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POLLUTED MARINE ECOSISISTEMS: RESERVOIR OF MICROBIAL RESOURCES

FOR HYDROCARBONS BIOREMEDIATION

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ABSTRACT

Hydrocarbon (HC) pollution is a worldwide threat to marine natural ecosystems due to the increasing exploitation of underground marine petroleum deposits in several areas and to the high traffic of oil tankers and the presence of submarine pipes that are main transport routes for crude oil and refined products. HCs spread in the marine environment is mainly due to accidental oil spills or inadequate practices and their release affects marine ecosystems causing severe ecological and economical damages. The Mediterranean Sea is particularly endangered by hydrocarbon pollution because of its physical nature – it is an enclosed basin with a slow water exchange – and because it hosts about 20% of the global oil tanker traffic in its waters and tens of oil-related sites along its coastline. The conventional remediation strategies, comprising chemical and physical methods, are extremely expensive and invasive, therefore the development of cheaper and eco-friendly approaches is crucial to preserve human and ecosystem health. In this perspective, bioremediation (i.e. the use of living organisms to remove pollutants from a contaminated area) is a promising technology which, taking advantage of microbes' metabolic potential to degrade a wide range of pollutants, can both reduce the costs and may represent a permanent solution. Nevertheless, there is still a scarce knowledge of the processes and the microorganisms involved in the clean-up of hydrocarbons from marine environments, hence some problems still exist concerning the in-field application of bioremediation.
The aim of the present PhD thesis was to: i) investigate the overall prokaryotic diversity of pristine and oil polluted sites across the whole Mediterranean Sea; ii) depict the phylogenetic and functional diversity of hydrocarbonoclastic bacteria inhabiting pristine and polluted sites; iii) establish a large collection of bacteria showing degrading activities toward hydrocarbon compounds; iv) set up microcosm experiments to investigate the potential of bacterial bioaugmentation in bioremediation processes under laboratory scale conditions, v) test the degrading potential of selected bacterial strains and consortia under different pressure values, simulating different depths along the water column.
The diversity of planktonic bacterial communities in the Mediterranean Sea was firstly evaluated on open seawater samples collected at different depths in a transect covering the main oil tanker route across the whole basin, from the Levantine Sea to the Gibraltar strait. Automated Ribosomal Intergenic Spacer Analysis (ARISA) showed that the microbiome inhabiting deep and surface water samples were sharply separated. Furthermore, the composition of the bacterial communities described in the surface layers of the water columns at different sampling stations has been significantly correlated, beside to their geographical position and depth, to the temperature and salinity values recorded for each sample.
Denaturing Gradient Gel Electrophoresis (DGGE) and ARISA fingerprinting were also applied to depict the bacterial composition of highly polluted sediments collected at the Ancona harbor (Italy) and El-Max district (Egypt), showing the significant influence of the different pollutants' concentration (i.e. hydrocarbons, heavy metals) in the selection of peculiar bacterial assemblages . This molecular approach led to the identification of bacterial species potentially useful for site-tailored bioremediation purposes. A large collection of hydrocarbon degrading bacterial strains was hence established from enrichments using contaminated sediments as inoculum and diesel, crude oil and naphthalene as unique carbon sources. The cultivation approaches adopted to enrich and isolate hydrocarbonoclastic bacteria from chronically polluted area, like the Ancona harbor, permitted to evaluate the influence of different hydrocarbon pollutants used as single carbon source in the selection of specific marine bacteria populations. The results obtained taking advantage of DGGE fingerprinting and 16S rRNA pyrosequencing applied on the enrichments showed that, under laboratory conditions, the supply of different hydrocarbon compounds led to the selection of different, and specialized, bacterial communities.
A total of 248 bacterial strains have been isolated from open sea surface water collected along oil tanker routes and the chronically polluted sediments, and have been identified by 16S rRNA gene sequencing. *Alcanivorax* and *Marinobacter*, two ubiquitous marine hydrocarbonoclastic genera, were the most abundant within the established collection, representing respectively 67% and 23% of the isolates. Due to the great importance of the *Alcanivorax* genus for hydrocarbon remediation of marine polluted sites, all the isolates belonging to this genus were investigated at a finer level in terms of phylogenetic and functional diversity. This sub-collection, comprising 179 isolates belonging to the 4 species *A. borkumensis*, *A. jadensis*, *A. venustensis* and *A. dieselolei*, were genotyped using two different fingerprinting techniques: Internal Transcribed Spacer (ITS)-PCR and BOX-PCR. The combination of the applied techniques allowed the identification of 85 genotypes, distributed among the different sites investigated, showing clear evidence of geographic divergence. The functional diversity of these strains was furthermore investigated through the PCR amplification of the *alkB* gene, encoding for an alkane monooxigenase involved in the first step of hydrocarbons degradation, and subsequent Restriction Fragment Length Polymorphism (RFLP) analysis of the amplicons, allowing the identification of 16 different polymorphisms. The results demostrated the existence of a high degree of geographical divergence within the *Alcanivorax* genus, suggesting a potentially high metabolic diversity that could be exploited for site-tailored bioremediation interventions.

Recently, the Deepwater Horizon break in the Gulf of Mexico (2010) and the subsequent huge oil spill occurred at a depth of 1500 meters, highlighted the need to get more insight on bioremediation processes occurring at high depth. This accident represents a milestone and shed a light on the importance to investigate the effect of pressure, an environmental parameter that might hamper the activity of oil-degrading strains, on growth and degradation capabilities. The capability of selected hydrocarbonoclastic strains, belonging to the species *A. jadensis*, *A. dieselolei* and *M. hydrocarbonoclasticus,* to adapt and degrade a model alkane molecule (dodecane) at high pressure was therefore tested. The growth of the strains at increasing hydrostatic pressure and their physiologic activities were evaluated, comparing the results with the type strain *A. borkumensis* SK2. Overall, the results showed a detrimental effect of pressure for all the strains in terms of growth rates, O_2 consumption and CO_2 production. The potential adaptation of *A. borkumensis* and *A.dieselolei* was evaluated also with less recalcitrant carbon source than alkanes (pyruvate), without showing substantial differences, except for the higher consumption of pyruvate by *A. borkumensis* SK2. This investigation pinpointed that the tested bacteria can survive at high hydrostatic pressures, even though both their growth and degradation capability were mostly inhibited with the increase in hydrostatic pressure. Moreover, aiming to create a baseline for future transcriptomic analyses, the complete genome of this 4 strains was sequenced and annotated: all the strains owned multiple copies of the genes involved in the degradation of hydrocarbons (alkane monoossigenase, *alk* and cytochrome p450, *cyp450*), apparently belonging to different families, highlighting the great functional potential of these strains.

A second sub-collection of hydrocarbonoclastic bacteria, isolated from chronically polluted sediments, was screened for the presence of functional genes involved in the degradation/detoxification of specific pollutants (alkanes and heavy metals), the ability to grow on different HCs and the ability to produce biosurfactant and from biofilm. The results showed that several isolates, mainly belonging to the *Marinobacter* genus, were positive for the investigated traits, hence they could be potentially exploited for autochthonous bioaugmentation (ABA) purposes in the sites of provenience.

Finally, the bacterial community response in a biodegradation process based on an ex-situ landfarming set-up was evaluated. Landfarming was performed, using a combination of biostimulation and bioaugmentation, to remediate oil-polluted sediment collected at Elefsina bay (Greece). This work was realized to determine the effect of bioaugmentation by four allochthonous oil-degrading bacterial consortia, previously isolated from 4 polluted areas located in the Southern Mediterranean, in relation to the degradation efficiency of the indigenous community. DGGE fingerprinting analysis allowed the characterization of the bacterial community dynamics, evaluating the dominant taxa through time and at each treatment. The results showed that the added allochthonous bacteria quickly perished and were rarely detected, furthermore their addition induced minimal shifts in the community structure. These data, together with the measurement of HC degradation over the experimental time, suggested that, during the landfarming, biodegradation was mostly performed by the autochthonous populations rather than by the allochthonous ones. Furthermore, biostimulation, in contrast to bioaugmentation, was proved to enhance the HCs degradation when compared to the control treatment.

To conclude, the results obtained this Ph.D. project emphasized the high bacterial diversity of the Mediterranean Sea in both pristine and polluted sites and the occurrence of distribution patterns which were significantly related to several environmental parameters, including the concentration of hydrocarbons and heavy metals. Moreover, this study confirmed the great potential of the Mediterranean Sea as a source of bacterial strains harbouring degradation capabilities toward different hydrocarbon molecules and, through the *ex-situ* application of different bioremediation strategies (bioaugmentation and biostimulation), it demonstrated the great importance of autochthonous microbial community in remediating polluted environments.

RIASSUNTO

L'inquinamento da idrocarburi è una minaccia per gli ecosistemi marini di tutto il mondo a causa dell'aumento delle esplorazioni dei fondali alla ricerca di depositi di petrolio, del grande traffico di petroliere e dello sviluppo di numerose condutture sottomarine per il trasporto di petrolio. La diffusione di idrocarburi in ambiente marino è dovuta principalmente a incidenti o azioni illegali. Il loro rilascio ha un'influenza negativa sugli ecosistemi marini provocando gravi danni ecologici ed economici. Il Mar Mediterraneo è particolarmente minacciato dall'inquinamento da idrocarburi a causa della sua natura geofisica - si tratta di un bacino semichiuso con un lento ricambio di acqua - e perché è interessato dal 20% del traffico mondiale di petroliere e lungo le sue coste sono presenti decine di siti industriali dedicati alla lavorazione e al traffico di petrolio. Le strategie di risanamento classiche, che comprendono metodi fisici e chimici, sono estremamente costose e invasive, quindi è di fondamentale importanza lo sviluppo di metodologie più economiche ed ecocompatibili, in modo da preservare la salute umana e ambientale. E' proprio in questa prospettiva che il biorisanamento - l'utilizzo di organismi viventi per la rimozione degli inquinanti da un'area inquinata - si sta rivelando una tecnologia promettente, soprattutto se basata sullo sfruttamento del potenziale metabolico dei microorganismi per degradare un ampio spettro di inquinanti, in modo da abbattere i costi e rappresentare una soluzione permanente. Cio' nonostante, la conoscenza dei processi e dei microorganismi coinvolti nella degradazione degli idrocarburi in ambienti marini sono tutt'ora limitati, di conseguenza l'applicazione in campo delle tecniche di biorisanamento è ancora problematica. Lo scopo di questa tesi di dottorato è quello di: i) indagare la diversità procariotica totale di ambienti marini, sia puliti che inquinati da petrolio, dislocati in tutto il Mar Mediterraneo; ii) decrivere la diversità filogentica e funzionaledei batteri idrocarbonoclastici che risiedono nelle suddette aree; iii) allestire una collezione di isolati batterici in grado di degradare molecole idrocarburiche; iv) allestire microcosmi che permettano di investigare in laboratorio l'efficienza di biorisanamento dell'aggiunta di inoculi batterici in ambienti inquinati; v) valutare il potenziale di degradazione di ceppi batterici selezionati a diversi valori di pressione idrostatica, in modo da simulare diverse profondità marine.

La diversità della comunità batterica planctonica del Mar Mediterraneo è stata esaminata innanzitutto in campioni di acque campionate in mare aperto e a diverse profondità, lungo un transetto che copre le principali rotte seguite dalle petroliere attraverso l'intero bacino, dal Levantino allo stretto di Gibilterra. L' analisi "Automated Ribosomal Intergenic Spacer Analyses" (ARISA) mostra che i microbiomi che risiedono nei campioni di acque sia superficiali che profonde sono nettamente separati. Inoltre, la composizione delle comunità batteriche descritta negli strati più superficiali della colonna d'acqua è significativamente correlata alla loro posizione geografica, profondità, e ai valori di temperatura e salinità registrati in ogni campione. Le metodologie di fingerprinting molecolari ARISA e "Denaturing Gradient Gel Elechtrophoresis" (DGGE) sono state inoltre applicate per descrivere la composizione batterica di sedimenti altamente inquinati campionati nel porto di Ancona (Italia) e neldistretto di El-Max (Egitto). Queste analisi hanno mostrato l'influenza significativa delle diverse concentrazioni di inquinanti (per esempio di idrocarburi e metalli pesanti) nel selezionare popolazioni batteriche tipiche di ogni sito indagato. Questo approccio ha portato all'identificazione di specie batteriche potenzialmente utili in processi di biorisanamento sito-specifici, per questo motivo è stata allestita una grande collezione di ceppi batterici idrocarburo degradanti isolati tramite l'arricchimento di colture cellulari utilizzando diesel, petrolio e naftalene come unica fonte di carbonio. Gli approcci di coltivazione adottati per arricchire e isolare batteri idrocarbonoclastici a partire da aree cronicamente inquinate, comeilporto di Ancona, hanno permesso di valutare l'influenza di diversi inquinanti idrocarburici (petrolio, diesel e naftalene) usati come uniche fonti di carbonio nella selezione di popolazioni batteriche marine specifiche. Il risultato ottenuto sfruttando le tecniche di DGGE fingerprinting e 16S rRNA pyrotag ha mostrato che, in condizioni di laboratorio, il supplemento di diversi idrocarburi, ha portato alla selezione di comunità batteriche diverse e specializzate.

248 ceppi batterici sono stati isolati da acque superficiali campionati lungo le rotte delle petroliere e da sedimenti inquinati e sono stati identificati tramite il sequenziamento del gene 16S rRNA.

All'interno della collezione, i generi più abbondanti sono risultati essere *Marinobacter* e *Alcanivorax* - entrambi batteri marini idrocarbonoclasti ubiquitari - rappresentando rispettivamente il 67% e il 23% degli isolati. A causa della grande rilevanza del genere *Alcanivorax* per il risanamento degli ambienti marini inquinati da idrocarburi, tutti gli isolati afferenti a questo genere sono stati ulteriormente investigati in termini di diversità filogenetica e funzionale. Questa sub-collezione comprende 179 isolati appartenenti alle specie: *A. borkumensis*, *A. jadensis*, *A. venustensis* e *A. dieseloei*. Questi sono stati genotipizzati tramite due diverse tecniche di fingerprinting: "Internal Trancriber Spacer" (ITS)-PCR e BOX-PCR. Entrambe queste tecniche applicate in combinazione hanno permesso di identificare 85 diversi genotipi, distribuiti nei diversi siti indagati, mostrando chiare evidenze di divergenza geografica. La diversità funzionale di questi stessi ceppi è stata investigata tramite l'amplificazione PCR del gene *alkB*, codificante per un' alcano monoossigenasi coinvolta nella prima reazione di degradazione degli alcani, in seguito analizzato tramite

"Restriction Fragment Lenght Polymophism" (RFLP), identificando cosi' 16 diversi polimorfismi. Quest'analisi ha confermato l'esistenza di un alto livello di divergenza geografica, suggerendo un'altrettanto alta diversità metabolica che potrebbe essere sfruttata per l'implementazione di interventi di biorisanamento sito specifici.

Recentemente, l'incidente che ha visto protagonista la piattaforma di estrazione di petrolio da alta profondità Deepwater Horizon nel Golfo del Messico (2010) e la susseguente enorme perdita di petrolio avvenuta a 1500 m di profondità, ha portato alla luce il bisogno di approfondire le conoscenze sui processi di biorisanamento che hanno luogo ad alte profondità. Quest'incidente rappresenta una pietra miliare nella storia degli incidenti marini e accentua l'importanza di investigare gli effetti della pressione, un parametro ambientale che potrebbe ostacolare l'attività dei ceppi idrocarburo-degradanti, sulla loro crescita e sulle loro capacità degradanti. Per cui, è stata valutata la capacità di selezionati batteri idrocarbonoclastici, appartenenti alle specie *A. jadensis*, *A. dieselolei*, *M. hydrocarbonoclasticus*, di adattarsi e degradare un alcano modello (dodecano) ad alta pressione. Sono state valuate la crescita e le loro attività fisiologiche a pressioni idrostatiche crescenti, comparando i risultati con il ceppo *A. borkumensis* SK2. Complessivamente, i risultati evidenziano un'influenza negativa della pressione su tutti i ceppi in termini di crescita, consumo di O_2 e produzione di CO_2. Il potenziale di adattamento di *A. borkumensis* SK2 e di *A. dieseloeli* è stato inoltre valutato anche per l'utilizzo di diverse fonti di carbonio (piruvato e dodecano), senza mostrare differenze sostanziali quando i batteri crescono in presenza delle diverse fonti, con l'unica eccezione del maggior consumo di piruvato da parte di *A. borkumensis* SK2. Questo studio indica che i batteri indagati possono sopravvivere ad alte pressioni idrostatiche, anche se sia la loro crescita che la degradazione di dodecano sono inibite. In aggiunta, allo scopo di creare una piattaforma di base per future analisi di trascrittomica, il genoma completo di questi quattro ceppi è stato sequenziato e annotato: tutti i ceppi hanno mostrato di possedere copie multiple dei geni coinvolti nella degradazione degli idrocarburi (l'alcano monoossigenasi, *alk* e il citocromo p450, *cyp450*), in apparenza appartenenti a diverse famiglie, evidenziando l'elevato potenziale funzionale di questi ceppi.

Una seconda sub-collezione di batteri idrocarbonoclastici, isolati da sedimenti cronicamente inquinati, è stata analizzata per valutare: i) la presenza di geni funzionali coinvolti nella degradazione/detossificazione di specifici inquinanti (alcani e metalli pesanti); ii) la capacità di crescere utilizzando diversi idrocarburi e iii) la capacità di produrre biosurfattanti e biofilm. Molti isolati, soprattutto appartenenti al genere *Marinobacter*, risultano positivi per le caratteristiche indagate e potrebbero quindi essere sfruttati per tecniche di bioaggiunta autoctona (ABA) nel loro sito di provenienza.

Infine, è stata valutata la risposta della comunità batterica a processi di biorisanamento basati su un set up di landfarming *ex situ* . La tecnica del landfarming è stata applicata usando una combinazione di biostimolazione e bioaggiunta, per risanare sedimenti inquinati da petrolio provenienti dalla baia di Elefsina (Grecia). Questo lavoro è stato realizzato per determinare gli effetti dell' aggiunta di 4 consorzi batterici petrolio degradanti alloctoni, precedentemente isolati da 4 aree inquinate situate nel sud del Mediterraneo, confrontandoli con l'efficienza di degradazione della comunità microbica indigena. Analisi di DGGE fingerprinting, hanno permesso la caratterizzazione delle dinamiche della comunità batterica, valutandone i taxa dominanti a seguito di ogni trattamento. I risultati hanno mostrato che i batteri alloctoni aggiunti non erano in grado di sopravvivere ed erano individuati di rado, inoltre il loro inoculo induceva solo minimi cambiamenti all'interno della comunità microbica. Questi risultati, insieme alla misura della degradazione degli idrocarburi durante tutta la durata della sperimentazione, suggeriscono che, durante il landfarming, la biodegradazione avviene soprattutto a carico delle popolazioni autoctone. Inoltre la biostimolazione, contrariamente a quanto osservato per la bioaggiunta, è stata provata aumentare la degradazione di idrocarburi, se comparata con i trattamenti di controllo.

In conclusione, i risultati ottenuti nel corso dello svolgimento di questo dottorato di ricerca, enfatizzano l'alta diversità batterica del Mar Mediterraneo, sia in siti puliti che in siti inquinati da idrocarburi, e la presenza di pattern di distribuzione significativamente correlati a numerosi parametri ambientali, incluse le concentrazioni di idrocarburi e metalli pesanti. Inoltre, questo studio conferma le grandi potenzialità del Mar Mediterraneo come serbatoio di ceppi batterici con diverse capacità di degradazione di numerose molecole idrocarburiche e, attraverso l'applicazione *ex-situ* di diverse strategie di biorisanamento (bioaggiunta e bistimolazione), ha dimostrato la grande importanza della comunità microbica autoctona nel risanamento degli ambienti inquinati da idrocarburi.

Chapter

I

RATIONALE AND AIM OF THE WORK

Hydrocarbon pollution is a worldwide threat to marine natural ecosystems due to the increasing exploitation of underground marine petroleum deposits in several areas and to the high traffic of oil tankers and the presence of submarine pipes that are main transport routes for crude oil and refined products. Pollution in marine environments can be due to both accidents and deliberate illegal discharging of hydrocarbon wastes. The Mediterranean Sea is particularly endangered by hydrocarbon pollution because of its physical nature – it is an enclosed basin with a slow water exchange – and because it hosts about 20% of the global oil tanker traffic in its waters and tens of oil-related sites along its coastline.

Microbial communities play a fundamental role in pollutant biodegradation and since decades their exploitation in bioremediation processes, has been proposed to detoxify polluted sites as a cheaper and possibly more effective alternative to classical remediation techniques, which are extremely expensive and invasive (Atlas, 1995). The exploitation of the microbes' metabolic potential toward pollutants has been successfully applied, for instance, in remediation of aquifer polluted by chlorinated xenobiotics (Dick et al., 2001) and in radioactive and heavy metals contaminated soils (Georgiev, 2014).

Nevertheless, there is still a scarce knowledge of the processes and the microorganisms involved in clean-up of hydrocarbons from marine environments, therefore some problems still exist concerning the in-field application of this promising technology. To overcome these problems, it is of primary importance to get more insight in the phylogenetic and functional diversity of the microbial communities potentially involved in hydrocarbons remediation and their adaptation to the environmental niches were hydrocarbons might accumulate, including chronically polluted areas that are present in the Mediterranean Sea and can operate as natural laboratory for the selection of the best oil degrader microbes.

The aim of the present PhD thesis was to: i) investigate the overall prokaryotic diversity of pristine and oil polluted sites across the whole Mediterranean Sea; ii) depict the phylogenetic and functional diversity of hydrocarbonoclastic bacteria inhabiting pristine and polluted sites; iii) establish a large collection of bacteria showing degrading activities toward hydrocarbon molecules; iv) set up microcosm experiments to investigate the potential of bacterial bioaugmentation in bioremediation processes under laboratory scale conditions, v) test the degrading potential of selected bacterial strains and consortia at different pressure values, simulating different depths.

The environmental samples analyzed in this work have been collected from several sites located across the whole Mediterranean basin: seawater samples were collected from an East to West transect covering the major oil tanker routes, while sediments were sampled from the Ancona harbor (Italy) and El-Max district (Alexandria, Egypt).

Chapter I of this PhD thesis is the present rationale and aim of the work.

Chapter II is a review highlighting the need to better investigate the oil-degrading potential of microorganisms inhabiting the Mediterranean Sea, which holds a high microbial diversity already adapted to thrive in hydrocarbons polluted environments. All the following chapters are research works focused on the bacterial diversity and biogeographic patterns depicted across the Mediterranean basin (chapters III and IV) and on the degrading potential of isolated bacterial strains and consortia enriched from polluted sites of the Mediterranean Sea (chapters V, VI, VII and VIII).

Chapter III focuses on the description of the structure of planktonic bacterial communities inhabiting both deep and surface seawater from the Eastern to the Western borders of the Mediterranean basin, identifying which are the main environmental parameters involved in its shaping.

Chapter IV focuses on the phylogenetic and functional diversity of 4 bacterial species belonging to the *Alcanivorax* genus, one of the best known marine hydrocarbonoclastic genera, isolated from both seawater and sediments across the Mediterranean Sea. This research shows the occurrence of geographical

divergence within the genus *Alcanivorax*, highlighting a differential distribution of *Alcanivorax* ecotypes in the different investigated sites.

Chapter V describes and compares the adaptation capability of 4 hydrocarbonoclastic bacteria, belonging to the genera *Alcanivorax* and *Marinobacter*, to grow and degrade a model alkane molecule (dodecane) at increasing hydrostatic pressure. Pressure is an environmental parameter that might hamper the activity of oil-degrading strains, and which became an issue of great interest after the Deep Water Horizon oil-spill occurred in 2010 in the Gulf of Mexico (Hazen et al., 2010).

To obtain preliminary data in order to further address transcriptomic studies at different pressure values, alkane degrading capability was compared with more conventional carbon source (pyruvate) for two strains belonging to the species *A. dieselеolei* and *A. borkumensis*.

Chapter VI and VII investigate the physical and chemical parameters which characterize the hydrocarbon and heavy metals highly contaminated sediments collected respectively at El-Max bay (Egypt) and Ancona harbor (Italy). These chapters provide a snapshot of the bacterial communities inhabiting those sediments, investigating their composition and correlating its diversity with the environmental parameters to provide deeper knowledge about the drivers of bacterial communities' structure in marine contaminated sediments. Furthermore, hydrocarbonoclastic bacterial strains were isolated from the two sites and screened for metabolic traits of interest in the ambit of bioremediation potential.

Chapter VIII, investigates the application of different bacterial consortia during an *ex-situ* landfarming experiment setup for the bioremediation of polluted sediments collected near an oil refinery in the Elefsina bay (Greece). Here, biostimulation and allocthonous bioaugmentation were combined to optimize the best physico-chemical and biological conditions influencing the biological processes involved in hydrocarbons degradation. The structure of the bacterial communities were monitored using molecular methods during the landfarming process to clarify the relative effect of bioaugmentation with allocthonous bacteria *versus* the role of indigenous degrading population.

Chapter IX summarizes the general conclusions of this Ph.D. thesis and indicates new perspectives for future work.

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Chapter

II

THE MEDITERRANEAN SEA: AN UNEXPLOITED RESERVOIR OF MICROBES FOR SITE-TAILORED HYDROCARBON BIOREMEDIATION STRATEGIES

1. INTRODUCTION

Crude oil pollution is a worldwide threat to natural marine ecosystem, but its dangerousness is even higher in the Mediterranean Sea due to its physical nature, the high number of pipelines and the intense oil traffic that interest the whole basin, which host several oil-producing countries on its Southern coastlines. The 2010 Deepwater Horizon disaster in the Gulf of Mexico (Franci et al., 2014; Lamendella et al., 2014) is an example of the danger that marine habitats could face because of the increasing exploitation of deep marine petroleum reservoirs. Considering the semi-enclosed nature of the Mediterranean basin and the occurring relatively slow water exchange processes, the impact of a similar pollution event would be even stronger and more difficult to face, causing enormous economical losses in addition to health consequences for humans, animals and the environment as a whole.

Current remediation technologies are not adequate and, basing mostly on mechanical techniques, they simply remove the oil to another compartment without really solving the problem. In the last decades, new remediation approaches based on biotechnologies have been developed, but they are still far from being effective. Such approaches are defined as bioremediation and take advantage of the metabolic potential of living organisms, especially microbes, to remove pollutants from a contaminated area. Biological systems, from bacteria to plants, can be used for different aims in environmental biotechnologies, comprising remediation, pollutants prevention and monitoring of environmental contaminants (Sanabria, 2014). Microorganisms, in particular, play a key role in all biogeochemical cycles, therefore they are also pivotal in natural pollution attenuation and bioremediation.

Thanks to the advances in molecular biology and biochemical methods and in the knowledge of the ecological, genetic and metabolic diversity of organisms, marine bioremediation strategies underwent considerable advances but are still far from being effective under in field conditions. Because of its high biodiversity (Coll et al., 2010) and the presence of a high number of chronically petroleum polluted sites (Danovaro, 2002) the Mediterranean Sea is a potentially ideal source of bacteria exploitable for bioremediation purposes. Such strategies should rely on the naturally occurring catalytic activity of microbes already existing in the specific environment to transform environmental pollutants under a site-tailored approach.

The present review covers several aspects related to the oil pollution issue in the Mediterranean Sea, providing an overview of the peculiarities of such basin and including: i) the main causes responsible for the high degree of pollution; ii) the extent of oil pollution and the effort of the Mediterranean countries to counteract the problem; iii) the effect of oil pollution on the ecosystems, animals, and microorganisms and iv) the available technologies to remediate polluted sites through conventional and innovative methods.

2. OIL POLLUTION IN THE MEDITERRANEAN SEA

Human being started to civilize the coastline of Mediterranean Sea since the Neolithic era (Paschou et al.2014), therefore this basin became of fundamental importance for merchants and travelers since ancient times and its coastline became very densely inhabited, reaching a population of about 460 million peoples (UNEP/MAP, 2014). With the advent of the industrial revolution, the discovery and exploitation of fossil fuels started and, especially after the end of World War II, the demand and consumption of petroleum grew and it is continuously increasing (Steffen et al., 2014).

The huge exploitation of resources from the marine subsurface including mineral, oil and gas extraction, the intense maritime traffic and the presence of many industrial poles on both the North and South side of the Mediterranean Sea, have resulted in a rapid increase of the number of polluted coastal and offshore areas. The situation is further worsened by the physical nature of this semi-enclosed basin that has only limited water circulation and therefore low dilution of pollutants.
The Mediterranean Sea face a high risk of oil pollution since it hosts about 20% of the total oil tanker traffic, even if its waters account for less than 1% of the world marine waters, and its coasts host about 80 petroleum harbors and the same number of oil related facilities (FIG.1).

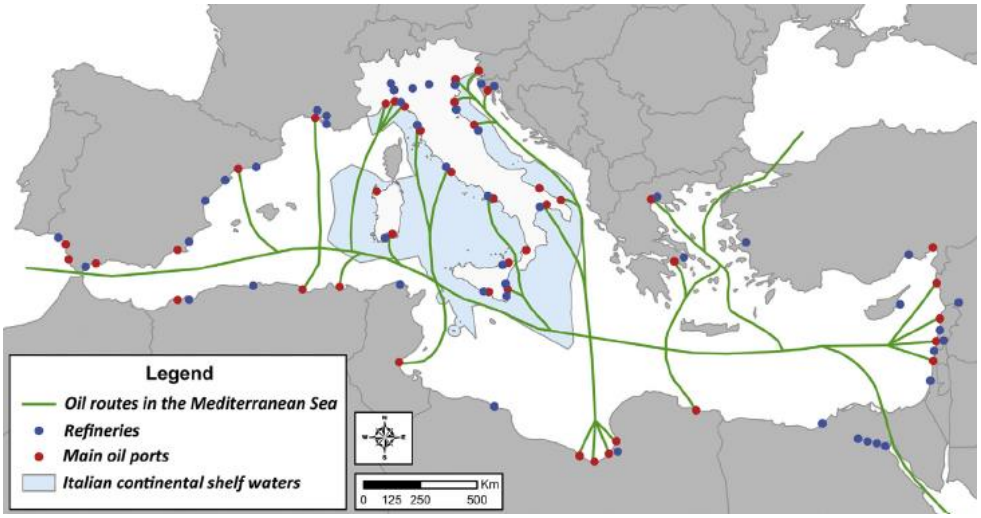

Fig. 1 **Map of the handling of oil in the Mediterranean Sea (main routes, refineries and main oil ports), from Garcia et al., 2013**

Every year, up to 100-150,000 tons of hydrocarbons are transported in the Mediterranean Sea (UNEP/MAP) and it has been estimated that 8,780,326 oil barrels are refined every day in this region, more than 10% of the total world amount (Legambiente dossier, 2007). Pollution of a restricted, but highly populated and exploited basin, like the Mediterranean Sea, could lead to tremendous socio-economic issues that can affect the lifestyle of its inhabitants. Besides impacts on biodiversity and wild life, marine oil spills can in fact impact the inhabitants of the affected areas by reducing tourism and fishing activities, with consequent strong economic loss.
Environmental problems receives little to no attention until the last decades, when the scientific community started to prove the effects of human activities on the environment, but meanwhile a process of decline of the global biodiversity already started (Butchart et al, 2010) and although the creation of protected areas that nowadays cover about the 12% of the global surface, any decreasing in the trend of the biodiversity loss has been observed (Eken et al, 2004; Steffen et al., 2014).
The Mediterranean countries were between the first to recognize these problems and in 1975, 16 of them and the European Community adopted the Mediterranean Action Plan (MAP), the first-ever Regional Seas Program under United Nations Environment Program's umbrella. The next year, these parties adopted the Convention for the Protection of the Mediterranean Sea Against Pollution (Barcelona Convention) that comprises seven protocols addressing specific aspects of Mediterranean environmental conservation to complete the MAP legal framework. Later, in November 1995, Ministers of Foreign Affairs of EU members and Mediterranean partners, launched the Barcelona Process to manage both bilateral and regional relations forming the basis of the Euro-Mediterranean Partnership, which has expanded and evolved into the Union for the Mediterranean, an innovative alliance aimed to create a Mediterranean region of peace, security and shared prosperity.

2.1 - Characteristics of the Mediterranean Sea

The Mediterranean Sea is a semi-enclosed basin, surrounded by three different continents -Europe, Africa and Asia- and with only narrow connections with other marine basins: with the Atlantic Ocean through the Gibraltar Strait, with the Black Sea trough the Bosphorus Strait and, since 1869, with the Red Sea through the Suez Canal. The Sicilian Channel divides this basin in the Western and Eastern sub-basins that are characterized by a very pronounced gradients: from West to East the temperature of the surface water ranges between 15.6°C to 23.2°C and the salinity increases because of the high rate of evaporation of the eastern waters (Coll et al., 2010).

Overall, the Mediterranean Sea is considered a deep sea with unusual characteristics like the absence of thermal stratification from 300–500 m deep to the bottom (with a temperature range between 12.8°C and 13.5°C in the western basin and between 13.5°C and 15.5°C in the eastern one) and high salinity (between 37.5 and 39.5 psu) (Coll et al., 2010).

Its waters are mostly oligotrophic, a state more pronounced in the eastern side, where this condition is defined as ultra-oligotrophic. The nutrient gradient is due to the fact that waters entering from the Atlantic Ocean are very poor in nutrients, while the Mediterranean ones are relatively nutrient rich, and this cause a constant loss of nutrients (Bas, 2009). The gradient of nutrients, like nitrogen and phosphorus, determine also a decreasing gradient in ocean productive capacity, particularly pronounced in the Eastern side (Thingstad et al., 2005) hence, the Levantine basin is one of the most oligotrophic marine environment all over the world, characterized by low nutrients, low chlorophyll amount and a very low primary production (Krom et al.,1991, 2003, 2005; Kres and Herut, 2001)

Despite this state of oligotrophy, the Mediterranean Sea host a very high biodiversity and many endemic species. It has been calculated that within its water lives between 4% and 18% of the world marine diversity (Coll et al., 2010). Furthermore, the Mediterranean Sea contains some very rare niches with peculiar geomorphological structures like submarine canyons, seamounts, mud volcanoes, hypersaline anoxic lakes, deep trenches and, most of all, it is one of the few deep sea basin of the world (Madron et al., 2011).

The Mediterranean Sea host a wide range of biodiversity also at the microbiological level, estimated in 17,000 different species inhabiting its waters (Coll et al., 2010), probably an underestimation of its actual richness since it is particularly difficult to define the biodiversity of environmental microorganisms. Furthermore, marine microbial diversity has been suggested to be even higher because of the presence of the so called “rare biosphere” composed by very few individual belonging to many different phylogenetic groups (Sogin et al., 2006).

Molecular fingerprinting studies covering the whole Mediterranean Sea highlighted that environmental parameters like longitude, latitude, pressure, salinity, temperature and oxygen are significant drivers that concur in shaping the composition of bacteria communities inhabiting the basin (Mapelli et al., 2013). Other studies tried to ascertain the bacterial diversity across the Mediterranean Sea. Alonso-Sàez and colleagues (2006) evaluated the seasonal variability of the microbial communities of western Mediterranean Sea surface water highlighting the dominance of α-*proteobacteria* along the year. In this study SAR11-like bacteria were the most abundant taxa during spring and summer, while *Roseobacter* predominated in winter and summer. The *Bacterioidetes* phylum was the second in abundance and its amount was stable along the year, while γ-*proteobacteria* showed a peak during summer. Feingersh and coauthors (2010) investigated the microbial assemblages of the eastern surface waters microbial communities indicating α-*proteobacteria* as the most abundant class. In this case α-*proteobacteria* were followed by *Cyanobacteria* and γ-*proteobacteria.* In a recent pyrosequencing study on the prokaryotic diversity of the Eastern deep waters, most of the analyzed 16S rRNA sequences were classified as unidentified bacteria (34%) while the Proteobacteria phylum comprised 32% of the sequences and was the most represented phylum, including the classes of Alpha-, Beta-, Gamma-, Delta- and Epsilon-Proteobacteria. Acidobacteria phylum accounted for 7% of the total sequences while Thaumarcheaota for 6% (Sevastou et al., 2013). Different studies showed the existence of biogeographic pattern of both whole prokaryotic communities (Mapelli et al., 2013) or specific bacterial taxa (Lamy et al., 2011) across the Mediterranean basin. Overall, the available studies on the microbial diversity and dynamics across the whole basin let suppose that a similar high

diversity and divergence can be found in its polluted sites, which could therefore contain still unknown microbiological resources exploitable for site-tailored bioremediation interventions.

2.2 - Hydrocarbons Polluted areas in the Mediterranean Sea

The Strategic Action Program (SAP) of United Nations Environment Program (UNEP) reported that numerous coastal areas located in the Mediterranean Sea are very polluted and individuated 131 "pollution hot spots" that are point of pollution sources or polluted coastal areas which may affect human health, ecosystem, biodiversity, sustainability, or economy. In addition, 59 sensitive areas have been identified, representing marine environments under the threat of becoming pollution hot spot (UNEP/WHO, 2003). It is noteworthy that most of the oil related sites present along the Mediterranean coastlines overlap with the Specially Protected Areas of Mediterranean Importance (SPAMIs) individuated in 2012 by MedPAN (The Network of Managers of Marine Protected Areas in the Mediterranean) (Fig.2).

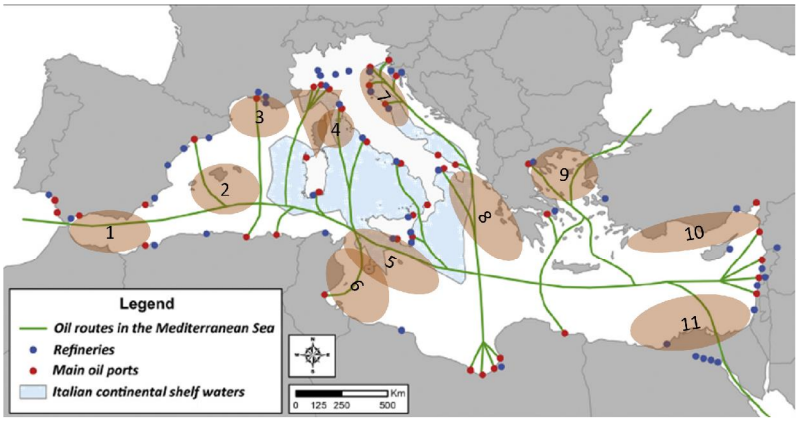

Fig. 2 **Map of the Medietrranean Sea including: refineries, main oil ports, main oil routes and SPAMIs areas. SPAMIs areas are numbered: 1. Alboran Sea; 2. South of Balearic Islands; 3. Gulf of Lions slope; 4. Central Tyrrenian Sea; 5. Sicily channel; 6. Tunisian plateu; 7. North-Central Adriatic Sea; 8. North-Central Ionic Sea; 9. North Aegean Sea; 10. North Levantine Sea; 11. Nyle hydrotermal area Modified from Garcia et al., 2013 and MedPAN, 2012**

SPAMIs are marine and coastal sites of the Mediterranean, created under the Barcelona Convention Protocol for Specially Protected Areas and Biological Diversity in the Mediterranean (SPA/BD Protocol) to conserve: *ñthe components of biological diversity in the Mediterranean; ecosystems specific to the Mediterranean area or the habitats of endangered species; areas of special interest at the scientific, aesthetic, cultural or educational levelsò*. Therefore, SPAMIs should be a shelter for endangered species, preventing the deterioration of habitats and allowing the development of natural biological communities, a function that is obviously greatly endangered by the close presence of oil related sites.

Since oil facilities (like harbors and refineries) and oil tanker routes are spread along the whole Mediterranean Sea, hydrocarbon pollution is a problem common to all the countries surrounding this basin. In particular, in 2006, the European Environment Agency (EEA) recognized oily effluents as an important problem in 4 Mediterranean countries (Algeria, Croatia, Italy and Syria) and as a problem of medium importance in other 5 States (Egypt, Israel, Libya, Malta and Turkey).

Hydrocarbon pollution regards a large variety of ecosystems, from costal lagoons like the Venice lagoon (Borin et al., 2009a) to costal saline systems (Lakhdar et al., 2006) and deep sea oil seeping systems (Borin et al, 2009b). The Mediterranean basin hosts a large variety of ecosystems affected by petroleum pollution that present extreme conditions for microbial life, hence it can be considered a natural laboratory for the selection of microbial resources adapted to such conditions. The bioremediation of extreme polluted sites in fact cannot take advantage of the exploitation of consolidated processes and microbial catalysts known to be effective under conventional conditions and, from the other side, microbial extremophiles have been widely recognized as important sources for the development of biotechnological products (Venter et al., 2004; Antranikian et al., 2005). Thus, several polluted sites in the Mediterranean Sea, being subjected to different environmental constrains, are considered invaluable sources of unexplored microorganisms with the potential for the development of new bioremediation products and processes.

Among the coastal sites, examples of hot spot for hydrocarbon pollution are: El Max district, an area located near Alexandria, Egypt; the Ancona harbor in the Adriatic Sea, Italy; and the Elefsina Gulf, Aegean Sea, Greece.

El-Max bay is an industrial zone located at west of Alexandria City. In the last years many heavy industries (petrochemicals, pulp metal planting, industrial dyes, and textiles) developed here with an uncontrolled disposal of the resulting waste. Consequently, the costal water of El-Max Bay receives huge amounts of untreated industrial wastes (Fahmy et al., 1997; Shriadah and Emara, 1996, Amer at al., 2014). El-Max Bay receives heavy load of wastewater (i.e. 2.4×109 m^3/year) both directly into the sea from industrial outfalls as well as indirectly from Lake Marriott via El-Max (Said et al., 1994). A pumping station is situated about1 km upstream from El-Umum Drain for disposal of agricultural overflow of Lake Marriott (Okbah et al., 2012 ; Samir and El-Din, 2001). In addition, shipping activities along the harbor contribute to the overall bay pollution (Samir and El-Din, 2001).

The Ancona harbor is located in the Northern Adriatic Sea. It is a commercial harbor with an intense ferryboat and merchant activity and subjected to high organic waste dumping due to the activities of fishing boat. The area is also subjected to a strong industrial pollution due to the presence of shipyards and industrial plants. High concentration of chemical pollutants were found in the sediments (Dell'Anno et al., 2009). In particular, high concentration of total aliphatic hydrocarbons (500 µg g^{-1} d.w.), total polyciclyc aromatic hydrocarbons (PAH, up to 7473 µg g^{-1} d.w.) and naphthalene (2852 µg g^{-1}) occur in the sediments of the Ancona harbor (Mirto and Danovaro, 2004). Moreover, high concentration of heavy metals like Cu, Mn, Ni and Zn are also present (Mirto and Danovaro 2004; Dell'Anno et al., 2009). Thus, the Ancona Harbor can be considered a highly impacted site, receiving large inputs of the most concerning chemical pollutants (heavy metals and hydrocarbons) and subjected to a strong organic enrichment.

The Elfsina Bay is a major industrial area situated in the West of Athens. It is a closed basin between the Attika coast and the Salamis Island. For several decades, the industrial and shipping activities along its coast have introduced high amount of wastes in the bay waters and sediments. Both the United Nations Environment Program/ Mediterranean Action Plan (UNEP/MAP) and the EEA recognized the Elefsina Bay as an area of major concern. The sediments close to the ELPE refinery of Elefsina are contaminated with the heavy fraction of petroleum because of a rapture of a crude oil transport pipeline occurred several years ago (REF su ulixes).

