ARCHIVES OF ENVIRONMENTAL PROTECTION
ARCHIWUM OCHRONY ŚRODOWISKA
vol. 32 no. 1 pp. 43 - 58 2006

PL ISSN 0324-8461

DEGRADATION OF CRUDE OIL IN SEAWATER AND SAND BY MIXED BACTERIAL CULTURES

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Keywords: crude oil, sand remediation, seawater, bioaugmentation, biodegradation, marine bacteria.

ROZKŁAD ROPY NAFTOWEJ W WODZIE MORSKIEJ I W PRZYBRZEŻNYM PIASKU PRZEZ MIESZANE KULTURY BAKTERYJNE

Przypadkowe wycieki ropy na otwartym morzu są częstym problemem środowiskowym prowadzącym do degradacji życia morskiego jak i życia na wybrzeżu. Obecnie techniki są skoncentrowane na zebraniu oleju, wypaleniu *in-situ* pozostałości ropy, zbieraniu zanieczyszczonego piasku i likwidowaniu zanieczyszczeń lub magazynowaniu go na terenach przyległych. W ciągu ostatnich lat wzrosło zainteresowanie bioremediacją z użyciem mikroorganizmów aktywnych w rozkładzie zanieczyszczeń ropopochodnych.

Celem tej pracy było sprawdzenie zdolności mikroorganizmów wyizolowanych ze środowisk zanieczyszczonych do biodegradacji ropy naftowej w wodzie morskiej oraz w piasku pobranym z wybrzeża. Sztuczna woda morska wzbogacona biogenami została zaszczepiona bakteriami wyizolowanymi z osadów rafineryjnych. Te same kultury zostały użyte również w doświadczeniu z piaskiem pobranym z wybrzeża Krety. Badania z użyciem piasku zanieczyszczonego 5% (v/w) ropy naftowej były przeprowadzone po jego uprzednim wysterylizowaniu i bez poddania go sterylizacji. Takie modyfikacje miały na celu: wykazanie, czy wyizolowane kultury potrafią sobie radzić z zanieczyszczeniem w warunkach semi-naturalnych oraz scharakteryzowanie ich wzrostu i możliwości wystąpienia reakcji antagonistycznych pomiędzy inokulowanymi szczepami a mikroflorą naturalną. W trakcie eksperymentu mierzono zużycie tlenu, liczebność bakterii (cfu) i gęstość optyczną prób. Po zakończeniu eksperymentów (po 14 dniach) zmierzono zawartość węglowodorów ropopochodnych (TPH - total petroleum hydrocarbons) przy użyciu spektrofotometrii w podczerwieni (IR).

Wszystkie testowane kultury mieszane posiadały zdolność do rozkładu węglowodorów w wodzie morskiej i piasku. Po dwóch tygodniach eksperymentu usunięcie TPH w wodzie morskiej zanieczyszczonej ropą naftową było pomiędzy 56,8% (dla A2) i 64,4% (dla A1). Stwierdzono, iż kultury najefektywniej usuwające węglowodory w wodzie morskiej nie są najlepszymi dla piasku. W piasku najlepszy rozkład węglowodorów obserwowano w próbach zaszczepionych mieszaniną bakterii wyizolowanych z osadów rafinerii w Koryncie. Zawartość węglowodorów była odpowiednio o ponad 70% niższa niż w odpowiadającym im kontrolach nieinokulowanych. Zaobserwowano, iż dodatek wody morskiej wzbogaconej biogenami do piasku miał również pozytywny wpływ na usunięcie węglowodorów przez naturalnie występujące w piasku mikroorganizmy (48%). Otrzymane wyniki wskazują na konieczność przeprowadzania analiz w warunkach semi-naturalnych zwłaszcza, gdy autochtoniczna mikroflora może posiadać wysoki potencjał degradacyjny. Obca mikroflora wprowadzona do środowiska może nie tylko nie przyspieszyć rozkładu zanieczyszczeń, ale i go spowolnić.

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Summary

Accidental oil spills at open sea is a common environmental problem. They lead to degradation of sea and shoreline life. In the last ten years there has been an increased interest in bioremediation using the enzymatic activity of the naturally occurring microorganisms. In this work the potential of mixed microbial cultures for biodegradation of crude oil in seawater and sand has been examined. Artificial seawater supplemented with nitrogen and phosphorus was inoculated with cultures isolated from refinery sludge. The same cultures were used for experiments in sand polluted by 5% (v/w) of crude oil. These experiments were performed in sterile and semi-natural (not sterile) conditions to see the degradation potential of isolated cultures, their growth characteristics and possible antagonisms between supplemented microorganisms and natural microflora. During the experiments the oxygen demand, number of bacteria (cfu) and optical density (OD_{660}) were monitored. After 14 days of cultivation, the concentration of total petroleum hydrocarbons (TPH) in all samples was measured.

