Effective bioremediation strategy for rapid in situ cleanup of anoxic marine sediments in mesocosm oil spill simulation

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The purpose of present study was the simulation of an oil spill accompanied by burial of significant amount of petroleum hydrocarbons (PHs) in coastal sediments. Approximately 1000 kg of sediments collected in Messina harbor were spiked with Bunker C furnace fuel oil (6500 ppm). The rapid consumption of oxygen by aerobic heterotrophs created highly reduced conditions in the sediments with subsequent recession of biodegradation rates. As follows, after 3 months of ageing, the anaerobic sediments did not exhibit any significant levels of biodegradation and more than 80% of added Bunker C fuel oil remained buried. Anaerobic microbial community exhibited a strong enrichment in sulfate-reducing PHs-degrading and PHs-associated Deltaproteobacteria. As an effective bioremediation strategy to clean up these contaminated sediments, we applied a Modular Slurry System (MSS) allowing the containment of sediments and their physical–chemical treatment, e.g., aeration. Aeration for 3 months has increased the removal of main PHs contaminants up to 98%. As revealed by CARD-FISH, qPCR, and 16S rRNA gene clone library analyses, addition of Bunker C fuel oil initially affected the activity of autochthonous aerobic obligate marine hydrocarbonoclastic bacteria (OMHCB), and after 1 month more than the third of microbial population was represented by Alcanivorax-, Cycloclasticus-, and Marinobacter-related organisms. In the end of the experiment, the microbial community composition has returned to a status typically observed in pristine marine ecosystems with no detectable OMHCB present. Eco-toxicological bioassay revealed that the toxicity of sediments after treatment was substantially decreased. Thus, our studies demonstrated that petroleum-contaminated anaerobic marine sediments could efficiently be cleaned through an in situ oxygenation which stimulates their self-cleaning potential due to reawakening of allochthonous aerobic OMHCB.

Keywords: marine anoxic sediments, crude oil pollution, hydrocarbonoclastic bacteria, in situ bioremediation, aerated slurry system

INTRODUCTION

The worldwide production of crude oil and natural gas is at the peak, with an estimated worldwide production of 89 million barrels per day in 2011 (International Energy Agency, http://omrpublic.iea.org/). Approximately, a half of this amount is transported by the sea (Gertler et al., 2010). As follows, worldwide marine coastal areas are exposed to the oil spills occurring as a result of accidents or illegal practices (Psarros et al., 2010). The release of thousands of tons of petroleum hydrocarbons (PHs) affects the marine environment and causes severe ecological and economical damage. For example, only the pollution resulting from the tanker washing or ballast water has been estimated to contribute about 2 million tons per year worldwide (Ferraro et al., 2007; Gertler et al., 2010). The recent spillage of 780,000 m³ of oil into the Gulf of Mexico proved again that human activities might cause a contamination without precedents. This accident presented a huge challenge to existing oil spill treatment methods, and current technologies were not able to cope with the size and nature of the Deepwater Horizon oil spill. Therefore, there is an urgent demand for development and optimization of bioremediation techniques that can play a central role in marine oil spill response contingency plans.

One of the most important issues in bioremediation is the application (or stimulation) of autochthonous hydrocarbon-degrading microbial populations. Some marine gammaproteobacteria have a high affinity toward PHs. Species such as Alcanivorax borkumensis (Yakimov et al., 1998), Cycloclasticus pugetii (Dyksterhouse et al., 1995), Oleispira antarctica (Yakimov
et al., 2003), *Oleiphilus messinensis* (Golyshin et al., 2002), and *Thalassolituus oleivorans* (Yakimov et al., 2004) constitute a distinct group of obligate marine hydrocarbon-degrading bacteria (OMHCB). Following a sudden oil spill event, these organisms outcompete most of the naturally occurring oligotrophic marine microorganisms (Hara et al., 2003; Yakimov et al., 2007). Growing on PHs, aerobic OMHCB use oxygen not only as the terminal electron acceptor for respiratory energy conservation, but also as an indispensable reactant in the PHs activation mechanism. Thus, the stimulation of the OMHCBs degradation activity in the contaminated site can significantly improve the self-cleaning potential. Unfortunately, due to metabolic requirements of the OMHCBs, this type of bioremediation is restricted to either seawater column or superficial sediments. Due to a high biological oxygen demand and its slow diffusion into marine sediments, these compartments below the surface are typically highly reduced (Engelen and Cypionka, 2009). The realization that activated oxygen is used to overcome the chemical sluggishness of hydrocarbons has for some decades favored the view that hydrocarbons are not biodegradable under anoxic conditions (Widdel and Rabus, 2001). Although recently, a number of strictly anaerobic microorganisms have been shown to utilize PHs as growth substrates, this process is extremely slow compared to aerobic degradation and can not be considered as rapid bioremediation scenario.

