

BIOREMEDIATION OF PETROLEUM CONTAMINATED SANDS WITH BACTERIA CULTURES

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Received: 01.03.2010; Revised: 08.04.2010; Accepted: 15.04.2010

Abstract

Accidental oil spills at open sea is a common environmental problem. However, we are faced with a much more severe problem once the oil spill reaches the shoreline. Current oil spill combating practice is to collect manually the polluted sand and proceed with disposal or temporary storage in nearby sites.

Addition of mixed bacterial cultures into petroleum contaminated sand was examined. Experiments with sand polluted by 5% (v/w) of crude oil were performed in sterile and non-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 was monitored. Correlation between high oxygen demand and high depletion of hydrocarbons was observed. The best TPH removal in sterile conditions was found in samples with the mixed cultures isolated from waste sludge from petroleum refinery Motor Oil Hellas (Korinth Refineries, Greece). For this culture concentration of hydrocarbons in sterile sand was 73.2% lower than in control sample. In non-sterile sand concentration of TPH after 14 days was 70.5% lower than in control (sterile sand) without bioaugmentation. The lowest depletion of hydrocarbons was observed in sample with addition of mixed culture (AM) of *Alcanivorax borcumensis* (DSM 11573) and *Marinobacter hydrocarbonoclasticus* (DSM 8798) (32.9%). Finally, the addition of artificial seawater and fertilizers had also a positive influence on contaminants depletion by naturally occurring microorganisms (48%).

Streszczenie

Powszechnym problemem środowiskowym są przypadkowe wycieki paliw na otwartym morzu. Z jeszcze bardziej uciążliwym problemem spotykamy się jednakże w momencie dotarcia wycieku do wybrzeża. Obecne praktyki rekultywacyjne polegają na zebraniu zanieczyszczonego piasku i jego usunięciu lub okresowym składowaniu na pobliskich terenach. Celem badań było określenie wpływu inokulacji mieszanymi kulturami bakteryjnymi piasku zanieczyszczonego surową ropą naftową. Badania prowadzono na piasku zanieczyszczonym 5% (v/w) surowej ropy naftowej w warunkach sterylnych i niesterylnych tak, aby zaobserwować zdolność wyizolowanych kultur do degradacji zanieczyszczenia, scharakteryzować ich wzrost oraz możliwe antagonizmy między wprowadzonymi mikroorganizmami a naturalną mikroflorą. Zaobserwowano dodatnią korelację między ubytkiem tlenu i wysokim ubytkiem węglowodorów. Najwyższy stopień usunięcia TPH w warunkach sterylnych zaobserwowano w próbach z mieszaną kulturą wyizolowaną z osadu pochodzącego z rafinerii Motor Oil Hellas (Korinth Refineries, Grecja). Stężenie ropopochodnych w próbce z tą kulturą i sterylnym piaskiem było o 72.3% niższe niż w kontroli. W warunkach niesterylnych stężenie TPH po 14 dniach było niższe niż w kontroli (piasek sterylny) o 70.5%. Najniższy ubytek węglowodorów zaobserwowano w próbach zaszczerpionych mieszaniną kultur (AM) zawierających *Alcanivorax borcumensis* (DSM 11573) i *Marinobacter hydrocarbonoclasticus* (DSM 8798) (32.9%). Wreszcie zaobserwowano, iż dodatek sztucznie spreparowanej wody morskiej i biogenów miały również pozytywny wpływ na ubytek zanieczyszczeń spowodowany działalnością autochtonicznych mikroorganizmów (48%).

Keywords: Crude oil; Sand remediation; Bioaugmentation; Marine bacteria.

1. INTRODUCTION

Open sea and seashore are environments, which are frequently exposed to crude oil pollution. 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 [1,2]. Crude oil and petroleum products introduced to the marine environment are immediately subjected to a variety of physical, chemical and biological changes. Abiotic weathering processes include evaporation, dissolution, dispersion, photochemical oxidation, water-in-oil emulsification, adsorption onto suspended particle material, sinking, sedimentation and biological processes including ingestion by organisms as well as microbial degradation. These processes occur simultaneously and cause important changes in the chemical composition and physical properties of the original pollutant [1-4].

Increased environmental pollution necessitates the development of new cost-effective and fast method of remediation. Among them biological methods using naturally occurring bacteria are expected to have the smallest negative influence on the environment during the remediation process. They mostly involve minimal physical disruption of a site, appear to have no or only minor and short-lived adverse effects when properly used, often they can be carried out on-site, and they offer a simpler and more economical solution for the remediation of polluted areas. They are cost-effective because they are less labor intensive and simpler to use, however, they must be specifically tailored to each polluted site [3, 5, 6].

Bacteria able to degrade hydrocarbons occur in all environments but in different numbers and community structures [7-9]. Generally hydrocarbon utilizers constitute less than 0.1% of the microbial community in unpolluted ecosystems. The highest concentration even up to 100% is observed in environments constantly exposed to hydrocarbon pollution as refinery areas [8-14]. Adaptation to high concentration of pollutants results in high resistance to toxic compounds and high degradation potential of microorganisms especially when these sites are exposed to pollution for long time. Communities exposed to hydrocarbons become adapted exhibiting selective enrichment and genetic changes [10].