All tested cultures had a potential for degradation of hydrocarbons in seawater and sand. After two weeks of experiment, loss of hydrocarbons in seawater polluted with crude oil was between 56.8% (A2 culture) and 64.4% (A1 culture). The most effective culture for bioaugmentation of seawater does not have to be the best solution for bioaugmentation of sand. In sand the best degraders in sterile and semi-natural conditions were found in the mixed cultures isolated from Corinth refinery sludge. For this culture concentration of hydrocarbons in sterile sand was 73.2% lower than in control sample and in non-sterile sand 70.5% lower than in control (sterile sand) without bioaugmentation. Finally, the addition of seawater and fertilizers to sand had also a positive influence on contaminants degradation by naturally occurring microorganisms (48%).

Experiments performed with different environments (seawater and sand) and under different conditions (sterilized material and semi-natural conditions) confirmed that cultures should be tested in semi-natural conditions especially when indigenous microflora cans posse's high degradation potential. Allochtonic cultures, very active in sterile conditions, after inoculation to natural environment can even slow down the degradation.

INTRODUCTION

Open sea and seashore are environments frequently exposed to pollution from petroleum hydrocarbons [4, 9]. Explosions, spills, fires and blowouts occur frequently during drilling operations. Offshore oil platforms produce a wide variety of liquid, solid and gaseous wastes and some of them are discharged directly into the ocean. Platform collapses or collisions with ships, pipeline ruptures, leaks and accidents in transferring oil and gas between facilities, fires and explosions are connected with all steps of extraction and hardly with transportation of petroleum [4, 9]. The most important source of hydrocarbons in the sea is the marine transportation of crude oil (45.5%) and runoff from land-based activities (29.0%). Other sources include natural, like terrestrial plant waxes, marine phytoplankton and bacteria, biomass combustion and digenetic transformation of biogenic precursors, all of them contributing only by 9% [12, 31]. Petroleum hydrocarbons introduced into the marine environment are immediately subjected to a variety of physical, chemical and biological changes. These weathering processes result in important changes in the chemical composition and physical properties of the original pollutant [4, 9, 32].

Following an oil spill in the open sea, several methods can be employed including placement of floating booms around the spill, use of skimmer boats to collect oil from the surface, and addition of dispersants to break the surface slick. Natural bioremediation of marine oil spills can be enhanced either (i) by stimulation of the indigenous microorganisms through the addition of nutrients (i.e., fertilizers) or (ii) by bioaugmentation with special mixtures of naturally occurring oil-degrading microorganisms or by the introduction of genetically engineered ones with enhanced oil-degrading capabilities [5, 17, 19, 26, 32]. Biodegradation takes place at the oil-water interface. Creation of oil droplets by natural or

chemical dispersion increases the surface area of the oil which in turn increases the surface area accessible by the microorganisms for biodegradation [9].

One of the most important factors, that complicate bioremediation of crude oil spills at open sea and coastal areas, is salinity [27]. A significant number of oil-polluted ecosystems are characterized by moderate, high or extreme salinities like those found in Mediterranean beaches and salt marshes [6]. High salinities or wide salinity variations make oil pollution more difficult to treat using conventional bioremediation methods. High or variable salinity disrupts cell membranes, denatures enzymes, and results in reduction of metabolic activity. For example, desiccation due to high osmotic pressure is lethal to many conventional wastewater treatment microorganisms [6, 7, 27]. To treat these petroleum residues with biological methods, halophilic or halotolerant organisms that can tolerate these environments with increased salinity must be used and the optimal physicochemical conditions should be determined to enable successful bioremediation [6, 7]. Marine aerobic bacteria used for bioremediation of marine ecosystems belong to the genera: *Alteromonas*, *Pseudomonas*, *Moraxella*, *Bacillus*, *Flavobacterium*, *Mycobacterium*, *Cycloclasticus*, *Marinobacter*, *Sphingomonas* and *Vibrio* [16, 17].

Successful bioremediation of oil spills in marine and saline environments has often been observed [22, 29]. Microorganisms able to grow in the presence of salt are found in all domains of life: Prokaryota (Archaea, Bacteria) and Eukaryota (Fungi, Algae, Plantae etc.) [22]. Yakimov et al. [35] isolated biosurfactant-producing, n-alkane-degrading, Gram-negative, aerobic, rod-shaped marine bacteria. *Marinobacter hydrocarbonoclasticus* is another marine bacterium, isolated from the Mediterranean Sea near a petroleum refinery by Gauthier et al. [11] with the ability to use various hydrocarbons as the sole carbon and energy source. Dutta and Harayama [8] found that *Alcanivorax* sp. transformed alkyled hydrocarbons. The results of Kasai et al. [16] showed that *Alcanivorax* sp. and *Cycloclasticus* sp. are two major populations in chronically polluted environments with petroleum hydrocarbons. Shelton et al. [29] showed that artificially weathered crude oil can be degraded by four diverse cultures of mixed marine bacteria under optimized conditions for 7 and 14 days.

The goal of this study was to find the best group of crude oil degraders isolated from hydrocarbon polluted areas which enhance biodegradation of crude oil in seawater and in coastal sand. These microorganisms should survive competition with natural microoflora. Changes in concentrations of microorganisms and degradation rates of selected isolates and communities were also monitored.

MATERIALS AND METHODS

Artificial seawater

Experiments were performed with artificial seawater (a recipe based on Difco Artificial Sea Water) which contained: NaCl (24 g/dm^3), $MgCl_2 \cdot 6H_2O$ (11 g/dm^3), Na_2SO_4 (4 g/dm^3), $CaCl_2 \cdot 6H_2O$ (2 g/dm^3), KCl (0.7 g/dm^3), KBr (0.1 g/dm^3), H_3BO_3 (0.03 g/dm^3).

