota in both sites. Depending to local conditions, their abundance can vary between 10^1-10^6 bacteria/g (Henriksen & Kemp, 1988, Isnansetyo et al., 2011).

Ammonia oxidation is primarly a pathway to nitrogen sinking. Nitrates produced are converted by denitrifiers, mostly to volatile compounds. Among the main factors that influence nitrification are available oxygen, available hydrogen sulfide and competition with other organisms for ammonia (Herbert, 1999).

The large majority of nitrifiers are Gram-negative, so as 64% of those cultured and observed in the current study. The most common genera are *Nitrosomonas*, *Nitrosovibrio*, *Nitrosococcus* etc. (Henriksen & Kemp, 1988, Herbert, 1999). Often, nitrifiers form consortia with Gram-positive heterotrophs (Achuthan et al., 2006), which would explain their relatively high numbers observed.

Denitrifying microorganisms had similar densities and a similar proportion in both sites. For comparison, several authors found 10^1-10^6 denitirifiers/g, in various types of marine sediments (Heitzer & Ottow, 1976, Sugahara et al., 1988, Shieh & Yang, 1997, Kannan, 2004).

The most common denitrifiers belong to the *Pseudomonas* genus, but there are also species of *Achromobacter*, *Aeromonas*, *Agrobacterium*, *Alcaligenes*, *Chromobacterium*, *Flavobacterium*, *Hyphomicrobium* (Gram-negative), *Bacillus*, *Streptomyces* (Gram-positive; Takeuchi, 2005, Rezaee et al., 2010). Most bacteria observed in this experiment were rod-shaped or filamentous Gram-positives.

In conclusion, microorgansims involved in all phases of nitrogen biogeochemical cycle are present in littoral sediments. Tha most numerous, especially in fine sands, are ammonifiers. Usually the ratio of ammonifiers to other groups involved is much higher (for example, Kannan, 2004, or Podgórska & Mudryk, 2007). This seems to indicate either a dependence of sediment microbiota on planktonic ammonification, regarding the source of ammonium, either the preferential cycling of nitrogen by other easily metabolized compounds, such as urea and uric acid (Herbert, 1999, Podgórska & Mudryk, 2007).

Regarding the estimative numbers of sulfate-reducers, there were huge differences between the two sites. While in site A, compared to the total density, they formed less than 0.006%, in site C, they reach 0.17%. Generally, these bacteria have densities between 10^2-10^6 bacterii/cm^3 in littoral sediments (Hines & Buck, 1982, Chandrika et al., 1990, Bussmann & Reichardt, 1991, Phuong et al., 2006).

Most sulfate-reducers belong to Deltaproteobacteria (Gram-negative), the most common in marine sediments being *Desulfovibrio*, *Desulfobacter*, *Desulfobacterium*, (rod or vibrio-shaped) and *Desulfococcus* (usually spherical). *Desulfotomaculum*, *Desulfosporosinus* and other few related genera are Gram-positive, rod-shaped sulfate-reducers, also common in sediments (Bussmann & Reichardt, 1991, Castro et al., 2000, Phuong et al., 2006). In this experiment, most bacteria observed (59%) were Gram-negative and rod-shaped, followed by Gram-positive rods and Gram-negative cocci.

5.5. Bioremediation potential of littoral benthic microbiota

5.5.1. The effect of hydrocarbon pollution on littoral benthic microbiota

While in the control microcosm microbial density and structure remained relatively constant throughout the experiment, diesel oil addition had significant effects on microorganisms in the other microcosm. First, a major decrease in bacterial abundance (by around 28%; fig. 11) was observed during the first four days. The microbiota steadily recovered, at the end of the two weeks, desnities being close to the initial ones. The structure of microbial communities in the contaminated microcosm also changed, mostly by an increase in Gram-positive rod-shaped bacterial proportion (fig. 12; also see 5.5.3).

