NRC Research Press

ARTICLE

Bacterial communities in sediment of a Mediterranean marine protected area

Valentina Catania, Gianluca Sarà, Luca Settanni, and Paola Quatrini

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 with MPA sediment was compared with that from sediment of an adjacent harbour exposed to intense nautical traffic. The MPA sediment showed higher diversity with 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%). Analysis of the hydrocarbon (HC)-degrading bacteria was performed using enrichment cultures. Most of the MPA sediment isolates were affiliated with Gram-positive G+C rich bacteria, whereas the majority of taxa in the harbour sediment clustered with *Alpha*- and *Gammaproteobacteria*; no Gram-positive HC degraders were isolated from the harbour sediment. Our results show that protection probably has an influence on bacterial diversity, and suggest the importance of monitoring the effects of protection at microbial level as well. This study creates a baseline of data that can be used to assess changes over time in bacterial communities associated with a Mediterranean MPA.

Key words: bacterial communities, hydrocarbon-degrading bacteria, marine protected areas, *Bacteriovorax*, DGGE analysis.

Résumé : La biodiversité est cruciale à la préservation des écosystèmes, et les communautés bactériennes jouent un rôle indispensable dans le fonctionnement des écosystèmes marins. La Zone de Protection Marine de la Méditerranée (ZPM) « Capo Gallo–Isola delle Femmine » a été établie afin de préserver la biodiversité marine. On a comparé la diversité bactérienne associée aux sédiments de la ZPM à celle de sédiments d'un port adjacent exposé à la circulation nautique. Les sédiments de la ZPM ont révélé une plus grande diversité par rapport au site touché par la circulation. Une banque de clones d'ADN ribosomal 16S des sédiments de la ZPM a permis de recenser 7 phyla, soit *Proteobacteria* (78 %), *Firmicutes* (11 %), *Acidobacteria* (3 %), *Actinobacteria* (3 %), *Bacteroidetes* (2 %), *Planctomycetes* (2 %) et *Cyanobacteria* (1 %). L'analyse des bactéries dégradant les hydrocarbures a été réalisée au moyen de 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 se regroupaient dans les *Alpha* et *Gamma Proteobacteria*; aucun Gram positif dégradant les HC n'a été isolé des sédiments du port. Nos résultats montrent que la protection a probablement une influence sur la diversité bactérienne, et mettent en relief l'importance de surveiller les effets de la protection au nuveau microbien. Cette étude fournit des données de référence qui pourront être utilisées pour évaluer les changements chronologiques dans les communautés microbiennes associées à une ZPM méditerranéenne.

Mots-clés : communautés bactériennes, bactéries dégradant les hydrocarbures, zones de protection marines, *Bacteriovorax*, analyse DGGE.

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 Protista 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

Received 27 June 2016. Revision received 15 November 2016. Accepted 25 November 2016.

V. Catania and P. Quatrini. Dipartimento di Scienze e Tecnologie Biologiche, Chimiche e Farmaceutiche (STEBICEF), Università degli Studi di Palermo, Viale delle Scienze Ed.16 - 90128 Palermo, Italia.
G. Sarà. Dipartimento di Scienze della Terra e del Mare (DISTEM), Università degli Studi di Palermo, Viale delle Scienze Ed.16 - 90128 Palermo, Italia.
L. Settanni. Dipartimento di Scienze Agrarie e Forestali (SAF), Università degli Studi di Palermo, Viale delle Scienze Ed.4 - 90128 Palermo, Italia.
Corresponding author: Paola Quatrini (email: paola.quatrini@unipa.it).

Copyright remains with the author(s) or their institution(s). Permission for reuse (free in most cases) can be obtained from RightsLink.

Published at www.nrcresearchpress.com/cjm on 8 December 2016.

PROVIDED FOR NON-COMMERCIAL RESEARCH USE AND EDUCATION USE. NOT FOR REPRODUCTION, DISTRIBUTION OR COMMERCIAL USE.

occur in the Mediterranean Sea, and of these, at least 26% are prokaryotic (*Bacteria* and *Archaea*) and eukaryotic (Protista) marine microbes (Coll et al. 2010).

The need to protect 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, overexploitation, and species invasions (Fraschetti et al. 2002). The knowledge of the ecological effects of protection is still limited because of 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 plant and animal diversity, 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 taxonomic diversity and functional diversity, based on functional gene and (or) 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 MPAs (Yeung et al. 2010), and to our knowledge, there are no reports on microbial diversity of sediment from MPAs in the Mediterranean Sea. The Mediterranean marine protected area "Capo Gallo–Isola delle Femmine" was 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 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 (HCs), attention has been devoted to analyse the cultivable fraction of HC-degrading bacteria 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) Capo Gallo–Isola delle Femmine (northwest Sicily, Italy) (Fig. 1). This MPA is an area of approximately 20 km², with 3 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.