Many indigenous microorganisms are capable to degrade petroleum hydrocarbons such as alkanes, paraffins and aromatics, but in marine environment biodegradation rates are low and often limited by such environmental factors as bioavailability, molecular oxygen, and mostly by phosphate and nitrogenous nutrients [15]. Stimulation of indigenous organisms by addition of nutrients is the most promising alternative to combat most of sea and coast oil spills [3, 12, 16-20]. Also introduction of microbes is beneficial in areas where native organisms grow slowly or are unable to degrade a particular hydrocarbon [3]. However, it is necessary to remember that addition of the microorganisms in products sometimes neither inhibits nor enhances contaminant removal [21]. For soil remediation, a good tool is slurry-phase bioremediation where contaminated soil is combined with water and other additives in a large bioreactor and mixed to keep the microorganisms – which are already present in the soil or added – in contact with the contaminants in the soil [22]. This slurry-phase biological treatment can be a relatively rapid process compared to other biological treatment processes, particularly for contaminated clays. This technology is particularly useful where rapid remediation is a high priority as it provides better conditions for mass transfer by facilitating better contact between contaminated soil and the microorganism [22, 23].

Environmental conditions play a major role in the level of the biological activity of indigenous microorganisms or microorganisms transferred from other sites, or pre-cultured microorganisms returned to the contaminated site. These conditions fall into two general categories: those that affect directly the microbial activity, such as temperature, humidity and ionic strength, and those that alter the mass transfer rate of the compound from the bulk to the surface of the microorganisms, for example, clay and organic-matter content, nutrients content, bioavailability and toxicity of pollutant [24, 25]. In sandy beaches, the particle size of sand grains influences decomposition of the contaminating hydrocarbons. A lower decomposition was observed in sand with finer sand particles probably due to poorer drainage and persistent anaerobic conditions. The porosity of sand in connection with moisture content and gas pressure may affect the oxygen concentration [7, 26]. Löser et al. [27] showed that hydrocarbons may be strongly adsorbed even on coarse-grained and organic-free soils in microporous spaces and hence, they are no longer bioavailable to hydrocarbon-degrading microorganisms. This portion of hydrocarbons

remains in place after biological remediation [7, 24]. Salinity is another factor particularly for contaminated sea and coastal areas. It is mostly connected with the reduction of the metabolic activity of many microorganisms [28]. High salinities or wide salinity ranges make oil pollution difficult to treat using conventional bioremediation methods. It disrupts cell membranes, denaturates enzymes, and results in desiccating osmotic forces that are lethal to many microorganisms. In these cases halotolerant or halophilic microorganisms must be used and the physicochemical factors must be optimized to allow successful bioremediation [11, 15, 25, 28, 29]. Diaz et al. [11, 29] proved that crude oil can be degraded even at NaCl concentrations of up to 100 g/L.

Successful bioremediation of oil spills in marine and saline environments has often been observed [30, 31]. Microorganisms able to grow in the presence of salt are found in all three domains of life: Archea, Bacteria and Eukarya [30]. Yakimov et al. [32] isolated biosurfactant-producing, n-alkane-degrading, Gram-negative, aerobic, rod-shaped marine bacteria. *Marinobacter hydrocarbonoclasticus* is another marine bacterium, isolated from Mediterranean Sea near a petroleum refinery by Gauthier et al. [33] with the ability to use various hydrocarbons as the sole carbon and energy source. Dutta and Harayama [34] found that *Alcanivorax* sp. transformed alkylated hydrocarbons. The results of Kasai et al. [19] showed that *Alcanivorax* sp. and *Cycloclasticus* sp. are two major populations in chronically polluted environments with petroleum hydrocarbons. The first one is able to degrade alkanes whereas the second one is also involved in the degradation of aromatic compounds. Both of them mixed with other microorganisms can and have been used in bioremediation techniques [19]. This confirms Watanabe [35] suggestion that some groups of bacteria commonly occur in oil-contaminated marine environment, although other populations change under different environmental conditions. Marine aerobic bacteria used for bioremediation purposes of marine contaminated sites were found among the following genera: *Alteromonas*, *Pseudomonas*, *Moraxella*, *Bacillus*, *Flavobacterium*, *Mycobacterium* and *Vibrio* [36]. Marine bacteria including members of genera *Cycloclasticus*, *Flavobacterium*, *Marinobacter*, *Moraxella*, *Pseudomonas*, *Sphingomonas* and *Vibrio* are also well known as PAHs degraders [19].

The aim of this research was to find the best combination of microorganisms to use for bioaugmentation of crude oil polluted sandy beaches.

2. MATERIALS AND METHODS

Unpolluted sand was collected from one of the beaches in northwestern Crete. Experiments were performed in triplicate in dark sterile BOD bottles (1 L.) equipped with OxiTop measuring head. Two base modifications of experiments were using sterile sand and not-sterile sand to see biodegradation of crude oil and changes in microorganisms' activity also in non-sterile conditions.

To each bottle 50 g of sand was added. To each of bottle, 2.5 ml of autoclaved (121°C at 1.5 atm. for 25 min.) crude oil (1.91 g) and 20 ml of autoclaved artificial seawater supplemented with nitrogen N and phosphorus were added. Crude oil was purchased from petroleum refinery Motor Oil Hellas (Korinth Refineries, Greece).

Artificial seawater was prepared with recipe based on Difco Artificial Sea Water. Media contained: NaCl (24g/L), MgCl₂*6H₂O (11g/L), Na₂SO₄ (4g/L), CaCl₂*6H₂O (2g/L), KCl (0.7g/L), KBr (0.1g/L), H₃BO₃ (0.03g/L) (all for preparing artificial seawater). As source of N and P (NH₄)₂SO₄ in concentration 1g/L, K₂HPO₄ in concentration 0.8g/L and KH₂PO₄ in concentration 0.2g/L were used, pH was adjusted to 7.0.