The three coastal polluted areas briefly described in the previous paragraphs are extreme environments with occurring different environmental conditions that might host novel microbial resources for the setup of site-tailored bioremediation strategies.

2.3 - Spills in the Mediterranean Sea

Oil spills that concur to pollute seawaters have two main origins: accidents or illegal discharges. From the 1985, the 27 biggest accidents occurred resulted in more than 270,000 tons of hydrocarbons released in the environment (Legambiente dossier, 2007). Fortunately this kind of accidents do not occur frequently, hence they represent only a fraction of the total hydrocarbon pollution. Besides pollution due to accidents of ships, there is pollution due to routine ship operation like tank washing, ballast waters and engine room effluent discharges. The International Tanker Owners Pollution Federation (ITOPF), a not-for-profit organization with the aim ***ñ****to promote an effective response to marine spills of oil chemicals and other substances****ò*** showed that most of the oil spillages originate from these routine operations. Interestingly, not only oil tankers are the responsible for this pollution, but also cargo ships, fishing boats, leisure crafts and naval vessels (ITOPF, 2001). New methods to locate possible spills are based on space-borne instruments (synthetic aperture radar - SAR- imagery) and showed higher concentrations of oil spills along the major shipping routes, particularly in the Ligurian Sea and the Gulf of Lion and close to the eastern coast of Corsica and in the Strait of Sicily. Taking advantage of this method, a total area of spills of 17,141 km^2 was detected in 1999 in the Mediterranean basin (Abdulla & Linden, 2008). More recently, a revised estimation by Ferraro and coauthors (2007) brought the estimation up to an annual discharge of 13,858 metric tonnes.

Since 1976, Mediterranean counties activated themselves against illegal oil discharges and in order to prevent and reduce pollution from ships and combat pollution in case of emergency the Regional Marine

Pollution Emergency Response Centre for the Mediterranean Sea (REMPEC) was established in 1989. According to the REMPEC database a total of 415 accidents occurred from 2000 to today and 155 out of the total resolved in an oil spill (Fig. 3). Greece seems to be the country with the highest number of total accidents (174, 81 involving spills), followed by Italy, Turkey and Spain (Fig.3).
One of the worst accident ever occurred in the Mediterranean Sea, and more precisely in the Ligurian Sea, is that of the Haven ship. In 1991 the Haven petroleum cargo transporting a total of 115,490 tons of oil underwent an onboard explosion, after which the cargo sank causing the immediate release of about all the oil transported. A high percentage (66-77%) of the oil is thought to have been burned during the fire and dispersed in the atmosphere; 25-33,000 tons (17-23%) were released in the sea and about 1,550 tons of crude oil reached the coasts. In 1997, six year after the disaster, a marine biomonitoring program has been developed by the Ligurian District Authorities, in order to assess the long term-effects of the discharged pollutants on marine organisms living in the ecosystem (www.haven.it). Mussels and benthic fish used as bioindicator still showed stress signals due to the presence of the bitumen formed during the Haven disaster (Vinarengo et al., 2007) and the environmental recovery seems to be still in progress, confirming the slow recovery rate previously reported for environments involved in accidental oil spills (Dauvin, 1998).

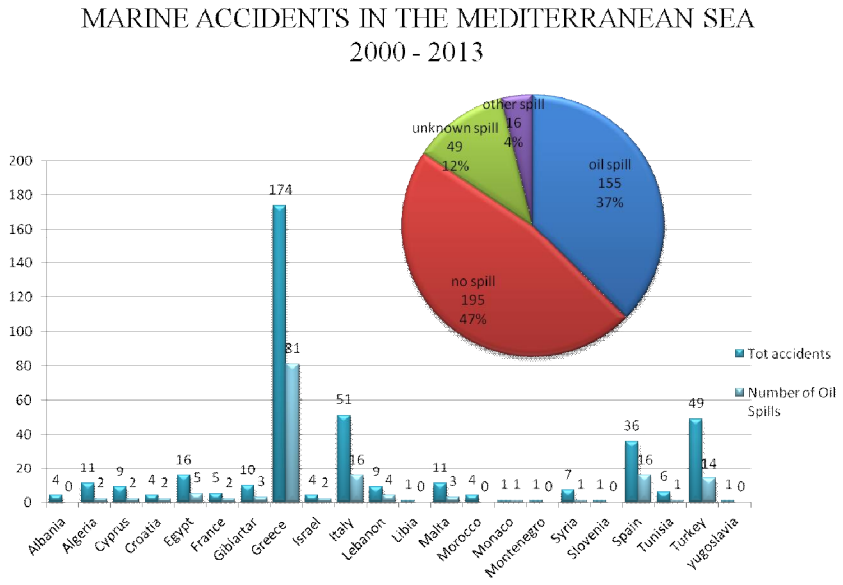

Fig. 3 **Accidents in the Mediterranean Sea from 2000 to 2013. Data from the REMPEC database**

2.4 - Scientific efforts of Mediterranean countries

Since the year 2000, more than a thousand of scientific papers focused on marine pollution have been published on peer review journal from the countries surrounding the Mediterranean Sea (Fig.4. This huge number indicates a high interest of the Mediterranean countries concerning the problem of pollution in the Mediterranean Sea, yet only a small part of these articles is focused on hydrocarbon pollution (about 200) and very few on the actual application of bioremediation strategies (Fig.4).
Until now, the efforts of the scientific community were devoted mainly to the mere description of the polluted areas, describing the level of pollution (Brekke et al., 2005; Coll et al, 2010; and references therein), how the pollutants influence the organisms and natural environments (Abdulla & Linden, 2008; Simonato el al., 2008; Viarengo et al., 2007) and the mechanisms of actions of the autochthonous microorganisms involved in the pollutants' degradation (Fuentes et al., 2014 and references therein). This type of scientific articles provide an illustrative knowledge of the Mediterranean polluted areas that is a first unavoidable step to discover new resources potentially useful for bioremediation but, on the other hand, it is not sufficient to improve and apply bioremediation strategies in the field. The very few studies carried on in the Mediterranean Sea under *in-situ* conditions (Nikolopoulou et al, 2013; Vezzulli et al., 2004) demonstrated how the complexity of the natural environment and of the autochthonous microbial community, play major roles in the efficacy of the applied remediation strategies.

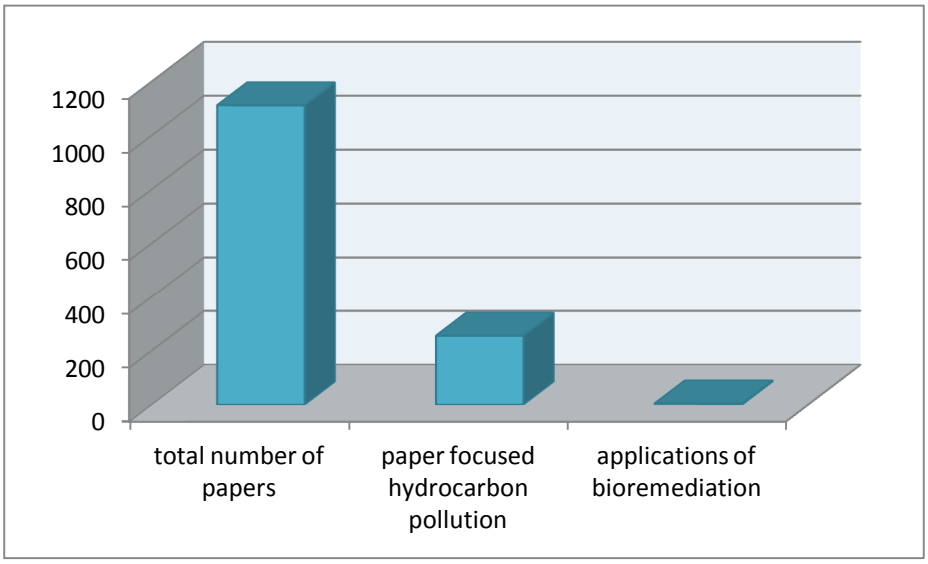

Fig. 4 **Number of paper published on peer review journals from 2000 to 2014. Search performed on Web of Science database.**

Despite several countries located along the Southern side of the Mediterranean Sea are major oil and gas producers, and the oil terminals located on their shorelines represent major pollution hot-spots in the Mediterranean basin, these areas have been largely neglected by the scientific studies. This fact represents a wide gap of knowledge in the exploitation of the Mediterranean Sea microbial diversity for bioremediation purposes (Fig.5). Recently, the research European Project "ULIXES" (www.ulixes/unimi.it) focused efforts on the study of specific highly polluted areas located in the Southern Mediterranean identifying different sites of interests along the Moroccan, Tunisian, Egyptian coastlines (Daffonchio et al., 2013). An integrated approach based on the application of molecular ecology, metagenomics, metabolomics has been undertaken by the "ULIXES" consortium, that comprises universities and research centers of both the sides of the basin. Such approach allowed to unravel the high metabolic and degradation potential of 3 sites located on the Southern Mediterranean and 1 site located on the Red Sea coast in Jordan, representing a reference site for the possible future scenario in the Mediterranean due to global warming (Bargiela et al., personal communication).

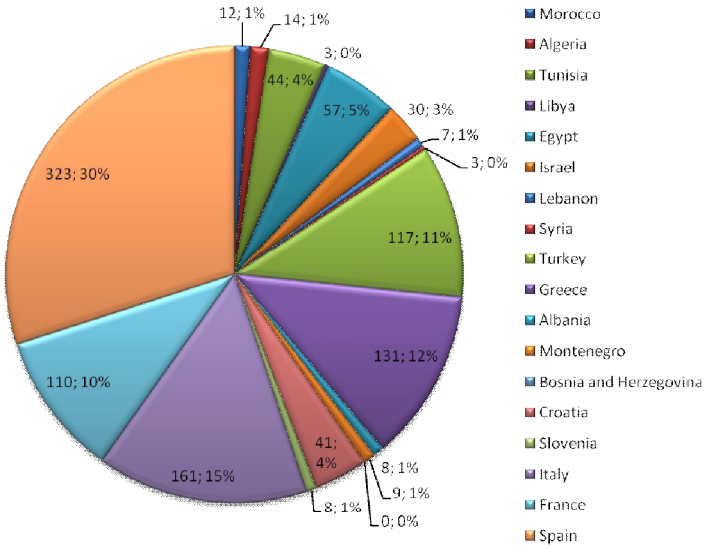

Fig. 5 **Scientific papers published on peer review journals from 2000 to 2014. Bibliografy search was performed on the Web of Science database, using the following entries: *marine pollution ñname of the countryò***

3. EFFECTS OF HYDROCARBON POLLUTION

Petroleum is a natural occurring, oily, flammable liquid that is present under the surface of the Earth. It originates in geological deposits from the organic decomposition of ancient plants and animals under high temperature and pressure. Its composition is very complex and its physical properties are highly variable and peculiar of every different geographical source. Petroleum is composed by a wide variety of low and high molecular weight hydrocarbons. It contains saturated alkanes, branched alkanes, alkenes, naphtenes (homo-cyclics and hetero-cyclics), aromatics (containing hetero atoms like sulfur, oxygen, nitrogen and other heavy metal complex), naptheno-aromatics, large aromatic molecules like resins, asphaltenes and hydrocarbon containing different functional groups like carboxylic acid and ethers (Abha and Singh, 2012).
The first effects of crude oil entering in the environment are the reduction of the oxygen levels and the changes of the sediments property. Usually hypoxia is only temporary and affect areas close to the spill, while sediments act as an hydrocarbons sink, resulting in benthic microorganism to be surrounded by petroleum contaminants (Abha and Singh, 2012).
After an oil spill, the volatile and light aliphatic factions of crude oil are the first to disappear from the released petroleum because of vaporization, photo-oxidation and biodegradation, whereas the heavier fractions, composed mainly by poly-aromatic hydrocarbons (PAHs) and asphaltenes, remains present as recalcitrant contaminants entering in the open sea and sediments. This kind of pollution is the major problem after an oil spill because this compounds are highly toxic and bioaccumulate in the food chain.
Volatile and soluble oil fractions vaporize in the air and leak fast into groundwater, therefore living organisms are easily exposed to these oil fractions by breathing contaminated air and/or drinking contaminated water (Welch et al., 1999). On the other side, the heavy fraction of crude oil are absorbed by sediment and can persist in the site of release for years. Furthermore, petroleum hydrocarbon can migrate from the release site and affect also terrestrial environment.
Petroleum hydrocarbon molecules cause different toxic effects depending on their chemical and physical nature, on their bioavailability and on the way and time of exposure. They can affect living being at every level, from DNA to organs, organisms and even behavior and communities (Danovaro et al. 1995; Meyer et al., 1998; Carvallo et al, 2006; Franci et al., 2014).

3.1 - Oil spills effects on higher organisms

Several studies linked the presence of hydrocarbons - especially PAHs - with genetic damage: DNA mutation are induced and, in Vertebrata, they often result in chromosomal aberrations, birth defects and cancer (Carvallo et al, 2006; Bolognesi et al., 2006a; Cebulska-Wasilewska et al., 2005). In cells, hydrocarbons can accumulate in membrane and lipid compartments (Di Toro et al., 2001), potentially affecting their biochemical pathway and physiological function and an increase in oxidative stress was observed in those organisms chronically exposed to oil pollutants (Di Giulio et al, 1993). Others studies related the exposure to petroleum components to haematic and immunological deficits (Simonato et al., 2008; Reynaud & Deschaux, 2006). Liver is one of the most sensitive organ, developing pre-neoplastic, neoplastic and necrotic lesions (Meyer et al., 1998; Moor &Myers, 1994): such kind of damages have been found in fish even 9 years after the Heaven accident in Ligurian Sea (Pietrapiana et al, 2002).
Seabird are among the most affected animals after an oil spill. Since they depend on marine water and environment for different activities, from feed to rest, they are particularly exposed to the released toxic compounds. The ingestion and the chronic exposure to PAHs, for instance, can cause oxidative stress in liver and kidney, neurological problem, endocrine alterations and immune suppression in seabird (Alonso-Alvarez et al., 2007; Balseiro et al., 2005; Chastel et al., 2005; Golet et al., 2002; Neilson, 1963; Troisi and Borjesson, 2005). Particularly, hormonal dysfunction can lead to behavioral changes with consequence on the reproductive abilities of these animals (Franci et al., 2014).
Changes in the animal communities inhabiting impacted area were documented after the Agip Abruzzo oil spill, in 1991, Livorno (Danovaro et al. 1995). In addition, the primary production and the phytoplankton biomass change after a spill, as observed after the Tsesis accident, near Stockolm, in 1977, when both increased (Johansson et al., 1980).

The overall animal composition of pristine and polluted environments was shown to be different: pristine environments are composed by f crustaceans (10-20%), mollusks (15-20%), echinoderms (5-8%), of polychaetes (about 58%) and other species (5-10%), while in highly polluted sites polychaetes and nematodes can reach up to 90% of the total animals (Bas, 2009).
The International Agency for research on Cancer (IARC) has indicated gasoline as possibly carcinogenic to humans (IARC 2000; Mortelmans et al, 1986). Furthermore, in human body, crude oil compounds can have detrimental effects in any organ and their effects can be either immediate or appearing several years after the exposure (Costello, 1979; Singh et al., 2004).

3.2 - Oil spills effects on marine microbial communities

Microbial organisms are also affected by hydrocarbon pollution representing, through their degradative metabolism, the main route of removal of hydrocarbons from the environment. The bacteria response to hydrocarbons pollution differ on the basis of the initial indigenous communities, nonetheless previous studies identified common patterns of reaction, indicating that the total bacteria abundance increases because hydrocarbons act as a source of carbon and electrons in an otherwise very poor environment (Kostka et al., 2011; Alonso-Gutierres et al., 2009). On the opposite, the bacteria diversity decrease, and a strong selection of hydrocarbon degrading organisms, involved in the cleaning of oil polluted environments, is normally recorded (Danovaro, 2003; Kasai et al, 2002a, 2002b; Maruyama et al., 2003; Roling at al., 2004). The bacterial genera that more commonly bloom after an oil spill in marine environment are *Alcanivorax*, *Cycloclasticus*, *Marinobacter*, *Neptunomonas*, *Oleiphilus*, *Oleispira* and *Thalassolituus* (Miralles et al., 2007) and the flourishing of each of these genera can be favored by specific environmental conditions (McGenity, 2012). In addition to seawater, shorelines are environments particularly sensitive to oil pollution. Studies carried out on beach ecosystems impacted by the Deepwater Horizon oil spill identified *Alcanivorax* genus as not only the more abundant, but also the more active within the oil degrading bacterial community, highlighting its role with the early stages of hydrocarbon degradation (Kostka et al., 2011; Martins dos Santos et al, 2010). When the first steps of oil degradation, namely alkanes removal, is accomplished leaving more recalcitrant molecules in the polluted environment, a new shift in the microbial community can be observed. Microbial population assemblages change toward taxonomic groups involved in the degradation of recalcitrant compounds, like α-*proteobacteria* and Gram positive bacteria (Alonso-Gutierres et al., 2009; Kasai et al, 2001).

4. HYDROCARBON REMEDIATION OF THE MEDITERRANEAN SEA

The inability to prevent oil spills, in spite of the legislative efforts of the countries surrounding the Mediterranean Sea, and to avoid that petroleum reach sensitive areas, can lead to economic, social and environmental damages. This highlight the need for remediation technologies that can swiftly respond to mitigate such potential damages. Several techniques have been developed for oil spill remediation of marine sites including mechanical recovery, use of dispersants and solidifiers, burning and bioremediation. Mechanical recovery, use of dispersant and burning alone are the classical remediation strategies, so far the most used to counteract marine oil spills, but these remediation strategies display some impacts on the ecosystem, so their benefit and potential damages toward the environment need to be carefully evaluated. Bioremediation is more environment friendly, but it started to be considered as an actual option for remediate polluted environments only in the last decade. Both these techniques alone seem to be inadequate to remediate oil spills, thus an integrated approach that consider metabolic requirement of degrading organisms, oil properties, environmental limitation on oil biodegradation and innovative delivery mechanisms that reduce these bottleneck is essential.

4.1 - Classical remedation

4.1.1 - Mechanical and physical strategies

Through this approach, which requires the use of oil booms, skimmers and adsorbent materials, the petroleum released in the environment is physically blocked or removed to another compartment, but not degraded. Booms are floating barriers that control the movement of surface oil slicks and that can improve the recovery of oil through skimmers(Dave and Ghaly, 2011). This kind of device is very efficient in blocking the oil to spread in calm waters, but are mostly inefficient in difficult weather conditions (Ventikos at al., 2004; Potter and Morrison, 2008; OSS, 2010).

Skimmers are mechanical devices designed to recover oil from the water surface and are often used in conjunction with booms. The oil recovered in this way can be reprocessed and reused since its properties are maintained (Dave and Ghaly, 2011).

Adsorbent materials are hydrophobic sorbents that entrap oil on their surface. They are usually exploited in the final steps of cleanup for the removal of residual oil after operations that involve skimmers and booms, and they can be used in both open water or shorelines (Wei et al, 2003). Recently many research efforts focused on developing new or improved sorbent materials (Gertler et al, 2009; Klavins & Porshnov, 2012; Simons et al., 2013) and it was also proposed to couple such materials with microorganisms able to degrade hydrocarbons to improve their efficiency (Gertler et al, 2009).

Even if the use of these methods is the preferred response option in many contingency plans, it is not completely effective in removing the oil pollutants and have many disadvantages. Their effectiveness is hampered by the oil tendency to spread, fragment and disperse under the influence of wind waves and currents. For instance, in rough sea, a spill of oil of low viscosity can be scattered for many km^2 in just few hours. It has been estimated that mechanical recovery in open sea results in the removal of only about 10-15% of the total spill (ITOPF, 2010). Finally, the high costs and the problems concerning the storage and disposal of the recovered oil, which need to be further processed, are additional disadvantages of these techniques.

4.1.2 - Chemicals

Chemical methods are commonly used in combination with physical methods which help to restrict the spreading of oil to protect shorelines and sensitive marine habitats. Various chemicals can be added and their action is to change the physical and chemical properties of the spilled oil (Vergetis, 2002): dispersant and solidifiers are the main classes used.

Dispersants consist of surfactants dissolved in a solvent sprayed onto oil spills. They are able to brake the slick in small droplets that enter in the column waters and, most important, render the oil more easily accessible to microorganism able to degrade it. They could be used also to help and enhance *in-situ* bioremediation, however these compounds can be very toxic for the environment and can cause human health hazard during the application (Dave & Ghaly, 2011). They have also been found to be responsible for fouling of shoreline and contamination of drinking waters (NRC, 1989). The new dispersant agents nowadays available are less toxic and more effective of the compounds used in the past (Lessard and Demarco, 2000), but still they are not free of dangers since they can also have acute toxic effects on living organisms (Hansen et al., 2014; Niu et al, 2010). Despite of their disadvantages, the addition of dispersants to remediate an oil spill has been proved to be effective in removing up to 90% of an oil spills and are cheaper than the physical methods (Holakoo, 2011). Moreover, differently from mechanical and physical methods, dispersants can be used in all weather and sea conditions, they render the oil less likely to stick to surfaces (including animals skin), and they accelerate the rate of natural oil biodegradation.

Solidifiers are hydrophobic polymers that can react with oil and change its liquid state into solid rubber that can be easily removed using mechanical methods, but their efficiency strictly depends on the oil composition. These compounds are difficult to manage, more expensive and less efficient of all the other available options (Fingas et al, 1990).

Overall, chemical agents have the great advantages that can be used with any sea condition, they are quick and they have an important role in accelerating oil degradation by natural processes. On the other hand their

actual efficacy depends on the oil composition, since they are not effective on highly viscous and waxy oils (Dave and Ghaly, 2011), and they can have toxic effects on benthic communities (Hansen et al., 2014).

4.1.3 - In-situ burning

In-situ burning requires a first step of containing the spill through a boom, followed by a controlled burn. Often burns need to be sustained through addition of burning agents (Fingas et al, 1990) and oxygen (Buist et al, 1999), but it allows a higher efficient oil removal. This method is far from being ideal because of the possibility of developing of second fires and, moreover, because of the risks for environment and human health and due to the byproducts of burning, like PAHs (Buist et al, 1999; Dave and Ghaly, 2011). Furthermore, both vegetation and aquatic animals near the burning site can be affected by the detrimental effects of *in-situ* burning, leading to long term alteration in aquatic life (Dave and Ghaly, 2011).

4.2 ï Bioremediation

The aim of bioremediation is to overcome the limiting factors that slow down natural pollutants' degradation rates in a certain environment. It is an environmental friendly approach for the clean-up of polluted environments (Head and Swanell, 1999), and it is generally considered non invasive and cost effective (April et al., 2000). This option could be the most reliable and less expensive method in solving different chemical pollution problems (Mesarch et al, 2000) and its application has been proposed to remediate numerous oil polluted marine environments after spills from ships or pipelines (Head et al., 2006).

Bioremediation can be accomplished either by *in-situ* or *ex-situ* treatments. During *in-situ* applications, the pollutant is degraded directly at the contaminated sites. This task is usually accomplished through bioaugmentation and biostimulation. The *ex-situ* technologies involve the transport of the polluted environmental matrix to a place where a suitable treatment system can be engineered. This kind of treatments comprise biopiles, composting and landfarming (Fuentes et al, 2014). Usually the *ex-situ* treatments are more effective in the degradation of pollutants because they occur under more controlled environmental parameters, nonetheless the transport of the polluted matrix in another place to be treated is often very difficult to accomplish.

4.2.1- Bioaugmentation

Bioaugmentation consists in the addition of selected microbial species, harboring specific catabolic abilities, into a contaminated environment in order to speed up or enable the degradation of pollutants. This technique can be classified as "allocthonous" or "autochthonous" bioaugmentation according to the origin of the applied microbial strains. The main problem associated to allocthonous bioaugmentation is that, usually, new organisms, selected and growth in laboratory conditions, do not survive when enter in a natural environment, due to their low adaptation to the occurring environmental conditions and because they can be easily outcompeted by the autochthonous populations (REF). It has been observed that bioaugmentation do not enhance oil degradation significantly if compared to natural attenuation (Tam and Wong, 2008). Its effectiveness can be inhibited by a variety of factors including pH, redox potential, the presence of toxic contaminants, the concentration and bioavailability of the target contaminants, or the absence of key growth substrates (Perelo, 2010), therefore the selection of the most appropriate microbial strains is of fundamental importance to successfully apply this techniques . Normally, bioaugmentation is performed by using mixed consortia instead of pure bacterial cultures. This is due to the fact that different bacterial species are involved in the successional steps of degradation processes toward mixture of hydrocarbons. While the less recalcitrant compounds (e.g. alkanes) are promptly metabolized by hydrocarbonoclastic bacteria like *Alcanivorax* sp., the degradation of the most recalcitrant compounds like PAHs requires the presence of different bacterial taxa such as *Bacillaceae* (REF, Kostka et al?). Moreover, the consortia used for bioaugmentation can contain microbial species that are not directly involved in the degradation reactions but play a crucial role within the biodegrading network by producing molecules such as biosurfactant, that are extremely important to enhance the availability of the target oil pollutants (Head et al., 2006).

Interesting results were obtained when microbes isolated from the same site to be remediated were selected, enriched under laboratory conditions, and then added back to the polluted area (autochthonous bioaugmentation). Diaz and colleagues (2000) isolated a petroleum degrading consortium of bacteria able to tolerate high salinity range, a common restriction in applying bioaugmentation to marine sediments. The consortium, isolated from the same polluted sediments to be treated and from crude oil, showed both degradation activity and high resistance to salt stress. Bioaugmentation has been proposed as a good remediation strategies when the limiting factor of natural attenuation is the absence, within the indigenous microbial community, of catabolic genes codifying for enzymes involved in the degradation of a certain pollutant, so that the degrading abilities would be provided by the supplemented strains (Thompson at al., 2005). Genetic information codifying for degrading abilities may also be introduced by donor species, which themselves do not survive a long time in the environment but enough to transfer the lacking genetic information to autochthonous microorganisms through horizontal gene transfer (Perelo, 2010).

4.2.2 - Biostimulation

Biostimulation consists in the addition of different substances, mainly chemicals, to contaminated environments to induce an acceleration of the natural biodegradation process performed by indigenous degrading populations. The biodegradation of oil spills in the marine environment is often limited by low oxygen, nitrogen, phosphorous and iron concentration, as well as by the low bioavailability of pollutants (Chandra et al., 2013): hence, the addition of appropriate molecules can help enhancing the biodegradation rates. Biodegradation of hydrocarbons is mainly an aerobic process and, since the first studies on hydrocarbon biodegradation, the concentration of oxygen has been recognized as a rate-limiting factor in soils and groundwater (vonWedel et al., 1988; Jamison et al., 1975). Its addition is often achieved through bioventing: air is delivered though pipes, either passively or by forced aeration (Fuentes et al., 2014). Nitrogen and phosphorus are nutrients essential for the growth of hydrocarbons degraders and they are always present in low concentration in marine environments. After an oil spill the ratio between carbon and the other nutrients become unbalanced and the low amount of nitrogen and phosphorous become a limiting factor for hydrocarbon biodegradation, slowing the natural attenuation of oil spills (Atlas, 1985; Chandra et al., 2013). The necessity to supply the lacking nutrients need to be carefully defined basing on how much carbon can be metabolized, depending on the oxygen-limiting condition, in order to avoid overapplication of nitrogen and phosphorous fertilizers. In fact, it has been demonstrated that disproportionated nutrients concentration can also inhibit the biodegradation activity (Chaillan et al, 2006). A safe approach could be the use of oleophilic biostimulants that remain preferentially at the oil phase/seawater interface, thus being available to hydrocarbon-degraders for enhancing their growth and metabolism. Attempts have been made to use less toxic and biodegradable fertilizers of natural origin, like uric acid and lecithin (Nikolopoulou et al, 2013). Uric acid has a low solubility in water and is the major component of guano fertilizer and hennery wastes. Natural phospholipids such as lecithin are in fact oil soluble, easy to get at low cost as by-products of the oil seeds industry and have good dispersant properties (Ron & Rosenberg, 2014).

A general problem in biodegradation of crude oil is the availability of the contaminant to the degrading organisms. Bioavailability depend on the physical and chemical characteristics of the molecules composing the mixture or petroleum: the more complex and big, the less soluble is the substance and therefore more recalcitrant to biodegradation. For this reasons surfactant molecules can be added to enhance the solubility of oil components and to help the access of oil pollutants by the degrading microorganisms (Perleo et al., 2010).

A new approach is the use of compounds of biological origin, like biosurfactants, bioemulsifiers and dispersants of natural origin (Banat et al., 2010; Satpute et al., 2010). The production of these compounds is one of the numerous adaptations of biological systems to metabolize hydrocarbons or mitigating their impact (Perfumo et al., 2010). Like synthetic dispersants, biosurfactants induce the formation of droplets from the oil slick, but they show the advantages to be degradable and much less toxic (Marti et al., 2014). On the other hand, their production and availability are still quite limited, thus they are very expensive. For instance, rhamnolipid - one of the best investigated class of biosurfactants (Chen et al., 2010) - can be produced by *Pseudomonas aeruginosa*, a potential pathogen, therefore not suitable for large scale production and release in the environment (Banat et al., 2010). Certain marine microorganisms adapted to

live in hydrocarbons polluted environments are already able to produce biosurfactants under natural condition, and they could be exploitable to produce such molecules in a cheap and safe way, however today only few methods for their cost effective production are available (Makkar et al., 2011). The availability of cheap and effective biological surfactants is pivotal to extensive future commercial applications which may lead to a significant increase in use for oil mobilization, dispersion and cleaning of surfaces. Recently, a method for the production and recover of biosurfactants useful for oil spill remediation in marine environments has been developed by Marti and colleagues (2014): two biosurfactants, surfactin and fatty acyl-glutamate, were produced from genetically-modified strains of *Bacillus subtilis*, producing efficient biodispersants with very low environmental impact and using green chemistry.
An in-field experiment aimed to compare different strategies of bioremediation of organic-rich sediments was carried out nearby Agrigento, in the Sicily channel (Vezzulli et al., 2004). The authors tested both biostimulation (oxygen release compounds—ORC) and bioaugmentation protocols. Biostimulation resulted in an increased mineralisation and net carbon loss via respiration, therefore it contributed to enhance bacterial degradation efficiency. On the other hand, even if the microbial consortium added through bioaugmentation did not enhance directly the rate of pollutant degradation, it survived in the treated natural environment and stimulated pollutant mobilisation processes. In fact, the debate about the effectiveness of bioaugmentation versus biostimulation is still open.

4.2.3 - Biopiles and composting

Both biopiles and composting involve the mixing of polluted matrix - mainly soil or sediment - with organic matter as a bulking agent, promoting microbial activity by improving matrix texture, aeration, and moisture maintenance (Jørgensen et al., 2000). Organic matter can be a substrate for microbial growth or even a source of microorganisms (Pèrez-Armendáriz et al., 2004). The main difference between biopiles and composting is the aeration methodology: composts are aerated by turning the soil/bulking agent mixture periodically with a modified windrow turner, whereas in biopiles a pipe network delivers air. Biopiles and composting have been successfully used for the remediation of a wide range of contaminants from contaminated soils (Namkoong et al. 2002; Van Gestel et al., 2003).

4.2.4 - Landfarming

Landfarming is mainly used for soils remediation. It consists in an aboveground remediation technology that reduces concentrations of petroleum constituents through volatilization and biodegradation.
It is based on the controlled spreading of organic waste on the soil surface to allow native microorganisms to aerobically degrade the present pollutants. Usually the excavated contaminated soils is spread in a thin layer on the ground surface and the aerobic microbial activity is promoted within the soils by aeration and/or addition of minerals, nutrients, and moisture (Anon, 1994).
This method has been widely used in soil remediation for over a century due to its low cost, simplicity in use, compliance with governmental regulations and potential application in a variety of environments. Landfarming is particularly adapt to remediate heavy petroleum hydrocarbons like lubricating oils and diesel fuel that do not evaporate and which removal is therefore predominantly due to microbial degradation (Khan et al., 2004). Anyway, a successful landfarming approach needs the help of biostimulation and bioaugmentation strategies: it requires the addition of nitrogen and phosphorous based-nutrients and other growth-limiting substrates like oxygen, which is provided by regular tiling. Recent studies also suggest to supply specialized pollutant-degrading prokaryotes during the startup phase of a landfarming facility for remediate marine sediments (Nikolopoulou & Kalogerakis, 2010)(Kalogerakis et al., 2005). All these factors concur to influence the activity of the degrading microbial community inhabiting the landfarming site, therefore identifying the key organisms that play role in different bioremediation treatments is very important for understanding, evaluating and further decide on the best remediation strategy.

5. CONCLUSIONS

In the last decades, many studies have been published on the state of the polluted areas of the Mediterranean Sea, describing both the extent of pollution and the living organisms inhabiting these areas. Furthermore, several studies focused on the function and role of specific microorganisms in remediating hydrocarbon pollution, representing an essential baseline that still is not sufficient to develop effective remediation strategies. It has been recognized that autochthonous microorganism already thriving in a polluted environment are the best candidate to effectively remediate it, because they are already adapted to the specific environment conditions. Nonetheless, the natural degrading activity of the autochthonous microbial community alone is not sufficient to accomplish remediation of polluted.

Thanks to the high biodiversity of the Mediterranean Sea, the study of its chronically polluted sites should bring to discover new biological resources exploitable for bioremediation approaches. The availability of new bioremediation strategies based on the exploitation of *in-situ* microbial communities and the enhancement of their metabolism is the key point for facing the pollution problem in the Mediterranean basin, because bioremediation technologies today are still far from fully satisfy the need of specific and efficient products and technologies tailored over specific sites or pollution types. Therefore, further holistic and multidisciplinary studies are crucial to comply with the urgent need of developing new products (i.e. microorganisms and their products) for the remediation of marine polluted sites in an economical, environmental and social sustainable manner.

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Chapter

III

BIOGEOGRAPHY OF PLANKTONIC BACTERIAL COMMUNITIES ACROSS THE WHOLE MEDITERRANEAN SEA

Abstract

Bacterial population distribution in the Mediterranean Sea has been mainly studied by considering small geographical areas or specific phylogenetic groups. In the present study, a molecular microbiology investigation aimed to identify the environmental factors driving total bacterioplankton community composition of seawater samples collected along a transect covering the whole Mediterranean Sea. We performed Automated Ribosomal Intergenic Sequence Analysis (ARISA) and microscope evaluation of prokaryotic abundance of seawater sampled across both vertical profiles and longitudinal transects in the whole basin. Prokaryotic abundance decreased with depth at all the stations and presented similar values in epi-, meso- and bathypelagic layers across the whole Mediterranean Sea. On the contrary, peculiar bacterial assemblages were selected along a longitudinal transect in the epipelagic layers of the eastern and western sub-basins. Vertical differences of the bacterial communities was observed only considering the epi- and bathypelagic waters, while the study of the structure of bacterial communities at a finer scale across the water column displayed higher variability at the intermediate layers. Nonetheless, different physico-chemical factors were significantly related to the distribution of bacterial populations, both according to geographic position and down the water column in the whole Mediterranean Sea. These results demonstrated that bacterial assemblages are putatively correlated to different water masses of the complex hydrographical systems of the eastern and western Mediterranean sub-basins.

1. INTRODUCTION

Prokaryotes are key players in sea ecosystems both in terms of biomass and phylogenetic diversity and for their role in biogeochemical cycles. Only recently, due to the development and advancement of molecular tools, have marine scientists carried out studies of the distribution and adaptation of microbial populations to specific environmental conditions in oceans (Agogué et al., 2011; Fuhrman et al., 2008; Galand et al., 2009; Hewson et al., 2006; Lovejoy et al., 2002; Varela et al., 2008a; Yilmaz et al., 2012). Nevertheless, some studies have focused on the ecological structuring of prokaryotes in the Mediterranean Sea, mainly through investigation of the eastern sub-basin (Feingersch et al., 2010; Moesender et al., 2001; De Corte et al., 2009; Yokokawa et al., 2010). The Mediterranean Sea has been proposed as a biodiversity hot spot, hosting about 17000 marine species belonging to the Eukarya domain and a number, at present impossible to estimate, of Bacteria and Archaea species (Coll et al., 2010). The Mediterranean Sea is a semi-enclosed basin divided into two main sub-basins by the Sicily channel, the western and eastern Mediterranean Sea. A typical trait of Mediterranean seawater is its oligotrophic nature, exceptionally pronounced in the easternmost area, where the conditions can be defined as ultra-oligotrophic. The low amount of inorganic phosphorous has been reported as a limiting factor of primary productivity in the eastern Mediterranean basin (Thingstad et al., 2005). The lack of thermal confines in the Mediterranean deep waters results in warm bottom layers and renders this marine ecosystem suitable for the study of partitioning of bacterioplankton throughout the water column, although the effect of pressure must be considered. Recent reports indicate that it is possible to correlate specific microbial community structure to different water masses in the ocean (Agogué et al., 2011; Galand et al., 2009; Varela et al., 2008a;b). However, similar studies performed in the Mediterranean Sea could not draw unequivocal conclusions (Tamburini et al., 2009; Yokokawa et al., 2010), in some cases indicating a depth-related distribution of specific groups of prokaryotes (De Corte et al., 2009, Winter et al., 2009). Indeed, water masses circulation and dynamics is extremely complex in the Mediterranean Sea (Bensi et al., 2012; Hecht et al., 1988; Pinardi et al., 2000 and references therein; Rubino et al., 2007) and a pronounced spatial and temporal variability of water masses composition in the different sub-basins can be related to local geographic peculiarities, such as deep water formation in the Adriatic and the Gulf of Lions, and the water input entering the basin from the Strait of Gibraltar, the Black Sea and the Suez Canal. Most of the microbiological investigations carried out in the

Mediterranean Sea focus on a small number of stations, generally located in a narrow area (De Corte et al., 2009; Tamburini et al., 2009; Yokokawa et al., 2010). Taking advantage of the M83/4 cruise, held onboard the R/V Meteor during April 2011, we examined the vertical and longitudinal distribution of bacterial populations at different sampling stations located across the whole Mediterranean Sea, from the Levantine to Alboran basin.
Automated Ribosomal Intergenic Sequence Analysis (ARISA) has proven to be a valuable tool for describing the bacterial community structure in marine ecosystems (Borin et al., 2009a;b; Fuhrman et al., 2008; Hewson et al., 2006; Zehr et al., 2009), and it was applied here to depict the bacterial community structure of epi-, meso- and bathypelagic waters. The aims of the present study were: i) to identify distinct or common patterns of bacterial community structure in epipelagic and deep waters sampled at twenty three stations along a longitudinal transect in the whole Mediterranean Sea, and ii) to describe the microbiome composition of seawater collected throughout the water column at eight stations located in the eastern and the western Mediterranean basins.
To the best of our knowledge this is one of the most comprehensive investigations of bacterioplankton distribution realized along a longitudinal transect in the oligotrophic surface and deep Mediterranean seawater.

2. MATERIALS and METHODS

2.1 - Study site, sample collection and oceanographic data

Sampling was carried out during the cruise M84/3 held on April 2011 on the R/V Meteor. Sampling stations were located in the main sub-basins (eastern and western) of the Mediterranean Sea, across a longitudinal transect of about 3500 Km (Figure.2). Water samples were collected from epi- (0-100 m), meso- (100-1000 m) and bathypelagic (> 1000 m) layers of the water column in order to investigate prokaryotic abundance and bacterial community composition: i) in epipelagic and deep waters along the whole longitudinal transect, and ii) at a finer vertical scale in the water column at eight stations to elucidate the vertical profiling of prokaryotic communities in the eastern and western sub-basins. The eight stations were chosen in order to cover the basin-wide variability of the epipelagic water layers (Atlantic Surface Water), the salinity maximum associated with Levantine Intermediate Water and the deep waters in the eastern, western, Adriatic and Aegean Sea (Table.1, Figure.1).
Water samples were retrieved at different depths (from 5 m to a maximum depth of 4190 m) of the water column by using Niskin bottles housed on a cable-connected rosette sampler under the control of a CTD (Conductivity-Temperature-Depth) probe (Seabird 19 Plus), providing the measurements of salinity, temperature and pressure. Inorganic nutrient concentration was measured on-board using a segmented flow SkalarSANplus System Instrument (Rahav et al., 2013; Tanhua et al., 2013a). Dissolved oxygen was measured following the Winkler potentiometric method as described by Tanhua et al. (2013a).

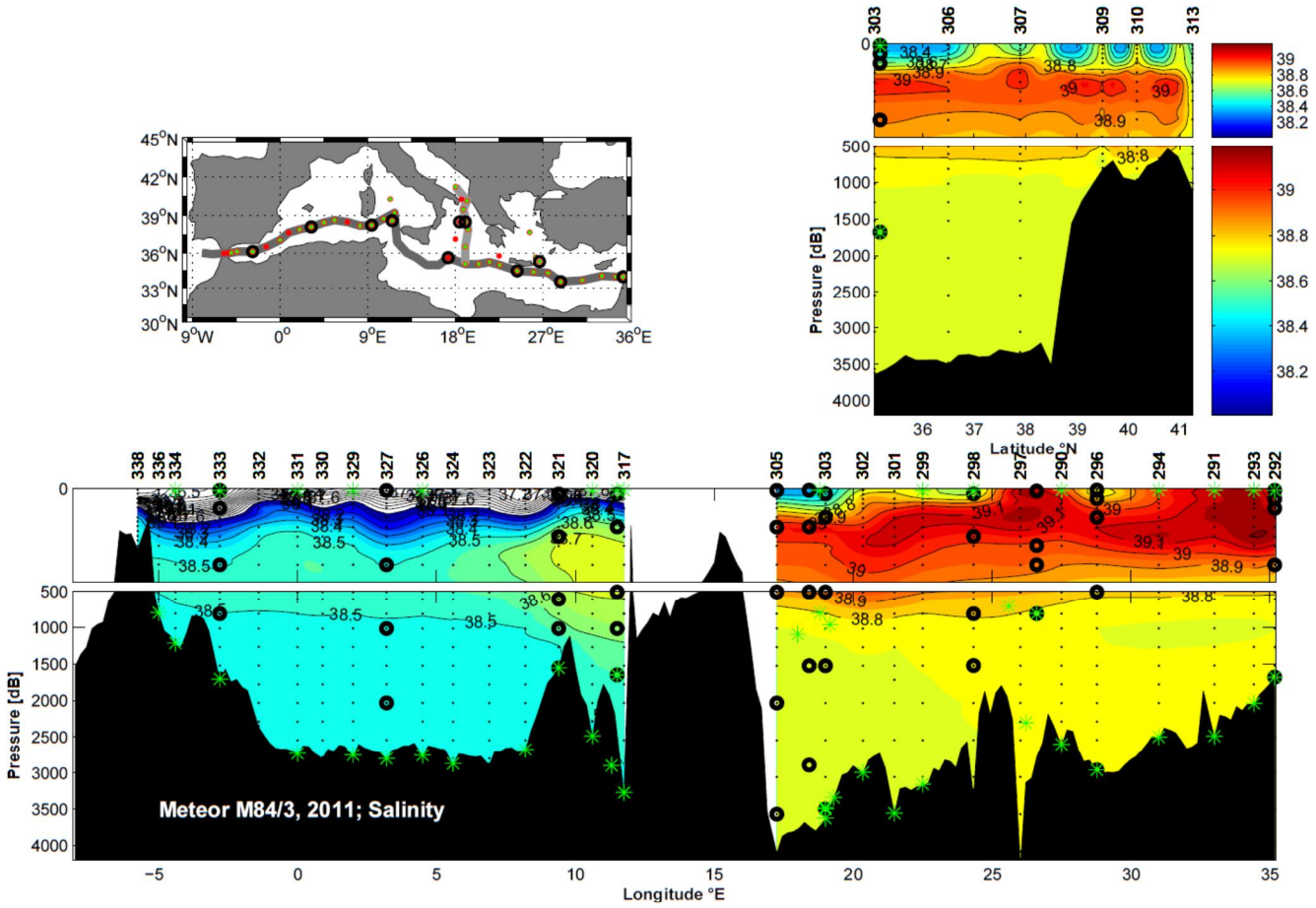

Figure 1 Sections of salinity in the Mediterranean Sea from the Meteor cruise M84/3. The top left panel shows the Mediterranean Sea map with the hydrographic stations sampled during the cruise (red dots), stations sampled by the University of Milan for epi- and bathypelagic waters (green dots) and stations sampled by IEO at a finer scale in the vertical profiles (black circles). The top right panel is a meridional section from the Adriatic Sea to the Ionian Sea (shown as a light gray line on the map). The lower panel is the zonal section from the coast of Lebanon in the Eastern Mediterranean Sea to the Strait of Gibraltar (shown as a dark gray line on the map). The depth and color scales are identical for both panels, where the top 500 m in each section is slightly expanded and the collected samples are marked by green stars (University of Milan dataset) and black circles (IEO dataset).