Growth supplements

As a source of nitrogen and phosphor mineral basal salt medium was used [28]. The medium contained $(NH_4)_2SO_4$, K_2HPO_4 and KH_2PO_4 (in concentrations 1 g/dm^3, 0.8 g/dm^3 and 0.2 g/dm^3 respectively). Seawater as well as crude oil contain some other compounds

that should be add to this medium (Mg, S, $CaCl_2 \cdot 6H_2O$, etc.). To avoid increase of concentration of other salts the rest of compounds of mineral salt medium was not added. The concentration of N and P was the same as in medium on which bacteria were isolated. As sole carbon source crude oil was added to the medium. Before addition, crude oil was separately sterilized by autoclaving for 20 minutes at a temperature of 121°C and pressure 1.2 atm. The pH of the prepared media was adjusted to 7.0.

Microbial cultures (inocula)

In our experiments mixed cultures enriched on different types of media were used. Culture A1 was isolated from a refinery sludge (Hellenic Petroleum Co. – Aspropyrgos Refineries, Greece) on media saturated with gas oil, culture A2 was enriched from the same refinery sludge on artificial sea water supplemented with N and P and with crude oil as the sole carbon source; culture C1 was isolated from the sludge of a trickling filter (Motor Oil Co. – Corinth Refineries, Greece) on the same media as the other cultures but with higher concentration of petroleum hydrocarbons (crude oil – 1% v/v). Cultures were not characterized and not classified. Type of growth of cultures colonies on solid media (shape, color, structure etc.) suggested that it were mixtures of bacteria.

Prior to use, cultures stored in refrigerator as suspension in physiological salt with glycerol, were pre-grown on Marine Broth with 1% of crude oil. Seven-day old cultures were centrifuged and resuspended in physiological salt (8.5 g/dm^3).

BOD measurements

Experiments were performed in 1 dm^3 dark glass BOD bottles equipped with OxiTop (WTW) measuring heads. Bottles were being first sterilized to avoid contamination by other microorganisms. Samples were stirred all the time. BOD values were taken each day. Calculation was performed with the formula:

$$BOD = \frac{MW_{O2}}{RT_m}\left[\frac{V_{tot} - V_s}{V_s} + \alpha\frac{T_m}{T_r}\right]\Delta P_{O2}$$

where: MW_{O2} is the molecular weight of oxygen (32 000 mg/mol), R is the gas constant (83.144 dm^3·mbar/mol·K), T_r is the reference temperature (273.15K), T_m is the measured temperature, V_{tot} is the total volume of the BOD bottle [cm^3], V_s is the volume of sample [cm^3], α is the Bunsen absorption coefficient (0.03103) and ΔP_{O2} is the difference in the partial pressure of oxygen [hPa].

Microorganisms concentration measurements

The number of bacteria measured as colony forming units per ml of liquid sample (cfu/cm^3) was checked on Petri plates on Marine Agar (Difco) at different dilutions. The dilutions were done in physiological salt (8.5 g/dm^3 NaCl). In addition, the optical density at wavelength 660 nm (OD_{660}) was measured spectrophotometricaly to estimate microorganism's concentration. As a background, microbial-free medium was used.

Total Petroleum Hydrocarbons (TPH) Analysis

TPH extraction from the liquid samples was done with 10 cm^3 CCl_4 by vigorously shaking for 15 minutes. Extraction of sand samples was done using sonication with guidelines that for 10 g sand sample 10 cm^3 of CCl_4 (with 3 g of Na_2SO_4) is used and extraction is done for 15 minutes following the method 8015AZ (Office of Laboratory Licensure, Certification & Training – Arizona Department of Health Service, Revision 1.0, 1998). THP concentration in samples was measured by FT-IR (Perkin Elmer Spectrum 1000 with variable pathlength liquid cell Graseby Specac ZnSe 7009) with pathlength 0.05 cm. Spectrum for TPH concentration calculation was taken between 3100 and 2750 cm^{-1}.

Degradation experiments in seawater

BOD bottles, equipped with OxiTop measuring heads, were filled with 50 cm^3 of sterilized artificial seawater, supplemented with N and P: $(NH_4)_2SO_4$ – 1 g/dm^3, K_2HPO_4 – 0.8 g/dm^3, KH_2PO_4 – 0.2 g/dm^3. A volume of 2.5 cm^3 of sterilized crude oil (1.87 g) was added to each bottle. Seven-day old cultures C1, A1 and A2 pre-grown on Marine Broth (Difco) with 1% of crude oil (v/v) were centrifuged and resuspended in physiological salt (8.5 g/dm^3 NaCl). Initial OD_{660} of each culture was about 0.89. One cm^3 of appropriate suspension was added to the bottles. The control sample contained only artificial seawater. Samples were stirred and incubated for 14 days at 27°C. At the beginning and at the end of the experiment the number of bacteria (cfu/cm^3) was measured on Marine Agar (Difco). In addition, at the end of the experiment, the optical density (OD_{660}), the concentration of Total Petroleum Hydrocarbons (TPH) and the production of biosurfactants were measured.