The aim of this study was to monitor both immediate and long-term responses of indigenous microbial consortia to a simulated oil spill and during bioremediation treatment. Keeping in mind that the decontamination of PHs-polluted anoxic sediments is a very sluggish process, the stimulation of indigenous aerobic OMHCBs was performed by the *in situ* aeration of sediments without their removal from contaminated side to avoid the re-contamination of adjacent aquifer. The reactor was inserted into the sediment. Sediment directly beneath the MSS were treated by the reactor, and those sediments outside of it were undisturbed and served as a control. All experimentations have been conducted for 3 months. To monitor the succession of 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 (T0, T1, T29, and T90) at six different points inside and outside the MSS. Additionally, measurements of the biochemical oxygen demand (BOD5), reduction potential (Eh), and eco-toxicological assays (Microtox® and *Corophium orientale* mortality test) were monitored. The Eh of sediment was measured by a Waterproof CyberScan PCD 650

![FIGURE 1](image1.png) **FIGURE 1** (A,B) Schematic representation (A) and detail (B) of “Modular Slurry System” used throughout this study. Abbreviation used: A, Modular Slurry System; 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.
multivariate (Eutech Instruments) according to the manufacturer’s instructions at aforementioned time intervals. BOD5 was measured using a BOD sensor (VELP Scientifica) after 5 days of incubation in accordance with the manufacturer’s instructions.

ANALYSIS OF PETROLEUM HYDROCARBONS

Efficiency of petroleum degradation was estimated by the analysis of total extracted and resolved hydrocarbons (TERHC). At the fixed time points, TERCH were extracted from sediments following the 3550C EPA (Environmental Protection Agency) procedure. Briefly, 500 ml mixture of CH2Cl2:CH3COCH3 (1:1, vol/vol) was added to 1000 g of pooled and dried sediments or external sediments, collected from six different sites (see above). Mixture was sonicated for 2 min in ultrasound bath (Branson 1200 Ultrasonic Cleaner, Branson USA). Samples were further shaken at 150 rpm for 30 min, centrifuged for 10 min at 5000 × g and supernatant was passed through a ceramic column filled with anhydrous Na2SO4 (Sigma-Aldrich, Milan). Same treatment of pooled and dried sediments was repeated with 500 ml of CH2Cl2 and the obtained extracts were combined and volatilized to the dryness. Residues were re-suspended in CH2Cl2 prior to the gas chromatography (GC) analysis (Rocchetti et al., 2011). All measures were performed using a Master GC DANI Instruments (Development ANalytical Instruments), equipped with SSL injector and FID detector. Sample (1 μl) was injected in splitless mode at 330°C. The analytical column was a Restek Rxi-5 Sil MS with Integra-Guard, 30 m × 0.25 mm (ID × 0.25 μm film thickness). Helium carrier gas was maintained at a constant flow of 1.5 ml/min. TERCH were calculated using the mean response factors of n-alkanes, i.e., individual n-alkane concentrations from n-C15 to n-C30, pristane and phytane were calculated for each sample. To estimate the biodegradation of aliphatic fraction, the evaluation indices n-C17/pristane and n-C18/phytane were selected for this study. The amount of analyzed TERCH was expressed as ppm (part per million) or mg kg−1.