Station	Depth (m)	Longitude (E)	Latitude (N)	Pressure	Salinity	Temperature (°C)	Oxygen (µmol/kg)	Prokaryotic abundance (cells/l)	ARISA fingerprinting	PO_4 (µmol/kg)	NO_3+NO_2 (µmol/kg)
287	799	25,6	37,66	806,8	39,09	14,51	210,6	7,40E+07	-	0,06	1,19
288	2273	26,21	35,65	2302,6	39,05	14,36	208,8	6,85E+07	-	0,13	3,77
289	10	26,603	35,274	10,1	39,2301	17,653	230,7	-	X	0	0,14
289	300	26,603	35,274	302,3	39,0917	15,516	201,2	-	X	0,08	3,16
289	402	26,603	35,274	404,7	38,9906	14,822	192,7	-	X	0,14	4,37
289	801	26,6	35,27	809,3	39,02	14,6	206,9	8,08E+06	X	0,11	3,27
290	5	27,5	34,33	5,4	38,9699	17,247	232,2	-	X	0	0
290	2565	27,5	34,33	2600,2	38,7528	13,93	189,6	4,04E+07	X	0,17	4,71
291	5	33	34,06	5,3	39,1516	18,193	228,9	3,27E+08	X	0	0
291	2453	33	34,06	2485,9	38,7859	14,026	186,9	7,94E+07	X	0,18	4,7
292	7	35,17	33,99	6,9	38,9753	18,754	229,2	8,15E+08	X	0	0
292	52	35,17	33,99	52,3	39,03	17,91	224,5	3,54E+08	X	0,01	0,15
292	102	35,17	33,99	102,5	39,15	18	223,4	4,34E+08	X	0,01	0,27
292	402	35,17	33,99	405,7	38,87	14,31	178	1,27E+08	X	0,22	5,45
292	1659	35,17	33,99	1677,9	38,78	13,89	108,8	5,62E+07	X	0,18	4,89
293	6	34,42	34	6,6	39,1667	18,751	226,1	2,96E+08	X	0	0
293	2013	34,42	34	2037,8	38,7881	13,96	185,3	8,35E+07	X	0,18	4,71
294	6	31	33,7	6,2	39,0284	18,138	228,8	2,74E+08	X	0	0,07
294	2464	31	33,7	2497	38,7826	14,016	188,2	5,75E+07	X	0,19	4,79
296	5	28,77	33,58	5,4	38,7	17,47	231,3	2,55E+08	-	0,01	0,06
296	51	28,77	33,58	51,3	38,79	17,37	230,2	2,66E+08	-	0	0,06
296	150	28,77	33,58	151,5	39,03	16,23	207,4	1,58E+08	-	0,03	1,52
296	502	28,77	33,58	506,7	38,81	13,97	175,4	7,26E+07	-	0,24	5,89
296	2914	28,77	33,58	2956,3	38,77	14,07	187,7	3,96E+07	-	0,18	4,85
297	4190	26,01	34,39	4262,8	38,75	14,22	193,1	2,88E+06	-	0,16	4,75
298	5	24,33	34,5	5,7	38,5803	17,122	235,7	-	X	0	0
298	25	24,333	34,5	25,5	38,6785	16,605	-	-	X	-	-
298	251	24,333	34,5	253,5	39,0166	14,889	196,1	4,94E+07	X	0,11	4,1
298	803	24,333	34,5	810,8	38,815	13,939	186,2	6,45E+07	X	0,19	5,08
298	1505	24,333	34,5	1521,7	38,7604	13,78	188,1	4,66E+07	X	0,17	4,88
298	3266	24,333	34,5	3316,1	38,7376	13,992	192,5	3,97E+07	X	0,16	4,69
299	6	22,5	35	6,3	38,7433	16,791	233,6	-	X	0	0,02
299	3106	22,5	35	3152,6	38,7368	13,954	191,9	-	X	0,18	4,51
301	3497	21,48	35,23	3552,6	38,7433	16,447	-	-	X	1,29	0
302	2948	20,35	35,06	2990,8	38,7258	13,915	192,6	9,86E+06	X	0,18	4,67
303	3565	19	35,06	3622	38,72	14,01	199,4	4,38E+06	-	0,16	4,43
305	6	17,25	35,6	6,3	38,34	17,05	237	2,35E+08	-	0,01	0

Table 1 List of the samples analyzed for prokaryotic abundance and/or bacterioplankton community composition, reporting the corresponding available spatial and physico-chemical characteristics.

2.2 - Abundance of prokaryotes

Prokaryotic abundance was determined in seawater collected from the Niskin bottles and fixed with sterile formaldehyde (4% final concentration) in the dark. Subsequently, the samples were filtered on 0.22 □m pore size black polycarbonate filters (Millipore, USA), frozen and kept at -20ºC until analysis. Prokaryotic abundance was evaluated by 4',6-diamidino-2-phenylindole (DAPI) staining (excitation 340/360 nm, emission 440/470 nm). Prokaryotic cells positively stained by DAPI were counted with an epifluorescence microscope. For each sample, 30 microscope fields and more than 3000 DAPI-stained cells were counted. From a sub-set of samples analyzed in triplicate, the counting error was estimated to be less than 25.6% (average 13.8%).

2.3 - DNA extraction and ARISA fingerprinting

A volume of 4-10 l of water was filtered through sterile GWSP 0.22 □m pore size filters (Millipore, USA). Subsequently, 1.8 ml of lysis buffer (40 mM EDTA, 50 mMTris-HCl, 0.75 M sucrose) was immediately added to the filters before storing them at -20°C until extraction. DNA extraction from the filters was performed following the protocol described in Borin et al. (2009b) by the addition of lysozyme and sodium-dodecyl-sulphate (SDS) to lyse the cells. Proteins were removed from the lysis mixture by proteinase K and chloroform/phenol extraction before DNA precipitation by isopropanol. The pellet was washed with 70% ethanol and resuspended in sterile TE buffer. ARISA-PCR was conducted on a standard amount of DNA on each sample by using the primer set ITSF, 5´-GTC GTA ACA AGG TAG GCC GTA-3´ and ITSReub, 5´-GCC AAG GCA TCC ACC 3´, as previously described (Cardinale et al., 2004). ARISA fragments were separated using the ABI3730XL genetic analyzer applying the internal standard 1200-LIZ (Macrogen, Korea) or Peak Scanner Software - Applied Biosystems and the internal standard 1500- ROX. The ARISA analyses were performed separately on i) epi-and bathypelagic samples across the whole longitudinal transect, and ii) samples from eight stations across a vertical profile covering epi-, meso- and bathypelagic zones of the water column, performed respectively at the University of Milan and Instituto Español de Oceanografía (IEO). The results obtained were analyzed as two different datasets and are discussed separately in the manuscript in sections 3.2-3.3 and 3.4, respectively. The output peak matrix was transferred to Microsoft Excel for the following analysis. Peaks showing a height value < 50 fluorescence units were removed from the output peak matrix before statistical analyses. Each polymorphic ARISA peak was defined as a different Operational Taxonomic Unit (OTU). To account for variability in size associated with standards, ARISA fingerprints were binned ± 1 bp from 150 to 300 bp, ± 3bp from 300 to 500 bp and ± 10bp > 500 bp.

2.4 - Statistical analysis

Non-metric multidimensional scaling (nMDS) was carried out to explore similarities between OTUs, based on the resemblance matrix generated using Bray–Curtis similarity on the presence/absence of the OTUs within each sample. Principal component analysis (PCA) was performed on the environmental data matrix (latitude, longitude, pressure, temperature, salinity and oxygen concentration) to visualize the relationship among the samples. The same set of environmental data was used in the distance-based multivariate analysis for a linear model (DistLM, Anderson, 2002) to determine which significant environmental variables explained the observed similarity among the samples. The Akaike Information Criterion (AIC) was used to select the predictor variables. The contribution of each environmental variable was assessed, firstly using 'marginal tests' to assess the statistical significance and percentage contribution of each variable taken alone, and secondly a 'sequential test' was employed to evaluate the cumulative effect of the environmental variables explaining biotic similarity. A distance-based redundancy analysis (dbRDA) was used for graphical visualization of the DistLM results. Significant differences in microbial community composition were investigated by permutational analysis of variance (PERMANOVA, Anderson, 2001),

considering the sampling area as a fixed and orthogonal factor. Ecological diversity indices were calculated from the matrix of ARISA OTUs. All the statistical tests were performed by PRIMER v. 6.1 (Clarke et al., 2006), PERMANOVA+ for PRIMER routines (Anderson et al., 2008) and PAST software.

3. RESULTS and DISCUSSION

3.1- Environmental parameters

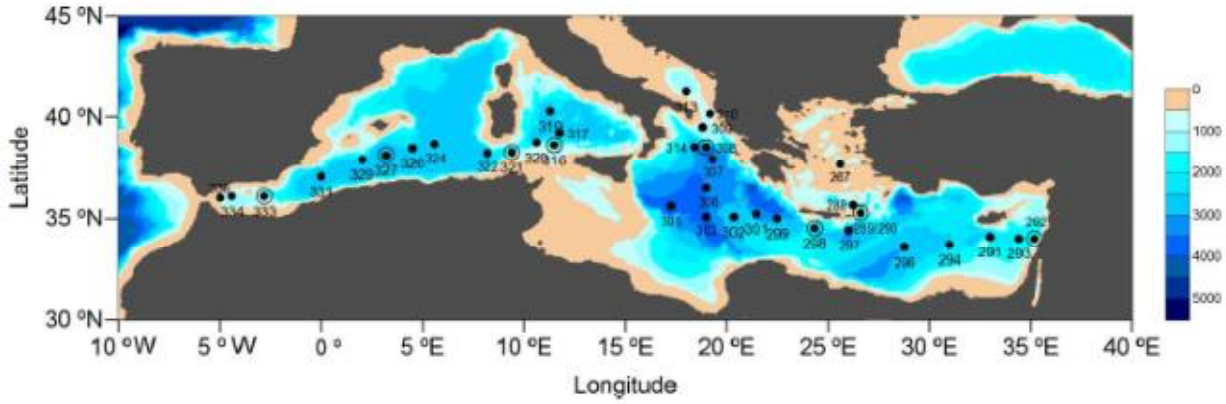

Figure 2 Location of sampling stations during the M84/3 cruise. Map of the study area of the Mediterranean Sea with the stations occupied during the METEOR cruise indicated by dots. Station where bacterial abundance was measured are indicated by encircle dots. Stations numbers indicate stations where the community composition (ARISA fingerprinting) was determined.

The longitudinal and vertical distribution of the main physical (temperature and salinity) and chemical (inorganic nutrients and dissolved oxygen) variables measured across the Mediterranean Sea during the M84/3 cruise is described by Tanhua and co-authors (2013b). The physico-chemical data shown in Table.1 and Figure.1 provide the parameters for the characterization of the samples specifically investigated here by microbiological analyses.

Briefly, epipelagic samples were characterized by a high temperature (>16ºC) and salinity (>36), both increasing eastwards due to the salinification of surface Atlantic water as it travels eastwards in the Mediterranean Sea (Table.2). Both nitrate and phosphate were depleted in the epipelagic layer. The physical and chemical values measured in each sample are reported in detail in Table.1. The vertical distribution of salinity basin-wide and the location of the samples collected for microbiological analyses are indicated in Figure.1. Bottom samples in the western basin presented homogenous temperature and salinity (Table.2 and Figure.1), lower than in the eastern basin. Within the bottom samples, those collected from the Aegean Sea showed the highest temperature and salinity values while the higher nutrient values were recorded in the western basin samples.

Region	Layer (m)	Salinity	Temperature (ºC)	Dissolved Oxygen ($\mu mol\ l^{-1}$)	PO_4 ($\mu mol\ l^{-1}$)	NO_3 ($\mu mol\ l^{-1}$)	NO_2 ($\mu mol\ l^{-1}$)
Eastern	<100	38.80 ± 0.29 (26)	16.58 ± 0.99 (26)	220.46 ± 1.25 (26)	0.01 ± 0.002 (15)	0.18 ± 0.04 (25)	0.02 ± 0.01 (20)
	100-1000	38.93 ± 0.03 (28)	14.93 ± 0.13 (28)	189.48 ± 1.97 (25)	0.10 ± 0.007 (25)	3.27 ± 0.18 (25)	0.02 ± 0.005 (22)
	>1000	38.76 ± 0.01 (23)	13.89 ± 0.04 (23)	175.02 ± 1.24 (23)	0.18 ± 0.008 (21)	4.69 ± 0.08 (21)	0.006 ± 0.001 (15)
Western	<100	37.15 ± 0.12 (22)	15.77 ± 0.14 (22)	218.77 ± 1.90 (22)	0.08 ± 0.01 (21)	1.79 ± 0.37 (22)	0.04 ± 0.006 (22)
	100-1000	38.10 ± 0.17 (26)	13.42 ± 0.07 (26)	172.25 ± 1.58 (26)	0.40 ± 0.03 (19)	8.02 ± 0.45 (22)	0.01 ± 0.001 (21)
	>1000	38.36 ± 0.12	13.05 ± 0.20	174.94 ± 0.88	0.41 ± 0.03	8.57 ± 0.55	0.008 ± 0.003

	(20)	(20)	(19)	(17)	(17)	(13)

Table 2 Region name, layer, physical parameters (salinity and temperature) and chemical parameters (phosphate, nitrate and nitrite) of the stations sampled during the M84/3 METEOR cruise. Mean ± SD are given for the epi-, meso- and bathypelagic waters. Numbers between brackets indicate the number of stations.

3.2 - Prokaryotic abundance decrease throughout the water column of eastern and western Mediterranean basins

Prokaryotic abundance was estimated at different stations distributed along the two sub-basins (western and eastern) from the epi-, meso- and bathypelagic waters of the Mediterranean Sea (Figure.3). Prokaryotic abundance ranged between 1.94 x 108 and 8.15 x 108 cell L-1 (3.24 x 108 ± 1.40 x 108, mean ± SE) in the epipelagic layer up to 100 m depth and decreased exponentially with depth to 4.82 x 107 - 3.65 x 108 cell L-1 (9.81 x 107 ± 6.60 x 107, mean ± SE) in the mesopelagic layers and to 2.88 x 106 - 1 x 108 cell L-1 (4.55 x 107 ± 2.50 x 107, mean ± SE) in the bathypelagic waters. Total microbial abundances reported in this study are in agreement with previously published data for both epipelagic and deeper layers of the water column in different oceanic regions, including the North Atlantic (Varela et al., 2008a, Arístegui et al., 2009), eastern (Borin et al., 2009b; Yokokawa et al., 2010) and western Mediterranean Sea (Luna et al., 2012) and the whole Mediterranean Sea (Zaccone et al., 2012).

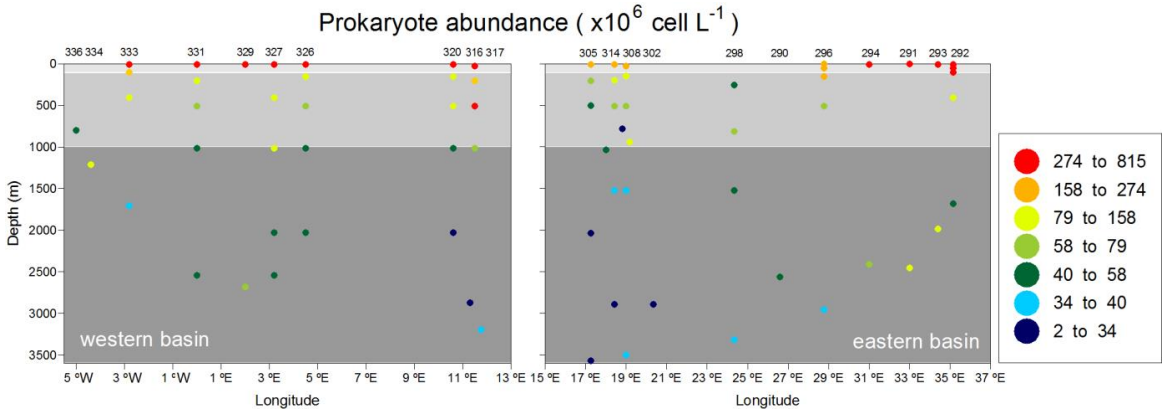

Figure 3 Prokaryotic abundance across the water column in the eastern and western Mediterranean Sea. Prokaryotic abundance in each of the three water layers (epi-: ≤100 m; meso-: 100-1000 m and bathypelagic: >1000 m) in the eastern and western basin of the Mediterranean Sea during the METEOR cruise. Station name labels are reported only for those stations where prokaryotic abundance was evaluated at two or more depths.The Figure was generated by SURFER 11- Golden Software.

The PERMANOVA analysis indicated that prokaryote abundance in the epi-, meso- and bathypelagic zones was significantly different (p=0.0001). On the contrary, the comparison of the prokaryotic abundance obtained along the water column between the eastern and western basins of the Mediterranean Sea revealed no significant differences in the epi- (p=0.3215), meso- (p=0.0628) and bathypelagic (p=0.4274) layers of the two sub-basins. The statistical approach adopted in this study showed that differences in the prokaryotic abundance values are ascribable to a depth-related decline and excluded any correlation between total cell abundance and specific water masses conditions characterizing the different sub-basins of the Mediterranean Sea.

3.3 - Surface and deep water layers host significantly different bacterial communities

ARISA fingerprinting was applied to detect spatial patterns in the structure of bacterial communities, aiming to identify the correlation between structure and the environmental conditions that characterize the epi- and bathypelagic waters of the Mediterranean Sea. The use of molecular methods based on the PCR amplification of regions of the ribosomal operon is particularly suitable to depict the bacterial community composition in marine oligotrophic ecosystems (Brown et al., 2005; Moeseneder et al., 2001), since bacteria growing in nutrient-poor water generally have single or few identical operon copies (Brown et al., 2005; Fegatella et al., 1998).

The ARISA profiles of the bacterial communities inhabiting the epi- and bathypelagic waters in the eastern and western Mediterranean Sea showed 194 polymorphic peaks in total, ranging between 157-1600 bp, considered as different bacterial taxa (OTUs). None of the detected OTUs were ubiquitously present in the epi-and bathypelagic layers of the analyzed stations, while 18% of the OTUs were unique. The ARISA profiles of the epipelagic layers comprised 32-68 OTUs (56.1 ± 8.9, mean ± SE) and displayed Shannon diversity index values ranging between 2.91 and 3.73 (Table.4). On the contrary, the ARISA patterns in the bathypelagic layers showed slightly lower numbers of taxa (45 ± 13.7, mean ± SE) and lower taxonomic diversity (Shannon index values: 1.29 - 3.67) (Table.4).Of the total OTUs retrieved, 134 out of 194 (69%) were present in the deep layers, whereas only 3 OTUs (2%) were shared among all the stations and 31 OTUs (23%) were singletons. Non-metric Multidimensional Scaling (NMDS) analysis was applied on the ARISA fingerprints, showing a clear separation between bacterial communities inhabiting the epi- and bathypelagic layers (Figure.4a). The NMDS analysis, characterized by a low stress value, and PERMANOVA test indicated that the bacterial communities in seawater samples collected from the epi- and bathypelagic layers were significantly different (p=0.0001), indicating the occurrence of strong selecting forces that differentiate these water column layers (Figure.4a).

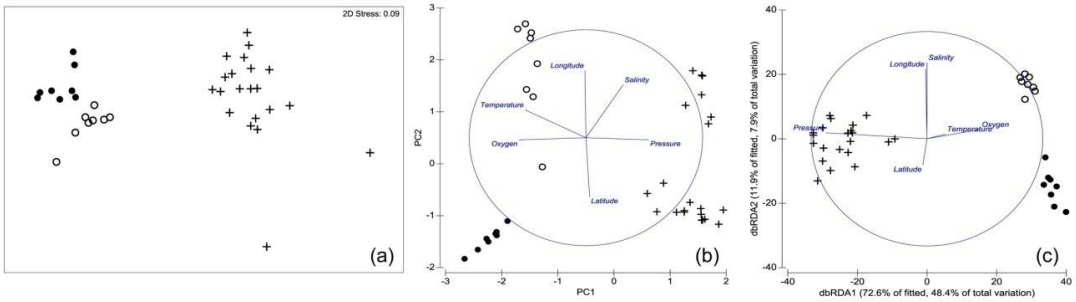

Figure 4 Distribution of the bacterial communities in the epi-and bathypelagic waters according to biotic and environmental data. (a) Non-metric multidimensional scaling (NMDS) results based on qualitative ARISA fingerprinting showing a sharp clustering of epi-(open and filled circles corresponding respectively to eastern and western epipelagic samples) and bathypelagic (crosses) seawater samples. (b) Principal Component Analysis performed on the environmental data reported in Table.1. (c) dbRDA ordinations of the presence/absence ARISA dataset overlaid with the partial correlations of the tested environmental variables explaining the clustering of epi- (open and filled circles) and bathypelagic (crosses) seawater samples.

Axis	% Explained variation (fitted model)		% Explained variation (total variation)	
	Individual	Cumulative	Individual	Cumulative
dbRDA1	71.44	71.44	48.85	48.85
dbRDA2	11.03	82.47	7.54	56.39
dbRDA3	9.29	91.76	6.35	62.74
dbRDA4	4.65	96.41	3.18	65.92
dbRDA5	2.54	98.95	1.74	67.65
dbRDA6	1.05	100.00	0.72	68.37

Table 3 Results of distance-based multivariate linear model (DistLM) for bacterial community composition in epipelagic and deep water layers, showing the % variation explained by individual axes. The first two columns relate to the percentage explained out of the fitted model (individuated with the DistLM analysis). The second two columns relate to the percentage explained out of the total variation in the resemblance matrix used to build up the DistLM model.

A Principal Component Analysis (PCA) was applied to the available environmental data (Table.2), to illustrate the distribution of the samples according to their physico-chemical and geographical parameters (Figure.4b). Both the epi-and bathypelagic samples were divided according to the longitude and latitude values, whereas the pressure, representing a proxy for depth, mainly determined the segregation of

epipelagic and deep samples. A distance-based multivariate analysis for a linear model (DistLM) was applied, with the aim to identify the environmental variables shaping the bacterial community composition in epipelagic and deep waters across a wide transect (about 3500 Km) in the Mediterranean Sea. The DistLM analysis identified that 68.37% of total variation was related to five significant environmental variables (Table.3), responsible for the observed spatial distribution of bacterial communities in epi- and bathypelagic zones.

The DistLM analysis indicated that longitude (p=0.04), pressure (p=0.0001), salinity (p=0.0001), temperature (p=0.0001) and oxygen concentration (p=0.004) were significant variables, while the latitude was not significantly related (p=0.36) to the variation in bacterial community composition. The two axes of the distance-based redundancy analysis (db-RDA) displayed 56.39% of the total variation (Figure.4c) overlapping with the bacterial population distribution detected in the epi- and bathypelagic layers with the environmental variables, represented as vectors. Epi- and bathypelagic samples clustered separately according to pressure, temperature and oxygen values, which represent the primary factors driving bacteria partitioning in such different zones of the water column. Microbial zonation according to depth has been previously reported both in the Mediterranean Sea and in the oceans by applying molecular fingerprinting (De Corte et al., 2009; Moeseneder et al., 2001), 16S rRNA pyrotag sequencing (Agogué et al., 2011) and metagenomics (DeLong et al., 2006). In the Mediterranean Sea, the water temperature of the deep layer is only slightly lower than that measured in the epipelagic waters (see Table.2 in this study). Still, the differences in the microbial community structure in the epi- and bathypelagic biomes in the Mediterranean Sea can be ascribed to factors of remarkable influence including the hydrostatic pressure effect (Tamburini et al., 2009), selection for piezophilic populations in deep water, light penetration and nutrient profiles. Our study confirms, for the first time on a transect covering the whole Mediterranean Sea, the bacterioplankton communities pattern previously reported at single stations and/or shorter transects in different oceanographic regions around the world.

Sample	OTUs	Shannon index
290-E	59	3,504
291-E	62	3,389
292-E	59	3,429
293-E	51	3,186
294-E	63	3,431
298-E	68	3,295
299-E	60	3,297
309-E	32	2,911
316-E	61	3,735
317-E	59	3,606
320-E	64	3,502
326-E	53	3,523
327-E	48	3,421
329-E	46	3,298
331-E	62	3,378
333-E	51	3,042
290-B	44	2,9
291-B	55	3,149
293-B	30	2,889
294-B	44	2,539
299-B	45	3,135
301-B	24	1,47
302-B	14	1,29
309-B	45	3,349
310-B	51	3,341
313-B	71	3,676
316-B	51	2,799
317-B	69	3,557
319-B	51	3,484
320-B	40	3,233
326-B	29	2,907
327-B	47	3,339
329-B	40	3,216
331-B	62	3,572
333-B	49	3,29
334-B	37	2,945
336-B	47	3,075

Table 4 Total number of OTUs and Shannon index of the samples analyzed by ARISA fingerprinting at the University of Milan.

3.4 - Surface bacterial community composition is patterned according to longitude

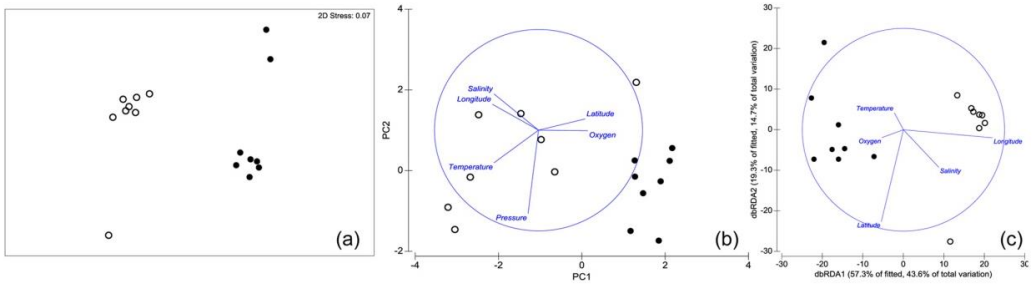

Figure 5 Distribution of the bacterial communities in the Mediterranean Sea epipelagic waters according to biotic and environmental data. (a) Non-metric multidimensional scaling (NMDS) results based on qualitative ARISA fingerprinting illustrating a clear clustering of eastern (open circles) and western (filled circles) Mediterranean Sea epipelagic water. (b) Principal Component Analysis performed on the environmental data reported in Table.1. (c) dbRDA ordinations of the presence / absence ARISA dataset overlaid with the partial correlations of the tested environmental variables explaining the clustering of eastern (open circles) and western (filled circles) Mediterranean Sea epipelagic water.

The occurrence of a distribution pattern of planktonic bacterial populations within the epipelagic seawater was gathered from the db-RDA analyses performed on the ARISA profiles of epipelagic and deep waters (Figure.4c). To investigate in detail the existence of different bacterial assemblages in the epipelagic water sampled across the Mediterranean Sea transect during the M84-3 cruise, the ARISA profiles of epipelagic samples were analyzed separately adopting the same approach described above for the whole ARISA dataset. ARISA fingerprinting detected 140 OTUs in epipelagic samples, including 13 ubiquitous OTUs (9%) and 26 OTUs (18.5%) present as singletons. NMDS analysis demonstrated the separation of planktonic bacterial communities inhabiting epipelagic waters in two distinct clusters of samples (Figure.5a), corresponding to the stations located in the eastern and western Mediterranean Sea. The results of NMDS analysis (stress value =0.07) was strengthened by PERMANOVA test, showing that the bacterial community structure in the eastern and western sub-basins of the Mediterranean Sea was significantly different (p=0.0001). PCA of the epipelagic samples according to the environmental variables showed a less clear separation among the epipelagic seawaters (Figure.5b); nonetheless, the DistLM analysis indicated that four significant explaining variables accounted for up to 76.12% of the total variation detected in the bacterial community composition (Table.5).

Axis	% Explained variation (fitted model)		% Explained variation (total variation)	
	Individual	Cumulative	Individual	Cumulative
dbRDA1	57.33	57.33	43.65	43.65
dbRDA2	19.25	76.59	14.65	58.30
dbRDA3	13.44	90.03	10.23	68.53
dbRDA4	6.54	96.57	4.98	73.51
dbRDA5	3.43	100.00	2.61	76.12

Table 5 Results of distance-based multivariate linear model (DistLM) for bacterial community composition in epipelagic waters showing the % variation explained by individual axes. The first two columns relate to the percentage explained out of the fitted model (individuated with the DistLM analysis). The second two columns relate to the percentage explained out of the total variation in the resemblance matrix used to build up the DistLM model.

Longitude (p=0.0001), latitude (p=0.0017), salinity (p=0.0021) and oxygen concentration (p=0.047) were identified as significant variables, while temperature (p=0.068) was not significantly related to the observed biodiversity pattern. The low importance of temperature in determining the distribution of bacterial epipelagic populations was expected, considering that the recorded temperature values were within a narrow range (14.83-18.19 ºC at approximately 5 m depth) across the epipelagic waters of the eastern and western Mediterranean Sea (Table.2, Table.1). The two axes of the db-RDA displayed 58.3% of the total

variation of bacterial distribution (Figure.5c), indicating that bacterial zonation in epipelagic samples is unambiguously related to longitude, as already inferred by NMDS.
The existence of biogeography patterns in the composition of bacterioplankton communities in epipelagic seawaters was recently demonstrated in the Mediterranean Sea for specific bacterial taxa. The abundance of aerobic anoxygenic phototrophs was shown to be inversely linked to the nutrient concentrations in epipelagic waters of the Mediterranean Sea along a transect covering the whole eastern and part of the western Mediterranean basins (Lamy et al., 2011). Similarly, two diversity studies on Cyanobacteria showed that the Synechococcus (Mazard et al., 2007) and Prochlorohococcus (Mella-Flores et al., 2011) clades were differently distributed in the epipelagic layers of the eastern and western Mediterranean Sea, questioning the possible influence of water inputs from the Suez channel (Feingersch et al., 2010). About 600 exogenous species belonging to the Plantae and Animalia kingdoms have been introduced into the Mediterranean Sea, mainly from the Suez channel (Coll et al., 2010 and references therein). This is a partial estimation since unicellular organisms are not included in the number of allochthonous species constituting the core of a novel biodiversity imported into the Mediterranean basin. Exogenous species are spatially distributed along the Mediterranean Sea coasts, displaying a higher abundance in correspondence with the Levantine basin (Coll et al., 2010). Possibly, a similar distribution model is followed by planktonic microorganisms, which would diversely impact on the epipelagic water of the eastern and western Mediterranean Sea, thus corroborating the results of the present study. Epipelagic seawater samples were collected by Rahav and co-authors (2013) during the M84/3 cruise, in parallel with the samples analyzed in the present study, and the role of diazotrophy on total primary productivity was estimated. The lowest N2 fixation values were measured in the Levantine basin, while the highest N2 fixation rate was observed at the Gibraltar strait, corresponding to an east-west trend of increased contribution of N2 fixation to primary productivity, as shown for chlorophyll concentration (Rahav et al., 2013). N2 fixing measurements allowed the inference of the main role of autotrophic and heterotrophic N2 fixing bacteria respectively in the eastern and western basins (Rahav et al., 2013), an aspect that might affect the total bacterial community structure in epipelagic seawater. Indeed, the correlation between picophytoplankton and specific groups of bacteria, namely aerobic anoxygenic phototrophic bacteria, was reported in the Mediterranean Sea (Lamy et al., 2011), while bacterioplankton and phytoplankton abundance in the Arctic Ocean showed a similar trend (Li et al., 2009). The influence of latitude on the microbiome inhabiting surface waters has already been reported by Fuhrman et al. (2008), investigating the microbial community assemblages by ARISA fingerprinting in 57 different locations around the world. Salinity values were higher in the eastern Mediterranean epipelagic water analyzed in this study compared to those collected in the western basin (Table.2, Figure.1). Salinity, together with temperature and oxygen content is a signature of specific water masses. Its different value in the two sub-basins of the Mediterranean Sea typically mirrors the influence of distinct water masses in the epipelagic layers of the Mediterranean basin due to, for example, the intrusion of cold and less saline seawater from the Gibraltar Strait into the Alboran Sea and the intense evaporation phenomena in the Levantine basin. The detection of a significant relationship between salinity and oxygen content values and planktonic bacterial assemblages in epipelagic waters collected from the eastern and westernmost areas of the Mediterranean Sea (Figure.2) suggests that specific water masses in the epipelagic seawater layers influence the impact of certain bacterial taxa in the total planktonic community structure. These data support the concept that specific water masses host a peculiar microbial community, as demonstrated in the North Atlantic (Varela et al., 2008a;b) and the high Arctic (Galand et al., 2009; Hamilton et al., 2008). Moreover, future measurements on quantity and quality of dissolved organic matter might enhance the insight required to explain the variables that determine bacterioplankton composition along the eastern and western Mediterranean Sea.

3.5 - Bacterial populations are stratified across the water column according to environmental variables

The vertical bacterioplankton community composition at eight stations was assessed by ARISA fingerprinting, showing the occurrence of a vertical profile across the water column (Figure.6). As illustrated by NMDS analysis (Figure.6a), the pattern of bacterial community composition was less distinct than previously observed taking into account only the epipelagic and bottom waters (Figure.4a);

nevertheless, epipelagic samples clustered differently to those from meso- and bathypelagic depths. The structure of bacterial communities of the upper meso- and bathypelagic layers was distinguishable, while the samples collected at the lower mesopelagic depths presented a higher variability (Figure.6a). A certain degree of variability of sample distribution was detected within the categories, also according to spatial and physico-chemical parameters (Figure.6b). In spite of the reported unevenness, DistLM analysis showed that four significant variables, longitude (p=0.0166), pressure (p=0.0014), salinity (p=0.024) and temperature (p=0.0007) explained only 32.79% of the total variation of the bacterial community composition along the investigated vertical profile (Table.6). Distance-based redundancy analysis (db-RDA) displayed 24.20% of the total variation (Figure.6c), showing that bacterioplankton communities are shaped by longitude, pressure, salinity and temperature.

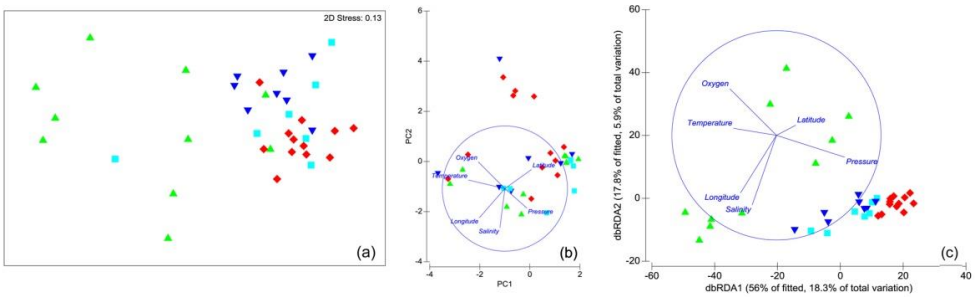

Figure 6 Distribution of the bacterial communities throughout the water column according to biotic and environmental data. (a) Non-metric multidimensional scaling (NMDS) results based on qualitative ARISA fingerprinting of the samples across the water column, showing the distribution of epipelagic (green triangles), upper mesopelagic (blue triangles), lower mesopelagic (light blue squares) and bathypelagic (red diamonds) samples. (b) Principal Component Analysis performed on the environmental data reported in Table.1. (c) dbRDA ordinations of the presence / absence ARISA dataset overlaid with the partial correlations of the tested environmental variables.

Axis	% Explained variation (fitted model)		% Explained variation (total variation)	
	Individual	Cumulative	Individual	Cumulative
dbRDA1	55.96	55.96	18.35	18.35
dbRDA2	17.84	73.81	5.85	24.20
dbRDA3	11.58	85.39	3.8	28.00
dbRDA4	7.87	93.27	2.58	30.58
dbRDA5	4.55	97.82	1.49	32.07
dbRDA6	2.18	100.00	0.72	32.79

Table 6 Results of distance-based multivariate linear model (DistLM) for bacterial community composition in epi-, meso- and bathypelagic seawater layers showing the % variation explained by individual axes. The first two columns relate to the percentage explained out of the fitted model (individuated with the DistLM analysis). The second two columns relate to the percentage explained out of the total variation in the resemblance matrix used to build up the DistLM model.

The investigation of structure of bacterial communities along the depth gradient by ARISA fingerprinting and NMDS analysis (stress value: 0.13) was consistent with the results of the PERMANOVA statistical test. The bacterial species composition in the epipelagic samples was significantly diverse from those collected in the upper (p=0.0085) and lower (p=0.033) meso- and bathypelagic layers (p=0.0005). The upper meso- and bathypelagic strata were also different (p=0.0017). As inferable by NMDS and DistLM analyses, where the distribution of lower mesopelagic samples partially overlapped with the upper mesopelagic and bathypelagic depths, PERMANOVA test showed that bacterial community structure of the lower mesopelagic samples was not significantly different from upper mesopelagic (p=0.1553) and bathypelagic (p=0.1296) bacterial assemblages.

Several recent studies provided hints of a correlation between prokaryotic community composition and water masses, defined as “bio-oceanographic islands” (Agogué et al., 2011; Galand et al., 2009) which carry their own specific microbiome. Fingerprinting methods applied to seawater collected along a north-

south transect in the Aegean Sea demonstrated that both free-living and attached bacterial communities clustered according to the region and depth of sampling, highlighting the existence of horizontal and vertical profiling of bacteria in the Aegean Sea (Moesender et al., 2001). The samples analyzed in the present study were collected on a larger scale, along a wider transect covering both the eastern and western Mediterranean Sea, making the interpretation of bacteria distribution data across the water column more difficult. The absence of a sharp profiling of bacterial communities according to depth in the intermediate layers of the water column could possibly be influenced by the extremely hydrographically complex nature of the system, where several water masses at different spatial and temporal scales concur with the water composition of meso- and bathypelagic layers (Gačić et al., 2010; Rubino et al., 2012).
The observed spatial pattern of bacterial communities throughout the water column was related to different variables, specifically the site of sampling (longitude), the depth of collection (pressure) and the water salinity and temperature values. Our results regarding bacterial stratification across the water column are in agreement with previous studies carried out in the Mediterranean Sea and different oceanic regions (De Corte et al., 2009; DeLong et al., 2006; Winter et al., 2008). Further studies are necessary to correlate bacterial zonation according to water masses composition, particularly in light of the high variability of bacterial microbiome composition in samples characterized by the same T-S values in the eastern Mediterranean Sea (Yokokawa et al., 2010). These findings are consistent with the notion that physical conditions of deep ocean environments are more stable than those in the ocean surface, while concentration and composition of organic constituents could display relatively high variability (Nagata et al., 2010 and references therein).

4. CONCLUSIONS

Our study provides a comprehensive depiction of bacterioplankton community structure across the water column over a longitudinal transect covering the whole Mediterranean Sea. As recently emerged from the study of specific bacterial taxa (Feingersch et al., 2010; Lamy et al., 2011; Mazard et al., 2007), our investigation demonstrated that the overall planktonic bacterial community inhabiting epipelagic seawater was also spatially patterned in the Mediterranean Sea, exhibiting different populations in the eastern and western basins. The bacterial community distribution shifted along the epi-, upper and lower meso- and bathypelagic zones of the Mediterranean Sea according to spatial and physico-chemical parameters including longitude, pressure, temperature and salinity. Bacterial zonation according to depth was also demonstrated. Partial evidence was provided, concerning the relationship between bacterial community structure and water masses in the different water column layers. Due to the distribution patchiness of the intermediate levels of the water column and the interleaving phenomena of water masses (Rubino et al., 2012), the hypothesis of a correlation between communities and water masses should be confirmed by large-scale studies of the whole Mediterranean Sea. Global warming is estimated to have an impact on oceanic circulation and on the entrance of exotic species into the Mediterranean Sea. These factors that are putatively involved in shifts of the biogeochemical cycles, which are triggered by microbial activity. Therefore, a deeper knowledge of the mechanisms driving bacterial diversity in the whole Mediterranean basin may be crucial in the climate change perspective.

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Chapter

IV

GENOTYPING OF *Alcanivorax* GENUS ISOLATES FROM THE MEDITERRANEAN SEA HIGHLIGHT GEOGRAPHICAL DIVERGENCE

Abstract

Alcanivorax, an ubiquitous marine bacteria, is one of the main organisms involved in aerobic degradation of aliphatic hydrocarbons in marine environments. The first step of alkane aerobic degradation requires specific classes of enzymes, one of the best characterized is alkane hydroxylase alkB. Genes encoding for this enzyme have been found also in *Alcanivorax* genus. Even in the "omics era", pure culture collections are the basis for advances in basic and applied microbial science, therefore in this work a large collection of *Alcanivorax* strains was established. Through the analysis of natural populations genetic variation it is possible to evaluate the basis for potential metabolic differences that could be useful to biotechnological applications. Molecular fingerprinting analyses based on polymerase chain reaction (PCR) – Internal Transcribed Sequence (ITS-PCR), BOX-PCR and Restriction Fragment Length Polymorphism (RFLP) of *alkB* gene – were applied in order to discriminate different bacterial strains within the collection. The aim of this work was to evaluate the existence of geographical divergence within the collection of *Alcanivorax* spp. isolates coming from nine different sites located across the whole Mediterranean Sea. The phylogenetic and functional diversity within collection were respectively investigated through i) ITS-PCR and BOX-PCR fingerprinting and ii) RFLP analyses of the functional gene *alkB*. The results revealed that Alcanivorax isolates undergo to phylogenetic geographic divergence, furthermore the polymorphisms of *alkB* gene appeared to be correlated to the strains geographic origin.

