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Bioaugmentation strategy employing a microbial consortium immobilized in chitosan beads for oil degradation in mesocosm scale

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ABSTRACT

A bacterial consortium composed by four metagenomic clones and *Bacillus subtilis* strain CBMAI 707, all derived from petroleum reservoirs, was entrapped in chitosan beads and evaluated regarding hydrocarbon degradation capability. Experiments were carried out in mesocosm scale (3000 L) with seawater artificially polluted with crude oil. At different time intervals, mesocosms were sampled and subjected to GC-FID and microbiological analyses, as total and heterotrophic culturable bacterial abundance (DAPI and CFU count), biological oxygen demand (BOD) and taxonomic diversity (massive sequencing of 16S rRNA genes). The results obtained showed that degradation of n-alkane hydrocarbons was similar between both treatments. However, aromatic compound degradation was more efficient in bioaugmentation treatment, with biodegradation percentages reaching up to 99% in 30 days. Community dynamics was different between treatments and the consortium used in the bioaugmentation treatment contributed to a significant increase in aromatic hydrocarbon degradation.

1. Introduction

Environmental pollution is a worldwide concern, being mostly associated with anthropic activities. Heavy metals, pesticides, solvents, as well as industrial wastes, when submitted to an improper management can turn into pollution sources harmful to environment and human health (Mulligan, 2005). Petroleum and other petroleum-derived products constitute potential sources of contamination when released in soils, sediments or sea, as result of oil spill accidents, for example, frequently reported in literature (Peterson et al., 2003; Caselles et al., 2008; Chen and Denison, 2011; Macaulay and Rees, 2014). Some remediation techniques only remove or restrain physically the pollution source, being applied according to a specific contamination scenario. Bioremediation is a well described technique (Dua et al., 2002; Okoh and Trejo-Hernandez, 2006; Luqueño et al., 2011) which involves the utilization of microorganisms in order to transform some organic toxic pollutants in inert or non-toxic compounds. However, this technique is considered an efficient method when the microorganisms employed are capable to survive in the contaminated area and completely degrade the contaminants (Kosaric, 2001). There are two main approaches in bioremediation: i) *Biostimulation*, based on nutrient addition, like nitrogen or phosphorus to stimulate the growth of the indigenous microbial community, consequently improving the biodegradation process; and ii) *Bioaugmentation*, based on the introduction of specific microbial strains or consortia known as competent degraders for accelerating xenobiotic compound degradation (Silva and Alvarez, 2010; Luqueño et al., 2011).

Evaluating distinct microorganisms in bioremediation studies at non-sterile conditions, similar to natural environment is highly relevant, once it allows demonstrating the capability to degrade xenobiotic and/or recalcitrant compounds under the pressure of biotic and abiotic environmental factors (Pandey et al., 2009). From this perspective, mesocosms assays are a useful tool to investigate the microbial response in a polluted area and provides a larger scale analysis in comparison to microcosms, allowing a broader comprehension of the influence of several ecological parameters. Studies performed at mesocosm scale offer some advantages, such as the control of diverse variables (chemical, physical and biological), besides the possibility of replication and reproducibility when compared to natural ecosystems (Cappello and Yakimov, 2010).

In order to enhance the effectiveness of microorganisms in natural environments, the microbial immobilization in biological matrices is an alternative technique that has been evaluated (Gentry et al., 2004; Le-Tien et al., 2004; Siripattanakul and Khan, 2010). Immobilization or encapsulation process can provide a physical support for biofilm formation and slow release of microbial cells in the surrounding medium, resulting in an increased capacity to support stressful environmental conditions, consequently enabling more efficient biodegradation

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process when compared to those performed by free cells (Chen et al., 2007). In addition, encapsulation may allow the control of nutrient flow, lowering the concentration of toxic compounds in the microenvironment of the cells and minimizing cell membrane damage, besides protecting from predation and competition; thereby mimicking a miniature bioreactor in the environment (Tyagi et al., 2011). Immobilization process has been employed not only to entrap microorganisms, but also to enzymes (Juang et al., 2002; Altun and Cetinus, 2007), food supplementation with probiotics (Le-Tien et al., 2004), and bioremediation approaches (Bashan and Bashan, 2010; Gentili et al., 2006). The choice of a proper matrix can influence the success of the microbial response and some natural polymers have been evaluated elsewhere, like alginate, agarose and agar (Vassileva et al., 2003; Ahamad and Kunhi, 2011). Chitosan is a polymer obtained from deacetylation reactions of chitin, a polysaccharide present in crustaceans, as shrimps and lobsters. Due to some intrinsic features, this material presents some advantages when compared with synthetic matrix, such as biodegradability, non-toxicity and availability in the natural environment (Angelim et al., 2013). Entrapment process enables a high concentration of microorganisms in chitosan beads even when used in continuous bioreactors, with no significant decrease of cells when compared with free cell condition (Hsieh et al., 2008).

