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Bioremediation (bioaugmentation/biostimulation) trials of oil polluted seawater: A mesocosm simulation study

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ABSTRACT

Bioaugmentation (amendment with selected bacterial strains) and/or biostimulation (nutrients addition and/or air supply) are relatively new fields in environmental microbiology for preventing pollution and cleanup contamination. In this study, the efficiency of application of bioaugmentation/biostimulation treatments, for recovery of crude oil-polluted seawater, was evaluated. Three different series of experiments were performed in a "Mesocosm Facility" (10.000 L). Natural seawater was artificially polluted with crude oil (1000 ppm) and was amended with inorganic nutrients (Mesocosm 1, M1), inorganic nutrient and an inoculum of *Alcanivorax borkumensis* SK2^T (Mesocosm 2, M2) and inorganic nutrient and an inoculum of *A. borkumensis* SK2^T and *Thalassolituus oleivorans* MIL-1^T (Mesocosm 3, M3), respectively. During the experimental period (20 days) bacterial abundance (DAPI count), culturable heterotrophic bacteria (CFU count), MPN, microbial metabolic activity [Biochemical Oxygen Demand and enzymatic activity (leucine aminopeptidase LAP, β-glucosidase BG, alkaline phosphatase AP)] and quali-, quantitative analysis of the composition of total extracted and resolved hydrocarbons and their derivates (TERHCs) were carried out. The microbiological and physiological analysis of marine microbial community found during the three different biostimulation and bioaugmentation assays performed in mesocosms show that the load of crude oil increases total microbial abundance, inhibits the activity of some enzymes such as LAP while stimulates both AP and BG activities. The biodegradation results show that bioaugmentation with *A. borkumensis* SK2^T alone is able to produce the highest percentage of degradation (95%) in comparison with the biostimulation treatment (80%) and bioaugmentation using an *Alcanivorax-Thalassolituus* bacterial consortium (70%). This result highlights the reduced biodegradation capability of the consortium used in this study, suggesting an unfavourable interaction between the two bacterial genera.

1. Introduction

Petroleum hydrocarbons are the most widespread contaminants in the environment (Santas et al., 1999; Yakimov et al., 2007) entering into aquatic environments because of catastrophic accidents (shipping disasters or pipeline failures), chronic pollution (ships, ports, oil terminals, freshwater runoff, rivers and sewage systems), natural oil seepages and natural sources (Floodgate, 1972; Cappello et al., 2007a; Hassanshahian et al., 2012a).

Many physical, chemical and biological technologies have been developed to remove hydrocarbon pollutants from soils and marine environments. However, these techniques often are not able to fully remove pollutants from environments. Bioremediation, including biostimulation and bioaugmentation, has proven to be an effective method for cleaning up residual oil in a variety of environments (Van Hamme et al., 2003) and has been proposed as the only viable management option that can be implemented on a large scale in marine environments (Snape et al., 2001; Hassanshahian et al., 2012b).

At sea, petroleum hydrocarbon degradation is mainly performed by microorganisms, and the microbial communities of marine ecosystems involved in this process have been extensively studied (Harayama et al., 1999; Yakimov et al., 2004).

Different studies have shown that marine communities of hydrocarbon-degrading bacteria are composed of indigenous members (Cappello et al., 2007b; Gertler et al., 2012) able to degrade mixtures of hydrocarbons, like crude oil. It has been well-documented that the addition of nitrogen and phosphorus significantly enhances the growth of hydrocarbon-degrading bacteria, with

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consequent stimulation of metabolic processes involved in oil biodegradation (Gertler et al., 2012). Moreover, as a single species can metabolize only a limited range of hydrocarbon substrates, a consortium of many different bacterial species, with broad enzymatic capacities, is usually involved in oil degradation (Rölling et al., 2002). To understand how the composition of microbial community affects the process of biodegradation, it is necessary to analyse the microbial response to oil pollution both at genetic and metabolic levels. It is also important to develop a multidisciplinary approach for the study of microorganisms having high specificity for recalcitrant compounds, dealing with their structure and function. Knowledge of microbial diversity and metabolism in oil-polluted sites can be helpful for bioremediation of oil spills, as human intervention by using specific microbial consortia can be planned for cleaning up oil pollution (Denaro et al., 2005; Hassanshahian and Emtiazi, 2008).

The application of bioremediation techniques (bioaugmentation and/or biostimulation) for the recovery of polluted marine environments relies on the knowledge of oil weathering processes, biological dynamics (e.g. variation of the microbial community) and rates of degradation of hydrocarbons (Genovese et al., 2013). However, despite its proven utility, natural and non-destructive character, bioremediation remains a controversial technology (Lee and Merlin, 1999; Genovese et al., 2013; Hassanshahian et al., 2013). The effectiveness of bioremediation practice depends on several factors, such as the presence of suitable hydrocarbon-degrading microbial consortia as well as of favourable environmental conditions (Hassanshahian and Emtiazi, 2008). Moreover, advancements in this topic are often difficult due to the insufficient consistency between data obtained from laboratory experiments and those obtained in "in situ" experiments (Reilly, 1999). The "*in vitro*" experiments sometime produce few realistic data, which cannot be exported to natural environments. On the other hand, "in situ" experiments are difficult to be carried out and the obtained data are not simple to be interpreted because of the complexity of the analysed biological processes (Cappello and Yakimov, 2010). As previously indicated (Cappello and Yakimov, 2010) the need to link the "*in vitro*" experiments – that can be statistically replicated but are not performed under real conditions – and the experiments performed in large-scale – in which intrinsic difficulties are related to the inability to control environmental factors-, has lead to the increasing use of medium-scale systems, like microcosms and/or mesocosms. Different studies have demonstrated the great versatility and potentiality of mesocosm systems in reproducing the same processes occurring in nature (Alldredge et al., 1995; Reilly, 1999; Takeuchi et al., 2000; Lebaron et al., 2001) through the control over both dependent/independent variables and the interactions among the chemical, physical and biological parameters (Cappello and Yakimov, 2010).