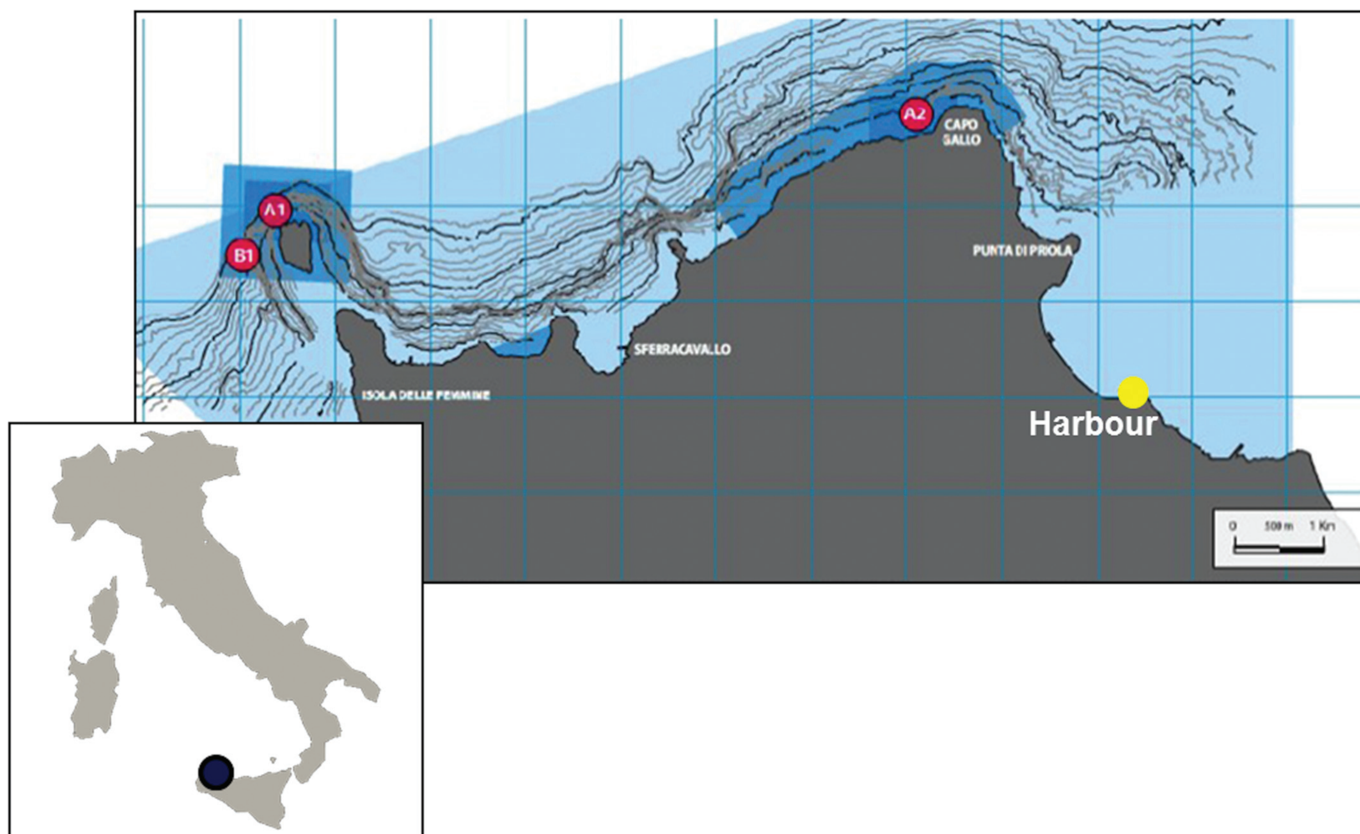
Superficial sediment cores for bacterial analysis were collected by scuba divers along the MPA protection gradient: 2 sites were chosen in the no-take A-zone (A1: 38°12′46.2″N, 13°14′10.8″E — depth 9 m; A2: 38°13′19.1″N, 13°18′26.3″E — depth 8 m); 1 site was chosen in the general partially protected B-zone (38°12′25.7″N, 13°14′03.5″E — depth 8.6 m). The impacted site was chosen within the area of an external adjacent small fishing harbour (the C-zone; Arenella: 38°09′07.4″N, 13°22′30.5″E — depth 7 m) with a high number of fishery activities and intense nautical traffic. Owing to intensive boating activities, a complex mixture containing a broad spectrum of HCs is released into the harbour environment and in the proximal areas by combustion of fossil fuels. Accordingly, sediments were highly contaminated, as they showed total polycyclic aromatic hydrocarbons (ΣPAHs) concentrations that would classify them as highly contaminated (total PAH concentrations exceeding 0.500 $mg \cdot kg^{-1}$; 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 the no-take A-zone, no sedimentary contamination due to local nautical traffic should be present and, coherently, ΣPAHs 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 sand near the coast and coarse and medium sand beyond the coast (up to 2 mm; G. Sarà, Università degli Studi di Palermo, personal communication). Sediment samples were collected in triplicate from all sites (0–5 cm) using sterilized tube cores (50 mL) and were immediately stored on board in freezer storage bags. Once brought back to the laboratory within 2 h, sedimentary samples from the same site were pooled together and immediately centrifuged at 6000*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 mass) using the FastDNA Spin kit for soil (MP Biomedicals, USA) according to the manufacturer's instructions, except in the last step, where the elution was repeated twice with 50 μL of DNase- and RNase-free water. The DNA samples were analysed by electrophoresis in 1% (*m*/*v*) agarose gel with

PROVIDED FOR NON-COMMERCIAL RESEARCH USE AND EDUCATION USE. NOT FOR REPRODUCTION, DISTRIBUTION OR COMMERCIAL USE.