ECOTOXICOCLOGICAL ASSAYS

The Microtox® luminescence assay was performed on sediment pore water. Sub-samples of sediment were centrifuged (5000× g for 45 min, 4°C) and filtered (0.45 μm nitrate cellulose membrane) to remove the fine suspended particles and maintained at 4°C until used in assays. Microtox® toxicity tests were conducted according to the standard procedures EN12457 with the following modifications. As far as the Solid Phase Test deals with fine particles that affect the bioluminescence of bacteria (Bulich et al., 1992; Benton et al., 1995; Ringwood et al., 1997), each experimental sediment sample was compared with a reference sediment sample with the same granulometry, collected from a pristine site. Toxicities were reported as effective concentration of toxicant resulting in a 50% decrease in bioluminescence (EC50). EC50 with 95% of confidence intervals were calculated following the procedures outlined in the Microtox® System Operating Manual (Microtox, 1982). Amphipods Corophium orientale were delivered from CIBM (Livorno, Italy). The animals were used following the procedure reported by Onorati et al. (1999). Briefly, the juveniles and young adults, which passed through 1000 μm and retained by 710 μm mesh sieve, were selected for ecotoxicology experiments. The test was carried out inside 2.5-l glass flasks containing approximately 2 cm layer of sediments and filled with 1000 ml of filtered seawater. The seawater was aerated and kept at a constant temperature (16 ± 2°C). Flasks were illuminated during 12 h daily by a lamp system consisted of six tubes (36W, 120 cm). One hundred amphipods were randomly selected and introduced into each flask. No food was added to the test and control chambers. At the end of exposition (10 days), amount of survived organisms were counted. Missing amphipods were assumed as dead animals. The sensitivity of the populations was estimated as a fraction of dead organisms to the initial amount of added amphipods. All the experiments were replicated twice.

TOTAL RNA EXTRACTION, REVERSE TRANSCRIPTION-PCR (RT-PCR) AND 16S rRNA CLONE LIBRARIES

Total RNA was extracted from sediment sample (5 g) using the FastRNA® Pro soil direct Kit (MP Biomedicals™) as previously described (Roussel et al., 2009). Extracted RNA from three different samples was pooled and further converted to cDNA using First-Strand cDNA Synthesis SuperScript™ II Reverse Transcriptase (Life Technologies). RT reaction mixtures (20 μl) contained 1 μl the random hexamer primer mix (Bioline), 30 ng of RNA, 1 μl 10 mM dNTPs (Gibco, Invitrogen Co., Carlsbad, CA) and sterile distilled water (14 μl). After heating to 65°C for 5 min, and incubate on ice for 1 min, the mixture was supplemented with 4 μl of 5 × First-Strand Buffer, 1 μl of DTT and 1 μl of SuperScript™. The mixture was shaken and incubated at 50°C for 30 min. Finally, SuperScript™ enzyme was inactivated by heating at 70°C for 15 min. 16S rRNA genes were amplified from total cDNA using the universal primers (530F [5′-GTGCGCAGCM GCCGCGG-3′] and 1492R [5′-TACGGYTACCTTGTGTAAGACT-3′]) (Lane, 1991). The PCR was performed in 50 μl mixture (total volume) containing 1× solution Q (Qiagen), 1× Qiagen reaction buffer, 1 μl of each forward and reverse primer, 10 μM dNTPs (Gibco, Invitrogen Co.), 2.0 ml (50–100 ng) of template and 2.0 U of Qiagen Taq Polymerase (Qiagen). The reaction (3 min hot-start at 95°C; 1 min at 94°C, 1 min at 50°C, 2 min at 72°C, 30 cycles; final extension 10 min at 72°C) was performed with GeneAmp 5700 (PE Applied Biosystems). The quality of amplification products was checked by agarose electrophoresis and purified using Qiaquick Gel Extraction kit (Qiagen). Purified 16S crDNA amplicons were further cloned into the pGEM T-easy Vector II (Promega), transformed into E. coli DH10B cells and subsequently amplified with primers, specific for the pGEM T-easy vector (M13F [5′-GTGAAAACAGCCGGCCGT-3′] and M13R [5′-TACACAGGAAAAACAGCTATGAC-3′]). Positive products were purified and sequenced by Macrogen (Amsterdam, The Netherlands). Sequences were checked for possible chimeric origin using Pintail 1.1 software (Ashelford et al., 2005). For the 16S rRNA gene sequences, initial alignment of amplified sequences and close relatives identified with BLAST (Altschul et al., 1997) were performed using the SILVA alignment tool (Pruesse et al., 2007) and manually aligned with ARB (Ludwig et al., 2004). After alignment, the neighbor-joining algorithm of ARB package was used to generate the phylogenetic trees based on distance analysis for 16S rRNA genes. The robustness of inferred topologies

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was verified by bootstrap re-sampling analysis, using the same distance model (1000 replicates).