Degradation experiments in sand

Unpolluted sand was collected from one of the beaches in northwestern Crete. Experiments were performed in sterile BOD bottles (1 dm^3) equipped with OxiTop measuring head. Two base modifications of experiments were: usage of sterile sand and usage of not-sterile. These modifications should show potential of cultures for degradation of hydrocarbons (sterile sand) and changes in microorganisms' activity and possible interactions between microorganisms in semi-natural conditions.

To each bottle, 50 g of artificial seawater supplemented with N and P sources was added. To each bottle, 2.5 cm^3 of sterilized crude oil (1.91 g) and 20 cm^3 of artificial seawater supplemented with nitrogen N and P were added. Biological oxygen demand was measured every day. At the end of the experiment the concentration of Total Petroleum Hydrocarbons (TPH) was measured.

Table 1. Initial OD_{660} of water with cultures added to sand

	C	C1	A1	A2
Sterilized sand	0.00	0.5724	0.4040	0.4300
Non-sterilized sand	0.00	0.2650	0.2397	0.2238

Drop-collapsing test

Biosurfactant production was checked by drop-collapsing test [14, 20]. The method is based on changes of surface tension. Cultures used for test were firstly pregrown for 14 days on artificial seawater supplemented with N and P sources as previously and with 5% of crude oil (v/v). A 10-ml drop of enriched bacterial culture was placed on the surface of the top of a Petri dish immersed in paraffin. After 2 minutes, the diameter of the drop was measured with scale. At the same time a picture of the drop profile was taken with digital camera to see differences between samples and control. As a control pure sterile medium was used. The percent increase of the diameter of each drop was then calculated.

Adhesion of cultures to sand

Additional test of adhesion of bacteria to sand was done by method described by Huysman and Verstraete [13] and Mehmannavaz et al. [23]. All cell cultures were firstly pregrown on Nutrient Broth (Fluka) prepared with addition of NaCl to get the end concentration of this salt of about 2.5%. After 24 h of cultivation the cultures were centrifuged and resuspended in physiological salt to get the end OD_{660} of about 0.34–0.39. 10 cm^3 of microorganism's suspension was added to 1 g of sterile sand and vigorously shaken for 1 minute. As a control 1 g of sterilized sand with 10 cm^3 of physiological salt was used. All tests were done in triplicate. After 15 minutes of settlement OD_{660} was measured taking one milliliters of the aqueous layer from the top of sample. The following formula was used for percentage adhesion calculation:

$$\frac{[ODi - (Ode - ODc)]}{ODi} * 100 \ [\%]$$

ODi – OD_{660} of initial suspension,
ODe – average OD_{660} of end suspension,
ODc – average OD_{660} of control suspension.

Statistical analyses were done with the Statistica 5.1 software package.

EXPERIMENTAL RESULTS

A series of experiments was designed and conducted to examine the effectiveness of the chosen microbial mixtures to degrade crude oil at open sea and in sand conditions. All experiments were done in BOD bottles to determine changes in activity of microorganisms during degradation of pollutant. Artificial seawater supplemented with nitrogen and phosphorous and three types of cultures (A1, A2 and C1) enriched on different media and with different concentration and types of pollutant (crude oil and gas oil) were used.

All tested cultures degraded hydrocarbons in seawater by more than 50% as shown in Fig. 1. The highest removal of TPH was observed for culture A1 (64.4% after 14 days) and culture C1 (61.2% after 14 days). Culture A2 resulted in a TPH degradation of about 57%.

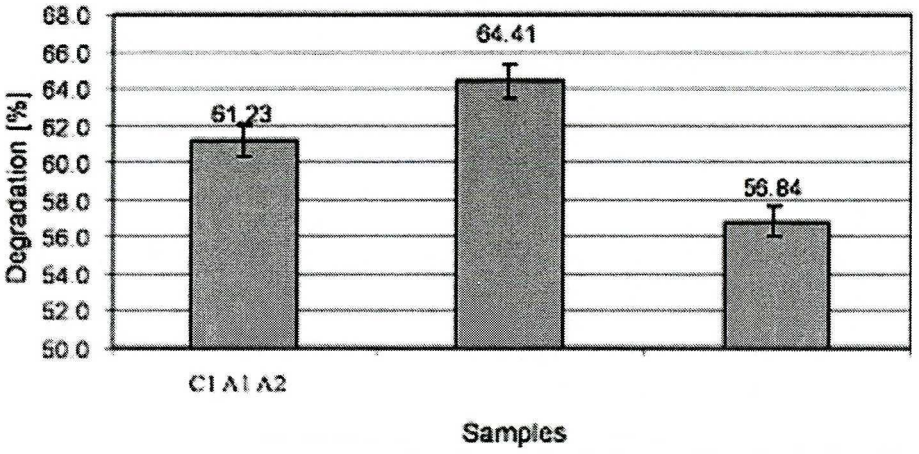

Fig. 1. Degradation of hydrocarbons (TPH) in seawater by mixed cultures of bacteria

The highest degradation was correlated with the highest biological oxygen demand activity, and was observed in samples with cultures A1 as seen in Fig. 2. After the 5th day the measured value exceeded the measuring capability of the OxiTop head. Last measurable value of BOD was 950.0 mg O_2/dm^3 on the 4th day. Very high activity was also observed in the sample with culture C1 (682.1 mg O_2/dm^3 on the 4th day and overvalued on 5th day). Culture A2 was characterized by lower activity at the beginning of the experiment and a quick increase after the 7th day up to 1144 mg O_2/dm^3 on 10th day (the last day when value was possible to be measured). In the control sample no changes in biochemical oxygen demand were observed.