Fig. 1. The study area — the marine protected area Capo Gallo–Isola delle Femmine, southern Mediterranean, Italy. The map shows the sampling sites B1, A1, A2, and the Arenella harbour.



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 (polymerase chain reaction – denaturing gradient gel electrophoresis) 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 harbour 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 0.8 μL of Phire Hot Start II DNA Polymerase (Thermo Scientific), 1× PCR buffer, 500 nmol·L^{-1} (each) primer, 0.30 mmol·L^{-1} 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% (*m*/*v*) agarose gels with ethidium bromide. DGGE was performed with the INGENY phor-U2 system instrument (Ingeny, Leiden, the Netherlands); PCR samples were applied directly onto the 8% (*m*/*v*) acrylamide:bisacrylamide (37.5:1) gel in a 1× TAE buffer with a 35%–70% denaturing gradient (100% denaturant corresponding to 7 mol·L^{-1} urea and 40% (*v*/*v*) deionised formamide). Electrophoresis was performed at 60 °C with 1× TAE for 17 h. Gels were stained with SYBR Gold nucleic acid gel stain (Molecular Probes, Invitrogen) in accordance with 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 (version 3.0) 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 fD1 and rD1 (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. PCR product (150 ng) 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 Luria–Bertani agar, clones were archived at

PROVIDED FOR NON-COMMERCIAL RESEARCH USE AND EDUCATION USE. NOT FOR REPRODUCTION, DISTRIBUTION OR COMMERCIAL USE.

−80 °C in 20% glycerol. Plasmid DNA was extracted from 140 candidate-positive colonies using the alkaline lysis procedure (Sambrook et al. 1989); about 100 ng of each plasmid DNA was digested using the enzymes *Eco*RI and *Pst*I (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 the fDl and rDl primers as described above. The amplified 16S rRNA gene was bidirectionally sequenced and sequences were compared with sequences in the EMBL–SwissProt–GenBank nonredundant 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 (Cole et al. 2014).

Sequences were submitted to the DDBJ–EMBL–GenBank database under accession Nos. KT906693–KT906712.

Isolation and identification of HC-degrading bacteria

Enrichment cultures were set up in the presence of HCs as the sole carbon source to isolate the HC-degrading bacteria. For the enrichments, 1 g of sediment from the A1 site of the MPA and from the Arenella harbour were added to 250 mL Erlenmeyer flasks containing 50 mL of Bushnell Haas Mineral Salts (BHSM) medium (Difco). A mixture of the *n*-alkanes C_{12}, C_{15}, C_{16}, (1 g·L^{-1}) or Diesel oil (1 g·L^{-1}) was then added to the medium (Quatrini et al. 2008). Flasks were incubated without shaking for 15 days at room temperature. Subcultures were prepared by transferring 1 mL from the enrichment cultures to flasks containing 50 mL of fresh BHSM medium and the HCs. 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 room temperature, 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 characteristic different from the others were further characterized by PCR analysis of the internally transcribed spacer (ITS) 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 positions 38 and 23 of the 23S rRNA of *E. coli* respectively (Cardinale et al. 2004). The amplification reaction mixture consisted of 1× PCR reaction buffer, 1.5 mmol·L^{-1} $MgCl_2$, 0.25 mmol·L^{-1} dNTP, 0.2 μmol·L^{-1} (each) primer, 1 U of 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 by electrophoresis on a 1% agarose gel. Strains having overlapping ITS-PCR amplification patterns were assigned to the same OTUs. The 16S rRNA gene of 1 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 with sequences in the EMBL–SwissProt–GenBank nonredundant 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 (Cole et al. 2014).

Sequences were submitted to the DDBJ–EMBL–GenBank database under accession Nos. KT933187–KT933197.

Results

Bacterial diversity of the sediment

Sedimentary bacterial diversity of the MPA and the adjacent harbour was analysed by 16S rDNA PCR–DGGE (Fig. 2). The 3 replicates, obtained by 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 harbour 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 B1 sites) shared 18 OTUs, while only 11 OTUs were common between the MPA and harbour sediment. Richness and diversity indices are shown in Table 1; all the indices related to the MPA are higher than those related to the harbour, and within the MPA, the B zone hosts slightly higher diversity than the no-take A zones.

Composition of the bacterial community of MPA sediment

One hundred and forty 16S rRNA clones were clustered into 20 OTUs by ARDRA. The OTUs were affiliated with 7 phyla: *Proteobacteria* (78%), *Firmicutes* (11%), *Acidobacteria* (3%), *Actinobacteria* (3%), *Bacteroidetes* (2%), *Planctomycetes* (2%), and *Cyanobacteria* (1%). Within the dominating phylum *Proteobacteria*, *Deltaproteobacteria* was the most abundant class (61%), followed by *Alphaproteobacteria* (29%) and

PROVIDED FOR NON-COMMERCIAL RESEARCH USE AND EDUCATION USE ONLY. NOT FOR REPRODUCTION, DISTRIBUTION OR COMMERCIAL USE.