1. INTRODUCTION

Alcanivorax is a marine bacterial genus belonging to the γ-proteobacteria class, order *Oceanospirillales*. This ubiquitous genus, encompassing eight different species, was proposed for the first time in 1998 by Yakimov and coauthors and includes some of the most studied alkane-degrading bacteria typical of marine environment. The members of genus *Alcanivorax* are aerobic bacteria that can use aliphatic hydrocarbons as the sole source of carbon and energy (Yakimov et al., 1998) and are defined as obligate marine hydrocarbonoclastic bacteria (OMHB, Yakimov 2007). Such peculiar metabolism, together with their ubiquity, makes them pivotal in biodegradation of hydrocarbon pollutants in marine habitats: representatives of this genus were found to be the firsts to bloom after an oil spill (Head et al., 2006) and they are the major responsible for degradation of a wide range of oil components such as aliphatic hydrocarbons with a short-medium chain length and branched-chain alkanes (Head et al., 2006; Yakimov et al., 2007). *Alcanivorax* spp. normally represent a minor fraction of the overall bacterial community, blooming only after a big amount of hydrocarbons enter the environment, hence their presence has been reported in several types of oil-impacted marine environments all over the world including surface waters, shallow and deep sea water bodies, sediments (Head et al., 2006 and references therein), hydrothermal vents and mud volcanoes and also in association with other organisms, like coral, sponges and dinoflagellates (Yakimov at al., 2007).

The first step of alkane aerobic degradation is their oxidation into a primary alcohol, a reaction that requires the activation of the substrate molecule through a class of enzymes named monooxygenases, which allows to overcome the low chemical reactivity of the hydrocarbon generating reactive oxygen species (Rojo, 2009). Among the enzymes involved in the activation step, one of the best characterized is AlkB, an alkane hydroxylase firstly discovered in *Pseudomonas putida* GPo1 (Kok et al., 1989) and later found also in the majority of alkane degrading bacteria. This enzyme allows the degradation of medium to long chain hydrocarbons (van Beilen et al., 2003) and today more than 250 *alkB* gene homologues, in at least 45 bacterial species, have been discovered in both Gram-positive and Gram-negative bacteria (Wang et al., 2010). Within the latter *alkB* gene has been described also in bacteria belonging to the *Alcanivorax* genus (Smits et al., 2002, van Bailen et al., 2003, 2004).

Even in the "omics era", pure culture collections are the basis for advances in basic and applied microbial science including physiology, biochemistry and biotechnology (Prakash et al., 2013) hence biotechnological application like bioremediation should not disregard cultivation techniques that are essential to obtain, study and manipulate microbial resources aiming to design site-tailored strategies for crude oil compounds clean-up. Through the analysis of natural populations genetic variation it is possible to evaluate the basis for potential metabolic differences that could be useful to biotechnological applications, as already proved in food microbiology for *Lactobacillus* spp. (Bautista-Gallego et al., 2014). Molecular fingerprinting analyses based on polymerase chain reaction (PCR) like Internal Transcribed Sequence (ITS-PCR), BOX-PCR and Restriction Fragment Length Polymorphism (RFLP) of specific genes are commonly used, both individually and combined together, to discriminate different bacterial strains within the same species (Chouaia et al, 2010; Yadav et al., 2012; Guesmi et al., 2013). Moreover, geographical divergence within a single species can lead to different functional and metabolic activities between the strains, as already observed for different species. Geographical segregation has been recently related to the diversity of human epidemiology of the pathogen *Escherichia coli* O157 between US and Australians strains (Mellor et al., 2013) and to the different virulence level of strains affiliated to the subspecies *Pectobacterium carotovorum* ssp. *carotovorum* and associated to different host plants (Yishai et al., 2008).

The Mediterranean Sea is an environment highly exposed to hydrocarbon pollution – hosting 20% of the global oil tanker traffic and due to the high number of oil related facilities (Daffonchio et al., 2013) – nonetheless it host a high biodiversity (Coll et al., 2010) that points out its potentiality as a source of microbes exploitable for biotechnological purposes. The occurrence of a sharp distribution pattern for the planktonic bacterial communities inhabiting the surface waters of the Mediterranean sub-basins water and the role of environmental parameters in shaping the bacterial community structure in its different geographical areas was recently reported (Mapelli et al., 2013).

Therefore, in the present study, a collection of *Alcanivorax* isolates was established from surface waters collected along the main oil tanker routes and from highly polluted sediments collected from the Ancona harbor, one of the main Italian cargo harbor, whose sediments are highly contaminated by hydrocarbons (Dell'Anno et al., 2009). Our collection encompasses four out of the eight species of the genus *Alcanivorax,* namely *A. borkumensis, A. jadensis, A. venustensis* and *A. dieselolei.*

Aim of this work was to evaluate the existence of geographical divergence within a collection of *Alcanivorax* spp. isolates coming from nine different sites located across the whole Mediterranean Sea, providing valuable information for future site-specific clean-up interventions. The phylogenetic and functional diversity within the *Alcanivorax* collection were respectively investigated through i) ITS-PCR and BOX-PCR fingerprinting and ii) RFLP analyses of the functional gene *alkB*.

2. MATERIALS AND METHODS

2.1. Isolation of hydrocarbonoclastic bacteria

Alcanivorax strains were isolated from 9 different located across the whole Mediterranean Sea. Surface water samples were collected along the main oil tanker routes (7 sites) (Tab.1) while polluted sediments were collected from the Ancona harbor (2 sites) (Tab.1). Enrichment cultures were established using the minimal mineral media ONR7a, adding: Diesel Oil (1% v/v) or Crude Oil (1% v/v) as the sole carbon source. Cycloheximide (0.01% w/v) was added to the medium to avoid mold growth. The samples inoculum was 1% v/v in the case of water samples or 1% w/v in the case of sediment samples. The enrichments were incubated at 30°C under shaking condition, until the liquid media showed turbidity. Ten-fold dilution were prepared to isolate the strains on ONR7a plates. The isolates were streaked with and without carbon source (diesel and crude oil) and only those able to grow exclusively in presence of the carbon source were selected for further characterization.

Fig.1 ï **Localization of sampling site. Sample from: Levantine basin, Alboran Sea and Gibraltar Strict are surface water; Ancona Harnor are sediment.**

Station Code	Matrix	Lat	Long	Enrichment	n° isolates	n° species
291	Surface water	34° 3.995 N	32°59.995 E	Diesel oil	20	1
292	Surface water	33°59.386 N	35°10.346 E	Diesel oil	11	2
293	Surface water	33°59.945 N	34°25.346 E	Diesel oil	27	2
294	Surface water	33°42.002 N	31°0.003 E	Diesel oil	22	1
329	Surface water	37°54.004 N	2°00.001 W	Diesel oil	10	2
334	Surface water	36°6.021 N	4°23.975 W	Diesel oil	24	2
339	Surface water	35°54005 N	7°00.005 W	Diesel oil	12	1
AN5	Sediment	43°36'55.16"N	13°30'9.98"E	Diesel oil or Crude oil	25	1
AN7	Sediment	43°37'22.63"N	13°30'13.64"E	Diesel oil or Crude oil	28	1

Tab. 1 Geographical coordinates of the sampling sites, carbon source supplied during the microorganisms enrichment and isolation and number of isolates strains and identified species within each sampling site.

2.2. Genomic DNA extraction

The strains were inoculated in 1.5mL of the same medium used for isolation and incubated at 30°C with shaking for 3 days. The cells were than pelletted discharging the supernatant and subsequently resuspended with TE. The genomic DNA was extracted using the cetyltrimethylammonium bromide (CTAB) method described by Jara et al., 2008, with minor modification: a prior enzymatic cell lysis step was performed using lysozyme and proteinase K.

2.3 Strain identification

*16S rRNA.*The 16S rRNA gene was amplified using the universal bacterial primers 27F (5'-AGAGTTTGATCCTGGCTCAG-3') and 1492R (5'-CTACGGCTACCTTGTTACGA-3'). The PCR was performed with the following conditions: the 25 µL PCR mixture contained Buffer 1x, $MgCl_2$ 1.5 mM, dNTP mix 0.12 mM, primers 27F and 1492R 0.3 µM, Taq 1U and DNA template 1 µL. The DNA was amplified with the following thermal conditions: initial denaturing at 94°C, 5 min; 35 cycles of 45 sec denaturation at 94°C, 1 min annealing at 55°C and 1 min of elongation time at 72°C; a final extension step for 7 min at 72°C.

2.4 PCR typing of *Alcanivorax* isolates

2.4.1. Internal transcribed spacer (ITS)

ITS regions were amplified using the primers ITS F (3'-GTCGTAACAAGGTAGCCGTA-5) and ITS R (3'-CTACGGCTACCTTGTTACGA-5') according to Daffonchio et al., 1998. The PCR was performed with the following conditions: the 25µL PCR mixture contained Buffer 1x, $MgCl_2$ 1.50mM, dNTP mix

0.20mM, primers ITS F and ITS R 0.3µM, Taq 1U and DNA template 1 µL. The DNA was amplified with the following thermal conditions: initial denaturing at 94°C, 4 min; 35 cycles of 45 sec denaturation at 94°C, 1 min annealing at 55°C and 2 min of elongation time at 72°C; a final extension step for 10 min at 72°C.

2.4.2. BOX PCR.

BOX PCR was performed with the primer BOX A1 R (5'-CTACGGCAAGGCGACGCTGACG-3') according to Urzì et al., 2001. The PCR was performed with the following conditions: the 25µL PCR mixture contained Buffer 1x, $MgCl_2$ 2.00mM, dNTP mix 0.20mM, primer BOX A1 R 0.80µM, DMSO 5%, Taq 1.3U and DNA template 1 µL. The DNA was amplified with the following thermal conditions: initial denaturing at 95°C, 5 min; 35 cycles of 1 min denaturation at 95°C, 1 min annealing at 45°C and 2 min of elongation time at 72°C; a final extension step for 10 min at 72°C.

2.4.3. alkB.

Partial *alkB* genes were amplified using the primers alkB F (5'-AAYACNGCNCAYGARCTNG GNCAY A-3') and alkB R (5'-GCRTGRTGRTCNGARTGNCGNTG-3') according to Kloos et al., 2006. The PCR was performed with the following conditions: the 25µL PCR mixture contained Buffer 1x, $MgCl_2$ 2.00mM, dNTP mix 0.20mM, primer alkB F and alkB R 0.1µM, DMSO 5%, Taq 1U and DNA template 1.5µL. The DNA was amplified with the following thermal conditions: initial denaturing at 95°C, 5 min; 30 cycles of 45 sec denaturation at 94°C, 45 sec annealing at 54°C and 1 min of elongation time at 72°C; a final extension step for 10 min at 72°C.

2.4.4. Restriction Fragment Length Polymorphism (RFLP) on alkB gene

alkB gene amplicons obtained as described above, were digested using the restriction enzyme MspI (SibEnzyme). The reaction was performed in a total volume of 20µL with: 10 U of restriction enzyme, 2µL of buffer, 10µL of PCR product and bringing to the final volume with milliQ water. The samples were digested for 3 h at 37°C. To stop the reaction 5µL of loading dye were added and the samples were left overnight at -20°C. The restriction profiles were resolved by gel electrophoresis (2% agarose gel) and visualized using a Gel Doc 2000 apparatus (Bio-Rad, Milan, Italy).

2.5 Fingerprinting image analysis and Statistical analysis

Band profiles were analyzed using the Quantity One software, allowing the construction of a matrix of presence/absence of each band. This matrix was finally analyzed to evaluate the profile similarity of each isolate and a cluster analysis was performed using the Microsoft Excel XLSTAT software (Addinsoft Inc.,New York, NY, USA). The single profiles were clusterd using the Jaccard similarity coefficient. The similarity threshold value was 60%.

2.6 Statistical analyses

Significant differences in the similarity of the ITS-BOX profiles was investigated by permutational analysis of variance (PERMANOVA, Anderson, 2001). Principal component analyses (PCA) was used to visualize the relationship among the samples.

3. RESULTS and DISCUSSION

3.1. All the bacterial strains isolated from surface seawater and polluted sediments belong to the genus *Alcanivorax*

179 bacterial strains were isolated from 9 sampling sites comprising both offshore seawater collected along the main oil tanker routes across the Mediterranean Sea and polluted sediments collected inside the Ancona harbor. 16S rRNA gene amplification and sequencing allowed the identification of all the isolates that were all affiliated to the genus *Alcanivorax,* and divided in 4 different species (Table 2). Seventy-five isolates belonged to the specie *A. borkumensis* that was the only one isolated from both sediments (An-U5 and AN-U7) and surface seawater (site 334, in the Alboran Sea). The other species were isolated solely from surface seawater. *A. venustensis,* including eight isolates, was isolated from sites 329 in the Alboran Sea and 292 in the Levantine basin. *A. jadensis* is the most abundant genus in our collection, being represented by 88 isolates isolated from sites 329 and 334 in the Alboran Sea, 339 in the Gibraltar Strait and from all the sites

located in the Levantine basin (Table 2). Finally, the eight isolates belonging to the specie *A. dieselolei* were all retrieved from site 293, located in the Levantine basin.
The dominance of the genus *Alcanivorax* within the collection is probably due to the application of very strict isolation conditions: the only carbon source available for the bacterial growth were hydrocarbon molecules, therefore only hydrocarbon degrading bacteria have been selected. Moreover, *Alcanivorax* is a genus involved in the first steps of oil compounds degradation hence it is further favored under the applied cultivation conditions. The established *Alcanivorax* collection was further investigated through different molecular fingerprinting analyses in order to evaluate the occurring diversity at the species and intra-specie level of this important hydrocarbonoclastic bacteria.

Strain code	Closest described species	ITS profile	BOX profile	ITS-BOX profile	alkB profile
291/1	*Alcanivorax jadensis*	13	10	13.10	-
291/10	*Alcanivorax jadensis*	17	15	17.15	1
291/11	*Alcanivorax jadensis*	17	25	17.25	1
291/12	*Alcanivorax jadensis*	18	16	18.16	1
291/13	*Alcanivorax jadensis*	17	15	17.15	1
291/15	*Alcanivorax jadensis*	17	17	17.17	1
291/16	*Alcanivorax jadensis*	18	17	18.17	1
291/17	*Alcanivorax jadensis*	18	18	18.18	1
291/18	*Alcanivorax jadensis*	18	16	18.16	1
291/2	*Alcanivorax jadensis*	13	11	13.11	1
291/20	*Alcanivorax jadensis*	17	25	17.25	1
291/23	*Alcanivorax jadensis*	17	18	17.18	1
291/25	*Alcanivorax jadensis*	17	19	17.19	1
291/26	*Alcanivorax jadensis*	17	16	17.16	1
291/27	*Alcanivorax jadensis*	17	17	17.17	1
291/3	*Alcanivorax jadensis*	17	12	17.12	1
291/4	*Alcanivorax jadensis*	17	25	17.25	1
291/5	*Alcanivorax jadensis*	37	44	37.44	-
291/7	*Alcanivorax jadensis*	17	13	17.13	1
291/9	*Alcanivorax jadensis*	17	14	17.14	1
292/1	*Alcanivorax jadensis*	19	20	19.20	2
292/11	*Alcanivorax jadensis*	14	21	14.21	3
292/12	*Alcanivorax jadensis*	20	22	20.22	1
292/14	*Alcanivorax jadensis*	20	23	20.23	1
292/15	*Alcanivorax jadensis*	20	22	20.22	1
292/16	*Alcanivorax jadensis*	20	22	20.22	1
292/17	*Alcanivorax venustensis*	15	8	15.8	12
292/3	*Alcanivorax venustensis*	34	45	34.45	11
292/4	*Alcanivorax venustensis*	14	8	14.8	11
292/5	*Alcanivorax venustensis*	14	8	14.8	11
292/9	*Alcanivorax jadensis*	20	25	20.25	1
293/1	*Alcanivorax dieselolei*	10	8	10.8	5
293/10	*Alcanivorax jadensis*	22	26	22.26	7
293/11	*Alcanivorax dieselolei*	12	8	12.8	5

293/12	*Alcanivorax jadensis*	12	26	12.26	5
293/13	*Alcanivorax dieselolei*	10	8	10.8	5
293/14	*Alcanivorax jadensis*	10	25	10.25	5
293/16	*Alcanivorax jadensis*	12	26	12.26	5
293/18	*Alcanivorax jadensis*	16	26	16.26	5
293/19	*Alcanivorax jadensis*	12	26	12.26	5
293/20	*Alcanivorax jadensis*	23	42	23.42	9
293/21	*Alcanivorax jadensis*	22	43	22.43	7
293/22	*Alcanivorax dieselolei*	10	8	10.8	5
293/23	*Alcanivorax jadensis*	31	25	31.25	7
293/24	*Alcanivorax jadensis*	22	25	22.25	7
293/25	*Alcanivorax jadensis*	10	26	10.26	5
293/26	*Alcanivorax dieselolei*	10	8	10.8	5
293/27	*Alcanivorax jadensis*	12	40	12.40	5
293/28	*Alcanivorax jadensis*	22	26	22.26	7
293/29	*Alcanivorax dieselolei*	12	8	12.8	5
293/3	*Alcanivorax dieselolei*	11	8	11.8	4
293/30	*Alcanivorax dieselolei*	21	8	21.8	5
293/4	*Alcanivorax jadensis*	12	26	12.26	4
293/5	*Alcanivorax jadensis*	22	27	22.27	6
293/6	*Alcanivorax jadensis*	10	26	10.26	5
293/7	*Alcanivorax jadensis*	22	28	22.28	7
293/8	*Alcanivorax jadensis*	22	25	22.25	7
293/9	*Alcanivorax jadensis*	35	46	35.46	7
294/1	*Alcanivorax jadensis*	24	25	24.25	1
294/10	*Alcanivorax jadensis*	24	25	24.25	1
294/12	*Alcanivorax jadensis*	26	30	26.30	1
294/13	*Alcanivorax jadensis*	26	41	26.41	1
294/14	*Alcanivorax jadensis*	26	41	26.41	1
294/16	*Alcanivorax jadensis*	26	49	26.49	1
294/18	*Alcanivorax jadensis*	26	41	26.41	1
294/2	*Alcanivorax jadensis*	26	31	26.31	1
294/20	*Alcanivorax jadensis*	26	31	26.31	1
294/21	*Alcanivorax jadensis*	26	31	26.31	1
294/22	*Alcanivorax jadensis*	26	31	26.31	1
294/24	*Alcanivorax jadensis*	26	31	26.31	1
294/25	*Alcanivorax jadensis*	35	32	35.32	1
294/26	*Alcanivorax jadensis*	26	33	26.33	1
294/27	*Alcanivorax jadensis*	26	31	26.31	1
294/3	*Alcanivorax jadensis*	26	31	26.31	1
294/4	*Alcanivorax jadensis*	26	31	26.31	1
294/5	*Alcanivorax jadensis*	27	31	27.31	1
294/6	*Alcanivorax jadensis*	26	31	26.31	1
294/7	*Alcanivorax jadensis*	28	31	28.31	1

294/8	*Alcanivorax jadensis*	27	46	27.46	1
294/9	*Alcanivorax jadensis*	27	33	27.33	1
329/1	*Alcanivorax venustensis*	16	8	16.8	13
329/10	*Alcanivorax jadensis*	29	34	29.34	13
329/11	*Alcanivorax jadensis*	30	35	30.35	15
329/12	*Alcanivorax jadensis*	30	35	30.35	15
329/2	*Alcanivorax venustensis*	16	8	16.8	13
329/3	*Alcanivorax venustensis*	16	8	16.8	13
329/4	*Alcanivorax jadensis*	30	35	30.35	15
329/7	*Alcanivorax venustensis*	16	8	16.8	13
329/8	*Alcanivorax jadensis*	30	36	30.36	15
329/9	*Alcanivorax jadensis*	30	37	30.37	15
334/1	*Alcanivorax borkumensis*	7	8	7.8	10
334/10	*Alcanivorax borkumensis*	7	8	7.8	10
334/11	*Alcanivorax borkumensis*	7	1	7.1	10
334/12	*Alcanivorax jadensis*	36	38	36.38	15
334/13	*Alcanivorax borkumensis*	7	8	7.8	10
334/14	*Alcanivorax borkumensis*	8	7	8.7	8
334/15	*Alcanivorax borkumensis*	8	7	8.7	8
334/16	*Alcanivorax borkumensis*	7	8	7.8	10
334/17	*Alcanivorax borkumensis*	7	8	7.8	10
334/18	*Alcanivorax borkumensis*	7	1	7.1	10
334/19	*Alcanivorax borkumensis*	8	24	8.24	8
334/2	*Alcanivorax borkumensis*	8	7	8.7	8
334/20	*Alcanivorax borkumensis*	7	1	7.1	10
334/21	*Alcanivorax borkumensis*	7	1	7.1	10
334/22	*Alcanivorax borkumensis*	7	1	7.1	10
334/23	*Alcanivorax borkumensis*	7	8	7.8	10
334/24	*Alcanivorax borkumensis*	9	9	9.9	-
334/26	*Alcanivorax borkumensis*	7	1	7.1	10
334/3	*Alcanivorax borkumensis*	25	29	25.29	-
334/4	*Alcanivorax jadensis*	36	39	36.39	14
334/5	*Alcanivorax borkumensis*	8	7	8.7	8
334/6	*Alcanivorax borkumensis*	8	7	8.7	8
334/8	*Alcanivorax borkumensis*	7	8	7.8	10
334/9	*Alcanivorax borkumensis*	7	1	7.1	10
339/1	*Alcanivorax jadensis*	32	37	32.37	1
339/10	*Alcanivorax jadensis*	32	49	32.49	1
339/11	*Alcanivorax jadensis*	32	40	32.40	1
339/12	*Alcanivorax jadensis*	33	48	33.48	1
339/2	*Alcanivorax jadensis*	32	41	32.41	1
339/3	*Alcanivorax jadensis*	38	47	38.47	-
339/4	*Alcanivorax jadensis*	32	48	32.48	1
339/5	*Alcanivorax jadensis*	33	41	33.41	1

339/6	*Alcanivorax jadensis*	32	40	32.40	1
339/7	*Alcanivorax jadensis*	34	41	34.41	1
339/8	*Alcanivorax jadensis*	34	40	34.40	1
339/9	*Alcanivorax jadensis*	34	40	34.40	1
AN5_CO1	*Alcanivorax borkumensis*	2	1	2.1	16
AN5_CO11	*Alcanivorax borkumensis*	1	1	1.1	16
AN5_CO12	*Alcanivorax borkumensis*	2	1	2.1	16
AN5_CO13	*Alcanivorax borkumensis*	2	1	2.1	16
AN5_CO14	*Alcanivorax borkumensis*	2	1	2.1	16
AN5_CO15	*Alcanivorax borkumensis*	1	1	1.1	16
AN5_CO16	*Alcanivorax borkumensis*	1	1	1.1	16
AN5_CO18	*Alcanivorax borkumensis*	2	1	2.1	16
AN5_CO2	*Alcanivorax borkumensis*	2	1	2.1	16
AN5_CO3	*Alcanivorax borkumensis*	1	1	1.1	16
AN5_CO6	*Alcanivorax borkumensis*	4	1	4.1	16
AN5_CO8	*Alcanivorax borkumensis*	4	1	4.1	16
AN5_CO9	*Alcanivorax borkumensis*	1	1	1.1	16
AN5_GF1	*Alcanivorax borkumensis*	2	1	2.1	16
AN5_GF11	*Alcanivorax borkumensis*	2	1	2.1	16
AN5_GF4	*Alcanivorax borkumensis*	2	1	2.1	16
AN5_GF5	*Alcanivorax borkumensis*	2	1	2.1	16
AN5_GF6	*Alcanivorax borkumensis*	3	2	3.2	16
AN5_GF7	*Alcanivorax borkumensis*	2	1	2.1	16
AN5_GF9	*Alcanivorax borkumensis*	2	1	2.1	16
AN5_GS10	*Alcanivorax borkumensis*	2	1	2.1	16
AN5_GS16	*Alcanivorax borkumensis*	2	1	2.1	16
AN5_GS4	*Alcanivorax borkumensis*	2	1	2.1	16
AN5_GS8	*Alcanivorax borkumensis*	2	1	2.1	16
AN5_GS9	*Alcanivorax borkumensis*	2	1	2.1	16
AN7_CO14	*Alcanivorax borkumensis*	6	4	6.4	16
AN7_CO17	*Alcanivorax borkumensis*	6	1	6.1	16
AN7_CO18	*Alcanivorax borkumensis*	6	3	6.3	16
AN7_CO6	*Alcanivorax borkumensis*	5	3	5.3	16
AN7_CO7	*Alcanivorax borkumensis*	6	3	6.3	16
AN7_CO9	*Alcanivorax borkumensis*	6	4	6.4	16
AN7_GF10	*Alcanivorax borkumensis*	6	4	6.4	16
AN7_GF11	*Alcanivorax borkumensis*	6	4	6.4	16
AN7_GF12	*Alcanivorax borkumensis*	6	4	6.4	16
AN7_GF15	*Alcanivorax borkumensis*	6	4	6.4	16
AN7_GF17	*Alcanivorax borkumensis*	6	4	6.4	16
AN7_GF18	*Alcanivorax borkumensis*	6	5	6.5	16
AN7_GF2	*Alcanivorax borkumensis*	6	3	6.3	16
AN7_GF3	*Alcanivorax borkumensis*	6	4	6.4	16
AN7_GF6	*Alcanivorax borkumensis*	6	4	6.4	16

AN7_GF7	*Alcanivorax borkumensis*	6	3	6.3	16
AN7_GS1	*Alcanivorax borkumensis*	6	4	6.4	16
AN7_GS10	*Alcanivorax borkumensis*	6	6	6.6	16
AN7_GS11	*Alcanivorax borkumensis*	6	6	6.6	16
AN7_GS13	*Alcanivorax borkumensis*	6	6	6.6	16
AN7_GS16	*Alcanivorax borkumensis*	5	6	5.6	16
AN7_GS17	*Alcanivorax borkumensis*	6	6	6.6	16
AN7_GS2	*Alcanivorax borkumensis*	6	1	6.1	16
AN7_GS3	*Alcanivorax borkumensis*	5	6	5.6	16
AN7_GS6	*Alcanivorax borkumensis*	6	6	6.6	16
AN7_GS7	*Alcanivorax borkumensis*	6	6	6.6	16
AN7_GS8	*Alcanivorax borkumensis*	6	6	6.6	16
AN7_GS9	*Alcanivorax borkumensis*	6	6	6.6	16

Tab. 2 List of the isolated strains, with identification and affiliation to the ITS, BOX, ITS-BOX, alkB profiles

3.2. ITS-PCR fingerprinting provides specie-specific but not site-specific profiles

ITS-PCR fingerprinting is frequently used to discriminate bacterial strains at the species and intra-species levels and it is based on the polymorphisms of the genome region between the 16S and 23S rRNA genetic loci. This analysis allowed to identify 49 different profiles within the collection (Table 2). The distribution of the profiles within the different species was highly specific. With the only exception of the profile ITS 8 – observed in bacteria belonging to *A. borkumensis*, *A. jadensis* and *A. dieselolei* species – representative of each ITS profiles belonged only to one specie, confirming the reliability of this method for the screening of vast bacteria collection. ITS-PCR indicated *A. jadensis* as the more heterogenic specie encompassing 37 profiles out of 88 isolates, followed, in the order, by *A. venustensis* (2 profiles/8 isolates), *A. borkumensis* (11 profiles/75 isolates) and *A. dieselolei* (1 profile/8 isolates). Nevertheless, any clear geographic divergence was highlighted investigating the distribution of the ITS profiles within the sampling sites, since many of them were observed in bacterial isolates retrieved from different sites. For instance, profile ITS 1 was identified in *A. borkumensis* isolated from both sites An-U5 and An-U7 (Ancona harbor sediments) and 334 (surface seawater near the Gibraltar strait).

3.3. BOX-PCR generated profiles that are not specie-specific but allowed the detection of site-specificity

BOX-PCR fingerprinting is based on the amplification of specific genomic regions delimited by BOX elements, namely 154bp palindrome sequences, randomly located in the whole genome (Lee & Wong, 2009). Band profiles generated through BOX fingerprinting are useful to determine and cluster organisms at the inter and intra-specific level (Lee & Wong, 2009).

38 different BOX profiles have been identified within the bacteria studied collection (Table 2). In contrast to what observed analyzing the distribution of the ITS profiles across species and sites, the BOX patterns didn't appear to be specie-specific. Many of the BOX profiles in fact have been detected in more than one specie, for instance profile BOX-10 has been observed in both *A. dieselolei*, *A. jadensis* species. On the contrary, the distribution of the BOX profiles appear to be site-specific, since each profile was observed exclusively in one sampling station. The only exception were profiles BOX-16, BOX-34 and BOX-35, whose isolates where identified in more than one site. Similarly to what observed for the ITS profiles, the sites showing the higher diversity were those located in the Levantine basin. Here, the sites 292 and 293 comprised respectively 5 and 9 profiles distributed between 11 and 27 isolates. However, also the station 339 (near the Gibraltar strait) showed a high diversity: the 12 isolates affiliated as *A. jadensis* were divided in 4 different profiles. On the other side, the 2 sampling stations situated inside the Ancona harbor were characterized by the lowest level of genotypic diversity, being represented exclusively by bacteria belonging the *A. borkumensis* specie.

3.4. Merging of ITS and BOX profiles highlighted the geographical divergence within the genus *Alcanivorax* in the Mediterranean Sea

The separate analyses of ITS and BOX fingerprinting did not allowed to define inequivocal patterns of geographic divergence within the genus *Alcanivorax* hence the obtained profiles were merged, as previously reported by Chouaia and colleagues (2010). This approach was proved as a valuable method to get more insight in the genotypic diversity of different species (Chouaia et al. 2010) and was applied in this study to investigate the biogeography of the *Alcanivorax* species in different Mediterranean Sea areas comprising highly polluted sites like the Ancona harbor. The merging of ITS and BOX patterns generated 85 profiles, corresponding to 85 different genotypes (Table 2), which showed both specie and site specificity. Indeed, each profile was detected only in a single specie and in a single sampling site (Fig.2).

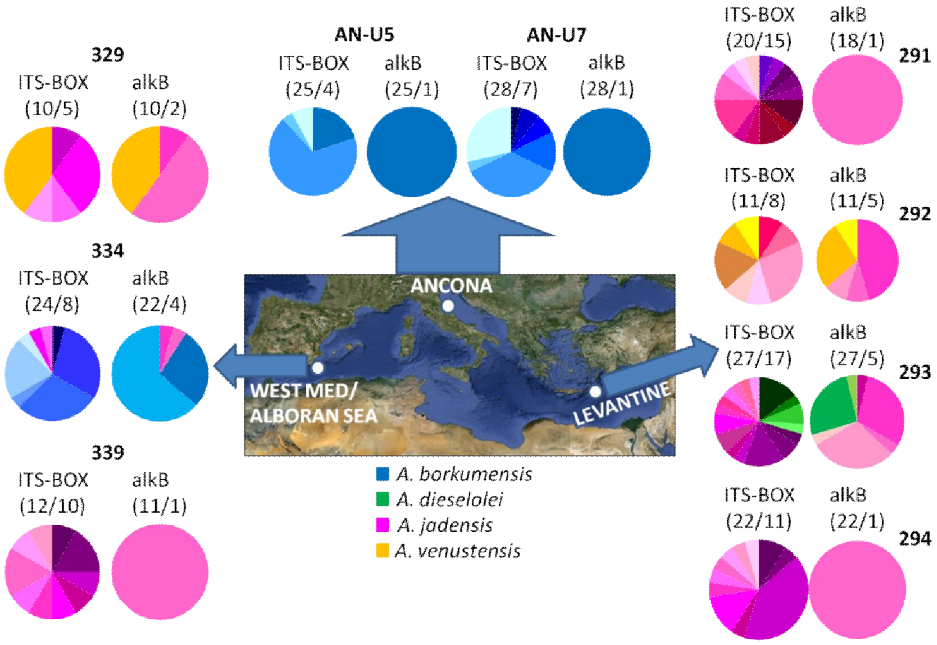

Fig. 2 – Geographical distribution of ITS-BOX and alkB profiles. Between parenthesis are indicated, in the order, the number of isolates and the number of profiles.

The distribution of ITS-BOX profiles within the single species (Tab.2, Fig.2) indicated *A. borkumensis,* as the species characterized by lowest diversity, comprising 75 bacteria isolates subdivided between 17 profiles ITS-BOX. The analysis showed that *A. jadensis,* with 88 isolates subdivided between 60 different ITS-BOX profiles was not only the most abundant specie of the collection, but also the specie hosting the highest genotypic diversity. Finally, although the species *A. diselolei* and *A. venustensis* were the less represented species in the analyzed collection, including only 8 isolates each, they showed a relatively high diversity, both comprising 4 ITS-BOX profiles.

The distribution of the ITS-BOX profiles within the different sampling sites (Tab.2, Fig.2) indicated that site 339, near the Gibraltar strait, is the station hosting the highest diversity: the 12 strains isolated here displayed 10 different genotypes. Interestingly, the bacteria isolated from site 291, in the Levantine basin, showed an high intra specific diversity: altough all the 20 isolates belonged to one specie (*A. jadensis*), 15 different ITS-BOX profiles were described in this station.

In order to get more insight on the geographical divergence of the genus *Alcanivorax*, demonstrated by the distribution of the ITS-BOX profiles detected in this collection of strains, the similarity of the different ITS-BOX profiles individuated in each geographic area, sampling station and specie, was analyzed. The similarity, in terms of bands composition, of the ITS-BOX profiles was studied through the PERMANOVA analyses performed on the qualitative matrix of ITS-BOX bands occurring in each profiles.

The three geographic areas considered were the Ancona harbor, the Alboran Sea and the Levantine basin (Fig.1). The performed statistical analyses showed that the ITS-BOX profiles were significantly different between the three areas ($p<0.005$) (Table 3), further confirming the occurrence of geographical divergence

of *Alcanivorax* spp. in the Mediterranean. The ITS-BOX profiles distribution was further investigated at a finer level, taking into account each single sampling site. Again, the geographical divergence was confirmed between most of the analyzed sites ($p<0.005$) (Table 3). Exceptions were represented by sites 334 and 329. The former was not significantly different from other 4 station (An-U5, 293, 292 and 329) and the latter to 3 (An-U5, 292, 334) (Table 3).

PAIR-WISE TESTS	
Similarity between geographic areas	
	P(MC)
Ancona Harbor, Alboran Sea	0,0004
Ancona Harbor, Levantine	0,0001
Alboran Sea, Levantine	0,0005
Similarity between sampling station	
	P(MC)
AN5, AN7	0,0149
AN5, 334	0,466
AN5, 293	0,0391
AN5, 291	0,0079
AN5, 292	0,0487
AN5, 329	0,0811
AN5, 294	0,0011
AN5, 339	0,0075
AN7, 334	0,0056
AN7, 293	0,0002
AN7, 291	0,0004
AN7, 292	0,0016
AN7, 329	0,0044
AN7, 294	0,0001
AN7, 339	0,0001
334, 293	0,1049
334, 291	0,0006
334, 292	0,1315
334, 329	0,1427
334, 294	0,0001
334, 339	0,0007
293, 291	0,0001
293, 292	0,0151
293, 329	0,0224
293, 294	0,0001
293, 339	0,0001
291, 292	0,0002
291, 329	0,0046
291, 294	0,0001
291, 339	0,0001
292, 329	0,1045
292, 294	0,0003

	292, 339	0,0029
	329, 294	0,0008
	329, 339	0,0022
	294, 339	0,0001

Tab.3 p values resulted from PERMANOVA analyses**.**

A Principal Coordinates Analyses (PCoA) was applied to the ITS-BOX profiles to represent their diversity correlated according to the sampling sites in which they were detected (Fig.3).

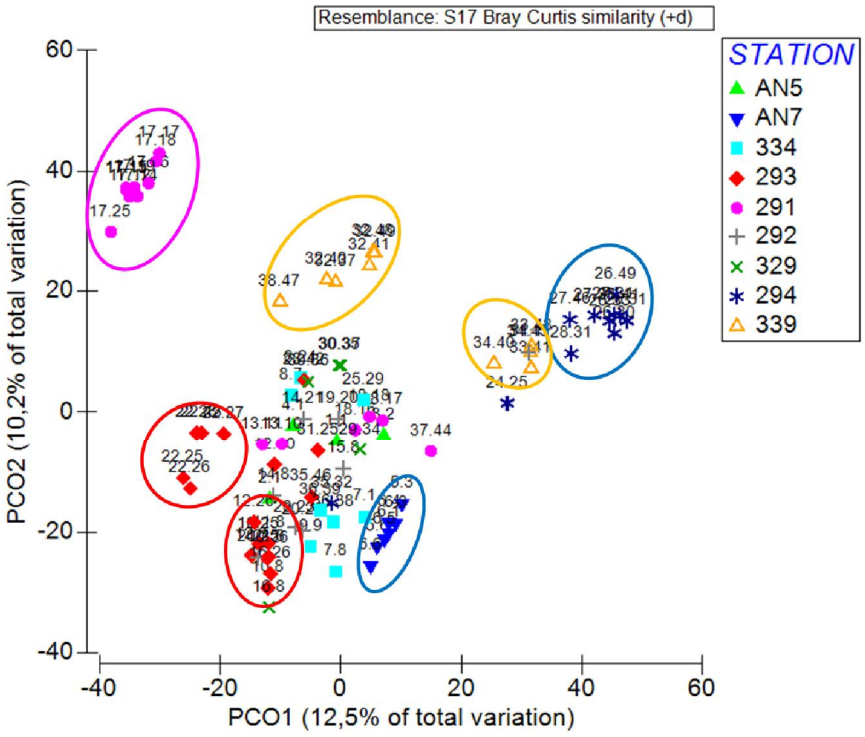

Fig. 3 ï **PCA analyses of the deiversity of the ITS-BOX profiles on the basis of the different sampling sites**

Although the PCoA analysis explained a low percentage of the total diversity (PCO1 12.5%; PCO2 10.2%), it was possible to identify a partial clustering between the different profiles basing on the isolation sites (Fig.3). Most of the isolates from 291, 294 and An-U7 clustered together, while most of the profiles retrieved at stations 339 and 293 clustered in 2 different groups.

These results concur to highlight the high level of bacteria diversity hosted within the Mediterranean Sea, as showed by previous studies based on molecular analysis of its microbial communities (Mapelli et al., 2013, Coll et al. 2010 and references therein). A spatial pattern of diversity was demonstrated for specific taxa also by previous studies, realized on different groups of interest such as for example anoxygenic phototrophic bacteria (Lamy et al, 2011; Mella-Flores et al., 2011; Feingersh et al., 2010). Their studies showed how different environmental parameters, like longitude, salinity and nutrient amount can concur to shape different bacterial communities. Likely, some of them could be involved also in the genotypic diversity observed inside the strain collection here investigated. Furthermore, environmental pollution could play a role in the divergence of the *Alcanivorax* genotypes identified in the present collection, since this environmental parametr has been already demonstrated, also in the next chapters of this thesis (Chapters VI and VII), to be a driving factor shaping different bacteria communities (Kostka et al., 2011;. Roling at al., 2004)

3.5. *Alcanivorax* typing ï *alkB* RFLP

The functional diversity of the *Alcanivorax* collection has been investigated through RFLP analysis of the *alkB* gene, amplified from the isolates' genome. A total of 16 different profiles were identified within the 174 isolates analyzed (Table 2, Fig.4). Any PCR amplicons could be obtained from 5 isolates belonging to *A. jadensis* and *A. borkumensis* species, in spite of several trials. Such isolates belonged to different ITS-BOX genotypes of which they were the sole representatives: 9.9, 13.10, 25.29, 37.44 and 38.47.
Most of the *alkB* profiles depicted by RFLP correlated to more than one genotype previously identified through ITS-BOX fingerprinting, but conversely each ITS-BOX group was always characterize by only one *alkB* profile.
Profile alkB-1, the most abundant in the collection with 56 representative, is characteristics of *A. jadensis* and it has been identified in 3 sites of the Levantine basin (291,292 and 294) and in the site 339, near the Gibraltar strait. Profile alkB-16, with 53 representative, has been observed only in *A. borkumensis* isolated from the Ancona harbor sediments (both An-U5 and An-U7) where it also represent the unique profile, and it was not present in the isolates of the same species retrieved from surface water samples,.
Most of the profiles were site-specific, nonetheless also the profiles alkB-15 and alkB-16, observed in more than one site, could be referred to the same geographic area, since they have been identified from surface water of the Alboran Sea and from Ancona harbor sediments respectively. Profile alkB-1 is the only exception, being present in both eastern and western basins of the Mediterranean Sea.
On the other hand, three profiles were sheared between more than one specie: profiles alkB-4 and alkB-5, by *A. jadensis* and *A. borkumensis* isolated from site 293, and profile alkB-13 by both *A. jadensis* and *A. venustensis* isolated from site 329. This result is in accordance with previous a report (Wang et al., 2010) showing, through the analyses of phylogenetic trees built basing on *alkB* gene sequences, that the same *alkB* genotype could be present in different taxa. The retrieval of the same *alkB* genotype in different species isolated from the same sampling site reinforce the possibility that phenomena of horizontal gene transfer may have an important role in the distribution of this gene among bacterial communities, as already suggested by several authors (Smits et al., 2002; van Beilen et al., 2003; Heiss-Blanquet et al., 2005; Tourova et al., 2008 and Wang et al., 2010; Giebler et al., 2013). Furthermore, *alkB* genes are known to be encoded not only by chromosomal DNA, but also by plasmids (Chakrabarty et al., 1973).
The distribution of the alkB profiles was also evaluated between the different sampling sites (Fig.2, Table 2). The site having the highest *alkB* diversity was station 292, located in the Levantine basin, hosting 5 different profiles distributed between 11 isolates of 2 different species, partially reflecting the higher diversity depicted through the ITS-BOX genotype analysis (Fig.2 and 3).
Furthermore, *alkB* gene polymorphism did not seem to be directly correlated to ITS-BOX profiles divergence. Generally, the high diversity observed at the phylogenetic level through ITS-BOX profiles, was not reflected by *alkB* RFLP analysis. Confuting previous observation stating a strict correlation between phylogenetic and *alkB* diversity (Hamamura et al., 2008 and Kuhn et al., 2009), and accordingly with the finding of Giebler et al., 2013 and Smith et al., 2013, the present work showed that the functional diversity of *alkB* gene is not strictly correlated with the phylogenic diversity, also at the intra-specific level.

4. CONCLUSIONS

The phylogenetic diversity of a collection of 179 *Alcanivorax* isolates obtained from different three areas located in the whole Mediterranean basin was investigated. ITS and BOX molecular fingerprinting were applied both individually and subsequently combined to obtain univocal correlation to the species and the site of origin. ITS-BOX profiles allowed to individuate 85 different ITS-BOX profiles within the strains collection and showed both specie-specificity and site-specificity. Furthermore, statistical analyses on the similarity of the profiles confirmed as significant the evidence of phylogenetic geographical divergence of the genus Alcanivorax across the Mediterranean Sea.
To study the functional diversity of these strains, the polymorphism of the gene *alkB* was investigated through RFLP analyses. alkB profiles showed an high diversity that did not seem to be correlated with the phylogenic divergence of the strains, as much as their geographical origin.

Overall, this observation indicated that the studied *Alcanivorax* strains could be specifically adapted to the environmental condition occurring at the sampling sites. This high phylogenetic and functional diversity has to be taken into account while exploiting cultivable environmental bacteria for the development of bioremediation technologies.

