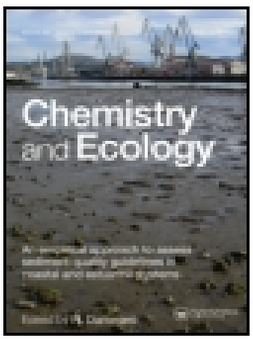

Chemistry and Ecology

ISSN: 0275-7540 (Print) 1029-0370 (Online) Journal homepage: http://www.tandfonline.com/loi/gche20

Presence of hydrocarbon-degrading bacteria in the gills of mussel Mytilus galloprovincialis in a contaminated environment: a mesoscale simulation study

Simone Cappello , Daniela Russo , Santina Santisi , Rosario Calogero , Christoph Gertler , Francesca Crisafi , Mariolina De Domenico & Michail M. Yakimov

To cite this article: Simone Cappello , Daniela Russo , Santina Santisi , Rosario Calogero , Christoph Gertler , Francesca Crisafi , Mariolina De Domenico & Michail M. Yakimov (2012) Presence of hydrocarbon-degrading bacteria in the gills of mussel Mytilus galloprovincialis in a contaminated environment: a mesoscale simulation study, Chemistry and Ecology, 28:3, 239-252, DOI: 10.1080/02757540.2011.639768

To link to this article: http://dx.doi.org/10.1080/02757540.2011.639768

Published online: 23 Dec 2011.

Chemistry and Ecology
Vol. 28, No. 3, June 2012, 239–252

Taylor & Francis
Taylor & Francis Group

Presence of hydrocarbon-degrading bacteria in the gills of mussel *Mytilus galloprovincialis* in a contaminated environment: a mesoscale simulation study

Simone Cappello[a]†, Daniela Russo[a,b]*†, Santina Santisi[a,b], Rosario Calogero[a,b], Christoph Gertler[c], Francesca Crisafi[a], Mariolina De Domenico[b] and Michail M. Yakimov[a]

[a]*Istituto per l'Ambiente Marino Costiero (IAMC) – CNR UOS, Messina, Italy;* [b]*Università degli Studi di Messina, Dip. Biologia Animale ed Ecologia Marina, Messina, Italy;* [c]*School of Biological Sciences, Bangor University, Gwynedd, UK*

(*Received 21 March 2011; final version received 8 November 2011*)

A number of previous studies have shown that the relationships of symbiosis existing between mussels and microorganisms are directly dependent on the environmental conditions. However, little is known about existing relationships between mussels and bacteria in hydrocarbon-impacted marine environments. The aim of this preliminary study is to investigate the presence of oil-degrading bacteria in the mussel *Mytilus galloprovincialis* during growth in polluted ecosystems. All the experiments were carried out in a mesocosm system designed to simulate chronic pollution and to enable direct exposure of mussels to chemicals. Quantitative (4′,6-diamidino-2-phenylindole, colony-forming units, Most Probable Number) analyses and screening (presence/absence) of metabolic functional genes were performed to analyse bacterial populations inside the gills of mussels exposed and not exposed to hydrocarbons. The data obtained show that the presence of hydrocarbons affected the abundance of bacteria inside the gills of specimens and determines selection for specific (hydrocarbon-degrading) bacteria (i.e. *Alcanivorax* sp. and *Marinobacter* sp.). However, is not yet clear whether the presence of such genera of bacteria inside the mussel is due to symbiosis or as a result of filtration.

Keywords: *Mytilus galloprovincialis*; hydrocarbonoclastic bacteria; symbiont; *Alcanivorax*; *Marinobacter*

1. Introduction

Petroleum hydrocarbons are the most widespread contaminants in the environment [1], entering into aquatic environments due to catastrophic accidents (shipping disasters or pipeline failures), chronic pollution (ships, ports, oil terminals, freshwater run-off, rivers and sewage systems), natural oil seepages and natural sources (biota) [2].

Marine organisms can take up contaminants from bottom sediments, suspended particulate material and food sources. Incorporation rates depend on both the availability of the contaminants and also on several biotic and abiotic factors. Bivalves are ecologically important members of

*Corresponding author. Email: darusso@unime.it
†Simone Cappello and Daniela Russo contributed equally to this work.

ISSN 0275-7540 print/ISSN 1029-0370 online

http://dx.doi.org/10.1080/02757540.2011.639768
http://www.tandfonline.com

coastal and estuarine communities [3]. In polluted environments, indicator organisms such as *Mytilus galloprovincialis* can accumulate hydrophobic contaminants. These bivalves filter large volumes of water to meet their food requirements and accumulate dissolved oil components and particles containing hydrocarbons present in oil-polluted water columns. By contrast, active suspension feeders with powerful water-filtration mechanisms (e.g. bivalve molluscs) may also affect the levels of the bacterioplankton in the water column [4]. In nearly all polluted marine environments, hydrocarbon-degrading bacteria [5–7] are able to multiply and degrade even in complex hydrocarbon mixtures such as crude oil. In fact, given a supply of essential nutrients (as nitrogen and phosphorous), these bacteria can significantly decrease the amount of pollutants [7–10]. The relationship between heterotrophic and hydrocarbon-degrading bacteria and benthic filter feeders may be functionally important to aquatic ecosystems, especially polluted ones. Several studies have shown that the mussels' filter-feeding activity may exert control on microbial communities in terms of both abundance and biodiversity [11]. No study to date has investigated the relationship between hydrocarbon-degrading bacteria and mussels in a polluted environment.

This study represents a first approach to investigate the presence of hydrocarbon-degrading bacteria in *M. galloprovincialis* during growth in polluted ecosystems.

Quantitative analyses of heterotrophic and hydrocarbon-degrading bacteria [4′,6-diamidino-2-phenylindole (DAPI), colony-forming units (CFU), Most Probable Number (MPN)] and screening (presence/absence) of metabolic functional genes were performed in both seawater samples and mussels collected (for 35 days; 5 weeks) from a mesocosm system. Hydrocarbon-degrading bacteria in seawater and mussels were investigated and compared in both contaminated and pristine mesocosms.

2. Materials and methods

2.1. *Sampling and storage*

Mytilus galloprovincialis specimens were harvested in September 2008 from Lake Faro (38°15′59.95″N; 15°38′19.56″E), located on the north-eastern point of Sicily (Messina, Italy). Lake Faro is a meromictic lake with a surface area of 0.263 km^2 and reaches a maximum depth of ∼30 m at its centre. GC-MS analysis was used to reveal the presence of chemicals in lake water (data not shown). Mussels were collected by hand and transported to the laboratory within 30 min.

After 3 days housed in deprivation tanks, the organisms were transferred to experimental mesocosms. In total, 150 (±10) organisms were included in each mesocosm.

2.2. *Experimental mesocosms*

The experiments were performed into two rectangular fibreglass tanks of 768 L capacity (120 × 80 × 80 cm). Each mesocosm was filled with 500 L of natural seawater collected directly from the Straits of Messina (Italy).

Before introduction into the mesocosms, seawater was filtered through a 200-μm nylon mesh (Millipore, Milan, Italy) to screen out large metazoans and detritus. To ensure a constant level of water inside the mesocosm tank, the flow of water was controlled by an overflow (a vertical conduct placed on the bottom of the tank) that discharges excess of seawater; the microcosms were filled continuously for the entire experimental period (Figure 1).