Fig. 2. Denaturing gradient gel electrophoresis (DGGE) analysis. (*a*) Cluster analysis dendrogram displaying the bacterial community of sediment samples collected from the integral reserve zone of the marine protected area of Capo Gallo–Isola delle Femmine A1 site (samples 1, 2, 3) and A2 site (samples 4, 5, 6), from the general reserve site B1 (samples 7, 8, 9) and from 1 site of impacted Arenella harbour (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.

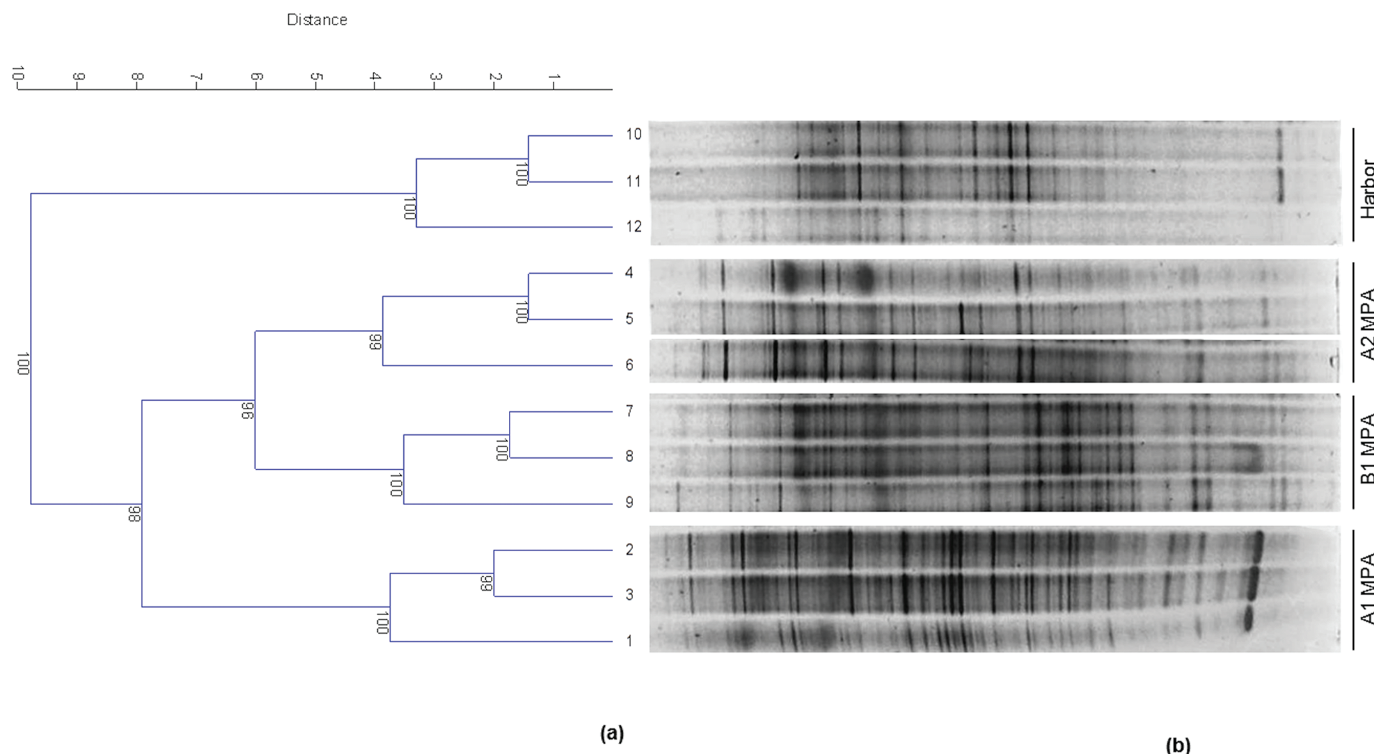


Table 1. Bacterial diversity indices of the marine sediment based on DGGE patterns.

Index	Area B1	Area A1	Area A2	Harbour
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
Chao1	1932	1903	1659	848

Note: Areas A1, A2, B1, and Harbour are indicated in Fig. 1.

Gammaproteobacteria (10%). Most of the sequences displayed the highest identity with uncultivated marine bacteria and a 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%), and *Oceanirhabdus* (8%). A large number of sequences (23%) were distantly related to the genus *Lutibaculum* (92% nucleotide 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*, which 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 sulfate-reducing *Deltaproteobacteria*, 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 oxidizers (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 mud of a volcano (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 sequences of bacteria from MPA sediment samples, in particular, show high similarity with an uncultivated specimen detected in a deep-sea octacoral (Penn et al. 2006).

HC degraders of MPA and harbour

The HC-degrading bacteria isolated by enrichment cultures from the MPA sediment were remarkably different from those of the harbour sediment (Table 3). The MPA isolates were affiliated with 3 phyla: *Actinobacteria*, *Firmicutes*, and *Proteobacteria*, while all the harbour isolates belonged uniquely to the *Proteobacteria* phylum in the

PROVIDED FOR NON-COMMERCIAL RESEARCH USE AND EDUCATION USE ONLY. NO REPRODUCTION, DISTRIBUTION OR COMMERCIAL USE.

Table 2. Phylogenetic affiliation of the 16S rDNA clones in the A1 marine protected area library.