Seven days bacterial cultures pregrown on Marine Broth (Difco) with 1% of crude oil were centrifuged and resuspended in used media (artificial sea water). Before centrifugation crude oil was collected from the surface by pipette. OD₆₆₀ of media with cultures was measured (data shown in table 1). Modifications used in sterilized and non-sterilized conditions were the same: C (control without bioaugmentation), K (culture isolated from waste sludge from petroleum refinery Motor Oil Hellas (Korinth Refineries, Greece)), PR (culture isolated from refinery sludge from Athens on media saturated with gasoline), COR (culture isolated from refinery sludge from Athens on media saturated with crude oil), PM culture isolated from wastewater treatment plant in Chania on media saturated with gasoline), AM (mixture of pure cultures of *Alcanivorax borcumensis* and *Marinobacter hydrocarbonoclasticus* mixed in ratio 1:1). *Alcanivorax borcumensis* (DSM 11573) and *Marinobacter hydrocarbonoclasticus* (DSM 8798) strains were purchased from German Collection of Microorganisms and cell culture. Bottles with sand, artificial seawater and bacteria were stirred and incubated at 27°C for 14 days. Biological oxygen demand was measured every day and calculated with formula showed below. After the

end of experiment samples were collected and kept frozen till time of TPH (FT-IR) measurement.

$$BOD = \frac{M(O_2)}{R \cdot T_m} \cdot \left(\frac{V_{tot} - V_l}{V_l} + \alpha \frac{T_m}{T_0} \right) \cdot \Delta p(O_2) \quad (1)$$

$M(O_2)$ – molecular weight of oxygen (32 000 mg/mol),
 R – gas constant (83.144 l.mbar/mol.K),
 T_0 – Reference temperature (273.15 K),
 T_m – Measuring temperature,
 V_{tot} – Bottle volume (theoretical volume) [ml],
 V_l – Volume of sample [ml],
 α – Bunsen absorption coefficient (0.03103),
 $\Delta p(O_2)$ – Difference of the partial oxygen pressure [hPa].

Extraction of sand samples was done using sonication with guidelines that for 10g sand sample 10 ml of CCl_4 (with 3g 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 path-length liquid cell (Graseby Specac ZnSe 7009) with pathlength 0.05 cm. Spectrum for TPH concentration calculation was taken between 3100 and 2750 cm^{-1} .

2.1. Adhesion of cultures to sand and production of biosurfactants

Additional test of adhesion of bacteria to sand was done with the use of the method described by *Huysman and Verstraete* [37] and *Mehmannavaz et al.* [38]. All five mixed cultures of bacteria were used in this experiment: K, PR, PM, COR and A+M. All cell cultures were firstly pregrown on Nutrient Broth (Fluka) prepared with addition of NaCl to get the end concentration of this salt about 2.5% (w/v). After 24 h of cultivation cultures were centrifuged and resuspended in physiological salt (8.5 g NaCl/L) to get the end OD_{600} about 0.34-0.39. Ten milliliter of microorganism’s suspension was added to 1 g of ster-

ile sand and vigorously shaken for 1 minute. As a control 1 g of sterilized sand with 10 ml of physiological salt was used. All test were done in triplicate. After 15 minutes of settlement OD_{660} was measured taking one milliliter of the aqueous layer from the top of sample. Following formula was used for percentage adhesion calculation.

$$\frac{[OD_i - (OD_e - OD_c)]}{OD_i} * 100 \quad [\%] \quad (2)$$

OD_i – OD_{660} of initial suspension,
 OD_e – average OD_{660} of end suspension,
 OD_c – average OD_{660} of control suspension.

Three cultures of the best degraders (K and PR) and one of the worst degraders (culture COR) were tested for biosurfactants production with drop-collapsing test [39, 40]. Cultures used for test were firstly pregrown on artificial seawater supplemented with N and P sources as previously and with 5% of crude oil (v/v). After 14 days crude oil was collected from samples surface. Test was performed using a 10- μ l drop of each enriched bacterial culture placed on a surface of the top of a petri dish immersed in paraffin. The diameter of the each drop was compared to a diameter of water’s drop. The profile of each drop was also compared.

Statistical analyses were done with the Statistica 5.1 software package.

3. RESULTS

As shown in Fig. 1, the depletion of TPH after 14 days of incubation in sterile sand ranged between 27.5 and 73.2%. The highest depletion of crude oil in sand was observed for Korinth culture (K=73.2%). This culture was isolated with different method than cultures PM, COR and PR and on media with higher concentration of TPH. The lowest depletion was noticed for culture isolated during enriched experiment from Athens refinery sludge on crude oil (COR=27.5%). Another good culture of degraders was AM mixture of two pure strains (*Alcanivorax borcumensis* (DSM 11573) and *Marinobacter hydrocar-*

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

	C	A+M	K	PM	PR	COR
Sterilized sand	0.00	0.2035	0.5724	0.4550	0.4040	0.4300
Non-sterilized sand	0.00	0.0849	0.2650	0.2398	0.2397	0.2238

bonoclasticus (DSM 8798)) known as organisms with ability for pollutants removal. The loss of TPH after

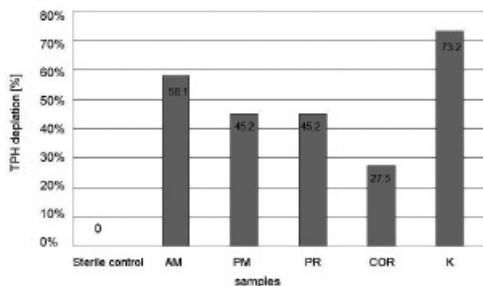


Figure 1.
Influence of bioaugmentation with mixed culture on TPH depletion [%] in sterile sand polluted with 5% of crude oil

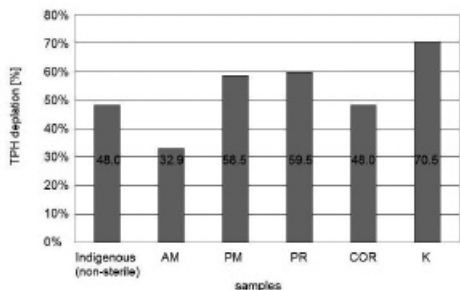


Figure 2.
Influence of bioaugmentation with mixed culture on TPH depletion in non-sterile sand polluted with 5% of crude oil

14 days of cultivation was 58.1%. Mixed cultures isolated on media with gas oil during enrichment, from municipal sludge (PM) and refinery sludge (PR), resulted in losses of TPH by about 45.2%.