However, no study has ever been carried out to test, in meso-scale systems in the size (10,000 L) similar to those used in this work, efficiency of bioremediation processes and biostimulation.

The aim of the present study was to monitor the efficiency of application of bioaugmentation/biostimulation strategies, in the mesoscale (mesocosm) systems, for the recovery of crude oil-polluted marine area. Some physiological, biochemical and enzymatic assays occurring in the structure and composition of seawater natural microbial communities were carried out to reach this goal.

2. Materials and methods

2.1. Site description and sampling

During mesocosm experiments, natural seawater (SW) was collected, in November 2011, from the station "Marisicilia" (38°12.23′N, 15°33.10′E) located in the harbour of Messina (Italy).

2.2. Set-up of experimental mesocosms systems

Experiments in mesocosms were performed in a "Mesocosm Facility" designed and built at the Institute for Coastal Marine Environment (IAMC) – CNR of Messina, Italy (Fig. 1). The experiments were carried out in a rectangular tank of 11250 L of capacity (5000 cm long, 150 cm deep, 150 cm wide) filled with 10000 L of natural seawater (Fig. 2). The mesocosm system was filled with natural sea water (SW) collected directly from the Straits of Messina through a direct pipeline. Before the introduction in the mesocosms, natural seawater was filtered through a 100 μm nylon mesh to remove large metazoans and detritus. During the study the seawater was aerated and kept under agitation during the all experimental period. Mesocosm water was mixed by a pump (35 l h^{-1}), placed at the exit of each tank, that takes water from two opposite bottom corners and drives it below the surface. Seawater temperature (18 ± 2 °C) was checked for all experimental period; pH values were also measured to detect possible variations.

2.3. Bacterial strains

Two hydrocarbonoclastic bacterial strains *Alcanivorax borkumensis* strain SK2^T (Genebank accession number Y12579; = DSM 11573^T; Yakimov et al. (1998) and *Thalassolituus oleivorans* strain MIL-1^T (Genebank accession number AJ431699; = DSM 14913^T, = LMG 21420^T; Yakimov et al. (2004) were used in this study (Fig. 3). Both strains belong to a collection of hydrocarbon-degrading bacteria hold at IAMC-Messina.

2.4. Growth conditions of the bacterial inocula amendments

Starting cultures of bacteria selected for bioaugmentation (*A. borkumensis* SK2^T and *T. oleivorans* MIL-1^T) were prepared separately for each strain by inoculating one loop of bacterial cells into 10 mL of ONR7a mineral medium (Dyksterhouse et al. (1995) containing 0.1% (w/v) of tetradecane ($C_{14}H_{30}$, Sigma–Aldrich, Milan, Italy) sterilized by filtration through a 0.2-μm syringe filter (Sartorius). After growth in a rotary shaker (New Brunswick C24KC, Edison NJ, USA; 150 rpm) at 25 °C for two days, 500 μL of the seed culture broth were transferred into a 250 mL Erlenmeyer flask containing 100 mL of ONR7a medium supplemented with 1% (w/v) of sterile Arabian Light Crude Oil (ENI Technology S.P.A.). The culture was incubated in a rotary shaker (150 rpm) at 25 °C for 5 days. At values of microbial abundance (measured by DAPI count) of 10^8 cell mL^{-1} cultures were added in experimental mesocosms (Beginning of experiment, t0).

2.5. Design of mesocosm experiments

Three different series of experiments were set up. In all the experiments, natural seawater was supplemented with Arabian Light Crude Oil and inorganic nutrients. 1 L of Arabian Light Crude Oil (ENI Technology S.P.A.) were added to all the mesocosms; 0.1% (v/v) of squalene ($C_{30}H_{50}$, Sigma–Aldrich, Milan) was also added to crude oil as an internal spike/control to monitor the biodegradation process. Inorganic nutrients were added to reach concentrations higher than those in natural water (final concentrations: KH_2PO_4 0.077 g L^{-1}, NH_4Cl 0.2 g L^{-1} and $NaNO_3$ 0.1 g L^{-1}). The "control" mesocosm, consisting of natural seawater with oil and inorganic nutrients, was indicated as "mesocosm 1 (M1)". The mesocosm amended with the isolate *A. borkumensis* SK2^T only was indicated as "mesocosm 2" (M2), while the mesocosm where *A. borkumensis* SK2^T and *T.oleivorans* MIL-1^T were simultaneously added was indicated as "mesocosm 3 (M3)".