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Chapter

V

BEHAVIOR OF DIFFERENT HYDROCARBONOCLASTIC BACTERIA STRAINS AT HIGH PRESSURE, SIMULATING OIL BIOREMEDIATION AT HIGH MARINE DEPTH

1. INTRODUCTION

In the last few years many seafloor explorations revealed a large amount of fossil fuel in the subsurface of the Mediterranean Sea and the recent advances in the extraction technologies rendered easier to get these previously inaccessible resources. For this reason, most of the Mediterranean countries started to further exploit this richness and new permissions for both exploration and drilling of the sea floor were given. For instance, in the last years Italian government approved 66 new drilling permission, 25 exploration concessions and evaluated a huge number of applications for new exploration along the Italian coastlines (Margottini, 2011). Such a high exploitation of the marine floor for oil extraction through off-shore platform will reasonably increase the risk of hydrocarbons spills at high depth. The Deepwater Horizon break in the Gulf of Mexico in 2010 was a milestone in "oil spills": the effects of such an accident would be even more detrimental if occurring in a semi-enclosed sea, like Mediterranean's.

Several studies focused on the fate of oil (Parinos et al., 2013) and on microbial community shifts in response to oil spills at high water depth (Joye et al., 2014, Lu et al, 2012, Mason et al., 2012; Hazen et al, 2010), but very few of them gave new insights on the physiological changes imposed by hydrocarbon degradation taking place in this extreme environment.

Community analysis of bacteria living near or within the deep oil plume originated after the Deepwater Horizon accident showed a high amount of bacteria belonging to the *Oceanospirellales* order (Joye et al., 2014), which comprise many well known hydrocarbonoclastic genera like *Alcanivorax*.

Bacteria belonging to the *Alcanivorax* genus appear to be responsible for the oxidation of hydrocarbon compounds not only in surface waters but also at high depth, as recently demonstrated by several studies (Bertrand et al., 2013; Lai et al., 2013; Tapilatu et al., 2009; Liu and Shao 2005). Involvement of other well-known oil-degrading bacteria genera (i.e. *Marinobacter*) at high-depth has also been claimed *(*Kaye et al., 2010; Grossi et al, 2010).

Deep ocean, the most extensive habitat of the biosphere (Whitman et al., 1998), is considered an extreme environment for the living beings because of permanent darkness, cold temperature, high inorganic nutrients and low organic carbon concentration. Furthermore, hydrostatic pressure is one of the main factors influencing the physiology of organisms living at high depth (Tamburini et al., 2013). In fact, bacteria can be classified on the basis of their capability to adapt and grow at pressure higher than the atmospheric pressure (1 bar): pressure-adapted bacteria can be defined as piezotolerant if they have similar growth rate at atmospheric and high pressure or piezophilic if they grow more rapidly at high pressure than atmospheric pressure, while organisms that grow best at atmospheric pressure, with little to no growth at higher pressure are termed piezosensitive (Fang et al., 2010).

Both *Alcanivorax* and *Marinobacter* genera comprise piezotolerant species, detected and isolated from deep-sea environments (Grossi et al, 2010; Tapilatu et al, 2009) but their capability to degrade hydrocarbons has been tested only at atmospheric pressure. It is therefore of great importance to understand the adaptation mechanisms and function of hydrocarbonoclastic bacteria at high pressure.

2. MATERIALS and METHODS

2.1 Hydrocarbonoclastic Strains

Isolates belonging to different bacteria species were selected from the wide collection of hydrocarbonoclastic bacteria established in the course of this PhD. *Marinobacter hydrocarbonoclasticus* (An_U5) was isolated from polluted sediment collected at the Ancona harbor. *Alcanivorax jadensis* (339) and *Alcanivorax dieselolei* (293) were isolated from surface waters collected respectively from the Gibraltar strict and the Levantine basin. The *Alcanivorax borkumensis* SK2 type strain was chosen as a control strain.

2.2 Genomic Analyses

2.2.1- Growth Conditions

For the genomic analyses, strains were grown with ONR7a media supplied with sodium pyruvate as the sole carbon source (1%, w/v). Strains were grown at 30°C under constant shaking.

2.2.2- Genome Extraction, Sequencing, Assembly, and Annotation

The whole genome DNAs of *A. jadensis* 339, *A.dieselolei* 293 and *M. hydrocarbonoclasticus* An_U5 were purified using the DNeasy Blood and Tissue kit (QIAGEN) and sequenced by GATC company (The Netherlands) using the Illumina Hiseq2000 platform generating 150-pb-long pair-end reads (~24 10^6 reads, ~3 Gb). Raw data were assembled into 14, 57 and 37 contigs, respectively, using Mira (version 4.1) (Chevreux et al., 1999); total coverage over the whole genomes reached ~500-fold. The draft genomes were respectively 3692567, 4790658 and 3977360 bp in length and contained 3439, 4445 and 3654 coding sequences. The G+C content of the three genomes was 59.51% for *A. jadensis* 339, 62.09% for *A. dieselolei* 293 and 58.03% for *M. hydrocarbonoclasticus* An_U5. The functional annotation of the predicted genes was performed using the RAST server (Aziz et al., 2008).

2.3 Experiments at High Hydrostatic Pressure

2.3.1- Growth Conditions

During high-pressure tests, strains were grown on ONR7a liquid media using dodecane (1%, v/v) as the sole carbon source for 4 days. Afterwards, cells were pelleted and re-suspended in ONR7a liquid media to obtain a starting optical density (OD) of 0.1, as evaluated at 610 nm (OD_{610}). Cell cultures were then inoculated in 10-mL sterile plastic syringes. Each syringe contained 3.5 mL of cell culture provided with 1% (v/v) dodecane as the sole carbon source. Syringes were split in 3 groups: the first group was incubated in High Pressure (HP) reactors (Parr, USA) at 100 bars; another group was incubated in HP reactors at 50 bars while the third was incubated at 1 bar. Cells grew 4 days at room temperature (about 20°C).

2.3.2- Growth evaluation

Cell growth was evaluated with OD_{610}. Cell number was also analyzed using flow cytometry (Cyan TM ADP LX, BD Accuri, Erembodegem, Belgium), where both total cell number and intact *vs.* damaged cell number was evaluated. For total cell number, cells were stained with fluorescent dye SYBER Green I. Samples were diluted in filtered PBS prior to staining as required. SYBER Green I solution was prepared as follows: commercial solution (Invitrogen-Thermo Fisher Scientific, USA) was diluted 100 times in 0.22 µm filtered dimethyl sulfoxide (DMSO). For the intact/damaged cell count, a second fluorescent dye, i.e., propidium iodide, was added. Propidium iodide was prepared as follows: 20 mM in DMSO was diluted 50 times. All samples were stained with 1% (v/v) of fluorescent dye. Stained samples were incubated for 13 min in the dark at 37°C prior to analysis at flow cytometer.

2.3.3- Biogas composition

Headspace gas composition of each syringe (i.e,. N_2, O_2 and CO_2) was evaluated at the end of the incubation in high-pressure experiments. Heasdspace gas was analyzed with a Compact GC (Global Analyzer Solutions, Breda, The Netherlands) equipped with 2 channels, both with a TCD detector. First channel was equipped with a pre-column (Molsieve 5A) and column (Porabond Q) to measure O_2 and N_2. Second

channel was equipped with a pre-column (Rt-QS-bond) and column (Rt-Q-bond) to measure CO_2. Carrier gas was helium.

2.3.4- pH

pH change was evaluated at the end of the growth using a Consort C532 pH meter.

2.3.5- Dodecane availability

Dodecane availability in the culture medium was evaluated at the end of each incubation by means of an extraction with an organic phase (i.e hexane). The latter was added in a ratio 1:1 (v/v) to the culture, mixed by hand for 1 min, then kept stable for 15 min to allow separation between the watery and the organic phase. Afterwards, the organic upper phase was sampled and injected in a GC Varian model CP-3800 equipped with a flame-ionization detector (FID). The column was a HP-5 (Agilent Technologies), 30 m length, 0.250 mm diameter, 0.25 µm film thickness. Carrier gas was helium. Oven was set at 135 °C, injector at 275 °C and detector at 325°C.

2.4 Experiments with different Carbon Sources

2.4.1- Growth Conditions

Growth comparison using different carbon sources of the isolates of *A. dieselolei* and *A. borkumensis* were performed by growing these strains in 250 mL glass bottles containing 100 mL of ONR7a media supplied with 1% dodecane (v/v) or pyruvate (w/v) as the sole carbon source. Strains were grown at room temperature for 7 days without mixing.

2.4.2- Growth and pH evaluation

Growth of the cells in the different conditions and changes in the pH of the growth medium were evaluated with the same methodologies previously described in the High Hydrostatic Pressure Section.

2.4.3- Dodecane degradation

Dodecane degradation was evaluated through GC-FID analyses with the same methodologies previously described for the measurement of dodecane availability in the High Hydrostatic Pressure Section.

2.4.4- Pyruvate degradation

Organic acids were analysed using a 930 Compact IC Flex (Metrohm, Switzerland) ion chromatography system with inline bicarbonate removal (MCS with 0.5 M LiCl as regeneration liquid). Separation was done on a Metrosep organic acids (250/7.8) column at 35°C behind a Metrosep organic acids (4.6) guard column. A conductivity detector (ProfIC Detector MF) was used for detection of eluted components. The eluent was 1 mM H_2SO_4. The sample aspiration needle was cleaned with acetone between each analysis. The lower limit of quantification was 1 mg/L.

2.4.5- Cell hydrophobicity

Cell hydrophobicity was evaluated through the Microbial Adesion To Hydrocarbon (MATH) test modified from Rosenberg et al. (1980). Cells were pelletted at 4000 rpm for 10 min and rinsed in PUM buffer ($K_2HPO_4.3H_2O$ (22.2 g/L), KH_2PO_4 (7.26 g/L), UREA (1.8 g/L) MgSO4.7H2O (0.2 g/L)). pH was adjusted to 7.1. Finally, cells were resuspended in PUM buffer to a final volume of 1.2 mL. Initial volumes of cell cultures varied in order to obtain an OD_{610} value of the resuspended cells of about 1. After measuring the rinsed-cells OD_{610} (ODi), cells were spiked with 0.1 mL hexadecane and incubated at 28°C for 10 min. Afterwards, cells were mixed for 2 min and left to rest for 15 min. Finally, the OD_{610} of the aqueous phase was measured again (ODf). The percentage of cell bound to the hexadecane was calculated as follows:

Equation 1 ((ODi-ODf)*100)/ODi

2.5 Statistical analysis

The statistical analysis of the data was carried out with GraphPad (La Jolla, CA, USA), using a Student *t*-test with a two-sided distribution having a 95% confidence interval (95% CI). The *t* values represented the percentile below 2.5 and above 97.5 in the Student *t* distribution with (n-1) degrees of freedom, according to the following equation:

Equation 2 95%CI: X ï t (0.025) x radq (s_X) ≤ µ ≤ X + t (0.975) x radq (s_X)

where the value of *t* depends on the degrees of freedom (n being the sample set), *X* is equal to the sample mean and s_X is equal to s^2/n (s being the sample standard deviation). The statistical significance of the data was assessed with GraphPad using a nonparametric test (the Mann–Whitney test), considering a two-sided distribution with 95% CI. The presented data are the results of experiments performed between 3 and 20 replicates, according to the samples set.

3. RESULT and DISCUSSION

The capability of 4 different hydrocarbonoclastic strains (*A. borkumensis* SK2, *A. jadendis* 339, *A. dieselolei* 293 and *M. hydrocarbonoclastic* An_U5) to grow and degrade hydrocarbon at different pressures was evaluated. *A. jadensis* 339 and *A. dieselolei* 293 were isolated from surface marine waters collected along oil tanker routes, while *M. hydrocarbonoclasticus* An_U5 was isolated from a highly polluted sediment collected inside the Ancona harbour, which depth is between 2 and 11 m. Some of these species are known to be able to live in deep-sea environment. *A. dieselolei* was isolated for the first time from deep-sea sediments collected at a depth of 5027 m in the east Pacific Ocean (Liu & Shao, 2005) while *M. hydrocarbonoclasticus,* firstly isolated from intertidal sediments collected near a petroleum refinery outlet (Gauthier et al., 1992), was found also in deep-sea samples, collected at different depths, between 2400 m and 3500 m (Daffonchio et al., 2006; Tapilatu et al., 2009; Grossi et al., 2010;). *A. borkumensis* SK2 was chosen as a reference strains being one of the most studied marine hydrocarbonosclastic bacteria.

3.1 Genomic analyses

Firstly, the genome of *A. dieselolei* 293, *A. jadensis* 339 and *M.hydrocarbonoclasticus* An_U5 was extracted, sequenced, assembled and annotated.
Genome annotation on the RAST (Rapid Annotation using Subsystem Technology) server version 2.0 allowed comparing the basic characteristics of our genomes of interest (Tab.1)

Organism	Genome size	Contigs	Coding Sequences	GC %
A. jadensis 339	3,692,567	14	3,439	59.51%
A. dieselolei 293	4,790,658	57	4,445	62.09%
M. hydrocarbonoclasticus An_U5	3,977,360	37	3,654	58.03%

Tab.1 General genome features of *A. jadensis* 339, *A. dieselolei* 293, *M. hydrocarbonoclasticus* An_U5

After annotation, the presence of *alk* and *cyp450* genes was evaluated. *alk* genes encode for alkane hydroxylases involved in the degradation of medium to long chain hydrocarbons (van Beilen et al., 2003), while *cyp450* encode for cytochrome P450 monooxigenases, hemoproteins able to hydroxylate a large number of compounds. *cyp450* are ubiquitous among all kingdoms of life, and it was found also in several bacterial strains able to degrade C5–C10 alkanes (Wang et al., 2010). All the strains showed the presence of both class of genes and preliminary genetic analyses on their sequence indicate high similarity within these functional genes (data not shown), even if they belong to different bacteria species, suggesting the same biodegrading potential in the four strains. The preliminary genomic studies reported here represent a baseline for future transcriptomic analyses that will be realized on the 4 hydrocarbonoclastic bacteria grown, as discussed in the following sections, at different high hydrostatic pressures and using different carbon sources to investigate their hydrocarbon degradation potential.

3.2 High Hydrostatic Pressure: Cell Growth and Dodecane Degradation

In order to simulate the deep marine environment, growth and capability to degrade hydrocarbons were evaluated at different pressures: 1 bar, reproducing the surface environment; 50 bars, reproducing a depth of

500 m; 100 bars reproducing a depth of 1000 m. The general growth trend of the four strains was investigated using both OD_{610} measurement and flow cytometry cell count, which showed a general decrease of growth correlated with the increase of pressure (Fig.1-2).

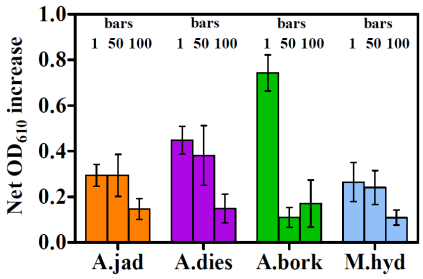

Fig.1: **Growth evaluation through OD610 measurment of the cells culture after incubation for 4 days at different pressure values tested . Column with different letters are statistically different on the basis of the Mann-Witheny test (p < 0.05), bars indicate the 95% confidence interval (CI).**

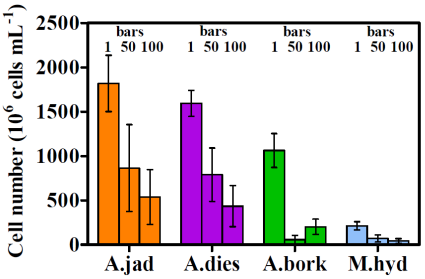

Fig.2: **Growth evaluation through total cells number count with flow cytometry measurment of the cells culture after incubation for 4 days at the different pressure values tested. Column with different letters are statistically different on the basis of the Mann-Witheny test (p < 0.05), bars indicate the 95% confidence interval (CI).**

This indicates that all the studied strains do not belong to the class of piezophilic bacteria, which show highest growth rate at higher hydrostatic pressures than atmospheric one. On the other side, they show growth at each of the tested pressure, hence they can be classified as piezotolerant, a category that includes strains able to grow with increasing hydrostatic pressures even if at a lower pace with respect to the atmospheric one. Indeed, by comparing microbial growth at 1, 50 and 100 bars detected through OD_{610} measurement (Fig.1), a significant decrease in cell growth was assessed only when a pressure of 100 bars was applied, with the only exception of *A. borkumensis* SK2, which seems to be very sensitive already to a pressure value of 50 bars. Contrasting results were obtained through flow cytometry, as they showed a significant decrease of the total cell numbers, for all the tested strains, at 50 bar (Fig.2). This apparent contradiction may be explained by the fact that OD can be affected by production of extracellular compounds, which may increase turbidity while it does not affect cell number.

As a decrease in cell growth does not necessarily correspond to cell damage or death, percentage of intact cells in each sample was evaluated. This analysis was carried out through flow cytometry, as populations of intact and damage cells can be stained with different fluorescent molecules (Hammes et al., 2012) (Fig.3). A significant impact of pressure on the number of intact cells was found exclusively for *A. borkumensis* SK2, in accordance with a decrease in its total cell number with increasing pressure. Most interestingly, this strain appears to be more sensitive to 50 rather than 100 bars, as the number of intact cells decreased at 50 bar but it increased significantly when pressure was further increased to 100 bars (Fig.3).

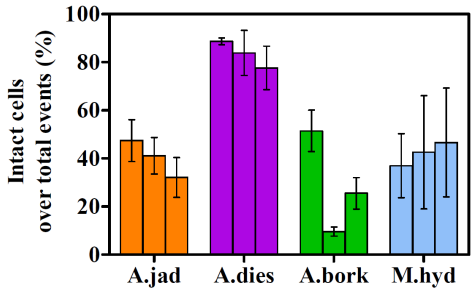

Fig.3: **Percentage of intact cells over total cells number count with flow cytometry measurement of the cells culture after incubation for 4 days at the different pressure values tested. First, second and third colums indicate 1, 50 and 100 bars respectively. Column with different letters are statistically different on the basis of the Mann-Witheny test (p < 0.05), bars indicate the 95% confidence interval (CI).**

Once demonstrated that cells integrity was not an issue for the three new isolates, question arose about their degradation potential. O_2 consumption and CO_2 production measurements were considered as a direct indicator of cells respiration and indirect indication of dodecane degradation, respectively (Fig.4-5), being dodecane the unique carbon source provided with the growth medium (thus, the only carbon source for CO_2

production). In all strains, O_2 consumption increased significantly already at 50 bars. Concomitantly, CO_2 production showed a significant decrease at increasing pressures, with the only exception of *A. jadensis* 339, for which the decrease of CO_2 production is significant only at a pressure of 100 bars.

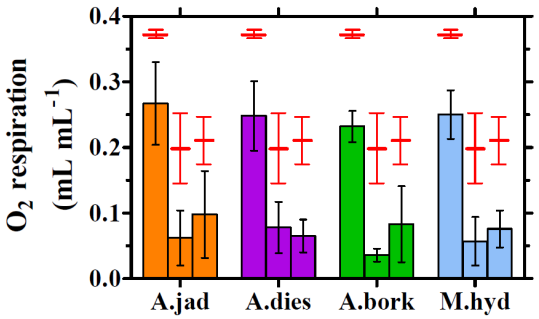

Fig.4: **GC measurement of the concentration of oxigen in the head space of the cells culture after incubation for 4 days at the different pressure values tested. First, second and third colums indicate 1, 50 and 100 bars respectively. Column with different letters are statistically different on the basis of the Mann-Witheny test (p < 0.05), bars indicate the 95% confidence interval (CI). Red bars are the abiotic controls values.**

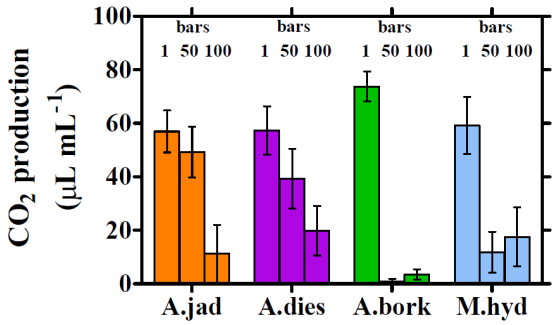

Fig.5: **GC measurment of the concentration of CO2 in the head space of the cells culture after incubation for 4 days at the different pressures. Column with different letters are statistically different on the basis of the Mann-Witheny test (p < 0.05), bars indicate the 95% confidence interval (CI).**

This trend was confirmed by pH analysis of the growth medium (Fig.6), which showed a significant higher acidification at 1 bar, corresponding to the higher production of CO_2, and more basic values as pressure increased. It must be considered that biogas solubility, including CO_2, increases at increasing hydrostatic pressures. Thus, the more basic values recorded at high pressure respect to atmospheric one indicate an effective lower metabolic activity of the tested bacteria at 50 and 100 bars.

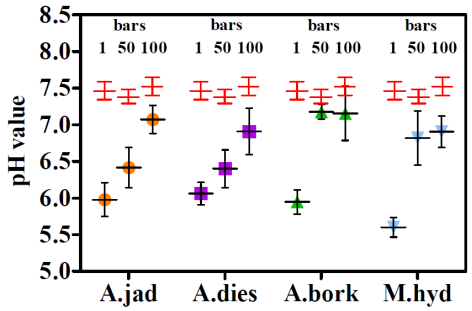

Fig.6: **pH measurment of the cells culture media after incubation for 4 days at the different pressure values tested. Column with different letters are statistically different on the basis of the Mann-Witheny test (p < 0.05), bars indicate the 95% confidence interval (CI). Red bars are the abiotic controls values.**

Dodecane availability was assessed at the end of each incubation. Provided that hydrocarbons tend to be adsorbed by plastics, biodegradation rates could not be evaluated with the present set-up, as the syringes used for cell incubation were indeed made of plastic. In fact, control syringes- only supplied with medium and dodecane- showed extremely low dodecane concentrations at the end of the experiment. Nonetheless, high concentrations of this hydrocarbon were found in the syringes supplied with cell cultures, meaning that each of the strains adopted in this study was able to partially solubilize dodecane in water (Fig. 7). Despite the fact that reproducibility was rather low, dodecane was found in concentrations up to 3 g/L (50 bars, *Marinobacter*), showing that bioavailability of the carbon source did not represent a limiting factor for cell growth (note that solubility of dodecane in water is equal to 8.42 µg/L at 25°C, Shaw and Maczynski, 2006).

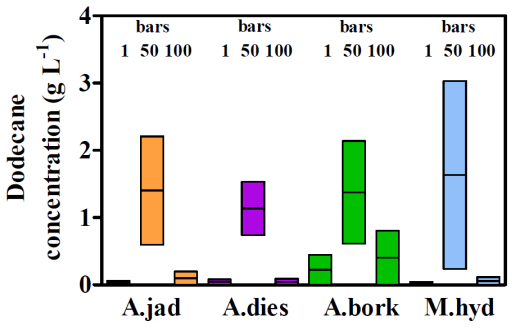

Fig.7: **GC-FID measurment of the concentration of dodecane in the cells culture after incubation for 4 days at the different pressure values tested. Bars in the middle of the column are the mean values, while the extremes of the columuns are the minimum and the maximum values measured.**

3.3 Carbon Sources: Cell Growth and Degradation of Pyruvate and Dodecane

In order to elucidate how different carbon sources may affect gene activation, the growth capability of *A. borkumensis* SK2 and *A. dieselolei* 293 was investigated supplying either pyruvate, one of the few non-alkane substrate that these hydrocarbonoclastic bacteria can metabolize, or dodecane as hydrocarbon source, under atmospheric pressure. The growth of *A. borkumensis* SK2 did not appear to be influenced by the different carbon source, neither evaluating it through OD_{610} measurement nor counting the total cell number with flow cytometry (Fig.8-9).

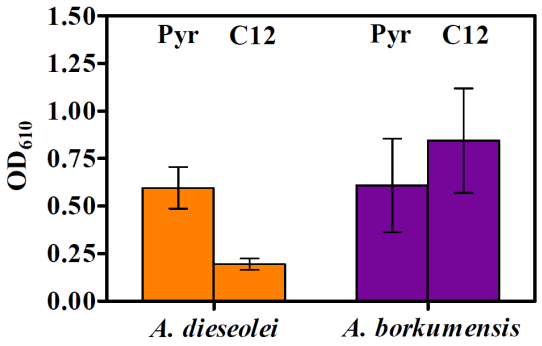

Fig.8: **Growth evaluation through OD610 measurment of the cells culture after incubation for 5 days with different carbon sources. Column with different letters are statistically different on the basis of the Mann-Witheny test ($p < 0.05$), bars indicate the 95% confidence interval (CI).**

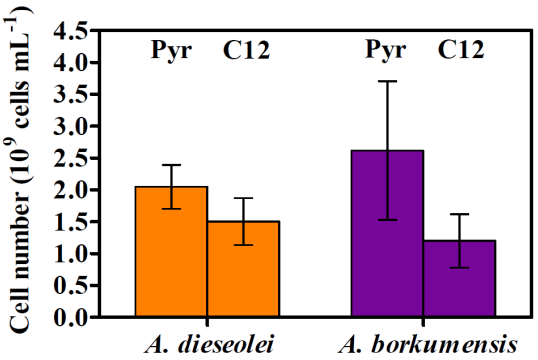

Fig.9: **Growth evaluation through total cells number count with flowcytometry measurment of the cells culture after incubation for 5 days with different carbon sources. Column with different letters are statistically different on the basis of the Mann-Witheny test ($p < 0.05$), bars indicate the 95% confidence interval (CI).**

On the contrary, OD_{610} measurement of *A. dieselolei* 293 showed a significant increase of growth when supplied with pyruvate. However, this data was not confirmed by flow cytometry, which shows only a slight increase in cell number when pyruvate was supplied in place of dodecane. A significant decrease of pH values was noted when compared with negative controls (where only media and carbon source, but no cells, were provided), indicating an actual activity on the part of the cells for both tested bacteria. Furthermore, when dodecane was supplied, the pH was significantly lower if compared with the values measured after the growth with pyruvate (Fig.10). This may indicate a higher degradation activity towards dodecane rather than pyruvate in both strains, even though the direct evaluation of the carbon source degradation through GC and HPLC analyses is in contrast with this hypothesis (Tab.2).

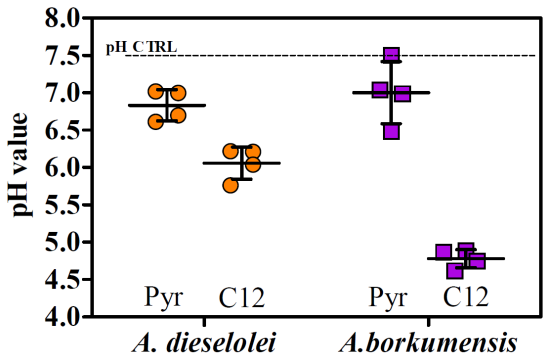

Fig.10: **pH measurment of the cells culture media after incubation for 5 days with different carbon sources. Values with different letters are statistically different on the basis of the Mann-Witheny test (p < 0.05), bars indicate the 95% confidence interval (CI).**

	***A. borkumensis* SK2**		***A. dieselolei* 293**	
	PYRUVATE	DODECANE	PYRUVATE	DODECANE
n° of replicates	3	8	3	3
consumption (mM)	100,58	19,89	100,23	35,73
Mann-Whitney test	0,0186		0,1	

Tab. 2 Average consumption of the different carbon source. Mann-Whitney test indicate a significative higher consumption of pyruvate from *A. borkumensis* SK2.

According to GC and HPLC analyses, the consumption of the two different carbon sources supplied in the medium was not significantly different in the case of *A. dieselolei* 293, while *A. borkumensis* SK2 degraded a significantly higher amount of pyruvate than dodecane. The more basic pH values recorded in the culture medium of *A. borkumensis* SK2 growing on pyruvate may be due to the cells capability to synthesize internal reserves. Storage of lipids are frequently observed in intracellular bodies in several hydrocarbonoclastic bacteria belonging to the genera *Alcanivorax, Marinobacter, Micrococcus, Pseudomonas and Thalassolituus* (Waltermann & Steinbuchel, 2005; Kalscheuer et al., 2007; Grossi et al., 2010).

Alkane molecules are highly hydrophobic and their bioavailability is a fundamental problem when they are the sole carbon source and energy available for bacterial growth. Frequently, bacterial surface becomes more hydrophobic to facilitate the access to this kind of organic substrate (Wick et al., 2002; Naether et al., 2013). When applying the MATH test (Rosenberg et al., 1980) to the studied strains we evaluated the changes in the capability of the cells to adhere to hexadecane, which reflect their grade of hydrophobicity. *A. dieselolei* 293 did not show significant changes of its hydrophobicity growing either on pyruvate or dodecane, while *A. borkumensis* SK2 behavior was completely different. When supplied with pyruvate as the sole carbon source, this strain showed to be remarkably hydrophilic (Fig. 11), while when supplied with dodecane its adhesion to hydrocarbons (ie hexadecane) was significantly high, thus confirming *A. borkumensis* capability to produce biosurfactants (Yakimov at al., 1998; Naether et al., 2013).

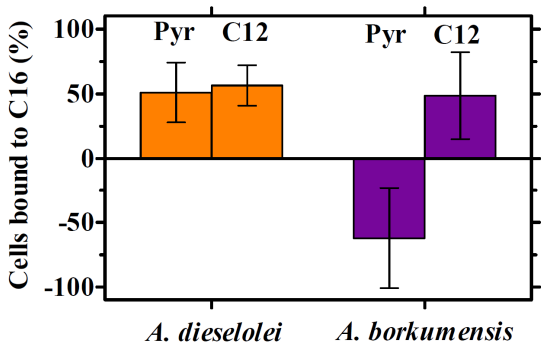

Fig.11: **MATH test (Rosenberg et al. 1980) performed on cell cultures after growth of 5 days with different carbon surces. Column with different letters are statistically different on the basis of the Mann-Witheny test (p < 0.05), bars indicate the 95% confidence interval (CI).**

3. CONCLUSIONS

The behavior of *A. borkumensis* SK2, *A. jadensis* 339, *A. dieselolei* 293 and *M. hydrcoacrboncolasticus* An_U5 at high hydrostatic pressure was evaluated. In general, hydrostatic pressure was perceived as a stress factor in all strains, as suggested by a decrease in growth, increase of O_2 consumption and decrease in CO_2 production. However, the only species showing a sign of cell damage was *A. borkumensis* SK2, while the 3 novel isolates reported in this study appear to be less affected, in terms of cells integrity, by high hydrostatic pressure. Due to the fact that the cultures were grown in plastic syringe during this experimental set up, it was not possible to evaluate directly the dodecane degradation. Nonetheless, indirect estimation through CO_2 and pH measurements suggested that dodecane consumption decrease as the hydrostatic pressure increase. In light of all the physiological data so far obtained, the strains selected for this experiments do not appear to be good hydrocarbon-degrader candidates at high hydrostatic pressure as much as they are at atmospheric pressure.

When different carbon sources were supplied (pyruvate vs dodecane), differences in the hydrophobicity of *A. borkumensis* SK2 cells were observed, probably indicating a change in the phospholipid layer of the cells membrane, more hydrophobic in the presence of hydrocarbons.

These differences in adaptation to high hydrostatic pressure and to different carbon sources need to be further investigated to better understand the actual mechanisms that take place in hydrocarbon degradation in marine environments. Analysis of the transcriptome of the four strains tested for growth and hydrocarbon consumption will be carried out to pinpoint the differences in the genes activation, possibly revealing the cellular response to hydrocarbon pollution.

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Chapter
VI

BACTERIAL DIVERSITY AND BIOREMEDIATION POTENTIAL OF THE HIGHLY CONTAMINATED MARINE SEDIMENTS AT EL-MAX DISTRICT (EGYPT, MEDITERRANEAN SEA)

Abstract

Coastal environments worldwide are threatened by the effects of pollution, a risk particularly high in semi-enclosed basins like the Mediterranean Sea that is poorly studied from bioremediation potential perspective especially in the Southern coast. Here, we investigated the physical, chemical and microbiological features of hydrocarbon and heavy metals contaminated sediments collected at El-Max Bay (Egypt). Molecular and statistical approaches assessing the structure of the sediment-dwelling bacterial communities showed correlations between the composition of bacterial assemblages and the associated environmental parameters. Fifty strains were isolated on mineral media supplemented by 1% crude oil and identified as a diverse range of hydrocarbon-degrading bacteria involved in different successional stages of biodegradation. We screened the collection for biotechnological potential studying biosurfactant production, biofilm formation and the capability to utilize different hydrocarbons. Some strains were able to grow on multiple hydrocarbons as unique carbon source and presented biosurfactant-like activities and/or capacity to form biofilm and owned genes involved in different detoxification/degradation processes. The El-Max sediments represent a promising reservoir of novel bacterial strains adapted to high hydrocarbon contamination loads. The potential of the strains for exploitation for *in-situ* intervention to combat pollution in coastal areas is discussed.

1. INTRODUCTION

The Mediterranean Sea is exposed to a high risk of pollution by petroleum hydrocarbons (HC), due to the presence of tens of sites related to their extraction, refinery and transport along its coastline [1]. This risk is exacerbated by several factors, including the semi-enclosed nature of this sea and the geographical location of most of the oil-producing and oil-consuming countries, placed respectively on the Southern and Northern sides of the basin, entailing the presence of pipeline terminal and oil tanker traffic. A recent analysis of the paper published in the last years about the microbiology of coastal and open-sea sites in the Mediterranean sea clearly showed that the Southern side of the basin has been largely neglected [2] although it hosts several polluted area along its coasts, such as the El-Max district area (Alexandria, Egypt). Due to the numerous industrial activities, the disposal of untreated waste effluents and the shipping activities, the El-Max bay is a coastal site chronically contaminated by crude oil and heavy metals [3] which clean-up represents a challenge for the Egyptian country and for the entire research community. Crude oil is a mixture of organic compound that may contain up to 20000 chemicals and it is hardly removable from polluted ecosystems by traditional methods. Bioremediation is an alternative to physical and chemical methods and takes advantage of the natural ability of certain microbes to degrade HC, buffering the effect of oil pollution in natural ecosystems. Bioremediation can be achieved by adding nutrients to the autochthonous biodegrading microbes (biostimulation) or adding a microorganism's inoculum in the polluted environment (bioaugmentation). The successfulness of such approaches is still under debate [4, 5, 6], however recent reports suggest the use of autochthonous bioaugmentation (ABA) as the best practice to restore polluted marine ecosystems [7]. The starting point for such approach is the detailed study of the diversity of microbial communities colonizing the polluted site of interest. Such survey should be accomplished through both molecular and cultivation dependent techniques that respectively

allow i) the correlation of the environmental parameters with the structure of the whole microbial communities and ii) the enrichment, identification and characterization of degrading microbes for traits of interest like the production of biosurfactant. Biosurfactant are molecules that have hydrophilic and hydrophobic moieties and, enhancing the bioavailability of oil hydrocarbons, are pivotal in microbial oil degradation network [8]. In this pipeline, the most promising microbes can be selected for subsequent laboratory scale experiments to test their degrading capability before ex-situ and in-situ field ABA trials.
This work represents the first holistic investigation of the bacterial communities inhabiting the marine sediments of different stations located in the El-Max district bay. It aims to unravel the pattern of bacterial diversity, ecology and degradation potential in polluted sediments and to obtain promising bacterial resources to be exploited for marine sites' clean-up. The chronically polluted El-Max district represents a very interesting site for this research topic since, due to the occurring of strong selective pressure, most of the autochthonous bacteria should be able to cope with the environmental stressors induced by oil contamination.

2. MATERIALS AND METHODS

2.1 - Sites description and sampling

The sampling areas is located at El Max Bay, which lies in the western side of Alexandria at longitude 29°78 E and latitude 31°13 N (Fig. 1a). The shoreline is mainly rocky with occurrences of narrow sandy beaches. There are pronounced differences in direction and intensity of marine currents in the bay near the outlets [9, 10]. Sediment samples were collected in triplicates at depth comprised between 3 and 16 meters, using a grab sampler, from 4 stations (Fig. 1b-c): P (31° 9'31.20"N, 29°50'28.20"E), Q (31° 9'28.40"N, 29°50'14.40"E), R (31° 9'18.56"N, 29°50'5.89"E) and S (31° 9'4.89"N, 29°50'2.49"E). Sediment samples were packed in aluminum foil for HC analysis and in plastic bags for the rest of the physic-chemical parameters. The water content, particle size and total organic carbon were determined immediately after sampling. Sediment samples were collected using sterile spoons and stored in sterile bags at 4 °C for bacteria isolation and - 20 °C for molecular analyses.

Figure 1. **Location of the study area and sampling stations. (a) Overall area of El Max district (Egypt) in the Mediterranean Sea and (b) satellite image of the sampling area, (c) showing the position of the four sampling sites.**

2.2 - Chemical characterization of sediment samples

Phosphorus extraction was performed according to Aspila et al. [11]. Total phosphorus was extracted by ashing the sample at 550˚C for 2.5 h and subsequent shaking with 1 N HCl for 16 hours while the inorganic phosphorus was extracted by shaking the oven - dried sediments (110 ˚C) with 1 N HCl for 16 hours. Phosphorus determination in the two extracts was made according to the method of Murphy and Riley [12]. Organic phosphorus was calculated subtracting the value of the inorganic phosphorous from the total phosphorus.
Total nitrogen content in the sediment samples was determined by using Kjeldahl apparatus (Raypa®, Model: DNP–1500, R. Espinar S.L., Barcelona, Spanish) according to standard method [13].
The total organic content (TOC) was determined by the loss on combustion technique after removal of carbonate with dilute (IN) HCl, a portion of sediments was weighed into a porcelain crucible and ignited in a muffle furnace at 550°C for two hours. The crucible was cooled in a

desiccator, re-weighed and the total organic content (TOC) was calculated as the weight loss in percentage [14]. The analysis of total pesticides and poly-chlorinated biphenyls (PCBs) were performed as previously described [15, 16, 17].
The presence and abundance of different n-alkanes was estimated by chromatographic techniques. The n-alkane concentration was analyzed by Agilent 7890 -USA. A HP-5 capillary chromatographic column (30 m x 0.32 mm I.D.) and a capillary column (30 m x 0.25 mm I.D.) were used for GC-FID and GC-MS analyses, respectively. Nitrogen was the carrier gas with 3 ml/min. Injector and detector temperature were maintained at 300 °C and 320 °C, respectively. The identification of n-paraffin peaks was established using a reference mixture of n-paraffin of known composition.
To determine the total content of heavy metals (copper Cu, iron Fe, zinc Zn, chromium Cr, nickel Ni, cadmium Cd, cobalt Co, lead Pb) and arsenic (As) in sediments, samples (0.1 g) were $HNO_3/HClO_4$ (4:1, v/v) digested in a microwave oven (CEM, MARS5). After digestion, the volume of each sample was adjusted to 20 mL using deionized water. Heavy metals and arsenic content was determined by Inductively Coupled Plasma-Mass Spectrometry (ICP-MS, Agilent Technologies, Santa Clara, CA, USA). Standards of heavy metals and of arsenic for concentrations ranging from 0 to 1 mg/L were prepared from multi-element calibration standard-2A solution (Agilent Technologies) and from sodium arsenite solution ($NaAsO_2$) (Sigma-Aldrich, St Louis, MO, USA) respectively. For all the measures by ICP-MS an aliquot of a 2 mg/L of an internal standard solution (45Sc, 89Y, 159Tb, Agilent Technologies) was added both to samples and a calibration curve to give a final concentration of 20 µg/L. The instrument was tuned daily with a multi-element tuning solution for optimized signal-to-noise ratio.

2.3 - Metagenome extraction and 16S rRNA amplification

Total DNA was extracted from 0.5 g of sediment using the "Power Soil" kit (MoBio Laboratories Inc., Carlsbad, CA, USA) following the manufacturer's instructions. DNA was quantified using a NanoDrop 1000 spectrophotometer (Thermo Scientific, Waltham, MA, USA). Bacterial 16S rRNA gene fragments (~550 bp) were amplified with Polymerase Chain Reaction (PCR) using primers 907R (3'-CCGTCAATTCCTTTGAGTTT- 5') and GC-357F (3'-CCTACGGGAGGCAGCAG- 5' with a 5'-end GC-clamp) targeting a portion of the 16S rRNA gene that includes the hypervariable V3-V5 regions[18]. PCR reactions were performed as previously described [19]. Presence and length of PCR products were checked by electrophoresis in 1% w/v agarose gel prior to Denaturing Gradient Gel Electrophoresis (DGGE) analysis.

2.4 - Denaturing Gradient Gel Electrophoresis

PCR products (~150 ng) were loaded in a 0.5 mm polyacrylamide gel (7% [w/v] acrylamide-bisacrylamide, 37.5:1) containing 43 to 56% urea-formamide denaturing gradient (100% corresponds to 7M urea and 40% [v/v] formamide). The gels were run for 16 h at 60 °C by applying a constant voltage of 90 V in 1X Tris-acetate-EDTA (TAE) buffer. After electrophoresis, the gels were stained for 30 min in 1X TAE buffer containing 1X SYBR Green (Molecular Probes, Leiden, the Netherlands) according to manufacturer's instructions and rinsed twice for 10 min with distilled water. Gels images were captured using a Gel Doc 2000 apparatus (Bio-Rad, Milan, Italy). The band patterns of the DGGE gel were analysed using Image J software (available for free download at http://rsb.info.nih.gov/ij/). A Principal Coordinates Analysis (PCO) was performed using PRIMER v. 6.1 [20]. DGGE bands were excided from the gels with a sterile scalpel and eluted in 50 µl of sterile Milli-Q water at 37 °C for 4 h. The eluted DNA was amplified by PCR using primers 357F and 907R and positive amplifications were sequenced by Macrogen Inc., Korea.

2.5 - PCR amplification of functional genes

The presence of *alkB* gene, encoding for alkane hydroxylase, in the metagenome extracted from the sediments was assessed using the primer D-alkF (5'-GCICAYGARYTIGGICAYAAR-3'), to which a 40 bp GC clamp was attached, and D-alkR (5'-GCRTGRTGRTCISWRTG-3') [21]. PCR amplification was performed in 50 µL reaction containing 1X buffer, 2mM MgCl2, 0.12mM of dNTPs mixture, 1 µM of each primer, 5% DMSO, 1.5 U Taq polymerase, and 10 ng of template, applying the following thermic protocol: 94 °C for 4', followed by 30 cycles of 94 °C for 45'', 55 °C for 1', and 72 °C for 1' and a final extension at 72 °C for 10min.

Primer nccA-F (5'-ACGCCGGACATCACGAACAAG-3') and nccA-R (5'-CCAGCGCACCGAGACTCATCA-3') were used as previously reported [22] to amplify the *nccA* gene that encode for nickel-cobalt-cadmium efflux pump. Primer dacr5F (5'-TGATCTGGGTCATGATCTTCCCVATGMTGVT-3') and dacr4R (5'-CGGCCACGGCCAGYTCRAARAARTT-3') were used for amplification of arsenite efflux pump (*ACR3(2)*) according to Achour et al. [23]. Primer Phn321F (5'-TTCTCGGTCGGG ACTTTCCAA-3') and Phn671R (5'-GGCAACCAGATCTGTCATG-3') were used for amplification of *phnA1* gene coding for 3,4-phenanthrene dioxygenase, according to Cavalca et al. [24]. PCR reactions were performed in a final volume of 25 μL containing 1X buffer, 1.75 mM $MgCl_2$, 0.2mM of dNTPs mixture, 0.4 μM of each primer, 1.5 U Taq polymerase, and 10 ng of total DNA.

