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Complete List of Authors:	Catania, Valentina; Universita degli Studi di Palermo Dipartimento di Scienze e Tecnologie Biologiche Chimiche e Farmaceutiche, Viale delle Scienze, bldg 16 Palermo, PA, IT 90128 09123897320 Sarà, Gianluca; Universita degli Studi di Palermo Dipartimento di Scienze della Terra e del Mare, Viale delle Scienze, bldg 16 Palermo, PA, IT 90128 Settanni, Luca; Universita degli Studi di Palermo Dipartimento di Scienze Agrarie e Forestali, Viale delle Scienze, bldg 5 Palermo, PA, IT 90128 Quatrini, Paola; Universita degli Studi di Palermo Dipartimento di Scienze e Tecnologie Biologiche Chimiche e Farmaceutiche, ; University of Palermo Dpt. STEBICEF
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Bacterial communities in sediment of a Mediterranean Marine Protected Area

Valentina Catania^a, Gianluca Sarà^b, Luca Settanni^c and Paola Quatrini^{a*}

^aDipartimento di Scienze e Tecnologie Biologiche, Chimiche e Farmaceutiche (STEBICEF), Università degli Studi di Palermo, Viale delle Scienze Ed.16 - 90128 Palermo - ITALIA

^bDipartimento di Scienze della Terra e del Mare (DISTEM), Università degli Studi di Palermo, Viale delle Scienze Ed.16 - 90128 Palermo – ITALIA

^cDipartimento di Scienze Agrarie e Forestali (SAF), Università degli Studi di Palermo, Viale delle Scienze Ed.4 - 90128 Palermo – ITALIA

*corresponding author. Paola Quatrini *phone.:* ++39 091 23897320 *E-mail address:* paola.quatrini@unipa.it

Abstract

Biodiversity is crucial in preservation of ecosystems, and bacterial communities play an indispensable role for the functioning of marine ecosystems. The Mediterranean Marine Protected Area (MPA) “Capo Gallo-Isola delle Femmine” was instituted to preserve marine biodiversity. The bacterial diversity associated to MPA sediment was compared with that from sediment of an adjacent harbor exposed to intense nautical traffic. MPA sediment showed higher diversity in respect to the impacted site. A 16S rDNA clone library of the MPA sediment allowed the identification of 7 phyla, Proteobacteria (78%), Firmicutes (11%), Acidobacteria (3%), Actinobacteria (3%), Bacteroidetes (2%), Planctomycetes (2%) and Cyanobacteria (1%). The analysis of the hydrocarbon-degrading bacteria was performed using enrichment cultures. Most of the MPA isolates were affiliated to Gram-positive G+C rich bacteria while the majority of taxa in the harbor clustered with Alpha and Gamma Proteobacteria; No Gram-positive HC-degraders were isolated from the harbor sediment.

Our results show that protection has probably an influence on bacterial diversity, and suggest the importance of monitoring the effects of protection also at microbial level. This study creates a baseline of data that can be used to assess changes in bacterial communities associated with a Mediterranean MPA, over time.

Resumée

La biodiversité est crucial dans la préservation des écosystèmes et les communautés bactériennes jouent un rôle indispensable pour le fonctionnement des écosystèmes marins. La zone de Protection Marine de la Méditerranée (ZPM) "Capo Gallo-Isola delle Femmine" a été instituée pour préserver la biodiversité marine. La diversité bactérienne associée aux sédiments de la ZPM a été comparée à celle des sédiments d'un port adjacent exposé à une circulation nautique intense. Les sédiments de la ZPM ont montré une plus grande diversité par rapport au port adjacent. Une collection de clones ADN ribosomal 16S des sédiments de la ZPM a permis d'identifier 7 Phyla, Proteobacteria (78%), Firmicutes (11%), Acidobacteria (3%), Actinobactéries (3%), Bacteroidetes (2%), Planctomycètes (2%) et de cyanobactéries (1%). L'analyse des bactéries dégradant les hydrocarbures a été effectuée en utilisant des cultures d'enrichissement. La plupart des isolats de la ZPM étaient affiliés aux Gram positifs riches en G + C tandis que la majorité des taxons dans le port sont affiliés avec Alpha et Gamma Proteobacteria; Aucun Gram positif HC-dégradeurs ont été isolés à partir des sédiments du port.

Nos résultats montrent que la protection a probablement une influence sur la diversité bactérienne, et suggèrent l'importance de surveiller les effets de la protection au niveau microbique. Cette étude crée une base de données qui peut être utilisée pour évaluer les changements dans les communautés microbiennes, associée à une ZPM méditerranéenne, au fil du temps.

Keywords: Bacterial communities; Hydrocarbon degrading bacteria; Marine protected areas; *Bacteriovorax*, DGGE analysis.

Introduction

The analysis of biodiversity is mainly devoted to plants and animals while the diversity of microbes is rarely considered. The data available for Bacteria, Archaea, and Protists are somewhat limited, and microbial diversity is substantially underestimated (Kemp and Aller 2004; Pontes et al. 2007). The Mediterranean Sea is a marine biodiversity hot spot. It has been estimated that 17,000 species occur in the Mediterranean Sea and of these, at least 26% are prokaryotic (Bacteria and Archaea) and eukaryotic (Protists) marine microbes (Coll et al. 2010).

