Marine Pollution Bulletin xxx (2015) xxx–xxx

Contents lists available at ScienceDirect

Marine Pollution Bulletin

journal homepage: www.elsevier.com/locate/marpolbul

# Intrinsic bioremediation potential of a chronically polluted marine coastal area

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ARTICLE INFO

*Article history:*
Received 10 June 2015
Accepted 18 July 2015
Available online xxxx

*Keywords:*
Sediment bacteria
Hydrocarbonoclastic bacteria
DGGE
PLFA
*Oleibacter*

ABSTRACT

A microbiological survey of the Priolo Bay (eastern coast of Sicily, Ionian Sea), a chronically polluted marine coastal area, was carried out in order to discern its intrinsic bioremediation potential. Microbiological analysis, 16S rDNA-based DGGE fingerprinting and PLFAs analysis were performed on seawater and sediment samples from six stations on two transects. Higher diversity and variability among stations was detected by DGGE in sediment than in water samples although seawater revealed higher diversity of culturable hydrocarbon-degrading bacteria. The most polluted sediment hosted higher total bacterial diversity and higher abundance and diversity of culturable HC degraders. Alkane- and PAH-degrading bacteria were isolated from all stations and assigned to *Alcanivorax*, *Marinobacter*, *Thalassospira*, *Alteromonas* and *Oleibacter* (first isolation from the Mediterranean area). High total microbial diversity associated to a large selection of HC degraders is believed to contribute to natural attenuation of the area, provided that new contaminant contributions are avoided.



## 1. Introduction

Petroleum hydrocarbons are among the most widespread environmental pollutants (Hassanshahian et al., 2012). Oil pollution may arise either accidentally or operationally; indeed, pollutants may enter into marine ecosystems following catastrophic accidents (shipping disasters or pipeline failures), chronic pollution (ships, ports, oil terminals, freshwater runoff, rivers and sewage systems), natural oil seepages and natural sources (biota) (Floodgate, 1972).

In many aquatic systems, sediment generally act as a sink of pollutants, which may afterwards move back up through the water column causing serious consequences. Indeed, compared to upper layers of the water column, the bottom ones are characterized by a higher concentration of pollutants because of the re-suspension of sediment and of associated pollutants (McGenity et al., 2012).

The presence of pollutants in marine sediment may produce adverse effects on marine ecosystems and human health. Benthic organisms can gather toxic substances in their body, affecting and poisoning predators at the highest level of the food chain (Moreno et al., 2013). As hydrocarbon components belong to the family of carcinogens and neurotoxic organic pollutants, harmful effects are expected on humans exposed to such contaminants (Jarvis et al., 2014).

Increased awareness of the importance of microbial communities in marine environmental processes, together with their ability to respond to environmental contamination, has intensified the attention on the relation between microbial community structure and contaminant concentration. Among the contaminants most commonly introduced into the marine environment, hydrocarbons can exert a selective pressure on microbial communities (e.g. Langworthy et al., 2002; Wang and Tam, 2012). Bacterial communities have therefore great potential to be used as sensitive indicators of contamination in marine sediment (Sun et al., 2012). On the other hand, microorganisms (bacteria and fungi) are the main degraders of organic pollutants in marine environments. Many marine bacteria endowed with biodegradative abilities have been described (Yakimov et al., 2007) and their role in the bioremediation of contaminated sites has been addressed (Ron and

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http://dx.doi.org/10.1016/j.marpolbul.2015.07.042
0025-326X/

Rosenberg, 2014). Techniques of bioremediation and utilization of oil degrading bacteria and hydrocarbon-degrading microbial consortia have a key role in these processes (Beolchini et al., 2010; Rocchetti et al., 2011). In general, bioremediation is based on in situ stimulation of the microbial community (biostimulation) or amending the microbial community with an inoculum of hydrocarbon-degrading bacteria (bioaugmentation).

Biological treatments using marine bacteria are becoming more important, mainly because of the low environmental impact, cheaper costs than other clean-up technologies, the capability to degrade organic contaminants and the possibility of beneficial use of treated sediment (Rulkens and Bruning, 2005).

Polluted sediment can be sanitized via in situ actions, which always require an accurate knowledge of the local biocenosis (Doni et al., 2013). In particular, marine obligate hydrocarbonoclastic bacteria exhibit peculiar properties on hydrocarbon degradation (Yakimov et al., 2007) and, when nutrients (as nitrogen and phosphorous) are available at not limiting concentrations, an increase of their growth rate is registered with the decrease of pollutants (Röling et al., 2004; Cappello et al., 2007a, 2007b; Yakimov et al., 2006, 2007). The complexity of contaminant transformation processes in the natural environment is due to the influence of the nature and amount of hydrocarbons, the structure and dynamics of the indigenous microbial community and the interplay of geochemical and biological factors at contaminated sites (Atlas, 1981; Leahy and Colwell, 1990; Gibson and Sayler, 1992).

A better understanding of the processes inherent to natural bioremediation requires enhancement of microbial ecology awareness. The first challenge is to overcome the complicated attribution of in situ microbe-mediated processes to the responsible organism(s), improving the knowledge on dynamics and functional activity together with identification of ecologically significant strains and genes in response to environmental stressors.

Characterization of microbial communities can provide important information to understand the extent of contamination in the sedimentary compartment and in the water column, and also to assess and predict the catabolic potential of environmental sites. DNA-based molecular analysis, such as 16S rDNA-based DGGE fingerprinting (Nocker et al., 2007), and biochemical analysis of phospholipid fatty acids (PLFA), that are essential component of viable bacterial cell membranes (Findlay et al., 1990; Kaur et al., 2005), are considered as robust tools to depict the bacterial communities in relation to environmental stressors.

The Augusta Bay, on the eastern coast of Sicily (Ionian Sea) is identified as an area with heavy industrialization (petrochemical and wastewater treatment plants) and high contamination due also to the presence of the Augusta Harbour in the northern part of the bay. Navigation and dredging of polluted sediment, in addition to direct discharge of contaminants from the industries occurred during the last century, have been identified as the main cause of the environmental contamination in the harbour and nearby (Bellucci et al., 2012). Indeed, severe inorganic and organic pollution has been detected both in and outside the harbour, as effect of dumping of dredged sediment in the surrounding area (ISPRA-APAT, 2007; ISPRA, 2010; Sprovieri et al., 2012; Di Leonardo et al., 2014) with important risks on ecosystem and human health (Ausili et al., 2008; Bonsignore et al., 2013; Genovese et al., 2014).

The goals of the present study are: (i) to characterize the organic contamination of surface sediment of the southern part of Augusta Bay, Priolo Bay (Sicily, Italy); (ii) to perform a molecular and biochemical analysis of the indigenous microbial communities associated with this area and (iii) to isolate oil degrading bacteria and hydrocarbonoclastic bacteria to discern the intrinsic bioremediation potential of the area.

## 2. Materials and methods

### 2.1. Sample collection

Seawater and sediment samples were collected in Priolo Bay (South-East Sicily, Italy, Mediterranean Sea) in July 2012 (Fig. 1). Surface sediment (0–5 cm, Fig. S1) samples with the upper seawater layer were collected in triplicate using sterile Plexiglas cores (20 cm long, 5 cm diameter), from three stations located along a northern transect (N1, N2 and N3) and three stations located along a southern transect (S4, S5 and S6) (Fig. 1).

After collection, samples were immediately transported to the laboratory in a cool box (4 ± 1 °C), where samples were used for immediate analysis or aliquots were stored at −80 ± 5 °C and at −20 ± 1 °C with glycerol (20% final concentration). Unfortunately, part of the water component of core samples of station S4 was lost during sampling and could only be analyzed for PLFAs, and isolation of HC degraders. The physico-chemical characteristics of the sediment are described elsewhere (Di Leonardo et al., 2014).