Phylogeny (RDPII)								
Phylum	Class	Family	No. of clones in the OTU	Sequenced clone	Closest relative (top blast hit)	% Similarity	Closest cultured relative	% Similarity
Acidobacteria	*Acidobacteria_Gp4*		2	A1s12	Uncultured bacterium clone D21011 16S JX193344.1	98	*Blastocatella fastidiosa* A2-16 NR_118350.1	89
	Acidobacteria_Gp10		1	A1s39	Uncultured *Acidobacteria* JQ580504.1 MP-01 NR_109681.1	98	*Thermoanaerobaculum aquaticum*	84
	Acidobacteria_Gp22		1	A1s153	Uncultured bacterium clone Cm1-2 16S GQ246339.1	97	*Geoalkalibacter ferrihydriticus* Z-0531 NR_043709.1	85
Actinobacteria	*Actinobacteria*	*Acidimicrobineae*	4	A1s9	Uncultured bacterium clone Q10904 16S JX193426.1	94	*Aciditerrimonas ferrireducens* IC-180 NR_112972.1	89
Bacteroidetes	*Sphingobacteria*	*Flammeovirgaceae*	3	A1s77	Uncultured *Flammovirgaceae* FJ516770.1	97	*Marivirga tractuosa* DSM 4126 NR_074493.1	89
Planctomycetes	*Planctomycetacia*	*Planctomycetaceae*	3	A1sH9	Uncultured bacterium clone SHFH484 16S FJ203440.1	97	*Bythopirellula goksoyri* Pr1d NR_118636.1	92
Cyanobacteria/ Chloroplast	*Cyanobacteria*		2	A1s127	Uncultured bacterium clone SHFG705 16S FJ203286.1	96	*Loriellopsis cavernicola* LF-B5 NR_117881.1	87
Proteobacteria	*Alphaproteobacteria*		32	A1sB11	Uncultured organism clone ctg_CGOAA69 16S DQ395471.1	98	*Lutibaculum baratangense* AMV1 NR_116954.1	92
	Gammaproteobacteria	*Ferrimonadaceae*	2	A1s139	Uncultured bacterium clone SHFH755 16S FJ203654.1	97	*Ferrimonas balearica* DSM 9799 NR_027602.1	96
		Ectothiorhodospiraceae	1	A1s112	Uncultured bacterium clone LC1446B-88 16S DQ270631.1	98	*Thioalkalivibrio sulfidophilus* HL-EbGR7 NR_074692.1	91
			2	A1sE6	Uncultured gammaproteobacterium JQ579797.1	99	*Thioprofundum hispidum* gps61 NR_112620.1	91
			5	A1s80	Uncultured gammaproteobacterium GU230336.1	99	*Thioalkalivibrio sulfidophilus HL-EbGR7NR_074692.1*	91
			1	A1s53	Uncultured gamma clone OXIC-100 JF344370.1	98	*Thioalkalivibrio sulfidophilus* HL-EbGR7 NR_074692.1	91
	Deltaproteobacteria	*Bacteriovoracaceae*	35	A1s125	Uncultured bacterium clone Woods-Hole_a4757 16S KF798850.1	96	*Bacteriovorax marinus* SJ NR_102485.1	90
		Desulfobulbaceae	2	A1s144	Uncultured bacterium clone 12_E01 16S KF268830.1	99	*Desulfotalea arctica* LSv514 NR_024949.1	94
		Desulfobacteraceae	29	A1s155	Uncultured bacterium clone FeSO4_B_3 16S GQ356986.1	96	*Desulfosarcina cetonica* 480 NR_028896.1	93
Firmicutes	*Bacilli*	*Pasteuriaceae*	1	A1s137	Uncultured bacterium clone FFCH6206 16S EU135048.1	94	*Bythopirellula goksoyri* Pr1dNR_118636.1	92
		Staphylococcaceae	1	A1s19	*Staphylococcus equorum* C2060 KF439734.1	99	*Staphylococcus equorum* subsp. *linens* RP29 NR_041926.1	99
	Clostridia	*Clostridiaceae 1*	12	A1s114	Uncultured bacterium clone CK_1C4_25 16S EU488051.1	97	*Oceanirhabdus sediminicola* NH-JN4 NR_109625.1	93
		Lachnospiraceae	1	A1s132	Uncultured bacterium clone V1SC07b171 16S HQ153989.1	98	*Vallitalea guaymasensis* Ra1766G1 NR_117645.1	93

Note: Sequenced clones, top blast hits to the nonreduntant 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.

PROVIDED FOR NON-COMMERCIAL RESEARCH USE AND EDUCATION USE. NOT FOR REPRODUCTION, DISTRIBUTION OR COMMERCIAL USE.

Table 3. Hydrocarbon-oxidizing bacteria isolated from the sediments of the marine protected area (MPA) Capo Gallo–Isola delle Femmine (A1 site) and Arenella harbour.