**ENUMERATION OF CELLS BY CARD-FISH**

The catalyzed reporter deposition fluorescence *in situ* hybridization (CARD-FISH) was performed using the protocol of Pernthaler et al. (2002) with some modifications. Briefly, 5 g of pooled sediments samples were fixed with formaldehyde (2% v/v final concentrations) and left for 12 h at 4°C. Fixed sediment samples were further incubated for at least 15 min with Tween 80 (final concentration, 1 mg l\(^{-1}\)) and than sonicated during 20 min in an Ultrasonic cleaner (Branson 1200, Milan) for the bacterial dispersion (Kuwae and Hosokawa, 1999). Supernatant samples were then filtered onto polycarbonate membrane filters (type 0.1\% wt/vol, Sigma-Aldrich, Milan), dried at 37°C for 20 min, and dehydrated with 95% ethanol. Embedded cells were permeabilized by 1 h of exposition with solution A (10 mg ml\(^{-1}\) of lysozyme (Sigma-Aldrich, Milan); 0.5 M EDTA, 0.1 M Tris-HCl [pH 8.0]) followed by the 30 min-long incubation with achromopeptidase (60 U ml\(^{-1}\); diameter, 2.5 cm; Sartorius, Göttingen, Germany). Filters for CARD-FISH counts were embedded in low-melting point agarose (0.1% wt/vol, Sigma-Aldrich, Milan), dried at 37°C for 20 min, and hybridized with 5'-horseradish peroxidase (HRP)-labeled oligonucleotide probes as described by Pernthaler et al. (2002). Probes used in this work are listed in Table 1. After the hybridization and amplification steps, slides were examined using a epifluorescence with Axioplan 2 Imaging (Zeiss; Carl Zeiss Inc., Thornwood, NY) microscope. All results were expressed as number of cells per gram of sediment.

**DETERMINATION OF CELL NUMBERS BY QUANTITATIVE PCR (qPCR)**

qPCR method was employed to determine the relative cell densities of *Alcanivorax*, *Marinobacter*, and *Cycloclasticus* in the sediment samples. Primers used through this study are listed in the Table 2. Primers were based on the sequences of *Alcanivorax* alkane hydroxylase gene (alkB2), *Marinobacter* alkane hydroxylase gene (alkB), and *Cycloclasticus* aromatic ring-hydroxylating dioxygenase (phnA) gene. Primers for alkB2 and phnA genes were previously designed and validated for qPCR elsewhere (McKew et al., 2007; Gray et al., 2011). The primers specific for the alkB gene of *Marinobacter* were designed through this study using Primer Express software Primer Express software, version 2.0 (Applied Biosystems, Foster City, Calif.) with reference to the *Marinobacter hydrocarbonoclasticus* (FO20363).

Total DNA was extracted from 2.0 g of sediment samples collected at selected time scales from three different points inside the MSS by using a Bio101 FastDNA SPIN kit as described by the manufacturer (Bio101, Inc., Vista, Calif.). Extracted DNA was dissolved in 50 µl of TE buffer (10 mM Tris-HCl, 1 mM EDTA [pH 7.5]) and quantified using a NanoDrop ND-1000 spectrophotometer (Celbio). The quality of the extracted DNA was analyzed by electrophoresis on a 1.0% agarose gel. The qPCR was performed with absolute quantification method in an ABI Prism 7300 Real-time PCR System (Applied Biosystems) in a total volume of 25 µl, consisting 12.5 µl of SYBR green master mix, 200 nM of each primers, and 50 ng of template DNA. The volume of each reaction was adjusted to 25 µl by adding DNase-free water. The cycling parameters for the qPCR amplification were as follows: an initial denaturation step at 95°C for 10 min, followed by 45 cycles of denaturation at 95°C for 15 s and annealing/elongation at 60°C for 60 s. A dissociation step was added to check for primer-dimer formation. A tenfold serial dilution series of genomic DNA ranging from 10 to 10\(^8\) copies per reaction was used in triplicate to create the standard curve for quantification. Serial dilutions were prepared once for each target and used for real-time quantification. From the slope of each curve, PCR amplification efficiency was calculated as it described elsewhere (Rasmussen, 2001). Obtained slope values, (−3.17 for alkB2, −3.29 for alkB, and −3.37 for phnA) fell within the optimal