In non-sterile conditions the highest depletion of crude oil was also observed with culture K. The depletion of TPH with culture K compared to the control was 70.5% (Fig. 2). The depletion observed with cultures PM and PR was about 58-60%, whereas bioaugmentation of sand with mixture of pure bacteria's cultures AM and mixed culture COR did not increase depletion. In sample inoculated with AM culture (*Alcanivorax borkumensis* (DSM 11573) and *Marinobacter hydrocarbonoclasticus* (DSM 8798)) depletion rate was lower than by indigenous culture with addition only of salt water, nitrogen and phosphorus sources (32.9 and 48.0 respectively).

Changes of BOD were observed during experiments. In sterile sand the lowest oxygen demand was observed in sample inoculated with COR culture (Fig. 3). Value was up to 61.6 mgO₂/l. A bit higher activity was measured in samples inoculated with cul-

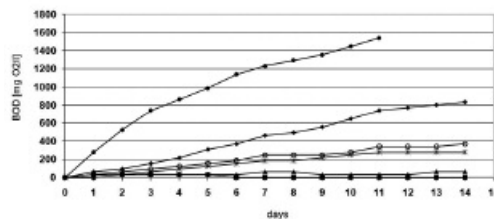


Figure 3.
Biological oxygen demand's changes during experiment in sterilized soil polluted with crude oil
* After 11th day for sample K value was higher than measurable by OxiTop measuring heads

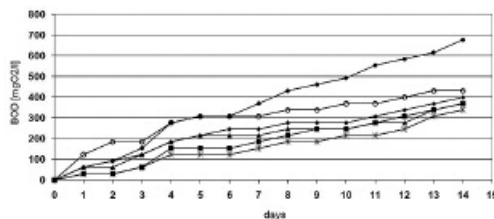


Figure 4.
Biological oxygen demand's changes during experiment in non-sterilized soil polluted with crude oil

ture AM ((A+M) of *Alcanivorax borkumensis* (DSM 11573) and *Marinobacter hydrocarbonoclasticus* (DSM 8798)) and PR (up to 277.2 and 369.6 mgO₂/l respectively). From the beginning of experiment the highest oxygen demand was noticed in sample inoculated with culture K, the culture with the highest depletion of TPH. After 1st day BOD for that culture was 4.5 times higher than in sample PM and 9 times higher that value noticed in the rest of samples. At 11th day of experiment BOD was 1540 mgO₂/l and later exceed measurable value of OxiTop head.

In non-sterile sand BOD was increasing also in control sample (Fig. 4). Measured BOD values during the experiment were the same till 3rd day of experiment and later even higher than in sample inoculated with mixture of *Alcanivorax borkumensis* and *Marinobacter hydrocarbonoclasticus*. Slightly higher oxygen demand was observed in sample with culture COR and PM. After first day the highest BOD value was noticed in sample inoculated with culture PR (123 mgO₂/l). At the end of experiment the highest oxygen demand was noticed in sample inoculated with culture K (616 mgO₂/l) and PR (431 mgO₂/l). BOD in the rest samples including the culture with only the indigenous population was about 308-370 mgO₂/l after 14 days.

3.1. 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 PR and K. No adhesion was observed for the rest of cultures (culture COR was adhered in 0.4%, and PM, AM (*Alcanivorax borcumensis* (DSM 11573) and *Marinobacter hydrocarbonoclasticus* (DSM 8798)) in less than 0.1%).

Cultures of the best degraders (K and PR) and one of the worst degrading cultures (COR) were tested with drop-collapsing test for ability of biosurfactants production (Fig. 6). All tested cultures had ability to produce surfactants. The highest increase of drop diameter was observed for culture PR (44%). Cultures K and COR were characterized by lower production of biosurfactants. The increased drop diameter was 32.4 and 35.3% respectively.

4. DISCUSSION

Sand is normally a nutrient-poor environment for microorganisms and the presence of polluting substances can decrease number of microorganisms even further, making natural attenuation extremely slow. The aim was to find one or two groups of high efficient hydrocarbon-degraders and introduce them with nutrient supplements (N & P) to polluted sandy beaches to enhance biodegradation. Sterile sand was used to see activity and ability of cultures for depletion of TPH and non-sterile sand to see behavior of applied cultures in non-sterile ecosystem. Artificial seawater was used as a medium for dilution and supplementation of biogens. Tested cultures were mostly from polluted areas as refinery sludge. For comparison purposes, an AM mixture of pure culture of *Alcanivorax borkumensis* (DSM 11573) and pure culture of *Marinobacter hydrocarbonoclasticus* (DSM 8798) were used. The best depletion of crude oil in sterile and non-sterile conditions characterized culture K isolated from refinery waste sludge from petroleum refinery Motor Oil Hellas (Korinth Refineries, Greece). It was 73.2% in sterile sand and 70.5 in non-sterile conditions. Depletion of crude oil in sterile sand was lowest for COR culture (27.5%). The same tendencies were observed in non-sterile conditions. High depletion of crude oil during 14 days of experiment was also observed in samples inoculated with mixture of two pure bacteria cultures (*A. borkumensis* (DSM 11573) and *M. hydrocarbonoclasticus* (DSM 8798) signed as AM and it was almost 60% in sterile sand. *Alcanivorax* is well known bacte-

