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Evaluation of autochthonous bioaugmentation and biostimulation during microcosm-simulated oil spills

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ABSTRACT

Oil spills are treated as a widespread problem that poses a great threat to any ecosystem. Following first response actions, bioremediation has emerged as the best strategy for combating oil spills and can be enhanced by the following two complementary approaches: bioaugmentation and biostimulation. Bioaugmentation is one of the most controversial issues of bioremediation. Studies that compare the relative performance of bioaugmentation and biostimulation suggest that nutrient addition alone has a greater effect on oil biodegradation than the addition of microbial products because the survival and degradation ability of microbes introduced to a contaminated site are highly dependent on environmental conditions. Microbial populations grown in rich media under laboratory conditions become stressed when exposed to field conditions in which nutrient concentrations are substantially lower. There is increasing evidence that the best approach to overcoming these barriers is the use of microorganisms from the polluted area, an approach proposed as autochthonous bioaugmentation (ABA) and defined as a bioaugmentation technology that exclusively uses microorganisms indigenous to the sites (soil, sand, and water) slated for decontamination. In this work, we examined the effectiveness of strategies combining autochthonous bioaugmentation with biostimulation for successful remediation of polluted marine environments. Seawater was collected from a pristine area (Agios Onoufrios Beach, Chania) and was placed in a bioreactor with 1% v/v crude oil to facilitate the adaptation of the indigenous microorganism population. The pre-adapted consortium and the indigenous population were tested in combination with inorganic or lipophilic nutrients in the presence (or absence) of biosurfactants (rhamnolipids) during 90-day long experiments. Chemical analysis (gas chromatography–mass spectrometry) of petroleum hydrocarbons confirmed the results of previous work demonstrating that the biodegradation processes were enhanced by the addition of lipophilic fertilizers (uric acid and lecithin) in combination with biosurfactants (rhamnolipids), resulting in increased removal of petroleum hydrocarbons as well as reduction of the lag phase within 15 days of treatment. Considering this outcome and examining the results, the use of biostimulation additives in combination with naturally pre-adapted hydrocarbon-degrading consortia (bioaugmentation) has proved to be an effective treatment and is a promising strategy that could be applied specifically when an oil spill approaches near a shore line and an immediate hydrocarbon degradation effort is needed.

1. Introduction

The recent Deep Horizon oil spill accident in the Gulf of Mexico provided an alert and a reminder that despite the stricter environmental regulations that have been adopted by most countries, oil spills still remain as a serious risk to marine ecosystems. In both socioeconomic and ecological terms, the impact of an oil spill on the marine environment can be quite significant. Most importantly, the loss of species richness, downgraded sediment quality and further negative impacts on offshore fish and crustacean fisheries represent a subset of the side effects of oil spills (Kirby and Law, 2008).

Conventional first response actions, such as physical removal with booms, skimmers and absorbent materials, cannot achieve complete clean-up of oil spills and must be deployed shortly after the oil spill occurs. However, when applicable, the use of chemical dispersants is only allowed when the coastline depth is at least 15 m due to their potential toxic effects on marine organisms; otherwise, their overall effectiveness is questionable.

In past years, bioremediation has emerged as an effective and environmentally friendly treatment for shorelines contaminated as a result of marine oil spills. The majority of compounds in crude oil and refined products are biodegradable and will eventually be

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removed from the environment through consumption by microbes. Enhanced bioremediation aims to stimulate the rate of this process with the following two complementary approaches: bioaugmentation and biostimulation. In bioaugmentation, the addition of oil-degrading bacteria boosts biodegradation rates, whereas in biostimulation, the growth of indigenous hydrocarbon degraders is stimulated by the addition of nutrients (mainly N and P) or other growth-limiting nutrients (Nikolopoulou and Kalogerakis, 2010).

Although the effectiveness of bioaugmentation in the marine environment is still under investigation, the addition of oil-degrading microorganisms has been proposed as a bioremediation strategy. Crude oil is composed of a wide range of different compounds, which makes it difficult for the indigenous population to cope with this broad variety of substrates, and hence, oil-degrading microorganisms could be added to supplement the indigenous population (Leahy and Colwell, 1990).

Although laboratory studies on bioaugmentation have shown an enhancement of oil biodegradation, the effectiveness of bioaugmentation has not been convincingly demonstrated in the field. Most of the field studies conducted thus far suggest that bioaugmentation is not effective in significantly enhancing oil biodegradation for most environments in the long run (Nikolopoulou and Kalogerakis, 2011). Generally, in most environments, it appears that indigenous oil-degrading microorganisms can degrade oil if they are not limited by the prevailing environmental conditions. Case studies support the potential of bioaugmentation as a remediation strategy to combat oil spills, but this promising technology is still in the experimental stage (El Fantroussi and Agathos, 2005).

