Kill•Spill

Integrated Biotechnological Solutions for Combating Marine Oil Spills

Deliverable D5.7

Report on application of a modular sediment remediation system and clean up strategies.

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1 About this deliverable

The activity of IAMC-CNR within WP5 focused on contribution to the development of 20-"Kill●Spill Sed-Cleaner" in subtask 5.4.1: "Optimization of *in situ* biostimulation and bioaugmentation treatments within modular systems, which can be placed in the targeted polluted region"

The aim of this deliverable was to develop and validate of *in situ* bioremediation techniques aimed at recovering of chronically polluted seawater and sediments. This has been achieved by stimulation of the autochthonous aerobic hydrocarbon-degrading bacteria in a modular sediment remediation system, specially designed to provide extensive oxygenation of otherwise anaerobic sediments.

Data presented here are of particular importance as the perspective of developing and testing new technologies which enhanced the biodegradation of hydrocarbon in contaminated sediment. Detailed protocol of this clean-up strategy was developed in frames of WP8 "Field Testing of the Most Promising Technologies and Benchmarking with existing products" and described in deliverables D8.1 and 8.2.Noteworthy, the results of this Delivery have been published in 2015 (Kill●Spill was acknowledged as the principal source of funding) in ***Frontiers in Microbiology*** (IF=3.941) as research article: Genovese, M., Crisafi, F., Denaro, R., Cappello, S., Russo, D., Calogero, R., Santisi, S., Catalfamo, M., Modica, A., Smedile, F., Genovese, L., Golyshin, P.N., Giuliano, L., and Yakimov, M.M. (2014) Effective bioremediation strategy for rapid in situ cleanup of anoxic marine sediments in mesocosm oil spill simulation*. Frontiers in Microbiology* 5:162. *doi: 10.3389/fmicb.2014.00162*.

Article has received great interest, which can be seen by the number of citations (21) as for May 2016.

2 Report on application of a modular sediment remediation system in experimental mesocosm

The experiment was carried out in rectangular tank of 3.75 m^3 capacity (166 cm long, 150 cm deep, 150 cm wide). This reservoir was filled with ca. 2000l of seawater taken directly from the harbor of Messina (38 11' 42.58'' N 15◦ 34' 25.19'' E). Prior to use, the seawater was filtered through a 200 µm nylon mesh to remove large metazoans and detritus. Approximately 1000 kg of sandy sediments were collected at the same place and artificially contaminated with Bunker C furnace fuel oil (6500 ppm) to simulate an oil spill accident. Temperature inside the mesocosm was maintained about 20 ± 1°C for the experimental period. The mesocosm continuously received seawater at a flow rate of 1 l min^{-1}. The modular sediment remediation used in mesocosm experiments is shown on Figure 1.

This remediation system was developed especially for *in situ* aeration (20 l min^{-1}) of polluted sediments without their removal from contaminated site to avoid the re-contamination of an adjacent aquifer. The reactor was inserted into the sediment. Sediment inside the system were treated by the reactor, and those sediments outside of it were undisturbed and served as a control.