	Phylogeny[a] (RDPII)					BLAST results (16S database)		
Site	Phylum	Class	Family	Sequenced isolate	Carbon source	Most closely related sequence	Id. %	Acc. No.
MPA	*Actinobacteria*	*Actinobacteria*	*Micrococcineae*	A1sdiesD4.1	Diesel	*Microbacterium aurantiacum* DSM 12506	99	NR_114991.1
			Nocardiaceae	A1sdiesD4.2	Diesel	*Dietzia maris* DSM 43672	99	NR_118596.1
	Proteobacteria	*Alphaproteobacteria*	*Brucellaceae*	A1sdiesD8	Diesel	*Ochrobactrum anthropi* ATCC 49188	99	NR_074243.1
			Rhizobiaceae	A1salkB8.1	*n*-Alkanes[b]	*Ensifer adhaerens* OV14	99	NR_121784.1
		Gammaproteobacteria	*Moraxellaceae*	A1salkA7	*n*-Alkanes[b]	*Acinetobacter venetianus* ATCC 31012	99	NR_042049.1
	Firmicutes	*Bacilli*		A1salkB5.2	*n*-Alkanes[b]	*Exiguobacterium mexicanum* 8 N	99	NR_042424.1
Harbour	*Proteobacteria*	*Alphaproteobacteria*	*Brucellaceae*	PsalkA1	*n*-Alkanes[b]	*Ochrobactrum anthropi strain* ATCC 49188	99	NR_074243.1
			Sphingomonadaceae	PsdiesE4a	Diesel	*Sphingobium lactosutens* DS20	99	NR_116408.1
		Betaproteobacteria	*Alcaligenaceae*	PsdiesE1a	Diesel	*Achromobacter xylosoxidans* A8	99	NR_074754.1
		Gammaproteobacteria	*Moraxellaceae*	PsdiesE1b	Diesel	*Acinetobacter venetianus* ATCC 31012	99	NR_042049.1
			Xanthomonadaceae	PsdiesE2	Diesel	*Stenotrophomonas pavanii LMG 25348*	99	NR_118008.1
				PsdiesE7.2	Diesel	*Stenotrophomonas maltophilia* 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: C_{12}, C_{15}, C_{16}.

classes *Alpha-*, *Beta-*, and *Gammaproteobacteria*. The Gram-positive MPA isolates had their closest relatives in the HC-degrading *Dietzia*, *Microbacterium*, and *Exiguobacterium* (Johnson and Hill 2003; Alonso-Gutiérrez et al. 2011; Ganesh Kumar et al. 2014). *Gammaproteobacteria* were affiliated with the HC-degrading genera *Acinetobacter* and *Stenotrophomonas* (McGenity et al. 2012). The HC-degrading bacteria affiliated with *Alphaproteobacteria* and *Betaproteobacteria* belong to the genera *Ochrobactrum*, *Sphingobium*, and *Achromobacter* (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 in both the MPA and harbour sediments.

Discussion

The analysis of bacterial communities associated with 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 with respect to the impacted site, suggesting that the higher the degree of protection, 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 20 OTUs from 7 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 that of the 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 the 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 the microbial scale (Buckling et al. 2000), and it is also corroborated by a further intriguing finding: the 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

PROVIDED FOR NON-COMMERCIAL RESEARCH USE AND EDUCATION USE ONLY. NO REPRODUCTION, DISTRIBUTION OR COMMERCIAL USE.

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 also hosted a more diverse group of potential HC degraders, including both Gram-positive and Gram-negative isolates, compared with the harbour sediment, 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 *Gammaproteobacteria* are generally associated with a first fast phase of petroleum degradation, where Gram-positive bacteria are never dominant (Kostka et al. 2011; Catania et al. 2015) The Arenella harbour is characterized by intense nautical traffic (Bracciali et al. 2012), which presumably causes the continuous HC 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 as such 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 up the possibility of wider use of bacterial diversity as a bioindicator of an ecosystem's health status in marine environments. As well, *Gammaproteobacteria* have the potential for use 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.

Acknowledgements

The authors thank E. Chiri, M.A. Gulli, and M. Sinacori for technical help. Funds were provided by the DINAUTIS project by the Environmental Minister of Italian Government (no numbers apply). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript. Author contributions: PQ conceived and designed the experiments, VC and GS performed the experiments. VC and PQ analysed the data. GS and LS contributed reagents, materials, and analysis tools. VC and PQ wrote the draft. All the authors corrected and approved the final version of the manuscript.

References

Alonso-Gutiérrez, J., Teramoto, M., Yamazoe, A., Harayama, S., Figueras, A., and 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**: 800–810. PMID: 21767337.

Beal, E.J., House, C.H., and Orphan, V.J. 2009. Manganese- and iron-dependent marine methane oxidation. Science, **325**(5937): 184–187. doi:10.1126/science.1169984. PMID:19589998.

Bodelier, P.L. 2011. Toward understanding, managing, and protecting microbial ecosystems. Front Microbiol. **25**: 2–80. doi: 10.3389/fmicb.2011.00080.

Bracciali, C., Campobello, D., Giacoma, C., and Sarà, 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. PMID:22792375.

Bruce, T., Meirelles, P.M., Garcia, G., Paranhos, R., Rezende, C.E., de Moura, R.L., et al. 2012. Abrolhos Bank Reef health evaluated by means of water quality, microbial diversity, benthic cover, and fish biomass data. PLoS One, **7**(6): e36687. doi:10.1371/journal.pone.0036687. PMID:22679480.

Buckling, A., Kassen, R., Bell, G., and Rainey, P.B. 2000. Disturbance and diversity in experimental microcosms. Nature, **408**(6815): 961–964. doi:10.1038/35050080. PMID:11140680.