There is increasing evidence that the best approach for overcoming these barriers is the use of microorganisms from the polluted area. A new concept in bioaugmentation, known as ‘‘autochthonous bioaugmentation’’ (ABA), has been proposed by Ueno et al. (2007) and is defined as a bioaugmentation technology that *exclusively* uses microorganisms indigenous to the sites (soil, sand, and water) slated for decontamination. Isolated single strains or enriched cultures, which are obtained ‘‘before’’ or ‘‘after’’ the contamination of the target sites, are administered to the sites once contamination occurs. The key concept is to conduct the enrichment of contaminant-degrading bacteria under the same or similar conditions as those present where the bioaugmentation will be performed. The ABA approach uses autochthonous microbial consortia or isolates that are highly enriched and much better adapted to chronically or artificially contaminated environments (Hosokawa et al., 2009). The success of oil spill bioremediation depends on the establishment and maintenance of physical, chemical and biological conditions that favor enhanced oil biodegradation rates in the marine environment. Through biostimulation, the growth of indigenous oil degraders is stimulated by the addition of nutrients (Nitrogen and Phosphorous) or other growth-limiting co-substrates and/or by alterations in environmental conditions (e.g., surf-washing, oxygen addition by plant growth, etc.). In this study, we examined the capabilities of an acclimated indigenous microbial consortium (ABA) sampled from a pristine environment in the presence or absence of other rate limiting factors (i.e., nutrients and biosurfactants) (biostimulation) as a potential strategy for the successful remediation of polluted marine environments.

2. Materials and methods

2.1. Experimental design

In this study, we examined the effectiveness of autochthonous bioaugmentation together with biostimulation versus biostimulation-only strategies for the successful remediation of polluted marine environments. Seawater was collected from a pristine environment in the Eastern Mediterranean Sea (Agios Onoufrios Beach, Chania, Crete) and was placed in a bioreactor with 1% v/v crude oil to grow and adapt the indigenous population for later use of this consortium for bioaugmentation purposes. Crude oil (compliments of Hellenic Petroleum Co., Aspropyrgos, Greece) was weathered artificially by distillation according to ASTM method D 86. Duplicate microcosms were established in sterile 40-ml vial bottles containing 20 ml of seawater and contaminated with 0.5% w/v weathered crude oil.

Three biostimulation treatments were designed: (i) seawater + oil, (ii) seawater + oil supplemented with KNO_3 and K_2HPO_4 (NPK) and (iii) seawater + oil supplemented with uric acid, lecithin and biosurfactant (rhamnolipids) (ULR). In addition, three autochthonous bioaugmentation treatments were established as shown in Table 1: (iv) seawater + oil supplemented with KNO_3, K_2HPO_4 and pre-adapted indigenous cultures (NPKM); (v) seawater + oil supplemented with KNO_3, K_2HPO_4, biosurfactant (rhamnolipids) and pre-adapted indigenous cultures (NPKMR); and (vi) seawater + oil supplemented with uric acid, lecithin, biosurfactant (rhamnolipids) and pre-adapted indigenous cultures (ULRM). Nutrients were added in an amount that resulted in a final concentration equivalent to a C:N:P molar ratio of 100:10:1. The microcosms were incubated under aerobic conditions at 20 °C with continuous agitation on an orbital shaker (200 rpm). The JBR210 biosurfactant of microbial origin (rhamnolipid) consisted of a blend of $C_{26}H_{48}O_9$ and $C_{32}H_{58}O_{13}$ and was composed of 10% active ingredient supplied by Jeneil Biosurfactants Co., USA. The growth of the oil degraders was measured by the most probable number (MPN) procedure, and hydrocarbons were analyzed via chromatographic techniques (solid-phase extraction followed by gas chromatography–mass spectrometry) after 0, 5, 15, 30, 60 and 90 days.

We investigated the effects of autochthonous bioaugmentation with these organisms on hydrocarbon degradation in seawater and also compared the role of bioaugmentation with biostimulation via different types of nutrients (organic and inorganic) with or without a rhamnolipid biosurfactant amendment.

2.2. Microbiological analyses

The amount of hydrocarbon degraders in the flasks was estimated by the most probable number (MPN) method according to Wrenn and Venosa (1996). The growth medium was a Bushnell–Hass minimal salts medium (BHS) supplemented with crude oil as the hydrocarbon substrate. The MPN plates were 96-well micro-titer tissue culture plates, with each well containing 180 μl BHS, 5 μL crude oil and 20 μL of the appropriate dilution of sample. One milliliter of each microcosm was diluted in a 9-mL aliquot of Bushnell–Hass solution (pH 7). Tenfold serial dilutions were carried out to 10^{-10}, and the plates were inoculated by adding 20 μL of each dilution to one of the 12 rows of eight wells. The inoculated plates were incubated at 20 °C for 2 weeks. At the end of the incubation period, 50 μL of p-iodonitrotetrazolium violet dye (INT 3 g/L) was added to each well of the tissue culture plates and allowed to stand at room temperature for 1 h. The dye turns from colorless to red (when reduced) in the presence of actively respiring microorganisms. The MPN values were calculated using the ‘‘MPN Calculator’’ software program by Klee (1993) of the EPA Risk Reduction Engineering Laboratory.

2.3. Chemical analysis

2.3.1. Reagents, materials and standards

Trace analysis (SupraSolv) dichloromethane (CH_2Cl_2) and *n*-hexane (C_6H_{14}) were obtained from Merck (Darmstadt, Germany). Solid-phase cartridges of silica/cyanopropyl (SiO_2/C_3–CN, 1.0/0.5 g, 6 ml) were obtained from Interchim (Best Buy Analyt-