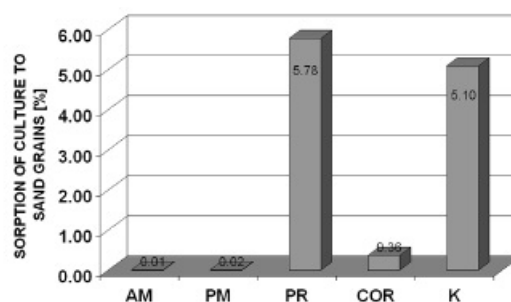


Figure 5. Adhesion of culture to sand grains [%]

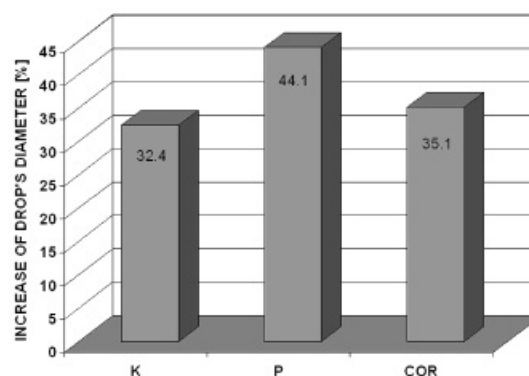


Figure 6. Biosurfactants' activity measured by drop-collapsing test

ria that take primary role during degradation of alkanes in saline conditions. Also *Marinobacter* can degrade hydrocarbons [25, 32, 33]. In not sterile sand depletion was lower than by indigenous cultures of microorganisms (32.9 and 48.0 respectively). *Rahman et al.* [8, 9] observed that individual bacteria cultures showed less growth and degradation than mixed bacterial consortia. An effective degradation of crude oil would require simultaneous action of several metabolically versatile microorganisms [8].

It is important to notice that different interactions between different microorganisms, as cometabolism and antagonism, can be observed under mixed culture conditions especially after bioaugmentation. These processes might be very important for bioremediation results. Mostly biodegradation of toxic organic compounds by the bacterial consortium is higher than by single strain. Intermediates produced by one organism can facilitate total removal of pollutant strain but antagonistic relations can make it lower [14].

Depletion of TPH by indigenous culture was 48.0% after 14 days of experiment. Microscopic studies of sand from samples showed that artificial seawater

washed out almost all crude oil from sand grains. The positive influence of simple aqueous suspension to enhance degradation was observed also by *Abbondanzi et al.* [41]. Addition of fertilizers results in stimulation of their activity and degradation [4,6].

Two other cultures PM and PR enriched on media saturated with petroleum hydrocarbons showed positive influence on TPH depletion in sand. In sterile condition depletion was about 45% and in non-sterile conditions it was about 59%. The high level of indigenous microbial activity suggests a potential for biodegradation, especially when addition of fertilizer can relieve environmental nutrient limitations [6, 24].

Bioaugmentation is technology which has got many advantages especially when pollutants are really toxic and lack of appropriate microorganisms is observed but determination of the potential success of bioaugmentation requires understanding of some factors like survival and activity of added microorganisms [17, 24]. 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 helpful method for evaluation of biological activity and valid alternative to classical biomass determination methods in soil [42, 43]. For some cultures increase of BOD values was correlated with increasing depletion rate. Such tendencies were also observed by *Michel et al.* [44] and *Löser et al.* [45] during degradation of diesel-fuel in sandy soil.

The highest oxygen demand was observed for K culture isolated from waste sludge from petroleum refinery Motor Oil Hellas (Korinth Refineries, Greece) characterized as culture of the best degraders. At the end of experiment BOD values for this culture were more than 1540 mgO₂/l and 677 mgO₂/l respectively for sterile and non-sterile conditions. It is also necessary to notice that biomass of microorganisms added to sand was always two times higher in sterile sand than in non-sterile sand. That could have influence of BOD values. More important than BOD values themselves are tendencies observed in both types of sand. The oxygen demand at the end of experiment in non-sterile sand inoculated with K culture was 1.8 times higher than in control. PM culture was the next very active culture in sterile sand but in natural sand PR culture was the most active. The activity of PM culture in sterilized sand resulted in BOD of about 801 mgO₂/l and in non-sterile conditions it was almost the same as in control without bioaugmentation (BOD 370 mgO₂/l and 339 mgO₂/l for culture and control respectively).

At the end of experiment BOD for culture PR was 339 and 431 mgO₂/l in sterile and non-sterile sand respectively. In both types of sand for AM mixture of cultures of *Alcanivorax borkumensis* (DSM 11573) and *Marinobacter hydrocarbonoclasticus* (DSM 8798) increase of BOD was not observed and all the time they were close to control sands. *Molina et al.* [18] noticed that abundance of oil-degrading bacteria was not consistent with the extent of oil degradation and even low number of bacteria was able to degrade hydrocarbons. Also *Aldrett et al.* [21] noticed that high microbial counts were not indicative of higher degradation extent.