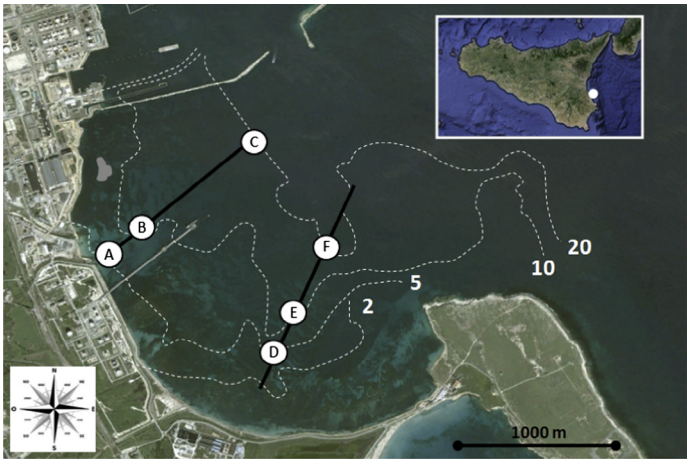
### 2.2. Analysis of hydrocarbons

Total hydrocarbons were extracted from sediment following the 3550C EPA (Environmental Protection Agency) procedure (Rocchetti et al., 2012). Briefly, a mixture of $CH_2Cl_2$:$CH_3COCH_3$ (1:1, v/v) was added to sediment samples. The mixture was sonicated for 2 min in ultrasound bath (Branson 1200 Ultrasonic Cleaner, Branson, USA). Samples were further shaken at 150*g* for 30 min, centrifuged for 10 min at 5000*g* and supernatant was passed through a ceramic column filled with anhydrous $Na_2SO_4$ (Sigma–Aldrich, Milan). Same treatment of pooled and dried sediment was repeated with $CH_2Cl_2$ and the obtained extracts were combined and volatilized to dryness. Residues were re-suspended in $CH_2Cl_2$ prior the gas chromatography (GC) analysis. All measures were performed using a Master GC DANI Instruments (Development Analytical Instruments), equipped with SSL injector and FID detector. Samples (1 μl) were 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 $min^{-1}$. Total hydrocarbons were also calculated for each sample (Genovese et al., 2014). Three samples for any stations in study were analyzed.

### 2.3. Phospholipids fatty acids (PLFA) extraction and analysis

Approximately 4 g of freeze-dried sediment was weighted in screw-cap Pyrex test tubes. Lipids were extracted in triplicate and separated into fractions following the method proposed by Bligh and Dyer (1959) and modified by White et al. (1979), that is with a mixture of chloroform:methanol:P-buffer solution (1:2:0.8 v/v/v). Phospholipids were separated from the other lipid fractions using silicic solid phase extraction (SPE) cartridges under low vacuum and derivatized to fatty acid methyl esters (FAME) by mild alkaline methanolysis. The organic phase was evaporated to dryness under gentle nitrogen stream and reconstituted with an *N*-hexane solution with internal standard (C 23:00). PLFAs were analyzed through a Shimadzu 2010 Gas Chromatograph equipped with a flame ionization detector (GC-FID) using a fused siloxane capillary column (BPX-70; 30 × 25 mm i.d.; film thickness 0.25 μm SGE, Inc., Austin, TX). Individual PLFAs were identified by comparison of retention times with commercially available standard mixtures (FAME and BAME mix, Supelco) and quantified by correlating the peak areas to that of the internal standard. Results were given as relative percentage abundance.

Please cite this article in press as: Catania, V., et al. Intrinsic bioremediation potential of a chronically polluted marine coastal area. Mar. Pollut. Bull. (2015), http://dx.doi.org/10.1016/j.marpolbul.2015.07.042




| Sampling Station | Station Code | Latitude | Longitude | Depth |
|---|---|---|---|---|
| A | N1 | 37.16619894 | 15.20074368 | 5 m |
| B | N2 | 37.16770367 | 15.20604372 | 10 m |
| C | N3 | 37.16927676 | 15.21497011 | 20 m |
| D | S4 | 37.15569923 | 15.21703005 | 5 m |
| E | S5 | 37.15850386 | 15.21939039 | 10 m |
| F | S6 | 37.1630184 | 15.22239447 | 20 m |

**Fig. 1.** Map showing the location of sampling stations in Priolo Bay (Augusta, Sicily, Italy). The bathymetry of the sampling area is indicated by white dashed lines and numbers.

Conventional nomenclature was used for PLFAs, namely A:Bn-C where A indicates the carbon chain length, B the degree of unsaturation, and C the position of the first double bond from the omega or aliphatic end of the molecule. Terminal branching was indicated by i (*iso*) and a (*anteiso*); presence of cyclopropane and hydroxyl groups in the PLFA molecule was indicated with the suffix *cy*- and OH-, respectively, plus the position from the carboxylic carbon. PLFAs were grouped into biomarkers of specific microbial groups according to Findlay et al. (1990) and Kaur et al. (2005).

PLFA data were analyzed using multivariate techniques by means of the software PRIMER 6 (Anderson et al., 2008). Cluster analysis (group average linkage method) and Principal Coordinate analysis (PCO; Anderson and Willis, 2003) were carried out on Bray Curtis similarities calculated from the arcsine-transformed relative abundance of the 34 PLFA identified. Selected PLFA biomarkers were superimposed onto the graph to detect their correspondence with the ordination obtained. Clusters defined at 91% of similarity were also superimposed.

### 2.4. Measures of microbial abundance

To monitor the abundance of microbial populations present in seawater and sediment, measures of direct bacterial count (DAPI), cultivable bacteria (CFU) and Most Probable Number (MPN) were carried out in triplicates.

#### 2.4.1. Total bacterial abundance (DAPI count)

For seawater samples, after a short-time (30″) ultrasonic treatment (Ultrasonic Bath Branson 1200, Branson, USA), the total bacterial cell counts were performed by DAPI (4′,6-diamidino -2-phenylindole 2HCl, Sigma–Aldrich S.r.L., Milan, Italy) staining of seawater samples fixed by formaldehyde (2% final concentration), according to Porter and Feig (1980) and Cappello et al. (2012).

For sediment, prior to dispersion, samples were incubated for at least 15 min with Tween 80 (final concentration, 1 mg $l^{-1}$). According to Kuwae and Hosokawa (1999) an ultrasonic cleaner (Branson 1200, Milan) was used for the bacterial dispersion from the sediment (20 min). After centrifugation (8 min at 8000*g*) and collection of water-tween 80 phase, cell counts were performed by DAPI as above described.

Slides were examined by epifluorescence by using an Axioplan 2 Imaging (Zeiss; Carl Zeiss Inc., Thornwood, N.Y.) microscope. Cell counts from 10 grid fields on each slide were tallied (Ni Chadhain et al., 2006). Results were expressed for seawater and sediment samples as number of cells $ml^{-1}$ and number of cells $g^{-1}$, respectively.

#### 2.4.2. Cultivable bacteria (CFU)

For enumeration of total heterotrophic bacteria and hydrocarbon-degrading bacteria, cells present in seawater and sediment samples (from bacterial dispersion carried out as previously

described) were serially diluted in sterile natural seawater (0.2 μM Millipore filter; Millipore, Milan, Italy) and plated (100 μl) on Marine 2216 agar medium (Difco S.p.a, Milan, Italy). All agar plates were incubated at 20 ± 1 °C for 7 days. Results were expressed as colony forming units (CFU) in ml (for seawater samples, CFU $ml^{-1}$) and grams (for sediment sample, CFU $g^{-1}$).

*2.4.3. Most Probable Number (MPN)*

Hydrocarbon-degrading bacteria from seawater and sediment samples (after dispersion procedures) were enumerated by a miniaturized Most Probable Number (MPN) method (Brown and Braddock, 1990), slightly modified (Cappello et al., 2007a). The MPN of hydrocarbon-degrading microorganisms was determined from the appropriate MPN tables according to American Public Health Association (A.P.H.A., 1992).

*2.5. PCR-DGGE*

Denaturing Gel Gradient Electrophoresis (DGGE) of PCR-amplified 16S rRNA gene was used to analyze the taxonomic structure of microbial population present in seawater and sediment samples.