Mean water temperature was 16.5–17.5 °C, with daily temperature fluctuations not exceeding 2 °C. Mesocosms were illuminated by a fluorescence lamp, which consisted of four light bulbs (36 W, 80 cm) providing light on a 14/10 h light/dark period.

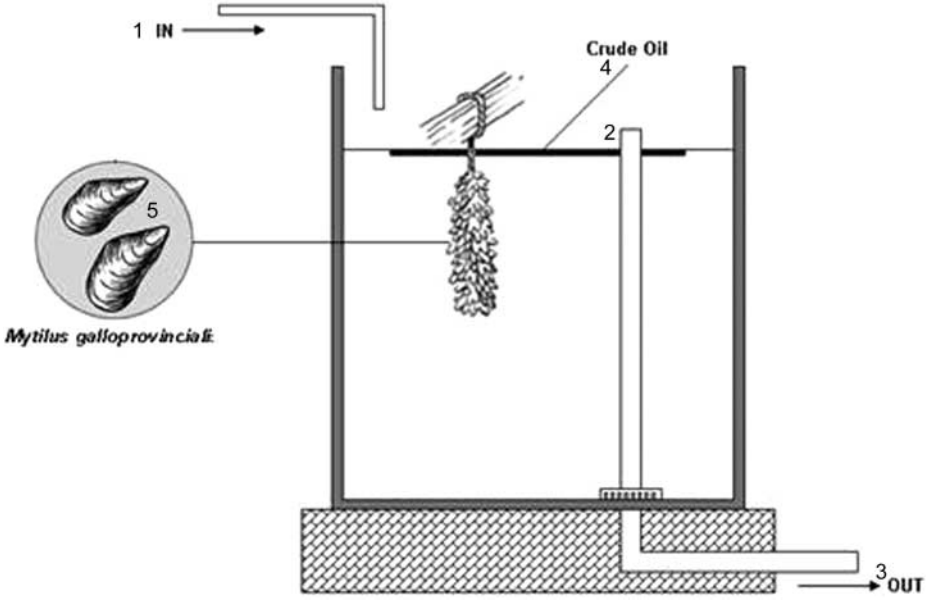

Figure 1. Schematic representation of the microcosm systems used in this study. (1) Tube for water filling, (2) overflow system for discharge of excess seawater, (3) system for seawater outflow, (4) crude oil, (5) *Mytilus galloprovincialis*.

The tank containing natural seawater without crude oil is referred to as mesocosm 'SW' and a second tank containing natural seawater spiked with 500 mL ($\sim$13 mg·L^{-1}) of Arabian Light Crude Oil is referred to as mesocosm 'OIL'.

2.3. *Sampling strategy and surveyed parameters*

The total duration of the experiment was 5 weeks (35 days). To monitor the dynamics of bacterial populations in seawater and the gills of mussels, 500 mL of seawater and/or 10 organisms, were taken aseptically, respectively, from each mesocosm (SW and OIL) every week (T_1, T_2, T_3, T_4 and T_5) from the time of oil spiking (time zero). Numbers of total bacteria (DAPI), cultivable bacteria (CFU) and MPN, as well as microbial metabolic activity (screening of functional genes) were measured.

All mussels analysed in this study were cleaned under running tap water with a stiff brush and aseptically shucked using a sterilised knife. After dissection, all gill samples were washed four times with 0.2-μm-filtered and autoclaved seawater to remove any loosely associated microbes.

Gills were processed immediately for microbial cell counts and DNA/RNA extraction. With the exception of the screening for bacterial functional genes, all parameters detected were measured in triplicate.

2.4. *Bacterial counts*

Samples of gill tissue were thawed and gently ground in 10% formaldehyde (Sigma-Aldrich, Milan, Italy) in artificial seawater using a ground-glass tissue grinder. After appropriate dilution, aliquots of the homogenates were stained with 1 mg·L^{-1} DAPI (Sigma-Aldrich) for 5 min and collected on a black, 0.2-μm Nuclepore filter (Millipore, Milan, Italy). The abundance of bacteria, expressed as the number of cells·g^{-1}, was determined by epifluorescence counting.

Bacteria present in samples of seawater were analysed and measured, as described by Cappello et al. [3] and Hueh and Chan [12], these results were expressed as the number of cells·mL^{-1}.

2.5. *Heterotrophic cultivable bacteria*

Heterotrophic culturable bacteria in both mussel gills and seawater were estimated by spreading 100 μL of 10-fold dilutions of either gill homogenate or seawater on plates of Marine Agar 2216 medium (Difco, Milan, Italy), followed by incubation at 20 °C for 7 days. The results obtained for bacteria from mussel gills were expressed as $CFU \cdot g^{-1}$; the results for heterotrophic cultivable bacteria in seawater were expressed as $CFU \cdot mL^{-1}$.

2.6. *Cultivable hydrocarbon-degrading bacteria*

Culturable oil-degrading bacteria present in seawater and mussel gills were estimated by spreading 100 μL of 10-fold dilutions of seawater or gill homogenate on plates of ONR7a medium [8], and incubating at 20 °C for 7 days. The results obtained for culturable bacteria from mussel gills were expressed as $CFU_{ONR7a} \cdot g^{-1}$: the results for culturable hydrocarbon-degrading bacteria from seawater were expressed as $CFU_{ONR7a} \cdot mL^{-1}$.

Samples of homogenate and seawater were spread simultaneously on plates of ONR7a medium with and without exposure to sterile crude oil as the only source of carbon and energy. This cultivation step is essential for the identification of obligate hydrocarbon-degrading bacteria.

2.7. *Most Probable Number*

Hydrocarbon-degrading bacteria in mussel gills and seawater were enumerated using a miniaturised MPN method, as described by Cappello et al. [3] and Brown et al. [13]. A 'five-tube' MPN was performed using sterile 24-well (3 mL each) tissue culture test plates containing 2 mL of sterile Bushnell–Hass (B–H) medium (Difco) with the addition of NaCl to final concentration of 2% (w/v) (pH 7.0) per well. Tenfold serial dilutions of samples (10^{-2} to 10^{-9}) were prepared in B–H medium. Wells were inoculated with 100 μL of sample dilutions. Following sample inoculation, 10 μL of sterile Arabian Light Crude Oil was applied at the centre of each well. The remaining four wells of each plate were used as a reference (B–H medium + oil). Plates were incubated at 20 ± 1 °C for 20 days. Wells were scored as positive when oil emulsification was clearly indicated by the disruption of the oil film and/or the oil 'ring' produced on the surface of the medium in contact with the wall of the well, accompanied by changes in the oil colour. Uncertain positive scores were confirmed by an emulsification test, performed by inoculating 100 μL of the well contents in a 15 × 150 mm test tube with 10 mL of the sterile culture medium, addition of 20 μL of sterile oil and incubation at 20 °C for 15 days in a thermostat under agitation. The MPN index was calculated according to American Public Health Association.