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**Table 1 | CARD-FISH specific probes used in the present study.**

| Probe                          | Sequence (5' to 3') of probe | FA (%) | Source                |
|-------------------------------|------------------------------|--------|-----------------------|
| Eub338                        | GCTGCTCGGTAGGGAGT            | 35     | Amann et al., 1990    |
| Marinobacter sp.               | ATGGTAGAAATCCTGACGTTAGT      | 20     | Karner and Fuhrman, 1997 |
| Cycloclasticus sp.             | GAAACCCGCCCAACAGT            | 20     | Karner and Fuhrman, 1997 |
| Alcanivorax sp.                | CGACCGAGCTCATCAGCTA          | 10     | Karner and Fuhrman, 1997 |

FA: percentage of formamide in hybridization buffer.

**Table 2 | qPCR primers used in the present study.**

| Gene      | Forward Reverse | Organism       | Amplification efficiency (E) | Source            |
|-----------|-----------------|----------------|-----------------------------|-------------------|
| alkB2     | CGCCGTGTGAAAGCAAGGG CGACGCTTGAGCTGTTAGCATG | Alcanivorax | 99.6%                        | McKew et al., 2007 |
| alkB      | TCTTTTGCTGAAAGCCGAGTT ACGATCCTGTTCAAGCCGAG | Marinobacter | 97.3%                        | This study        |
| phnA      | CGTGGGCGGTAGGAAGGTGCGG CTTGCCCCCCAATACCAGCC | Cycloclasticus | 96.2%                        | McKew et al., 2007 |
range corresponding to an efficiency of 99.6, 97.3, and 96.2%, respectively. Amplicon numbers were quantified against the standard curve using automatic analysis settings for the Ct values and baseline settings. Detected target genes were converted to cell density in sediments (cells gram$^{-1}$) assuming that all three genes present as a single copy per genome.

**STATISTICAL ANALYSIS**

For statistical analyses, clones from each 16S crDNA library were separately considered to define phylotypes, or operational taxonomic units (OTUs) at cutoff of either 95 or 97% of sequence identity (Kemp and Aller, 2004). Clustering of sequences was performed using Dotur program (Schloss and Handelsman, 2005). Various parameters for each clone library, such as diversity index, rarefaction analysis, taxa, total clones, singletons, Shannon dominance, equitability, Simpson and chao2 were calculated by PAST version 2.17c (http://folk.uio.no/ohammer/past; Hammer et al., 2001). Coverage values given as $C = 1 - (n_1/N)$, where $n_1$ is the number of clones which occurred only once in the library of N clones (Good, 1953), were calculated to determine how efficient clone libraries described the complexity of original bacterial community. The Primer 6 ecological software package developed by the Plymouth Marine Laboratory (Clarke and Gorley, 2006) was employed to perform the Hierarchical Cluster Analysis (HCA) (Clarke, 1993). We applied HCA on microbial biodiversity and Bray-Curtis similarity on relative abundance matrix of the OTUs detected at different sampling time. Significant difference of the microbial assemblages derived from both treated and control samples at the different sampling times was detected via the $P$-test significance and principal coordinates analysis (PCoA) using UniFrac program (http://bmf.colorado.edu/unifrac/index.psp, last access: 24 July 2008) (Lozupone et al., 2007). Differences in cell number per gram of sediment detected by DAPI, CARD, and qPCR between different samples was examined by analysis of variance (ANOVA) on ranks (Holm_Sindak method). Relative importance of each treatment group was investigated by Multiple Comparisons vs. Control Group comparison test. Calculations were carried out using SigmaStat software for Windows, ver. 3.1 (Copyright 1992–1995; Jandel Corporation). Differences were considered significant at $P < 0.05$.

**NUCLEOTIDE SEQUENCE ACCESSION NUMBERS**

To avoid the submission of identical sequences obtained among 411 analyzed clones, only 98 distinguishing 16S rRNA gene sequences have been deposited in the DDBJ/EMBL/GenBank databases under accession numbers KF896304-KF896401.