Need for protection of natural marine resources is forcing stronger conservation efforts, which are leading to an increasing number of marine protected areas (MPAs) worldwide. MPAs are essential for conservation of biodiversity by offering a protection from major anthropogenic threats such as chemical contamination, eutrophication, over-exploitation and species invasions (Fraschetti et al. 2002). The knowledge of the ecological effects of protection is still limited due to the complexity of processes and the difficulty in quantifying them (Jameson et al. 2002; La Manna et al. 2015). Microbial communities are the basis of life on earth and provide fundamental ecosystem services by catalysing biogeochemical reactions that drive global nutrient cycles and by directly interacting with the biota. Unlike plants and animals, microbial diversity is not considered under menace as bacteria are known for their redundancy, resistance and resilience (Bodelier 2011). However all these aspects have not been analysed enough because of inadequate methods to assess microbial diversity. Molecular culture-independent approaches are now available to overcome the previous limit of bacteria cultivability and knowledge of bacterial diversity has been rapidly expanding in recent decades. Phylogenetic and taxonomy diversity and functional diversity, based on functional gene/protein sequence diversity, are enhancing our ability to link microbial diversity to the functioning of microbial communities and ecosystems in the so-called Biodiversity-Ecosystem Functioning relationship (Krause et al. 2014).

Only a few studies have been carried out on microbial diversity of MPA (Yeung et al. 2010) and, to our knowledge, there are no reports on microbial diversity of sediment from MPA in the Mediterranean Sea.

The Mediterranean Marine Protected Area “Capo Gallo-Isola delle Femmine” instituted in 2002 in the gulf of Palermo, Italy.

The aim of this study was to analyse the bacterial diversity associated with sediment of the Mediterranean MPA “Capo Gallo-Isola delle Femmine” in order to evaluate the effect of protection on the microbial component of biodiversity. As one of the most threatening risks of coastal sediment is the contamination by hydrocarbons (HC), attention has been devoted to analyse the cultivable fraction of HC-degrading bacteria in order to evaluate the effect of protection on the distribution and composition of this important functional group of bacteria. Here, the effect of protection on sedimentary bacterial diversity was assessed through a comparison with an adjacent impacted area.

Materials and methods

Study area and design

We conducted this study in the Marine Protected Area (MPA) of Capo Gallo and Isola delle Femmine (Northwest Sicily, Italy) (Fig. 1). The MPA is an area of approximately 20 km² with three zones of different levels of protection: the A-zone, a no-take zone where no recreational use, including diving and fishing, is allowed and access is limited to boats for monitoring and scientific research; the B-zone, where recreational use is allowed, and the C-zone, where recreational use is allowed as well as fishing with permits from the local authorities. Recreational nautical traffic includes activities such as boat passages and mooring within buoy-fields. Superficial sediment cores for bacterial analysis were collected by scuba divers along the MPA protection gradient: 2 sites were chosen in the no-take A-zone (A1: N 38° 12' 46.2"; E 13° 14' 10.8" - depth 9 m; A2: N 38° 13' 19.1"; E 13° 18' 26.3" - depth 8m); one site was chosen in the general partially protected B-zone (N 38° 12' 25.7"; E 13° 14' 03.5" - depth 8.6 m). The impacted site was chosen within the area of an external adjacent fisherman small harbor (the C-zone; Arenella; N 38° 09' 07.4"; E 13° 22' 30.5"; depth 7 m) with a high number of fishery activities and intense nautical traffic. Due to intense boating activities, a complex mixture containing a broad spectrum of hydrocarbon is released into the harbor environment and in the

proximal areas by combustion of fossil fuels. Accordingly, sediment were highly contaminated as showed total polycyclic aromatic hydrocarbons (Σ PAHs) concentrations which allowed to classify sediment as highly contaminated (total PAH concentrations exceeding 0.500 mg kg⁻¹; Italian regulatory limits for marine sediment; G.U.R.I., D.M. 367/03, 2004) indicating the combustion as a major source for this geographic area (Di Leonardo et al. 2009). In no-take A-zone, no sedimentary contamination should be present due to local nautical traffic, No-take area is aside in respect to the rest of the MPA and as in a contiguous area of the Gulf of Palermo, Σ PAHs concentrations were under the Italian regulatory limits (Di Leonardo et al. 2009). Sediment composition is predominantly limestone with a biogenic component especially in coarse sediment. The particle size analysis shows fine sands near the coast and coarse and medium sand (up to 2 mm; Sarà pers. com.). Sediment samples were collected in triplicate from all sites (0-5 cm) using sterilized tube cores (50 ml), and immediately stored on board in fridge bags. Once brought back to the laboratory in not more than 2 hours, sedimentary samples from the same site were pooled together, immediately centrifuged at 6,000 g (4°C; 15 min) to eliminate seawater. Aliquots were used for immediate analysis or stored for future analyses (-20°C).

DNA extraction from the sediment

Total DNA was extracted from pooled sediment of each collected core (500 mg wet weight) using the FastDNA Spin Kit for Soil (MP Biomedicals, United States) according to the manufacturer's instructions, except in the last step, where the elution was repeated twice with 50µl of DNase/RNase free water. The DNA samples were analysed by electrophoresis in 1% (wt/vol) agarose gel with 1% ethidium bromide; nucleic acids were quantified using a NanoDrop ND-1000 Spectrophotometer (NanoDrop Technologies) and stored at -20 °C until further processed.