2.8. *RNA extraction*

Extraction of RNA from mussel gill samples was performed using a DNeasy Tissue kit (QIAGEN, Valencia, CA, USA) according to the manufacturer's protocol. Extraction of RNA from seawater samples was performed using a DNA/RNA extraction kit (QIAGEN).

2.9. *Reverse transcription and polymerase chain reaction amplification*

In order to be able to remove any genomic DNA contamination all samples were treated with DNase I [14]. Heteroduplex rRNA–crDNA was synthesised by reverse transcription (RT) using random hexamer primers and SuperScript II RNase H-free reverse transcriptase (Life Technologies Inc., Rockville, MD, USA) according to Yakimov et al. [15]. The crDNA end product was used

as the template for a standard amplification with a polymerase chain reaction (PCR). Possible DNA contamination of RNA templates was routinely monitored by PCR amplification of aliquots of RNA that were not reverse transcribed; no contaminating DNA was detected in any of these reactions.

Primers used for PCR included (1) the universal reverse primer (Uni_1492R) and 16S rRNA forward domain-specific bacteria, Bac27_F (5′-AGAGTTTGATCCTGGCTCAG-3′), [16]; (2) the primer for the functional gene for alkane hydroxylases of *Alcanivorax borkumensis*, AlkB1-R (5′-GCTTAGGAACAACGGTTCAGG-3′) and AlkB1-F (5′-AATTGGCCTATATCTC GTA-3′); (3) the primer for the functional gene for alkane hydroxylases of *Thalassolituus oleivorans*, AlkB-ThlR342 (5′-GGGCCATACAGAGCAAGCAA-3′) and Alkb-ThlF125 (5′-GACGTCGCCACA CCTGCC-3′); and (4) the primer for the functional gene for the biphenyl 2,3-dioxygenase of *Pseudomonas* sp. and *Rhodococcus* sp., Bph-R (5′-GCNGCRAAYTTCCARTTRCANGG-3′) and Bph-F (5′-TGCAGCTACCACGGCTGGGCCTA-3′).

PCR (initial denaturation: 5 min hot-start at 95 °C; 1 min at 94 °C, 1 min at 50 °C, for universal primers, or 1 min at 60 °C for functional primers; 2 min at 72 °C, 30 cycles; extension: 10 min at 72 °C) was performed with GeneAmp 5700 (PE Applied Biosystems, Foster City, CA, USA), using a 50 μL (total volume) mixture containing 10 μL Q solution (Qiagen, Hilden, Germany), 5 μL Qiagen reaction buffer, 1 μM of each forward and reverse primer, 10 μM dNTPs (Gibco, Invitrogen Co., Carlsbad, CA, USA), 2.0 mL (50–100 ng) of template and 2.0 U of Qiagen *Taq* pDNA polymerase (Qiagen).

2.10. *Taxonomic characterisation of isolates from mussel gills*

Bacteria obtained from spreading homogenate of mussel gills in ONR7a with oil as the carbon and energy source were isolated with regard to different colony morphology. 16S rDNA data were analysed to perform taxonomic characterisation of the isolated strains. Total DNA extraction of bacterial strains was performed with the CTAB method [5]. The 16S rDNA loci were amplified using one primer pair, the above reverse primer (Uni_1492R) and 16S rRNA forward domain-specific bacteria, Bac27_F, [16]. PCR amplification was performed in a total volume of 50 mL, as described above. The 16S rDNA amplified was sequenced with a BigDye Terminator v3.1 cycle sequencing kit on an automated capillary sequencer (model 3100 Avant Genetic Analyzer, Applied Biosystems). Analysis and phylogenetic affiliates of sequences was performed as described before [7,9,15]. The sequences similarity of individual inserts was analysed with the program FASTA Nucleotide Database Query available through the EMBL–European Bioinformatics Institute.

2.11. *Statistical analysis*

Statistically significant differences between data (DAPI count, CFU count, MPN) obtained from seawater and mussels of control (SW) and oil-polluted microcosms (OIL) were evaluated by analysis of variance (ANOVA).

3. Results

3.1. *Total bacterial abundance*

The total bacterial density in the seawater mesocosm (SW) remained constant over the course of the experiment, with values similar to the cell counts at the outset (2.5×10^5 mL^{-1}). Conversely, in

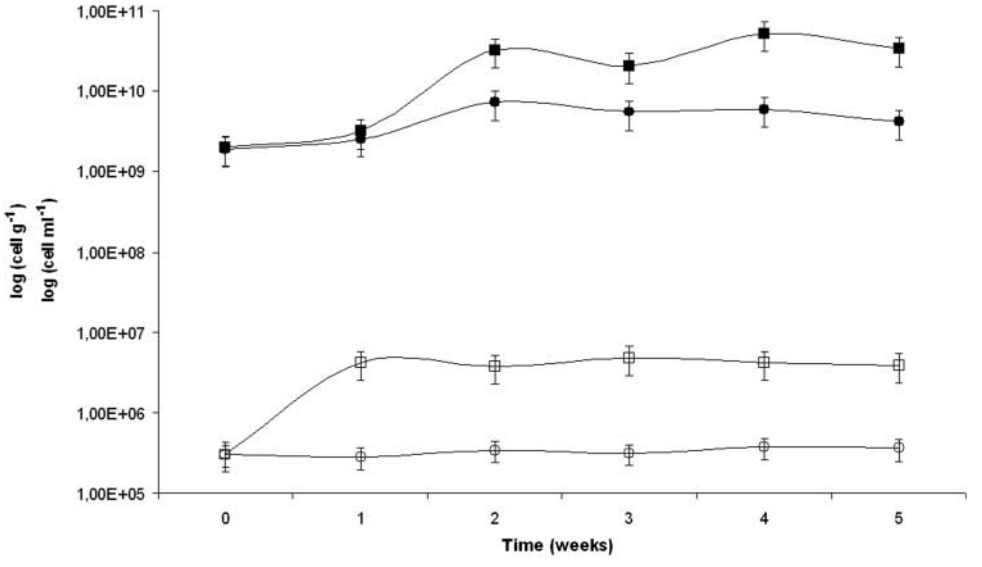

Figure 2. Bacterial abundance determined by DAPI staining. Concentration of the cells observed in the gills of mussels (cell·g^{-1} tissue) during exposure to hydrocarbons (OIL, filled squares) and in the control experiment (SW, filled circles). Bacteria from polluted and unpolluted seawater (cells·mL^{-1}) are depicted by empty squares and circles.

oil-spiked seawater of mesocosm OIL, total bacterial density increased by one order of magnitude in comparison with the beginning of the experiment, and remained constant with a maximum of 3.1×10^6 cells·mL^{-1} for the whole experiment (Figure 2).

Measurements of bacterial abundance within the gills of mussels in the unpolluted microcosm showed a mean value of 10^9 cell·g^{-1}. As can be seen in Figure 2, this value remains constant until the end of the experiment (T_5). This is in stark contrast to cell counts obtained from gills of mussels exposed to oil. Although total bacterial cell counts for the first week of the experiment (T_1) were similar to those found in the seawater control experiment, gills from mussel specimens in the oil-impacted mesocosm showed a general increase of one order of magnitude with values ranging from 3.2×10^9 cell·g^{-1} (T_2) to 3.2×10^{10} cell·g^{-1} (T_3) starting in the second week of the experiment (T_2).