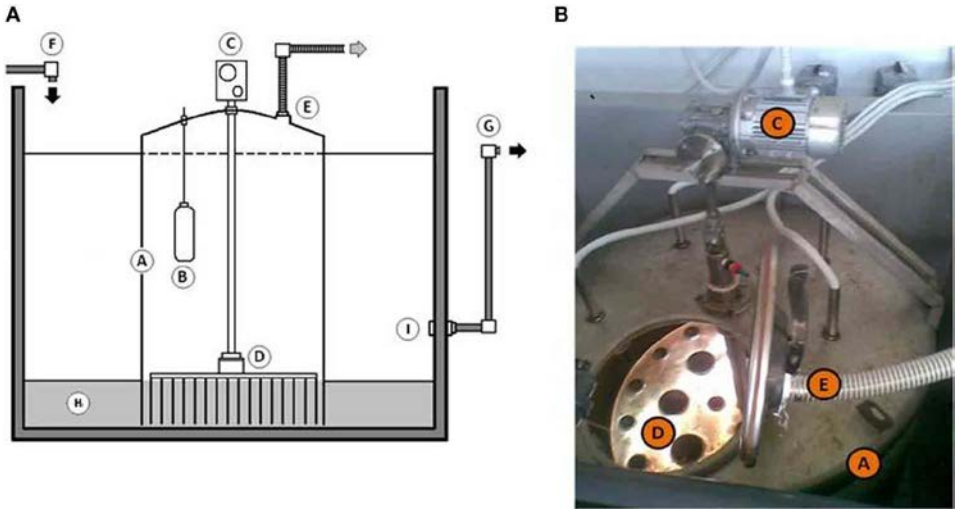

Figure 1 (A,B) Schematic representation (A) and detail (B) of "Modular Sediment System (MSS)" used throughout this study.
Abbreviation used: A, Modular Sediment Remediation; B, temperature controller; C, external air pump; D, steel plate with needles to supply an oxygen into deep sediments; E, exhaust tube; F, seawater inlet; G, seawater outlet; H, contaminated sediments; I, overflow regulation system.

2.1 Experimental procedure

All experiments were conducted for 3 months. To monitor the succession of the microbial population and the efficiency of petroleum degradation, 1.5–2.0 kg of sediments (up to 10 kg in total) were sampled on fixed days (T_0, T_1, T_{29}, and T_{90}) at six different points inside and outside the system. Additionally, measurements of the biochemical oxygen demand (BOD_5) (Delzer & McKenzie, 2003), reduction potential (E_h), and eco-toxicological assays (Microtox® and *Corophium orientale* mortality test) were monitored.

2.2 Results

2.2.1 Geochemical properties of the sediments

Oxygen consumption in the external superficial sediments (0– 5 cm) was monitored during all 3 months of experimentation. These values were compared with BOD in the internal sediments taken at the beginning (T0), after 1 day (T1), 1 month (T29), and after 3 months (T90) of experimentation (Figure 2). Sediments outside of the modular system remediation exhibited constant BOD rates of approximately 2.5 mg O_2 day^{-1} kg^{-1} during all period of experimentation, whereas the aerated sediments inside of the modular system demonstrated a progressive increment of oxygen consumption. Maximum of oxygen demand (16.0 mg O_2 day^{-1} kg^{-1}) was obtained at T29 and afterwards the BOD values trended to diminish, reaching 10.0 mg O_2 day^{-1} kg^{-1} at the end of experiment.

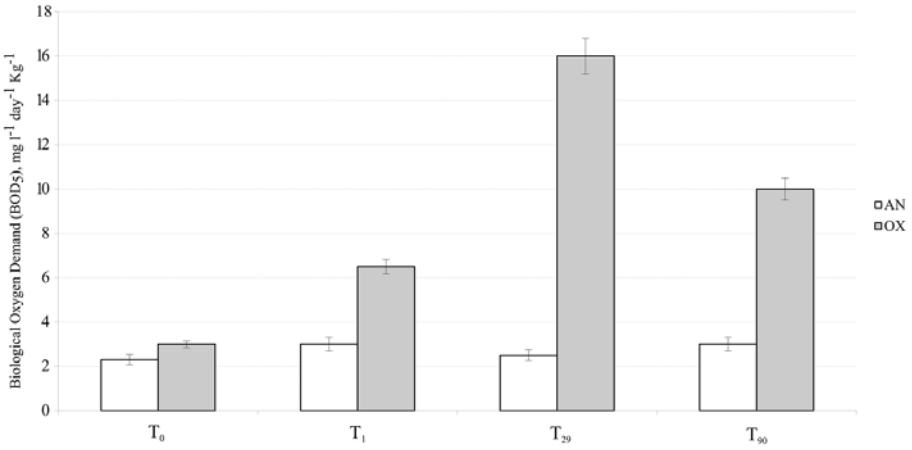

Figure 2 **Dynamic of oxygen consumption (BOD values) measured in external (untreated) superficial sediments (white bars) and internal sediments (grey bars).**
Error bar indicates the standard deviation of triplicate measurements.

