Microbial communities of polluted sub-surface marine sediments

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Abstract
Microbial communities of coastal marine sediment play a key role in degradation of petroleum contaminants. Here the bacterial and archaeal communities of sub-surface sediments (5-10 cm) of the chronically polluted Priolo Bay (eastern coast of Sicily, Italy), contaminated mainly by n-alkanes and biodegraded/weathered oils, were characterized by cultural and molecular approaches. 16S PCR-DGGE analysis at six stations, revealed that bacterial communities display lower phylogenetic diversity than the surface sediment, respond to oil supplementation in microcosms with a significant reduction in biodiversity and a shift in composition. They retain high biodegradation capacities and host hydrocarbon (HC) degraders that were isolated and identified. HC-degrading Alfa, Gamma and Epsilonproteobacteria together with Clostridia and Archaea are a common feature of sub-surface communities. These assemblages show similarities with that of subsurface petroleum reservoirs also characterized by the presence of biodegraded and weathered oils where anaerobic or microaerophilic syntrophic HC metabolism has been proposed.

Introduction
Coastal environments, especially the semi-enclosed basins characterized by low turnover of seawater such as the Mediterranean Sea, are exposed to high levels of contamination by petroleum hydrocarbons due to the intense maritime traffic and high presence of sources of pollution along its entire coastline (Bargiela et al., 2015; Duran et al., 2015; Daffonchio et al., 2013). Petroleum hydrocarbons (HC) are classified as persistent pollutants which remain trapped in the sediments for long periods of time making them a permanent pollution source (McGenity et al., 2012) which have adverse effects on human and environmental health (Varjani, 2017). A part of petroleum HC is degraded in seawater in relatively short time (days or months) while, when the oil reaches the coast, it has a longer residence time in the marine environment (Hazen at al., 2016). The oil contamination can prevent ventilation of upper layers resulting in the development of extensive anoxic zones. Bacterial communities of coastal sediments play a key role in the degradation of these contaminants under aerobic, microaerophilic and anaerobic conditions making bioremediation a concrete alternative to physical and chemical methods for minimizing coastal pollution (Xue et al., 2015). Understanding diversity, composition, and biodegradation ability of microbial communities that inhabit contaminated sediment is the starting point for bioremediation actions. Coastal sediments are heterogeneous habitats and show a high dissimilarity in the composition of bacterial communities among different sites (Acosta-González & Marqués 2016); much research in recent years has contributed to increasing the knowledge of diversity of indigenous microbial populations of contaminated sediment in order to exploit their metabolic potential (Daffonchio et al., 2013; Bargiela et al., 2015). Most studies have investigated the diversity of HC degrading microbial communities colonizing superficial sediment of polluted sites in the Mediterranean Sea (Amer et al., 2015, Catania

et al. 2015; Jeanbille et al., 2016). Sediments generally have a higher microbial abundance and diversity within the marine habitat and bacterial HC degrading communities in marine coastal environments reveal functional differences and different potential for the biodegradation of pollutants; each site is populated by different bacterial communities, generally dominated by Proteobacteria. Among the dominant taxa, specialized hydrocarbonoclastic bacteria were identified in most chronically polluted sites (Catania et al., 2015; Varjani, 2017). However, contamination levels and the differential bacterial distribution of sub- surface sediment is still poorly known. Most studies analyzing the effect of hydrocarbon pollution on subtidal sediments have generally analyzed the uppermost centimeters and in some cases the depth of sampled material has not even been detailed (Bargiela et al., 2015, Acosta-Gonzalez and Marques, 2016). The bacterial communities inhabiting anaerobic sediments are poorly known and their response to oiling merits particular attention (Miralles et al., 2007). The aim of this study was to explore the diversity and biodegradation potential of microbial communities in sub- surface marine sediments (5-10 cm below the sediment surface) of the Priolo Bay (eastern coast of Sicily, Italy, Central Mediterranean). The Priolo Bay is a Site of Community Importance (SIC) situated in proximity to the Augusta Harbour, affected for decades by pollution from industrial and petrochemical plants (Di Leonardo et al., 2014). The bioremediation potential of superficial sediment and seawater of the Priolo Bay was evaluated previously (Catania et al., 2015) and the most polluted superficial sediments (0-5 cm) were found to host higher bacterial diversity and higher abundance and diversity of cultivable HC degraders than in less polluted sites. Oil and oil-derived hydrocarbon degrading bacteria were isolated from superficial sediment and assigned to *Alcanivorax, Marinobacter*, *Halomonas* and *Vibrio* (Catania et al., 2015). In this work, the microbial communities of sub- surface sediments from 5 to 10 cm depth from the Priolo Bay were characterized by DGGE (Denaturing Gradient Gel Electrophoresis) fingerprinting analysis immediately after sampling and after enrichment in oil supplemented microcosms. The degrading capacity of sub-surface sediment microbial communities was analyzed by GC-FID analysis and isolation of HC degraders was carried out under aerobic and anaerobic conditions. This study provides the characterization of the bioremediation potential and describes microbial communities involved in degrading the hydrocarbons in rarely studied sub-surface sediments of a chronically polluted area.

Materials and methods

The study area

Priolo Bay is a coastal marine basin located along the South-East coast of Sicily (Italy, Central Mediterranean Sea) in the southern part of Augusta Bay; the northern sector of Augusta Bay includes the heavily-industrialized Augusta Harbour. The areas of Augusta and Priolo have been declared "areas of high risk of environmental crisis" due to the high levels of contamination andthe Priolo Bay was included among the site of national interest (SIN). Moreover the Priolo bay is part of the Saline di Priolo, a Site of Community Importance (SIC-ZPS ITA090013, Habitats Directive 92/43/EEC) characterized by a coastal wetland affected by water brackish subject to temporary summer drying.
The date of granulometric analysis show in the Augusta Bay a settling mainly pelitic and pelitic sandy almost everywhere, probably influenced by rocky outcrops and by the particular structure of the substrate in the area that determines the presence of more coarser and heterogeneous sediments in the band coastal area and close to the dams (ICRAM 2008).
The Bay of Augusta is subject to pollution by petrochemical plants and wastewater treatment plants, dense urbanization and intense tanker traffic transporting crude and refined oil to and from the refinery located at this site. The high concentrations of Hg, HCB and HC (C>12), identified within the Augusta bay are superior to any environmental quality standard defined at national and international level (ICRAM, 2008; Signa et al., 2017).
The southern sector of Augusta Harbour is in connection to the Priolo Bay by the Scirocco inlet (300m wide,13m deep) and the Priolo Bay is influenced by the release of contaminants from the Augusta Harbour being in direct connection with it (Di Leonardo et al., 2014). Total PAH, n-alkanes and trace element (mainly As, Cd, Hg, Ni) analysis performed on the superficial sediments of Priolo