PCR- DGGE analysis

The 16S rRNA PCR-DGGE analysis of the bacterial communities was performed with the total DNA extracted from the sediment of the MPA (A1, A2 and B1 sites) and the harbor in triplicate. Primers

341f-GC and 354r (corresponding to positions 341–534 in the *Escherichia coli* 16S rDNA sequence) were used to amplify the V3 variable region of the bacterial 16S rDNA gene (Muyzer et al. 1993). PCR amplification was performed in a 50 µl reaction volume with 0.8µl Phire Hot Start II DNA Polymerase (Thermo Scientific), 1X PCR buffer, 500 nM each primer, 0.30 mM dNTP and 100-150 ng of DNA. The PCR procedure was as follows: an initial cycle of 30 s at 98°C, followed by 40 cycles of 15 s at 98°C, 10 s at 66°C, 10 s at 72°C, with a final extension of 2 min at 72°C. Amplification products were analysed by electrophoresis in 1.5% (wt/vol) agarose gels with ethidium bromide. DGGE was performed with the INGENY phor-U2 system instrument (Ingeny, Leiden, The Netherlands); PCR samples were applied directly on to 8%(wt/vol) [acrylamide/bisacrylamide (37.5:1)] in a 1X TAE buffer with a 35-70% denaturing gradient [100% denaturant corresponding to 7 M urea and 40% (v/v) deionised formamide]. Electrophoresis was performed at 60°C with 1X TAE for 17h. Gels were stained with SYBR Gold nucleic acid gel stain (Molecular Probes, Invitrogen) used according to the manufacturer's instructions, and the gel image was captured with a Gel Doc 2000 (Bio-Rad). DGGE profiles were visually analysed and the relative position of each band was recorded in a resulting 0-1 matrix that was input into the PAST software package and analysed using multivariate cluster analysis based on Jaccard dissimilarity (Hammer et al. 2001).

Construction of a 16S rRNA gene library from the MPA sediment

The total DNA extracted from the sediment of the A1 site was used as a template to amplify the bacterial 16S rRNA gene with primers fDl and rDl (Weisburg et al. 1991) as described above. The amplicon was gel-purified using the QIAquick Gel Extraction Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. 150 ng of PCR product was cloned into the pCR 2.1-TOPO vector (Invitrogen) and transformed into One shot TOP10 chemically competent cells of *Escherichia coli* using the TOPO TA Cloning kit (Version M) as recommended by the manufacturer (Invitrogen). After selection on X-gal and kanamycin LB agar, clones were archived at -80°C in 20% glycerol. Plasmid DNA was extracted from 140 candidate-positive colonies using the alkaline lysis

procedure (Sambrook et al. 1989); about 100ng of each plasmid DNA was digested using the enzymes *EcoRI* and *PstI* (Invitrogen) at 37 °C for 60 min. Restriction digests were analysed by agarose gel electrophoresis (1.5% agarose, 1× TAE buffer) and the resulting RFLP 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 the Gen Elute Plasmid Miniprep Kit (SIGMA, ALDRICH) and its insert was sequenced using fDI and rDI primers as described above. The amplified 16S rRNA gene was bidirectionally sequenced and sequences were compared to the EMBL/SwissProt/GenBank non-redundant nucleotide 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. 2014).

Sequences were submitted to the DDBJ/EMBL/Genbank database under accession numbers KT906693-712.

Isolation and identification of HC-degrading bacteria

Enrichment cultures were set up in the presence of hydrocarbons as the sole carbon source to isolate the HC-degrading bacteria. For the enrichments, 1 g of sediment from A1 site of the MPA and from the Arenella harbor, were added to 250 ml Erlenmeyer flasks containing 50 ml Bushnell Haas Mineral Salts medium (BHSM, DIFCO). A mixture of the *n*-alkanes C₁₂, C₁₅, C₁₆, (1gr l⁻¹) or Diesel oil (1gr l⁻¹) were then added to the medium (Quatrini et al. 2008). Flasks were incubated without shaking for 15 days at RT. Sub-cultures were prepared by transferring 1ml from the enrichment cultures to flasks containing 50 ml of fresh BHSM medium and the hydrocarbons. The new enrichment cultures were cultivated under the same conditions for 1 month. Serial dilutions were then spread on BHSM agar medium plates, with diesel oil or *n*-alkanes, supplied on a filter paper on the lid of the Petri dish; the concentrations were the same as described previously. After incubation at RT, individual colonies with different morphologies were selected and purified by repeated streaking on the same medium. All the apparently different colonies growing on HC-plates were isolated in pure culture and analysed by

observation under a microscope (cell shape, mobility, Gram stain reaction). All the isolates, that were able to grow on diesel oil or alkanes as sole C source and showing at least one characteristic different from the others, were further characterized by PCR analysis of the internally-transcribed spacer between the 16S and the 23S rRNA genes (16S-23S ITS).

The ribosomal 16S–23S intergenic spacer region of the isolates was amplified by colony PCR for phylogenetic analysis. PCR amplification was performed by using primers ITSF (5'-GTCGTAACAAGGTAGCCGTA-3') and ITSReub (5'-GCCAAGGCATCCACC-3'); complementary to positions 1423 and 1443 of the 16S rRNA and 38 and 23 of the 23S rRNA of *E. coli* respectively (Cardinale et al. 2004). The amplification reaction mixture consisted of 1X PCR reaction buffer, 1.5 mM MgCl₂, 0.25 mM dNTP, 0.2 µM of each primer, 1 U Taq Hot Start DNA polymerase and 1 µl of colony TE lysate. The PCR program consisted of an initial activation step of the enzyme at 95°C for 15 min, followed by a denaturation step at 95°C for 5 min, 30 cycles of denaturation at 95°C for 1 min, annealing at 55°C for 1 min and elongation at 72°C for 1.5 min, followed by a final elongation step at 72°C for 7 min. The ITS-PCR amplification products were separated on 1% agarose gel electrophoresis. Strains having overlapping ITS-PCR amplification patterns were assigned to the same operational taxonomic units (OTUs). The 16S rRNA gene of one strain representative of each OTU was amplified using the universal primers fDl and rDl (Weisburg et al. 1991) using the same conditions described above. The amplified 16S rRNA gene was sequenced and compared to the EMBL/SwissProt/GenBank non-redundant nucleotide 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. 2014).

Sequences were submitted to the DDBJ/EMBL/Genbank database under accession numbers KT933187-197.