*2.5.1. Extraction of DNA*

The extraction of total DNA from seawater samples was performed using the MasterPure Complete DNA and RNA Purification Kit (Epicentre, USA). An aliquot of 100 ml of seawater samples was filtered with 0.2 μM Millipore filter (Millipore, Milan, Italy); the filters were transferred to 1.5 ml plastic tubes and 350 μl of Cell Lysis solution containing 50 μg of Proteinase K were added. Each sample was incubated at 65 ± 1 °C for 15 min, transferred on ice 5 min and the filter removed. The precipitation of total nucleic acids was performed following the manufacturer's instructions. The total nucleic acids were suspended in 50 μl of DNA/RNA free water (GIBCO).

0.5 g of frozen sediment sample from each core were used for direct extraction with the FastDNA™ SPIN Kit for Soil (MP Biomedicals, Germany), following the procedures described by the manufacturer except in the last step, where the nucleic acids were eluted with 50 μl of DNA/RNA free water (GIBCO). To homogenize sediment samples the FastPrep instrument was used according to the manufacturer's instructions. The quality and concentration of DNA extracted from seawater and sediment samples were checked on 1.0% agarose gel run in Tris–acetate–EDTA buffer stained with ethidium bromide and UV illuminated. Nucleic acids were quantified using NanoDrop ND-1000 Spectrophotometer (NanoDrop Technologies). DNAs were stored at −20 °C until required for PCR amplification.

*2.5.2. PCR-DGGE analysis*

Primers 341f-GC and 354r (Muyzer et al., 1993) were used to amplify the variable V3 region of bacterial 16S rRNA genes for DGGE analysis. PCR amplification was performed in a 50 μl final volume with Phire Hot Start II DNA Polymerase (Thermo Scientific), 1× PCR buffer, 500 nM each primer, 0.30 mM dNTP and 150–300 ng of DNA. The PCR procedure was as follows: an initial cycle of 30 s at 98 °C, followed by 35 cycles of 15 s at 98 °C, 10 s at 66 °C, 10 s at 72 °C, with a final extension of 1 min at 72 °C. PCR products were purified and the amplicons analyzed on 1.5% agarose gel. Amplicons were run in polyacrylamide gels (20 c m × 20 cm × 1 mm) using the Phor-U2 system (Ingeny, Leiden, The Netherlands). Gels were prepared with 8% (w/v) polyacrylamide acrylamide/bisacrylamide (37:5:1)] in a 1× TAE [40 mM Tris, 20 mM acetic acid, and 1 M EDTA (pH 8.0)] buffer with a 20% and 70% denaturing gradient [100% denaturant corresponding to 7 M urea and 40% (v/v) deionised formamide] increasing in the direction of electrophoresis. Electrophoresis was performed at 60 °C, at 80 V with 1× TAE for 17 h; after samples were loaded, it was run for 15 min at 20 V and then at 80 V for 17 h. Gel was stained for 30 min in SYBR® Gold I Nucleic Acid Gel Stain (Invitrogen CA, USA) at 37 °C, washed with sterile distilled water and captured with a Gel Doc 2000 (Bio-Rad Laboratories, Inc., CA, USA). Some dominant DGGE bands were excised with a sterile pipette tip and re-suspended overnight in 20 μl DNA/RNA free water (GIBCO) at 4 °C then frozen at −20 °C. A 2 μl aliquot was used as template for PCR using the 341f-GC and 354r primer set as described above. The PCR products were purified with NucleoSpin® Gel and PCR Clean-up kit (Macherey-Nagel) following the manufacturer's instructions and sequenced.

*2.6. Isolation of HC-degrading bacteria by enrichment cultures*

To isolate the aerobic hydrocarbon degrading bacteria, samples of seawater and superficial sediment were used as inoculum for enrichment cultures in the mineral medium ONR7a (Dyksterhouse et al., 1995).

Different enrichment sets were performed by supplementing the ONR7a medium, respectively, with 0.1% (v/v) *n*-alkanes mixture ($C_{16}$, $C_{18}$ and $C_{24}$), PAHs (phenanthrene, pyrene, biphenyl and dibenzothiophene) or crude oil (Arabian Light Crude Oil).

After incubation at 20 ± 1 °C with shaking (80 g) for 1 month, subcultures were prepared by transferring 1% v/v from the enrichment cultures to flasks containing fresh medium ONR7a and 0.1% of chemicals. The new enrichment cultures were cultivated under the same conditions for 1 month. All the enrichment cultures and subcultures were aliquoted and stored at −80 °C with 20% glycerol. After 1 month, the enrichment cultures were serially diluted with saline solution (0.9% NaCl), and spread on mineral ONR7a plates containing the same hydrocarbons.

Phenotypically different colonies obtained from the plates of ONR7a were transferred to fresh plates of ONR7a with and without carbon source to eliminate autotrophs and agar-utilizing bacteria. The procedure was repeated, and only isolates exhibiting pronounced growth on crude oil and/or other hydrocarbons were stored in stock media with glycerol at −20 °C for further characterization (Chaillan et al., 2004; Hassanshahian and Emtiazi, 2008).

*2.6.1. Biochemical characterization*

To identify and characterize the bacterial isolates, Gram staining and biochemical tests for oxidation/fermentation, the production of acid from carbohydrates and the hydrolysis of gelatin and citrate were performed according to the Bergey's Manual of Systematic Bacteriology (taxonomy) (Holt et al., 1998).

*2.6.2. Taxonomical analysis of 16S rRNA genes*

Analysis of the 16S rRNA gene was performed for the taxonomic characterization of the isolates. Extraction of total DNA from the isolates was performed using a DNA/RNA extraction kit (QIAGEN, Valencia, CA, USA), according to manufacturer's instructions. The bacterial 16S rRNA loci were amplified using the domain-specific (Lane, 1991) forward primer Bac27_F (5′-AGAGTTTGATCCTGGCTC AG-3′) and the universal reverse primer Uni_1492R (5′-TACGYTA CCTTGTTACGACTT-3′). The amplification reaction was performed in a total volume of 50 μl containing 1× solution Q (Qiagen, Hilden, Germany), 1× Qiagen reaction buffer, 1 mM of each forward and reverse primer, 10 mM dNTPs (Gibco, Invitrogen Co., Carlsbad, CA), and 2 U of Taq polymerase (Qiagen). Amplification for 35 cycles was performed in aGeneAmp5700 thermocycler (PE Applied Biosystems, Foster City, CA, USA). The temperature profile for PCR was 95 °C for 5 min (1 cycle); 94 °C for 1 min and 72 °C for 2 min (35 cycles); and 72 °C for 10 min after the final cycle (Troussellier et al., 2005). The amplified 16S rRNA fragment was

sequenced with a Big Dye Terminator v3.1 Cycle Sequencing Kit on an automated capillary sequencer (model 3100 Avant Genetic Analyzer, Applied Biosystems). The similarity rank from the Ribosomal Database Project RDP (Maidak et al., 1997) and FASTA Nucleotide Database Queries were used to estimate the degree of similarity to other 16S rRNA gene sequences. Phylogenetic analysis of the sequences was performed as previously described (Yakimov et al., 2006).

16S rDNA sequences were deposited in Genbank under accession numbers KT348211 – KT348365.

## 3. Results

### 3.1. Hydrocarbon contamination in sediment

All the sediment samples were contaminated by hydrocarbons. GC-FID analysis (Table 1) revealed that the highest concentration of total hydrocarbons was detected in station N3 (10,786 ng $g^{-1}$) from the northern transect and in stations S4 (10,364 ng $g^{-1}$) from the southern transect; the less contaminated station S5 (3733 ng $g^{-1}$). Stations N3 and S4 showed also the maximum concentration of linear hydrocarbons (*n*-alkanes from $C_8$ to $C_{30}$) with values of 710 and 524 ng $g^{-1}$, respectively (Table 1). High concentrations of PAHs (18 PAH congeners, including the 16 priority PAHs defined by the U.S. EPA) were previously detected in N3 sediment also by Di Leonardo et al. (2014), although the highest contamination of PAHs was recorded in N1 station.

### 3.2. Microbial abundance

Measures of microbial abundance (DAPI, CFU and MPN counts) (Fig. 2) from seawater and sediment samples showed that rich and diverse populations of bacteria, including HC degraders, thrived in this chronically polluted area. The abundance of HC degraders in the most polluted sediment (N3) was higher than that of total heterotrophs, indicating an active catabolic activity.