Cardinale, M., Brusetti, L., Quatrini, P., Borin, S., Puglia, A.M., Rizzi, A., et al. 2004. Comparison of different primer sets for use in automated ribosomal intergenic spacer analysis of complex bacterial communities. Appl. Environ. Microbiol. **70**(10): 6147–6156. doi:10.1128/AEM.70.10.6147-6156.2004. PMID: 15466561.

Catania, V., Santisi, S., Signa, G., Vizzini, S., Mazzola, A., Cappello, S., et al. 2015. Intrinsic bioremediation potential of a chronically polluted marine coastal area. Mar. Pollut. Bull. **99**(1–2): 138–149. doi:10.1016/j.marpolbul.2015.07.042. PMID:26248825.

Chen, H., Athar, R., Zheng, G., and Williams, H.N. 2011. Prey bacteria shape the community structure of their predators. ISME J. **5**: 1314–1322. doi:10.1038/ismej.2011.4. PMID:21326335.

Cole, J.R., Wang, Q., Fish, J.A., Chai, B., McGarrell, D.M., Sun, Y., et al. 2014. Ribosomal Database Project: data and tools for high throughput rRNA analysis. Nucleic Acids Res. **42**: D633–D642. doi:10.1093/nar/gkt1244. PMID:24288368.

Coll, M., Piroddi, C., Steenbeek, J., Kaschner, K., Ben, Rais, Lasram, F., Aguzzi, J., et al. 2010. The biodiversity of the Mediterranean Sea: estimates, patterns, and threats. PLoS One, **5**(8): e11842. doi:10.1371/journal.pone.0011842. PMID:20689844.

Crossman, L.C., Chen, H., Cerdeño-Tárraga, A.M., Brooks, K., Quail, M.A., Pineiro, A.S., et al. 2013. A small predatory core genome in the divergent marine *Bacteriovorax marinus* SJ and the terrestrial *Bdellovibrio bacteriovorus*. ISME J. **7**(1): 148–160. doi:10.1038/ismej.2012.90. PMID:22955231.

De Pasquale, C., Palazzolo, E., Lo Piccolo, L., and Quatrini, P. 2012. Degradation of long chain *n*-alkanes in soil microcosms by two Actinobacteria. J. Environ. Sci. Health, Part A: Toxic/Hazard. Subst. Environ. Eng. **47**(3): 374–381. doi:10.1080/10934529.2012.645786. PMID:22320689.

Di Leonardo, R., Vizzini, S., Bellanca, A., and Mazzola, A. 2009. Sedimentary record of anthropogenic contaminants (trace metals and PAHs) and organic matter in a Mediterranean

PROVIDED FOR NON-COMMERCIAL RESEARCH USE AND EDUCATION USE. NOT FOR REPRODUCTION, DISTRIBUTION OR COMMERCIAL USE.

coastal area (Gulf of Palermo, Italy). J. Mar. Syst. **78**: 136–145. doi:10.1016/j.jmarsys.2009.04.004.

Dishaw, L.J., Flores-Torres, J., Lax, S., Gemayel, K., Leigh, B., Melillo, D., et al. 2014. The gut of geographically disparate *Ciona intestinalis* harbors a core microbiota. PLoS One, **9**(4): e93386. doi:10.1371/journal.pone.0093386. PMID:24695540.

Dudášová, H., Lukáčová, L., Murínová, S., Puškárová, A., Pangallo, D., and Dercová, K. 2014. Bacterial strains isolated from PCB-contaminated sediments and their use for bioaugmentation strategy in microcosms. J. Basic Microbiol. **54**(4): 253–260. doi:10.1002/jobm.201200369. PMID:23553615.

Fraschetti, S., Terlizzi, A., Micheli, F., Benedetti-Cecchi, L., and Boero, F. 2002. Marine protected areas in the Mediterranean Sea: objectives, effectiveness and monitoring. Mar. Ecol. **23**(s1): 190–200. doi:10.1111/j.1439-0485.2002.tb00018.x.

Ganesh Kumar, A., Vijayakumar, L., Joshi, G., Magesh, Peter, D., Dharani, G., and Kirubagaran, R. 2014. Biodegradation of complex hydrocarbons in spent engine oil by novel bacterial consortium isolated from deep sea sediment. Bioresour. Technol. **170**: 556–564. doi:10.1016/j.biortech.2014.08.008. PMID:25171211.

Hammer, Ø., Harper, D.A.T., and Ryan, P.D. 2001. PAST: Paleontological statistics software package for education and data analysis. Palaeontologia Electronica. Available from http://palaeo-electronica.org/2001_1/past/issue1_01.htm.

Head, I.M., Jones, D.M., and Röling, W.F. 2006. Marine microorganisms make a meal of oil. Nat. Rev. Microbiol. **4**(3): 173–182. doi:10.1038/nrmicro1348. PMID:16489346.

Jameson, S.C., Tupper, M.H., and Ridley, J.M. 2002. The three screen doors: Can marine "protected" areas be effective? Mar. Pollut. Bull. **44**: 1177–1183. doi:10.1016/S0025-326X(02)00258-8. PMID:12523516.

Johnson, J.E., and Hill, R.T. 2003. Sediment microbes of deep-sea bioherms on the northwest shelf of Australia. Microb. Ecol. **46**(1): 55–61. doi:10.1007/s00248-002-2031-y. PMID:12739077.

