

Research Paper

## Quick stimulation of *Alcanivorax* sp. by bioemulsificant EPS<sub>2003</sub> on microcosm oil spill simulation

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### Abstract

Oil spill microcosms experiments were carried out to evaluate the effect of bioemulsificant exopolysaccharide (EPS<sub>2003</sub>) on quick stimulation of hydrocarbonoclastic bacteria. Early hours of oil spill, were stimulated using an experimental seawater microcosm, supplemented with crude oil and EPS<sub>2003</sub> (SW+OIL+EPS<sub>2003</sub>); this system was monitored for 2 days and compared to control microcosm (only oil-polluted seawater, SW+OIL). Determination of bacterial abundance, heterotrophic cultivable and hydrocarbon-degrading bacteria were carried out. Community composition of marine bacterioplankton was determined by 16S rRNA gene clone libraries. Data obtained indicated that bioemulsificant addition stimulated an increase of total bacterial abundance and, in particular, selection of bacteria related to *Alcanivorax* genus; confirming that EPS<sub>2003</sub> could be used for the dispersion of oil slicks and could stimulate the selection of marine hydrocarbon degraders thus increasing bioremediation process.

**Key words:** *Alcanivorax*, bioemulsificant exopolysaccharide (EPS<sub>2003</sub>), oil spill.

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### Introduction

Environmental pollution caused by oil spills is one of the major threat for the marine ecosystem. Anthropogenic activities (*i.e.* storing and transport operations) have been identified as critical point for catastrophic accidents and potential sources of chronic pollution (*i.e.* ships, ports, oil terminals, freshwater runoff, rivers and sewage systems). One of the main reasons for prolonged persistence of hydrocarbons in polluted environments is their low water solubility which limits their availability to biodegrading microorganisms (Barkay *et al.*, 1999). Biodegradation of crude oil spilled in water, is limited by the extent of the exchange phenomena occurring at the interface between the water phase, that hosts microbial life, and the insoluble hydrocarbons. Thus several methods to enhance oil biodegradation have been tested, in attempting to rise the apparent solubility of hydrophobic hydrocarbons by the addition of

emulsifier molecules of biological or synthetic origin (Hang *et al.*, 2009; Wen *et al.*, 2010). Currently, application of bioemulsificants is an effective tool which could be applied to increase oil recovery and hydrocarbon bioremediation in aquatic environments (Banat, 1995). Bioremediation is based upon the use of nutrients or aeration to enhance the activity of indigenous organisms (*biostimulation*) and/or the addition of microbial inocula (*bioaugmentation*) to enhance the clean-up pills (Supaphol *et al.*, 2006; Hassanshahian *et al.*, 2010; Cappello *et al.*, 2012). The addition of bioemulsificant (*biostimulation*) can stimulate the indigenous bacterial population to degrade hydrocarbons at higher rates and the use of these substances can be an effective tool in the management of crude oil spilled in aquatic environments. The understanding of the effect of these substances on microbial community dynamics and selection of bacteria able to participate at the

biodegradation process is essential for predicting long-term persistence following an oil spill (Lindstrom and Braddock, 2002) and optimized the use of these substances in these processes. Moreover the knowledge of microbial diversity and metabolism in oil-polluted sites are helpful for oil spill bioremediation activities, as the use of specific microbial consortia can be planned as part of cleaning up procedures upon oil pollution (Gertler *et al.*, 2012).

The aim of the present study has been to evaluate the shift of the composition of bacterial community structure and quick stimulation of hydrocarbonoclastic bacteria in first days of an oil spill phenomena consequently to rapid introduction of bioemulsifier EPS<sub>2003</sub>.

## Materials and Methods

### Set-up of experimental microcosms systems

The experiment was performed in two rectangular glass tanks (Figure 1, closed systems) of 90 L capacity (100 cm long, 30 cm deep, 30 cm wide). Each microcosm was filled with 70 L of natural surface seawater collected, in June 2005, from Messina Harbour (Italy). Natural seawater was filtered through a 200 µm nylon mesh to remove large metazoans and detritus. Mean water temperature was  $18 \pm 2^\circ\text{C}$ . Microcosm water was aerated and stirred by a pump ( $35 \text{ L h}^{-1}$ ), placed at the exit of each tank, that takes water from two opposite bottom corners and drives it below the surface (Cappello *et al.*, 2007).