### 3.3. Microbial diversity at Priolo Bay

#### 3.3.1. Total microbial diversity of the sediment

Thirty-four phospholipid fatty acids (PLFA) were identified in the sediment samples from Priolo Bay (Table 2). The most important biomarkers, indicative of the microbial community components, were computed and also listed in Table 2 to assess if there were significant changes in community composition in relation to sediment contamination. The highest relative abundance of PLFAs were made up of saturated (SFA: 45.46 ± 2.03%) and monounsaturated PLFAs (MUFA: 31.33 ± 1.54%) without substantial differences among stations. Sum of polyunsaturated PLFAs showed the highest mean concentration in stations S4 and S6 (PUFA: 12.33 ± 2.47% and 14.61 ± 3.47%, respectively) mainly driven by the high relative abundance of 20:4 n6 and 20:5 n3, biomarkers of macro- and microalgae (Kelly and Scheibling, 2012; Parrish, 2013). As regards biomarkers of specific microbial groups, the sum of MUFAs most representative of bacteria (bacterial MUFA: 16:1 n7; 18:1 n7; 19:1) was higher than 20% in all stations, indicating an overall dominance of Gram negative bacteria in the whole area. The sum of cyclopropyl and hydroxyl PLFAs, also indicative of Gram negative bacteria, showed the highest mean values in all three northern transect stations: N1, N2, N3 (Fig. 3, Table 2). Sum of terminal branched fatty acids, indicators of Gram positive and sulfate-reducing bacteria, were around 10–12% in all stations except in S6 (8.34 ± 0.21%). Fungal biomarkers (18:2 n6, 18:3 n3, 18:3 n6) and the ratio of the sum of fungal *vs.* bacterial biomarkers were slightly higher in S6 and S5, while the protozoan biomarkers (sum of all PUFAs 20 n6) were slightly higher in the three southern transect stations: S4, S5, S6 (Table 2). PCO ordination was performed using the whole PLFA profile to assess the similarity among sampling stations (Fig. 3). The northern transect stations clustered in the right side of the ordination, while the southern transect stations were distributed more along the centre/left side: S4 and S5 grouped in the below part and S6 sharply distinct in the upper part. Indeed, S6 site was featured by the exclusive presence of a number of PLFAs (17:1 n7, 20:1 n9, 24:1 n9, 22:4 n6) that caused this clear isolation and masked the separation between the two transects. In spite of the influence of S6, the clusters superimposed to the graph reveal that northern and southern transect are well distinct. PLFA biomarkers superimposed to the graph indicated that cyclopropyl, hydroxyl and bacterial MUFAs featured the northern transect stations, while fungal, protozoan, diatom biomarkers and the ratio of the sum of fungal *vs.* bacterial biomarkers determined the ordination of S4 and S5. The first axis (PCO1) explained 56.4% of the total variation, while the second axis (PCO2) 26.4%.

**Table 1**
Total hydrocarbons (HCs) and *n*-alkanes ($C_8$–$C_{30}$) (ng $g^{-1}$) (SD) in Priolo Bay sediment.

| | Sediment | | | | | |
|---|---|---|---|---|---|---|
| | N1 | N2 | N3 | S4 | S5 | S6 |
| Total HCs | 3975 (119.5) | 5329 (84.8) | 10,786 (428) | 10,364 (360.7) | 3733 (97.8) | 8905 (99.7) |
| *n*-Alkanes ($C_8$–$C_{30}$) | 245 (13.3) | 260 (21.2) | 710 (31.6) | 524 (41.8) | 167 (17.6) | 160 (8.5) |

#### 3.3.2. Bacterial diversity of sediment and water

To compare the bacterial diversity associated with seawater and surface sediment collected from Priolo Bay, 16S rDNA Polymerase Chain Reaction-Denaturing Gradient Gel Electrophoresis (PCR-DGGE) was performed (Fig. 4). Twenty-three to forty-one discernible bands were observed for each sample. The DGGE patterns revealed that the bacterial community composition of seawater and sediment was partially distinct, although a few identical bands were identified in all seawater and sediment profiles. Diversity indices calculated for each DGGE profile were in general higher and more variable in sediment than in water samples. Sediment from station N3 showed the highest Chao's index (861), while sediment from S6 the lowest one (276) (Table 3). The average Chao's index in seawater was 506. The dendrogram, applied to the DGGE profiles, clustered sediment and seawater bacterial communities on two different branches. Within each of these two branches, N1 and N2 profiles from the northern transect clustered together as well as N3, S4 and S5 (Fig. 4). The S6 banding patterns from both sediment and seawater were on a different branch, indicating a totally different bacterial community that reflects different environmental conditions. The sequence of a few common bands identified the presence of uncultured Alpha and Gammaproteobacteria, including the hydrocarbon degraders *Alcanivorax* (in sediment) and *Alteromonas* (in seawater), and of Firmicutes (order Clostridiales) in sediment samples (Table 4).

#### 3.3.3. Diversity of culturable HC-degrading bacteria

In order to isolate the HC-degrading bacteria, sediment and seawater were used to set enrichment cultures on mineral ONR7a medium with a *n*-alkanes mixture ($C_{16}$, $C_{18}$, $C_{24}$), different PAHs (phenanthrene, pyrene, biphenyl and dibenzothiophene) or crude oil (Arabian Light Crude Oil). A total of 258 isolates were obtained from seawater (100 isolates) and sediment (158 isolates) enrichments (Table 5). On the basis of different colony morphology, 158 isolates were identified by 16S rDNA analysis (Fig. 5,

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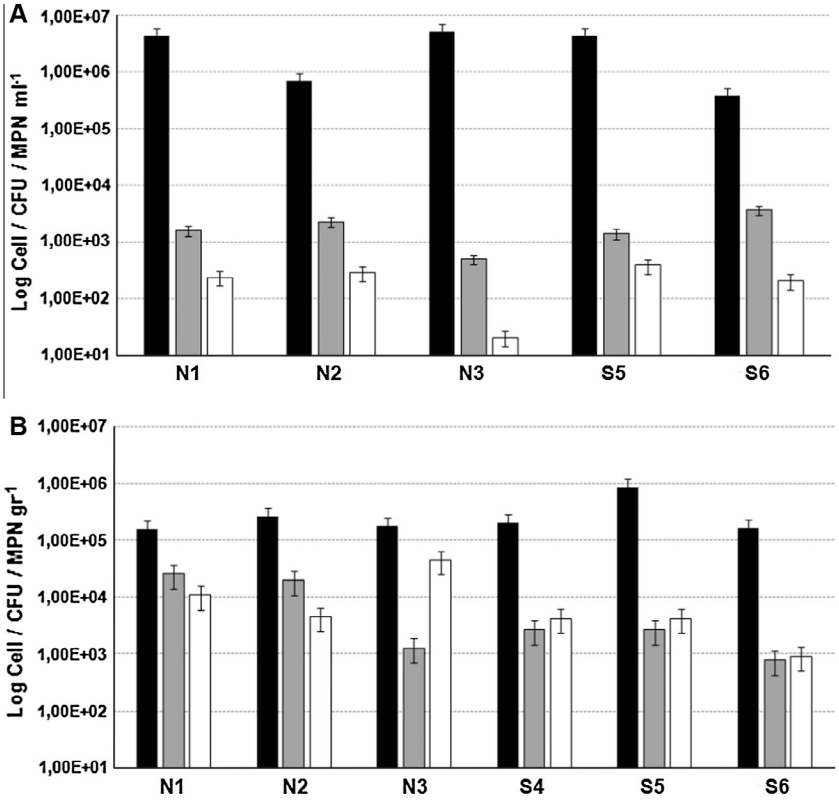


**Fig. 2.** Microbial abundance measured as DAPI total counts (black bars); CFU of heterotrophic cultivable bacteria (gray bars); Most Probable Number (MPN) (white bars) in seawater (A) and superficial sediment (B).

Table 6) and were affiliated mainly to genera of obligate hydrocarbonoclastic bacteria (*Alcanivorax*) and/or to generalist HC-degraders (*Thalassospira*, *Oleibacter*, *Vibrio*, *Marinobacter*, *Rhodococcus*, *Pseudomonas*).