Outside the remediation system, the amendment of the Bunker C furnace fuel oil turned initially oxygenated (T0, E_h = 77 ± 4 mV) sediments into highly reduced ones (Figure 3). The external sediments below 5 cm became oxygen-depleted already within 1 day after spiking, obviously due to the active respiration of aerobic heterotrophic microorganisms. The reduction potential of external sediments decreased continuously during the experiment and after 3 months reached the E_h values of −345 mV in the deepest layers. In contrary, treated internal sediments remained aerobic during all period of bioremediation effort.

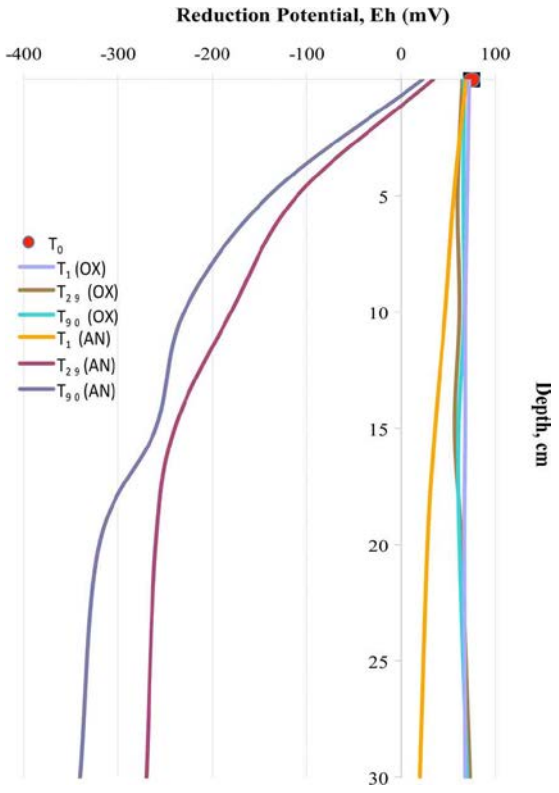

Figure 3 **Reduction potential (E_h) measured in external anaerobic sediments (AN) and in internal aerated sediments (OX) during 3 months of experimentation.**

2.2.2 *Hydrocarbon analysis*

Before the addition of 6500 ppm of Bunker C fuel oil into the mesocosm, the total hydrocarbon concentration in the original Messina harbour sediments was estimated at the level of 120 ppm. The petroleum hydrocarbon (PH)s fingerprint analysis showed a clear dominance of alkyl- aromatic derivatives (97%) over aliphatic hydrocarbons (3%) (data not shown). Once Bunker C fuel oil was added to the sediments, the total extracted and resolved hydrocarbons (TERHC) fraction mass balance was shifted toward the dominance of aliphatic and naphthenic hydrocarbons (70%) over aromatics (30%). The degree of the Bunker C fuel oil degradation in both, aerated sediments and in the untreated anoxic sediments was examined during all 3 months of experimentation. The concentration of PHs, especially aliphatic hydrocarbons was normalized using the pristane/phytane ratio, and the values obtained in triplicate sub- samples were averaged.

In the aerated internal sediments, the total degradation of Bunker C fuel oil TERHC fraction was 97.7 ± 0.9%. In contrast, external anoxic sediments contained more than 81.8 ± 1.2% of initially added Bunker C fuel oil (Figure 4). Additionally, we performed the gravimetrical analysis of total extracted hydrophobic fraction (TEHF) in the sediments. The TEHF values inside the remediation system accounted for 780 ± 80 mg kg^{-1} dry sediment weight, whereas in concordance with extremely slow biodegradation rates under anaerobic conditions, the external sediments contained more than 5400 ± 120mg kg^{-1} of hydrophobic material.