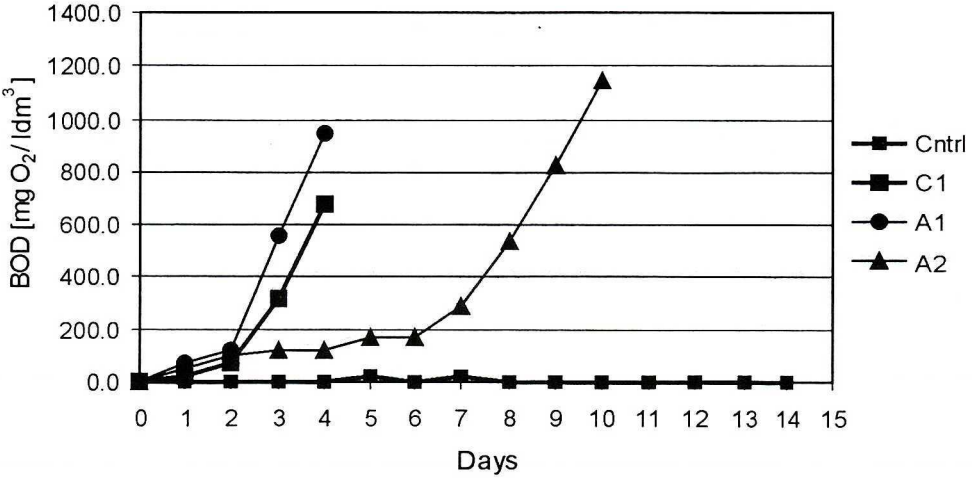

Fig. 2. Changes of biochemical oxygen demand during experiment in seawater during degradation of hydrocarbons

At the beginning and the end of the experiment the optical density of samples was measured (Table 2). At the end of the experiment the highest OD_{660} was noticed in the sample with culture A1 (3.07) and the lowest in sample with culture C1 (0.93). Initial OD_{660} in all samples except the controls was the same and about 0.018. Increase of colony forming bacteria was also observed. The number of bacteria on solid media (colony forming units) at the beginning of the experiment was the highest in sample inoculated with A2 culture (5×10^5 cfu/cm^3) and the lowest in sample with culture C1 (1.4×10^4 cfu/cm^3). At the end of the experiment the highest number of bacteria was found for culture A1 (1.28×10^9 cfu/cm^3). The lowest number of bacteria was found in sample with culture A2 (5.7×10^7cfu/cm^3). A correlation between degradation rate and OD was not observed. At the same time a correlation between degradation rate, oxygen demand and number of bacteria was observed. A correlation coefficient (r^2) was 0.76 between degradation rate and a number of bacteria and 0.99 for degradation rate and oxygen demand (oxygen demand on 4th day).

Table 2. Data collected during experiment with seawater

	OD_{660} of inoculum	OD_{660}		Number of bacteria [cfu/cm^3]		Drop collapsing test	
		Initial	Final	Initial	Final	Changes in drop's profile	Increase of drop's diameter [%]
Control		0.0032	0.0034	0.00	0.00	not observed	–
C1	0.8838	0.0177	0.9347	1.40×10^4	2.00×10^8	+ small	32.4
A1	0.8867	0.0177	3.0682	1.14×10^5	1.28×10^9	+++ large	44.1
A2	0.9012	0.0180	2.6581	5.00×10^5	5.70×10^7	+ small	35.3

All tested cultures were producing surfactants during degradation of hydrocarbons as seen in Table 2. Changes in drop's profile and increase in drop's diameter were the most significant in sample with culture A1 (increase of drop diameter by 44%). For other two cultures (C1 and A2) changes were smaller and the increase in drop's diameter was 32% and 35%, respectively.

As shown in Fig. 3, the degradation of TPH after 14 days of incubation in sterile sand ranged between 27.5 and 73.2%. The highest degradation of crude oil in sand was observed for Corinth culture (C1 = 73.2%). This culture was isolated by a different method than cultures A1 and A2 and on media with higher concentration of TPH. The lowest degradation was noticed for culture isolated during enriched experiment from Athens refinery sludge on crude oil (A2 = 27.5%). Mixed culture isolated on media with gas oil (A1) resulted in losses of TPH of about 45.2%. Also in non-sterile conditions the highest degradation of crude oil was also observed with culture C1. The degradation of TPH with this culture compared to control was 70.5% (Fig. 3). The degradation observed with cultures A1 was about 60%, whereas bioaugmentation of sand with culture A2 did not increase degradation.