**RESULTS**

**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 MSS 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 MSS 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 MSS 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. Outside the MSS, the amendment of the Bunker C furnace fuel oil turned initially oxygenated (T$_0$, 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, inside the MSS the sediments remained aerobic during all period of bioremediation effort.

**HYDROCARBON ANALYSIS**

Before the addition of 6500 ppm of Bunker C fuel oil into the mesocosm, the total hydrocarbon concentration in the original Messina harbor sediments was estimated at the level of 120 ppm.
The PHs 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 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 MSS and in the untreated anoxic sediments was examined at the end of experiment (T90). The concentration of PHs, especially aliphatic hydrocarbons was normalized using the pristane/phytane ratio, and the values obtained in triplicate subsamples were averaged. In the aerated internal MSS 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. Additionally, we performed the gravimetical analysis of total extracted hydrophobic fraction (TEHF) in the sediments. The TEHF values inside the MSS 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 ± 120 mg kg⁻¹ of hydrophobic material. As shown in subsequent section, TEH fraction seems be primarily responsible for the observed toxicity of Bunker C fuel oil.

**ECOTOXICOLOGICAL ANALYSIS**

According to standard guidelines of Italian Institute for Environmental Protection and Research (ISPRA, 2013), ecotoxicological 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 (vol/vol) and no significant level of bioluminescence decay has been observed. As it 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 4). At the same time, the toxicity of polluted internal MSS 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 MSS sediment at T90 was significantly less toxic than their external counterpart.

**DIVERSITY AND SUCCESSION OF BACTERIAL COMMUNITIES DURING BIOREMediation AND NATURAL SEDIMENT AGEING**

To monitor the succession of metabolically active microbial communities during the bioremediation treatment, five 16S rRNA transcript libraries were established. 450 clones from these libraries were randomly selected and analyzed. Among those, 411 sequences were included into phygenetic analysis (T0, 96 clones; T1, 83 clones; T29, 80 clones; T90, 90 clones and T90OUT, 62 clones). 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%), Chlororflexi (5%), Verrucomicrobia (3%), and Bacteroidetes (2%) (Figure 5). 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%). The analysis of 16S rRNA transcripts from T90OUT clone library revealed a notable prevalence of hydrocarbon-degrading or hydrocarbon contamination-associated *Deltaproteobacteria* (90.3%). In particular, almost two-thirds of all clones (40 out of 62 clones analyzed) were closely related to the uncultured bacterium RII-AN044 found in anoxic polluted sediments after the Prestige oil spill (Acosta González et al., 2013). More than 16% of clones revealed >98% of similarity to the deltaproteobacterium NaphS6, capable of naphthalene and 2-methylnaphthalene degradation (Wilkes et al., 2008). Almost 10% of T90OUT clones were closely related to the *n*-alkanes- and *n*-alkenes-degrading strain *Desulfatibacillum aliphaticivorans* (Cravo-Laureau et al., 2004) (Figure 6).
FIGURE 6 | Phylogenetic affiliation of the *Eubacteria* clones retrieved in Messina harbor native sediments and during mesocosm experiment.

In accordance with the type of the contaminant load, the majority of analyzed clones had highest Blastn homologies with sequences related to PH-degrading or petroleum contamination-associated organisms belonging to the *Gamma-, Alpha- and Deltaproteobacteria* (Figures 6–8). 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 5).

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 (Païssé et al., 2008, 2010).

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 ± 0.17 × 10⁶ cells gram⁻¹ (Table 3). 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 ± 0.12 × 10⁶ cells gram⁻¹). Noteworthy, a tenfold increase in the number of CARD-positive cells (2.95 ± 0.11 × 10⁷ 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...
FIGURE 7 | Phylogenetic affiliation of the Gammaproteobacteria clones retrieved in Messina harbor sediments and in mesocosm experimentation. Neighbor-joining analysis using 1000 bootstrap replicates was used to infer tree topology. The scale bar represents 10% of sequence divergence. Bootstrap values ≥50 are shown as open circles. Cenarchaeum symbiosum was used as the out-group. Sequences obtained in this study are indicated in bold.
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 MSS 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. Compared to an initial density, their population increased twofold at T29, from 4.5 ± 0.2 to 9.0 ± 0.1 × 10⁵ cells g⁻¹. 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 MSS) 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 ± 0.15 × 10⁶ 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 MSS microbial community at the end of experiment.
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 3). None of these hydrocarbon-degrading bacteria were detected by qPCR at the end of experiment.