103 Bay revealed an irregular spatial distribution of contaminants with sediments close to the Augusta
104 harbour recording greater contaminant concentrations (Di Leonardo et al 2014; Catania et al., 2015;
105 Signa et al., 2017).
106 Sedimentation rate was previously calculated in Priolo Bay through ^{210}Pb data (Di Leonardo et al.
107 2017). Nevertheless, analyses carried out in cores collected from bare sediment indicated highly
108 disturbed sedimentation probably related to sediment reworking and did not allow any estimation of
109 sedimentation rate. The only data available is from a core collected in dead matte of Posidonia
110 oceanica, where sediment accumulation rate was 1.2 mm yr-1 over the last 120 yr. A much higher
111 sediment accumulation rate was measured by Bellucci et al., 2012 in the Augusta Bay, but they could
112 be influenced by massive sediment dredging activities within the harbour and illegal dumping and
113 discharge offshore (Bellucci et al., 2012).
114 **Sediment collection**
115 The sediment samples were collected at the Priolo Bay, on the South-East coast of Sicily (Italy,
116 Central Mediterranean Sea), in July 2012. Sediment was sampled through sterile Plexiglas cores (20
117 cm long, 5 cm diameter) as described in Catania et al. (2015), from three stations located along a
118 northern transect (N1: N 37° 9' 58,3'', E 15° 12' 2.6''- depth 5 m; N2: N 37° 10' 3,7'', E 15° 12'
119 21.7''- depth 10 m; N3: N 37' 10' 9.3", E 15° 12' 53,8''- depth 20 m) and three stations located along
120 a southern transect (S4: N 37° 9' 20.5'', E-15° 13' 1.3''-depth 5 m; S5: N 37° 9' 30.6'', E 15° 13'
121 9.8''- depth 10 m; S6: N 37° 9' 46.8'', E 15° 13' 20.6'' - depth 20 m; Figure 1). The samples were
122 transported to the laboratory in a cool box and used for immediate chemical and microbiological
123 analysis and DNA extraction. Aliquots from the layer 5-10 cm were stored at -80° and at -20 C°
124 under glycerol (20% final concentration) for further analysis.
125 Grain size analysis was carried out on bulk sediment pre-treated with hydrogen peroxide and Na-
126 Hexametaphosphate to remove organic matter and avoid particle flocculation. Then it was wet-sieved
127 through a 63 µm net to separate mud from coarser particles, which were electromechanically dry-
128 sieved through a DIN ISO 3310-1 standard sieve set to obtain a grain size series with intervals of ½
129 Φ (Φ = - log2 Ø mm). The pH of sediment samples was measured using a pH meter and probe. The
130 probe was calibrated using the standard solutions (pH 4, 7, and 10 buffers) and was submerged
131 directly into the sample.
132
133 **Analysis of hydrocarbons**
134 Total hydrocarbons were extracted from sub-surface sediment following the 3550C EPA
135 (Environmental Protection Agency) procedure. Briefly, a mixture of CH_2Cl_2:CH_3COCH_3 (1:1, v/v)
136 was added to sediment samples. The mixture was sonicated for 30 min in ultrasound bath (Branson
137 1200 Ultrasonic Cleaner, Branson USA). Samples were further shaken at 150 ×g for 30 min,
138 centrifuged for 10 min at 5000 ×g and supernatant was passed through a ceramic column filled with
139 sodium sulphate anhydrous Na_2SO_4 (Sigma-Aldrich, Milan). Same treatment of pooled and dried
140 sediment was repeated with CH_2Cl_2 and the obtained extracts were combined and volatilized to
141 dryness. Residues were re-suspended in CH_2Cl_2 prior to gas chromatography (GC) analysis. All
142 measures were performed using a Master GC DANI Instruments (Development Analytical
143 Instruments), equipped with SSL injector and FID detector. Samples (1 µl) were injected in split less
144 mode at 330 °C. The analytical column was a Restek Rxi-5 Sil MS with Integra-Guard, 30 m × 0.25
145 mm (ID × 0.25µm film thickness). Helium carrier gas was maintained at a constant flow of 1.5 ml
146 min^{-1}. Total hydrocarbons were also calculated for each sample (Genovese et al., 2014). Three sub-
147 samples for each station were analyzed.
148
149 **Set-up of microcosms and biodegradation of HC**
150 Microcosms were set up in an anaerobic chamber to prevent exposure to the atmospheric oxygen, by
151 transferring of 0.5 g of each sub- surface sediment in 10 mL glass serum bottle containing 5 ml
152 mineral medium ONR7a supplemented with 1% (v/v) of crude oil (Arabian Light Crude Oil, ENI
153 S.p.A) as sole carbon source. Bottles were capped with a rubber stopper and sealed with aluminum

crimps, and incubated at 18±1°C without shaking for 1 month. Microcosms were set in triplicates together with abiotic controls. Total hydrocarbons and their derivates (TERHC) were extracted from microcosms and analyzed by GC–FID according to the procedure previously described (Denaro et al., 2005). TERHC were extracted at room temperature on a shaking table using dichloromethane–seawater (10% v/v). This procedure was repeated three times, and the CH_2Cl_2 (DMC; Sigma Aldrich, Milan, Italy) phase was combined and treated with anhydrous Na_2SO_4 (Sigma Aldrich, Milan, Italy) to remove residual water. Extracts were concentrated by rotary evaporation (Rotavapor model R110; Buchi Labortechnik, Flawil, Switzerland) at room temperature (<30°C), diluted 1:10 with DCM and then injected in split 1:50. The TERHC composition was analyzed by high-resolution Master GC Fast Gas Chromatograph System (DANI Instrument S.p.A., Milan). The identification of resolved aliphatic hydrocarbons was achieved by comparing retention times with the corresponding standards (Chem Service Inc, West Chester, PA, USA). Total resolved aliphatics (TRA) were calculated using the mean response factors of *n*-alkanes. Individual *n*-alkane concentrations from n-C_{10} to n-C_{28}, Pristane (Pr) and Phytane (Ph) isoprenoid levels, total resolved n-alkanes (Σnalk) and TRA were calculated for each sample. Hydrocarbon concentration values were expressed as ppm. Evaluation indices selected for this study to evaluate the relative biodegradation of n-alkanes were: n-C_{17}/Pristane (nC_{17}/Pr), n-C_{18}/Phytane (nC_{18}/Ph).

PCR-DGGE of bacterial and archaeal communities in sediment and microcosms

Total community DNA was extracted from sub-surface sediments immediately after sampling using the FastDNA™ SPIN Kit for Soil (MP Biomedicals, Germany) according to the manufacturer's instructions. Total genomic DNA was extracted from oil-supplemented microcosms using the QIAamp® DNA Stool Kit (Qiagen). DNA concentration was determined using a NanoDrop ND-1000 Spectrophotometer (NanoDrop Technologies). The DNA extracted was used as template to amplify the bacterial and archaeal V3 hypervariable region of the 16S rRNA gene. Bacterial 16S genes was amplified using primers 341f-GC, 354r (Muyzer et al., 1993). Archaeal 16S rDNA was amplified with primers ARCH 349 F-GC, A571R (Gagliano et al., 2016); the 5'-GC-clamp (Muyzer et al., 1993) was attached to the forward primer. The bacterial V3 region was amplified as described in Catania et al., 2016. The same reaction mixture was utilized to amplify the V3 region of the Archaeal 16S rRNA gene. PCR was carried out with the following conditions: initial denaturation for 30 s at 98°C, followed by 40 cycles of denaturation for 15 s at 98°C, annealing at 58°C for 10 s extension at 72°C for 10 s, final extension at 72°C for 2 min. PCR products were analyzed on 1.5% agarose gels stained with ethidium bromide. The DGGE analysis was conducted as described in Catania et al. (2016). Bands of interest on the DGGE gel were excised, transferred to a clean Eppendorf tube containing 20 µl of DNA/RNA free water (GIBCO). 2µl of the eluted DNA were used as the template and amplified under the same conditions for sequencing. The amplified hypervariable region V3 was compared to the EMBL/SwissProt/GenBank non-redundant nucleotide or 16S_ribosomal_RNA database using Mega BLAST algorithm (http://blast.ncbi.nlm.nih.gov/Blast.cgi) and the Naïve Bayesian rRNA Classifier Version 2.8, of the Ribosomal Database Project II (RDP) (Cole et al., 2013). The Bacterial and Archaeal diversity indices and the cluster analysis were obtained using the PAST software (Hammer et al., 2001).

16S rRNA gene clone library from microcosm S5

The total DNA extracted from the microcosm S5 was used as a template to amplify the ribosomal 16S rRNA gene using the universal bacterial primers 27F e 1492R. Reaction mixtures contained 20 ng of template DNA, 1x Invitrogen reaction buffer (20 mM Tris-HCl pH 8.4, 50 mM KCl), 50 mM of $MgCl_2$, 0.2 mM of each dNTPs, 200 nM of each primer and 0.5 U of Taq Polymerase. The following PCR conditions were used: an initial denaturation step at a 94° for 3 min, 30 cycles of 45s at 94°C, 60s at 50°C, and 90s at 72°C; and a final extension step at 72°C for 10 min. The PCR products were electrophoresed, stained as mentioned above and purified with NucleoSpin® Gel and PCR Clean-Up Kit (Macherey-Nagel) following the manufacturer's instructions. The purified PCR

fragments (about 150 ng) were then cloned as described above. Recombinant plasmids were extracted and restricted with *AfaI* (Biolabs New England). Restriction digests were analyzed on 1.5% agarose gels stained with ethidium bromide 1%. The resulting restriction patterns were used to assign the clones to operational taxonomic units (OTUs). One representative clone of each OTU was chosen, plasmid DNA was extracted using Gen Elute Plasmid Miniprep Kit (SIGMA, ALDRICH) and its insert was sequenced using the M13 primers. The sequences were compared to the EMBL/SwissProt/GenBank rRNA_typestrains/prokaryotic_16S_ribosomal_RNA database using Mega BLAST algorithm (http://blast.ncbi.nlm.nih.gov/ Blast.cgi) and the Naïve Bayesian rRNA Classifier Version 2.8, of the Ribosomal Database Project II (RDP) (Cole et al., 2013). Sequences were submitted to the DDBJ/EMBL/Genbank database under accession numbers The 16S rRNA sequences were deposited in the GenBank database under accession numbers KY595131/ KY595133.