2.6 - Bacteria isolation and identification

Bacteria were enriched and isolated using two different marine mineral media (Artificial Seawater [ASW] and ONR7a) supplemented with 1% crude oil. Enrichment vials were incubated at 30°C under rotation until turbidity was observed before to proceed with isolation. Twenty-five bacterial isolates have been obtained in pure cultures from both the media. DNA extraction was performed on each isolates by boiling lysis or using Thermo Scientific GeneJET Genomic DNA Purification Kit. The amplification of the bacterial 16S rRNA gene was performed using the universal primer 27F (3'-AGAGTTTGATCMTGGCTCAG-5') and 1492R (3'-CTACGGCTACCTTGTTACGA-5'). The PCR amplification conditions and thermal protocol were set up as previously described [19] providing a PCR amplicon of approximately 1400 bp.

2.7 - Nucleotide Sequence Analyses and Accession Numbers

Nucleotide sequences were edited in Chromas Lite 2.01 (http://www.technelysium.com.au) and subjected to BLAST search (http://blast.ncbi.nlm.nih.gov/Blast.cgi). The partial 16S rRNA gene sequences obtained from the bacterial isolates have been deposited in the GenBank and ENA (European Nucleotide Archive) databases and the related accession number are reported in Table 6. The sequences obtained from the excised DGGE bands are available at ENA under the accession number LN610485 - LN610498.

2.8 - Evaluation of metabolic traits, biofilm and biosurfactant production within the bacteria collection

The potential ability to produce biosurfactant has been assessed within the bacteria collection using different assays aimed to determine the surface tension reduction, hemolytic activity, and cell hydrophobicity as previously described [3]. Biofilm formation was evaluated according to published protocols [3, 25]. The capability of each bacteria to utilize different HC molecules (xylene, octane, pyrene, dibenzothiophene, phenanthrene and naphthalene) as sole carbon source was tested in ASW agar medium with a final concentration of 25 mg/l of the different HC. Xylene, octane and naphthalene were added in the inner side of the lids of petri-dishes and incubated upside down to allow the upwards diffusion of the HC through the medium, whereas the other HCs were spread on the medium surface. The plates were incubated at 30°C for two weeks: if colonies could be detected on the plates, the ability to grow in presence of a certain compound was considered positive [3].

2.9 - Statistical analyses

Significant differences in the bacterial community composition were analyzed by permutational analysis of variance [PERMANOVA, 26] considering the sampling stations and the type of sediment as and orthogonal fixed factor. All the statistical tests were performed by PRIMER v. 6.1 [20], PERMANOVA+ for PRIMER routines [27]. To assess the significance correlation between environmental data with the bacterial community composition obtained by DGGE, a Mantel Test was performed [R packageade4, mantel.rtest, 999 iterations; 28].

Furthermore, distance-based multivariate analysis for a linear model [DistLM; 29] was carried out to determine the significant environmental variables explaining the observed similarity among the samples. The Akaike information criterion (AIC) was used to select the significant predictor

variables. The contribution of each environmental variable was assessed using a "sequential test" to evaluate the cumulative effect of the environmental variables explaining biotic similarity.

3. RESULTS AND DISCUSSION

3.1 - Physico-chemical analyses indicate high level of pollution in the El-Max district sediments

Physical analyses showed that the sediments collected from the stations P, Q, R are mainly composed by sand (85.82-95.62%) while the sediment of station S displayed a different composition, containing approximately the same percentage of sand (39.41%) and silt (34.39%) and a higher proportion of clay (26.20 %) compared to the rest of the stations (0-5.49%). Grain size measurements of superficial sediment revealed that stations P and Q contained coarse sand whereas station R displayed medium size sand. The highest water content percentage was detected in station S (35 %) which contains a fine silty sediment type (Table 1).

Table 7 Physical characteristics of the El-Max district sediments samples.

Station	% Sand	% Silt	% Clay	Mean Size (phi)	Type of Sediment	Water Content %	Porosity %
P	95.18	4.82	0.00	0.11	coarse sand	15.00	6.90
Q	85.82	8.7	5.49	0.49	coarse sand	20.00	9.79
R	95.62	3.04	1.34	1.51	medium sand	28.00	15.02
S	39.41	34.39	26.20	4.84	coarse silt	35.00	20.22

Such differences in the water content and grain size are known to influence the solubility of elements and nutrients in marine sediments, ultimately affecting the distribution of metals and other pollutants that preferentially bind to fine particles [30], determining as a consequence that the four stations analyzed constitute different environmental niches. All the stations showed total nitrogen content below 0.2% w/v (Figure2a). Stations R and S showed a high content of total phosphorous with 0.83 and 0.59 ppm respectively (Figure 2b).

In the case of station R, which showed the highest concentration, this could be due to the close presence of the agricultural drain El-Umum. As shown in Figure 2c, sediments of the stations P and S displayed the highest concentrations of total organic carbon (0.56 and 0.634 ng/g respectively) and total pesticides (0.1362 and 0.1452 ng/g respectively). Moreover, sediments P and S contained high concentrations of PCBs (Figure 2c), which highest concentration was recorded in sediments of station Q (0.17 ng/g). The assessment of total PAH concentration in the sediment was performed by measuring the content of 16 different aromatic hydrocarbons and it indicated that station P has the highest PAH level (Figure 2d). Stations P and S, containing 81.6 and 11.6 μg/g of PAH, are active fishing area characterized by PAH concentration higher than the maximum indicated by the quality standards for marine water [31] and allowed by the EU (0.20 gL−1) and US (ΣPAHs = 0.030gL−1) Environmental Quality Criteria for protection of human consumers of aquatic life [32]. GC analysis of HC compounds in the analyzed stations (Figure 3) revealed that the dominant n-alkanes were *n*-C_{20} (Eicosanes), *n*-C_{26} (Hexaicosanes), *n*-C_{28} (Octaicosanes), *n*-C_{30} (Tricontanes) and *n*-C_{36} (Hexatricontanes). Sediment collected at station P contained the highest concentration of n-alkane of *n*-C_{22}, while the sediment of station S showed the highest concentrations of long chain n-alkanes of *n*-C_{26}, *n*-C_{28} and *n*-C_{30}. Overall, the sediment collected at station S showed the highest total n-alkanes content (Figure 3).

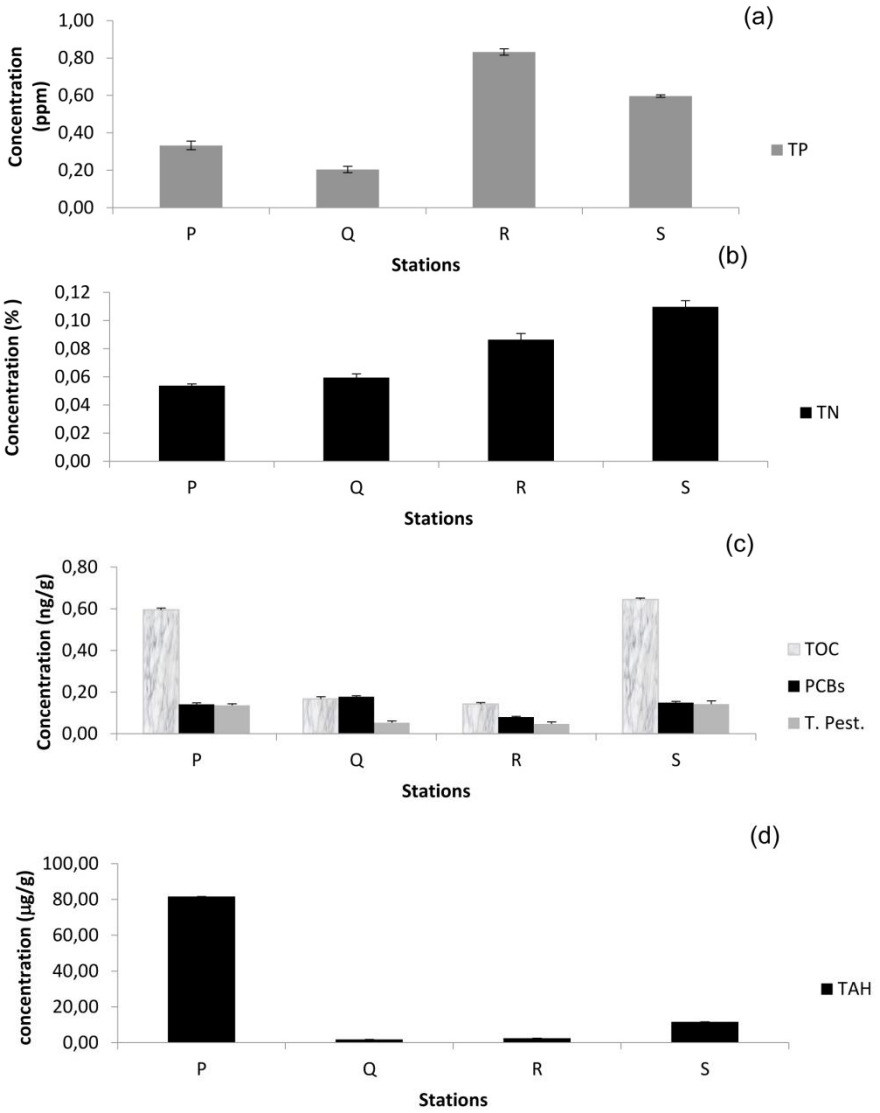

Figure 2. Chemical characterization of the sediments. **Concentration in the sediment of (a) total nitrogen (TN) ; (b) total phosphorous (TP); (c) total organic carbon (TOC), total pesticides (TPest) and poly-chlorinated biphenyls (PCBs); (d) total aromatic hydrocarbons.**

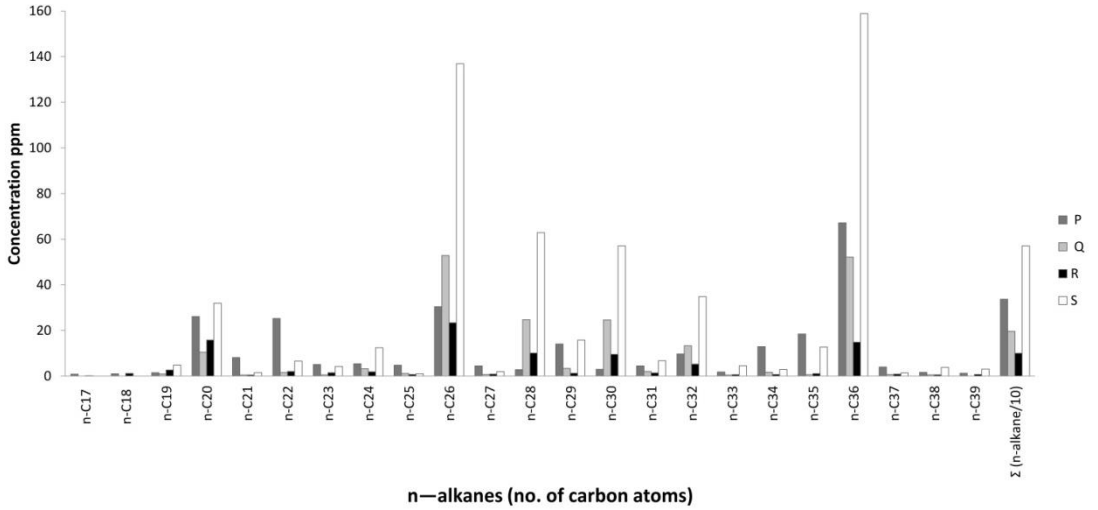

Figure 3. **Concentration of n-alkanes in the El Max district sediments.**

Table 8 Total heavy metal and arsenic content in El-Max district sediments.

	Cu (mg/kg)	Fe (g/kg)	Zn (mg/kg)	Cr (mg/kg)	Ni (mg/kg)	Cd (mg/kg)	Co (mg/kg)	Pb (mg/kg)	As (mg/kg)
P	22.63 ±4.09	4.389±0.21	45.77±10.42	19.21±1.27	7.83±2.12	1.19±1.93	1.58±0.03	19.27±4.87	3.31±0.57
Q	65.98±11.1	11.16±1.69	142.97±17.16	78.35±10.19	15.93±2.95	0.25 ±0.14	3.71±0.69	44.15 ±0.44	4.90±0.84
R	72.79±1.66	11.23±0.75	142.80±11.16	86.6±.3.66	19.18±0.74	0.28±0.05	4.11±0.31	45.57±2.47	5.17±0.93
S	118.15±12.14	11.66±0.93	247.71±22.59	105.08±7.69	26.37±2.08	0.58±0.11	4.45±0.54	59.40 ±5.01	7.06±1.25

Heavy metal and metalloids content in sediments was evaluated by measuring 8 different metals (Cu, Fe, Zn, Cr, Ni, Cd, Co, Pb) and As. It is noted that station S has the highest metal and As content while station P showed the lowest content (Table2). Unlike metal concentrations in surface water, where many countries adopted clear and unambiguous guidelines [i.e. 33, 34], there are no accepted international or local standards of metal levels in marine sediments. Only few countries (i.e. The Netherlands and Canada) have a long-standing legislative tradition developing criteria and regulations for sediment quality [35], while Egypt as the majority of the countries has not enforced any environmental protection laws or the existing legislation is not clear. Thus, the metals and arsenic levels assessed in this study were compared to the EU intervention limits imposed by the law for soil and subsoil of residential or industrial areas and to literature data. In all the sediments, metals content resulted below the threshold concentration of European Union Standard [36] and in particular, Ni, Pb and Cd were retrieved at a similar level present in rural soils of many countries [37]. In addition, arsenic content was present at a comparable level to the uncontaminated soils [38].

3.2 - El-Max district polluted sediments host complex bacterial communities which diversity is driven by physico-chemical parameters

Denaturing Gradient Gel Electrophoresis (DGGE) was applied to the metagenome extracted from the sediments to provide a snapshot of the bacterial communities' structure. From each station, total sediment DNA was extracted and analyzed by DGGE. Fingerprinting performed on triplicates demonstrated the reliability of the obtained DGGE profiles (Figure 4a). DGGE patterns showed the occurrence of complex bacterial communities in all the analyzed sediments (Figure 4a) indicating that the pollution level did not affect the taxonomic diversity of bacterial communities. A positive correlation between the environmental data available for the analyzed sediments and the detected DGGE pattern was indicated by the Mantel test ($r = 0.597$; $p < 0.05$), revealing that the physical parameters, together with the measured nutrients and pollutants concentration, are the main drivers of the overall composition of the bacterial communities. The DGGE patterns of the sediments P, Q and R appeared similar whereas differences could be observed with sediment S, concerning the presence of peculiar bands as well as differential abundance of some ubiquitous bands (Figure 4a). Principal Coordinates Analysis (PCO) of the DGGE fingerprints confirmed the observed differences showing a sharp clustering of the sediments S1-2-3 separately from the other sediments (Figure 4b). Basing on PCO1 (explaining 75.2% of the total variation) the bacterial community of the samples collected at station Q were also different from the sediments P and R (Figure 4b). Statistical analysis supported the PCO indications, confirming that both the bacterial community dwelling sediment S and Q were significantly different from those collected at stations P and R (see Table 3a for pairwise comparison). Moreover, PERMANOVA showed that the structure of sediments' bacterial community was influenced by the type of sediment (PERMANOVA, $df = 2$, $F = 12.7$, $p = 0.0001$), distinguishing sandy (P, Q, R) and silty (S) sediments as statistically different (see Table 3b for pairwise comparison).

Table 9 **PERMANOVA pair-wise results. (a) Groups correspond to the different stations. (b) Groups correspond to the different type/granulometry of the sediment. t: t statistic; p: statistical significance.** Bold = **groups significantly different.**

Groups	t	p
P, Q	**2,1697**	**0,0385**
P, R	1,6055	0,1077
P, S	**3,7151**	**0,0044**

Q, R	**2,6056**	**0,0146**
Q, S	**5,9167**	**0,0009**
R, S	**5,1805**	**0,002**

(a)

Groups	**t**	**p**
coarse sand, medium sand	1,2488	0,2166
coarse sand, coarse silt	**4,3187**	**0,0006**
medium sand, coarse silt	**5,1805**	**0,0021**

(b)

Physical and chemical parameters measured at the investigated stations were analyzed to assess their influence on the structure of the bacterial communities (Table 4). The sequential test showed that sand and clay percentage in the sediments are the statistically significant physical parameters involved in shaping the bacterial communities (Table 4a). Furthermore, distLM analysis was performed on chemical data considering separately the metal concentration and the other available chemical parameters. The sequential tests showed that, among metals, Cu, Fe and Zn concentration are the drivers of the bacterial community structure in the sediments (Table 4b) and that total organic carbon (TOC) and PCBs concentration were the others statistically significant parameters involved in the selection and assemblage of bacterial populations (Table 4c). Our data are in agreement with a recent study, which indicated both sediment particle size and the concentration of metals, including Fe and Zn, as pivotal factors in shaping the sediment's bacterial community [39].

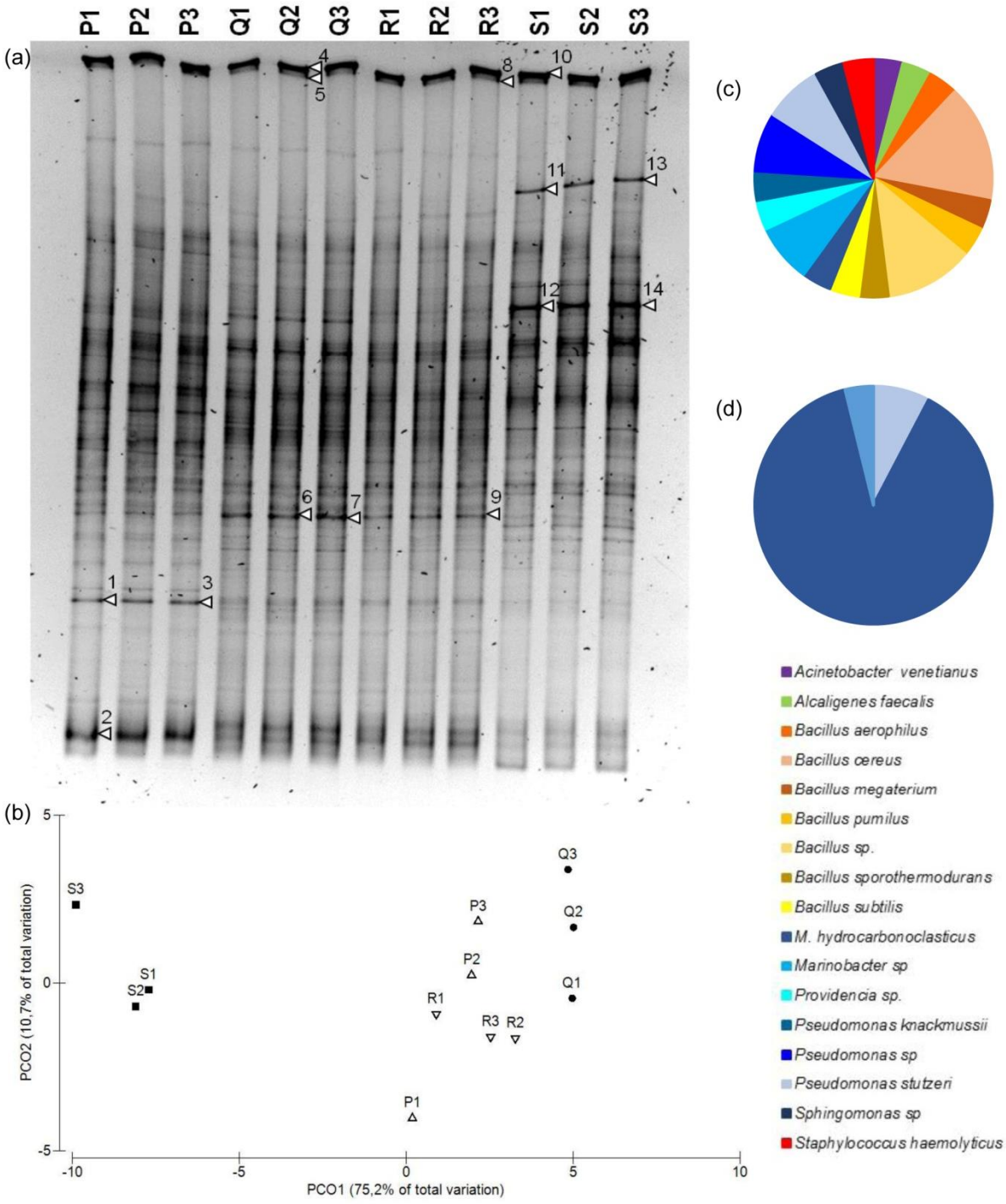

Figure 4. Cultivation dependent and independent analyses of the bacterial communities at El-Max district. **(a) DGGE analysis performed of the 16S rRNA on the sediment metagenome. The numbers in the name of the samples represented the three analyzed replicates. Bands numbered have been excised from the gel and their DNA content sequenced (results are reported in Table 5). (b) Principal Component Analysis based on the DGGE profiles of the 16S rRNA gene in the sediments. (c-d) Identification of the bacteria isolated respectively on ONR7a and ASW medium.**

Table 10 **(a) Sequential test (DistLM) explaining the total variation with the contribution of all the predictor variable accounted separately according to their division in (a) physical data, (b) metal/metalloid concentration, (c) nutrients/pollutants concentration. F = statistic; p = probability; Prop = proportion of total variation explained; Cumul = cumulative explained by the listed variables; Res df = residual degrees of freedom.** Bold = **statistically significant variables.**

Sequential tests						
Variable	**AIC**	**F**	**p**	**Prop.**	**Cumul.**	**res.df**
+% sand	35,267	16,445	0,0036	0,62186	0,62186	10
+% silt	34,458	2,3747	0,0738	0,078943	0,70081	9
+% clay	28,526	7,493	0,0008	0,1447	0,84551	8
mean size	**28,526**	**No test**		**-1,60E-14**	**0,84551**	**8**
water content	**28,526**	**No test**		**-1,59E-16**	**0,84551**	**8**
porosity	**28,526**	**No test**		**1,14E-15**	**0,84551**	**8**

(a)

Sequential tests						
Variable	**AIC**	**F**	**p**	**Prop.**	**Cumul.**	**res.df**
Cu	40,706	6,8084	0,0092	0,40506	0,40506	10
Fe	30	16,947	0,0009	0,38858	0,79364	9
Zn	28,526	2,686	0,055	0,051871	0,84551	8
Cr	**28,526**	**No test**		**-4,67E-16**	**0,84551**	**8**
Ni	**28,526**	**No test**		**-6,42E-16**	**0,84551**	**8**
Cd	**28,526**	**No test**		**7,80E-16**	**0,84551**	**8**
Co	**28,526**	**No test**		**-2,21E-16**	**0,84551**	**8**
Pb	**28,526**	**No test**		**5,79E-16**	**0,84551**	**8**
As	**28,526**	**No test**		**7,27E-16**	**0,84551**	**8**

(b)

Sequential tests						
Variable	**AIC**	**F**	**p**	**Prop.**	**Cumul.**	**res.df**
TAH	**46,138**	**0,68916**	**0,506**	**6,45E-02**	**6,45E-02**	**10**
TOC	31,775	26,19	0,0006	0,69626	0,76073	9
PCHs	28,526	4,3898	0,0099	8,48E-02	0,84551	8
Tpest	**28,526**	**No test**		**3,40E-16**	**0,84551**	**8**
TN	**28,526**	**No test**		**-9,57E-16**	**0,84551**	**8**
TP	**28,526**	**No test**		**8,47E-16**	**0,84551**	**8**

(c)

Aiming to identify the dominant taxa associated with the PCR-DGGE profiles several DGGE bands were excised from the gel. The successfully sequencing of partial 16S rRNA could be obtained only for 14 bands (Figure 4a), pointing out the presence of bacteria typical of marine ecosystems and characterized by low identity percentage with any known sequence in the public databases (Table 5). According to DGGE band sequencing, the main phylum associated to the El-Max district sediment was represented by Bacteroidetes, while Actinobacteria, Acidobacteria and Spirochaetes were retrieved in lower abundance (Table 5). The phylum Bacteroidetes was previously indicated among the main actors involved in PAH degradation in river sediment basing on DGGE analyses [40], and could possibly play a role in marine sediments. The phyla Actinobacteria, Acidobacteria and Bacteroidetes were also detected by high-throughput sequencing in estuarine sediments [39] while Spirochaetes were identified within the metabolically active bacterial communities in microcosms established using chronically polluted estuarine sediments [41].
The presence of putative HC-oxidizers in El Max district sediments was, moreover, demonstrated by the amplification of the *alkB* gene, codifying for the alkane monooxigenase enzyme, from all the sediment metagenomes (data not shown).

Table 11 Phylogenetic identification of bacteria from sequenced DGGE bands (see Figure 4 for band correspondence). The column "Environment" reports the habitat in which the "Closest relative" sequence present in NCBI database was detected.

Band	Sample	Closest relative (acc n°)	Identity (%)	Phylum	Environment
1	**P1**	**Unc. Bacterium (FR851749)**	**99**	**Actinobacteria**	**coral reef sands**
2	**P1**	**Unc. Acidobacteria (JF344347)**	**97**	**Acidobacteria**	**oil-polluted sediments**
3	**P3**	**Unc. Bacterium (FR851749)**	**99**	**Actinobacteria**	**coral reef sands**
4	**Q2**	**Unc. Bacterium (JN453366)**	**95**	**Bacteroidetes**	**hypersaline microbial mat**
5	**Q2**	**Unc. Bacterium (JN470103)**	**96**	**Bacteroidetes**	**hypersaline microbial mat**
6	**Q2**	**Unc. Bacterium (JN530286)**	**98**	**Spirochaetes**	**hypersaline microbial mat**
7	**Q3**	**Unc. Bacterium (JN530286)**	**97**	**Spirochaetes**	**hypersaline microbial mat**
8	**R3**	**Unc. Bacterium (KC574864)**	**95**	**Bacteroidetes**	**-**
9	**R3**	**Unc. Bacterium (KC574864)**	**94**	**Bacteroidetes**	**hypersaline microbial mat**
10	**S1**	**Unc. Bacterium (JN529047)**	**93**	**Bacteroidetes**	**hypersaline microbial mat**
11	**S1**	**Unc. Bacteroidetes (AF507860)**	**97**	**Bacteroidetes**	**meromictic soda lake**
12	**S1**	**Unc. Bacterium (KF268891)**	**99**	**Bacteroidetes**	**marine sediment**
13	**S3**	**Unc. Bacteroidetes (AF507860)**	**97**	**Bacteroidetes**	**meromictic soda lake**
14	**S3**	**Unc. Bacterium (KF268891)**	**99**	**Bacteroidetes**	**marine sediment**

3.3 - Phylogenetically different hydrocarbonoclastic bacterial isolates were obtained using different cultivation media

Besides molecular analysis, a cultivation approach was applied to obtain and characterize bacterial isolates according to the biotechnological potential for bioremediation applications. Twenty-five bacteria were isolated from the four stations on ASW medium supplemented with crude oil as the sole carbon source. This collection included Bacilli (13), Betaproteobacteria (1) and Gammaproteobacteria (11) divided in several families and genera (Table 6), witha high Shannon-Weaver index (2.69), calculated from the number of individuals per genus. The collection included bacterial genera widely studied for their ability to degrade oil hydrocarbons, such as *Acinetobacter venetianus* [42], *Pseudomonas stutzeri* [43] and *Marinobacter hydrocarbonoclasticus* [44] (Table 6, Figure 4c). Bacteria belonging to the genera *Acinetobacter, Pseudomonas* and *Marinobacter* were isolated from a variety of oil contaminated sites around the world. Such environments included coastal oil polluted site in Tunisia [45], intertidal sand

affected by oil pollution after the Prestige spill [46], the Gulf of Mexico beach sand [47] and deep hypersaline anoxic basins [48]. Kostka and coauthors [47] recently proposed that Gram-positive bacteria like those of the genus *Bacillus*, representing the 48% of the bacteria we isolated on ASW medium, could be used as sentinel for the later stages of oil degradation, when PAH dominate the composition of the residual oil. Accordingly, other authors [49, 50, 51] previously reported the presence of hydrocarbon degrading *Bacillus* strains from marine sediments and seawater. The fraction of El-Max sediment bacteria cultivable on ASW medium included representatives of the genera *Alcaligenes*, *Sphingomonas* and *Providencia* (Table 6, Figure 4d), the latter showing high potential for the bioremediation of heavy metals [52, 53], which are abundantly present in the samples analyzed in this study. Marine bacteria able to resist to high mercury concentrations and able to detoxify cadmium and lead were described also within the species *Bacillus pumilus* and *Alcaligenes faecalis* [54], present in our collection (Table 6). *A. faecalis* can perform phenanthrene degradation [55] and was also previously detected by DGGE analysis in weathered fuel enrichment established after the Prestige oil spill [56]. The ability of *Sphingomonas* spp. isolates to degrade a wide range of xenobiotics has been reported and their remediating capability have been assigned to a large plasmid harboring the genes codifying for degrading enzymes [55].

Sediment from station S, which host a peculiar bacterial community according to the DGGE analysis, was used to perform a second enrichment on ONR7a medium, adding crude oil as the sole carbon source. A second collection of twenty-five bacteria was obtained from the ONR7a enrichment, leading to the selection of three different species, thus showing a lower diversity compared to the ASW collection (Shannon- Weaver index: 0.44). All the bacteria isolated on ONR7a medium belonged to the known hydrocarbon-degrading species *Pseudomonas stutzeri* (2), *Marinobacter adhaerens* (1), and *Marinobacter hydrocarbonoclasticus* (22) (Table 6). It is worthy to note how much our perception of the cultivable fraction of oil-degrading bacteria in a certain environment can vary simply changing the medium applied for cultivation purposes. In fact, ONR7a medium selected exclusively Gammaproteobacteria from El-Max district polluted sediments, mainly represented by the well-known metabolic versatile *Marinobacter hydrocarbonoclasticus*. The cultivation approach based on the use of two different media provided a wider perspective on the cultivable fraction of the bacterial community present in the investigated sediments. Such strategy permitted to identify both i) specialist and versatile bacterial species involved in the first stages of the degradation of aliphatic and aromatic hydrocarbons (*i.e.* Gammaproteobacteria) and ii) bacterial species previously indicated as key players in the successional stages of the degradation process (*i.e. Bacillus*).

Table 12 Acc. No. Accession number of the 16S rRNA sequences amplified from the isolated strains and deposited in GenBank.
List of the bacterial strains isolated from the polluted sediments of El-Max district (Egypt) and their phylogenetic affiliation. The code of the strains isolated from station R are indicated in italic since their identification was previously reported by the same authors [75]

Strain code	Medium	Acc. No.	Class	Family	Closest described relative	Identity (%)
SCP2	ASW	KC573500	Bacilli	*Bacillaceae*	*Bacillus sporothermodurans*	96
SCuQ1	ASW	KC573503			*Bacillus megaterium*	99
SCR2[a]	ASW	KC573523			*Bacillus megaterium*	98
SCuR2[a]	ASW	KC573507			*Bacillus cereus*	98
SCR3[a]	ASW	KC573505			*Bacillus cereus*	99
*SC*R3*[a]	ASW	KF217252			*Bacillus cereus*	99
SCuR5[a]	ASW	KF217249			*Bacillus* sp.	99
SC*S1	ASW	KF217253			*Bacillus pumilus*	99
SCS2	ASW	KC573509			*Bacillus cereus*	99
SCS3	ASW	KC573510			*Bacillus subtilis*	99
SCS4	ASW	KF217259			*Bacillus* sp.	99
SC*S6	ASW	KF217254			*Bacillus aerophilus*	99
SCP1	ASW	KC573499		*Staphylococcaceae*	*Staphylococcus haemolyticus*	99
SC*CuP1	ASW	KC573501	Gammaproteobacteria	*Pseudomonadaceae*	*Pseudomonas xanthomarina*	98
SCuQ2	ASW	KC573504		*Pseudomonadaceae*	*Pseudomonas stutzeri*	99
SCuR3[a]	ASW	KC573508		*Pseudomonadaceae*	*Pseudomonas knackmussii*	98
SCuR4[a]	ASW	KC573524		*Pseudomonadaceae*	*Pseudomonas stutzeri*	99
SCS1	ASW	KC573525		*Pseudomonadaceae*	*Pseudomonas stutzeri*	100
SC*Q2	ASW	KC573520		*Moraxellaceae*	*Acinetobacter venetianus*	99
SCR1[a]	ASW	KC573522		*Alteromonadaceae*	*Marinobacter hydrocarbonoclasticus*	99
SC*Q3	ASW	KC573502			*Marinobacter hydrocarbonoclasticus*	98
SCS6	ASW	KC573526			*Marinobacter hydrocarbonoclasticus*	99

Strain code	Medium	Acc. No.	Class	Family	Closest described relative	Identity (%)
*SC*R2*[a]	ASW	KF217251		*Enterobacteriaceae*	*Providencia vermicola*	99
SCuR1[a]	ASW	KC573506		*Sphingomonadaceae*	*Sphingomonas* sp	95
SCP3	ASW	KF217258	Betaproteobacteria	*Alcaligenaceae*	*Alcaligenes faecalis*	99
S1_1	ONR7a	LN610460	Gammaproteobacteria	*Pseudomonadaceae*	*Pseudomonas stutzeri*	99
S1_24	ONR7a	LN610475			*Pseudomonas stutzeri*	99
S1_4	ONR7a	LN610461		*Alteromonadaceae*	*Marinobacter hydrocarbonoclastic us*	99
S1_5	ONR7a	LN610462			*Marinobacter hydrocarbonoclastic us*	100
S1_7	ONR7a	LN610463			*Marinobacter hydrocarbonoclastic us*	99
S1_9	ONR7a	LN610464			*Marinobacter hydrocarbonoclastic us*	99
S1_10	ONR7a	LN610465			*Marinobacter hydrocarbonoclastic us*	99
S1_11	ONR7a	LN610466			*Marinobacter hydrocarbonoclastic us*	99
S1_12	ONR7a	LN610467			*Marinobacter hydrocarbonoclastic us*	99
S1_13	ONR7a	LN610468			*Marinobacter hydrocarbonoclastic us*	100
S1_16	ONR7a	LN610469			*Marinobacter hydrocarbonoclastic us*	99
S1_17	ONR7a	LN610470			*Marinobacter hydrocarbonoclastic us*	100
S1_20	ONR7a	LN61047			*Marinobacter hydrocarbonoclastic us*	100
S1_21	ONR7a	LN610472			*Marinobacter hydrocarbonoclastic us*	100
S1_22	ONR7a	LN610473			*Marinobacter hydrocarbonoclastic us*	99
S1_23	ONR7a	LN610474			*Marinobacter hydrocarbonoclastic*	99

Strain code	Medium	Acc. No.	Class	Family	Closest described relative	Identity (%)
					us	
S1_26	ONR 7a	LN610476			*Marinobacter hydrocarbonoclastic us*	99
S1_28	ONR 7a	LN610477			*Marinobacter hydrocarbonoclastic us*	99
S1_29	ONR 7a	LN610478			*Marinobacter hydrocarbonoclastic us*	99
S1_30	ONR 7a	LN610479			*Marinobacter hydrocarbonoclastic us*	99
S1_31	ONR 7a	LN610480			*Marinobacter hydrocarbonoclastic us*	99
S1_32	ONR 7a	LN610481			*Marinobacter adhaerens*	100
S1_33	ONR 7a	LN610482			*Marinobacter hydrocarbonoclastic us*	99
S1_34	ONR 7a	LN610483			*Marinobacter hydrocarbonoclastic us*	99
S1_36	ONR 7a	LN610484			*Marinobacter hydrocarbonoclastic us*	99

3.4 - Hydrocarbonoclastic bacteria of El Max district possessed bioremediation potential traits

We screened the bacterial collection for the ability to grow on single hydrocarbon molecules as sole carbon source, showing that a variable percentage of the isolates was able to grow on the different tested HC (Table 7). A lower percentage of the strains was able to grow using pyrene (7%) and phenanthrene (8%) and naphthalene (9.5%). A higher portion of the collection could grow using dibenzothiophene (DBT, 11%), octane (15.5%) and xylene (36%). Both the collections obtained on ASW and ONR7a media included similar amounts of strains able to use all the tested hydrocarbons, with the exception of DBT and octane degrading bacteria that were mainly isolated on ASW and ONR7a medium respectively. The ability to grow using all the supplied HC molecules in minimal medium was recorded exclusively in few strains belonging to the species *Marinobacter hydrocarbonoclasticus* (Table 7), confirming the adaptable metabolisms of this species often isolated in marine contaminated environments. The low number of bacteria capable to utilize all the tested substrates corroborates the reports of several studies that indicate the need of microbial consortia to degrade complex mixtures of hydrocarbons, such as crude oil, in soil [57], fresh water [58] and marine environments [8].

To widen the characterization of the HC-degrading bacteria isolated from El-Max district we performed PCR assays looking for functional genes codifying for the 3,4-phenanthrene dioxygenase enzyme (*phnA*) and genes related to metal and metalloid detoxification systems, like the efflux pumps for arsenite (*ACR3(2)*) and for different heavy metals (*nccA*). Despite the sediments were not highly contaminated by metals and arsenic, isolates possessing arsenic and metals resistance genes were retrieved, confirming that bacteria capable of detoxification mechanism are widespread and their presence is not strictly related to metal and arsenic level [23, 59]. *ACR3(2)*, *nccA* and *phnA* genes were successfully amplified in 14, 10 and 4 bacterial isolated on ONR7a medium, respectively. On the contrary, the amplification of these genes was unsuccessful for all the bacteria isolated on ASW medium, with the exception of the strain *Alcaligenes faecalis* SCP3, which resulted positive for *nccA* gene amplification and belongs to a specie previously described as Cd and Pb detoxifying [54]. Overall, apart from strain *A. faecalis* SCP3, all the bacteria positive for *ACR3(2)*, *nccA* and *phnA* genes amplification belong to the specie *M. hydrocarbonoclasticus*. Only one out of the 4 strains harboring the *phnA* gene was able to grow on phenanthrene as the sole carbon source in the tested conditions. On the other hand, those that could grow on this HC failed to give positive amplification, probably due to mismatches between the tested primers and gene sequence [60].

The biotechnological potential of the strains inhabiting oil-polluted ecosystems does not rely exclusively on their ability to degrade a certain HC mixture but include additional features. Different microorganisms were shown to possess multiple adaptations to facilitate oil degradation procedures, such as the synthesis of biosurfactants or emulsifiers and biofilm formation [61, 62], processes that enhance the bacterial adhesion to hydrocarbons, increasing their solubility and thus promoting their degradation [63, 64]. Biosurfactants, in particular, reduce the surface tension at the interface of immiscible fluids, increasing the surface area of insoluble compounds like oil and water, which leads to increased bioavailability and subsequent biodegradation of the hydrocarbons [65, 66, 8]. In this study, we applied several methods to assess the ability of the isolated bacteria to produce biosurfactant molecules. One of the simplest methods used for screening the production of some types of biosurfactants is the blood haemolysis method [67]. In our study, 20 isolates (40 % of the collection) showed haemolytic activity on blood agar media (Table 7). They belong to the *Marinobacter* (11), *Bacillus* (5), *Pseudomonas* (3) and *Acinetobacter* (1) genera. The reduction of the surface tension (ST) of the medium, resulting from the emulsification of crude oil by the surfactants produced by microorganism, represents an alternative method for testing the biosurfactant production [68]. All the isolates in our collections were able to reduce surface tension (Table 7), in particular those isolated on the ONR7a medium (27.4 ± 10.2), namely *M. hydrocarbonoclasticus*, demonstrated higher ST reduction compared to those belonging to the ASW collection (47.25 ± 10.36). Furthermore, bacterial adhesion to hydrocarbons (BATH) test was applied to measure the cell surface hydrophobicity, a property related to the structure and composition of cell surface [69]. The uptake mechanism of hydrophobic substrate occurs by the direct contact between the hydrocarbon and cell surface and

can be thus dependent from its hydrophobicity [69, 70, 71]. The highest hydrophobicity (77.3 %) was recorded for the strain *M. hydrocarbonoclasticus* SCS6 (Table 7).
Hydrocarbonoclastic bacteria have been detected in both monospecies and multispecies biofilms developing on hydrocarbons [72]. Hence, the ability to produce biofilm was also investigated (Table 7) allowing the identification of 5 strains, representing 10% of the collection, as biofilm producers. Such strains belong to the *M. hydrocarbonoclasticus, A. faecalis, B. cereus* and *P. vermicola* species. According to the literature [73, 74] two strains were classified as weakly adherent, while two resulted strongly adherent (Table 7), the latter including the *M. hydrocarbonoclasticus* strain S1-21. A proteomic study realized on *M. hydrocarbonoclasticus* previously showed a differential protein expression for biofilm attached and detached cells, displaying the ability of recently detached cells to reinitiate the formation of a new biofilm at the hexadecane-water interface [72], a trait that might confer competitive advantage for hydrocarbon uptakes in the environment. The two *M. hydrocarbonoclasticus* isolates (S1-4 and S1-21) positive for biofilm formation were also able to grow using the entire set of hydrocarbons tested in this study, further demonstrating the high potential of this species for marine oil remediation.

Table 13. Screening of the bioremediation potential in the bacteria collection established from the sediment of El-Max district. +: growth; -: no growth
Xyl: Xylene; Oct: Octane; Pyr: Pyrene; DBT: dibenzothiophene; Phen: Phenanthrene; Naph: Naphthalene; oil: Crude oil
ST: surface tension; Hydro: hydrophobicity ratio (%)
(a) Ni, Co, Cd efflux pump, amplified with primer nccA (from Kamika and Momba 2013); (b) arsenite efflux pump (from Achour et al., 2007); (c) 3,4-phenanthrene dioxygenase large subunit amplified with primer Phn321F/P671R (from Cavalca et al. 2007).