### Experimental planning for microcosms and mesocosms experimentations

Both microcosms (SW+OIL and SW+OIL+EPS<sub>2003</sub>) were supplemented with 70 mL of Arabian Light Crude Oil (900 mg L<sup>-1</sup>). Microcosm SW+OIL+EPS<sub>2003</sub> was also sup-

plemented with dry EPS<sub>2003</sub> (45 mg L<sup>-1</sup>). As described (Crescenzi *et al.*, 2003; Cappello *et al.*, 2011), the molecular structure of the bioemulsificant EPS<sub>2003</sub> corresponds to a polysaccharidic chain with hydrophobic fatty acids substitutions of 12-18 carbon atoms length. EPS<sub>2003</sub> is obtained by *Acinetobacter calcoaceticus* CBS 962.97, a hydrocarbon-degrading bacterium isolated in laboratory.

### Sampling strategy

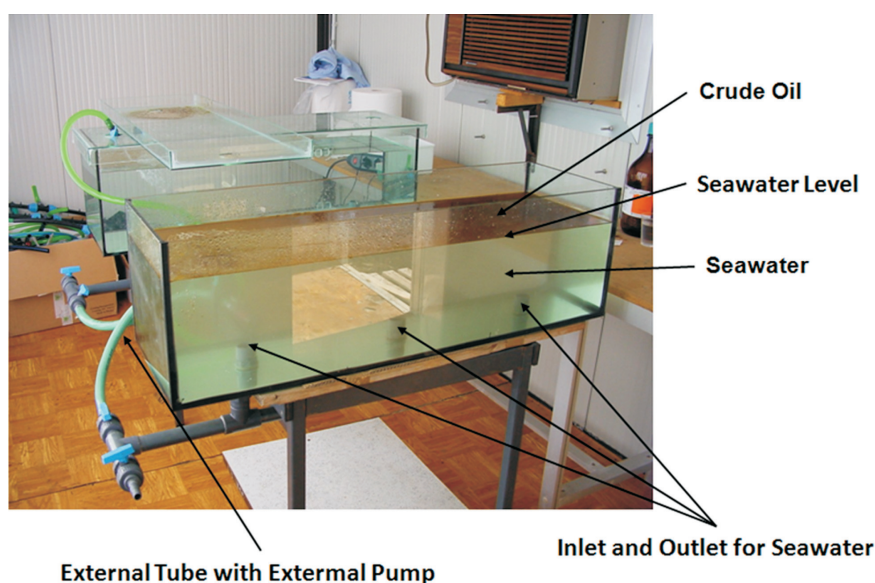
A total of two litres (2 L) of seawater have been collected, at the beginning (T<sub>0</sub>) and after two days (T<sub>2</sub>) of experimentation, from each experimental microcosm. Analysis of the total bacterial abundance [4',6-diamidino-2-phenylindole 2HCl, (DAPI count)], heterotrophic cultivable bacteria [Colony Forming Units, (CFU count)], enumeration of hydrocarbon-degrading bacteria [Most Probable Number, (MPN count)] and phylogenetic diversity [cloning library of 16S complementary rDNA (crDNA)] were carried out.

### Bacterial abundance (DAPI Count)

Cell counts were performed by DAPI (4',6-diamidino-2-phenylindole 2HCl, Sigma-Aldrich S.r.L., Milan, Italy) staining on samples (10 mL) fixed with formaldehyde (2% final concentration), according to Porter and Feig (1980). Slides were examined by epifluorescence with Axioplan 2 Imaging (Zeiss; Carl Zeiss Inc., Thornwood, N.Y.) microscope. All results were expressed as number of cells mL<sup>-1</sup>. All measures were repeated three times.

### Heterotrophic cultivable bacteria (CFU)

Enumeration of heterotrophic cultivable bacteria were estimated by spreading 100 µL of tenfold dilutions of microcosm samples on plates of Marine agar 2216 medium (Difco S.p.a, Milan, Italy), incubated at  $20 \pm 1^\circ\text{C}$  for 7



**Figure 1** - Schematic representation of the microcosm used through the study.

days. Results were expressed as colony forming units (CFU) mL<sup>-1</sup>. All measures were repeated three times.