Detection of catabolic genes

To detect the catabolic genes involved in the key step of hydrocarbon degradation, PCR amplification of monooxygenase and dioxygenase gene fragments were performed. The fragment of the terminal hydroxylase component of xylene monooxygenase encoded by the *xylM* gene, the electron transfer component of the xylene monooxygenase encoded by the *xylA* gene, and the catechol 2,3-dioxygenase encoded by *xylE1* gene were amplified using the primer sets TOL-F/TOL-R, XYLA-F/XYLA-R, XYLE1-F/XYLE1-R respectively as described by Hendrickx et al. (2006). A fragment of the alkane monooxygenase gene *alkB* was amplified using primers alkB-1f and alkB-1r (Kloos et al., 2006). PCR reactions were performed in a total volume of 20 µL containing OneTaq Standard Reaction Buffer 1 x (NEB), 0,25 mM of dNTPs mixture (Invitrogen), 100 nM of forward and reverse primers, 1 U of OneTaq DNA Polymerase (NEB), and 10-100 ng of template DNA extract from sediment immediately after sampling. The PCR program consisted of an initial denaturation step of 30 sec at 94°C, followed by 35 cycles of 30 sec at 94°C, 30 secs at 55°C, 1 min at 68°C, with a final extension of 5 min at 68°C. All amplicon was visualized by electrophoresis on 1,2% agarose gel. A library of fragment 550 bp size of *alkB* gene was construct. Amplicons were cloned into the pCR 2.1-TOPO vector and transformed into One shot TOP10 chemically competent Escherichia coli using TOPO® TA Cloning® Kit (Invitrogen) according to the manufacturer's instructions. Sequences of recombinant plasmids were analyzed using the NCBI BLAST database. The sequences were aligned with the MEGA 4 Software, and sequences showing >99% similarity were considered as one phylotype. Phylogenetic trees of *AlkB* sequences were constructed using the the neighbor-joining algorithm trough the Mega 4 program. Sequences were submitted to the DDBJ/EMBL/Genbank database under accession numbers KY595110/ KY595119, KY595121/ KY595130

Isolation and molecular identification of HC degrading bacteria in microcosms

Putative HC degrading bacteria were isolated from microcosms by spreading aliquots on ONR7-agar plates containing crude oil (Iranian Crude Oil; ENI S.p.A.) as described in Quatrini et al., 2008. Heterotrophic bacteria were isolated on Marine Agar plates (Pronadisa, Conda). All sets of plates were incubated at 18±1°C under both aerobic and anaerobic conditions, and inside anaerobic jars (Oxoid™ AnaeroJar™). After incubation, all colonies showing different phenotypes were selected and transferred to fresh ONR7-agar plates containing 100µl crude oil or fresh Marine Agar medium. The plates were incubated under the same conditions. The procedure was repeated to obtain pure cultures. Aliquots of bacterial cultures were stored with glycerol (20% final concentration) at -80°C for further characterization. In order to identify the isolates, the bacterial 16S rRNA genes were amplified and sequenced as described above. The 16S rRNA gene sequences of the isolates were deposited in the GenBank database under accession numbers KX604244/KX604249.

RESULTS

Chemical-physical characterization of sediments

255 Most sub-surface sediments of the Priolo Bay were classified as sandy with a higher component of
256 silt in N3 and gravel in S4 (Table 1). All sediments were contaminated by hydrocarbons represented
257 mainly by Unresolved Complex Mixtures (UCMs) and aliphatic hydrocarbons (Table 2). polycyclic
258 aromatic hydrocarbon (PAHs) were below the detection limits, except in N3 where they were detected
259 at low concentrations. UCM are composed of several recalcitrant petroleum aliphatic and aromatic
260 hydrocarbons that cannot be resolved by conventional gas chromatography; they are considered the
261 result of microbial degradation and weathering of petroleum hydrocarbons that accumulate in
262 sediments (Scarlett et al., 2007). Site N3 showed the highest level of contamination while N1 the
263 lowest.

264 **Oil biodegradation in microcosms**

265 To evaluate the HC degradation potential of sub-surface sediment in the Priolo Bay, microcosms were
266 set up on crude oil. After one-month of incubation, all microcosms showed moderate turbidity and
267 high HC degradation; almost complete degradation was observed for short and medium chain *n*-
268 alkanes (100% to 86%), while long-chain alkanes were degraded to a lesser extent (96% to 73%)
269 (Table 3). Oil degradation was similar for all microcosms, except for those containing sediment from
270 N3 where a lower rate of degradation was observed especially towards medium and long-chain
271 alkanes.
272

273 **Bacterial and Archaeal diversity**

274 The bacterial and Archaeal communities of sub-surface sediment were analyzed by 16S rDNA PCR-
275 DGGE, immediately after sampling and after oil-supplemented microcosm incubation. The DGGE
276 pattern of bacterial communities shows a variable number of discernible bands, between 12 and 37
277 (Figure 2); diversity indices, calculated for each bacterial DGGE profile, are higher in S5 station and
278 lower at N1 station (Table 4) and do not seem to be related to HC contamination level. After one-
279 month incubation in oil microcosm, bacterial diversity is reduced mainly in stations of the southern
280 transect. Cluster analysis based on the DGGE profiles separates on two different branches the
281 communities of sub-surface sediments from those of oil-supplemented microcosms. The bacterial
282 communities of microcosms N1 and N2 are closer to sediment communities indicating that
283 enrichment on oil had poor effect on them. The other microcosm communities cluster together with
284 the exception of S6 that is branched apart.
285 Sequences of 27 random bands from DGGE gel of microcosms were affiliated to Proteobacteria
286 (Alpha, Epsilon, Gamma) and Firmicutes (Table 5). Most of the sequences belong to generalist HC
287 degrader Gammaproteobacteria known to be directly involved in the degradation of *n*-alkanes and
288 PAHs (*Pseudoalteromonas* sp., *Pseudomonas* sp., *Marinobacter* sp., *Halomonas* sp. (McGenity et al.
289 2012) and to other genera frequently detected in hydrocarbon-degrading communities inhabiting oil-
290 rich marine environments (*Idiomarina sp., Colwellia sp.*) (McGenity 2014; Hazen et al., 2016). Two
291 other sequences belong to hydrocarbon degrading Alpha-proteobacteria (*Polymorphum*
292 *sp;Thalassospira sp.*). Among *Polymorphum gilvum* is able to emulsify crude oil and metabolize
293 aromatic compounds (Nie et al., 2012). A unique sequence affiliated with Epsilonproteobacteria was
294 detected only in microcosm S5. Firmicutes were assigned to anaerobic nitrate-reducers (*Vallitalea*)
295 and sulphate-reducers (*Peptoniphilus*). A lower abundance and diversity of archaeal communities
296 was observed in all sediment samples and in microcosm samples in respect to bacterial communities;
297 the discernible bands were between 6 and 20 (Figure 3). Furthermore, archaeal diversity seems less
298 influenced by supplementing of oil; no significant differences in the number of taxa were identified
299 in microcosms in respect to the sediment after sampling (Table 4). Sequence analysis of nine random
300 bands excised from the DGGE gel, revealed the presence of uncultured Crenarchaeota and
301 Thaumarchaeota (Table 6).
302

303 **Epsilonbacteria dominate microcosm S5**

304 Epsilonbacteria were detected by DGGE band sequencing only in S5 microcosm. To further
305 characterize the S5 microcosm community and estimate the relative abundance of Epsilonbacteria, a

16S rRNA gene clone library was constructed. Sixty clones, out of 600, were analyzed by amplified rDNA restriction analysis (ARDRA) and sequences. The clones were grouped in three operational taxonomic units (OTUs) and affiliated to Proteobacteria (two OTUs, 97% of clones) and Firmicutes (one OTU,3%). The Proteobacteria were almost exclusively represented by Epsilonproteobacteria of the genus *Arcobacter* (82%) and Gammaproteobacteria assigned to *Photobacterium* (15%). The Firmicutes were constituted of exclusively *Oceanirhabdus* (3%) (Table S1).