**STATISTICAL ANALYSIS OF BACTERIAL DIVERSITY**

As it shown in Table 4, the diversity index and coverage values have been calculated for each 16S rRNA transcript library. Based on sequence similarity threshold for OTU definition ≥ 97% (Rosselló-Mora and Amann, 2001), a total of 93 different OTUs were discerned. The rarefaction analysis did not demonstrate the saturation in any library (data not shown), however, the coverage values varying between 0.64 (T0 library) and 0.92 (T90OUT library) indicated that a satisfactory overview on bacterial community was obtained. As reflected by the high values of Simpson, Shannon and the number of missing species provided by Chao-2, the highest bacterial diversity was observed in original sediments (T0). Bacterial biodiversity was drastically affected by Bunker C fuel oil addition, decreasing the Simpson, Shannon and Equitability values, due to strong selection for both petroleum contamination-resilient and hydrocarbon-degrading bacteria (Harayama et al., 1999; Kasai et al., 2001, 2002; Syutsubo et al., 2001). Dominance index reached the highest value at T29 whereas at the end of experiment, the dominant Cluster 9 of the internal MSS sediments was dominated by OMHCBs, whereas at the end of experiment, the dominant Cluster 9 of the mesocosms experiment. These sediments chronically polluted with alkylnaphthalenes were chosen since the autochthonous microbial community is likely adapted to the steady presence of PHs and consequently may exhibit a higher biodegradation ability than those from pristine environments (Paisse et al., 2008, 2010). Accordingly, the fraction of the OMHCB genera *Alcanivorax*, *Cycloclasticus*, *Marinobacter* and *Thalassolituus*, usually imperceptible in pristine marine environments (Yakimov et al., 2007), accounted for a fifth part of the native microbial community of Messina harbor sediments. To simulate accidental oil spill we spiked 1000 kg sandy sediments with Bunker C fuel oil (6500 mg

**DISCUSSION**

The influence of the massive Bunker C fuel oil load upon the microbial population of coastal sediments collected in Messina harbor and their recovery was investigated during 3 months in the mesocosms experiment. These sediments chronically polluted with alkylnaphthalenes were chosen since the autochthonous microbial community is likely adapted to the steady presence of PHs and consequently may exhibit a higher biodegradation ability than those from pristine environments (Paisse et al., 2008, 2010). Accordingly, the fraction of the OMHCB genera *Alcanivorax*, *Cycloclasticus*, *Marinobacter*, and *Thalassolituus*, usually imperceptible in pristine marine environments (Yakimov et al., 2007), accounted for a fifth part of the native microbial community of Messina harbor sediments. To simulate accidental oil spill we spiked 1000 kg sandy sediments with Bunker C fuel oil (6500 mg

| Diversity index | T0 | T1 | T29 | T90 | T90OUT |
|-----------------|----|----|-----|-----|--------|
| Taxa/(OTUs)_S   | 46 | 16 | 14  | 12  | 5      |
| Number of clones| 96 | 83 | 80  | 90  | 62     |
| Dominance_D     | 0.04 | 0.15 | 0.22 | 0.24 | 0.44 |
| Simpson_1-D     | 0.96 | 0.85 | 0.78 | 0.76 | 0.55 |
| Shannon_H       | 3.50 | 2.31 | 2.08 | 1.89 | 1.09 |
| Equitability_J   | 0.91 | 0.84 | 0.78 | 0.76 | 0.68 |
| Chao-2          | 104.1 | 16 | 14  | 12  | 5      |
| Singletons      | 31  | 1  | 0   | 0   | 0      |
| Doubletones     | 7   | 10 | 8   | 6   | 2      |
| Coverage        | 0.64 | 0.74 | 0.77 | 0.87 | 0.92 |

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

Table 3 | CARD-FISH and qPCR cell number quantification in the MSS internal sediment during the bioremediation treatment.