Results

Bacterial diversity of the sediment

Sedimentary bacterial diversity of the MPA and the adjacent harbor was analysed by 16S rDNA Polymerase Chain Reaction-Denaturing Gradient Gel Electrophoresis (PCR-DGGE, Fig. 2). The three replicates, obtained pooling the sedimentary samples from the same site, showed almost identical DGGE profiles. Cluster analysis based on the DGGE profiles indicated that the bacterial communities from the MPA grouped together, while the harbor community was separated. The PCR-DGGE profiles of the MPA revealed the presence of 61, 57 and 62 putative OTUs, in the A1, A2 and B1 sites, respectively. Conversely only 41 OTUs were identified in the impacted site. The MPA bacterial communities (A1, A2 and B site) shared 18 OTUs while only 11 were common between the MPA and harbor sediment. Richness and diversity indices are shown in table 1; all the indices related to the MPA are higher than those related to the harbor and, within the MPA, the B zone hosts slightly higher diversity than no-take A zones.

Composition of the bacterial community of MPA sediment

One hundred and forty 16S rRNA clones were clustered into twenty operational taxonomic units (OTUs) by ARDRA; OTUs were affiliated to seven phyla, Proteobacteria (78%), Firmicutes (11%), Acidobacteria (3%), Actinobacteria (3%), Bacteroidetes (2%), Planctomycetes (2%) and Cyanobacteria (1%). Within the dominating phylum Proteobacteria, δ -Proteobacteria was the most abundant class (61%), followed by α -Proteobacteria (29%) and γ -Proteobacteria (10%). Most of the sequences displayed the highest identity with uncultivated marine bacteria and lower identity with the 16S rRNA genes of known bacteria (Table 2). The most abundant sequences had their closest relatives in the marine bacterial genera *Bacteriovorax* (25%) *Desulfosarcina* (21%) *Oceanirhabdus* (8%). A large number of sequences (23%) was distantly related to the genus *Lutibaculum* (92% nt identity). The *Bacteriovorax* sequences retrieved in the MPA have their closest relatives in the components of the microbiota of the sessile filter-feeding marine invertebrate *Ciona intestinalis* (Dishaw et. al., 2014). *Bacteriovorax marinus*, that is the closest cultivated relative, usually attaches to gram-negative bacteria and penetrates through the cell wall to form a bdelloplast in which it is

able to replicate (Crossman et al. 2013). The abundant presence of sulphate-reducing delta-proteobacteria, distantly related to *Desulfosarcina*, indicates anoxic conditions of the sediment. These bacteria have recently been described as having a role in anaerobic methane oxidation in association with archaeal methane oxidisers (Beal et al. 2009) and in alkanes degradation at marine seeps (Kleindienst et al. 2014).

The presence of bacteria distantly related to *Lutibaculum* appears quite unexpected, as the type strain of this novel genus, *L. baratangense* (gen. nov. sp. nov.), was isolated and described for the first time from a volcano mud (Singh et al. 2014). However, other genera in the order Rhizobiales close to *Lutibaculum*, such as *Amorphus* and *Bauldia*, are of marine origin, and the MPA sequences, in particular, show high similarity with an uncultivated specimen detected in a deep-sea octacoral (Penn et al. 2006).

HC-degraders of AMP and harbor

The HC-degrading bacteria isolated by enrichment cultures from the MPA sediment were remarkably different from those of the harbor sediment (Table 3). The MPA isolates were affiliated to three phyla Actinobacteria, Firmicutes and Proteobacteria, while all the harbor isolates belonged uniquely to the Proteobacteria phylum in the classes Alpha- Beta- and Gammaproteobacteria. The Gram positive MPA isolates had their closest relatives in the HC-degrading *Dietzia*, *Microbacterium* and *Exiguobacterium* (Alonso-Gutiérrez et al. 2011; Johnson and Hill, 2003; Ganesh Kumar et al. 2014). Gammaproteobacteria were affiliated to the HC-degrading genera *Acinetobacter*, *Stenotrophomonas* (McGenity et al. 2012). The HC degrading bacteria affiliated with Alphaproteobacteria and Betaproteobacteria, belong to the genera *Ochrobactrum*, *Sphingobium* and *Achromobacter* respectively (Dudášová et al. 2014). The Gram negative isolates from the MPA belonging to the genera *Acinetobacter* and *Ochrobactrum*, also known HC degraders (Jung et al. 2010), were detected both in the MPA and harbor sediment.

Discussion

The analysis of bacterial communities associated to sediment of a Mediterranean MPA was carried out here for the first time, with the ultimate aim of shedding light on the effects of protection on the microbial component of the benthic biota. Our culture-independent approach, based on 16S- rDNA PCR-DGGE analysis, revealed higher diversity in the MPA sediment in respect to the impacted site, suggesting that the higher the protection degree, the higher the bacterial diversity. Previous studies have demonstrated the effect of protection on the microbial communities; protected coral reefs for example, have higher microbial diversity, while the most degraded reefs show a marked reduction in microbial species richness (Bruce et al. 2012). The distribution of MPA bacteria among twenty OTUs from seven phyla reveals a complex community that can only partially be described in terms of functions. Seeking the link between the richness of bacterial species and the functional diversity is still an important goal for microbial ecologists. It is believed that higher number of bacterial species may account for a higher and more diverse number of functions in the marine ecosystem (Bodelier, 2011; Krause et al. 2014).