Identification of catabolic genes in sub- surface sediments

Mono- and dioxygenase encoding genes are considered molecular markers of HC biodegradation (Fuentes at al 2014). All sediment samples were positive to the *alkB* probe. No PCR products were obtained using primers targeting the aromatic catabolic genes *xylM, xylA, xylE1* in all samples (data not shown). To obtain a preliminary estimate of the potential of the microbial populations to degrade alkanes, the 550bp *alkB* gene fragment from sample N3 was cloned and sequenced. Twenty N3 *alk*B clones showed 76% to 98% identity with known *alkB* genes of mainly uncultivated bacteria detected in sediments with high concentration of hydrocarbons from Timor Sea, Australia (Wasmund et al., 2009), in the Berre lagoon (Paisse et al., 2011), in the Adriatic Sea (Korlević et al., 2015). From the original 20 sequenced clones, 18 with a sequences similarity <99% were used for phylogenetic analysis (Figure 4). The majority of the sequences were assigned to three large clusters in the phylogenetic tree. Three N3 *alkB* sequences formed a cluster with *Alcanivorax borkumensis*, *Pseudomonas* and *Rhodobacter*, all the other sequences clustered with sequences of Unc. clones and *Alcanivorax* sp S9-11. The clone sequences showed considerable evolutionary divergence from the sequences of *Oleibacter marinus*, *P. aeruginosa*, *Novosphingobium* and *Shewanella* included in the tree.

Isolation and molecular identification of HC degrading bacteria

A total of seventy-one isolates were obtained after one-month incubation from all oil-supplemented microcosms except microcosm N1 which gave no isolates under any of the growth conditions. Fifty isolates were obtained on Marine Agar in aerobic and anaerobic conditions, nineteen isolates grew aerobically in mineral medium with oil, while no growth was observed in mineral medium supplemented with oil under anaerobic conditions. A total of thirty-seven morphologically different colonies, considering all conditions, was selected for sequencing of PCR-amplified 16S rRNA gene. The results of phylogenetic identification are shown in Table 7. The isolates were affiliated to Proteobacteria and Firmicutes. Among Proteobacteria sequences assigned to known obligate and/or generalist aliphatic and PAHs-degrading Gammaproteobacteria (*Halomonas, Marinobacter, Pseudomonas, Pseudoalteromonas*) also known as biosurfactant producers, one phylotype, isolated from site S5 both on Marine Agar and crude oil, was affiliated to the genus *Idiomarina*, abundantly detected in oiled sediment (McGenety 2014) but poorly known for its catabolic capacity so far. Emulsification activity, bacterial adhesion to HC and ability of crude oil degradation have recently been described for *Idiomarina* strains isolated from Mactra mussels (Bayat et al., 2016), here the ability of free living *Idiomarina* isolates to grow on HC as unique C source was confirmed. Firmicutes were isolated from microcosms N3 and S4 and affiliated to the genus *Vallitalea*. Interestingly *Vallitalea* and most of the other genera isolated in pure culture, were also detected in DGGE bands.

The Hg−HCB correlation suggests that this situation is likely the effect of resuspension and redistribution of deep sediments by dredging and naval traffic (Bellucci et al., 2012). The environmental quality of Augusta area is strictly dependent on the intense naval traffic, with consequent risk handling and resuspension of contaminated sediments.

Analysis for depth distributions of radiotracers (210Pb and 137Cs), Hg, and main sediment parameters (magnetic susceptibility, grain size, dry bulk density, mineralogy, sedimentation rate and organic carbon and nitrogen contents) was also analyzed by Bellucci et al., 2012.

357
358
359 **DISCUSSION**

360 Microbial communities and metabolic potential in sub-surface sediments are still not well
361 characterized. We investigated HC contamination, bioremediation potential, and diversity of
362 indigenous microbial populations in sub-surface sediments of a chronically contaminated Site of
363 Community Importance (SIC), the Priolo Bay, in the Augusta basin located in SE Sicily (Italy, Central
364 Mediterranean). Previous studies carried out in the Augusta coastal area have reported contamination
365 by heavy metals (Sprovieri et al., 2011), Hexachlorobenzene (HCB) (Bellucci et al., 2012), PAHs (Di
366 Leonardo et al 2014) and *n*-alkanes (Catania et al., 2015) of both seawater and superficial sediments.
367 In this study, HC contaminants in the sub-surface sediments of the Priolo Bay were found mainly
368 represented by *n*-alkanes and UCM. The unresolved complex mixtures (UCM) component of
369 petroleum generally accumulates in sediments as a result of biodegradation and weathering of oils,
370 which originate from natural and/or anthropogenic sources. Their effects on the marine ecosystem
371 has only been partially studied; they show bioaccumulation potential and toxicity (Du et al., 2012)
372 and are persistent in sediments for decades, altering the bioavailability and toxicity of other
373 contaminants and acting as a sorbent (Scarlett et al. 2007). UCM are generally considered resistant to
374 bacterial degradation and bioremediation of UCM-contaminated sites by bacteria is a challenging
375 goal (Frenzel at al., 2009). The highest levels of UCM in sub-surface sediments were detected at
376 Station N3. This site was found highly contaminated by PAHs and total HCs also in the superficial
377 sediment (Di Leonardo et al 2014; Catania et al., 2015). N3 station is the closest to the Scirocco Inlet
378 that provides a connection between the Augusta Harbour and the Priolo Bay. Here, direct
379 contamination from the Augusta Harbour is associated to indirect contamination due to dredging
380 activities in the adjacent areas (Bellucci et al., 2012, Romano et al., 2013). Moreover, Station N3 has
381 a silty sediment type (mud 62.30%) that is known to positively affect binding of pollutants (Amer et
382 al., 2015). To characterize the microbial communities and to isolate/detect the microrganisms
383 involved in HC degradation in these sub-surface sediments, cultural method and classical culture-
384 independent approaches, such as 16S-PCR-DGGE and gene cloning of 16S and *alkB* gene fragments,
385 was performed.. All methods described have been successfully employed in marine microbial ecology
386 and represent methods commonly used for the study of microbial diversity in marine ecosystems
387 (Oberbeckmann et al., 2014, Bacosa et al., 2015, Lauer et al., 2016). The combined use of these
388 methods and cultivation techniques, provides information concerning community diversity, species
389 richness, and population structure, monitoring the shift resulting from changing environmental
390 parameters (oil supplementation)

391 Bacterial communities of contaminated sub-surface sediment at Priolo Bay show lower phylogenetic
392 diversity than those of the superficial layer 0-5 cm (Catania et al., 2015), but retain biodegradation
393 capacities towards crude oil *n*-alkanes as shown by oil-supplemented microcosm experiments. This
394 ability was also confirmed by isolation of HC degraders and molecular identification of alkane
395 monooxygenase gene sequences at the most polluted site N3. Most *alkB* gene sequences retrieved in
396 sub-surface sediments at N3 are closely related to organisms detected in similar polluted sites
397 suggesting a common trend in the structuring of communities in the presence of oil. The absence of
398 genes involved in aromatic pathway degradation at Priolo Bay suggests a low degradative capacity
399 of aromatic compounds, which, in fact, were below the detection level in all sub-surface sediments
400 except N3 where PAHs were detected at low levels.

401 Bacterial communities responded to oil supplementation in microcosms with a significant reduction
402 in biodiversity and a shift in the composition that was different among samples. Bacterial shift occurs
403 when hydrocarbon input causes a selective pressure that results in a loss of diversity concomitant with
404 the enrichment of members capable of tolerate and/or degrade HC (Acosta-González & Marqués
405 2016). Most sequences in oil-enriched sub-surface sediment belong to obligate or generalist aliphatic
406 hydrocarbon degraders (*Halomonas sp., Marinobacter sp., Pseudomonas sp., Pseudoalteromonas*
407 *sp.*,) (Piubeli et al., 2014), or genera inhabiting oiled sediments (*Thalassospira sp., Idiomarina sp.*).