Isolate	**biofilm formation**	**Blood hemolysis**	**ST (mN m^{-1})**	**Hydrophobicity (%)**	**Growth on different hydrocarbons**							**PCR amplification of gene markers**		
					Xyl	**Oct**	**Pyr**	**DBT**	**Phen**	**Naph**	**Oil**	***nccA*$^{(a)}$**	***ACR3(2)*$^{(b)}$**	***phnA*$^{(c)}$**
S1-1	0.0225	-	40	38	+	+	+	-	+	+	+	-	+	-
S1-4	0.16375	-	46	0	+	+	+	+	+	+	+	-	+	-
S1-5	0.024	-	10	5.9	-	+	-	-	-	-	+	-	-	+
S1-7	0.014	+	20	5.1	-	+	-	+	-	-	+	-	+	-
S1-9	0.0125	+	32	20.3	+	+	-	-	-	-	+	+	+	-
S1-10	0.01125	-	29	46.2	-	-	-	-	-	-	+	+	+	-
S1-11	0.0411429	+	19	10.4	-	+	-	-	-	-	+	+	+	-
S1-12	0.014	-	16	40.8	-	+	-	+	-	-	+	-	+	+
S1-13	0.024	+	13	0	-	+	-	-	-	+	+	-	+	-
S1-16	0.035	-	29	21.98	-	+	-	-	-	-	+	+/-	-	+
S1-17	0.01	+	29	9.3	-	+	-	+	-	-	+	+/-	+	-
S1-20	0.01	-	25	0	+	+	+	+	+	+	+	-	-	-
S1-21	0.41875	-	28	10.4	+	+	+	+	+	+	+	-	-	-
S1-22	0.0297143	-	20	0	+	+	-	-	-	-	+	-	-	-
S1-23	0.009	+	27	7.5	+	+	-	-	-	-	+	-	-	-
S1-24	0.0125714	-	24	0	+	+	+	+	+	+	+	+/-	-	-
S1-26	0.0525714	+	29	13.3	+	+	+	-	-	+	+	-	+	-
S1-28	0.01	-	40	16.3	-	+	-	-	-	-	+	-	+	-

Isolate	biofilm formation	Blood hemolysis	ST (mN m^{-1})	Hydrophobicity (%)	Growth on different hydrocarbons							PCR amplification of gene markers		
					Xyl	Oct	Pyr	DBT	Phen	Naph	Oil	*nccA*[(a)]	*ACR3(2)*[(b)]	*phnA*[(c)]
S1-29	0.004	+	21	14.2	-	+	-	-	+	+	+	-	-	+
S1-30	0.01	+	47	21.3	+	+	-	-	-	-	+	-	+	-
S1-31	0.0125	-	25	13.8	-	+	-	-	-	-	+	-	-	-
S1-32	0.0182857	-	20	15.8	+	+	-	-	-	-	+	+	-	-
S1-33	0.004	-	40	34.7	-	-	-	-	-	-	+	+/-	+	-
S1-34	0.01	-	41	22.2	-	+	-	-	-	-	+	-	+	-
S1-36	0.024	+	15	0	+	+	-	-	-	-	+	+	-	-
SCP1	0.011	-	47.16	0	-	-	-	-	-	-	-	-	-	-
SCP2	0.028	+	51.25	11.3	-	-	+	+	+	+	+	-	-	-
SCP3	0.417	-	48.30	31.6	+	+	-	+	+	+	-	+	-	-
SCR1[a]	0.046	+	53.50	77.3	n.d.	-	-	-	-	-	+	-	-	-
SCR2[a]	0.040	+	46.67	26.8	-	-	-	-	+	-	-	-	-	-
SCR3[a]	0.030	-	57.64	23.5	-	-	+	-	+	+	-	-	-	-
SCS1	0.037	-	52.52	10.2	-	-	-	-	-	+	-	-	-	-
SCS2	0.037	-	22.90	23.6	+	-	+	+	+	+	-	-	-	-
SCS3	0.065	+	41.56	0	+	+	-	+	-	-	++	-	-	-
SCS4	0.075	+	46.20	2.7	-	-	+	+	-	-	-	-	-	-
SCS6		-	44.35	n.d.	-	+	+	+	n.d.	n.d.	+	-	-	-
SCuP1	0.022	+	50.95	46.4	-	-	-	+	+	+	+	-	-	-
SCuQ1	0.081	-	52.30	0.96	-	-	-		+	-	+	-	-	-

Isolate	biofilm formation	Blood hemolysis	ST (mN m^{-1})	Hydrophobicity (%)	Growth on different hydrocarbons							PCR amplification of gene markers		
					Xyl	Oct	Pyr	DBT	Phen	Naph	Oil	*nccA*[(a)]	*ACR3(2)*[(b)]	*phnA*[(c)]
SCuQ2	0.007	-	59.82	28.8	-	-	-	-	-	-	-	-	-	-
SCuR1[a]	0.049	-	59.70	7.1	+	+	-	+	+	+	-	-	-	-
SCuR2[a]	0.003	-	50.09	6.7	-	-	-	-	-	-	-	-	-	-
SCuR3[a]		+	59.40	8.7	-	+	+	+	+	+	-	-	-	-
SCuR4[a]	0.051	+	35.87	29.1	+	+	+	+	-	-	+	-	-	-
SCuR5[a]	0.022		38.30	22.7	-	+	+	+	-	+	++	-	-	-
SC*Q2	0.032	+	47.84	9	-	-	-	+	-	+	-	-	-	-
SC*Q3	0.026	-	48.71	3	n.d.	-	-	+	-	-	-	-	-	-
SC*R2[a]	0.177	-	55.60	6.6	+	+	-	+	+	+	-	-	-	-
SC*R3[a]	0.366	+	42.6	n.d.	n.d.	n.d.	-	+	-	-	n.d.	-	-	-
SC*S1	0.067	-	16.88	36.6	-	-	-	-	+	+	++	-	-	-
SC*S6	0.012	-	51.25	18.3	-	-	-	-	-	-	-	-	-	-

4. CONCLUSIONS

This study represent the first holistic microbiological investigation of biodiversity occurring at El-Max district sediments taking advantage of both molecular and cultivation techniques. The adopted molecular approach, coupled with statistical analyses, clarified that a significant correlation exists between biotic and abiotic data in the polluted ecosystems, allowing to identify i) sand and clay composition, ii) TOC and PCBs and iii) the concentration of different heavy metals (Cu, Fe, Zn) as the driving forces shaping the structure of the bacterial microbiome. The establishment of a bacterial collection exploiting different growth media permitted to isolate species described for their pivotal role in the different successional stages of oil hydrocarbons' biodegradation, such as the highly abundant classes Gammaproteobacteria and Bacilli. Most of the isolates, belonging to different genera, showed one or more metabolic traits of interest for bioremediation purposes (e.g the capability to grow on single hydrocarbon molecules, presence of genes involved in detoxification systems and traits related to the production of biosurfactants). Our investigation contributed to fill the gap of knowledge on the microbial diversity of Southern Mediterranean sea sites, shedding a light on the potential of the contaminated sediments of El-Max district as a reservoir of microbial resources selected (and adapted) by the peculiar environmental conditions of the site and possibly exploitable for future *in-situ* intervention to combat pollution.

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Chapter

VII

SEDIMENT DWELLING MICROBIOME RICHNESS AND THE POTENTIAL FOR HYDROCARBON REMEDIATION ARE DRIVEN BY POLLUTANTS' TYPE AND CONCENTRATION: THE ANCONA HARBOR CASE-STUDY

Abstract

The Mediterranean Sea is subjected to high amounts of hydrocarbon pollutants because of the high oil related traffic. Environments exposed to hydrocarbons are often co-contaminated by both hydrocarbons and heavy metals, therefore are particularly difficult to bioremediate. Autoctonous bioaugmentation (ABA) – based on the inocula of autochthonous microbial population, previously enriched under laboratory conditions and later re-inoculated in the polluted environment to be treated – could be useful to treat this kind of problematic environments. Nonetheless, for ABA to be successful it is pivotal to gain a good knowledge of the indigenous microbial populations, of the occurring environmental parameters and their influence on survival and degradation rate of the microorganisms involved in the remediation processes. Therefore, the aim of this work was to depict which environmental parameters are the main driving factors influencing the bacterial communities inhabiting the Ancona harbor's sediments, establish enrichment cultures with different hydrocarbon pollutants (diesel oil, crude oil and naphthalene) aiming to evaluate their direct effects on the bacterial communities composition and finally, to establish a collection of hydrocarbon degrading bacteria isolates potentially exploitable in ABA. This study confirmed the high state of pollution of the Ancona harbor and individuated in the concentration of hydrocarbons and heavy metals the main factors driving the shape of the bacterial communities inhabiting its sediments. The correlation between the enrichments, under laboratory conditions, of different and specialized bacterial populations in the presence of diverse pollutants was also confirmed with molecular ecology analyses, coupled with culture dependant methods. Overall, this work improved the knowledge of the environmental drivers that influence the enrichment of different microbial communities involved in the degradation of the pollutants, an important step towards the implementation of bioremediation strategies, like ABA.

1. INTRODUCTION

The whole Mediterranean Sea is constantly exposed to a high amount of hydrocarbons pollutants deriving mainly from the transport of crude oil across its waters and from inputs of waste oil products close to harbors, refineries, chemical industries and pipelines. The situation is worsened by the physical nature of this semi-enclosed basin, characterized by a low water exchange that prevents the dilution and dispersion of the pollutants that enter its waters (Daffonchio et al., 2012). Sediments of coastal areas nearby oil related facilities accumulate high amount of the heavier fraction of petroleum pollutants. These sites are usually co-contaminated by hydrocarbons and heavy metals, the common components of crude oil, in which they are found associated to pyrrolic structures known as porphyrins (Abha & Singh, 2012). A previous report showed that hydrocarbons polluted sites co-contaminated by heavy metals are more difficult to bioremediate, since many microorganisms involved in the degradation of hydrocarbon compounds are sensitive to the presence of the heavy metals, which prevent them to perform the degradative metabolism toward oil pollutants or reducing their efficiency (Abha & Singh, 2012). The Ancona harbor (Italy) is a worthy example of a site co-contaminated by both hydrocarbons and heavy metals. It is located in the Northern Adriatic Sea and it is one of the biggest Mediterranean commercial harbor, with an intense ferryboat and merchant flux and surrounded by shipyards and important industrial plants. High concentration of chemicals like aliphatic hydrocarbons, polycyclic aromatic hydrocarbons and naphthalene

have been measured in its sediments, along with high concentration of many heavy metals like Cu, Mn, Ni and Zn (Mirto & Danovaro, 2004; Dell'Anno et al., 2009).
Since co-contamination hampers the bioremediation of polluted areas, it is imperative to find microbial resources able to degrade hydrocarbon contaminants and already adapted to survive under these harsh environmental conditions. This issue is especially important when bioaugmentation strategies – addition of degrading bacteria inocula to a polluted environment – are applied, since often allochthonous organisms cannot adapt to new environmental conditions. Recently, a new approach for bioaugmentation has been proposed and defined as autochthonous bioaugmentation (ABA) (Hosokawa et al., 2009). ABA consists in the employment of autochthonous microbial population, previously enriched under laboratory conditions and later re-inoculated in the polluted environment to be treated. To set-up successful ABA strategy, it is pivotal to gain a good knowledge of the indigenous microbial populations, of the occurring environmental parameters and their influence on survival and degradation rate of the microorganisms involved in the remediation processes.
In this perspective, the present work aimed to i) depict which environmental parameters are the main driving factors influencing the bacterial communities inhabiting the Ancona harbor's sediments and to ii) establish enrichment cultures with different hydrocarbon pollutants (diesel oil, crude oil and naphthalene) aiming to evaluate their direct effects on the bacterial communities composition. Finally, a collection of hydrocarbon degrading bacteria isolates potentially exploitable in ABA was established.

2. MATERIALS and METHODS

2.1 - Sites description and sampling

Sediment samples were collected in impacted and control stations, located respectively inside and outside the harbor area. In particular, samples were collected from 10 stations located in the harbor area (codes: An-U1 - An-U10) and from 5 addition stations (codes: An-C1 - An-C5) located up to 3.5 Km far from the harbor, along the dominant NW/SE current. Sampling depths ranged from 2.5 m in the port area up to 13 m in the control stations. Sampling station location is reported in Table. 1.

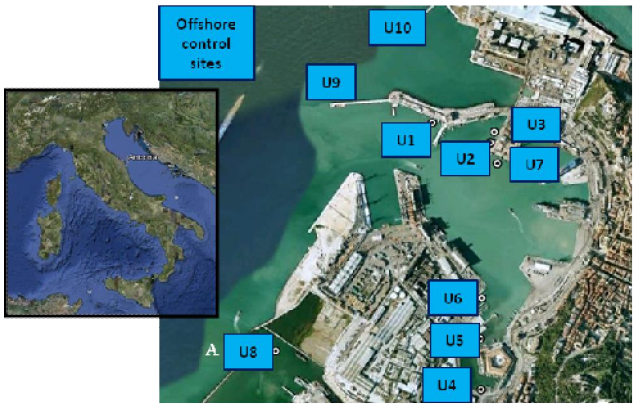


Fig. 1 **Sampling sites inside the Ancona harbor**

Station name	Latitude	Longitude	Water depth (m)
An-U1	**43°37'28,98'' N**	**13°29'59,85''E**	**4,5**
An-U2	**43°37'25,91'' N**	**13°30'12,21''E**	**4,4**
An-U3	**43°37'27,55'' N**	**13°30'13,07''E**	**3,8**
An-U4	**43°36'47,25'' N**	**13°30'10,17''E**	**2,9**
An-U5	**43°36'55,16'' N**	**13°30'9,98''E**	**3,6**
An-U6	**43°37'1,63'' N**	**13°30'10,37''E**	**5,5**
An-U7	**43°37'22,63'' N**	**13°30'13,64''E**	**6,8**
An-U8	**43°36'53,25'' N**	**13°29'36,60''E**	**4,9**
An-U9	**43°37'36,05'' N**	**13°29'37,41''E**	**6,5**
An-U10	**43°37'52,16'' N**	**13°29'51,71''E**	**7,1**

An-C1	**43°39'14,84'' N**	**13°29'9,79''E**	**12,5**
An-C2	**43°39'9,52'' N**	**13°28'5,26''E**	**11,8**
An-C3	**43°38'49,39'' N**	**13°30'16,13''E**	**9,4**
An-C4	**43°38'3,74'' N**	**13°32'37,44''E**	**10,6**
An-C5	**43°38'5,46'' N**	**13°34'30,15''E**	**10,2**

Tab.1**: Location of sampling site within the Ancona harbor.**

For each sample, pH and redox potential were measured. Samples for granulometric analyses (determination of percentages of gravel, sand and clay in the sediment), humidity and specific weight were transferred into plastic containers and stored at +4°C. Samples dedicated to analyses of trace metals (Hg, Cd, Pb, As, Cr, Cu, Ni, Zn, V), hydrocarbons C<12 and total polycyclic aromatic hydrocarbons were transferred into inert HDPE containers and stored at -18°C until laboratory analyses. Samples for the determination of total organic carbon (TOC) and molecular ecology analyses were transferred, respectively, into Petri dishes and sterile plastic containers, and stored at -20°C. For the determination of total bacteria, sediment slurries were prepared using sterile buffered formaldehyde (4% final concentration) while for bacterial isolation sediment sub-samples were collected using a sterile spoon and stored at +4°C.

2.3 ï Phisico-Chemical characterization of sediment samples

The total organic content (TOC) was determined by the loss on combustion technique after removal of carbonate with dilute (IN) HCl, a portion of sediments was weighed into a porcelain crucible and ignited in a muffle furnace at 550°C for two hours. The crucible was cooled in a desiccator, re-weighed and the total organic content (TOC) was calculated as the weight loss in percentage (Gaudette et al.,1974). The presence and abundance of different hydrocarbons (>C12 and PAHs) was estimated by chromatographic techniques. The HCs concentration was analyzed by Agilent 7890 -USA. A HP-5 capillary chromatographic column (30 m x 0.32 mm I.D.) and a capillary column (30 m x 0.25 mm I.D.) were used for GC-FID and GC-MS analyses, respectively. Nitrogen was the carrier gas with 3 ml/min. Injector and detector temperature was maintained at 300 °C and 320 °C, respectively. The identification of n-paraffin peaks was established using a reference mixture of n-paraffin of known composition.

To determine the total content of heavy metals (copper -Cu, zinc -Zn, chromium -Cr, nickel -Ni, cadmium -Cd, lead -Pb, mercury -Hg) vanadium -V and arsenic -As in sediments, samples (0.1 g) were HNO_3/$HClO_4$ (4:1, v/v) digested in a microwave oven (CEM, MARS5). After digestion, the volume of each sample was adjusted to 20 mL using deionized water. Heavy metals and As content was determined by Inductively Coupled Plasma-Mass Spectrometry (ICP-MS, Agilent Technologies, Santa Clara, CA, USA). Standards of heavy metals and of arsenic for concentrations ranging from 0 to 1 mg/L were prepared from multi-element calibration standard-2A solution (Agilent Technologies) and from sodium arsenite solution ($NaAsO_2$) (Sigma-Aldrich, St Louis, MO, USA) respectively. For all the measures by ICP-MS an aliquot of a 2 mg/L of an internal standard solution (45Sc, 89Y, 159Tb, Agilent Technologies) was added both to samples and a calibration curve to give a final concentration of 20 µg/L. The instrument was tuned daily with a multi-element tuning solution for optimized signal-to-noise ratio.

2.4 - Abundance of total prokaryotes and hydrocarbonoclastic bacteria

Total prokaryotic abundance was determined in samples fixed with sterile formaldehyde (4% final concentration) in the dark. Thereafter, the samples were filtered on 0.22 µm pore size black polycarbonate filters (Millipore, USA) and and kept at -20ºC until analysis. Prokaryotic abundance was evaluated by 4',6-diamidino-2-phenylindole (DAPI) staining (excitation 340/360 nm, emission 440/470 nm). Prokaryotic cells positively stained by DAPI were counted by epifluorescence microscope. For each sample, 30 microscope field and more than 3000 DAPI-stained cells were counted.

The number of hydrocarbon degrading bacteria was evaluated through the Most Probable Number (MPN) method. Serial dilutions of the sediments were performed (from 10^{-2} to 10^{-8}) and inoculated in triplicates (100 µL of dilution in 1 mL of growth medium) in ONR7a mineral medium added with crude oil (1% v/v) and cycloheximide (0.01% w/v) to inhibit eukaryotic growth. Bacterial growth was evaluated after 28 days.

2.5 - Metagenome extraction

Total DNA was extracted from 0.5 g of sediment using the "Power Soil" kit (MoBio Laboratories Inc., Carlsbad, CA, USA) following the manufacturer's instructions. DNA was quantified using a NanoDrop 1000 spectrophotometer (Thermo Scientific, Waltham, MA, USA).

2.6 - ARISA fingerprinting

ARISA-PCR was conducted on a standard amount of DNA on each sample by using the primer set ITSF, 5′-GTC GTA ACA AGG TAG GCC GTA-3′ and ITSReub, 5′-GCC AAG GCA TCC ACC 3′, as previously described (Cardinale et al., 2004). ARISA fragments were separated by using the ABI3730XL genetic analyzer applying the internal standard 1200-LIZ (Macrogen, Korea). The output peak matrix was transferred to Microsoft Excel for the following analysis. Peaks showing height value < 50 were removed from the output peak matrix before statistical analyses. Each polymorphic ARISA peak is defined as a different OTU. Richness is defined as the number of OTUs present in each sample. Shannon diversity and Evenness indices were calculated using PAST.

2.7 16S rRNA Pyrosequencing

Pyrotag assays were carried out using bacterial universal primers (27 F mod 5' – AGR GTT TGA TCM TGG CTC AG – 3'; 519 R mod bio 5' – GTN TTA CNG CGG CKG CTG – 3') targeting the variable regions of 16S rRNA V1-V3 and amplifying a fragment of approximately 400 bp. The amplified 16S rRNA regions contain enough nucleotide variability to be useful in identification of bacterial species (Van de Peer et al. 1996; Chakravorty et al., 2007). PCR reactions and next generation 454 pyrosequencing were performed as described in a previous work (Montagna et al., 2014) by the company MR DNA (Shallowater, TX – U.S.).

A total of 66365 raw, barcoded amplicons of the V1–V3 region of the 16S rRNA gene, were obtained. The reads were trimmed to remove pyrosequencing adaptors, low quality base calls (<30 Phred score) and size-selected (between 350 and 500 bp) using the QIIME (Caporaso et al, 2010) pipeline filtering scripts. High quality sequence reads that were not flagged as chimeras after screening with Chimeras layer were clustered into operational taxonomic units (OTUs), based on a sequence identity threshold of 97%, using Uclust (Edgar, 2010); drawing one sequence for each OTU, as representative, and then aligned to Greengenes (http://greengenes.lbl.gov/) using PyNast (Caporaso et al, 2010). Sequences representative of each OTU were taxonomically classified by BLASTn-based comparisons to the Greengenes and Silva databases within QIIME.

2.8 - Microcosm Enrichments

Different enrichment cultures were established using different sediments as inoculum and different hydrocarbon molecules as the sole carbon sources. 1 g of sediment collected either from sampling site An-U5 or An-U7 was inoculated in minimal mineral media ONR7a supplemented by 1% v/v crude oil (CO) or diesel oil (DB) or 1% w/v naphtalene (N). Cycloheximide (0.01% w/v) was added to the medium to avoid eukaryotic growth. The microcosms were incubated at 30°C with constant shaking until the turbidity appeared. In the case of diesel microcosm, 1 mL of the surface liquid culture was collected and re-inoculated in fresh ONR7a medium aiming to enrich those bacteria adhering on the diesel droplets floating on the medium surface: this newly established microcosm was definied as "diesel surface" (DS). Total DNA was extracted from 10 mL of each enrichments after filtering DNA extraction. The DNA extraction was performed from filters according to Murray et al. (1998).

2.9 - Bacteria isolation and identification

Bacterial isolates have been obtained in pure cultures exclusively from crude oil and diesel oil enrichments. Ten-fold dilutions were prepared to isolate the strains on ONR7a solid medium. After purification, the isolates were streaked with and without hydrocarbon compounds and those able to grow exclusively in presence of the carbon source were selected for further characterization.

The bacteria collection was dereplicated through Internal Transcribed Spacer (ITS) PCR. ITS PCR was performed using the primers ITS-F (3'-GTCGTAACAAGGTAGCCGTA-5) and ITS-R (3'-

CTACGGCTAC CTTGTTACGA-5') according to Daffonchio et al., 1998. The PCR was performed with the following conditions: the 25µL PCR mixture contained Buffer 1x, MgCl2 1.50mM, dNTP mix 0.20mM, primers ITS-F and ITS-R 0.3µM, Taq 1U and DNA template 1 µL. The DNA was amplified with the following thermal conditions: initial denaturing at 94°C, 4 min; 35 cycles of 45 sec denaturation at 94°C, 1 min annealing at 55°C and 2 min of elongation time at 72°C; a final extension step for 10 min at 72°C.
The amplification of the bacterial 16S rRNA gene was performed using the universal primer 27F (3'-AG AGTTTGATCMTGGCTCAG-5') and 1492R (3'-CTACGGCTACCTTGTTACGA-5').
The PCR amplification conditions and thermal protocol were set up as previously described (Mapelli et al., 2013) providing a PCR amplicon of approximately 1400 bp that were partially sequenced by Macrogen Inc., Korea.

2.10 - Denaturing Gradient Gel Electrophoresis

2.10.1 - 16S rRNA amplification

Bacterial 16S rRNA gene fragments (~550 bp) were amplified with Polymerase Chain Reaction (PCR) using primers 907R (3'-CCGTCAATTCCTTTGAGTTT- 5') and GC-357F (3'-CCTACGGGAGGCAGCAG- 5' with a 5'-end GC-clamp) targeting a portion of the 16S rRNA gene that includes the hypervariable V3-V5 regions (Muyzer et al., 1993). PCR reactions were performed as previously described (Mapelli et al., 2013).

2.10.2 - alkB amplification

PCR amplification was performed on the metagenome extracted from Ancona harbor enrichments (AN-U5-Crude Oil, ANU-5-Diesel Oil Bottom, AN-U5-Diesel Oil Surface, AN-U7-Crude Oil, AN-U7-Diesel Oil Bottom, AN-U7-Diesel Oil Surface) and on the metagenome extracted from An-U5 and An-U7 sediments. The PCR reaction was performed in a total volume of 50 ***ε***l containing: 1 × PCR reaction buffer, 2 mM MgCl2, 0,12 mM of each dNTP, 1 ***ε***M of each primer, 1,5U of Taq DNA polymerase, 5% DMSO and 2 ***ε***l of DNA template. The thermal PCR protocol consisted of: an initial denaturation of 3 min at 94°C; 30 cycle of denaturation of 45 seconds at 94°C, 1 min of annealing at 55°C, and elongation of 1 min at 72°C; final extension step of 10 min at 72°C (Li et al., 2013).

2.10.3 - DGGE

PCR products (~100 ng) were loaded in a 0.7 mm polyacrylamide gel (7% [w/v] acrylamide-bisacrylamide, 37.5:1) containing 38 to 63% and 40% to 70% urea-formamide denaturing gradient (100% corresponds to 7M urea and 40% [v/v] formamide), respectively for 16S rRNA and alkB genes. The gels were run for 17 h at 60 °C by applying a constant voltage of 90 V in 1X Tris-acetate-EDTA (TAE) buffer. After electrophoresis, the gels were stained for 15 min in 1X TAE buffer containing 1X SYBR Green (Molecular Probes, Leiden, the Netherlands) according to manufacturer's instructions and rinsed three times for 10 min with distilled water. Gels images were captured using a Gel Doc 2000 apparatus (Bio-Rad, Milan, Italy). The band patterns of the DGGE gel were analysed using Image J software (available for free download at http://rsb.info.nih.gov/ij/) and Microsoft Excel XLSTAT software (Addinsoft Inc.,New York, NY, USA) to perform a cluster analysis as previously described (Marasco et al., 2012). DGGE bands were excided from the gels with a sterile scalpel and eluted in 50 ?l of sterile Milli-Q water at 37 °C for 3 h. The eluted DNA was amplified by PCR using primers 357F and 907R and positive amplifications were partially sequenced by Macrogen Inc., Korea.

2.12 - Nucleotide Sequence Analyses

Nucleotide sequences were edited in Chromas Lite 2.01 (http://www.technelysium.com.au) and subjected to BLAST search (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

2.13 Statistical analyses

Significant differences in i) environmental chemical-physical parameter and ii) the bacterial community richness between control and polluted sediments were investigated by permutational analysis of variance (PERMANOVA, Anderson, 2001).

Non-metric multidimensional scaling (nMDS) was carried out as visual information to explore similarities between bacteria communities richness evaluated through ARISA fingerprinting. The same set of biotic data was used, together with the environmental parameters recorded at each site, in the distance-based multivariate analysis for a linear model (DistLM, Anderson, 2002) to determine which significant environmental variables explain the observed similarity among the samples. The Akaike Information Criterion (AIC) was used to select the predictor variables. The contribution of each environmental variable was assessed using 'marginal tests' to assess the statistical significance and percentage contribution of each variable. A distance-based redundancy analysis (dbRDA) was used for graphical visualization of the DistLM results. All the statistical tests were performed by PRIMER v. 6.1 (Clarke et al., 2006), PERMANOVA+ for PRIMER routines (Anderson et al., 2008).

4. RESULTS and DISCUSSION

4.1 - Physico-chemical analyses indicate high level of pollution in the Ancona harbor sediments

Only minor differences were found in terms of granulometric composition between all the sediments (Table 2), generally revealing the dominance of the clay fraction (on average 55%) and the reduced presence of gravel. The pH values in the sampling sites were within the typical pH range of coastal marine environments, which is between 7.5 and 8.4 (Chester at al., 2012), and the redox potential evidenced that sediments were oxygenated in the surface layers. (Table 2).

	Gravel (%)	Sand (%)	Silt (%)	Clay (%)	Humidity (%)	Specific weight (g cm^3)	pH	Eh (mV)
An-U01	**0**	**17,67**	**23,23**	**59,1**	**58,2**	**2,41**	**7,56**	**179,1**
An-U02	**3,42**	**15,68**	**18,26**	**62,65**	**58,5**	**2,43**	**7,7**	**197**
An-U03	**2,92**	**15,62**	**19,96**	**61,51**	**57,6**	**2,42**	**7,75**	**137,4**
An-U04	**0**	**18,51**	**33,1**	**48,39**	**59,1**	**2,73**	**7,76**	**247,3**
An-U05	**0**	**24,07**	**27,98**	**47,94**	**60,2**	**2,79**	**7,77**	**43,2**
An-U06	**0**	**25,59**	**25,14**	**49,27**	**61,8**	**2,77**	**7,8**	**161,4**
An-U07	**2,53**	**8,3**	**23,98**	**65,19**	**53,9**	**2,49**	**7,72**	**128,6**
An-U08	**0**	**12,75**	**24,15**	**63,1**	**63,7**	**2,53**	**7,64**	**121,4**
An-U09	**0**	**21,42**	**32,33**	**46,25**	**57,9**	**2,77**	**7,85**	**28**
An-U10	**0,16**	**22,24**	**27,2**	**50,4**	**66,2**	**2,79**	**7,5**	**142,2**
An-C1	**0**	**41,49**	**21,38**	**37,12**	**74,3**	**2,41**	**7,68**	**92,6**
An-C2	**0**	**16,11**	**31,34**	**52,55**	**61,7**	**2,69**	**7,47**	**28,4**
An-C3	**0**	**40,91**	**21,84**	**37,24**	**71,2**	**2,5**	**7,78**	**160,4**
An-C4	**0**	**18,9**	**33,22**	**47,88**	**70,1**	**2,82**	**7,72**	**101,3**
An-C5	**0**	**16,19**	**30,85**	**52,96**	**60**	**2,61**	**7,65**	**83,2**

Tab. 2 Physical **features of surface (0-1 cm) layer of sediments in the Ancona sampling sites**

Heavy metals can be dangerous to the environment and living organisms, and can also inhibit bioremediation of hydrocarbons polluted environment. Particularly, heavy metals tend to precipitate and to be absorbed by sediments particles, hence their concentration in the investigated sediments was measured. For instance, inorganic mercury is known to have adverse effects on microorganisms already at concentration of 5µg/L in a culture media (Boening, 1999; Orc et al., 2006).

Overall, the results of the chemical analyses (Table 3) indicated relatively low concentrations of mercury, lead, arsenic, zinc and vanadium in all the sampling stations. The measured concentration of chemical pollutants were compared with the Italian law limits stated by decree of 6 Nov 2003, n° 367.

Cadmium concentration was relatively high in all the stations located inside the harbor, displaying values above the Italian law limits (0.3 mg/kg d.s.) and revealing an evident difference comparing to the offshore stations. Chrome amount was very high in almost all samples independently from their location, being

under the law limits (50 mg/kg d.s.) only in 3 of the offshore sites, and it revealed two peaks at stations An-U2 and An-U3. Nickel was higher than Italian law limits (30 mg/kg d.s.) in all the sampling stations while lead was always below the low limits (30 mg/kg d.s.) with the only exception of site An-U1. All the sediments in the harbor area displayed high concentrations of heavy hydrocarbons (C>12), ranging from 65.3 mg/kg to 316.5 mg/kg, suggesting an input from ships and other harbor activities. In the offshore stations the heavy hydrocarbons concentration was much lower, with an average value of 34.98 mg/kg. Polycyclic aromatic hydrocarbons (PAHs) showed two important peaks at stations An-U1 and An-U4, probably due to the impact, in such sites, related to the presence of a fuel station.

	Hg	Cd	Pb	As	Cr	Cu	Ni	Zi	V	HC C>12.	Total PAH	Organic C
An-U01	**0,20**	**0,67**	**47,50**	**9,10**	**157,80**	**36,90**	**48,60**	**25,00**	**25,00**	**292,0**	**1,157**	**9,22**
An-U02	**0,10**	**0,80**	**18,90**	**6,20**	**517,30**	**39,00**	**61,60**	**107,00**	**60,30**	**252,0**	**0,359**	**8,58**
An-U03	**0,30**	**0,60**	**22,20**	**4,80**	**83,70**	**44,00**	**46,40**	**113,50**	**78,10**	**154,0**	**0,420**	**7,15**
An-U04	**0,05**	**0,60**	**15,10**	**5,50**	**105,10**	**23,90**	**52,90**	**88,90**	**73,40**	**65,3**	**3,697**	**9,8**
An-U05	**0,10**	**0,58**	**11,70**	**6,80**	**429,20**	**32,20**	**63,80**	**93,40**	**56,70**	**152,0**	**0,030**	**9,42**
An-U06	**0,10**	**0,57**	**12,00**	**6,20**	**130,60**	**32,30**	**52,10**	**89,90**	**57,70**	**165,6**	**0,087**	**9,63**
An-U07	**0,30**	**0,80**	**9,10**	**8,50**	**107,60**	**57,60**	**191,10**	**148,60**	**93,70**	**316,5**	**0,358**	**7,26**
An-U08	**0,10**	**0,56**	**25,50**	**7,90**	**88,40**	**28,70**	**47,00**	**106,50**	**62,40**	**152,0**	**0,487**	**8,23**
An-U09	**0,10**	**0,60**	**16,00**	**7,50**	**137,30**	**34,00**	**55,90**	**89,30**	**66,50**	**106,0**	**0,164**	**7,99**
An-U10	**0,10**	**0,60**	**17,40**	**8,20**	**84,40**	**23,70**	**54,00**	**86,60**	**51,80**	**119,0**	**0,584**	**8,32**
An-C1	**0,05**	**0,29**	**7,20**	**6,70**	**72,00**	**22,40**	**42,20**	**47,90**	**40,70**	**2,5**	**0,008**	**7,84**
An-C2	**0,05**	**0,21**	**20,30**	**5,00**	**45,10**	**23,40**	**38,50**	**97,40**	**62,70**	**82,7**	**0,203**	**5,24**
An-C3	**0,05**	**0,25**	**10,70**	**7,70**	**44,60**	**20,30**	**47,90**	**63,90**	**40,20**	**11,9**	**0,047**	**6,03**
An-C4	**0,10**	**0,24**	**10,20**	**7,80**	**89,20**	**18,70**	**48,90**	**66,70**	**57,80**	**19,0**	**0,106**	**7,01**
An-C5	**0,05**	**0,28**	**18,50**	**6,20**	**46,30**	**28,80**	**46,70**	**99,10**	**50,80**	**58,8**	**0,182**	**5,05**

Tab.3 **Concentrations of the main chemical contaminants in sediments of the 0-1 cm layer in the Ancona sampling site. All values are expressed as mg/kg d.s.**

PERMANOVA analysis on the chemical characterization showed a significant difference ($p = 0.0089$) between the samples collected from the stations located inside the harbor area and those located offshore. The result of the PERMANOVA analysis allowed to define such stations respectively as polluted and pristine stations.

4.2 - Microbial community richness is related to the chemical composition of sediments

Prokaryotic abundance in all the sampling sites was estimated through DAPI staining (Fig. 2). Prokaryotic abundance ranged between 4.04×10^8 and 1.13×10^9 cell/g d.s. in the sediments collected inside the harbor area, but decreased slightly in the offshore control sites, where their values ranged from 9.76×10^7 to 7.46×10^8 cell/g d.s. Total microbial abundances reported in this study are in accordance with previously published data for sediments collected at the sediment-water interface layers of the Adriatic sea (Molari et al., 2012).

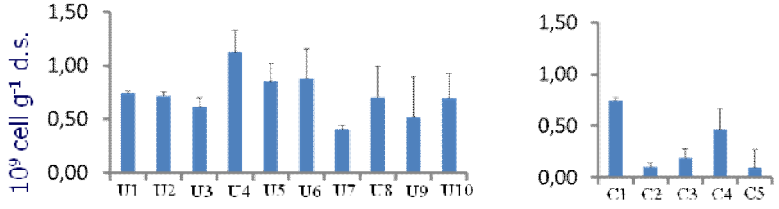


Fig. 2 **Total prokaryotic abundances evaluated through DAPI staining**

The total bacterial communities' structure of pristine and polluted sediments was investigated through ARISA fingerprinting, aiming to identify the environmental parameters shaping the bacterial communities, particularly to ascertain if the pollutants' content is actually a driving force of the bacterial communities' richness.
The polluted sediments comprised between 38 and 78 OTUs in the sites An-U2 and An-U3 respectively and displayed Shannon diversity index values ranging between 4.91 of site An-U6 and 5.18 of site An-U10 (Table 4), while the control sites located offshore showed an higher numbers of OTUs ranging between 87 (An-C4) and 94 (An-C2)

Site	Peaks number	Shannon index	Evenness
An-U1	60	4,93	0,86
An-U2	38	4,93	0,86
An-U3	78	5,01	0,81
An-U4	62	4,89	0,86
An-U5	54	4,96	0,89
An-U6	52	4,91	0,86
An-U7	58	4,93	0,86
An-U8	76	5,13	0,85
An-U9	51	5,02	0,82
An-U10	67	5,18	0,86
An-C1	89	5,63	0,87
An-C2	94	5,53	0,90
An-C3	93	5,56	0,89
An-C4	87	5,60	0,89
An-C5	90	5,43	0,91

Tab. 4 Number of arisa peaks and diversity index

nMDS analysis based on the ARISA patterns showed a clear separation between the bacterial communities inhabiting the polluted and control sites (Fig. 3). nMDS analysis, characterized by a stress value of 0.01, and PERMANOVA test indicated that the richness of the sediment dwelling bacterial communities from the pristine and polluted areas of the Ancona harbor was significantly different ($p = 0.0003$). This result highlighted the pivoltal role of pollutants' concentration in the selection of microbial communities already adapted to the conditions occurring at the polluted environments located inside the harbor area.

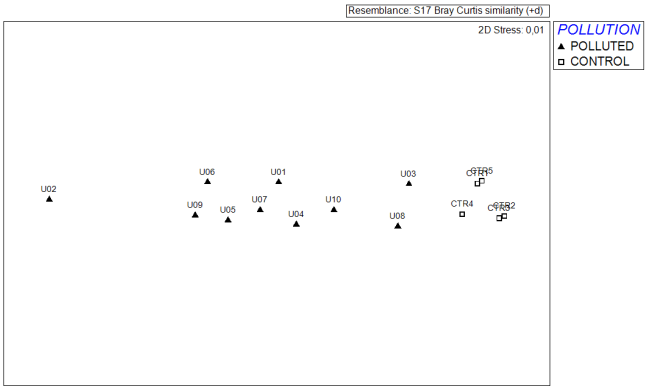


Fig. 3 **Distribution of the bacterial communities in the sediment sample. Nonmetric multidimensional scaling (nMDS) results based on qualitative ARISA fingerprinting.**

A DistLM analyses was applied with the aim to identify the environmental variables influencing the bacterial community richness in the polluted and control sites of the Ancona harbor. The results of DistLM showed that the observed variation was significantly related to five environmental variables, as shown in Figure 4.

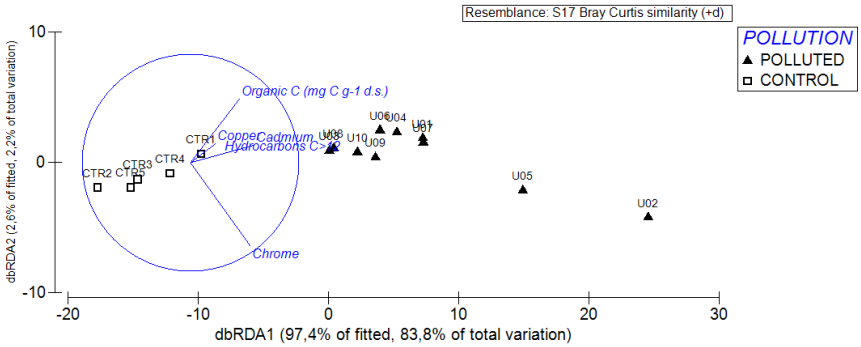


Fig. 4 **dbRDA ordinations of the ARISA dataset overlaid with the partial correlations of the tested environmental variables explaining the clustering of polluted and pristine sediment samples**

None of the physical properties of the sediments was significantly correlated to the richness values displayed by the bacterial communities while, among the chemical characteristics, cadmium (p = 0,0001), chrome (p =0,0001), copper (p = 0,0453), hydrocarbons C>12 (p =0,0055) and TOC (p =0,0043) were the significant variables. The two axes of the dbRDA displayed 86.0% of the total variation (Fig.4) overlapping the distribution of the samples, according to the communities' richness values, with the significant chemical parameters represented as vectors. Pristine and polluted samples clustered separately according to both heavy metals (cadmium, chrome and copper) and heavy hydrocarbons pollutants.

4.3 - Microcosm enrichment established with different carbon sources drive the selection of different microbial communities

Autochthonous bioaugmentation (ABA) is a new bioremediation strategies that involve the inocula in polluted sites of autochthonous microbial population able to degrade the pollutants and that are also able to survive in the specific contaminated sites. Hence, the investigation of the cultivable fraction of bacteria inhabiting polluted sites is an obligate step to find promising hydrocarbonoclastic bacteria exploitable for efficient bioremediation technologies.

In our case-study, the bacterial communities inhabiting the polluted sediments has proved to be influenced by the chemical parameters occurring in the sediments.

Two sites (An-U5 and An-U7), representative of two ideal sub-groups and individuated basing on the spatial distribution of the sampling sites within the harbor area, were chosen (Fig.1) to enrich hydrocarbon degrading bacterial cultures. An-U5 and An-U7 sites had the highest potential to provide different hydrocarbonoclastic populations since they differed in both physical characteristics and chemical content (Table 2 and Table 3). Site An-U7 had a higher percentage of gravel (2.53%) that was not present in site An-U5. Furthermore, the redox potential of site An-U7 was higher, reaching a value of 128,6 mV, than the value measured in site An-U5 (43,2 mV), indicating different level of oxygenation within the sediments.

To further characterize these two sites, the number of hydrocarbon degrading bacteria inhabiting them was evaluated through the MPN method. MPN is a culture dependant technique that allow to discriminate between organisms with different metabolic characteristics and to evaluate, in a certain sample, the number of individuals harbouring a specific activity. In the established test crude oil was the only carbon source supplied with the medium, thus only bacteria able to degrade it could grow. The number of hydrocarbon degrading bacteria evaluated was 7.3 x 10^6 cell/g of sediment in site An-U5 and 4.3 x 10^5 cell/g of sediment in site An-U7. The observed difference could be due to the higher (approximately double) amount of hydrocarbons in site An-U7 – 316.5 mg/kg d.s *versus* 152.0 mg/kg d.s. measured in site An-U5 – that possibly stimulated differentially the selection and growth of hydrocarbon degrading bacteria.

Sediments collected from both sites were exploited to establish microcosms with 3 different carbon sources: diesel oil (defined DS or DB according to the adopted enrichment procedures, see Materials and Method

section), crude oil (CO) and naphthalene (N). This allowed investigating the response of the cultivable bacterial popultaions to different pollutants under laboratory conditions.
The bacterial communities were evaluated applying two molecular techniques (DGGE fingerprinting and 16S rRNA pyrotag). Both the analyses confirmed the selection of specific bacterial communities after the addition of the different pollutants and a high specialization toward bacteria directly involved in their degradation, leading to an extreme simplification of the bacteria community compared to the environmental microbiome.

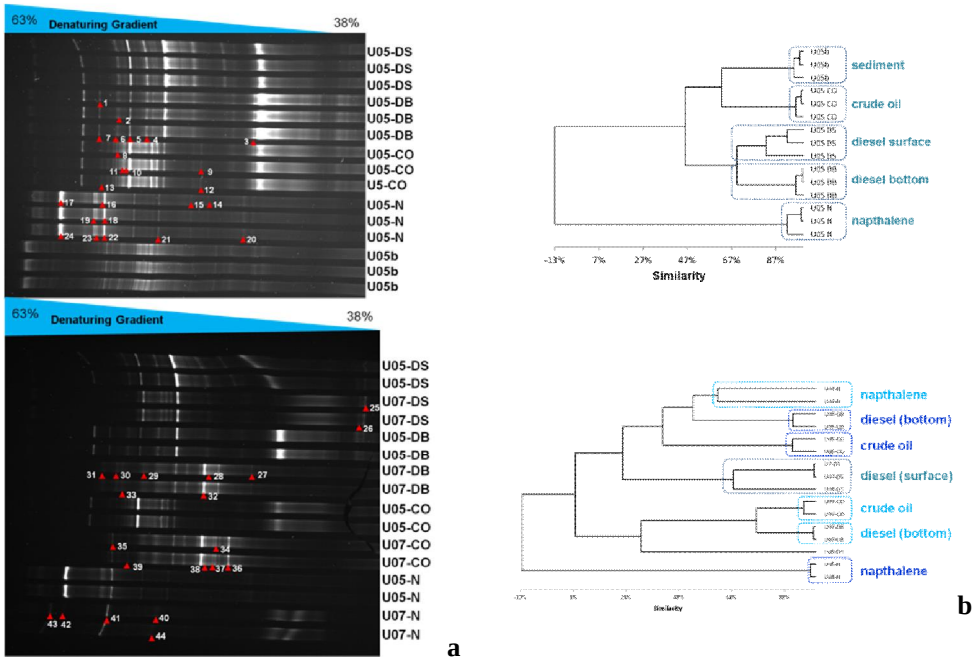


Fig 5 **16S rRNA DGGE fingerprinting analyses. a) Gel images. Red triangles indicate the cutted and identied bands. b) cluster analyses of DGGE bands profiles.**

16S rRNA DGGE analyses resulted in very different band patterns between the original sediments and the enriched samples (Fig.5a). An-U5 and An-U7 DGGE profiles were more complex than those observed for the respective enrichments. Overall, independently from the sediment of origin, the DGGE profiles appeared to be more similar when diesel oil or crude oil were added in the medium, while in the enrichment cultures supplied with naphthalene selection of specific bacterial populations was higher. This similarity was confirmed through a cluster analyses of the DGGE profiles (Fig.5b).
The sequencing and identification of partial 16S rRNA retrieved from DGGE bands have been successful for 43 bands (Table 5), allowing to identify some of the taxa detected in DGGE as different bands. The identified microorganisms belonged to γ- and α-Proteobacteria, Flavobacteria, Bacilli, Clostridia and Actinobacteria classes, and many of them were well known hydrocarbon degrading bacteria. For instance, *Marinobacter* genus that belong to the γ-proteobacteria class, was identified within most of the microcosms.
The high-throughput method 16S rRNA pyrotag allowed to get more insight on the alpha-diversity of the bacteria community of An-U5 and An-U7 sediments and microcosms established from the An-U5 sediment. The analyses of the rarefaction curves (Fig.6a) showed that all the libraries reached the saturation, therefore the pyrosequencing analysis reliably covered the whole bacterial diversity within the samples. Furthermore, both the sediment samples showed similar higher Shannon index (An-U5 = 8.201; An-U7 = 8.363) if compared with the enrichments obtained with the different pollutants (An-U5-N= 4.717; An-U5-CO= 4.589; An-U5-DB= 3.704), confirming the higher diversity of the environmental samples.