Some cultures were not adapted to such high pollution (5% of crude oil in sand (v/w)) as for example COR culture. In sterile and non-sterile sand activity of this culture was the same as of indigenous microorganisms. In non-sterile conditions biogenes and artificial seawater, which washed crude oil from sand grains, added to natural sand, resulted in high activity of sand indigenous microorganisms. It was also correlated with degradation of crude oil. Probably indigenous microorganisms were adapted to pollution by hydrocarbons and washing of sand with water resulted with dissolution of some hydrocarbons fraction. Addition of fertilizer is well known procedure during remediation of polluted sites. Hydrocarbons pollution causes 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.* [44] noticed that supplementation with N and P increase degradation rates. Results in soil supplemented with these two components were about 15% higher than without supplementation. *Venosa et al.* [46], *Bachoon et al.* [12] and *Oh et al.* [47] noticed that addition of sufficient amount of inorganic nutrients can be sometimes the most effective treatment for the enhancement of oil degradation. Initial degradation of aliphatic and aromatic hydrocarbons in microcosm with nutrients can be 17 to 40 times higher than in microcosm pure with nutrients [47].

One of the most important factors influencing biodegradation of contaminants in soil is adhesion. This process has influence on 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 useful process during bioaugmentation of polluted soil. The reversibility of bacterial adsorption to soil particles depends on their

properties and soil properties [38]. Short test allow to notice that cultures K and PR, the most active (BOD) cultures in natural sand, were characterized also as cultures that can to the highest extent adhere to sand grains. This adhesion to sand was only about 5-6%. Other cultures didn't adhere even in such low percentage. Adhesion of bacteria investigated by *Mehmannavaz* to garden soil was from 30-83%, much higher [38] Probably 5% adhesion will not influence on degradation of hydrocarbons in sand.

Production of surfactants by bacteria and fungi is often observed during cultivation on media with hydrocarbons [30, 32, 48-50]. This is important factor that can increase biodegradation of hydrocarbons. Biosurfactants enhance the remediation of oil-contaminated soil and water by decreasing surface tension. They increase the solubility and by thus mobility of hydrophobic hydrocarbons which may promote degradation [38, 50-53].

Drop collapsing test was performed for 3 cultures with different potential for degradation of petroleum hydrocarbons (K, COR and PR cultures). All of these cultures had ability to produce biosurfactants. The highest activity was observed for PR culture (44.1% increase of drop diameter) and the lowest for K culture (32.4% increase of drop diameter). Biosurfactant production can help microorganisms during biodegradation of pollutants but it is not the most important factor. Culture COR was good producer of biosurfactants but was the worst mixed culture of degraders. Another explanation is difference between soil and water environment. Microorganisms that can degrade hydrocarbons in seawater really well don't have to play dominant role in degradation of TPH in soil (data not shown).

This research should be complemented with field scale experiments using large bioreactors with full control capabilities. A fully instrumented slurry-phase bioreactor can offer such conditions. Slurry phase bioreactors improve the contact between microorganisms and contaminants, nutrients, terminal electron acceptors, substrate and microorganisms' distribution [54]. Also some toxicological tests should be performed. Addition of microbial cultures can have toxic effect because of interactions between native and introduced microorganisms and accumulation of chemicals (intermediates) of degradation with greater toxicity than those recoverable from untreated pollutants [21, 31].

5. CONCLUSIONS

The previous results and the subsequent discussion lead to the following conclusions:

1. Hydrocarbon degraders with different degradation potential and tolerance to high salinity can be isolated from different environments including places with very high concentration of pollutant as refinery sludge and also other sites less polluted with petroleum hydrocarbons as municipal wastewater treatment sludge.
2. Type and concentration of pollutant during the enrichment of hydrocarbon-degraders is important and have to be sufficient to create consortia with resistance to pollution and depletion potential. Bacteria enriched on media with higher concentration of pollutant were better degraders.
3. Mixed cultures of microorganisms isolated from high-polluted areas are better degraders of hydrocarbons than cultures containing one or mixture of two pure strains.
4. Cultures for bioaugmentation before application in environment should be examined for individual pollutant, environment, place and content of contaminants. Cultures should be tested in non-sterile conditions especially when indigenous microflora posses high depletion 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 in natural environment can even inhibit pollutant removal.

ACKNOWLEDGEMENT

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

REFERENCES

- [1] *Epstein P.R., Selber J.*; OIL - A Life cycle analysis of its health and environmental impacts, The Center for Health and the Global Environment Harvard Medical School, March 2002
- [2] *Alvinage E., Stoica D., Iversen K.*; Oil Pollution in the Baltic Sea and the Effects on Fish and Fisheries – Something to worry about?, Environmental Studies, Aarhus University, 2001