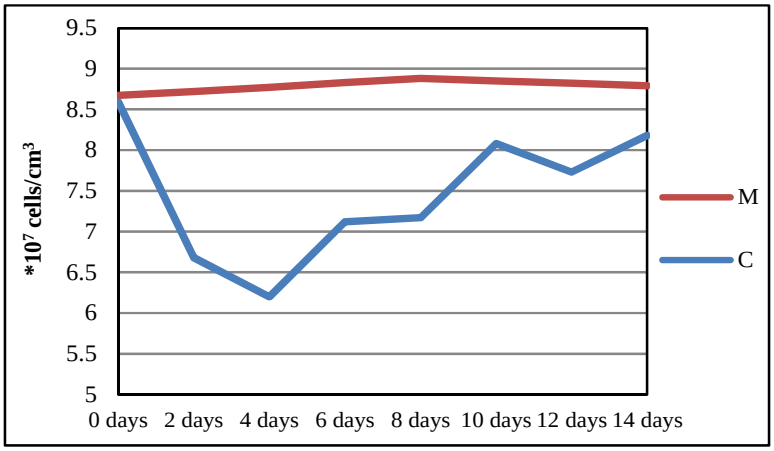
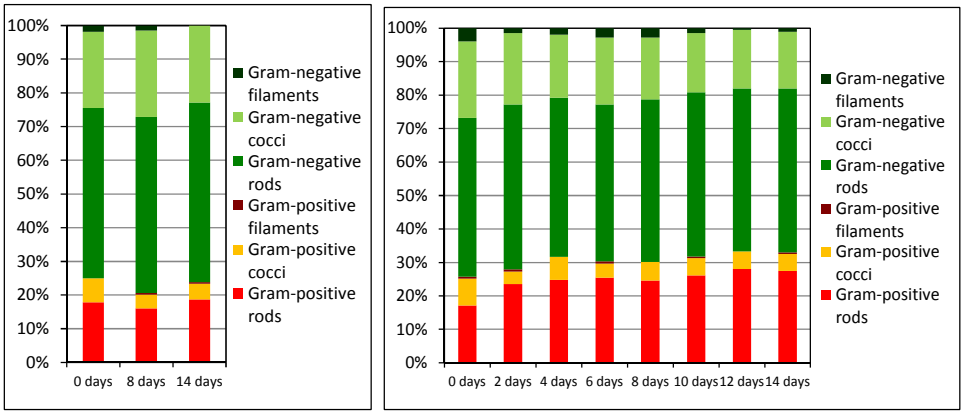


Fig. 11. Evolution of microbial density in both microcosms.



a) b)

Fig. 12. Evolution of the percentual proportion of different bacterial morpho-structural groups: a) control microcosm; b) contaminated microcosm.

Several researchers, studying the short-term impact of hydrocarbon pollution in various environments, noticed masive drops in bacterial densities during the first days, due to the contaminants' toxic effects, followed by recoveries, due to selecting hydrocarbon-tolerant microorganisms, usually in 2-3 week intervals (Delille & Vaillant, 1990, Delille & Delille, 2000, Akpoveta et al., 2011). Obviously, hydrocarbon contamination also affects community taxonomical composition (Røberg et al., 2011). The amplitude of such changes may vary from insignificant to major ones, depending on the pollutant's concentration (Alexander & Schwarz, 1980, Carman et al., 1996, Akpoveta et al., 2011).

5.5.2. Hydrocarbon and vegetable oil-degrading microbial density

Up to 0.01% of the total microorganisms in the sediments studied are able to use hydrocarbons as sole carbon sources and 0.002-0.025% can do this with vegetable oils (strictly culturable microorganisms; table 3). In site A, microorganisms able to oxidize light hydrocarbons (gasoline: C_4-C_{12}) dominate, while site C, with finer sediment, has more heavy hydrocarbon-degraders (C_{10}-C_{40}).