| Method          | Cell numbers, 10^5 x g sediments^-1 ± SD |
|-----------------|-----------------------------------------|
|                 | T0          | T1          | T29         | T90         |
| **CARD-FISH**   |             |             |             |             |
| *Eubacteria*    | 29.8 ± 1.7  | 23.6 ± 1.5  | 295.0 ± 11.0| 279, 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 ± 0.12 X 10^-3| 7.13 ± 0.28 | ND          |

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kg$^{-1}$). This type of heavy fuel oil is frequently used by the cargo and tanker ships and is generally more complex in composition and impurities than distilled fuels. Bunker C fuel oil includes about 25% polyaromatic, 15% aliphatic, 45% naphthenic and 15% non-hydrocarbon compounds (Clark et al., 1990). As it generally observed, after the oil spill reaches a shoreline, the oil burial is the main mechanism of the pollution dispersal. This results in intense oiling of subsurface sediments and consequent reduction in oxygen concentration due to initial activation of aerobic hydrocarbon-degrading microorganisms (Albaigés et al., 2006; Acosta González et al., 2013). Once O$_2$ concentration drops to zero, the degradation rates of both aliphatic and aromatic PHs decrease significantly. According with this observation, the oil cleanup was very marginal, and $>$80% of initially added Bunker C fuel oil was extracted from the anaerobic external sediments after 3 months of oil spill simulation. At the end of experiment, analysis of microbial community of the MSS external sediments revealed the overwhelming dominance of the Deltaproteobacteria.

The stimulation of autochthonous bacteria to tackle the pollution in contaminated environment is widely used in the remediation of aerobic sites (Harayama et al., 2004). However, this technology is hardly applicable to oxygen-depleted marine sediments. Additionally, due to high probability of contaminant spreading while removing, the application of ex-situ remediation technologies is severely limited. To initiate the self-cleaning process in sediments driven by indigenous aerobic OMHCB, we used in situ aeration of polluted anoxic sediments in specially designed MSS. It is generally assumed, that petroleum contamination induced drastic changes in the bacterial community structure associated with a decrease of diversity (Grötzschel et al., 2002; Röling et al., 2002; Yakimov et al., 2005, 2007; Head et al., 2006; Bordenave et al., 2007). These changes were referred to both toxic effect of PHs and a strong selection toward highly specialized hydrocarbon-degrading microorganisms (Harayama et al., 1999; Kasai et al., 2001; Grötzschel et al., 2002; Yakimov et al., 2005, 2007). Accordingly, the most drastic shifts in the MSS 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 a half of MSS microbial population at T$_{29}$, 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 (Harayama et al., 1999; Röling et al., 2002, 2004; Yakimov et al., 2007). Noteworthy, the dominance of these OMHCB genera was confirmed by both CARD-FISH and qPCR analyses. At the end of the treatment, the level of TERHC degradation in the MSS internal sediments was almost 98%, and the resulting microbial community was characterized by an almost complete extinction of OMHCBs. Both HCA and UniFrac PCoA statistic analyses of the OTUs abundance matrix indicated that the structure of T$_{90}$ microbial community was more similar to initial microbial community structures. As a consequence of successful bioremediation, the Corophium orientale eco-toxicological bioassay revealed that toxicity of the MSS internal sediments was substantially lower compared with the untreated external sediments. Thus, to the best of our knowledge, 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.

**ACKNOWLEDGMENTS**

This work was funded by the European Community Projects ULIXES (FP7-KBBE-2010-4 Project 266473 “Unravelling and
exploiting Mediterranean Sea microbial diversity and ecology for xenobiotics' and pollutants' clean-up') and KILL-SPILL (FP7-KBBE-2012.3.5-01-4 Project 312139 "Integrated Biotechnological Solutions for Combating Marine Oil Spills"). We would like to thank all partners of these projects for their useful discussions.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Received: 02 December 2013; accepted: 25 March 2014; published online: 14 April 2014.

Citation: Genovese M, Crisafi F, Denaro R, Cappello S, Russo D, Catalfamo M, Santisi S, Catalfamo M, Modica A, Smedile F, Genovese L, Golyshin PN, Giuliano L and Yakimov MM (2014) Effective bioremediation strategy for rapid in situ cleanup of anoxic marine sediments in mesocosm oil spill simulation. *Front. Microbiol.* 5:362. doi: 10.3389/fmicb.2014.00362

This article was submitted to Aquatic Microbiology, a section of the journal Frontiers in Microbiology.

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