A higher diversity of culturable hydrocarbon-degrading bacteria was detected in seawater samples (20 genera) in respect to sediment samples (12). The water sample N3 yielded the highest number of genera (8) and the sediment sample N1 the lowest (2).

Most sediment and water samples were dominated by bacteria related to *Alcanivorax* and *Marinobacter* genera. Other hydrocarbon degrading bacteria as those belonging to *Thalassospira* were preferentially isolated from a few water and sediment samples and *Oleibacter* from only one water sample (N3). *Oleibacter marinus* was described as a major degrader of petroleum aliphatic hydrocarbons spills in tropical seas (Teramoto et al., 2013) and particularly interesting appears that the isolation of this strain, in this work, is the first official isolation of a strain of *O. marinus* from the Mediterranean area.

## 4. Discussion

Augusta Bay is a wide natural bay with low water turnover that extends along 10 km of the eastern coast of Sicily. This region is characterized by heavy industrialization, dense urbanization and heavy large tanker traffic transporting crude and refined oil to and from the refinery located at this site. These anthropogenic factors have strongly affected the marine environment of the harbour (ISPRA, 2010).

The analysis of organic contaminants in surface sediment revealed high concentrations of total hydrocarbons clearly originating from human activities. The northern deepest station (N3, 20 m depth) and the southern stations (S4 and S6) result in the most intensively contaminated sites.

A large fraction of hydrocarbons is represented by mineral oils presumably derived from the distillation of petroleum to produce gasoline and other petroleum-based products; indeed, mineral oils have an important role in the petrochemical industry and are mostly used as chemical intermediates to produce a number of petrochemicals such as fatty alcohols, alkanolamides, sulfo-methyl esters, sucrose esters and other detergents. The relative high concentration of mineral oils compared to the concentration of other HC (*n*-alkanes) clearly indicates that the sediment is characterized by an old chronic presence of contaminants, where the most potentially degradable fractions have been degraded or reduced (both biologically and/or chemically), in respect to the fractions that have a lower degradation rate (Head et al., 2006). This deduction is confirmed by PAH molecular profiles obtained, on the same samples, by Di Leonardo et al. (2014), where a prevalence of high molecular weight PAHs (above three-ring) was detected in respect to a moderate concentration of two-ring PAHs.

The distribution of HC contaminants in the sediment was not homogeneous nor the chemical data do allow describing a contamination gradient from the coast to offshore that would reflect a contamination source from the coasts where refineries operate. The amount and nature of the HC varied at each station and N3, S4 and S6 stations contained the highest amount of total HC. The extreme heterogeneity of contaminant distribution in the sediment could be due to heterogeneity of the seabed at the bay. Bathymetry and direct observations by divers during sampling, revealed the presence of basins and depressions that work as catchments for hydrocarbons and contaminants. Moreover, this un-patterned distribution of contaminants within Priolo Bay may account for the dumping of contaminant sediment dredged within Augusta Harbour that consequently seems to act as a source of contaminants for nearby coastal and off-shore marine ecosystems (Di Leonardo et al., 2014).

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**Table 2**
Profiles of PLFAs (wt% of the total PLFAs; mean ± standard deviation) in the surface sediment. Sums and biomarker PLFAs are also indicated below.

| | N1 | | N2 | | N3 | | S4 | | S5 | | S6 | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Mean | sd | Mean | sd | Mean | sd | Mean | sd | Mean | sd | Mean | sd |
| *Saturated* | | | | | | | | | | | | |
| 12:00 | 5.36 | 0.38 | 5.60 | 0.09 | 5.39 | 0.29 | 4.65 | 0.55 | 4.98 | 0.62 | 4.51 | 0.71 |
| 14:00 | 5.28 | 0.12 | 5.57 | 0.53 | 5.32 | 0.19 | 4.91 | 0.19 | 4.90 | 0.10 | 4.47 | 0.18 |
| 15:00 | 2.45 | 0.13 | 2.20 | 0.25 | 2.53 | 0.07 | 1.96 | 0.11 | 1.86 | 0.26 | 1.82 | 0.15 |
| 16:00 | 15.42 | 0.17 | 15.20 | 0.15 | 15.12 | 0.46 | 15.49 | 0.81 | 14.48 | 1.21 | 13.74 | 0.38 |
| 17:00 | 1.92 | 0.14 | 1.86 | 0.07 | 1.82 | 0.10 | 1.64 | 0.11 | 1.70 | 0.20 | 1.80 | 0.35 |
| 18:00 | 8.80 | 0.56 | 9.22 | 0.20 | 8.78 | 0.52 | 8.03 | 0.55 | 8.53 | 0.53 | 8.20 | 1.35 |
| 20:00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 3.17 | 0.40 | 4.35 | 1.80 | 3.12 | 0.46 |
| 24:00 | 6.42 | 0.52 | 6.76 | 0.12 | 6.31 | 0.30 | 5.56 | 0.71 | 6.10 | 0.77 | 5.43 | 0.86 |
| Σ SFA | 45.66 | 1.40 | 46.42 | 0.79 | 45.28 | 1.48 | 45.41 | 1.74 | 46.89 | 1.76 | 43.09 | 3.36 |
| *Monounsaturated* | | | | | | | | | | | | |
| 16:1 n7 | 4.93 | 0.32 | 4.28 | 0.27 | 4.23 | 0.37 | 4.34 | 0.50 | 3.84 | 1.46 | 3.91 | 1.84 |
| 17:1 n7 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 1.43 | 0.05 |
| 18:1 n9 | 4.49 | 0.26 | 4.65 | 0.33 | 4.53 | 0.27 | 4.15 | 0.18 | 4.03 | 0.13 | 4.01 | 0.36 |
| 18:1 n7 | 12.43 | 0.49 | 12.16 | 0.47 | 12.23 | 0.28 | 11.93 | 0.49 | 11.72 | 0.40 | 10.57 | 0.31 |
| 19:1 n9 | 7.06 | 0.97 | 9.52 | 0.19 | 8.36 | 0.97 | 7.08 | 0.89 | 10.12 | 1.69 | 7.44 | 1.23 |
| 20:1 n9 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 1.29 | 0.11 |
| 20:1 n11 | 1.81 | 0.17 | 1.84 | 0.05 | 2.72 | 1.43 | 1.49 | 0.17 | 1.81 | 0.26 | 1.54 | 0.25 |
| 24:1 n9 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 2.02 | 0.30 |
| Σ MUFA | 30.73 | 1.22 | 32.43 | 0.59 | 32.08 | 2.11 | 29.00 | 1.09 | 31.51 | 0.23 | 32.21 | 0.16 |
| *Polyunsaturated* | | | | | | | | | | | | |
| 18:2 n6 | 1.34 | 1.19 | 1.44 | 1.27 | 1.72 | 0.31 | 1.93 | 0.36 | 1.74 | 0.41 | 1.34 | 0.12 |
| 18:3 n3 | 0.98 | 0.85 | 0.00 | 0.00 | 0.00 | 0.00 | 1.37 | 0.04 | 1.41 | 0.22 | 0.37 | 0.64 |
| 20:2 n6 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.83 | 0.73 | 0.40 | 0.69 | 0.40 | 0.69 |
| 20:4 n6 | 2.19 | 0.28 | 1.92 | 0.03 | 1.90 | 0.34 | 2.91 | 0.41 | 2.12 | 0.30 | 2.76 | 0.85 |
| 20:5 n3 | 2.84 | 0.33 | 3.08 | 0.50 | 3.07 | 0.52 | 4.24 | 0.38 | 2.73 | 0.34 | 3.98 | 1.47 |
| 22:4 n6 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 3.20 | 0.39 |
| 22:6 n3 | 2.06 | 0.08 | 2.01 | 0.22 | 2.05 | 0.44 | 1.03 | 0.90 | 0.50 | 0.86 | 2.56 | 0.46 |
| Σ PUFA | 9.41 | 2.53 | 8.45 | 1.96 | 8.74 | 0.92 | 12.33 | 2.47 | 8.89 | 1.13 | 14.61 | 3.47 |
| *Branched* | | | | | | | | | | | | |
| i-C14:00 | 1.15 | 0.06 | 1.20 | 0.06 | 1.25 | 0.05 | 1.11 | 0.06 | 1.17 | 0.04 | 0.97 | 0.16 |
| i-15:00 | 3.25 | 0.04 | 2.97 | 0.17 | 3.17 | 0.06 | 3.06 | 0.08 | 2.98 | 0.30 | 2.45 | 0.18 |
| a-15:00 | 3.04 | 0.06 | 2.64 | 0.31 | 3.27 | 0.15 | 3.64 | 0.37 | 3.06 | 0.34 | 2.22 | 0.07 |
| i-16:00 | 2.77 | 0.11 | 2.82 | 0.11 | 2.87 | 0.15 | 2.56 | 0.19 | 2.62 | 0.14 | 2.38 | 0.35 |
| i-17:00 | 0.13 | 0.09 | 0.02 | 0.03 | 0.06 | 0.11 | 0.23 | 0.04 | 0.08 | 0.14 | 0.09 | 0.11 |
| a-17:00 | 1.01 | 0.03 | 0.96 | 0.06 | 0.98 | 0.11 | 0.98 | 0.01 | 0.95 | 0.10 | 0.24 | 0.41 |
| Σ br | 11.35 | 0.08 | 10.60 | 0.59 | 11.60 | 0.46 | 11.58 | 0.28 | 10.86 | 0.66 | 8.34 | 0.21 |
| *Cyclopropyl* | | | | | | | | | | | | |
| cy-17:00 | 2.04 | 0.03 | 1.87 | 0.11 | 2.07 | 0.06 | 1.57 | 0.09 | 1.74 | 0.09 | 1.67 | 0.11 |
| cy-19:00 | 0.12 | 0.02 | 0.00 | 0.00 | 0.13 | 0.01 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 |
| Σ cy | 2.16 | 0.03 | 1.87 | 0.11 | 2.20 | 0.06 | 1.57 | 0.09 | 1.74 | 0.09 | 1.67 | 0.11 |
| *Hydroxy* | | | | | | | | | | | | |
| 2-OH 10:00 | 0.11 | 0.19 | 0.00 | 0.00 | 0.09 | 0.16 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 |
| 2-OH 12:00 | 0.18 | 0.09 | 0.23 | 0.04 | 0.14 | 0.01 | 0.11 | 0.02 | 0.10 | 0.09 | 0.08 | 0.04 |
| 2-OH 14:00 | 0.53 | 0.10 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 |
| Σ OH | 0.83 | 0.18 | 0.23 | 0.04 | 0.24 | 0.17 | 0.11 | 0.02 | 0.10 | 0.09 | 0.08 | 0.04 |
| Σ bact MUFA | 24.42 | 1.17 | 25.95 | 0.85 | 24.83 | 0.78 | 23.36 | 0.85 | 25.68 | 0.59 | 21.92 | 0.44 |
| Σ bact | 49.27 | 0.50 | 47.49 | 1.30 | 48.40 | 1.08 | 50.40 | 0.74 | 49.85 | 0.60 | 43.80 | 1.11 |
| Σ fung | 2.32 | 2.03 | 1.44 | 1.27 | 1.72 | 0.31 | 3.31 | 0.33 | 3.15 | 0.60 | 1.72 | 0.70 |
| Σ fung/Σ bact | 0.05 | 0.04 | 0.03 | 0.03 | 0.04 | 0.01 | 0.07 | 0.01 | 0.06 | 0.01 | 0.04 | 0.02 |
| Σ protozoa | 2.19 | 0.28 | 1.92 | 0.03 | 1.90 | 0.34 | 3.74 | 0.99 | 2.52 | 0.93 | 3.15 | 1.43 |