Sediment of the B-zone, where the nautical traffic was moderate and significantly larger than A-zones (Bracciali et al. 2012), hosted a more diverse bacterial community than the adjacent no-take-zones A1 and A2. Thus, intermediate levels of disturbance could directly or indirectly alter competitive hierarchy of microbial populations with more robust or competitive species increasing their mortality and making free resources for less competitive species. Such a pattern seems to meet the Intermediate Disturbance Hypothesis at microbial scale (Buckling et al. 2000) and it is also corroborated by a further intriguing finding: that of massive abundance of *Bacteriovorax* in the A-zone's sediment. Indeed, under pristine conditions (overall the A-zones), bacterial diversity should be reduced by bacterial predation, and in turn, predators would control bacterial mortality of other Gram-negative bacteria. *Bdellovibrio* and like-organisms (BALOs) in the MPA A-zones, could represent the competitive-dominant species in the absence of disturbance which plays a role in the control of bacterial abundance and diversity. While BALOs are often uncultivable (and

consequently their prey cannot be studied), the specificity of some isolates towards seafood-borne and human pathogens is of interest as they could act as controlling agents, shaping bacterial communities through selective mortality (Chen et. al., 2011). Further studies designed to test the cited hypotheses are needed.

The MPA sediment hosted also a more diverse group of potential HC degraders, including both Gram-positive and Gram-negative isolates, in respect to the harbour, where most of HC-degrading bacteria belong to the Gram-negative Gammaproteobacteria (Head et al. 2006) and no HC degrading Gram-positive bacteria were isolated. HC degrading Alpha and Gamma-Proteobacteria are generally associated with a first fast petroleum degradation phase, where Gram-positive bacteria are never dominant (Kostka et al. 2011; Catania et al. 2015). The Arenella harbor is characterized by intense nautical traffic (Bracciali et al. 2012) that presumably causes the continuous hydrocarbon accumulation that could reduce diversity by eliminating species or by enhancing the dominant species capable of degrading the environmental pollutants. Nevertheless, the presence of a population of HC degraders in the MPA sediment could suggest that, in the absence of new HC inputs due to closure of MPA boundaries, the most persistent components of the oil-derived old pollutants were buried in the sediment and subjected to slow degradation by the Gram-positive bacteria, known for their catabolic and enzymatic versatility (Quatrini et al. 2008; Lo Piccolo et al. 2011, De Pasquale et. al 2012). However, recent studies reported transboundary movements of chemicals into Italian marine environments, including MPAs (Perra et al. 2011), and such a fact may increase the complexity of the *scenario*.

The MPA microbial assemblage describes the overlooked potential of marine benthic microbiota to react to natural changes in seepage. Such an insight opens to a wider use of bacterial diversity as bio-indicator of ecosystem's health status in marine environments, and at the same time, Gammaproteobacteria have the potential for being used in bioremediation of marine oil pollution (Catania et al. 2015).

Our results show that protection has an influence on bacterial diversity and suggest the importance of monitoring the effects of protection also at a microbial scale. This study creates a baseline of data that can be used to monitor changes in bacterial diversity and composition, associated with a Mediterranean MPA, over time.

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Author Contributions

Conceived and designed the experiments: PQ. Performed the experiments: VC, GS. Analyzed the data: VC, PQ. Contributed reagents/materials/analysis tools: GS, LS. Wrote the draft: VC, PQ. All the authors corrected and approved the final version of the manuscript.

References

- Alonso-Gutiérrez J, Teramoto M, Yamazoe A, Harayama S, Figueras A, Novoa B. 2011. Alkane-degrading properties of *Dietzia* sp. H0B, a key player in the Prestige oil spill biodegradation (NW Spain). *J Appl Microbiol.* **111**:800-10.
- Beal EJ, House CH, Orphan VJ 2009. Manganese- and iron-dependent marine methane oxidation. *Science.* 325(5937):184-7.
- Bodelier PL 2011. Toward understanding, managing, and protecting microbial ecosystems. *Front Microbiol.* **25**:2-80. doi: 10.3389/fmicb.2011.00080.
- Bracciali C, Campobello D, Giacomini C, Sara` G 2012. Effects of Nautical Traffic and Noise on Foraging Patterns of Mediterranean Damselfish (*Chromis chromis*). *Plos One.*7:e40582; doi:10.1371/journal.pone.0040582

- Bruce T, Meirelles PM, Garcia G, et al. Abrolhos Bank Reef Health Evaluated by Means of Water Quality, Microbial Diversity, Benthic Cover, and Fish Biomass Data. Sandin SA, ed. PLoS ONE. 2012;7(6):e36687.doi:10.1371/journal.pone.0036687.
- Buckling A, Kassen R, Bell G, Rainey PB. 2000. Disturbance and diversity in experimental microcosms. *Nature*. **408**(6815):961-4.
- Cardinale M, Brusetti L, Quatrini P, Borin S, Puglia AM, Rizzi A, Zanardini E, Sorlini C, Corselli C, Daffonchio D. 2004. Comparison of different primer sets for use in automated ribosomal intergenic spacer analysis of complex bacterial communities. *Appl Environ Microbiol*. **70**(10):6147-56.
- Catania, V., Santisi, S., Signa, G., Vizzini, S., Mazzola, A., Cappello, S., ... & Quatrini, P. 2015. Intrinsic bioremediation potential of a chronically polluted marine coastal area. *Marine Pollution Bulletin*, **99**(1):138-149.
- Chen H, Rana A, Guili Z, Henry NW 2011. Prey bacteria shape the community structure of their predators. *The ISME Journal*. **5**:1314–1322.
- Cole JR, Wang Q, Fish JA, Chai B, McGarrell DM, Sun Y, Brown CT, Porras-Alfaro A, Kuske CR, Tiedje JM 2014. Ribosomal Database Project: data and tools for high throughput rRNA analysis *Nucl. Acids Res*. **41**(Database issue):D633-D642.
- Coll M, Piroddi C, Steenbeek J, Kaschner K, Ben Rais Lasram F, Aguzzi J, Ballesteros E,.... & Voultsiadou E. 2010. The Biodiversity of the Mediterranean Sea: Estimates, Patterns, and Threats. *PLoS ONE*. **5**(8):e11842. doi:10.1371/journal.pone.0011842.
- Crossman LC, Chen H, Cerdeño-Tárraga A, Brooks K, Michael AQ, Pineiro AS, Hobley L, Socket RE, Bentley SD, Parkhill J, Williams HN, Stine OC. 2013. A small predatory core genome in the divergent marine *Bacteriovorax marinus* SJ and the terrestrial *Bdellovibrio bacteriovorus*. *ISME J*. **7**(1):148-60.