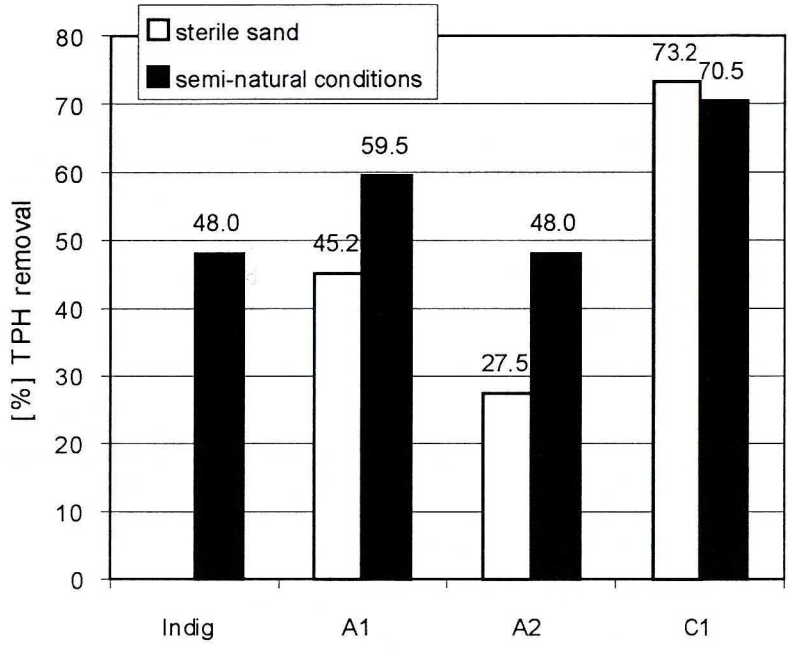

Fig. 3. Influence of bioaugmentation with mixed culture on TPH degradation in sterile and non-sterile sand polluted with 5% of crude oil

Changes of BOD were observed during experiments and were correlated with the removal rate. In sterile sand, the lowest oxygen demand was observed in sample inoculated with A2 culture (Fig. 4). The value was up to 61.6 mg O_2/dm^3. A bit higher activity was measured in sample inoculated with culture A1 (369.6 mg O_2/dm^3). From the beginning of the experiment the highest oxygen demand was noticed in sample inoculated with culture C1. After 1st day BOD for that culture was 9 times higher than value noticed in the rest of samples. On 11th day of experiment BOD was 1540 mg O_2/dm^3 and later exceeded measurable value of OxiTop head.

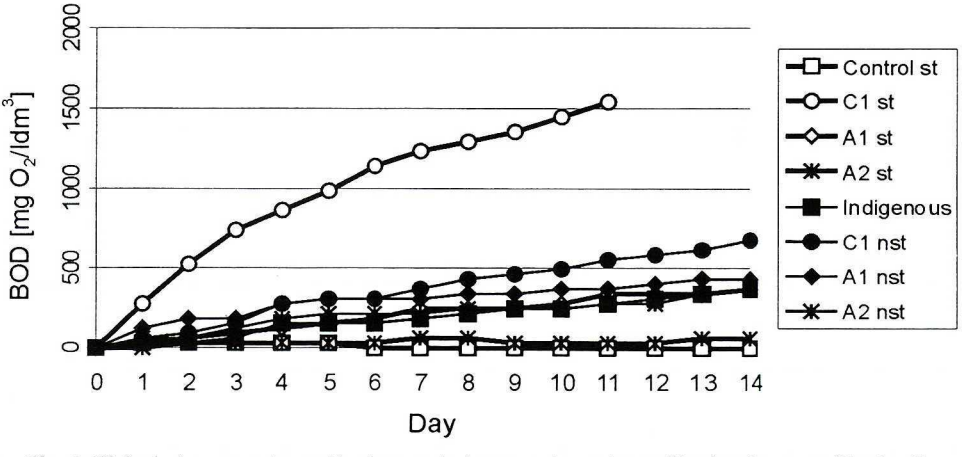

Fig. 4. Biological oxygen demand's changes during experiment in sterilized and non-sterilized soil polluted with crude oil

In non-sterile sand BOD was increasing also in the control sample (Fig. 4). Measured BOD values during the experiment were the same till 3rd day of experiment. A slightly higher oxygen demand was observed in sample with culture A2. After the first day the highest BOD value was noticed in sample inoculated with culture A1 (123 mg O_2/dm^3). At the end of the experiment the oxygen demand noticed in this sample was 431 mg O_2/dm^3 and the highest one was in the sample inoculated with culture C1 (616 mg O_2/dm^3).

Adhesion of cultures to sand and production of biosurfactants

Very low adhesion of cultures to sand grains was observed (Fig. 5). The highest value, about 5%, was noticed for culture A1 and C1 where no adhesion was observed for culture A2 (0.4%).