Microbial diversity of the sediment was analyzed by esther-linked phospholipid fatty acids (PLFA) analysis and denaturing gradient gel electrophoresis (DGGE) of the variable V3 region of bacterial 16S rRNA gene. Both PLFA profiles and biomarkers and DGGE analysis allowed to distinguish the microbial communities of the northern transect from those in the southern transect with the communities of station S6 always clustering apart from the others. Total bacterial diversity, however, seemed not influenced by depth nor by levels of contamination; the deepest stations, namely N3 and S6 showed the highest and lowest bacterial diversity levels, respectively, although the concentration of total hydrocarbons was the highest at both stations. The highest bacterial diversity at station N3 corresponds to the highest concentration of total HC, *n*-alkanes and Total Organic Carbon (TOC, Di Leonardo et al., 2014).

Microbial communities in the northern part of the bay showed PLFA profiles indicative of abundance of Gram-negative bacteria. In contrast, microeukaryotes (diatoms, fungi and protozoa) resulted more abundant in the southern stations, except from S6, where zooplanktonic biomarkers (20:1 n9, 24:1; Parrish, 2013) resulted abundant, probably due to the outward position along the transect that make it more influenced by the local currents. Increase of Gram-negative bacteria has been reported in marine sediment subjected to various stress conditions, among which PAH (Timmis et al., 2005; Castle et al., 2006; Wang and Tam, 2012) and trace element contamination (Córdova-Kreylos et al., 2006). The increased resistance and survival of Gram-negative bacteria to stressors is mainly attributable to the presence of cyclopropyl FAs that maintains higher membrane stability (Kaur et al., 2005). Conversely, microeukaryotes (diatoms,

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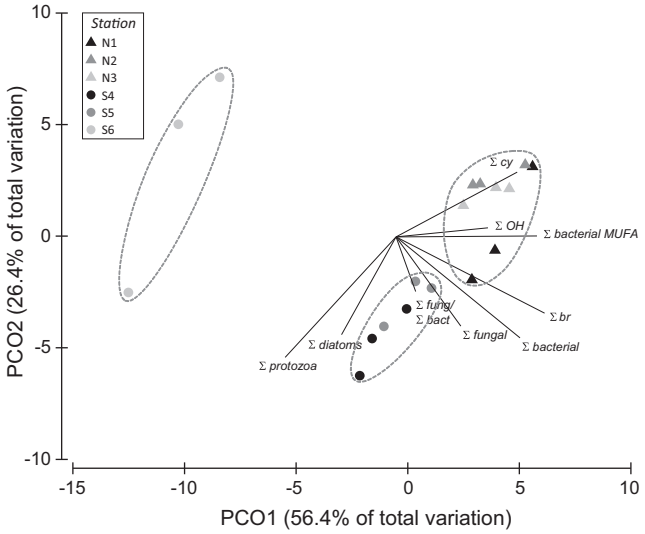


**Fig. 3.** Principal coordinates analysis (PCO) ordination based on Bray-Curtis similarity of arcsine-transformed PLFAs of surface sediment from Priolo Bay. The contribution of each axis (PCO1 and PCO2) to the total variation is shown. Vectors related to PLFA biomarkers of specific groups of microbial communities were superimposed to the graph. Clusters defined at 91% of similarity were superimposed too.

fungi, protozoa), characterized by high levels of PUFAs, seem to be more sensitive to physical and chemical disturbances. Similarly to our results, Napolitano et al. (1994) observed a decrease of 20:4 n6 and 20:5 n3 in streams polluted with chlorine and/or mixed industrial effluents, as well as Kelly et al. (2003) reported reduction of 18:2 n6 in soils contaminated by trace elements. Changes in microbial community composition could be also explained by indirect effect of HC pollution on phage-mediated lysis and on predation by protozoa; both effects tend to increase in response to pollution (Head et al., 2006).

High HC contamination has a direct effect on abundance of HC degraders (measured by MPN) that was higher in the most contaminated northern station N3 and in all the stations of the southern transect. The diversity of HC degraders isolated from seawater samples seemed also to be influenced by the sediment contamination and seawater samples reflected a similar, although not identical, species distribution of sediment samples.

Microbial communities within contaminated ecosystems tend to be dominated by the organisms capable of consuming and/or tolerating toxic organic compounds. Crude oil as a complex mixture of different classes of hydrocarbons can support the growth of various bacteria, each preferentially degrading one or several types of hydrocarbons. The presence of bacteria was strongly dependent on the nature of hydrocarbon pollutants and they had low inter-sample similarity.