408 A common trend of development in the shift of bacterial communities in highly contaminated sites,
409 results in the predominance of Gammaproteobacteria, generally considered as being the best-adapted
410 strains in polluted environments (Fuentes et al 2014). The common features of almost all the stations
411 was the presence of HC-degrading Alfa- and Gammaproteobacteria together with Clostridia. Most of
412 the bacteria detected as DGGE bands were also isolated in pure culture under different conditions.
413 HC-degrading Gammaproteobacteria were isolated aerobically on crude oil from stations N2, S4, S5
414 and S6. *Marinobacter, Halomonas, Pseudomonas* identified in Priolo sub-surface sediment are
415 reported to play important role in halophilic degradation of hydrocarbons and in microbial enhanced
416 oil recovery (MEOR) mechanisms in long-term water-flooding petroleum reservoirs (Gao et al.,
417 2015) and in hypersaline petroleum produced water (Piubeli et al., 2014). Alphaproteobacteria
418 *Idiomarina* and *Thalassospira* coexist with Gammaproteobacteria in the aforementioned habitats
419 characterized by high salinity and extensive crude oil contamination (Fathepure et al 2015). Similarly,
420 *Pseudomonas stutzeri, Arcobacter sp.* and *Clostridium* sp., were detected in production waters of a
421 low-temperature biodegraded oil reservoir (Grabowski, 2005).
422 Beyond HC-degrading Gamma- and Alphaproteobacteria, the presence of Clostridia in actively
423 biodegrading microcosms suggests that general anaerobic processes may occur in subsurface
424 sediments under anaerobic conditions that could be uncoupled with HC degradation (Bargiela et al.,
425 2015) or participate in methanogenic HC-degrading consortia (Piubeli et al., 2014). Some Clostridia
426 have also been proposed as HC degraders. *Vallitalea* in particular, an obligate anaerobic fermentative
427 heterotroph, here detected by DGGE and isolated from stations N2 N3 and S4, S5 might be fed by
428 small organic molecules including short chain HC (Postec et al., 2015). Clostridia at station S5, are
429 represented by the genera *Clostridium* and *Natraenovirga*. The closest sequence is *Natranaerovirga*
430 *pectinivora* strain AP3, recently proposed as *Abyssivirga alkaniphila*, a strictly anaerobic, mesophilic,
431 syntrophic, alkane-degrading strain, isolated from a biofilm sampled from a black smoker chimney
432 at the Loki's Castle vent field (Schouw et al., 2016).
433 The microbial assemblage of S5 sub-surface sediment was the only one characterized by the presence
434 of Epsilonbacteria together with fermenting and HC-degrading Clostridia, and aerobic and anaerobic
435 HC-degrading Gammaproteobacteria. Epsilonproteobacteria dominate different habitats and are
436 considered significant members of oil field communities in oil reservoirs (Grabowski 2005; Hubert
437 at al., 2012). There is increasing data on the presence and dominance of sulfide-oxidizing denitrifiers
438 belonging to the Epsilonproteobacteria clustering with *Arcobacter* group in oil-field waters.
439 *Arcobacter*, in particular, is suggested to have a role in syntrophic anaerobic degradation of HC
440 (Hubert, 2012). Some *Arcobacter* isolates, that have been physiologically characterized, are capable
441 of iron or manganese reduction and can grow under microaerophilic conditions (Hubert et al., 2012).
442 The whole assemblage inhabiting S5 sediment is somewhat similar to oil reservoir communities
443 where HC degradation is suggested to occur through syntrophic associations involving acetogenic
444 bacteria (*Arcobacter* sp.), which metabolize hydrocarbons to acetate and H_2, and both acetate- and
445 H_2/CO_2-utilizing methanogens (Grabowski et al., 2005). Although we did not detect methanogenic
446 Archaea in Priolo sediments, the dominance of *Arcobacter* may be explained by their ability to use
447 organic sulfur compost in crude oil as an electron donor and energy source (Hubert et al., 2012).
448 According to this model, the degradation of HC in the absence of oxygen or in microaerophilic
449 conditions could occur for co-metabolism most likely coupled with nitrate, iron or sulfate reduction,
450 rather than methanogenesis (Gieg et al., 2014).
451 Archaeal communities are generally poorly studied in oil-polluted environments (Stauffert ae al.,
452 2014). A low diversity was detected in all samples and Archaea community profiles do not shift
453 following exposure to oil in mesocosms; probably the presence of oil has negative effect only on the
454 abundance of Archaea rather than diversity when it is already low (Joshi et al., 2014). Archaea in the
455 Priolo Bay sediment are mainly represented by Crenarchaeota and the newly recognized phylum
456 Thaumarchaeota. Although this phylum is often associated with ammonia oxidation, Thaumarchaeota
457 were also detected in soil contaminated with petroleum HC, in oil refinery wastewater treatment
458 plants and other polluted matrices (Gao et al., 2015; Grabowski et al., 2005). It has recently been

hypothesized that they could not be obligate autotrophic ammonia oxidizers but they might actually be able to grow heterotrophically using crude oil-derived compounds (Mikkonen et al., 2014).
In conclusion, chemical analysis, community fingerprinting, clone libraries and cultural methods, described the sub-surface sediment (5-10 cm) of a chronically polluted Mediterranean SIC as highly divergent from the 0-5 cm above level, highly contaminated by n-alkanes and unresolved complex mixtures HC (UCMs) and hosting a microbial community characterized by oil HC biodegradation potential.
Although the 5-10 cm layer cannot be considered deep but still only a few studies have investigated this sediment layer. (Tapilatu et al., 2010; Dell'Anno et al., 2012; Yakimov et al., 2005). Nevertheless the 5-10 cm microbial communities of Prilo Bay, surprisingly, seem to change a lot in respect to the superficial layer even in a limited depth immediately below the surface. In the deeper layers we can expect other features that are of course worth to be further investigated. The composition of the microbial community is divergent from that above and similar to that of subsurface petroleum reservoirs and produced water where anaerobic or microaerophilic syntrophic HC metabolism has been proposed.
Both sub-surface contaminated sediments and produced waters from oil rigs share contamination with UCM and common features of the microbial assemblages hosted in such environments, suggesting that these communities could have a role in the biodegradation of the UCM fraction that is to be elucidated.
The results obtained allowed to analyze the microbial communities of sub-superficial sediment and compare the results with those obtained previously on microbial communities of seawater and superficial sediment of the same station in Priolo Bay (Catania et 2015). The results obtained were largely unexpected in terms of differences with the surface layer of sediment and also among the different stations of Prilo Bay. Considering the very thin layer of sediment explored, novel molecular approach, as well as the NGS sequencing, could be used to characterize the whole microbial communities that inhabit the sub-superficial sediments of Priolo bay in a future work.

Abbreviations

UCM: Unresolved Complex Mixtures
HC: Hydrocarbon
PAHs: polycyclic aromatic hydrocarbon
SIC: Site of Community Importance

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REFERENCES

Acosta-González, A., & Marqués, S. (2016). Bacterial diversity in oil-polluted marine coastal sediments. *Current opinion in biotechnology*, *38*, 24-32.

Amer, R. A., Mapelli, F., El Gendi, H. M., Barbato, M., Goda, D. A., Corsini, A., ... & Abdel-Fattah, Y. R. (2015). Bacterial diversity and bioremediation potential of the highly contaminated marine sediments at El-Max district (Egypt, Mediterranean Sea). BioMed research international, 2015.
Bacosa, H. P., & Inoue, C. (2015). Polycyclic aromatic hydrocarbons (PAHs) biodegradation potential and diversity of microbial consortia enriched from tsunami sediments in Miyagi, Japan. Journal of Hazardous Materials, 283, 689–697.