Besides assessing suitable alternatives to improve the persistence of microorganisms in environments, genetically engineered microorganisms (GEMs) have been considered for bioremediation purposes. Their utilization in many biotechnological processes has been reported since the 90 decade (Fulthorpe and Wyndham, 1991), as well as studies focusing on bioremediation of recalcitrant compounds with successful results (Ford et al., 1999; Sayler and Ripp, 2000; Pandey et al., 2005). However, despite GEMs having been widely studied on a laboratory scale, the long-term impacts and further consequences of their application on natural environments must be thoroughly evaluated. This work describes the evaluation of a bioaugmentation approach using a microbial consortium of metagenomic clones and *Bacillus subtilis* strain CBMAI 707 as a remediation tool to mitigate the impact of oil-pollution in seawater mesocosm systems.

2. Material and methods

2.1. Experimental mesocosms

All experiments were carried out in tanks of 3.75 m^3 capacity (166 cm long, 150 cm deep, 150 cm wide). The mesocosms were filled with ca. 3000 L of seawater taken directly from the harbor of Messina (38°11 42.58N 15°34 25.19E). Prior to use seawater was filtered through a 200 mm nylon mesh to remove large metazoans and detritus. During the study, seawater was aerated and kept under agitation during all experimental periods by using 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 monitored during the experimental period. Both mesocosm systems (bioaugmentation and control) were supplemented with 70 mL (900 mg L^{-1}) of sterilized Arabian Light Crude Oil to simulate a chronic pollution condition. Mesocosm containing bioaugmentation treatment (BT) was also supplemented with 250 g of chitosan beads containing microorganisms (Fig. 1).

2.2. Bacterial strains

The *Bacillus* strain and metagenomic clones used in the present work are listed in Table 1. All organisms were derived from Brazilian petroleum reservoirs (Campos Basin, RJ and Potiguar Basin, RN) and were previously evaluated for their ability to degrade different classes of hydrocarbons (Vasconcellos et al., 2009, 2010, 2011; Sierra-Garcıa et al., 2014) and/or crude oil in seawater microcosms (Dellagnezze et al., 2014). The only bacterial strain used (*B. subtilis* CBMAI 707) was deposited in the Brazilian Collection of Environmental and Industrial Microorganisms (CBMAI). The metagenomic clones consist of *Escherichia coli* host cells harboring a DNA vector (in this case, a fosmid vector) containing a large fragment (~40 kb) of environmental DNA (for details, see Vasconcellos et al., 2010).

2.3. Microbial growth and chitosan bead preparation

The microorganisms under study were previously tested regarding their resistance to the immobilization process in chitosan beads (data not shown). A pre-inoculum was prepared in Luria Broth (LB) medium for the metagenomic clones and in Nutrient Broth (NB) for *B. subtilis* strain, with an optical density (DO_{600}) above 1.0. The immobilization process was carried out following the protocol described by Angelim et al. (2013). In order to determine the number of viable cells entrapped in the beads, 4 g of beads were macerated, serially diluted in saline solution 0.9% (*w*/*v*) and plated onto the surface of LA (Luria Agar) plates for colony forming units (cfu)/g counting.

2.4. Sampling strategy and parameters assayed

To monitor the succession of bacterial assemblages and microbial dynamics in the mesocosm microbial community, 1 L of water from each mesocosm system was taken aseptically in triplicate, using sterile

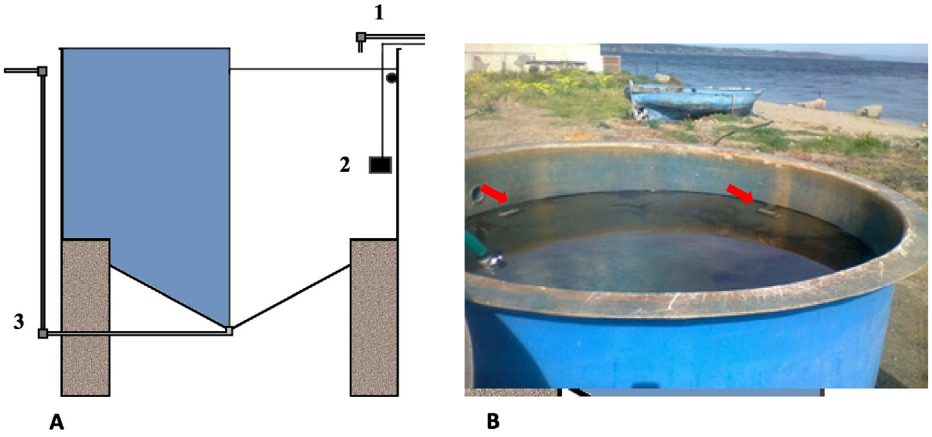

Fig. 1. Mesocosm tanks. A) Schematic representation of engineered and hydraulic system of Mesocosm System at IAMC-CNR (Messina, Italy) used in this study: 1) seawater input; 2) pump; and 3) seawater output; and B) picture depicting traps with chitosan beads adhered on the mesocosm walls, indicated by the red arrows.

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