These densities are similar to those determined by other authors in uncontaminated littoral sediments in various parts of the world: 10^1-10^5 MPN/g (Walker & Colwell, 1976, Roubal & Atlas, 1977, Higashihara et al., 1978, Venkateswaran et al., 1991, Delille & Delille, 2000, Ramsay et al., 2000, Swannell et al., 2000, Braddock et al., 2004, Røberg et al., 2007, Chikere et al., 2009).

Regarding vegetable oils, olive oil had the highest number of degraders. Few organisms grew on linseed oil, although there are studies showing it is easier degradable than sunflower oil, for instance (Pereira et al., 1998, cit. de Al-Darbi et al., 2005).

Tabel 3. MPN of hydrocarbon and vegtable lipid oil-degrading microorganisms ($\times 10^3$) 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

5.5.3. Nutrient influence on hydrocarbon-polluted sediment bioremediation

In all microcosms, the initial amount of diesel oil was 20,000 ppm. Preliminary tests showed that, using this gravimetric method, volatile fractions, impossible to determine due to evaporation accounted for up to 34% of the oil's mass.

Results show that a moderate nutrient addition of de 5-25 mg/L NH_4NO_3, respectively 0.5-2.5 mg/L KH_2PO_4 (microcosms 2 and 3; fig. 13) induced a fast and effective degradation within the water column. Overall, however, the fastest decrease in hydrocarbon concentration was determined in the control microcosm.

It is well established that nitrogen and phosphorus are often limiting factors to hydrocarbon biodegradation (Atlas, 1981). Biostimulation experiments in various environments have shown the favouring role that, within certain limits, addition of nitrate and phosphate compounds can have in accelerating the process (Horowitz & Atlas, 1977, Atlas, 1981, Leahy & Colwell, 1990, Harayama et al., 1999, Swannell et al., 2000, Röling et al., 2002, Adoki, 2007, Obahiagbon et al., 2009, Hazen, 2010, Okoro, 2010, Das & Chandran, 2011, Efeovbokhan et al., 2011).

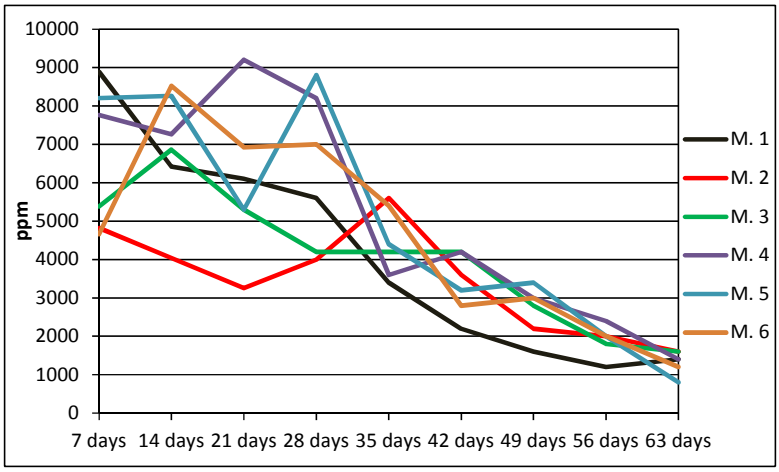


Fig. 13. Evolution of total hydrocarbon content in microcosm sediments.

However, there are situations when biostimulation with nitrates and phosphates can have little or no efficiency (Röling et al., 2002, Heitkamp & Cerniglia, 1989, Leahy & Colwell, 1990, Syafruddin et al., 2010).

In the current experiment, inorganic nitrogen and phosphorus addition led to a significant increase in potential biodegraders' numbers (table 4), but inhibited biodegradation itself. Such effects were encountered by other researchers as well (Fayad & Overton, 1995,

Walworth et al., 2007, Syafruddin et al., 2010). The main cause for bioremediation inhibition in marine environments would be eutrophication (Korda et al., 1997, Röling et al., 2002), leading to an excessive growth of primary producers. When there is no significant water dynamism, this would cause anoxia in sediments and, on the other hand, potential hydrocarbon-degraders would shift to a more easily assimilable trophic resource.