509 Bayat, Z., Hassanshahian, M., & Hesni, M. A. (2016). Study the symbiotic crude oil-degrading
510 bacteria in the mussel Mactra stultorum collected from the Persian Gulf. Marine pollution bulletin,
511 105(1), 120-124
512 Bargiela, R., Herbst, F. A., Martínez-Martínez, M., Seifert, J., Rojo, D., Cappello, S., ... & Barbas, C.
513 (2015). Metaproteomics and metabolomics analyses of chronically petroleum-polluted sites reveal
514 the importance of general anaerobic processes uncoupled with degradation. *Proteomics*, *15*(20),
515 3508-3520.
516 Bellucci, L. G., Giuliani, S., Romano, S., Albertazzi, S., Mugnai, C., & Frignani, M. (2012). An
517 integrated approach to the assessment of pollutant delivery chronologies to impacted areas: Hg in the
518 Augusta Bay (Italy). *Environmental science & technology*, *46*(4), 2040-2046.
519 Catania, V., Santisi, S., Signa, G., Vizzini, S., Mazzola, A., Cappello, S., ... & Quatrini, P. (2015).
520 Intrinsic bioremediation potential of a chronically polluted marine coastal area. *Marine Pollution*
521 *Bulletin*, 99(1), 138-149.
522 Catania, V., Sarà, G., Settanni, L., & Quatrini, P. (2016). Bacterial communities in sediment of a
523 Mediterranean Marine Protected Area. *Can J Microbiol. doi: 10.1139/cjm-2016-0406.*
524 Cole, J. R., Wang, Q., Fish, J. A., Chai, B., McGarrell, D. M., Sun, Y., ... & Tiedje, J. M. (2013).
525 Ribosomal Database Project: data and tools for high throughput rRNA analysis. *Nucleic acids*
526 *research*, gkt1244.
527 Daffonchio, D., Ferrer, M., Mapelli, F., Cherif, A., Lafraya, Á., Malkawi, H. I., ... & Kalogerakis, N.
528 (2013). Bioremediation of Southern Mediterranean oil polluted sites comes of age. *New*
529 *biotechnology*, *30*(6), 743-748.
530 Dell'Anno, A., Beolchini, F., Rocchetti, L., Luna, G. M., & Danovaro, R. (2012). High bacterial
531 biodiversity increases degradation performance of hydrocarbons during bioremediation of
532 contaminated harbor marine sediments. Environmental pollution, 167, 85-92.
533 Denaro, R., D'auria, G., Di Marco, G., Genovese, M., Troussellier, M., Yakimov, M. M., & Giuliano,
534 L. (2005). Assessing terminal restriction fragment length polymorphism suitability for the description
535 of bacterial community structure and dynamics in hydrocarbon-polluted marine environments.
536 Environmental Microbiology, 7(1), 78-87.
537 Di Leonardo, R., Mazzola, A., Tramati, C. D., Vaccaro, A., & Vizzini, S. (2014). Highly
538 contaminated areas as sources of pollution for adjoining ecosystems: the case of Augusta Bay (Central
539 Mediterranean). Marine pollution bulletin, 89(1), 417-426.
540 Di Leonardo, Mazzola A., Cundy A.B., Tramati C.D., Vizzini S. (2017) Trace element storage
541 capacity of sediments in dead posidonia oceanica mat from a chronically contaminated marine
542 ecosystem. Environmental Toxicology and Chemistry, Vol. 36, No. 1, pp. 49–58.
543 Du, J., Mehler, W. T., Lydy, M. J., & You, J. (2012). Toxicity of sediment-associated unresolved
544 complex mixture and its impact on bioavailability of polycyclic aromatic hydrocarbons. Journal of
545 hazardous materials, 203, 169-175.
546 Duran, R., Cuny, P., Bonin, P., & Cravo-Laureau, C. (2015). Microbial ecology of hydrocarbon-
547 polluted coastal sediments. Environ Sci Pollut Res 22(20), 15195–15199
548 Fathepure, B. Z. 2015,. Recent studies in microbial degradation of petroleum hydrocarbons in
549 hypersaline environments. In The Proceedings from Halophiles 2013, the International Congress on
550 Halophilic Microorganisms (Vol. 5, No. 12, p. 35). Frontiers Media SA.
551 Frenzel, M., James, P., Burton, S. K., Rowland, S. J., & Lappin-Scott, H. M. (2009). Towards
552 bioremediation of toxic unresolved complex mixtures of hydrocarbons: identification of bacteria
553 capable of rapid degradation of alkyltetralins. Journal of Soils and Sediments, 9(2), 129-136.
554 Fuentes, S., Méndez, V., Aguila, P., & Seeger, M. (2014). Bioremediation of petroleum
555 hydrocarbons: catabolic genes, microbial communities, and applications. Applied microbiology and
556 biotechnology, 98(11), 4781-4794.
557 Gagliano, A. L., Tagliavia, M., D'alessandro, W., Franzetti, A., Parello, F., & Quatrini, P. (2016). So
558 close, so different: geothermal flux shapes divergent soil microbial communities at neighboring sites.
559 *Geobiology*, *14*(2), 150-162.

560 Gao, P., Tian, H., Li, G., Sun, H., & Ma, T. (2015). Microbial diversity and abundance in the Xinjiang
561 Luliang long-term water-flooding petroleum reservoir. MicrobiologyOpen, 4(2), 332-342.
562 Genovese, M., Crisafi, F., Denaro, R., Cappello, S., Russo, D., Calogero, R., ... & Genovese, L.
563 (2014). Effective bioremediation strategy for rapid in situ clean up of anoxic marine sediments in
564 mesocosm oil spill simulation. Front. Microbiol. 14 (5), 162.
565 Gieg, L. M., Fowler, S. J., & Berdugo-Clavijo, C. (2014). Syntrophic biodegradation of hydrocarbon
566 contaminants. Current opinion in biotechnology, 27, 21-29.
567 Grabowski, A., Nercessian, O., Fayolle, F., Blanchet, D., & Jeanthon, C. (2005). Microbial diversity
568 in production waters of a low-temperature biodegraded oil reservoir. *FEMS Microbiology Ecology*,
569 *54*(3), 427-443.
570 Hammer, Ø., Harper, D. A. T., & Ryan, P. D. (2001). PAST-palaeontological statistics, ver. 1.89.
571 *Palaeontologia electronica*, *4*(9).
572 Hazen, T. C., Prince, R. C., & Mahmoudi, N. (2016). Marine oil biodegradation.
573 Hendrickx, B., Junca, H., Vosahlova, J., Lindner, A., Rüegg, I., Bucheli-Witschel, M., ... &
574 Brennerova, M. (2006). Alternative primer sets for PCR detection of genotypes involved in bacterial
575 aerobic BTEX degradation: distribution of the genes in BTEX degrading isolates and in subsurface
576 soils of a BTEX contaminated industrial site. *Journal of Microbiological Methods*, *64*(2), 250-265.
577 Hubert, C. R. J., Oldenburg, T. B. P., Fustic, M., Gray, N. D., Larter, S. R., Penn, K., … Head, I. M.
578 (2012). Massive dominance of Epsilonproteobacteria in formation waters from a Canadian oil sands
579 reservoir containing severely biodegraded oil. Environmental Microbiology, 14(2), 387–404.
580 Istituto Centrale per la Ricerca scientifica e tecnologica Applicata al Mare (ICRAM), 2008. Progetto
581 preliminare di bonifica dei fondali della rada di Augusta nel sito di interesse nazionale di Priolo—
582 Elaborazione definitiva, BoI-Pr-SI-PR-Rada di Augusta-03.22, p. 182.
583 Istituto Superiore per la Protezione e la Ricerca Ambientale (ISPRA), 2010. Elaborazione e
584 Valutazione dei Risultati della Caratterizzazione ai fini della individuazione degli opportuni
585 interventi di messa in sicurezza e bonifica del Litorale di Priolo dalla diga foranea della Rada di
586 Augusta al Porto Grande di Siracusa. Sito di Interesse Nazionale di Priolo. CII-El-SI-PR-Area
587 2_01.05, p. 87.
588 Jeanbille M, Gury J, Duran R, Tronczynski J, Ghiglione J-F, Agogué H, Saïd OB, Taïb N, Debroas
589 D, Garnier C and Auguet J-C (2016) Chronic Polyaromatic Hydrocarbon (PAH) Contamination Is a
590 Marginal Driver for Community Diversity and Prokaryotic Predicted Functioning in Coastal
591 Sediments.Front. Microbiol. 7:1303.
592 Joshi, M. N., Dhebar, S. V., Dhebar, S. V., Bhargava, P., Pandit, A., Patel, R. P., ... & Bagatharia, S.
593 B. (2014). Metagenomics of petroleum muck: revealing microbial diversity and depicting microbial
594 syntrophy. Archives of microbiology, 196(8), 531-544.
595 Kloos, K., Munch, J. C., & Schloter, M. (2006). A new method for the detection of alkane-
596 monooxygenase homologous genes (alkB) in soils based on PCR-hybridization. *Journal of*
597 *Microbiological Methods*, *66*(3), 486-496.
598 Korlević, M., Zucko, J., Dragić, M. N., Blažina, M., Pustijanac, E., Zeljko, T. V., ... & Long, P. F.
599 (2015). Bacterial diversity of polluted surface sediments in the northern Adriatic Sea. *Systematic and*
600 *applied microbiology*, *38*(3), 189-197.
601 Lauer et al., 2016 Phylogenetic Characterization of Marine Benthic Archaea in Organic-Poor
602 Sediments of the Eastern Equatorial Pacific Ocean (ODP Site 1225). Microorganisms, 4(3), 32.
603 McGenity, T. J. (2014). Hydrocarbon biodegradation in intertidal wetland sediments. Current opinion
604 in biotechnology, 27, 46-54.
605 McGenity, T. J., Folwell, B. D., McKew, B. A., & Sanni, G. O. (2012). Marine crude-oil
606 biodegradation: a central role for interspecies interactions. Aquatic Biosystems, 8(1), 10.
607 Mikkonen, A., Santalahti, M., Lappi, K., Pulkkinen, A.-M., Montonen, L. and Suominen, L. (2014),
608 Bacterial and archaeal communities in long-term contaminated surface and subsurface soil evaluated
609 through co-extracted RNA and DNA. FEMS Microbiol Ecol, 90: 103–114. doi:10.1111/1574-
610 6941.12376.