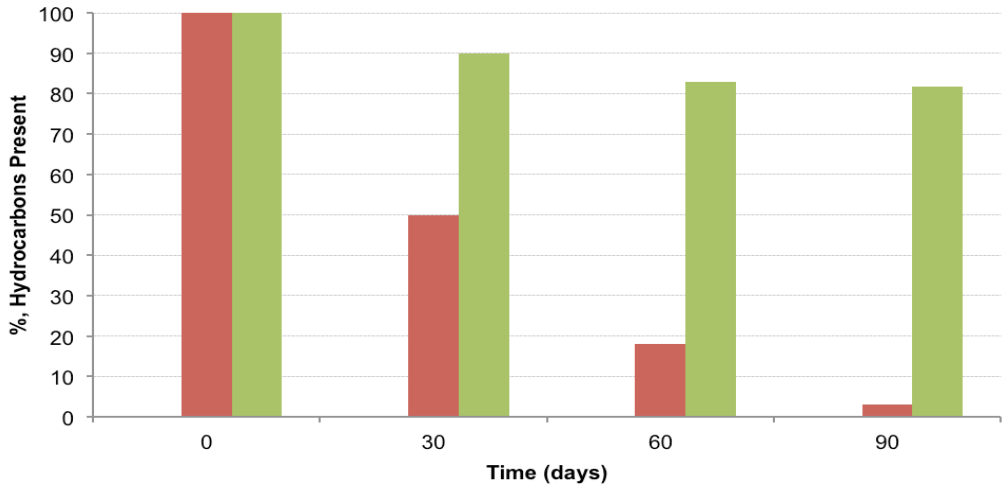

Figure 4 **Dynamic of hydrocarbon consumption measured in external (untreated) superficial sediments (green bars) and internal sediments (red bars).**

2.2.3 *Ecotoxicological analysis*

According to standard guidelines of Italian Institute for Environmental Protection and Research (ISPRA, 2013), eco- toxicological analysis of hydrocarbon-contaminated sediments were carried out using the Microtox® luminescence and amphipod *Corophium orientale* bioassays. Eventual decrease in Microtox® bioluminescence was measured on sediment pore water, whereas the rate of amphipods mortality was tested by direct exposition of *C. orientale* with the petroleum-contaminated sediments during 10 days. Following the EN12457 protocol, we combined the sediment pore water with sterile seawater in both 1:2 and 1:10 ratios (v/v) and no significant level of bioluminescence decay has been observed. As is reported in EN12457 protocol, Microtox® bioluminescent assay tested on sediment pore water typically exhibits an underestimated sensitivity against highly hydrophobic contaminants, such as PHs. This is mainly due to both extremely low

solubility of these compounds in water and an almost irreversible adsorption to a sedimentary matrix. Corresponding to the standardized protocol described by Onorati et al. (1999), the toxicological analysis of the sediments was performed with amphipods *C. orientale.* Addition of Bunker C fuel oil to the sediments (T0) caused the mortality in almost all organisms (98 ± 2%). The petroleum-contaminated external sediments remained highly toxic during all 3 months of experimentation with mortality indices exceeding 90% (Figure 5). At the same time, the toxicity of polluted internal sediments dropped almost twice after 1 month of aeration (T29) and continued to decrease till the end of bioremediation treatment, approaching the vitality of 62% of amphipods exposed to T90 sediments. This indicated that the internal sediment at T90 was significantly less toxic than their external counterpart.