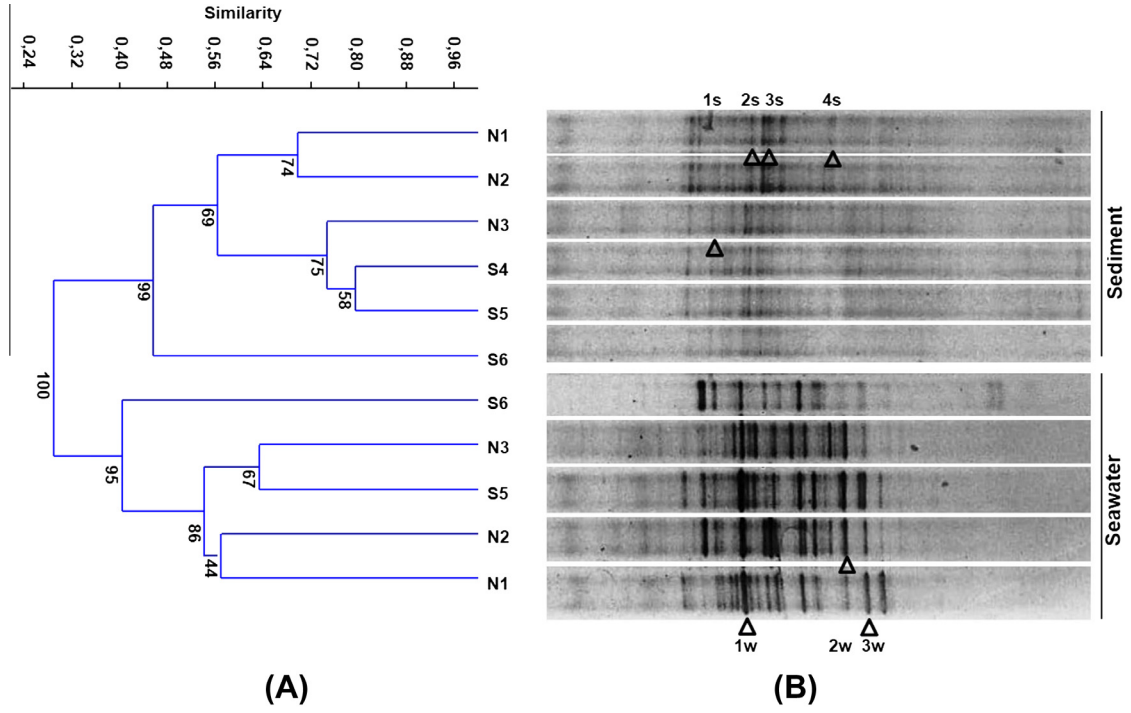


**Fig. 4.** Bacterial diversity of seawater and surface sediment of Priolo Bay. (A) Cluster analysis dendrogram displaying the bacterial community of seawater and surface sediment samples. Jaccard's coefficient and 100 bootstrap analysis was used to measure similarity among the eleven DGGE banding patterns. (B) DGGE profiles of PCR-amplified 16S rDNA V3 region from seawater and surface sediment samples. Arrowheads indicate the predominant band shared by all samples that were reamplified and sequenced (Table 4) S4 water sample is missing.

**Table 3**
Bacterial diversity indices of sediment and water samples in Priolo Bay based on DGGE profiles.

| | Seawater | | | | | | Sediment | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | N1 | N2 | N3 | S4 | S5 | S6 | N1 | N2 | N3 | S4 | S5 | S6 |
| Taxa_S | 28 | 33 | 33 | n.d. | 34 | 28 | 34 | 39 | 41 | 34 | 36 | 23 |
| Shannon | 3.332 | 3.497 | 3.497 | – | 3.526 | 3.332 | 3.526 | 3.664 | 3.714 | 3.526 | 3.584 | 3.135 |
| Chao-1 | 406 | 561 | 561 | – | 595 | 406 | 595 | 780 | 861 | 595 | 666 | 276 |

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**Table 4**
List of bacteria detected by PCR-DGGE in the seawater and sediment samples.

| Band no. | Sample | Sequence length (bp) | Closest sequence match (accession no) | Score (% sequence similarity) | *E* value |
|---|---|---|---|---|---|
| *Sediment* | | | | | |
| 1s | N3 | 125 | Uncultured bacterium (*Alcanivorax* sp.) (GU797076.1) | 149 (86) | 7e−33 |
| 2s | N1 | 131 | Uncultured *Thermoanaerobacterium* (JF819847.1) | 134 (83) | 2e−28 |
| 3s | N1 | 142 | Uncultured bacterium (Gammaproteobacteria) (FM214477.1) | 185 (90) | 1e−43 |
| 4s | N1 | 124 | Uncultured bacterium (Gammaproteobacteria) (KF741563.1) | 185 (94) | 9e−44 |
| *Seawater* | | | | | |
| 1w | N1 | 162 | Uncultured bacterium (*Alteromonas*) (GQ377785.1) | 230 (93) | 4e−57 |
| 2w | N2 | 134 | Uncultured *Thalassobius* (HQ836410.1) | 165 (88) | 1e−37 |
| 3w | N1 | 116 | Uncultured *Alteromonas* (HQ836379.1) | 268 (96) | 1e−68 |

**Table 5**
Total bacterial isolates obtained from the enrichment cultures of seawater and sediment and total hydrocarbon-degrading bacteria (HB) growing exclusively on hydrocarbons.

| Substrate | Sediment | Seawater | HB |
|---|---|---|---|
| *n*-Alkanes[a] | 62 | 31 | 93 |
| Crude oil[b] | 55 | 37 | 92 |
| BPH[c] | 9 | 14 | 23 |
| PYR[d] | 13 | 7 | 20 |
| DBT[e] | 6 | 7 | 13 |
| PHEN[f] | 13 | 4 | 17 |
| Total | 158 | 100 | 258 |

[a] Mixtures of *n*-alkanes ($C_{16}$, $C_{18}$, $C_{24}$).
[b] Arabian Light Crude Oil.
[c] Byphenile.
[d] Pyrene.
[e] Dibenzothiophene.
[f] Phenanthrene.

Most HC degraders isolated from Priolo Bay are Gram negative (specialized or generalist) alkane- and PAH-degraders and their isolation is consistent with the detection of Gram negative bacteria by PLFA analysis. HC degraders were not only cultivated after enrichment cultures on HC, but were also directly detected in the sediment and seawater samples, by sequencing of random DGGE bands. The sequencing of DGGE bands confirms that both sediment and seawater contain known HC degraders as *Alcanivorax* (alkanes) and *Alteromonas* (aromatics, Gutierrez et al., 2013) or putative, such as *Thalassobius* (Iwaki et al., 2012) and *Thermoanaerobacterium*, often dominant in methanogenic alkanes degrading enrichment culture microcosms (Wang et al., 2014).

Alkanes are the major constituents of crude oil, which explains the massive presence of *Alcanivorax* strains among all the sediment and water isolates (Hara et al., 2003). Capability of *Alcanivorax* genus to use hydrocarbons as the only sources of energy and organic carbon was widely described (Yakimov et al., 1998; Schneiker et al., 2006). Kasai (2002) and Cappello et al. (2012) explain these characteristics in ability of this strain to produce a lipidic bio-surfactant that increases the bioavailability of contaminants and the ability to use them (Yakimov et al., 1998; Schneiker et al., 2006). *Alcanivorax borkumensis* SK2 surfactant is proposed as one of the most efficient of bacterial surfactants. Abundance of *Alkanivorax* isolates was obtained from all the water (11%) and sediment samples (87.5%) of Priolo Bay and their abundance seems to have a negative effect on the diversity of other HC degrading genera (McGenity et al., 2012; Newton et al., 2013). In two water samples (N3 and S4) where *Alkanivorax*