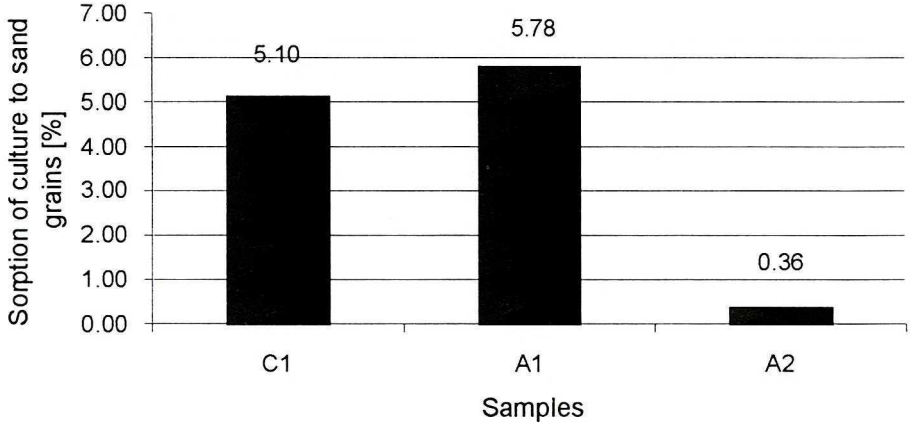

Fig. 5. Adhesion of culture to sand grains [%]

DISCUSSION

The goal of this research was to find the best group of crude oil degraders taken from different sources which enhance biodegradation in the marine and coastal environments. Three different mixed cultures were used. The first experiment was done with artificial seawater and showed that culture A1 isolated from the refinery sludge from Aspropygros is the culture that contains the best degraders. Degradation in seawater was 64.4% during 14 days of experiment. This high degradation was correlated with very high oxygen demand observed after the 3rd day (950 mg O_2/dm^3 on the 4th day), final OD_{660} value of 3.1, increase in number of bacteria (from 1.14×10^5 cfu/cm^3 at the beginning up to 1.28×10^9 cfu/cm^3 on day 14th) and high biosurfactant productivity (increase of drop's diameter taken by drop-collapsing test was 44%). The lowest values and degradation rate, despite high increase of OD (up to 2.7), during this experiment was observed for culture A2. Degradation was 57% and increase of bacteria number was from 5.00×10^5 to 5.70×10^7 cfu/cm^3. These results show that except OD it is necessary to follow other parameters. A high degradation (61.2%) and oxygen demand were observed also in sample inoculated with culture C1 and correlation with increase of bacteria's number, form 1.40×10^4 to 2.00×10^8 cfu/cm^3, was noticed. During the experiment a biosurfactant production was observed in all tested cultures. Also this culture, like A1 and A2, showed biosurfactant production but an increase of drop diameter was only 32%.

A production of surfactants by bacteria and fungi is often observed during cultivation on media with hydrocarbons [18, 22, 30, 35, 37]. This is an important factor that can increase biodegradation of hydrocarbons and usage of surfactants is becoming a very popular technique. The application of surfactants increases the solubilization of hydrophobic compounds as well as improves biodegradation in the nonaqueous phase [36]. By decreasing surface tension, the solubility and thus mobility of hydrophobic hydrocarbons is increased which in turn promotes degradation [22].

Sand is normally a nutrient-poor environment for microorganisms and the presence of polluting substances can decrease a number of microorganisms even further, making natural attenuation extremely slow. The aim was to find a group of high efficient hydrocarbon-degraders and introduce them with nutrient supplements (N and P) to polluted sandy beaches in order to enhance biodegradation. Sterile sand was used to see activity and ability of cultures for degradation of TPH and non-sterile sand to see behavior of applied cultures in semi-natural ecosystem. Seawater was used as a medium for dilution and supplementation of biogens. In sand the best degradation of crude oil in sterile and non-sterile conditions characterized culture C1. It was 73.2% in sterile sand and 70.5% in semi-natural conditions. Degradation of crude oil in sterile sand was the lowest for A2 culture (27.5%). The same tendencies were observed in semi-natural conditions.

Addition of seawater and fertilizers to sand had a positive influence on naturally occurring microorganisms. Degradation of TPH by indigenous culture was 48.0% after 14 days of experiment. Seawater washed out crude oil from sand grains and this way made it more accessible for microorganisms. A high level of indigenous microbial activity suggests a potential for biodegradation, especially when addition of fertilizer can relieve environmental nutrient limitations. The positive influence of simple aqueous suspension to enhance degradation was also observed by Abbondanzi et al. [1]. Addition of fertilizers resulted in stimulation of their activity and degradation.

Culture A1 showed a positive influence on TPH degradation in sand. In sterile condition degradation was about 45% and in semi-natural conditions it was about 59%. Bioaugmentation with culture A2 did not increase removal of TPH. It is important to notice that different interactions between different microorganisms as cooperation and antagonism can be observed. These processes might be very important for bioremediation results.

Bioaugmentation is technology which posses a lot of advantages, especially when pollutants are really toxic and a lack of appropriate microorganisms is observed but determination of the potential success of bioaugmentation requires an understanding of some factors such as the survival and activity of the added microorganisms [34]. Experiments with sand were performed in BOD bottles to see oxygen demand during the degradation of hydrocarbons in soil slurry. Respirometry of polluted soil can be a helpful method for evaluation of biological activity and valid alternative to classical biomass determination methods in soil [2, 10]. For some cultures increase of BOD values was correlated with increase of degradation rate. Such tendencies were also observed by Michel et al. [24] and Löser et al. [21] during degradation of diesel-fuel in sandy soil.