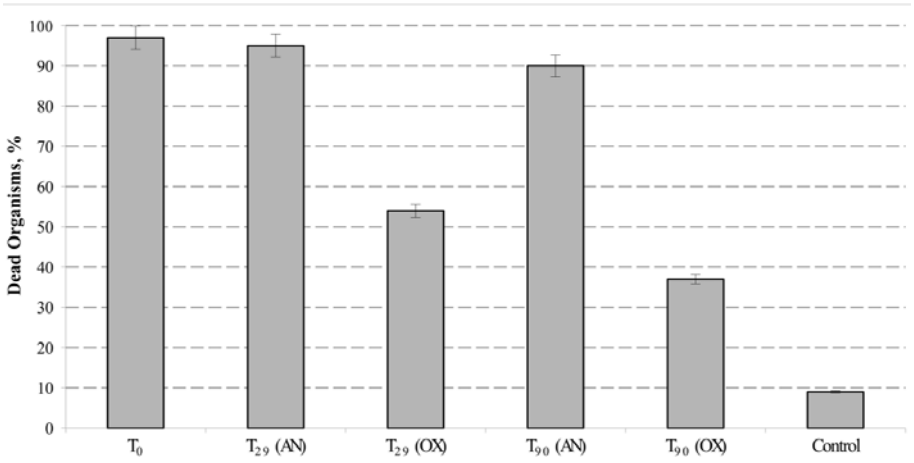

Figure 5 Mortality of *Corophium orientale* organisms in polluted (AN), treated (OX), and control (native) sediments.
Error bar indicates the standard deviation of duplicate measurements.

2.2.4 Diversity and succession of bacterial communities during bioremediation strategy

To monitor the succession of metabolically active microbial communities during the bioremediation treatment, five 16S rRNA transcript libraries were established.

The majority of native T0 clones were affiliated with the *Gammaproteobacteria* (51%), followed by the *Alphaproteobacteria* (22%). Other proteobacteria, belonging to microaerophilic and anaerobic *Epsilon*- and *Deltaproteobacteria* were also present, although in significantly lower numbers (6 and 2% of all clones analyzed, respectively). Remaining fraction of T0 microbial community consisted of the members of *Cyanobacteria* (7%), *Chloroflexi* (5%), *Verrucomicrobia* (3%), and *Bacteroidetes* (2%) (Figure 6). At the level of the Class, the derived from members of *Gammaproteobacteria* were predominant in all analyzed MSS internal sediments, with percentage ranging from 73 to 88%. There was a 50%-reduction of *Alphaproteobacteria* observed during first month of sediment treatment (decrease from 22 to 11%). Further on, their numbers returned to the initial (T0) values by the end of experiment. No *Deltaproteobacteria*– related organisms were detected in the internal MSS sediments throughout the experiment

A completely different scenario was observed regarding the succession of microbial community thriving in the external anoxic sediments. As we mentioned above, after loading the Bunker C fuel oil, the sediments became highly reduced within a short period of time and were inhabited mainly by the members of *Deltaproteobacteria* (96.8%).

At the genus and species level, more than 10% of initial microbial population was attributed to obligate marine hydrocarbonoclastic bacteria *Thalassolituus oleivorans* (Yakimov et al., 2004). This organism seemed to be sensitive to Bunker C fuel oil, since its abundance decreased to 2% after 1 day of oil exposition and disappeared afterwards. Three other OMHCB belonging to genera *Alcanivorax, Cycloclasticus,* and *Marinobacter* demonstrated similar dynamics, i.e., being in relative minority in the beginning of the experiment, they became predominant in T29 microbial community and disappeared in the T90 library (Figure 6).