- [3] U.S. Congress, Office of Technology Assessment. Bioremediation for Marine Oil Spills-Background Paper, OTA-BP-O-70 Washington, DC: U.S. Government Printing Office, 1991
- [4] *Otremba Z., Toczek H.*; Degradation of Crude Oil Film on the Surface of Seawater: the Role of Luminous, Biological and Aquatorial Factors, Polish Journal of Environmental Studies Vol.11, No.5, 2002; p.555-559
- [5] *Jones W.R.*; Practical application of marine bioremediation, Current Opinion in Biotechnology 9, 1998; p.300-304
- [6] *Piehler M.F., Swistak J.G., Pinckney J.L., Pearl H.W.*; Stimulation of diesel fuel biodegradation by indigenous nitrogen fixing bacterial consortia, Microbial Ecology 38, 1999; p.69-78
- [7] *Rowland A.P., Lindley D.K., Hall G.H., Rossall M.J., Wilson D.R., Benham D.G., Harrison A.F., Daniels R.E.*; Effects of beach sand properties, temperature and rainfall on the degradation rates of oil in buried oil/beach sand mixtures, Environmental Pollution 109, 2000; p.109-118
- [8] *Rahman K.S.M., Rahman T., Lakshmanaperumalsamy P., Banat I.M.*; Occurrence of crude oil degrading bacteria in gasoline and diesel station soils, J. Basic Microbiol. 42, 4, 2002; p.284-291
- [9] *Rahman K.S.M., Thahira-Rahman J., Lakshmanaperumalsamy P., Banat I.M.*; Towards efficient crude oil degradation by a mixed bacterial consortium, Biosource Technology 85, 2002; p.257-261
- [10] *Mohandass C., David J.J., Nair S., Loka Bharathi P.A., Chandramohan D.*; Behavior of marine oil-degrading bacterial population in a continuous culture system, J. Mar. Biotechnol. 5, 1997; p.168-171
- [11] *Diaz M.P., Grigson S.J.W., Peppiatt Ch.J., Burgess J.G.*; Isolation and Characterization of Novel Hydrocarbon-Degrading Euryhaline Consortia from Crude oil and Mangrove Sediments, Marine Biotechnology 2, 2000; p.522-532
- [12] *Bachoon D.S., Araujo R., Molina M., Hodson R.E.*; Microbial community dynamics and evaluation of bioremediation strategies in oil-impacted salt marsh sediment microcosms, Journal of Industrial Microbiology and Biotechnology 27, 2001; p.72-79
- [13] *Del'Arco J.P., Franca F.P.*; Influence of oil contamination levels on hydrocarbon biodegradation in sandy sediment, Environmental Pollution 110, 2001; p.515-519
- [14] *Tam N.F.Y., Guo C.L., Tau W.Y., Wong Y.S.*; Preliminary study on biodegradation of phenanthrene by bacteria isolated from mangrove sediments in Hong Kong, Marine Pollution Bulletin 45, 2002; p.316-324
- [15] *Yang L., Lai C., Sheieh W.K.*; Biodegradation of dispersed diesel fuel under high salinity conditions, Water Res. 34, no 13, 2000; p.3303-3314
- [16] *Piepre D.H., Reineke W.*; Engineering bacteria for bioremediation, Current Opinion in Biotechnology 11, 2000; p.262-270
- [17] *Kotwzan B.*; Assesment and choice of inoculants for the bioremediation of soil contaminated with petroleum products, Ochrona Srodowiska 30, no4, 2008; p.3
- [18] *Molina M., Araujo R., Bond J.R.*; Dynamics of Oil Degradation in Coastal Environments – Effect of bioremediation Products and Some Environmental Parameters, Proceedings of the 1995 U.S. EPA – Annual Symposium on Bioremediation of Hazardous Wastes, 1995; p.32-34
- [19] *Kasai Y., Kishira H., Harayama S.*; Bacteria belonging to the genus Cycloclasticus play a primary role in the degradation of aromatic hydrocarbons released in marine environment, Applied and Environmental Microbiology, November 2002; p.5625-5633
- [20] *Li G., Huang W., Lerner D.N., Zhang X.*; Enrichment of degrading microbes and bioremediation of petrochemical contaminants in polluted soil, Wat. Res. 34, No15; 2000, p.3845-3853
- [21] *Aldrett S., Bonner J.S., Mills M.A., Autenreith R.L., Stephens F.L.*; Microbial degradation of crude oil in marine environments tested in a flask experiment, Wat. Res 31, No.11, 1997; p.2840-2848
- [22] U.S. EPA. A citizen's guide to bioremediation, Solid Waste Emergency Response Agency (5102G), EPA 542-F-96-007, 1996
- [23] *Puskas K., Al-Awadhi N., Abdullah F., Literathy P.*; Remediation of oil-contaminated sandy soil in a slurry reactor, Environment International 21, no4, 1995; p.413-421
- [24] *Vogel T.M.*; Bioaugmentation as a soil bioremediation approach, Current Opinion in Biotechnology 7, 1996; p.311-316
- [25] *Saner M., Bollier D., Schneider K., Bachofen R.*; Mass transfer improvement of contaminants and nutrients in soil in a new type of closed bioreactor, Journal of Biotechnology 48, 1996; p.25-35
- [26] *Garcia-Rivero M., Saucedo-Castaneda G., De Hoyos, Gutierrez-Rojas M.*; Mass Transfer and hydrocarbon biodegradation of aged soil in slurry phase, Biotechnol. Prog. 18, 2002; p.728-733
- [27] *Löser C., Seidel H., Hoffmann P., Zehnsdorf A.*; Bioavailability of hydrocarbons during microbial remediation of a sandy soil, Appl. Microbiol. Biotechnol. 51, 1999; p.105-111
- [28] *Rhykerd R.L., Weaver R.W., McInnes K.J.*; Influence of salinity on bioremediation of oil in soil, Environmental Pollution vol.90 no. 1, 1995; p.127-130
- [29] *Diaz P.M., Boyd K.G., Grinson S.J.W., Burgess J.G.*; Biodegradation of Crude Oil across a Wide Range of Salinities by an Extremely Halotolerant Bacterial Consortium MPD-M, Immobilized onto Polypropylene Fibries, Biotechnology and Bioengineering, vol.79, no. 2, 2002; p.145-153