611 Miralles, G., Nérini, D., Manté, C., Acquaviva, M., Doumenq, P., Michotey, V., ... & Cuny, P. (2007).
612 Effects of spilled oil on bacterial communities of Mediterranean coastal anoxic sediments chronically
613 subjected to oil hydrocarbon contamination. Microbial ecology, 54(4), 646-661.
614 Muyzer, G., De Waal, E. C., & Uitterlinden, A. G. (1993). Profiling of complex microbial populations
615 by denaturing gradient gel electrophoresis analysis of polymerase chain reaction-amplified genes
616 coding for 16S rRNA. Applied and environmental microbiology, 59(3), 695-700.
617 Nie, Y., Tang, Y. Q., Li, Y., Chi, C. Q., Cai, M., & Wu, X. L. (2012). The genome sequence of
618 Polymorphum gilvum SL003B-26A1 T reveals its genetic basis for crude oil degradation and
619 adaptation to the saline soil. PLoS one, 7(2), e31261.
620 Oberbeckmann, S., Loeder, M. G.J., Gerdts, G. and Osborn, A. M. (2014), Spatial and seasonal
621 variation in diversity and structure of microbial biofilms on marine plastics in Northern European
622 waters. FEMS Microbiol Ecol, 90: 478–492.
623 Paisse, S., Duran, R., Coulon, F., & Goñi-Urriza, M. (2011). Are alkane hydroxylase genes (alkB)
624 relevant to assess petroleum bioremediation processes in chronically polluted coastal sediments?.
625 *Applied microbiology and biotechnology*, *92*(4), 835-844.
626 Piubeli, F., Grossman, M. J., Fantinatti-Garboggini, F., & Durrant, L. R. (2014). Phylogenetic
627 analysis of the microbial community in hypersaline petroleum produced water from the Campos
628 Basin. Environmental Science and Pollution Research, 21(20), 12006-12016.
629 Postec, A., Quéméneur, M., Méline Bes, N. M., Benaïssa, F., Payri, C., Pelletier, B., ... & Pisapia, C.
630 (2015). Microbial diversity in a submarine carbonate edifice from the serpentinizing hydrothermal
631 system of the Prony Bay (New Caledonia) over a 6-year period. Frontiers in microbiology, 6.
632 Quatrini, P., Scaglione, G., De Pasquale, C., Riela, S. E. R. E. N. A., & Puglia, A. M. (2008). Isolation
633 of Gram-positive n-alkane degraders from a hydrocarbon-contaminated Mediterranean shoreline.
634 Journal of applied microbiology, 104(1), 251-259.
635 Romano, E., Bergamin, L., Magno, M. C., & Ausili, A. (2013). Sediment characterization of the
636 highly impacted Augusta harbour (Sicily, Italy): modern benthic foraminifera in relation to grain-size
637 and sediment geochemistry. Environmental Science: Processes & Impacts, 15(5), 930-946.
638 Scarlett A., T.S. Galloway, S.J. Rowland, (2007) Chronic toxicity of unresolved complex mixtures
639 (UCM) of hydrocarbons in marine sediments, J. Soils Sediments 7 (4), 200-206
640 Schouw A, Leiknes Eide T, Stokke R, Pedersen R, Steen I, Bødtker G., (2016) *Abyssivirga*
641 alkaniphila gen. nov., sp. nov., an alkane-degrading, anaerobic bacterium from a deep-sea
642 hydrothermal vent system, and emended descriptions of Natranaerovirga pectinivora and
643 *Natranaerovirga hydrolytica*, Int J Syst Evol Microbiol 66(4):1724-1734
644 doi:10.1099/ijsem.0.000934
645 Signa, G., Mazzola, A., Di Leonardo, R., & Vizzini, S. (2017). Element-specific behaviour and
646 sediment properties modulate transfer and bioaccumulation of trace elements in a highly-
647 contaminated area (Augusta Bay, Central Mediterranean Sea). *Chemosphere*, *187*, 230-239.
648 Sprovieri, M., Oliveri, E., Di Leonardo, R., Romano, E., Ausili, A., Gabellini, M., ... & Budillon, F.
649 (2011). The key role played by the Augusta basin (southern Italy) in the mercury contamination of
650 the Mediterranean Sea. Journal of Environmental Monitoring, 13(6), 1753-1760.
651 Stauffert, M., Duran, R., Gassie, C., & Cravo-Laureau, C. (2014). Response of archaeal communities
652 to oil spill in bioturbated mudflat sediments. *Microbial ecology*, *67*(1), 108-119.
653 Varjani, S. J. (2017). Microbial degradation of petroleum hydrocarbons. Bioresource Technology,
654 223, 277-286.
655 Tapilatu, Y., Acquaviva, M., Guigue, C., Miralles, G., Bertrand, J. C., & Cuny, P. (2010). Isolation
656 of alkane-degrading bacteria from deep-sea Mediterranean sediments. Letters in applied
657 microbiology, 50(2), 234-236.
658 Wasmund, K., Burns, K. A., Kurtböke, D. I., & Bourne, D. G. (2009). Novel alkane hydroxylase gene
659 (alkB) diversity in sediments associated with hydrocarbon seeps in the Timor Sea, Australia. *Applied*
660 *and environmental microbiology*, *75*(23), 7391-7398.

661 Xue, J., Yu, Y., Bai, Y., Wang, L., & Wu, Y. (2015). Marine oil-degrading microorganisms and
662 biodegradation process of petroleum hydrocarbon in marine environments: a review. Current
663 microbiology, 71(2), 220-228.
664 Yakimov, M. M., Denaro, R., Genovese, M., Cappello, S., D'Auria, G., Chernikova, T. N., ... &
665 Giluliano, L. (2005). Natural microbial diversity in superficial sediments of Milazzo Harbor (Sicily)
666 and community successions during microcosm enrichment with various hydrocarbons.
667 Environmental microbiology, 7(9), 1426-1441.
668

669

670 **Table 1** pH and grain size distribution of sub-surface sediments.

671

Sediment sample		Gravel %		Sand %		Mud %		Sediment type
	pH	mean	sd	mean	sd	mean	sd	
N1	8.35	0.15	0.17	91.73	1.27	8.12	1.44	sand
N2	8.13	0.09	0.02	97.33	0.63	2.58	0.65	sand
N3	8.06	0.58	0.22	37.12	10.16	62.30	10.38	sandy silt
S4	8.33	12.09	1.35	75.88	2.28	12.03	0.93	gravelly sand
S5	8.01	4.43	3.13	81.87	1.63	13.70	1.49	sand
S6	8.25	1.86	1.70	96.98	2.64	1.16	0.94	sand

672

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674

675 **Table 2** Total hydrocarbons (HCs), polycyclic aromatic hydrocarbon (PAH), *n*-alkanes and 676 Unresolved complex mixtures (UCMs) (mg Kg^{-1} dry sediment) detected in sub-surface sediments of 677 the Priolo Bay.