### Most Probable Number (MPN)

Hydrocarbon-degrading bacteria were enumerated, in sterile Bushnell-Hass (B-H) medium (Difco Products) added with 2% NaCl (pH 7.0) and 10 µL of sterile Arabian light crude oil, by a miniaturized Most Probable Number (MPN) method (Brown *et al.*, 1990), slightly modified (Cappello *et al.*, 2007). The MPN of hydrocarbon-degrading microorganisms was determined, from the appropriate MPN tables according to the American Public Health Association (A.P.H.A., 1992). All measures were repeated three times.

### Molecular and taxonomic analysis (cloning library of 16S crDNA)

Total RNA extraction of each seawater samples were performed by Master Pure Complete DNA & RNA Purification Kit (Epicentre Biotechnologies, Madison, WI) in accordance with the manufacturer's instructions. RNA was converted to cDNA using First-Strand cDNA Synthesis SuperScript™ II Reverse Transcriptase (Life Technologies, Carlsbad, CA, USA). RT reaction mixtures (20 µL) contained 1 µL using the Random Hexamere Primer Mix (Bioline, Italy), 30 ng of RNA, 1 µL 10 mM dNTPs (Gibco, Invitrogen Co., Carlsbad, CA) and sterile distilled water to 14 µL. After heating to 65 °C for 5 min, and incubate on ice for 1 min, the mixture was added with 4 µL of 5 x First-Strand Buffer, 1 µL of DTT and 1 µL of SuperScript™. The mixture was mixed and incubated at 50 °C for 30 min. Finally enzyme was inactivated by heating at 70 °C for 15 min. PCR reaction was performed as above described. The quality of amplification products were examined by agarose electrophoresis and purified using Qiaquick Gel Extraction kit (QIAGEN, Valencia, CA).

16S rRNA genes were amplified from cDNA total community with the 530F (5'-GTGCCAGCMGCCGCGG-3') and universal primer Uni-1492R (5'-TACGGYTACCTTGTTACGACT-3'). The PCR was performed using a 50-µL (total volume) mixture containing 1 x solution Q (Qiagen, Hilden, Germany), 1 x Qiagen reaction buffer, 1 µM of each forward and reverse primer, 10 µM dNTPs (Gibco, Invitrogen Co., Carlsbad, CA), 2.0 mL (50-100 ng) of template and 2.0 U of Qiagen Taq Polymerase (Qiagen). The reaction started with a 3 min hot-start at 95 °C; 1 min at 94 °C, 1 min at 50 °C, 2 min at 72 °C, 35 cycles; extension: 10 min at 72 °C was performed with GeneAmp 5700 (PE Applied Biosystems, Foster City, CA, USA).

After gel electrophoresis of PCR products, purified amplicons representing 16S crDNA sequences were cloned into the pGEM T-easy Vector II (Promega, Madison, Wis., USA), and inserts from the transformed *E. coli* DH10β col-

onies were subsequently PCR amplified with primers specific for the vector, M13F (5'-TGAAAACGACGGCCAGT-3') and M13R (5'-TCACACAGGAAACAGCTATGA C-3'). The PCR products, after purification, were sequenced by Macrogen (Korea).

The sequences similarity was analysed with the program FASTA Nucleotide Database Query available through the EMBL- European Bioinformatics Institute. The phylogenetic affiliation of the sequenced was performed as described by Yakimov *et al.* (2005).

### Rarefaction analysis, diversity index and coverage values

For statistical analyses clones of each library were separately considered to define phylotypes at 97% of similarity, using DNADIST of the Philips package and Sequencer software previously described. PAST (PAleontological STatistics v1.19 software from <http://folk.uio.no/ohammer/past/>) website was used to perform different diversity indices [Rarefaction analysis, taxa, total clones, singletons, Dominance (D), Coverage (C), Shannon (H) and Simpson (D')] for each clone library. To perform rarefaction analysis, total number of obtained clones compared with the number of clones representing each unique phylotype was used to produce the rarefaction curves.