- De Pasquale C, Palazzolo E, Lo Piccolo L, Quatrini P. 2012. Degradation of long chain n-alkanes in soil microcosms by two Actinobacteria. *J Environ Sci Health A Tox Hazard Subst Environ Eng* **47**(3):374-381
- Di Leonardo R, Vizzini, S, Bellanca A, Mazzola A. 2009. Sedimentary record of anthropogenic contaminants (trace metals and PAHs) and organic matter in a Mediterranean coastal area (Gulf of Palermo, Italy), *Journal of Marine Systems*, **78**: 136-145
- Dishaw LJ, Flores-Torres J, Lax S, Gemayel K, Leigh B, Melillo D, Mueller MG, Natale L, Zucchetti I, De Santis R, Pinto MR, Litman GW, Gilbert JA. 2014. The Gut of Geographically Disparate *Ciona intestinalis* Harbors a Core Microbiota. *PLoS One*. **9**(4):e93386. doi: 10.1371/journal.pone.0093386
- Dudášová H, Lukáčová L, Murínová S, Puškárová A, Pangallo D, Dercová K. 2014. Bacterial strains isolated from PCB-contaminated sediment and their use for bioaugmentation strategy in microcosms. *J Basic Microbiol*. **54**(4):253-6; doi: 10.1002/jobm.201200369.
- Fraschetti S, Terlizzi A, Micheli F, Benedetti C, Cecchi L, Boero F 2002. Marine protected areas in the Mediterranean Sea: objectives, effectiveness and monitoring. *Mar Ecol*. **23**(s1):190-200.
- Ganesh Kumar A, Vijayakumar L, Joshi G, Magesh Peter D, Dharani G, Kirubakaran R. 2014. Biodegradation of complex hydrocarbons in spent engine oil by novel bacterial consortium isolated from deep sea sediment. *Bioresour Technol*. **170**:556-64.
- Hammer O, Harper DAT, Ryan PD 200. PAST: PALEONTOLOGICAL STATISTICS SOFTWARE PACKAGE FOR EDUCATION AND DATA ANALYSIS. *Palaeontologia Electronica* <http://palaeo-electronica.org>Hana
- Head IM, Jones DM, Röling WF. 2006. Marine microorganisms make a meal of oil. *Nat Rev Microbiol*. **4**(3):173-82.
- Jameson SC, Tupper MH, Ridley JM 2002. The three screen doors: Can marine “protected” areas be effective? *Marine Poll Bull*. **44**:1177–1183.

- Johnson JE, Hill RT 2003. Sediment microbes of deep-sea bioherms on the northwest shelf of Australia. *Microb Ecol.* **46**(1):55-61.
- Jung J, Baek JH, Park W. 2010. Complete genome sequence of the diesel-degrading *Acinetobacter* sp. strain DR1. *J Bacteriol.* **192**(18):4794-5.
- Kemp PF, Aller JY. 2004. Bacterial diversity in aquatic and other environments: what 16S rDNA libraries can tell us. *FEMS Microbiol Ecology.* **47**:161–177. doi: 10.1016/S0168-6496(03)00257-5.
- Kleindienst S, Herbst FA, Stagars M, von Netzer F, von Bergen M, Seifert J, Peplies J, Amann R, Musat F, Lueders T, Knittel K. 2014. Diverse sulfate-reducing bacteria of the *Desulfosarcina/Desulfococcus* clade are the key alkane degraders at marine seeps. *ISME J.* **8**(10):2029-44.
- Kostka, J. E., Prakash, O., Overholt, W. A., Green, S. J., Freyer, G., Canion, A., Jonathan Delgadio,¹ Nikita Norton,¹ Terry C. Hazen,⁴ ... Huettel, M. (2011). Hydrocarbon-Degrading Bacteria and the Bacterial Community Response in Gulf of Mexico Beach Sands Impacted by the Deepwater Horizon Oil Spill. *Applied and Environmental Microbiology*, 77(22), 7962–7974. <http://doi.org/10.1128/AEM.05402-11>
- Krause S, Le Roux X, Niklaus PA, Van Bodegom PM, Lennon JT, Bertilsson S, Grossart HP, Philippot L, Bodelier PL. 2014. Trait-based approaches for understanding microbial biodiversity and ecosystem functioning. *Front Microbiol.* **5**:251. doi: 10.3389/fmicb.2014.00251
- La Manna G, Donno Y, Sarà G. and Ceccherelli G 2015. The detrimental consequences for seagrass of ineffective marine park management related to boat anchoring. *Marine Poll Bull.* **90**:160-166.
- Lo Piccolo L, De Pasquale C, Fodale R, Puglia AM and Quatrini, P. 2011. Involvement of an alkane hydroxylase system of *Gordonia* sp. strain SoCg in degradation of solid n-alkanes. *Applied Environmental Microbiology* **77** (4) 1204-1213