Jung, J., Baek, J.H., and Park, W. 2010. Complete genome sequence of the diesel-degrading *Acinetobacter* sp. strain DR1. J. Bacteriol. **192**(18): 4794–4795. PMID:20639327.

Kemp, P.F., and Aller, J.Y. 2004. Bacterial diversity in aquatic and other environments: what 16S rDNA libraries can tell us. FEMS Microbiol. Ecol. **47**: 161–177. doi:10.1016/S0168-6496(03)00257-5. PMID:19712332.

Kleindienst, S., Herbst, F.A., Stagars, M., von Netzer, F., von Bergen, M., Seifert, J., et al. 2014. Diverse sulfate-reducing bacteria of the *Desulfosarcina*/*Desulfococcus* clade are the key alkane degraders at marine seeps. ISME J. **8**(10): 2029–2044. doi:10.1038/ismej.2014.51. PMID:24722631.

Kostka, J.E., Prakash, O., Overholt, W.A., Green, S.J., Freyer, G., Canion, A., et al. 2011. Hydrocarbon-degrading bacteria and the bacterial community response in Gulf of Mexico beach sands impacted by the Deepwater Horizon oil spill. Appl. Environ. Microbiol. **77**(22): 7962–7974. doi:10.1128/AEM.05402-11. PMID:21948834.

Krause, S., Le Roux, X., Niklaus, P.A., Van Bodegom, P.M., Lennon, J.T., Bertilsson, S., et al. 2014. Trait-based approaches for understanding microbial biodiversity and ecosystem functioning. Front Microbiol. **5**: 251. doi:10.3389/fmicb.2014.00251. PMID:24904563.

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. Mar. Pollut. Bull. **90**: 160–166. doi:10.1016/j.marpolbul.2014.11.001. PMID:25467874.

Lo Piccolo, L., De Pasquale, C., Fodale, R., Puglia, A.M., and Quatrini, P. 2011. Involvement of an alkane hydroxylase system of *Gordonia* sp. strain SoCg in degradation of solid n-alkanes. Appl. Environ. Microbiol. **77**(4): 1204–1213. PMID:21183636.

McGenity, T.J., Folwell, B.D., McKew, B.A., and Sanni, G.O. 2012. Marine crude-oil biodegradation: a central role for interspecies interactions. Aquat. Biosyst. **8**(1): 10. doi:10.1186/2046-9063-8-10. PMID:22591596.

Muyzer, G., de Waal, E.C., and Uitterlinden, A.G. 1993. Profiling of complex microbial populations by denaturing gradient gel electrophoresis analysis of polymerase chain reaction amplified genes encoding for 16S rRNA. Appl. Environ. Microbiol. **59**: 695–700. PMID:7683183.

Penn, K., Wu, D., Eisen, J.A., and Ward, N. 2006. Characterization of bacterial communities associated with deep-sea corals on Gulf of Alaska seamounts. Appl. Environ. Microbiol. **72**(2): 1680–1683. doi:10.1128/AEM.72.2.1680-1683.2006. PMID:16461727.

Perra, G., Pozo, K., Guerranti, C., Lazzeri, D., Volpi, V., Corsolini, S., and 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): 874–877. doi:10.1016/j.marpolbul.2011.01.023. 21310440.

Pontes, D.S., Lima-Bittencourt, C.I., Chartone-Souza, E., and Amaral, Nascimento, A.M. 2007. Molecular approaches: advantages and artifacts in assessing bacterial diversity. J. Ind. Microbiol. Biotechnol. **34**(7): 463–473. doi:10.1007/s10295-007-0219-3. PMID:17476541.

Quatrini, P., Scaglione, G., De Pasquale, C., Riela, S., and Puglia, A.M. 2008. Isolation of Gram positive *n*-alkane degraders from a hydrocarbon contaminated Mediterranean shoreline. J. Appl. Microbiol. **104**: 251–259. PMID:17922832.

Sambrook, J., Fritsch, E.F., and Maniatis, T. 1989. Molecular cloning, a laboratory manual. 2nd ed. Cold Spring Harbor Laboratory Press, New York, USA.

Singh, A., Sreenivas, A., Sathyanarayana Reddy, G., Pinnaka, A.K., and 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–e00714. PMID:25059877.

Weisburg, W.G., Barns, S.M., Pelletier, D.A., and Lane, D.J. 1991. 16S ribosomal DNA amplification for phylogenetic study. J. Bacteriol. **173**: 697–703. doi:10.1128/jb.173.2.697-703.1991. PMID:1987160.

Yeung, C.W., Lee, K., Whyte, L.G., and Greer, C.W. 2010. Microbial community characterization of the Gully: a marine protected area. Can. J. Microbiol. **56**(5): 421–431. doi:10.1139/W10-028. PMID:20555404.

e·lab PROVIDED FOR NON-COMMERCIAL RESEARCH USE AND EDUCATION USE. NOT FOR REPRODUCTION, DISTRIBUTION OR COMMERCIAL USE.

Copyright of Canadian Journal of Microbiology is the property of Canadian Science Publishing and its content may not be copied or emailed to multiple sites or posted to a listserv without the copyright holder's express written permission. However, users may print, download, or email articles for individual use.