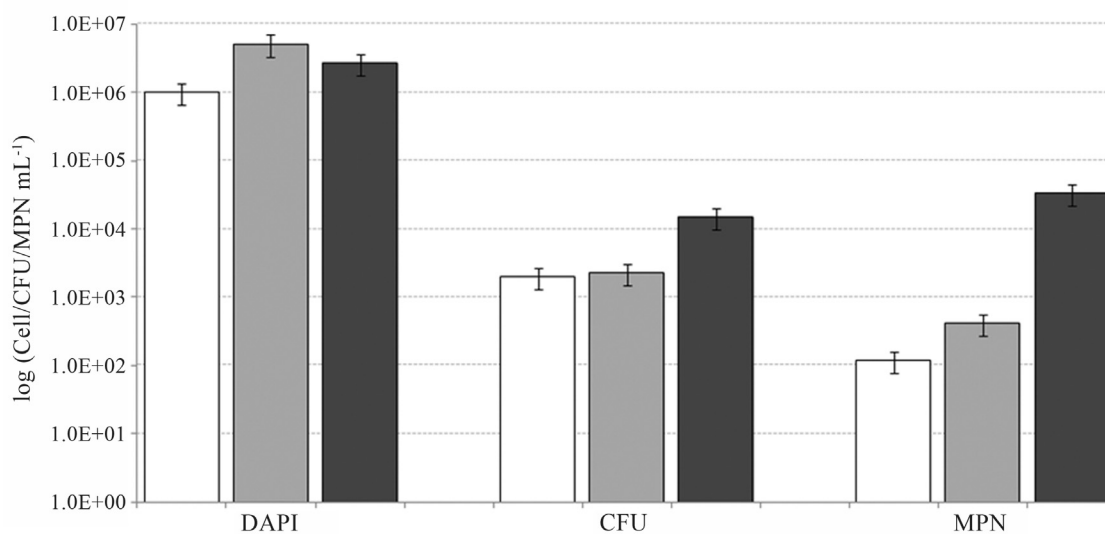
Coverage values were calculated to determine how efficient our clones libraries described the complexity of a theoretical community such as original bacterial community. The coverage value is given as  $C = 1 - (n1/N)$  where  $n1$  is the number of clones which occurred only once in the library.

## Results

### Total cell abundance (DAPI count), heterotrophic cultivable (CFU) and hydrocarbon-degrading bacteria (MPN) counting

The bacterial abundance were measured by DAPI staining (Figure 2). After two days of experimentation, the value of microbial abundance shown an increment of one order of magnitude (value  $\sim 10^6$  cell mL<sup>-1</sup>) in SW+OIL and SW+OIL+EPS<sub>2003</sub> microcosm.

In two days of experimentation, stable values ( $\sim 10^3$  CFU mL<sup>-1</sup>) in the amount of heterotrophic bacteria (CFU) were observed. In microcosm SW+OIL, the addition of bioemulsificant EPS<sub>2003</sub> determined a progressive increment with values that passing from  $2.1 \times 10^3$  CFU mL<sup>-1</sup> (T<sub>0</sub>) to  $1.5 \times 10^4$  CFU mL<sup>-1</sup> (T<sub>2</sub>). Values of  $5.4 \times 10^4$  MPN mL<sup>-1</sup> were observed after two days in experimentation performed with the introduction of EPS<sub>2003</sub>. In control experiment (SW+OIL) values of MPN ( $\sim 10^2$  MPN mL<sup>-1</sup>) are similar to those observed at the beginning of analysis ( $\sim 10^2$  MPN mL<sup>-1</sup>, T<sub>0</sub>).



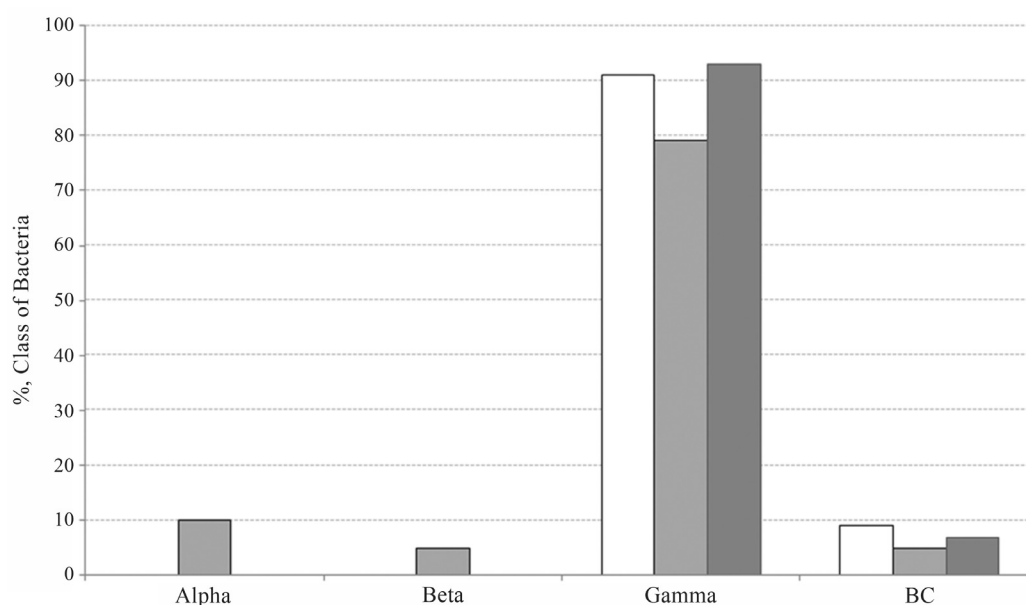
**Figure 2** - Bacterial abundance (DAPI staining  $\text{mL}^{-1}$ ), distribution of heterotrophic cultivable bacteria (Colony Forming Units, CFU  $\text{mL}^{-1}$ ) and distribution of bacteria degrading hydrocarbons (Most probable Number, MPN) measured during experimentation in study. All data were obtained at the beginning ( $T_0$ , SW; white bars) and after two days ( $T_2$ ) of experimentation (SW+OIL and SW+OIL+EPS<sub>2003</sub>; grey and dark bars respectively).