3.2. *Counts of heterotrophic culturable bacteria (CFU)*

Heterotrophic culturable bacteria cell numbers were determined by CFU plate counts on Marine Agar.

In seawater of mesocosm SW, the concentration of heterotrophic culturable bacteria increased from T_0 to T_5 (3.3×10^3 CFU·mL^{-1} and 8.8×10^4 CFU·mL^{-1}, respectively). A onefold increase in CFU was observed in seawater from the oil-spiked mesocosm, where heterotrophic culturable bacteria cell numbers increased to 4.1×10^5 CFU·mL^{-1} after 5 weeks (Figure 3).

Microbial populations on the gills of *M. galloprovincialis* from mesocosm SW were constant ($\sim 10^6$CFU·g^{-1}) over the whole course of this study (Figure 3). The number of CFUs enriched from the gills of *M. galloprovincialis* exposed to the hydrocarbons increased by two orders of magnitude after the first 2 weeks of the experiment, with values ranging from 4.9×10^6 CFU·g^{-1} (T_2) to 2.7×10^7 CFU·g^{-1} (T_3). These values remained constant until the end of the experiment (Figure 3).

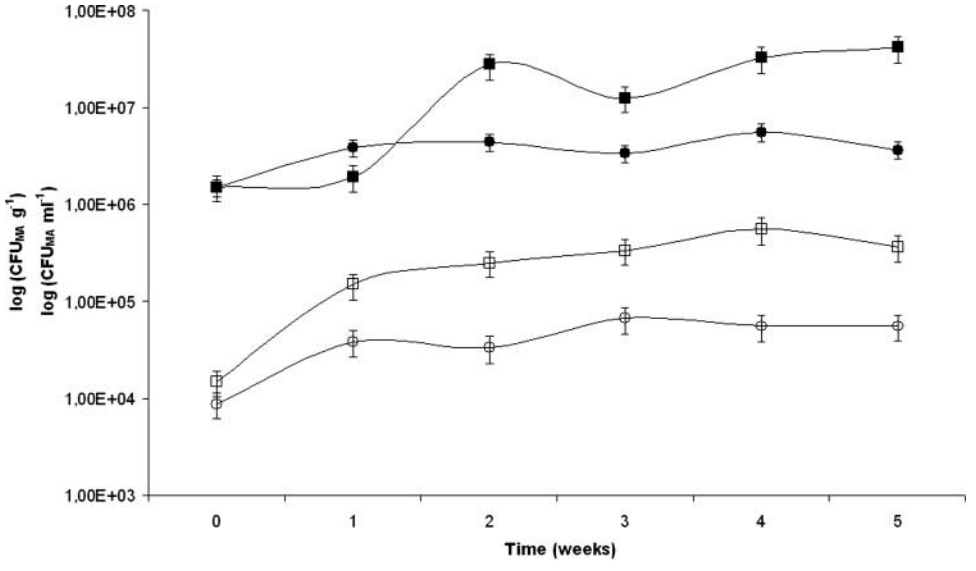

Figure 3. Measure of culturable heterotrophic bacteria on marine agar (CFU). The numbers of CFU·g^{-1} found in gills of mussels from polluted and unpolluted systems are indicated as squares and filled circles, respectively. The CFU·mL^{-1} of water for SW and OIL mesocosms are indicated by empty squares and circles, respectively.

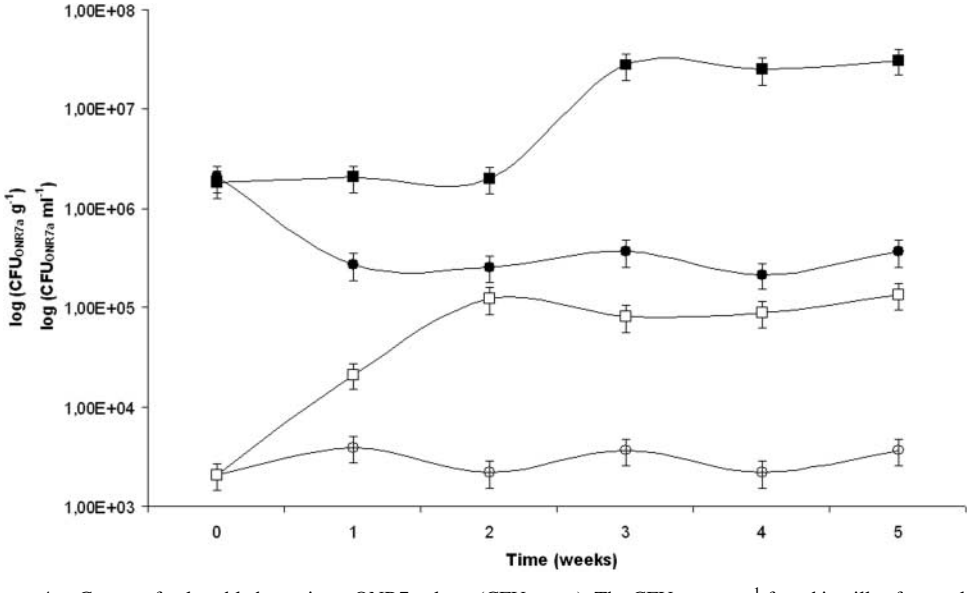

Figure 4. Counts of culturable bacteria on ONR7a plates (CFU_{ONR7a}). The CFU_{ONR7a}·g^{-1} found in gills of mussels in polluted (OIL) and unpolluted (SW) mesocosms are shown by filled squares and circles, respectively. The CFU_{ONR7a}·mL^{-1} of water from SW and OIL mesocosms are shown by empty squares and circles, respectively.

3.3. *Counts of the culturable bacteria on ONR7a (CFU_{ONR7a})*

As can be seen in Figure 4, the abundance of culturable hydrocarbon-degrading bacteria in the SW mesocosm remained constant over the whole experiment ($\sim 10^3$ CFU_{ONR7a}·mL^{-1}). An increase in CFU_{ONR7a} was observed in seawater from the polluted mesocosm (OIL). Values of 4.21×10^5 CFU_{ONR7a}·mL^{-1} were detected after 5 weeks (T_5).