- McGenity TJ, Folwell BD, McKew BA, Sanni GO 2012. Marine crude-oil biodegradation: a central role for interspecies interactions. *Aquat Biosyst.* **8**(1):10. doi: 10.1186/2046-9063-8-10.
- Muyzer G, de Wall EC, Uitterlinden AG. 1993. Profiling of complex microbial populations by denaturing gradient gel electrophoresis analysis of polymerase chain reaction amplified genes encoding for 16S rRNA. *App Environ Microbiol.* **59**:695–700.
- Penn K, Wu D, Eisen JA, Ward N. 2006. Characterization of bacterial communities associated with deep-sea corals on Gulf of Alaska seamounts. *Appl Environ Microbiol.* **72**(2):1680-3.
- Perra G, Pozo K, Guerranti C, Lazzeri D, Volpi V, Corsolini S, Focardi S. 2011. Levels and spatial distribution of polycyclic aromatic hydrocarbons (PAHs) in superficial sediment from 15 Italian marine protected areas (MPA). *Mar Pollut Bull.* **62**(4):874-7.
- Pontes DS, Lima-Bittencourt CI, Chartone-Souza E, Amaral Nascimento AM, 2007. Molecular approaches: advantages and artifacts in assessing bacterial diversity. *J Ind Microbiol Biotechnol.* **34**(7):463-73.
- Quatrini P, Scaglione G, De Pasquale C, Riela S, Puglia AM 2008. Isolation of Gram positive *n*-alkane degraders from a hydrocarbon contaminated Mediterranean shoreline. *J Appl Microbiol.* **104**:251–259.
- Sambrook J, Fritsch EF, Maniatis T. 1989. *Molecular Cloning, a Laboratory Manual*. 2nd edn. Cold Spring Harbor Laboratory Press, New York.
- Singh A, Sreenivas A, Sathyanarayana Reddy G, Pinnaka AK, Shivaji S 2014. Draft genome sequence of *Lutibaculum baratangense* strain AMV1^T, isolated from a mud volcano in Andamans, India. *Genome Announc.* **2**(4):e00735-14. doi:10.1128/genomeA00735-14.
- Weisburg WG, Barns SM, Pelletier DA, Lane DJ 1991. 16S Ribosomal DNA Amplification for Phylogenetic Study. *J Bacteriol.* **173**:697-703.
- Yeung CW, Lee K, Whyte LG, Greer CW 2010. Microbial community characterization of the Gully: a marine protected area. *Can J Microbiol.* **56**(5):421-31.

Figure legends

Fig. 1 The study area - MPA of Capo Gallo and Isola delle Femmine, Southern Mediterranean, Italy. The map shows the sampling sites B1, A1, A2 and the Arenella harbor

Fig. 2 Denaturing Gradient Gel Electrophoresis analysis. (a) Cluster analysis dendrogram displaying the bacterial community of sediment samples collected from the integral reserve zone of the MPA of Capo Gallo- Isola delle Femmine A1 site (line 1, 2, 3) and A2 site (samples 4, 5, 6), from the general reserve site B1 (samples 7, 8, 9) and from one site of impacted Arenella harbor (samples 10, 11, 12). PAST Jaccard's 100 bootstrap analysis was used to measure similarity among the samples banding patterns. (b) DGGE profile (35%–70% denaturant) of PCR-amplified V3 region fragments of the 16S rDNA gene from sediment samples

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Fig. 1
180x112mm (300 x 300 DPI)

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Fig. 2
241x132mm (300 x 300 DPI)

Table 1. Bacterial diversity indices of the marine sediment based on DGGE patterns. Areas A1, A2, B1 and Harbor are indicated in Figure 1.

Indices	Area B1	Area A1	Area A2	Harbor
Taxa_S	61,66	61	57	40,66
Simpson_1-D	0,98	1	0,98	0,97
Shannon_H	4,12	4,10	4,04	3,70
Brillouin	3,17	3,15	3,09	2,77
Menhinick	7,85	7,80	7,54	6,37
Margalef	14,72	14,59	13,85	10,70
Berger-Parker	0,016	0,016	0,018	0,025
Chao-1	1932	1903	1659	848

Table 2. Phylogenetic affiliation of the 16S rDNA clones in the A1 MPA library. Sequenced clones, top blast hits to the non-redundant database (closest relative) and best hit among cultured bacteria (closest cultured relative). Phylogeny was obtained using RDP Naive Bayesian rRNA Classifier Version 2.8, June 2014.