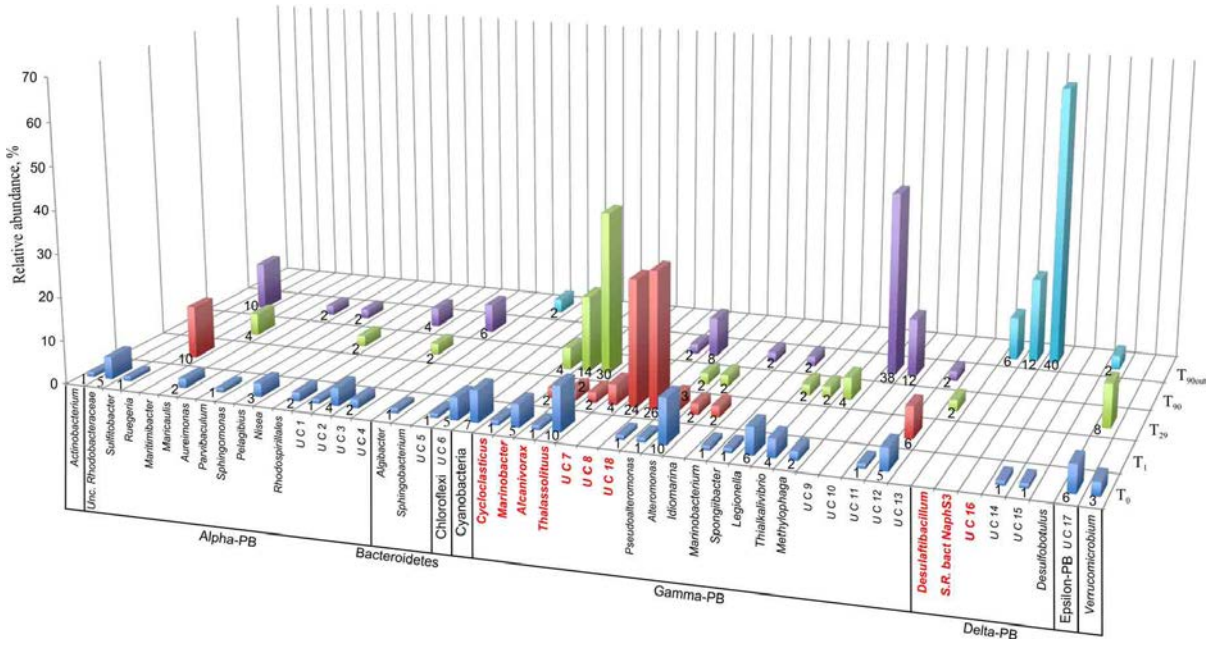

Figure 6 Dendrogram representation of taxonomic analysis of 16S crDNA clones retrieved from five libraries

The numbers at the base of columns represent the percentage of clones in corresponding libraries. Abbreviations used: PB, *Proteobacteria* divisions; UC, Unaffiliated cluster. Bacterial groups involved in, or associated to hydrocarbon degradation are marked in bold and red.

Noteworthy, the addition of Bunker C fuel oil to sediments drastically changed the structure of T1 microbial community and the proportion of aforementioned *Gammaproteobacteria-related* OMHCB decreased threefold compared with the initial population. In contrast, previously undetected organisms related to dinoflagellate- associated *Rugeria* sp. and to three deep-branching clusters of *Gammaproteobacteria* accounted for 77% of all analyzed T1 clones. Having only 16S rRNA gene sequences at our disposal, we cannot state that these uncultured organisms were involved in biodegradation activity, but they definitely possessed a remarkable resilience to the toxicity of Bunker C fuel oil.

Additionally to 16S rRNA-based analysis, the diversity and succession of both, total bacterial population and hydrocarbon-degrading bacteria, was assessed through the combined application of CARD-FISH and qPCR. Using the *Eubacteria-specific* probe Eub338, the concentration of CARD-positive cells at the beginning of experiment was estimated at $2.98 \pm 0.17 \times 10^6$ cells gram^{-1} (Table **1**) Their numbers decreased by 20% within 1 day after the oil spill simulation ($P < 0.001$, $n = 10$) and reached initial values at the end of experiment ($2.79 \pm 0.12 \times 10^6$ cells gram^{-1}). Noteworthy, a tenfold increase in the number of CARD-positive cells ($2.95 \pm 0.11 \times 10^7$ cells gram^{-1}) was detected after 29 days of oil spill, which fully corresponded to the observed dynamics of the BOD and clone libraries' values (Figure 2). Before the oil spill simulation (T0), the fraction of *Alcanivorax-related* cells,

detected with the CARD-FISH genus-specific probe, accounted for 7.7% of all Eub338-positive cells. After the addition of Bunker C fuel oil, their abundance increased within 1 month up to 27.5%. According to the analysis of 16S crDNA clone libraries, *Alcanivorax* became extinct in microbial community thriving in the modular remedation system internal sediments at the end of experiment. Dynamics of the *Marinobacter-related* bacteria was comparable with that of *Alcanivorax* population, with the only exception that the *Marinobacter* cells decreased their abundance by 44% at T1, likely due to the higher sensitivity to the load of fuel oil. Although due to the overwhelming growth of *Alcanivorax,* the relative abundance of *Marinobacter* during bioremediation experiment has never exceeded its initial values (15.2% of all Eub338-stained cells at T0) and at T29 (corresponding to the maximum of cell density in system) accounted for only 3% of total microbial community. Bacteria stained with *Cycloclasticus*- specific CARD-FISH probe, initially present in mesocosm sediments at concentration of $1.14 \pm 0.15 \times 10^5$ cells $gram^{-1}$, were not detected at T1, whereas their concentration increased four times after 1 month of the oil spill simulation. Similarly to the dynamics of *Alcanivorax,* neither *Marinobacter,* nor *Cycloclasticus* were present in the system microbial community at the end of experiment. (Genovese *et al.,* 2014)