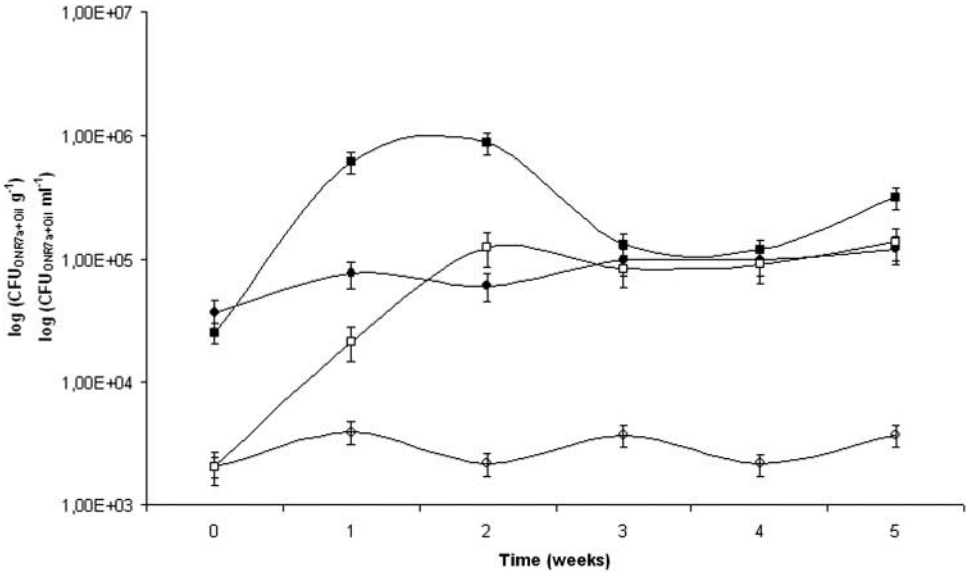

Figure 5. Count of culturable bacteria grown on ONR7a plates amended with Arabian Light Crude Oil ($CFU_{ONR7a+OIL}$). The $CFU_{ONR7a+OIL}$ in gills of mussels collected from polluted (OIL) and unpolluted (SW) mesocosms are shown by squares and circles filled, respectively, and were expressed as $CFU_{ONR7a+OIL}\cdot g^{-1}$. The $CFU_{ONR7a+OIL}\cdot mL^{-1}$ of water from OIL and SW mesocosms are shown by empty squares and circles, respectively.

In the SW mesocosm, the abundance of colony-forming bacteria inside the mussel gills decreased by one order of magnitude, with values ranging from 2.70×10^6 $CFU_{ONR7a}\cdot g^{-1}$ (T_0) to 2.72×10^5 $CFU_{ONR7a}\cdot g^{-1}$ (T_1). These values ($\sim 10^5$ $CFU_{ONR7a}\cdot g^{-1}$) remained constant until the end of the experiment. In the gills of *M. galloprovincialis* exposed to hydrocarbons (mesocosm OIL), the numbers of colony-forming oil-degrading microbes showed average values of 10^6 $CFU_{ONR7a}\cdot g^{-1}$ in the first 2 weeks of the experiment. However, these values increase by one order of magnitude in the third week, with values ranging from 2×10^6 $CFU_{ONR7a}\cdot g^{-1}$ (T_2) to 2.78×10^7 $CFU_{ONR7a}\cdot g^{-1}$ (T_3). As shown in Figure 4, these values remained stable until the end of the experiment.

3.4. *Calculation of the culturable bacteria on ONR7a with oil ($CFU_{ONR7a+OIL}$)*

In the control mesocosm (SW), the abundance of oil-degrading bacteria was stable over the whole course of the experiment, whereas a twofold increase in $CFU_{ONR7a+OIL}$ was observed after 5 weeks in the OIL mesocosm, with final values of 1.9×10^5 $CFU_{ONR7a+OIL}\cdot mL^{-1}$.

Counts of oil-degrading culturable bacteria in mussel gills from the unpolluted mesocosm (SW) were $\sim 10^4$ $CFU_{ONR7a+OIL}\cdot g^{-1}$ for the entire experiment. In the OIL mesocosm, an increase in these bacteria was observed during the first week (from 2.5×10^4 to 6×10^5 $CFU_{ONR7a+OIL}\cdot g^{-1}$) and numbers remained constant for the whole study (Figure 5).

3.5. *Most Probable Number*

The numbers of hydrocarbon-degrading bacteria were evaluated using the MPN method performed on B–H broth amended with 2% (w/v) of NaCl.

In samples of seawater collected from mesocosms SW and OIL, the populations of hydrocarbon-degrading bacteria (MPN) were constant to the end of the experiments and reached

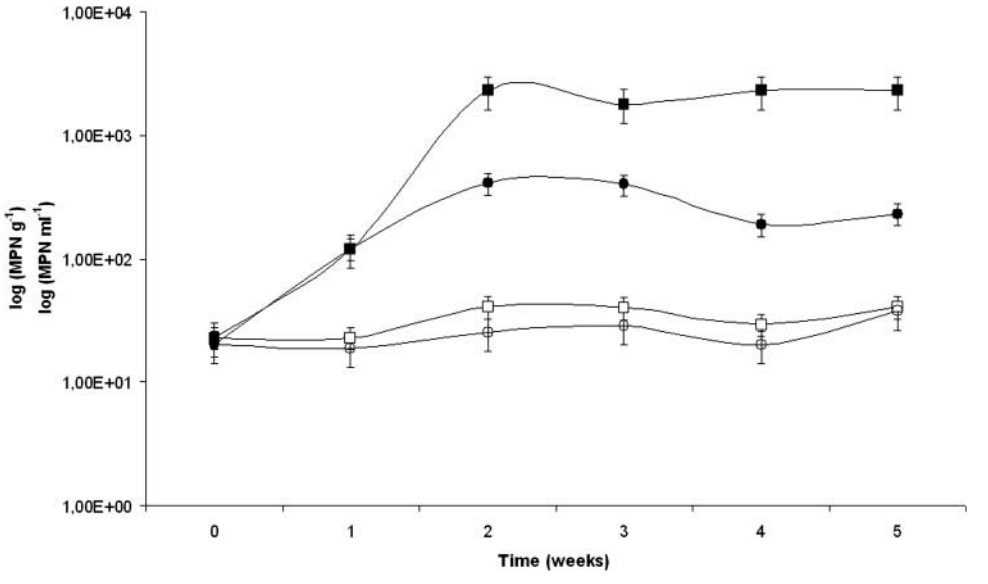

Figure 6. MPN of bacteria from seawater and mussel gills are displayed as MPN·mL^{-1} and MPN·g^{-1}, respectively. MPN observed in mussel gills of OIL and SW microcosms are indicated by filled squares and circles, respectively. Bacteria present in polluted and unpolluted seawater are depicted by empty squares and circles, respectively.

3.2×10^1 MPN·mL^{-1} (Figure 6). In mussels obtained from the unpolluted tank, values were 10^{-1} MPN·g^{-1} and were stable to the end of the experiment. The MPN in bacteria enriched from the gills of *M. galloprovincialis* in the polluted mesocosm increased from 2.3×10^3 MPN·g^{-1} (T_0) to 1.20×10^2 MPN·g^{-1} and 2.3×10^3 MPN·g^{-1}, respectively, after the first and the second week of the experiment (T_1 and T_2). These values ($\sim 10^3$ MPN·g^{-1}), remained constant to the end of the experiment (T_5) (Figure 6).

3.6. *Screening of functional genes*

Screening (presence/absence) of gene expression linked to hydrocarbon degradation was performed on bacterial crDNA extracted from seawater and mussel gills in both unpolluted and polluted mesocosms. As shown in Table 1, the PCR amplification using universal bacterial primers (27F-1492R) gave positive results for all samples obtained in this study. In seawater samples collected from the unpolluted mesocosm (SW), no positive amplification was detected for any of the

Table 1. Expression of functional genes of the microbial population present in seawater and from the gills of mussels during mesocosm (SW and OIL) experiments.