Tabel 4. MPN of hydrocarbon-degrading microorganisms ($\times 10^3$) per cm^3 in microcosm sediments (after 35 days).

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

Thus, at least in sediments less exposed to water dynamism, using nitrates and phosphates as biostimulants may negatively affect the degradation of oil products. In such situations, bioventilation or other means of oxygenation would be a priority (Syafruddin et al., 2010).

Microscopic analysis showed that most culturable hydrocarbon-oxidizing bacteria were Gram-positive rods (45,8%), closely followed by Gram-negative ones (42%). Of the most common hydrocarbon degraders found in aquatic environments, species of *Nocardia*, *Arthrobacter*, *Brevibacterium* and *Bacillus* would fall in the first category, while *Alcanivorax*, *Pseudomonas*, some Enterobacteria, *Achromobacter*, *Alcaligenes*, *Flavobacterium* and some cyanobacteria would fall in the latter (Atlas, 1981, Leahy & Colwell, 1990, Harayama et al., 1999).

CONCLUSIONS

1. Sandy sediments collected from various sites on along the Romanian coast had different microbial densities, all in the range of 10^7-10^8 cells/cm^3 (extreme values being 4.32-12.64 × 10^7 cell/scm^3).
2. Microbial biomasses in the sediments studied had values between 1.63 and 6.99 µg/cm^3.
3. Rod-shaped Gram-negative bacteria are dominant in littoral sediments (38-60%) and the mean biovolume ranges between 0.07 and 0.14 µm^3. Of all the cells counted, 30-42% had compromised membrane integrity (were not viable).
4. Both observations on natural samples and microcosm experiments confirmed the existence of a negative relation between the quantitative characteristics of benthic microbiota and sediment grain size; this relation is nor an absolute, neither a proportional one.
5. Granulometry also influences the distribution of various bacterial morpho-structural groups (the proportion of sphere-shaped bacteria tends to increase with the grain size) and the percentage of viable cells (higher in coarser sediments).
6. Under laboratory microcosm conditions, bacterial colonization of sterile sediments takes place very rapidly (densities similar to natural ones can be reached in only two weeks), which seems to indicate a planktonic origin for most benthic microorganisms.
7. There were obvious seasonal variations regarding the density, biomass and structure of littoral microbiobenthos. Cell density and biomass were significantly correlated to water temperature and sediment chlorophyll concentration (an indicator of primary producers' density).
8. Unfavourable environmental conditions (low temperatures, low primary production, coarser sediments) are associated with an increase in percentages of Gram-positives and small-size Gram-negative cocci.
9. Chlorophyll concentrations also depend on sediment characteristics, and the ratio between various chlorophylls confirms the main role diatoms play in littoral sediment primary production.
10. Using MPN methods, microorganisms involved in different stages of the nitrogen cycle were isolated and their density estimated: aerobic nitrogen-fixers, ammonifiers, nitrosifiers and denitrifiers. The rather low density of ammonifiers suggests the existence of alternative sources of ammonium for sediment microbiota. Microscopic analysis showed that

diazotrophs and nitrifiers are mostly Gram-negative, while ammonifiers and denitrifiers are mostly Gram-positive.

11. There are big differences in sulfate-reducers' densities (mostly Gram-negative), depending on the sediment type, with very high values in fine and weakly oxygenated sands.

12. Although obviously affected by the experimental hydrocarbon contamination, the fast recovery of benthic microbiota and the presence of microorganisms able to degrade various types of hydrocarbon mixtures (generally 10^2-10^3 degraders/cm^3, depending on the sediment and hydrocarbon used) or vegetable oils (up to over 10^4 culturable degraders per cm^3) illustrate an important bioremediation potential in regard to this kind of pollution.

13. Microcosm experiments showed that, although nitrate and phosphate nutrient addition leads to an increase in potential degraders' densities, this does not necessarly accelerate biodegradation, the effect may even be negative. Thus, in regard to biostimulation other factors, like available oxygen, should also be taken into account.

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