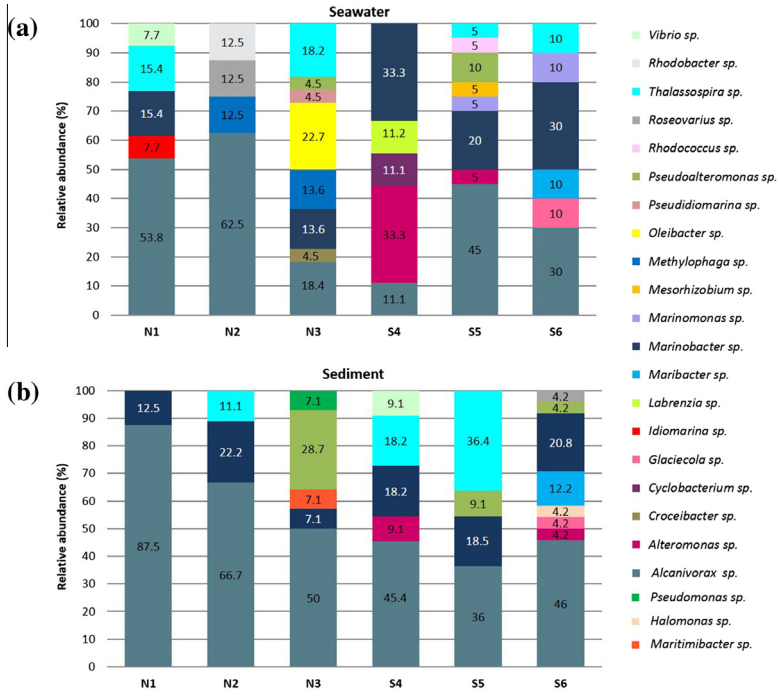


**Fig. 5.** Relative abundance of HC-degrading bacterial genera isolated from (a) seawater and (b) sediment of Priolo Bay by enrichment cultures.

**Table 6**
Bacterial genera isolated from seawater and sediment enrichment cultures on different hydrocarbons as energy/carbon sources.

| | Seawater | | | Sediment | | |
|---|---|---|---|---|---|---|
| | *n*-Alkanes[a] | Crude oil[b] | PAHs[c] | *n*-Alkanes[a] | Crude oil[b] | PAHs[c] |
| *Alcanivorax* | + | + | + | + | + | + |
| *Alteromonas* | | | + | + | | |
| *Marinobacter* | | | + | + | | + |
| *Marinomonas* | + | | | | | |
| *Oleibacter* | + | | | | | |
| *Pseudoalteromonas* | + | + | + | | | + |
| *Thalassospira* | + | + | + | | + | |
| *Vibrio* | + | | | | + | |
| *Croceibacter* | | | + | | | |
| *Cyclobacterium* | | | + | | | |
| *Glaciecola* | | | + | | | + |
| *Idiomarina* | | | + | | | |
| *Labrenzia* | | | + | | | |
| *Mesorhizobium* | | | + | | | |
| *Methylophaga* | | | + | | | |
| *Pseudidiomarina* | | | + | | | |
| *Rhodococcus* | | | + | | | |
| *Rhodobacter* | | | + | | | |
| *Halomonas* | | | | | | + |
| *Roseovarius* | | | + | | | + |
| *Pseudomonas* | | | | | | + |
| *Maritimibacter* | | | | | | + |
| *Maribacter* | | | | | | + |

[a] Mixtures of *n*-alkanes: $C_{16}$, $C_{18}$, $C_{24}$.
[b] Arabian Light Crude Oil.
[c] Polycyclic aromatic hydrocarbons: byphenile, pyrene, dibenzothiophene, phenanthrene.

was below 20%, it is probably not just by chance that two companion genera, *Oleibacter* and *Alteromonas*, prevail in the assemblage. Competition among HCB could depend on resources, but also on the production of inhibiting molecules by *Alkanivorax* (Kanoh et al., 2008).

In this study, the isolation of *Oleibacter* from the Mediterranean Sea was reported for the first time. *O. marinus* is a recently discovered species of OHCB, phylogenetically related to *Oceanobacter* sp. and *Thalassolituus* sp. that was isolated from a tropical marine environment. It shows high *n*-alkane and branched alkanes degrading activity (Teramoto et al., 2011) that is comparable to that of the genus *Alcanivorax*, suggesting that *Oleibacter* plays a key role in the degradation of petroleum hydrocarbons in the marine environment. *Oleibacter* was identified in biofilms associated with phototrophic and diazotrophic bacteria, suggesting that this interaction may be important for hydrocarbon degradation (Al-Bader et al., 2013; McGenity, 2014).

A higher diversity of PAH-degrading bacteria isolated from sediment of the southern transect mainly affiliated to the genera *Thalassospira* (Cui et al., 2008; Guibert et al., 2012), *Marinobacter* and *Alteromonas* (Cui et al., 2008; Jin et al., 2012), seems to be related to higher LPAH/HPAH ratio identified in the southern transect (Di Leonardo et al., 2014).

All together chemical and microbiological data allowed depicting a complex environmental scenario of the Priolo Bay where the release of pollutants from the adjacent Augusta Harbour adds to chronic contamination of the area. This hypothesis is supported by the higher contamination of the stations closer to Augusta Harbour (or directly influenced by the currents) and also by the massive presence of OHCB and *Alcanivorax* in particular, that is expected to decline after the removal of the bulk saturated hydrocarbons (Head et al., 2006).

New trends of environmental ecology are focused in the use of innovative techniques for the recovery of impacted ecosystems, rather than simply reducing the massive volume of oil released into the sea (clean up), or the removal of the contaminated matrix (sediment). Special attention is devoted to self-cleaning processes occurring in the sea after oil spill events as well as to the physical, chemical and biological factors influencing such power.

Biodegradation by natural populations of microorganisms is the central and most reliable mechanism by which thousands of pollutants, including crude oil, are eliminated from the environment (Prince et al., 2013). "Biostimulation" (growth stimulation of indigenous microorganisms) and "bioaugmentation" (direct inoculation of allochthonous and/or autochthonous oil-degrading bacteria) are promising strategies of accelerating the detoxifying and degrading activities into polluted site with minimal impact on natural environment (Cappello et al., 2007b; Emtiazi et al., 2005; Hassanshahian et al., 2012). Recovery of the polluted matrix (seawater and/or sediment) is possible using "passive" methods such as bioremediation, which uses bacteria to break down the hydrocarbons in the sediment into harmless by-products.

Priolo Bay shows high levels of total HC, saturated HC and PAHs in concomitance with high abundance and diversity of OHCB that are known to play a key role in HC degradation. The high total microbial diversity associated to a large selection of HC degraders is believed to contribute to natural attenuation of the area (Dell'Anno et al., 2012). The rich and diverse community of HC degraders is endowed with a very high potential of application in the bioremediation of this area. Several isolates obtained from enrichment cultures from Priolo Bay showed high degradation rates (98–99%) of crude oil (Santisi and Catania, unpublished). Levels of contamination in the bay are expected to be reduced by natural attenuation by autochthonous HC degraders provided that new contaminant contributions are avoided.

The use of natural microbial communities to clean the marine polluted sediment is identified as "green technology" because it does not introduce exogenous bacteria into the waters to be restored. Knowledge of microbial community dynamics and activities and efficiency of bacteria in oil-polluted sites may be helpful to promote bioremediation of contaminated sites since human actions can be planned in order to promote clean up oil pollution (Denaro et al., 2005).

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## Author contribution

Conceived and designed the experiments: PQ, SC, SV. Performed the experiments: VC, SS, GS. Analyzed the data and wrote the MS: PQ, SC, SV. Supervised and revised the MS: AM, MY.

## Acknowledgments

This work was supported by grants of (i) University of Palermo (Italy), (ii) Istituto per l'Ambiente Marino Costiero (IAMC) – CNR of Messina, Italy, (iii) National Operative Project PON R&C 2007-2013 "Tecnologie avanzate eco-sostenibili finalizzate alla bonifica ed al ripristino di aree marine costiere degradate (PON01_03112 – TETIDE), and (iv) Flagship Project RITMARE—Italian Research for the Sea—funded by the Italian Ministry of Education, University and Research. The authors are grateful to A. Savona and A.M. Vaccaro for help in field activity.

## Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.marpolbul.2015.07.042.

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Please cite this article in press as: Catania, V., et al. Intrinsic bioremediation potential of a chronically polluted marine coastal area. Mar. Pollut. Bull. (2015), http://dx.doi.org/10.1016/j.marpolbul.2015.07.042