	Non-polluted microcosm (Microcosm SW)						Polluted microcosm (Microcosm OIL)					
						Seawater						
16 crDNA	+	+	+	+	+	+	+	+	+	+	+	+
AlkB-1	−	−	−	−	−	−	+	+	+	+	+	
AlkB-Thal	−	−	−	−	−	−	−	+	+	−	−	−
BHF	−	−	−	−	−	−	−	−	−	−	−	−
						Gills						
16 crDNA	+	+	+	+	+	+	+	+	+	+	+	+
AlkB-1	−	−	−	−	−	−	−	−	−	−	−	−
AlkB-Thal	−	−	−	−	−	−	−	−	−	−	−	−
BHF	−	−	−	−	−	−	−	−	−	−	−	−

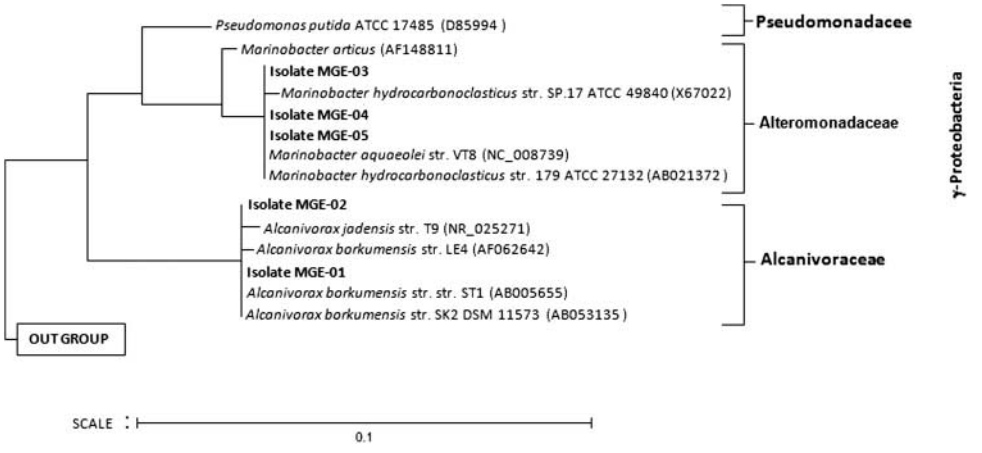

Figure 7. Phylogenetic tree of 16S rRNA sequences of the bacteria isolates from gills of mussels (cultivation in plate of ONR7a with Arabian Light Crude Oil). The tree was constructed using sequences of a comparable region of the 16S rRNA gene sequences available in public databases. Neighbour-joining analysis using 1000 bootstrap replicates was used to infer tree topology. The bar represents 0.1% sequence divergence.

functional genes analysed; by contrast, in seawater samples collected from the polluted mesocosm (OIL), positive amplification was observed for the alkane monoxygenase gene of *Alcanivorax* sp. After 2 weeks of incubation (T_2 and T_3), positive amplification of the *Thalassolituus* sp. alkane monoxygenase gene was observed. By contrast, no amplification was registered for biphenyl monoxigenase of *Pseudomonas* sp. and *Rhodococcus* sp. (BHP) in any sample within this study.

As shown in Figure 7, in samples of gills from both unpolluted and polluted systems, no positive PCR amplification was obtained when the primers for the amplification of the specific functional genes in study were used [alkane monoxigenase of *Alcanivorax* sp. (Alkb1); alkane monoxigenase of *Thalassolituus* sp. (Alk-B); biphenyl monoxigenase of *Pseudomonas* sp. and *Rhodococcus* sp. (BHP)].

3.7. *Taxonomic analysis of microbial isolates from mussel gills*

At the end of the experiment (T_5), 32 bacterial strains were isolated from ONR7a plates spiked with crude oil. Of these, 12 strains were isolated from mussels in the unpolluted system and 20 strains were isolated from mussels exposed to the oil. All of the isolates were chosen based on variations in colony morphology. To determine the capacity to degrade hydrocarbons, these bacteria were replated on ONR7a with and without oil. All bacteria obtained from the SW mesocosm were capable of growing in both the presence or absence of crude oil. Conversely, only 5 of the 20 strains obtained from the OIL mesocosm showed exclusive capacity to grow in the presence of hydrocarbons. The five isolates were identified as MGE-01, -02, -03, -04 and -05. As shown in Figure 7, phylogenetic analysis of 16S crDNA showed that all isolates to belong to the γ-Proteobacteria. Two of these strains (MGE-01 and -02) were related to the genus *Alcanivorax* (98 and 99% identity, respectively) and three strains (MGE-03, -04 and -05) were related to the genus *Marinobacter hydrocarbonoclasticus* (100, 98 and 100% identity, respectively).

4. Discussion

In recent years, there has been growing interest in the study of marine organisms living in oil-impacted ecosystems. However, little research has been conducted on the relationship

between complex metazoan organisms (e.g. mussels) and prokaryotes in polluted ecosystems. This study focuses, for first time, on bacterial abundance on the gills of filter-feeding organisms (*M. galloprovincialis*) during exposure to hydrocarbons.

Several studies reported that under certain environmental conditions, symbiosis between mussels and bacteria can be found in specific structures of the gills named 'bacteriosomes' [17]. The abundance of symbionts in mussels is not species specific, but can vary considerably among mussel populations [18]. Both qualitative and quantitative differences in microbes prevailing in or on mussels are related to the different chemical–physical characteristics of the sites from which these mussels are collected [19].

As reported previously [20,21], mussel filtration activity can affect microbial communities in terms of both abundance and biodiversity. Some studies [11,12,20–22] have shown that mussels are natural habitats for some bacterial species such as *Vibrio*, *Pseudomonas*, *Acinetobacter* and *Aeromonas* [3,23]. However, as stated above, to date, very little research has aimed at understanding the processes of symbiosis between bacteria and mussels in oil-contaminated ecosystems.

The total bacteria (DAPI) counts in this study show that microbial abundance is significantly higher in the gills of mussels extracted from polluted waters than those sampled in unpolluted waters.

This is in accordance with data obtained by Cavallo et al. [4], which showed that the total bacterial concentration detected in the mussels is directly related to bacterial cell counts in water samples, and highlights the ability of *M. galloprovincialis* to accumulate bacteria from the surrounding environment. This hypothesis is also supported by other studies, which revealed the ability of mussels to accumulate bacteria such as *Vibrio* spp. or other potential pathogens [23]. Our data highlight how the abundance of bacteria in the gills of organisms exposed to oil contamination is greater ($\sim 10^{10}$ cell$\cdot$g^{-1}) than that found in mussels not exposed to hydrocarbon pollution ($\sim 10^{6}$ cell$\cdot$g^{-1}).

Similar results were obtained for the abundance of heterotrophic culturable bacteria. The concentration of cells capable of forming colonies (CFU) on plates of Marine Agar was greater than the concentration found in the SW mesocosms. At the beginning of the experiment, the number of CFU obtained from mussels ($\sim 10^{6}$ CFU$\cdot$g^{-1}) was comparable with that obtained in previous studies [3]. In contrast to these findings, the abundance of colony-forming microbes in the gills of the mussels exposed to oil was about five times higher than in the gills of control organisms. The abundance of colony-forming microbes in the mussel gills may depend directly on the different concentrations of bacteria present in the two mesocosms (polluted and unpolluted).