Phylogeny (RDP11)			N. of clones in the OTU	Sequenced Clone	Closest relative (top blast hit)	%similarity	closest cultured relative	%similarity
phylum	class	family						
Acidobacteria	Acidobacteria_Gp4		2	A1s12	Uncultured bacterium clone D21011 16S JX193344.1	98	<i>Blastocatella fastidiosa</i> A2-16 NR_118350.1	89
	Acidobacteria_Gp10		1	A1s39	Uncultured <i>Acidobacteria</i> JQ580504.1	98	<i>Thermoanaerobaculum aquaticum</i> MP-01 NR_109681.1	84
	Acidobacteria_Gp22		1	A1s153	Uncultured bacterium clone Cm1-2 16S GQ246339.1	97	<i>Geoalkalibacter ferrihydriticus</i> Z-0531 NR_043709.1	85
Actinobacteria	Actinobacteria	Acidimicrobinae	4	A1s9	Uncultured bacterium clone Q10904 16S JX193426.1	94	<i>Aciditerrimonas ferrireducens</i> IC-180 NR_112972.1	89
Bacteroidetes	Sphingobacteria	Flammeovirgaceae	3	A1s77	Uncultured <i>Flammeovirgaceae</i> FJ516770.1	97	<i>Marivirga tractuosa</i> DSM 4126 NR_074493.1	89
Planctomycetes	Planctomycetacia	Planctomycetaceae	3	A1sH9	Uncultured bacterium clone SHFH484 16S FJ203440.1	97	<i>Bythopirellula goksoyri</i> Pr1d NR_118636.1	92
Cyanobacteria/Chloroplast	Cyanobacteria		2	A1s127	Uncultured bacterium clone SHFG705 16S FJ203286.1	96	<i>Loriellopsis cavernicola</i> LF-B5 NR_117881.1	87
Proteobacteria	Alphaproteobacteria		32	A1sB11	Uncultured organism clone ctg_CGOAA69 16S DQ395471.1	98	<i>Lutibaculum baratangense</i> AMV1 NR_116954.1	92
		Gammaproteobacteria	2	A1s139	Uncultured bacterium clone SHFH755 16S FJ203654.1	97	<i>Ferrimonas balearica</i> DSM 9799 NR_027602.1	96
		Ectothiorhodospiraceae	1	A1s112	Uncultured bacterium clone LC1446B-88 16S DQ270631.1	98	<i>Thioalkalivibrio sulfidophilus</i> HL-EbGR7 NR_074692.1	91
			2	A1sE6	Uncultured <i>Gammaproteobacterium</i> JQ579797.1	99	<i>Thiopfundum hispidum</i> gps61 NR_112620.1	91
			5	A1s80	Uncultured <i>Gammaproteobacterium</i> GU230336.1	99	<i>Thioalkalivibrio sulfidophilus</i> HL-EbGR7 NR_074692.1	91
			1	A1s53	Uncultured gamma clone OXIC-100 JF344370.1	98	<i>Thioalkalivibrio sulfidophilus</i> HL-EbGR7 NR_074692.1	91
	Deltaproteobacteria	Bacteriovoraceae	35	A1s125	Uncultured bacterium clone Woods-Hole_a4757 16S	96	<i>Bacteriovorax marinus</i> SJ NR_102485.1	90

KF798850.1									
Firmicutes	Bacilli	Desulfobulbaceae	2	A1s144	Uncultured bacterium clone 12_E01 16S KF268830.1	99	<i>Desulfotalea arctica</i> LSv514 NR_024949.1	94	
		Desulfobacteraceae	29	A1s155	Uncultured bacterium clone FeSO4_B_3 16S GQ356986.1	96	<i>Desulfosarcina cetonica</i> 480 NR_028896.1	93	
		Pasteuriaceae	1	A1s137	Uncultured bacterium clone FFCH6206 16S EU135048.1	94	<i>Bythopirellula goksoyri</i> Pr1d NR_118636.1	92	
		Staphylococcaceae	1	A1s19	<i>Staphylococcus equorum</i> C2060 KF439734.1	99	<i>Staphylococcus equorum subsp. linens</i> RP29 NR_041926.1	99	
	Clostridia	Clostridiaceae 1	12	A1s114	Uncultured bacterium clone CK_1C4_25 16S EU488051.1	97	<i>Oceanirhabdus sediminicola</i> NH-JN4 NR_109625.1	93	
		Lachnospiraceae	1	A1s132	Uncultured bacterium clone V1SC07b171 16S HQ153989.1	98	<i>Vallitalea guaymasensis</i> Ra1766G1 NR_117645.1	93	

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Table 3. HC-oxidating bacteria isolated from the sediments of the MPA Capogallo Isola delle Femmine (A1 site) and the Arenella harbor

Phylogeny (RDPII)				Sequenced isolate	Carbon source	BLAST results (16S database)		
Site	phylum	class	family			Most closely related sequence	Id. %	Acc. Number
MPA	Actinobacteria	Actinobacteria	Micrococcineae	A1sdiesD4.1	Diesel	<i>Microbacterium aurantiacum</i> DSM 12506	99	NR_114991.1
			Nocardiaceae	A1sdiesD4.2	Diesel	<i>Dietzia maris</i> DSM 43672	99	NR_118596.1
	Proteobacteria	Alphaproteobacteria	Brucellaceae	A1sdiesD8	Diesel	<i>Ochrobactrum anthropi</i> ATCC 49188	99	NR_074243.1
			Rhizobiaceae	A1salkB8.1	<i>n</i> -alkanes ^b	<i>Ensifer adhaerens</i> OV14	99	NR_121784.1
	Firmicutes	Bacilli	Moraxellaceae	A1salkA7	<i>n</i> -alkanes ^b	<i>Acinetobacter venetianus</i> ATCC 31012	99	NR_042049.1
				A1salkB5.2	<i>n</i> -alkanes ^b	<i>Exiguobacterium mexicanum</i> 8N	99	NR_042424.1
Harbor	Proteobacteria	Alphaproteobacteria	Brucellaceae	PsalkA1	<i>n</i> -alkanes ^b	<i>Ochrobactrum anthropi</i> strain ATCC 49188	99	NR_074243.1
			Sphingomonadaceae	PsdiesE4a	Diesel	<i>Sphingobium lactosutens</i> DS20	99	NR_116408.1
		Betaproteobacteria	Alcaligenaceae	PsdiesE1a	Diesel	<i>Achromobacter xylosoxidans</i> A8	99	NR_074754.1
		Gammaproteobacteria	Moraxellaceae	PsdiesE1b	Diesel	<i>Acinetobacter venetianus</i> ATCC 31012	99	NR_042049.1
			Xanthomonadaceae	PsdiesE2	Diesel	<i>Stenotrophomonas pavanii</i> LMG 25348	99	NR_118008.1
				PsdiesE7.2	Diesel	<i>Stenotrophomonas maltophilia</i> IAM 12423	98	NR_041577.1

^a Phylogeny was obtained using RDP Naive Bayesian rRNA Classifier Version 2.8, June 2014

^b Mixture of *n*-alkanes: C₁₂, C₁₅, C₁₆