#### Taxonomic analysis of clone libraries.

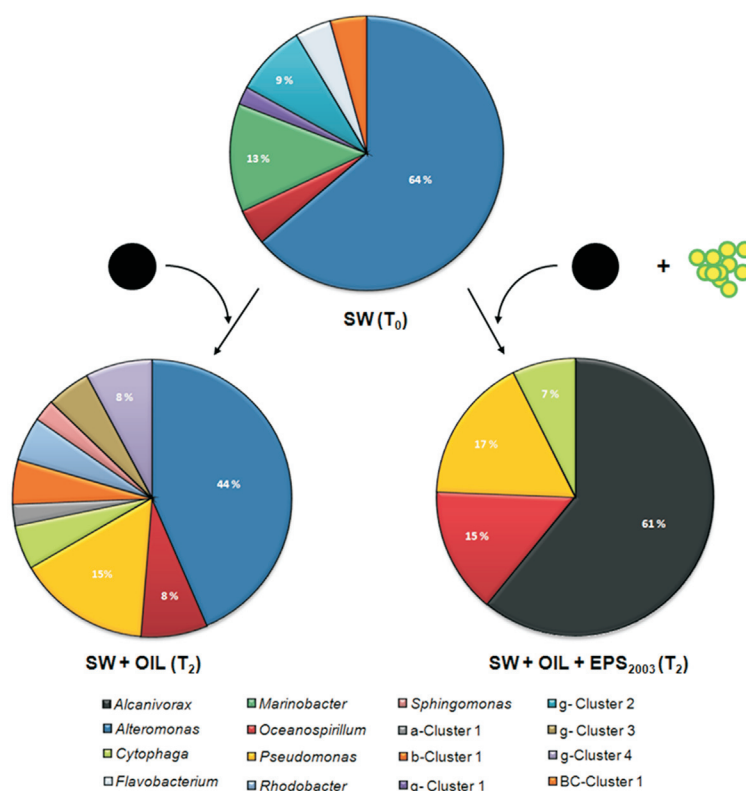
Bacterial community at the beginning ( $T_0$ ) and after two days ( $T_2$ ) of experimentation from microcosms SW+OIL and SW+OIL+EPS<sub>2003</sub> was subjected to 16S rRNA clone libraries taxonomic analysis. A total of 300 randomly picked clones containing 16S crDNA inserts have been sequenced and their phylogenetic affiliation was determined. No chimeric sequences were detected among the clones. Sequences that were > 97% similar were considered to have the same phylotype, or operational taxonomic units (OTUs).

At the beginning of experimentation (SW,  $T_0$ ), in terms of absolute number of clones and the diversity of their sequences, Gammaproteobacteria and Bacteroidetes/Chlorobi (B/C) were the most prominent groups bacteria present in the 16S crDNA clone library (Figures 3 and 4).

The group of the Gammaproteobacteria is the predominant bigger group (92%) with sequences related to the genus of *Alteromonas* (Alteromonadaceae, 64%), *Marinobacter* (Alteromonadaceae, 13%) and *Oceanospirillum* (Oceanospirillaceae, 4%) were detected.