Data acquired in this study indicate a predominance of hydrocarbon-degrading microbes in mussels exposed to oil-polluted seawater. As demonstrated by previous studies [8,9] bacteria capable of degrading hydrocarbons are widely distributed in the marine environment. Therefore, culture-dependent and -independent methods are required for an accurate analysis of the microbial population. Cultivation-based techniques for the identification of microbes are restricted to a small fraction of marine bacteria which are able to grow on artificial medium [24]. By contrast, culture-independent molecular biology techniques (e.g. qualitative/quantitative analysis of functional genes and genomic analysis of 16S DNA) overcome the limitations mentioned above and enable analysis of microbial characteristics regardless of their ability to grow in pure cultures.

One of the aims of this study was to demonstrate the presence of hydrocarbon-degrading bacteria in mussel gills as a direct result of the oil pollution to which these organisms have been exposed. The presence of these bacteria might effectively explain specific processes that lead to an adaptive function required for the mussel's resistance to hydrocarbon contamination.

Further evidence of the increased fraction of bacteria able to degrade hydrocarbons is given by the MPN method. Comparing the data obtained drom gills of non-exposed and exposed mussels, it is possible to observe a 46$\times$ increase in these bacteria in oil-impacted mussels ($\sim 10^{3}$ MPN$\cdot$g^{-1})

compared with controls ($\sim$10 MPN·g^{-1}). However, to verify whether the abundance of oil-degrading microbes on mussel gills is accompanied by microbial oil degradation, the data were backed up by expression analysis of genes related to bacterial degradation of hydrocarbons using crDNA and PCR amplification in combination with specific functional primers to identify the active fraction of microbial population. The primers chosen for expression analysis were specific for the alkane dehydrogenases (or alkane mono-oxygenase) of both *Alcanivorax* sp. (alk-B1) and *Thalassolituus* (alk-B/Thal), because these genes encode central enzymes within the aliphatic hydrocarbon-degradation processes of obligate hydrocarbonoclastic bacteria (OHCB) species, which are both ubiquitous and dominant in oil-contaminated environments. Furthermore, both species were detected in several comparable studies performed at the Messina Strait using a very similar type of crude oil [3,9,15]. In parallel, gene expression analysis of biphenyl dioxygenase (BHP) of *Pseudomonas* and *Rhodococcus* was conducted. These enzymes are essential for the degradation of aromatic fractions. However, PCR amplification for samples of crDNA from the gills of both control and exposed organisms showed negative results (no amplification), which indicates that these genes were not expressed under the chosen experimental conditions or their action was inhibited by co-purified substances. Similar to the gene expression of microbial communities on mussel gills, results obtained from seawater samples in the SW control experiment were negative. In contrast to this, positive PCR amplifications were obtained for the expression of the *alkB* genes for both *Alcanivorax* sp. and *Thalassolituus* sp. in polluted mesocosms (OIL). In all experiments, possible DNA contamination of RNA templates was routinely monitored by PCR amplification of aliquots of RNA that were not reverse transcribed. No contaminating DNA was detected in any of these reactions. Similarly, positive controls of templates were monitored by amplification with the universal reverse primer for 16S rRNA.

Quantitative evaluation of the microbial population (DAPI, CFU and MPN) showed that, although it was possible to identify an increase in the number of hydrocarbon-degrading microbes in the gills of organisms exposed to hydrocarbons, no metabolic activity of bacteria related to the genus *Alcanivorax*, *Thalassolituus*, *Pseudomonas* and *Rhodococcus* could be detected using molecular analysis.

Three hypotheses may explain these results. First, we can assume that the functional primers used in this phase of the study are too specific. Although a population of active hydrocarbon-degrading bacteria in mussel gills could be detected using cultivation-based approaches according to the numbers of microbes cultivated on ONR7a and MPN plate counts, these cells could not be identified by PCR because the primers used were not specific for this population. This would imply that hydrocarbon-degrading microbes present in oil-impacted mussels are different from the OHCB commonly found in oil-polluted seawater, i.e. *Alcanivorax borkumensis* and *Thalassolituus oleivorans*. The second possible explanation for this phenomenon is based on the idea that although a population of hydrocarbon-degrading bacteria was present in the mussel gills, this would be not directly identifiable using molecular screening methods because of a lack of mRNA for the metabolic genes of oil-degrading microbes. In this explanation, hydrocarbonoclastic bacteria are present within the gills of the mussels for an effect of natural water filtration, therefore, although they are accumulated and increasing in number, they do not have a degradative function, are not so metabolically active and therefore cannot be detected using the expression of functional genes.

Both assumptions can be explained by cultivation-based methods. In fact, during the cultivation of hydrocarbon-degrading microbes in oil-impacted mussels (CFU with oil), five isolates which had the specific ability to use oil as a sole source of carbon and energy were obtained. Among these isolates, three were related to the genus of *Alcanivorax* and two were related to genus *Marinobacter*. Because the primers used for gene expression analysis were specific for organisms other than these two species, expression of neither gene could be detected.

A. borkumensis is the predominant free-living oil-degrading microbe in all temperate water bodies. Its presence in seawater at the specific Messina site and using the same type of oil has

been shown previously [3,9,15]. The presence of cultivable bacteria related to the *Alcanivorax* genus and not detected by expression of *alkB* genes in mussel gills – an essential gene for hydrocarbon degradation and thus growth of this microbe – implies that these bacteria may well have been present in the gills in a dormant or starvation state and might have been reactivated upon hydrocarbon availability in seawater during filtration by mussels.

Although these findings indicate the presence of a possible dormant, mussel-associated population of hydrocarbonoclastic microbes, which has wider implications for both mussel farming and the use of mussels as pollution indicators, further investigations are required, especially of mussel populations in chronically polluted areas.

5. Conclusions

The data obtained fulfilled the following objectives of this study: (1) to analyse the relative abundance and diversity of oil-degrading bacteria in the gills of mussels; and (2) to study the correlation between these bacteria and their host in relation to environmental conditions (presence/absence of oil).

In this study, it was possible to demonstrate how the effect of the presence/absence of hydrocarbons may quantitatively influence the abundance of microbial populations within mussel gills. This presence/absence can also influence the selection of bacteria specific for the environmental conditions (presence of oil-degrading bacteria) to which the mussels are subjected.

However, it remains unclear whether a symbiotic process between hydrocarbon-degrading bacteria and filter organisms (mussels) exists or whether the presence of these bacteria in the mussels is a result of filter feeding. To shed more light on this extremely interesting process, further analysis is required, for example, (1) genetic screening using functional primers specific for other microbial groups (e.g. *Marinobacter*), (2) direct study of microbial population structure (e.g. by 16S clones libraries), and (3) the use of techniques for the direct functional identification of mussel-associated bacteria (e.g. fluorescence in situ hybridisation, electronic microscopy of gills, and fluorescent antibodies labelling and fluorescent microscopy).

Acknowledgements

The authors wish to thank Dr Renata Denaro and Maria Genovese of IAMC-CNR of Messina (Italy) for their valuable discussions and Prof. Emilio De Domenico of University of Messina (Italy) for his friendly collaboration/contribution to this article.