678

	N1	N2	N3	S4	S5	S6
Total HCs	7,27	144,40	216,64	23,76	140,90	119,66
PAHs	0	0	1,57	0	0	0
n-alkanes	2.57	20.36	50.50	2.83	7.51	6.55
UCMs	4.70	124.0	164.6	20.9	133.4	113.1

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682 **Table 3** Degradative capability of microbial community in microcosms performed by inoculum of
683 sub-surface sediment in mineral medium ONR7a supplementing with 1% of crude oil. The GC- FID
684 analysis was conducted after one month of incubation in anaerobic condition. *n*-alkanes Short-chain:
685 C_{10}, Σ Medium-chain: range between C_{11}-C_{16}; Long chain range between C_{17}-C_{28}.
686

n-alkanes (% degraded)		Northern transect			Southern transect		
		N1	N2	N3	S4	S5	S6
Short-chain	C_{10}	100	100	98	100	98	99
Σ Medium-chain	C_{11}- C_{16}	97	100	86	99	91	97
Σ Long-chain	C_{17}-C_{28}	92	98	73	96	79	91

687

688 **Table 4** Bacterial and Archaeal diversity indices based on DGGE profiles of sub-surface sediment immediately after sampling and after supplementing whit oil
689 (microcosms).

690

		Sub-surface sediment						Microcosm					
		N1	N2	N3	S4	S5	S6	N1	N2	N3	S4	S5	S6
Bacteria	Taxa_S	18	20	28	35	37	32	17	21	20	21	22	12
	Shannon_H	2.890	2.996	3.332	3.555	3.611	3.466	2.833	3.045	2.996	3.045	3.091	2.485
	Chao-1	171	210	406	630	703	528	153	231	210	231	253	78
Archaea	Taxa_S	15	9	13	15	18	6	6	15	15	20	14	6
	Shannon_H	2,708	2,197	2,565	2,708	2,89	1,792	1,792	2,708	2,708	2,996	2,639	1,792
	Chao-1	120	45	91	120	171	21	21	120	120	210	105	21

691

692 **Table 5** Bacterial sequences detected by PCR-DGGE of microcosms set up by inoculum of sub-
693 surface sediment of Priolo Bay in mineral medium and crude oil.
694
695

			Phylogeny (RDPII database)		Blast 16S (megablast)		
Site	DGGE band°	Lenght (bp)	Phylum	Class	Closet sequence match	Id. (%)	Accession number
N1	1	150	Proteobacteria	Gammaproteobacteria	*Vibrio sp.*	99	NR_119058.1
	2	156			*Vibrio sp.*	92	NR_113182.1
	3	150			*Vibrio sp.*	97	NR_122060.1
	4	156			*Vibrio sp.*	99	NR_122060.1
N2	5	90	Firmicutes	Clostridia	*Clostridium sp.*	97	NR_125713.1
	6	101	Proteobacteria	Alphaproteobacteria	*Thalassospira sp.*	100	NR_136875.1
	7	100		Gammaproteobacteria	*Colwellia sp.*	96	NR_136835.1
	8	173			*Pseudoalteromonas sp.*	99	NR_113220.1
	9	151			*Pseudoalteromonas sp.*	92	NR_044837.2
N3	10	130	Firmicutes	Clostridia	*Clostridium sp.*	99	NR_104889.1
	11	135			*Clostridium sp.*	94	NR_104889.1
	12	118			*Peptoniphilus sp.*	92	NR_108213.1
	13	158	Proteobacteria	Gammaproteobacteria	*Amphritea sp.*	99	NR_042455.1
S4	14	130	Firmicutes	Clostridia	*Acetivibrio sp.*	93	NR_025917.1
	15	123			*Sporobacterium sp.*	98	NR_024967.1
	16	110			*Vallitalea sp.*	99	NR_125677.1
	17	134			*Clostridium sp.*	90	NR_029164.1
S5	18	130	Firmicutes	Clostridia	*Clostridium sp.*	97	NR_104889.1
	19	137			*Clostridium sp.*	90	NR_104889.1
	20	122			*Natranaerovirga sp.*	91	NR_108636.1
	21	127	Proteobacteria	Epsilonproteobacteria	*Arcobacter sp.*	96	NR_136420.1
	22	166		Gammaproteobacteria	*Idiomarina sp.*	94	NR_074933.1
	23	117			*Marinobacter sp.*	94	NR_025116.1
	24	89			*Pseudomonas sp.*	94	NR_074829.1
S6	25	192	Proteobacteria	Alphaproteobacteria	*Polymorphum sp.*	97	NR_074240.1
	26	143			*Roseibium sp.*	97	NR_025885.1
	27	95		Gammaproteobacteria	*Halomonas sp.*	95	NR_125612.1

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698 **Table 6** Archaeal sequences detected by PCR-DGGE in sub-surface sediment immediately after sampling (site N1) and in microcosms (site N2-S6).

699

			Phylogeny (RDPII database)		Blast nr (megablast)		
Site	DGGE band°	Lenght (bp)	Phylum	Class	Closet sequence match	Id. (%)	Accession number
N1	1	140	Crenarchaeota	Thermoprotei	Uncultured *Sulfolobales* archaeon A980	91	LN896654.1
	2	142			Uncultured archaeon R124.5-9	89	JQ750558.1
	3	154			Uncultured *Desulfurococcales* Kam37_lr270106f07	92	JF305865.1
N2	4	163	Thaumarchaeote	Nitrososphaeria	Uncultured thaumarchaeote OTU 140	91	LT625952.1
N3	5	154	Thaumarchaeote	Nitrososphaeria	Uncultured archaeon QL-15-OTU21	99	KU297806.1
S4	6	161	Crenarchaeota	Thermoprotei	Uncultured crenarchaeote 01F7_S56	98	HG931461.1
	7	72	Thaumarchaeote	Nitrososphaeria	Uncultured archaeon OTU253	96	KX077511.1
S5	8	162	Thaumarchaeote	Nitrososphaeria	Uncultured archaeon AS36	93	KT225016.1
S6	9	143	Crenarchaeota	Thermoprotei	Uncultured Thermoprotei archaeon OTU_121	98	LT624873.1

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701 **Table 7** Bacterial strains isolated from microcosms in different growth conditions (+/- presence or absence of colonies on solid medium); Air: aerobic

702 conditions; Ana: anaerobic conditions in jar.

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					Phylogeny (RDPII database)		Blast 16S (megablast)	
Sediment Sample	Growth conditions				Phylum	Class	Closet sequence match	Accession no. and similarity (%)
	Marine Agar		Onr7a + Crude oil					
	Air	Ana	Air	Ana				
N1	-	-	-	-				
N2	-	-	+	-	Proteobacteria	Gammaproteobacteria	*Pseudoalteromonas shioyasakiensis*	NR_125458.1(99)
	-	+	+	-			*Marinobacter hydrocarbonoclasticus*	NR_027551.1(99)
N3	-	+	-	-	Firmicutes	Clostridia	*Vallitalea guaymasensis*	NR_117645.1(99)
S4	-	+	-	-	Firmicutes	Clostridia	*Vallitalea guaymasensis*	NR_117645.1(99)
S5	+	+	+	-	Proteobacteria	Gammaproteobacteria	*Pseudomonas stutzeri*	NR_074829.1(99)
	-	-	+	-			*Marinobacter hydrocarbonoclasticus*	NR_027551.1(99)
	+	-	+	-			*Idiomarina baltica*	NR_027560.1 (99)
S6	-	-	+	-	Proteobacteria	Gammaproteobacteria	*Halomonas*	NR_027219.1(99)
	-	+	-	-			*Marinobacter hydrocarbonoclasticus*	NR_027551.1 (99)

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705

706 **Figure legends**

707 **Figure 1** Location of the study area and sampling stations in the Priolo Bay. The white dashed line
708 shows the bathymetry.

709 **Figure 2** Bacterial diversity of sub-surface sediment of Priolo Bay. (A) Cluster analysis dendrogram;
710 (B) DGGE profiles of PCR-amplified 16S rDNA V3 region from sediment and from sediment
711 enriched on oil. Numbers indicate the bands that were sequenced. See Table 5 for identification.

712 **Figure 3** Archaeal diversity of sediment of Priolo Bay. (A) Cluster analysis dendrogram; (B) DGGE
713 profiles of PCR-amplified 16S rDNA V3 region from sediment and from sediment enriched on oil.
714 Numbers indicate the band that were amplified and sequenced (Table 4)

715 **Figure 4** Phylogenetic tree based on deduced AlkB amino acid sequences retrieved from S5 sediment
716 *alkB* clone library and reference sequences from cultured bacteria degraders and sequences obtained
717 from other *alkB* clone library. Sequences from the Priolo Bay *alkB* clone library are in bold type.

718 **Figure S1** Priolo Bay sub- surface sediment samples.