The highest oxygen demand was observed for C1 culture isolated from Corinth sludge characterized as culture of the best degraders. At the end of the experiment BOD values for this culture were more than 1540 mg O_2/dm^3 and 677 mg O_2/dm^3, respectively, for sterile and non-sterile conditions. It is necessary to notice also that biomass of microorganisms added to sand was always two times higher in sterile sand than in not-sterile sand. That could

have influenced BOD values. More important than BOD values themselves are tendencies observed in both types of sand. The oxygen demand at the end of the experiment in semi-natural sand inoculated with C1 culture was 1.8 times higher than in control. A1 culture was the next very active culture in sand.

Culture A2 was not adapted to such high pollution – 5% of crude oil in sand (v/w). This culture was isolated on medium containing the lowest concentration of TPH. These results show how important the choice of medium for isolation of microorganisms can be. In sterile and non-sterile sand an activity of this culture was the same like of indigenous microorganisms. In semi-natural conditions addition to natural sand of biogenes and seawater, which washed crude oil from sand grains, resulted in high activity of sand's indigenous microorganisms. It was also correlated with degradation of crude oil. Probably indigenous microorganisms were adapted to pollution by hydrocarbons and contained microorganism degrading hydrocarbons. On the other hand, washing of sand with water resulted with dissolution of some light hydrocarbons fraction. Addition of fertilizer is a well known procedure during remediation of polluted sites. Hydrocarbons pollution changes C:N ratio and leads to the lack of nitrogen and phosphorus that are necessary for biomass production. Addition of these two biogens results in increase of microorganisms' population. Michel et al. [24] noticed that supplementation with N and P increases degradation rates. Results in soil supplemented with these two components were about 15% higher than those without supplementation. Venosa et al. [32], Bachoon et al. [5] and Oh et al. [25] noticed that addition of sufficient amount of inorganic nutrients can be sometimes the most effective treatment for the enhancement of oil degradation. An initial degradation of aliphatic and aromatic hydrocarbons in microcosm with nutrients can be 17 to 40 times higher than in microcosm pure with nutrients [25].

One of the most important factors influencing biodegradation of contaminants in soil is adhesion. This process affects vertical transport, distribution and survival of microorganisms in soil environment. Microorganisms adhere to soil particles by electrostatic interactions and high adhesion is connected with low mobility of strains in environment. Sometimes it can be a useful process during bioaugmentation of polluted soil. The reversibility of bacterial adsorption to soil particles depends on their properties and soil properties [23]. Short test allows noticing that cultures C1 and A1, the most active (BOD) cultures in natural sand, were characterized also as cultures that can adhere to sand grains. This adhesion to sand was only about 5–6%. Culture A2 didn't adhere even in such low percentage. Adhesion of bacteria investigated by Mehmannavaz to garden soil was from 30–83%, so much higher [23]. Probably 5% adhesion will not influence degradation of hydrocarbons in sand.

These researches should be complemented with field scale experiments with natural seawater and coastal sand using large bioreactors with full control capabilities. Slurry phase bioreactors could better improve the contact between microorganisms and contaminants, nutrients, terminal electron acceptors, substrate and microorganisms' distribution [15]. Also some toxicological tests should be performed because addition of microbial cultures can have toxic effect because of interactions between native and introduced microorganisms and accumulation of chemicals (semi-finished products) of degradation with greater toxicity than those recoverable from untreated pollutants [3, 29].

CONCLUSIONS

Results of all performed experiments showed that all consortia isolated from refinery sludge survived in high concentrations of crude oil (5%) in seawater and they were able to degrade partly within the 14 days of culture period. The most effective in seawater after a crude oil spill is culture A1 isolated from refinery sludge from Aspropyrgos (Greece). This culture after 14 days of experiment degraded crude oil by 65–71%, even when concentration of nitrogen and phosphorus was lower than suggested C:N:P 100:10:1 for bioremediation of sites polluted with hydrocarbons. The most effective culture for bioaugmentation of seawater does not have to be the best solution for bioaugmentation of sand. The highest elimination of TPH in sand was observed after inoculation with culture C1 isolated from Corinth sludge.

Concentration of pollutant in media during the enrichment of hydrocarbon-degraders was found to be an important factor. It has to be sufficient to create consortia with sufficient resistance to pollution and degradation potential. Bacteria enriched on media with higher concentration of pollutant were characterized as better degraders and producers of biosurfactants.

The analysis of the parameters governing the growth of cultures led to conclusion that oxygen demand is a good tool to show activity of microorganisms and portrays well the degradation capabilities of microorganisms during preliminary tests.

Experiments performed with different environments (seawater and sand) and different conditions (sterilized material and semi-natural conditions) confirmed the results of other authors. Cultures should be tested in semi-natural conditions, especially when indigenous microfloras can posse's high degradation potential. In such cases, it is enough to stimulate this indigenous microflora by addition of sufficient amount of fertilizers. Allochtonic cultures, very active in sterile conditions, after inoculation to natural environment can even slow down degradation.

ACKNOWLEDGMENT

This research has been supported by the Marie Curie Development Host Fellowship program "Ex-situ Bioremediation of Petroleum-Contaminated Sands Using Hydrocarbon-Degrading Marine Microbes" (contract number HPMD-CT-2001-00060).

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Received: November 18, 2005; accepted: February 13, 2006.