References

[1] R. Santas, A. Korda, A. Tenente, K. Buchholz, and P.H. Santas, *Mesocosm assays of oil spill bioremediation with oleophilic fertilizers: Inipol, F1 or both?*, Mar. Pollut. Bull. 38(1) (1999), pp. 44–48.

[2] G.D. Floodgate, *Biodegradation of hydrocarbons in the sea*, in *Water Pollution Microbiology*, R. Mitchell, ed., Wiley-Interscience, New York, 1972, pp. 153–171.

[3] S. Cappello, R. Denaro, M. Genovese, L. Giuliano, and M.M. Yakimov, *Predominant growth of* Alcanivorax *during experiments on 'oil spill bioremediation' in mesocosms*, Microbiol. Res. 162 (2007), pp. 185–190.

[4] R.A. Cavallo, M.I. Acquaviva, and L. Stabili, *Culturable heterotrophic bacteria in seawater and* Mytilus galloprovincialis *from a Mediterranean area (northern Ionian Sea – Italy)*, Environ. Monit. Assess. 149(1) (2008), pp. 465–475.

[5] B. Winnepenninckx, T. Backeljau, and R. De Wachter, *Extraction of high molecular weight DNA from molluscs*, Trends Genet. 9 (1993), p. 407.

[6] S.E. Dyksterhouse, J.P. Gray, R.P. Herwig, J.C. Lara, and J.T. Staley, Cycloclasticus pugetii *gen. nov., sp. nov., an aromatic hydrocarbon-degrading bacterium from marine sediment*, Int. J. Syst. Bacteriol. 45 (1995), pp. 116–123.

[7] M.M. Yakimov, G. Gentile, V. Bruni, S. Cappello, G. D'Auria, P.N. Golyshin, and L. Giuliano, *Crude oil-induced structural shift of coastal bacterial communities of Rod Bay (Terra Nova Bay, Ross Sea, Antarctica) and*

characterization of cultured cold-adapted hydrocarbonoclastic bacteria, FEMS Microbiol. Ecol. 49(3) (2004), pp. 419–432.

[8] W.F.M. Röling, M.G. Milner, D. Martin Jones, F. Fratepietro, R.D.J. Swannell, F. Daniel, and I.M. Head, *Bacterial community dynamics and hydrocarbon degradation during a field-scale evaluation of bioremediation on a mudflat beach contaminated with buried oil*, Appl. Environ. Microbiol. 70 (2004), pp. 2603–2613.

[9] M.M. Yakimov, R. Denaro, M. Genovese, S. Cappello, G. D'Auria, T.N. Chernikova, K.T. Timmis, P.N. Golyshin, and L. Giuliano, *Natural microbial diversity in superficial sediments of Milazzo Harbour (Sicily) and community successions during microcosm enrichment with various hydrocarbons*, Environ. Microbiol. 7(9) (2005), pp. 1426–1441.

[10] S. Cappello and M.M. Yakimov, *Alvanivorax, in Handbook of Hydrocarbon and Lipid Microbiology*, K.N. Timmis, T. McGenity, J.R. van der Meer, and V. de Lorenzo, eds., Springer, Berlin, 2010, pp. 1737–1748.

[11] T. Kiorboe, F. Mohlenberg, and O. Nohr, *Effect of suspended bottom material on growth and energetics in* Mytilus edulis, Mar. Biol. 61 (1981), pp. 283–288.

[12] C.S. Hueh and K.Y. Chan, *Bacteria in bivalve shellfish with special reference to the oyster*, J. Appl. Microb.59 (1985), pp. 41–47.

[13] E.J. Brown and J.F. Braddock, *Sheen Screen, a miniaturized Most-Probable-Number method for enumeration of oil-degrading microorganisms*, Appl. Environ. Microbiol. 56(12) (1990), pp. 3895–3896.

[14] H.J. Mills, R.J. Martinez, S. Story, and P.A. Sobecky, *Identification of members of the metabolically active microbial populations associated with* Beggiatoa *species mat communities from Gulf of Mexico cold-seep sediments*, Appl. Environ. Microbiol. 70(9) (2004), pp. 5447–5458.

[15] M.M. Yakimov, S. Cappello, E. Crisafi, A. Tursi, C. Corsellim, S. Scarfì, and L. Giuliano, *Phylogenetic survey of metabolically active microbial communities associated with the deep-sea coral* Lophelia pertusa *from the Apulian Plateau, central Mediterran* Sea, Deep Sea Res I 53 (2006), pp. 62–75.

[16] D.J. Lane, *16/23S rRNA sequencing*, in: *Nucleic Acid Techniques in Bacterial Systematics*, E. Stackerbrandt and M. Goodfellow, eds., Wiley, New York, 1991, pp. 115–175.

[17] M. Le Pennac, P.G. Beninger, and A. Herry, *Feeding and digestive adaptations of bivalve molluscs to sulphide-rich habitats*, Comp. Biochem. Physiol. 111 (1995), pp. 183–189.

[18] A. Fiala-Mdioni, Z.P. McKiness, P. Dando, J. Boulengue, A. Mariotti, and M.A. Alayse-Danet, *Ultrastructural, biochemical and immunological charaterisation of two populations of the mytilid mussel* Bathymodiolus azoricus *from the Mid Atlantic Ridge: Evidence for a dual symbiosis*, Mar. Biol. 141 (2002), pp. 1035–1043.

[19] E. Starliper Clifford, R.J. Neves, S. Hanlon, and P. Whittington, *A survey of the indigenous microbiota (bacteria) in three species of mussels from the Clinch and Holston Rivers, Virginia*, J. Shellfish Res. 27(5) (2008), pp. 1311–1317.

[20] C.W. Kaspar and M.L. Tamplin, *Effects of temperature and salinity on the survival of* Vibrio vulnificus *in seawater and shellfish*, Appl. Environ. Microb. 29 (1993), pp. 269–274.

[21] L. Hoi, J.L. Larsen, I. Dalsgaard, and A. Dalsgaard, *Occurrence of* Vibrio vulnificus *biotypes in Danish marine environments*, Appl. Environm. Microb. 64(1) (1998), pp. 7–13.

[22] C.A. Kaysner, M.L. Tamplin, M.M. Wekell, R.F. Stott, and K.G. Colburn, *Survival of* Vibrio vulnificus *in shellstock and shucked oysters (*Crassostrea gigas *and* Crassostrea virginica*) and effects of isolation medium on recovery*, Appl. Environ. Microb. 55 (1989), pp. 3072–3079.

[23] L. Stabili, M.I. Acquaviva, and R.A. Cavallo, Mytilus galloprovincialis *filter feeding on the bacterial community in a Mediterranean coastal area (Northen Ionia Sea, Italy)*, Water Res. 39 (2005), pp. 469–477.

[24] S.J. MacNaughton, L.R. Stephen, A.D. Venosa, G.A. Davis, Y.J. Chang, and D.C. With, *Microbial population changes during bioremediation of an experimental oil spill*, Appl. Environ. Microbiol. 65 (1999), pp. 3566–3574.