**Figure 3** - Relative abundance (%) of principal bacterial class obtained with 16S crDNA clone libraries at time zero (white bars) and after 20 days of experimentation in SW+OIL (grey bars) and SW+OIL+EPS<sub>2003</sub> (dark grey bars) system. Alpha, Alphaproteobacteria, Beta, betaproteobacteria, Gamma, gammaproteobacteria and BC, Bacteroidetes/Chlorobi.



**Figure 4** - The overview of the prokaryotic diversity and relative abundance of representatives of phylogenetic groups recovered (by 16S rRNA clone libraries) at begging (T<sub>0</sub>) and after two days (T<sub>2</sub>) of microcosm oil spill experimentations. a-, Alphaproteobacteria; b-, Betaproteobacteria; g-, Gammaproteobacteria; BC, Bacteroidetes Chlorobi Group. Black circles and little yellow circles, represent, respectively the Crude Oil and the biosurfactant EPS<sub>2003</sub> insert in the systems in study.

Sequences related to unclassified and uncultured Gammaproteobacteria (g-Cluster 1 and g-Cluster 2) were also detected. The Bacteroidetes/Chlorobi group was made up of one genus represented principally by *Flavobacterium* (Flavobacteriaceae, 4%).

After two days from introduction of crude oil in experimental system (SW+OIL) the structure of microbial population is represented principally for Gammaproteobacteria (69%). During the first two days of analysis it was possible to observe a disappearance of sequences correlated to the *Alteromonas* genus. The absence of such sequences coincided with the increment of those associates to the genus of *Pseudomonas* (15%), *Alteromonas* (44%) and *Oceanospirillum* (8%). Sequences related to Alphaproteobacteria (12%) and B/C group (5%) are also detected.

The taxonomic analysis of the 16S crDNA among the 100 sequences obtained from SW+OIL+EPS<sub>2003</sub> microcosm revealed as the more abundant clones (93%) are affiliated to the Gammaproteobacteria group. The disappearance of sequences correlated to the *Alteromonas* genus coincided with the increment of those associates to the genus of *Pseudomonas* (17%), *Alcanivorax* (61%) and chemio-organotrophic *Oceanospirillum* (15%). Sequences

related to the *Cytophaga* (7%) genus are also present (Figures 3 and 4).

Measure of “Diversity indices” of SW+OIL and SW+OIL+EPS<sub>2003</sub> experiment show as the abundance of sequences related to *Alcanivorax* genus determined high values of Dominance (D) were shown in Table 1.

## Discussion

The aim of the present study has been to evaluate the shift of the composition of bacterial community structure and quick stimulation of hydrocarbonoclastic bacteria in

**Table 1** - Diversity indices calculated for the five clone libraries from different time of experimentation in microcosms SW+OIL and SW+OIL+EPS<sub>2003</sub>.

	SW	Microcosm SW+OIL	Microcosm SW+EPS <sub>2003</sub> +OIL
	T <sub>0</sub>	T <sub>2</sub>	T <sub>2</sub>
Taxa (S)	7	10	4
Total clones	100	100	100
Single clones	1	2	0
Dominance (D)	0.43	0.23	0.42