Succession of hydrocarbon-degrading bacteria during the sediment bioremediation was additionally quantified by qPCR. Based on the a priori higher sensitivity of qPCR approach, obtained numbers were slightly higher than those from taxon-specific CARD-FISH data. Nevertheless, obtained results remarkably corroborated with CARD-FISH counts and the general trend in *Alcanivorax, Marinobacter,* and *Cycloclasticus* dynamics was identical (Table 1). None of these hydrocarbon-degrading bacteria were detected by qPCR at the end of experiment.

Table 1 CARD-FISH and qPCR cell number quantification in internal (oxygenated) sediment during the bioremediation treatment.

Method	Cell numbers, 10^5 x gram sediments $^{-1}$ ± SD				
	Probe/Primers	T0	T1	T29	T90
CARD-FISH	Eubacteria	29.8 ± 1.7	23.6 ± 1.5	295.0 ± 11.0	27.9 ± 1.2
	Alcanivorax sp	2.3 ± 0.1	3.1 ± 0.2	81.0 ± 1.3	ND
	Marinobacter sp	4.5 ± 0.2	2.6 ± 0.1	9.0 ± 0.1	ND
	Cycloclasticus sp	1.2 ± 0.2	ND	4.9 ± 0.1	ND
qPCR*	alkB2	5.17 ± 0.17	4.73 ± 0.18	94.60 ± 3.80	ND
	alkB	7.90 ± 0.23	3.50 ± 0.20	15.30 ± 2.00	ND
	phnA	2.10 ± 0.16	$3.78 \pm 0.12 \times 10^{-3}$	7.13 ± 0.28	ND

* These values mean the average number of cells detected in triplicate from three individual subsamples of sediments collected in different parts of MSS.

3 Conclusion

The stimulation of autochthonous bacteria to tackle the pollution in contaminated environment is widely used in the remediation of aerobic sites but the innovation of this technology consist in the application to oxygen-depleted marine sediments. For this reason, to initiate the self-cleaning process in sediments driven by indigenous aerobic obligate marine hydrocarbonoclastic bacteria (OMHCB), we used *in situ* aeration of polluted anoxic sediments in specially designed modular sediment remediation. It is generally assumed, that petroleum contamination induces drastic changes in the bacterial community structure associated with a decrease of diversity (Yakimov *et al*.,

2005, 2007; Head *et al* 2006). These changes were referred to both toxic effect of oil and a strong selection toward highly specialized hydrocarbon-degrading microorganisms Accordingly, the most drastic shifts in the remediation system bacterial community dynamic was observed at the beginning and after 1 month of the oil spill. As revealed by 16S crDNA clone library analysis, more than half of the modular remediation system microbial population at T29 belonged to *Alcanivorax* (43%), *Cycloclasticus* (7%) and *Marinobacter* (5%), the genera of OMHCB, known to play a pivotal role in petroleum degradation in marine environments. At the end of the treatment, the level of TERHC degradation in the internal (aerated) sediments was almost 98%, and the resulting microbial community was characterized by an almost complete extinction of OMHCBs As a consequence of successful bioremediation, the *Corophium orientale* eco-toxicological bioassay revealed that toxicity of the treated sediments was substantially lower compared with the untreated external sediments. Thus, our studies for the first time demonstrated that petroleum-contaminated anaerobic marine sediments could be efficiently recovered by their capping and *in situ* aeration, thus stimulating the self-cleaning potential due to reawakening of residing aerobic OMHCBs.

4 References

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