- [30] Margesin R., Schinner F.; Potential of halotolerant and halophilic microorganisms for biotechnology, *Extremophiles* 5, 2001; p.73-83
- [31] Shelton M.E., Chapman P.J., Foss S.S., Fisher W.S.; Degradation of weathered oil by mix marine bacteria and the toxicity of accumulated water-soluble material to two marine crustacea, *Arch. Environ. Contam. Toxicol.* 36, 1999; p.13-20
- [32] Yakimov M.M., Golynshin P.N., Moore E.R.B., Abraham W.R., Lünsdorf H., Timmis K.N.; *Alcanivorax borkumensis* gen. Nov., sp.nov., a new hydrocarbon-degrading and surfactant-producing marine bacterium, *International Journal of Systematic Bacteriology* 48, 1998; p.339-348
- [33] Gauthier M.J., Lafay B., Christen R., Fernandez L., Acquaviva M., Bonin P., Bertrand J.C.; *Marinobacter hydrocarbonoclasticus* gen. nov., sp. nov., a new, extremely halotolerant, hydrocarbon-degrading marine bacterium, *Int. J. Syst. Bacteriol.* 42(4), 1992; p.568-76
- [34] Dutta T.T., Harayama S.; Biodegradation of n-alkylcycloalkanes and n-alkylbenzenes via new pathways in *Alcanivorax* sp. Strain MBIC 4326, *Applied and Environmental Microbiology*, vol 67, no. 4, 2001; p.1970-1974
- [35] Watanabe K.; Microorganisms relevant to bioremediation, *Current Opinion in Biotechnology* 12, 2001; p.237-241
- [36] Korda A., Santas P., Tenente A., Santas R.; Petroleum hydrocarbon bioremediation: sampling and analytical techniques, in situ treatments and commercial microorganisms currently used, *Appl. Microbiol. Biotechnol.* 48, 1997; p.677-686.
- [37] Huysman F, Verstraete W.; Water-facilitated transport of bacteria in saturated soil columns; influence of cell surface hydrophobicity and soil properties, *Soil Biol. Biochem.* 25, 1993; p.83-90
- [38] Mehmannaavaz R., Prasher S.O., Ahmad D.; Cell surface properties of rhizobial strains isolated from soils contaminated with hydrocarbon: hydrophobicity and adhesion to sandy soil, *Process Biochemistry* 36, 2001; p.683-688.
- [39] Jain D.K., Collins-Thompson D.L., Lee H., Trevors J.T.; A drop-collapsing test for screening surfactant-producing microorganisms, *Journal of Microbiological Methods* 13, Issue 4, 1991; p.271-279
- [40] Lindum P.W., Anthoni U., Christophersen C., Eberl L., Molin S., Givskov M.; N-Acyl-L-homoserine lactone autoinducers control production of an extracellular lipopeptide biosurfactant required from swarming motility of *Serratia liquefaciens* MG1, *J. Bacteriol.* 180, 1998; p.6384-6388
- [41] Abbondanza F, et al.; Microbial degradation of PAHs in soils – bioaugmentation study in slurry reactor, *Proceedings of the Second European Bioremediation Conference, Chania – Crete, Greece 2003*; p. 512-515
- [42] Fiuza A., Vila C.; Bioremediation of sandy soil polluted by petroleum hydrocarbons – respirometric studies, *Proceedings of the Second European Bioremediation Conference, Chania – Crete, Greece 2003*; p.55-58
- [43] Aichberger H., Braun R., Loibner A.P.; Hydrocarbon contamination of the vadose zone – preliminary testing to predict remediation performance, *Proceedings of the Second European Bioremediation Conference, Chania – Crete, Greece 2003*; p.67-70
- [44] Michel E.M.B., Sokolovska I., Agathos S.N.; Biodegradation of diesel fuel in soil at low temperature by *Rhodococcus erythropolis*, *Proceedings of the Second European Bioremediation Conference, Chania – Crete, Greece 2003*; p.59-62
- [45] Löser C., Seidel H., Zehnsdorf A., Stottmeister U.; Microbial degradation of hydrocarbons in soil during aerobic/anaerobic changes and under purely aerobic conditions, *Appl. Microbiol. Biotechnol.* 49, 1998; p.631-636
- [46] Venosa D.A.D., et al.; Bioremediation of crude oil intentionally released on the shoreline of flower beach, Delaware, *Proceedings of the 1995 U.S. EPA – Annual Symposium on Bioremediation of Hazardous Wastes, 1995*; p.29-31
- [47] Oh Young-Sook, Sim Doo-Seup, Kim Sang-Jin; Effects of Nutrients on Crude Oil Biodegradation in the Upper Intertidal Zone, *Marine Pollution Bulletin* 42 No.12, 2001; p.1367-1372
- [48] Singer M.E., Finnerty W.R.; *Microbial Metabolism of Straight-Chain and Branched Alkanes*, *Petroleum Microbiology* edited by M. Atlas, Macmalian Publishing Company, United States 1984; p. 1-59
- [49] Zhang Y, Miller R.M.; Enhanced octadecane dispersion and biodegradation by a *Pseudomonas* Rhamnolipid surfactant (biosurfactant), *Applied and Environmental Microbiology* 58/10, 1992; p.3276-3282
- [50] Couto H.J.B., Massarani G., Biscaia E.C., Sant'Anna G.L.; Remediation of sandy soils using surfactant solutions and foams, *Journal of Hazardous Materials* 164, 2009; p.1325-1334
- [51] Seok-Whan Kang, Young-Bum Kim, Jae-Dong Shin, Eun-Ki Kim; Enhanced Biodegradation of Hydrocarbons in Soil by Microbial Biosurfactant, Sophorolipid, *Appl Biochem Biotechnol* 160, 2010; p.780-790
- [52] Schippers C., Geßner K., Müller T., Scheper T.; Microbial degradation of phenanthrene by addition of a sophorolipid mixture, *Journal of Biotechnology* 83, 2000; p.189-198
- [53] Paria S.; Surfactant-enhanced remediation of organic contaminated soil and water, *Advances in Colloid and Interface Science* 138, 2008; p. 24-58
- [54] Juneson Ch., Ward O.P., Singh A.; Biodegradation of bis (2-ethylhexyl)phthalate in a soil slurry sequencing batch reactor, *Process Biochemistry* 37, 2001; p.305-313