first days of an oil spill phenomena consequently to rapid introduction of bioemulsifier EPS<sub>2003</sub>. In marine environments the structure and function of the bacterial population change fast due to anthropogenic impacts, adapting to the new conditions. In fact, microbial community response to an environmental stressor (*e.g.* the toxic effect of oil) is the transition to a specific surviving condition (Cappello and Guglielmino, 2006) which favours hydrocarbon tolerating species or hydrocarbonoclastic bacteria that consuming it (Cappello *et al.*, 2007). Study of the structure of microbial population, during the SW+OIL+EPS<sub>2003</sub> experimentation, showed a rapid increment of the microbial population with increasing from 10<sup>5</sup> cell mL<sup>-1</sup> to 10<sup>6</sup> cell mL<sup>-1</sup>. Also in mesure of heterotrophic cultivable bacteria (CFU) shown as the introduction of oil determined a toxic effect; this effect already described in literature (MacNaughton *et al.*, 1999) as “a phase of adaptation”, characterized by a reduction of bacterial taxonomical group with selection of genera able to tolerate and/or to degrade the oil. In the early phase of simulation of oil spill it was possible to observe a variation of microbial community with selection of bacteria belonging to  $\gamma$ -Proteobacteria and Bacteroidetes/Chloroby group which are affiliates whit hydrocarbons degrading bacteria. Inside  $\gamma$ -Proteobacteria group, some genera (*i.e.* *Oceanospirillum* and *Pseudomonas*) persisted by the start until the end of the experimentation. The Bacteroidetes/Chloroby group present a relative increase over time with sequences related to *Cytophaga* genus (belonging to the Flavobacteriaceae family). Bacteria belonging to this group are suggested to contribute to the biodegradation phenomena occurring in natural environment, with particular reference to the degradation of cyclical hydrocarbons and polycyclic aromatic hydrocarbons (PAHs). The 16S rRNA clone library reveal as the most fraction of sequences obtained after two day from the SW+OIL system are affiliated to the *Alteromonas*, *Oceanospirillum*, *Pseudomonas* genera; simultaneously did not allowed the presence of sequences belonging to marine obligate hydrocarbonoclastic bacteria (OHCB) group (*i.e.* *Alcanivorax sp.*). As already observed by other authors (Kasai *et al.*, 2002; MacNaughton *et al.*, 2009), the development of OHCB bacteria in petroleum polluted environments poor of nutrients (N and P) is limited respect to other heterotrophic microbial genera, able to tolerate (and/or to degrade) the presence of oil. Bacteria developed in the SW+OIL system have probably obviated the lack of nutrients using as alternative sources of additional nitrogen and phosphorus the compounds released by the microbial biomass died as a result of the toxic effect of the crude oil. The measure of the number of vital cells (data not shown), during the first 2 days of incubation, supports such hypothesis. This hypothesis is consistent with the taxonomic analysis of microbial community at the beginning of experimentation (T<sub>0</sub>), which does not reveal the presence of OHCB bacteria that have been suggested as bioindicator of regressed pollution (Yakimov *et al.*, 2008). These results are in according to data

obtained by Roling *et al.* (2002) that evidenced that in oil polluted systems, any process of degradation takes place without addition of the nourishing. According to the data present in literature, the “adaptation” to oil presence is a very complex process that requires a variable time (from several days to weeks) just at the moment that the composition of the oil continue “to force” the system. Soon after the occurring of “stress” conditions (determined by the presence of oil), the microbial population tries to restore the original diversity (Mac Naughton *et al.*, 1999).

Overall results indicated that the addition of bio-surfactant EPS<sub>2003</sub> determine quali-, quantitative changes in the microbial population and enhance processes of oil degradation. These results are in line with observation of Yoshida *et al.* (2006). Data particularly interesting is obtained from analysis of 16S crRNA clone libraries after introduction of biosurfactant. Data shown as sequences belonging to the Alcanivoraceae family become, in two days, quantitatively dominant in the system in study (61%). The increase of bacteria related to *Alcanivorax spp.* was presumably favoured by the greater availability of inorganic nourishing present in the system from the bacterial cells died due to toxic effect of the bioemulsificant and oil (Cappello *et al.*, 2007).

As reported bacteria closely related to *Alcanivorax* became a dominant bacterial population in petroleum-contaminated seawater when nitrogen and phosphorus nutrients were supplied in adequate quantity; different study demonstrate as *Alcanivorax* plays a fundamental role in the degradation of linear hydrocarbons in environments polluted by oil and different studies have confirmed as bacteria related to *Alcanivorax* are major players in the bioremediation of oil-contaminated marine environments (Kasai *et al.*, 2002; Roling *et al.*, 2002; Yakimov *et al.*, 2004).

The growth of *Alcanivorax* in crude or heavy oil was not particularly rapid in comparison with other oil-degrading bacteria inhabiting seawater; however the EPS<sub>2003</sub> seem determine a massive increment of this bacterium in system in study. These findings further confirm that *Alcanivorax* not only degrades oil hydrocarbons *in vitro*, but seems to play a crucial role in the natural cleaning of oil-polluted marine systems. Our results concerning EPS<sub>2003</sub> effectiveness are in agreement with findings of Bruheim *et al.* (1999) who suggests that the efficacy of a biosurfactant in the oxidation of paraffins does not depend exclusively on the physico-chemical characteristic of chemicals but also on the link whit microbial community (interaction, stimulation etc.) and in this case from selection of hydrocarbonoclastic bacteria with high degradative capability.

## Conflict of Interest

None of the authors of this paper has a direct financial relation with the commercial identities mentioned in this paper that might lead to a conflict of interest.

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