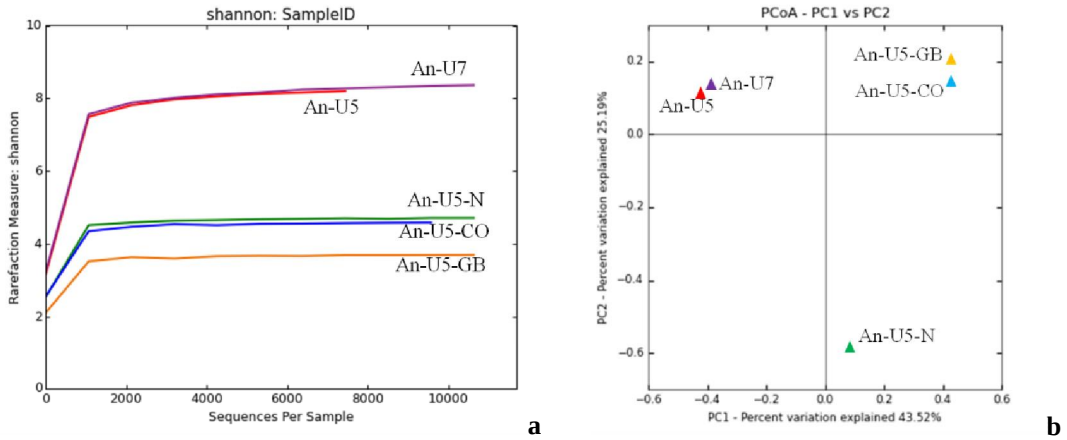
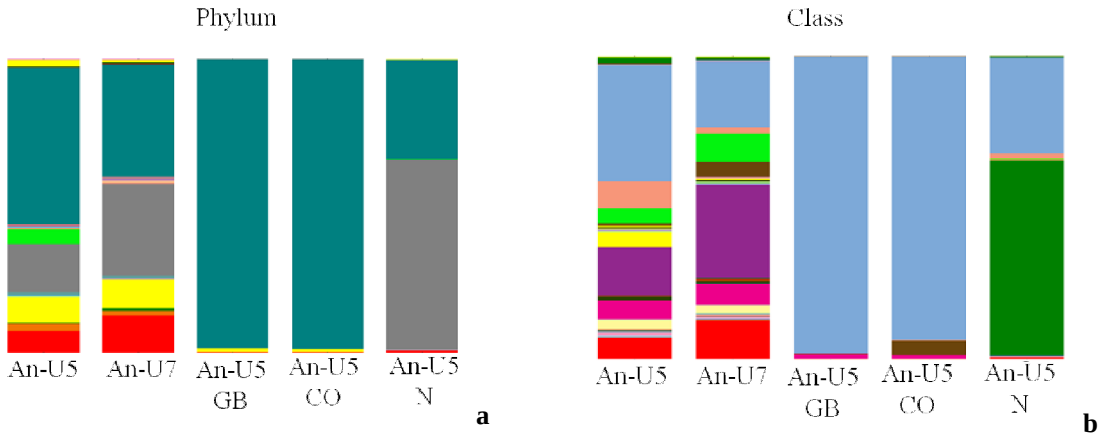


Fig.6 **16S rRNA pyrosequencing. a)Refraction curves.**

Principal component analyses (PCoA), explaining 68.71% of the diversity observed between the bacterial communities (Fig.6b) showed the differential clustering of environmental and cultivated bacterial communities. In particular PC1 explained 43.52% and along this axis it is possible to identify two groups, represented by i) the two environmental samples An-U5 and An-U7 and ii) diesel, crude oil and naphthalene microcosms. On the other side, along PC2, the enrichment culture An-U5-N selected using naphthalene is well separated from the other two enrichments, as previously observed by DGGE analysis (Fig. 5).

The bacterial community of site An-U5 was dominated by four different phylum (Proteobacteria, 52.82%, that cover more than half of the total diversity; Firmicutes, 15.98%; Bacteroidetes, 9.4% and Fusobacteria, 5.17%) while An-U7 was dominated only by three different phyla (Proteobacteria, 38.21%; Firmicutes, 31.9% and Bacteroidetes, 9.7%). Both Proteobacteria and Firmicutes phyla comprise several phylogenetic groups involved in the aerobic degradation of hydrocarbons. Indeed, these sediments, were dominated by γ-proteobacteria and Clostridia (Fig.7), coherently with the high pollution level of the Ancona harbor sediments. γ-proteobacteria represented 38% of the bacteria population of An-U5 and 23% of An-U7, while Clostridia represented 16% and 31% respectively. These classes comprise many well known hydrocarbon degrading bacteria (Head et al., 2006), nonetheless only few of the major genera involved in the hydrocarbons' degradation have been identified in our 16S rRNA libraries. For example, *Oleispira* was detected only in the site An-U7, where it represented 1% of the total bacterial community, while members of the family *Alteromonadaceae* - comprising the genus *Marinobacter* - were detected in the site An-U5 at a very low percentage (about 0.01%). Moreover, *Alcanivorax* was not detected neither in An-U5 nor in An-U7 sediments. Interestingly, due to the selective pressure imposed in the laboratory by the addition of specific pollutants in the established enrichments, An-U5 diesel and crude oil microcosms were dominated by *Alcanivorax* and *Marinobacter* genera pointing out that they were present in the original An-U5 sediment but below the detection limit of the technique.



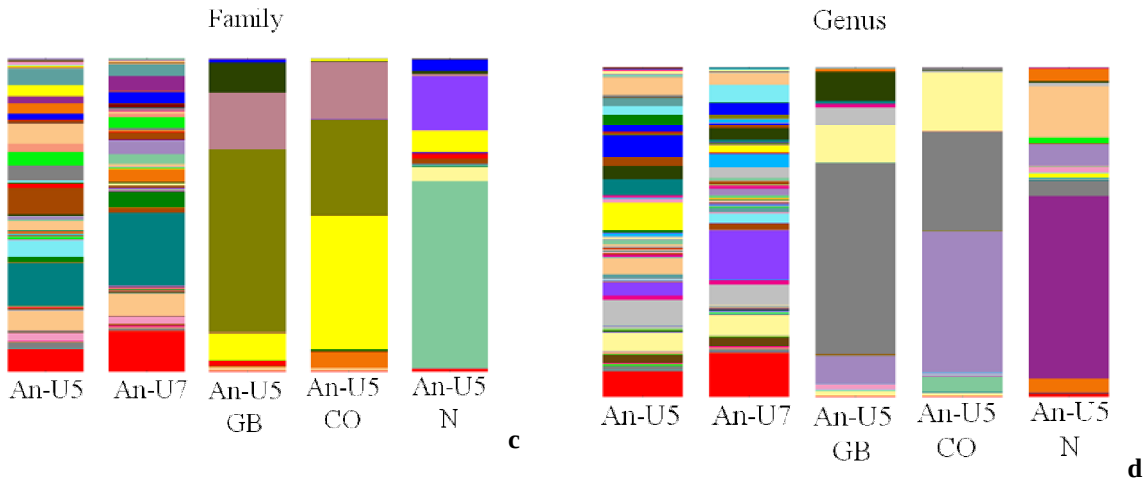


Fig. 7 **Graphical visualization of the bacteria diversity achieved through 16S rRNA pyrotag sequencing.**

The established microcosms hosted a less rich bacterial community: of the 20 different phylum identified in An-U5 sediment only 4 were present in the enrichment An-U5-DB, 3 in An-U5-CO and 4 in An-U5-N. Diesel and crude oil microcosms were dominated by Proteobacteria (98%), while the most abundant phylum in the naphthalene enrichment was Firmicutes (64%), followed by Proteobacteria (34%). A low bacterial diversity can be observed also at finer phylogenetic levels: γ-proteobacteria dominated both diesel oil and crude oil enrichments (98%), while naphthalene enrichment comprised mostly Bacilli (64.5%) and γ-proteobacteria (31%). The dominance of γ-proteobacteria is expected since the hydrocarbonoclastic bacteria responsible for the first step of hydrocarbons degradation belong to this class (Head et al., 2006, Yakimov et al., 2007). Furthermore, the genera that dominated diesel and crude oil microcosms were *Alcanivorax* and *Marinobacter* that together covered about 70% of the bacterial communities. Naphthalene enrichment was dominated by *Bacillus* (55.57%) and *Halomonas* (15.52%) genera, also known to be involved in the degradation of PAHs molecules (McGenity et al., 2012; Kostka et al., 2011).
In addition, to evaluate the communities functional diversity responsible for alkane degradation, the presence of the *alkB* gene – that encode for a monooxigenase involved in the first step of alkane degradation – was investigated through DGGE fingerprinting both in the sediments (An-U5 and An-U7) and in the diesel and crude oil microcosms (Fig.8).

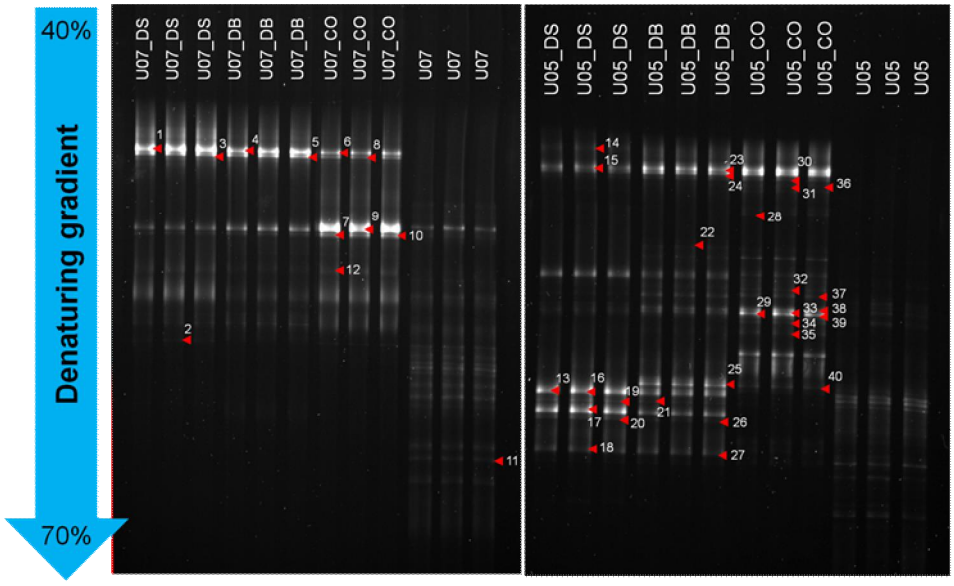


Fig. 8 ***alkB* DGGE fingerprinting analyses**

Also in this case the DGGE profiles of the sediments was more complex, indicating a richer hydrocarbons degrading microbial community compared to the enrichment cultures. All the 40 identified DGGE band belonged to few hydrocarbonoclastic bacterial species: *M. hydrocarbonoclasticus*, *M. adhearence*, *A. hodgenensis* and *A. borkumensis,* confirming their important role in hydrocarbon degradation in the polluted sediments of the Ancona harbor (Table. 6).

BAND	INOCULA	SAMPLE	CLOSEST RELATIVE	%	CLOSEST DESCRIBED SPECIE	LENGHT	ACC N	%	PHYLUM	CLASS	ORDER	FAMILY	GENUS
8	U05	CO_1	Marinobacter sp	99	Marinobacter guineae	513/519	AM503092	99	Proteobacteria	Gammaproteobacteria	Alteromonadales	Alteromonadaceae	Marinobacter
9	U05	CO_2	Uncultured Phaeobacter sp.	100	Oceanobacterium insulare	490/494	AB681665	99	Proteobacteria	Alphaproteobacteria	Rhodobacterales	Rhodobacteraceae	Roseovarius
10	U05	CO_2	Uncultured Phaeobacter sp.	100	Oceanobacterium insulare	490/494	AB681665	99	Proteobacteria	Alphaproteobacteria	Rhodobacterales	Rhodobacteraceae	Roseovarius
11	U05	CO_2	Uncultured Phaeobacter sp.	100	Oceanobacterium insulare	490/494	AB681665	99	Proteobacteria	Alphaproteobacteria	Rhodobacterales	Rhodobacteraceae	Roseovarius
13	U05	CO_3	Marinobacter sp.	99	Marinobacter litoralis	517/519	NR_028841	99	Proteobacteria	Gammaproteobacteria	Alteromonadales	Alteromonadaceae	Marinobacter
12	U05	CO_3	Uncultured Phaeobacter sp.	100	Oceanobacterium insulare	490/494	AB681665	99	Proteobacteria	Alphaproteobacteria	Rhodobacterales	Rhodobacteraceae	Roseovarius
1	U05	GF_1	Uncultured bacterium	99	Marinobacterium halophilum	518/520	NR_042980	99	Proteobacteria	Gammaproteobacteria	Alteromonadales	Alteromonadaceae	Marinobacterium
2	U05	GF_2	Uncultured bacterium	99	Marinobacter guineae	515/518	AM503092	99	Proteobacteria	Gammaproteobacteria	Alteromonadales	Alteromonadaceae	Marinobacter
3	U05	GF_3	Uncultured Bacteroidetes bacterium	99	Lutibacter litoralis	499/504	HM032816	99	Bacteroidetes	Flavobacteria	Flavobacteriales	Flavobacteriaceae	Lutibacter
6	U05	GF_3	Uncultured bacterium	99	Marinobacter hydrocarbonoclasticus	487/493	JN160761	99	Proteobacteria	Gammaproteobacteria	Alteromonadales	Alteromonadaceae	Marinobacter
7	U05	GF_3	Uncultured bacterium	100	Marinobacterium halophilum	519/520	NR_042980	99	Proteobacteria	Gammaproteobacteria	Alteromonadales	Alteromonadaceae	Marinobacterium
5	U05	GF_3	Uncultured Phaeobacter sp.	100	Oceanobacterium insulare	500/504	AB681665	99	Proteobacteria	Alphaproteobacteria	Rhodobacterales	Rhodobacteraceae	-
4	U05	GF_3	Uncultured bacterium	96	Pseudomonas pachastrellae	378/395	HQ425676	96	Proteobacteria	Gammaproteobacteria	Oceanospirillales	Oceanospirillaceae	-
14	U05	NAFT_1	Bacillus boroniphilus	97	Bacillus boroniphilus	492/508	FJ161333	97	Firmicutes	Bacilli	Bacillales	-	-
16	U05	NAFT_1	Bacillus firmus	97	Bacillus firmus	504/517	FJ897758	97	Firmicutes	Bacilli	Bacillales	-	-
17	U05	NAFT_1	Bacillus sp.	99	Bacillus selenatarsenatis	506/508	JN624922	99	Firmicutes	Bacilli	Bacillales	Bacillaceae 2	Bacillus
15	U05	NAFT_1	Bacillus sp.	99	Bacillus thioparans	506/507	JN999834	99	Firmicutes	Bacilli	Bacillales	Bacillaceae 1	Bacillus
19	U05	NAFT_2	Bacillus oceanisediminis	100	Bacillus oceanisediminis	505/505	HQ699494	100	Firmicutes	Bacilli	Bacillales	Bacillaceae 1	Bacillus
18	U05	NAFT_2	Thalassobacillus devorans	100	Thalassobacillus devorans	518/518	JQ799100	100	Firmicutes	Bacilli	Bacillales	Bacillaceae 2	Thalassobacillus
21	U05	NAFT_3	Uncultured bacterium	99	Acetivibrio multivorans	476/492	FR749900	97	Firmicutes	Clostridia	Clostridiales	Clostridiaceae 1	Proteiniclasticum
23	U05	NAFT_3	Bacillus firmus	99	Bacillus firmus	502/503	FJ613310	99	Firmicutes	Bacilli	Bacillales	Bacillaceae 1	Bacillus
24	U05	NAFT_3	Bacillus sp.	99	Bacillus selenatarsenatis	515/517	JN624922	99	Firmicutes	Bacilli	Bacillales	Bacillaceae 1	Bacillus
20	U05	NAFT_3	Uncultured Bacillaceae bacterium	99	Bacillus subterraneus	516/517	JQ684234	99	Firmicutes	Bacilli	Bacillales	Bacillaceae 1	Bacillus
22	U05	NAFT_3	Thalassobacillus devorans	100	Thalassobacillus devorans	518/518	JQ799100	100	Firmicutes	Bacilli	Bacillales	Bacillaceae 2	Thalassobacillus
35	U07	CO-7_1	Uncultured Clostridia bacterium	95	Clostridium halophilum	466/499	X77837	93	Firmicutes	Clostridia	Clostridiales	-	-
34	U07	CO-7_1	Uncultured bacterium	91	Owenweeksia hongkongensis		NR_040990	88	Bacteroidetes	-	-	-	-
37	U07	CO-7_2	Uncultured bacterium	92	-	-	-	-	Bacteroidetes	-	-	-	-
39	U07	CO-7_2	Uncultured organism	98	Alkaliphilus peptidofermentans	409/426	EF382660	96	Firmicutes	Clostridia	Clostridiales	Clostridiaceae 3	Sporosalibacterium
36	U07	CO-7_2	Uncultured Bacteroidetes	99	Marinifilum fragile	475/514	NR_044597	92	Bacteroidetes	"Bacteroidetes"_incertae_sedis	-	-	Marinifilum
38	U07	CO-7_2	Uncultured bacterium	91	Owenweeksia hongkongensis		CP003156	89	Bacteroidetes	-	-	-	-
27	U07	GF-7_1	Uncultured bacterium	95	-	-	-	-	Bacteroidetes	-	-	-	-
28	U07	GF-7_1	Uncultured bacterium	92	-	-	-	-	Bacteroidetes	-	-	-	-
30	U07	GF-7_1	Defluviitaleaceae bacterium	98	-	-	-	-	Firmicutes	Clostridia	Clostridiales	Lachnospiraceae	-
31	U07	GF-7_1	Uncultured Halanaerobiales bacterium	94	Clostridium thermosuccinogenes	413/467	Y18180	88	Firmicutes	Clostridia	-	-	-
29	U07	GF-7_1	Uncultured bacterium	98	Geosporobacter subterraneus	477/490	DQ643978	97	Firmicutes	Clostridia	Clostridiales	-	-

32	U07	GF-7_2	Uncultured bacterium	92	-	-	-	-	Bacteroidetes	-	-	-	-
33	U07	GF-7_2	Uncultured bacterium	92	-	-	-	-	Bacteroidetes	-	-	-	-
25	U07	GS-7_1	Bizionia sp.	100	Bizionia paragorgiae	522/522	HQ538734	100	Bacteroidetes	Flavobacteria	Flavobacteriales	Flavobacteriaceae	Bizionia
26	U07	GS-7_2	Bizionia sp.	100	Bizionia paragorgiae	428/428	HQ538734	100	Bacteroidetes	Flavobacteria	Flavobacteriales	Flavobacteriaceae	Bizionia
41	U07	NAFT-7_1	Bacillus sp.	99	Bacillus foraminis	516/532	JX203248	97	Firmicutes	Bacilli	Bacillales	Bacillaceae 1	Bacillus
42	U07	NAFT-7_1	Bacillus sp.	99	Bacillus thioparans	506/508	JN999834	99	Firmicutes	Bacilli	Bacillales	Bacillaceae 1	Bacillus
43	U07	NAFT-7_1	Microbacterium sp.	100	Microbacterium jejuense	522/522	EU419935	100	Actinobacteria	Actinobacteria	Actinomycetales	Microbacteriaceae	Microbacterium
40	U07	NAFT-7_1	Sulfitobacter delicatus	99	Sulfitobacter delicatus	470/476	EU939703	99	Proteobacteria	Alphaproteobacteria	Rhodobacterales	Rhodobacteraceae	Sulfitobacter
44	U07	NAFT-7_2	Sulfitobacter delicatus	98	Sulfitobacter delicatus	445/454	EU939703	98	Proteobacteria	Alphaproteobacteria	Rhodobacterales	Rhodobacteraceae	Sulfitobacter

Tab. 5 16s rRNA DGGE bands identification

INOCULA	SAMPLE	BAND	CLOSEST RELATIVE	GENE	LENGHT	ACC N	%	SPECIE	GENE	LENGHT	ACC N	%
An-U7	ANU7_CO	1	Uncultured bacterium	alkane hydroxylase B (alkB) gene	369/453	JQ279687	81	Marinobacter adhaerens	complete genome	364/449	CP001978	81
An-U7	ANU7_CO	2	Uncultured bacterium	alkane hydroxylase B (alkB) gene	364/443	JQ279687	82	Marinobacter adhaerens	complete genome	359/441	CP001978	81
An-U7	ANU7_CO	3	Uncultured bacterium	alkane hydroxylase (alkB) gene	364/434	JN986869	84	Marinobacter adhaerens	complete genome	357/428	CP001978	83
An-U7	ANU7_CO	4	Uncultured bacterium	alkane hydroxylase (alkB) gene	384/452	JN986869	85	Marinobacter adhaerens	complete genome	375/451	CP001978	83
An-U7	ANU7	5	Uncultured bacterium	alkane monooxygenase (alkB) gene	328/431	GQ184418	76	Simiduia agarivorans	complete genome	134/173	CP003746	77
An-U7	ANU7_DS	6	Uncultured bacterium	alkane monooxygenase (alkB) gene	316/343	JX276499	92	Marinobacter hydrocarbonoclasticus	alkane hydroxylase (alkB) gene	281/344	EU853368	82
An-U7	ANU7_CO	7	Uncultured bacterium	alkane monooxygenase (alkB) gene	368/449	JQ279687	82	Marinobacter adhaerens	complete genome	364/449	CP001978	81
An-U7	ANU7_GB	8	Uncultured bacterium	alkane monooxygenase (alkB) gene	366/449	JQ279687	82	Marinobacter adhaerens	complete genome	355/437	CP001978	81
An-U7	ANU7_CO	9	Uncultured bacterium	putative alkane monooxygenase (alkB) gene	235/281	JQ437623	84	Alcanivorax borkumensis	alkane hydroxylase (alkB) gene	97/123	EU853322	79
An-U7	ANU7_CO	10	Uncultured bacterium	alkane hydroxylase B (alkB) gene	381/451	JQ279685	84	Marinobacter adhaerens	complete genome	376/449	CP001978	84
An-U7	ANU7_DS	11	Uncultured bacterium	alkane hydroxylase B (alkB) gene	372/455	JQ279687	82	Marinobacter adhaerens	complete genome	363/449	CP001978	81
An-U7	ANU7_DS	12	Uncultured bacterium	alkane hydroxylase B (alkB) gene	383/470	JQ279687	81	Marinobacter adhaerens	complete genome	379/466	CP001978	82
An-U5	ANU5_CO	13	Uncultured bacterium	alkane hydroxylase (alkB) gene	295/344	JN986861	86	Alcanivorax hongdengensis	alkane hydroxylase (alkB) gene	186/230	EU438898	81
An-U5	ANU5_CO	14	Uncultured bacterium	alkane hydroxylase (alkB) gene	358/381	JN986869	94	Marinobacter adhaerens	complete genome	323/377	CP001978	86
An-U5	ANU5_CO	15	Uncultured bacterium	alkane hydroxylase (alkB) gene	352/374	JQ279685	94	Marinobacter adhaerens	complete genome	319/375	CP001978	85
An-U5	ANU5_DB	16	Marinobacter adhaerens	complete genome	250/316	CP001978	79	Marinobacter adhaerens	complete genome	250/316	CP001978	79
An-U5	ANU5_CO	17	Uncultured bacterium	alkane hydroxylase (alkB) gene	402/418	JQ279685	96	Marinobacter adhaerens	complete genome	362/416	CP001978	87
An-U5	ANU5_DS	18	Uncultured bacterium	alkane hydroxylase (alkB) gene	405/418	JQ279685	97	Marinobacter adhaerens	complete genome	364/416	CP001978	88
An-U5	ANU5_DB	19	Uncultured bacterium	alkane hydroxylase (alkB) gene	417/434	JN986869	96	Marinobacter adhaerens	complete genome	372/428	CP001978	87
An-U5	ANU5_GS	20	Uncultured bacterium	alkane hydroxylase (alkB) gene	403/418	JQ279685	96	Marinobacter adhaerens	complete genome	360/416	CP001978	87
An-U5	ANU5_DB	21	Uncultured bacterium	alkane hydroxylase (alkB) gene	408/427	JQ279685	96	Marinobacter adhaerens	complete genome	366/427	CP001978	86
An-U7	ANU5_CO	22	Uncultured bacterium	alkane hydroxylase (alkB) gene	294/333	JQ279685	88	Marinobacter hydrocarbonoclasticus	alkane hydroxylase (alkB) gene	281/335	EU853368	84
An-U5	ANU5_CO	23	Uncultured bacterium	alkane hydroxylase (alkB) gene	409/434	JN986869	94	Marinobacter adhaerens	complete genome	367/428	CP001978	86
An-U5	ANU5_CO	24	Uncultured bacterium	alkane hydroxylase (alkB) gene	408/431	JQ279685	95	Marinobacter adhaerens	complete genome	368/429	CP001978	86
An-U7	ANU5_DB	25	Uncultured bacterium	alkane hydroxylase (alkB) gene	342/424	JQ279687	81	Marinobacter hydrocarbonoclasticus	alkane hydroxylase (alkB) gene	331/415	EU853368	80
An-U5	ANU5_DB	26	Marinobacter sp. EPR21	hydroxylase (alkB) gene	370/403	KC610508	92	Alcanivorax hongdengensis	alkane hydroxylase (alkB) gene	243/295	EU438898	82
An-U5	ANU5_DS	27	Uncultured bacterium	alkane hydroxylase (alkB) gene	383/468	JX276502	82	Marinobacter adhaerens	complete genome	381/476	CP001978	80
An-U5	ANU5_DB	28	Uncultured bacterium	alkane hydroxylase (alkB) gene	377/455	JQ279687	83	Marinobacter adhaerens	complete genome	365/449	CP001978	81
An-U5	ANU5_DB	29	Uncultured bacterium	alkane hydroxylase B (alkB) gene	390/472	JQ279687	83	Marinobacter adhaerens	complete genome	379/466	CP001978	81
An-U5	ANU5_CO	30	Uncultured bacterium	alkane hydroxylase B (alkB) gene	385/472	JQ279687	81	Marinobacter adhaerens	complete genome	377/466	CP001978	81
An-U5	ANU5_CO	31	Uncultured bacterium	alkane hydroxylase B (alkB) gene	393/435	JN986869	90	Marinobacter adhaerens	complete genome	360/425	CP001978	85
An-U5	ANU5_CO	32	Uncultured bacterium	alkane hydroxylase B (alkB) gene	381/419	JQ279685	91	Marinobacter adhaerens	complete genome	349/414	CP001978	84
An-U5	ANU5_CO	33	Uncultured bacterium	alkane hydroxylase B (alkB) gene	357/381	JN986869	94	Marinobacter adhaerens	complete genome	321/377	CP001978	85

An-U5	ANU5_CO	34	Uncultured bacterium	alkane hydroxylase B (alkB) gene	364/389	JQ279685	94	Marinobacter adhaerens	complete genome	333/392	CP001978	85
An-U5	ANU5_CO	35	Uncultured bacterium	alkane hydroxylase B (alkB) gene	358/382	JQ279685	94	Marinobacter adhaerens	complete genome	321/377	CP001978	85
An-U5	ANU5_DS	36	Uncultured bacterium	alkane hydroxylase B (alkB) gene	461/484	JQ279685	95	Marinobacter adhaerens	complete genome	420/482	CP001978	87
An-U5	ANU5_DS	37	Uncultured bacterium	alkane hydroxylase B (alkB) gene	417/434	JN986869	96	Marinobacter adhaerens	complete genome	368/428	CP001978	86
An-U5	ANU5_DS	38	Uncultured bacterium	alkane hydroxylase B (alkB) gene	417/434	JN986869	96	Marinobacter adhaerens	complete genome	368/428	CP001978	86
An-U5	ANU5_DS	39	Uncultured bacterium	alkane hydroxylase B (alkB) gene	345/367	JQ279685	94	Marinobacter adhaerens	complete genome	315/367	CP001978	86
An-U5	ANU5_DS	40	Uncultured bacterium	alkane hydroxylase B (alkB) gene	419/436	JQ279685	96	Marinobacter adhaerens	complete genome	368/428	CP001978	86

Tab. 6 alkB DGGE bands identification

4.4 - Isolated strains from the microcosms show a very low diversity, in spite of the rich bacteria community of the sediments.

A collection of hydrocarbon degrading bacteria was established from the microcosms previously established with the polluted sediments collected at the An-U5 and An-U7 sites and different hydrocarbon sources, namely diesel oil and crude oil. Even if the starting bacteria communities showed a promising high diversity, all the 60 isolated strains belonged to few bacteria species. The isolates obtained from An-U5 sediments were *Alcanivorax borkumenis* (26 isolates), *Marinobacter hydrocarbonoclasticus* (3) and *Pseudomonas pachastrellae* (1) while from An-U7 sediment only *Alcanivorax borkumenis* (29) and *Marinobacter excellens* (1) were obtained in pure cultures.

The low phylogenetic diversity of the isolated strains is probably due to the strict isolation conditions, which favour few organisms, not only able to use hydrocarbons as a carbon source, but reported as the first to bloom after an oil spill (Head et al., 2006 and references therein). One of the physiologic characteristic that concur to make the *Alcanivorax* genus prevalent respect other bacterial species is its capability to produce biosurfactants, molecules that lower the hydrophobicity of hydrocarbons rendering them more bioavailable. Similarly, *Marinobacter* sp. representatives are able to use a wide variety of hydrocarbon molecules for their growth (Gorshkova et al. 2003; Gauthier et al., 1992). *Pseudomonas* are widespread microorganisms, which have been isolated from a variety of natural sources, soil, plants, mineral waters and clinical specimens, and they are characterized by a high level of metabolic diversity (Rossellò et al., 1991; Moore et al., 1996) including an important role as hydrocarbon consumers in marine environments (Mulet et al 2011).

5 CONCLUSIONS

This study confirms the high pollution level of the Ancona harbor sediments identifying polluted and pristine sites and indicating that different class of pollutants, namely heavy metals and hydrocarbons, determine the richness of bacteria communities inhabiting both polluted and pristine sites located in the harbor area. Furthermore, molecular ecology analyses, coupled with culture dependant methods, confirmed a correlation between the enrichments, under laboratory conditions, of different and specialized bacterial populations in the presence of diverse pollutants, strengthening the observation performed on the environmental samples. This work improved the knowledge of the environmental drivers that influence the enrichment of different microbial communities involved in the degradation of the pollutants, an important step towards the implementation of bioremediation strategies, like ABA. Nonetheless, the results obtained in this work remark how culture dependant strategies are still lacking for the ability to retrieve a large spectrum of the marine bacteria diversity, therefore losing many potential useful organisms exploitable for subsequent bioremediation of highly co-contaminated marine sites like the Ancona harbor.

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Chapter

VIII

ALLOCHTHONOUS BIOAUGMENTATION IN *EX-SITU* TREATMENT OF CRUDE OIL POLLUTED SEDIMENTS IN THE PRESENCE OF AN EFFECTIVE DEGRADING INDIGENOUS MICROBIOME

Abstract

Oil-polluted sediment bioremediation depends on both physicochemical and biological parameters, but the effect of the latter cannot be evaluated without the optimization of the former. We aimed in optimizing the physicochemical parameters related to biodegradation by applying an ex-situ landfarming set-up combined with biostimulation to oil-polluted sediment, in order to determine the added effect of bioaugmentation by four allochthonous oil-degrading bacterial consortia in relation to the degradation efficiency of the indigenous community. We monitored hydrocarbon degradation, sediment ecotoxicity and hydrolytic activity, bacterial population sizes and bacterial community dynamics, characterizing the dominant taxa through time and at each treatment. We observed no significant differences in total degradation, but increased ecotoxicity between the different treatments receiving both biostimulation and bioaugmentation and the biostimulated-only control. Moreover, the added allochthonous bacteria quickly perished and were rarely detected, their addition inducing minimal shifts in community structure although it altered the distribution of the residual hydrocarbons in two treatments. Therefore, we concluded that biodegradation was mostly performed by the autochthonous populations while bioaugmentation, in contrast to biostimulation, did not enhance the remediation process. Our results indicate that when environmental conditions are optimized, the indigenous microbiome at a polluted site will likely outperform any allochthonous consortium.

1.INTRODUCTION

Marine sands and sediments that are exposed to crude oil contamination are of major concern worldwide [1, 2]. Although both physicochemical and biological treatment methods exist for the remediation of such polluted environments, the use of the latter is continuously increasing due to their much lower costs and environmental friendly nature [3-5]. The remediation of oil-polluted sediments through biological means (bioremediation) involves the use of microorganisms that are able to degrade the pollutants. Currently, studies focus on enhancing the degradation potential of the indigenous microorganisms by altering the physicochemical parameters that could potentially stimulate the community's metabolic capacity (biostimulation), on increasing the number of microorganisms that are capable of degrading the pollutants (bioaugmentation), or on a combination of the two. Many studies have examined the effectiveness of each approach showing often contrasting results, with an ongoing scientific debate of how physicochemical (e.g. temperature, humidity, sediment oxygenation and grain size or organic matter content) or biological parameters (e.g. long term survival of the added microorganisms, antagonism with the indigenous populations, origin of the added strains) are determining the efficiency of each approach [3, 6-9]. One of the major bottlenecks of any applied method that is based on degradation by aerobic microorganisms in situ is the often-poor oxygen availability within the sediments that can severely limit the degradation potential of the community. The simplest method used to overcome oxygen limitation is ex situ treatment by landfarming, a technique that involves spreading of contaminated soils (including beach sand), petroleum sludges or sediments in a thin layer (typically less than 25-30 cm) and regular tilling, combined with the addition of nutrients and water to maintain moisture typically about 20%. This method has been widelyused in soil remediation for over a century due to its low cost, simplicity in use, compliance with governmental regulations and potential application in a variety of environments [1]. The aim of the present study was to evaluate the effectiveness of bioaugmentation with allochthonous bacterial consortia in the bioremediation of oil-contaminated sediments treated ex situ by landfarming. We setup an experimental design aimed to clarify the real advantage of bioaugmentation during landfarming practices through the evaluation of degradation performance between the different tested consortia and between allochthonous and indigenous degrading populations. An integrated approach was adopted, allowing to monitor the degradation of

different crude oil compounds, and the dynamics -in terms of enzymatic activity, community structure and population sizes-of the bacterial communities in each treatment.

2. MATERIALS and METHODS

2.1 - Sediment sampling and experimental setup

Polluted sediment (c.a. 30 L) was collected on January 14, 2013, from a coast adjacent to an oil refinery (Elefsina bay, Greece, 38° 2'16.28"N, 23°30'45.85"E), where hydrocarbon releases occur intermittently, and was transferred to the lab overnight, in a 50 L plastic barrel. Air temperature during sampling was 13°C and water temperature was 14°C. The initial sediment contamination was estimated at 5,000 ppm of crude oil per gram of sediment, using soxhlet extraction (see Supplementary Material). Upon arrival to the lab on the next day, sediment was filtered through a 5 mm sieve in order to remove large particles that could interfere with all downstream analyses, and was air-dried down to 20% humidity (w/w). Sediment temperature was monitored constantly, remaining within the 10-20 °C range. When sediment reached the desired humidity (20%), total heterotrophic bacterial colony forming units (CFUs) per gram of sediment were determined (see below) and sediment was placed within nine glass microcosms (1.4 L, 19 cm x 18.5 cm x 4 cm, open top) with 1.5 kg of sediment in each. Every microcosm represented a different treatment in terms of biostimulation and/or bioaugmentation. Biostimulation was performed with the addition of N/P in the form of KNO3/ KH2PO4 to a final ratio of C:N:P (100:10:1) while bioaugmentation with the addition of allochthonous bacteria to a final ratio of 10:1 (in terms of CFUs) to the indigenous. The addition of allochthonous consortia was performed by centrifuging the appropriate amount of liquid culture and then diluting the resulting pellet in 10 ml of sterile seawater. The resulting dilution was finally sprinkled over each treatment, subsequently mixed thoroughly for homogenization. The treatment codes and the actual treatments were as follows: C1: sediment with biostimulation without bioaugmentation. C2: sediment with the addition of killed allochthonous bacteria to a final ratio as in bioaugmented treatments. Bacteria were killed by means of UV-C irradiation -radiation wavelength 253.7 nm, 1 hour using a TUV PL-S 11W/2P 1 CT lamp (Philips, Amsterdam, Netherlands). This control treatment was performed in order to monitor degradation and bacterial community dynamics in the sediment where the added dead biomass is available as nutrients' source. C3: sediment acidified to pH=2 by means of HCl 37% (Sigma-Aldrich) plus biostimulation. This treatment was performed in order to monitor hydrocarbon losses by any means other than biodegradation. All reported degradation rates have been corrected taking into account this control treatment. C4: sediment without biostimulation or bioaugmentation. MUCSATB: sediment with bioaugmentation with the MUCSATB consortium plus biostimulation, UH2C2: sediment with bioaugmentation with the UH2C2 consortium plus biostimulation, UTUNB: sediment with bioaugmentation with the UTUNB consortium plus biostimulation, YUOW: sediment with bioaugmentation with the YUOW consortium plus biostimulation (Table 1). For additional information regarding the origin and isolation of the applied bacterial consortia see Supplementary Material. The overall experimental time was 56 days. Sediment humidity was monitored constantly and was kept between 8-20% by the addition of sterile distilled water when needed. Sediment was tilled every one or two days throughout the experiment for aeration by means of sterile plastic spatulas. Air temperature during the experiment was 10-15°C and sediment temperature was 12-13°C. Sediment for the determination of the saturated and aromatic hydrocarbons degradation rates, the humidity, and the total bacterial population sizes were collected on days 0 (7-8 hours after the addition of the allochthonous consortia), 7, 14, 28, 42 and 56. Sediment for total hydrolytic activity monitoring was collected with 1-day lag period, i.e., on days 1, 8, 15, 29, 43 and 57. Sediment for bacterial community characterization and dynamics was collected on days 0, 7, 28 and 42, in triplicates. Sedimentfor ecotoxicity analyses was collected at the beginning (Day 0) and at the end (Day 56) of the experiment.

Table 14 Sample codes and treatment description. N/P addition refers to the addition of N/P in the form of KNO3/ KH2PO4 to a final ratio of C:N:P (100:10:1). Sediment acidification refers to sediment pH fixation to pH=2.0 by HCl 37%. Added/indigenous ratio refers to the CFUs ratio of allochthonous to indigenous bacteria.

Sample code	Allochthonous consortium addition	N/P addition	Sediment Acidification	Added/indigenous ratio (in CFUs)

C1	no	yes	no	n/a
C2	yes, killed by UV irradiation	yes	no	10 to 1
C3	no	yes	yes	n/a
C4	no	no	no	n/a
MUCSATB	yes	yes	no	10 to 1
UH2C2	yes	yes	no	10 to 1
UTUNB	yes	yes	no	10 to 1
YUOW	yes	yes	no	10 to 1

2.2 Degradation of petroleum hydrocarbons monitoring

Sediment samples were initially treated with soxhlet extraction to extract the total petroleum hydrocarbons (TPH) from the sediment, then the extracts were separated in saturated and aromatic hydrocarbons fractions with solid phase extraction (SPE), and the aliphatic and aromatic components of the fractions were separated and quantified by Gas Chromatography -Mass Spectroscopy (GC-MS). For more details please refer to Supplementary Material.

2.3 Total bacterial populations

Total heterotrophic bacterial CFUs at the beginning of the experiment were determined by plate counts on nutrient-rich agar (see Supplementary Material) and during the experiment by flow cytometry. For flow cytometry, triplicate sediment aliquots (0.25 ml) were fixed before proceeding to extraction, by adding glutaraldehyde (4% final concentration) and keeping at room temperature for 30 min. Bacterial cells were detached from sediment particles with a combination of chemical and physical treatments, according to Amalfitano and Fazi [10] with some modifications (see Supplementary Material).

2.4 Sediment total hydrolytic activity

Total hydrolytic activity in the sediment was measured by fluorescein diacetate (FDA) hydrolysis using a slightly modified protocol described elsewhere [11] (see Supplementary Material).

2.5 Sediment ecotoxicity

Sediment elutriates were prepared as described elsewhere [12]. Two different target species were used for the determination of sediment toxicity: Vibrio fischeri and Paracentrotus lividus. Toxicity assays on V. fischeri were performed through standard Microtox® tests (UNI EN ISO 11348-3), using a Model 500 analyzer. Toxicity assays on P. lividus were performed considering the inhibition potential of sediment elutriates on egg fertilization rates (spermiotoxicity), following the standard methodology described elsewhere [13]. Both tests were performed on the initially collected, untreated, sediment and on treatments C1, UTUNB, MUCSATB and UH2C2 at the end of the incubation (day 56). Results of Microtox test were expressed as Toxic Units 50 (TU 50), calculated as 100/EC50, where EC50 is the elutriate dilution (%) resulting in a 50% decrease in bioluminescence after 5 and 15 min of incubation. Results of spermiotoxicity assays on P. lividus gamets were expressed as % of unfertilized eggs. Further details for each ecotoxicity test are given at the Supplementary Material.

2.6 Bacterial community characterization and dynamics

DNA was extracted from 0.5 g of sediment using the "Power Soil" kit (MoBio Laboratories Inc., Carlsbad, CA, USA) following the manufacturer's instructions. DNA was quantified using a NanoDrop 1000 spectrophotometer (Thermo Scientific, Waltham, MA, USA). Bacterial 16S rRNA gene fragments (~550 bp) were amplified with Polymerase Chain Reaction (PCR) using primers 907R (3'-CCGTCAATTCCTTTGAGTTT-5') and GC-357F (3'-CCTACGGGAGGCAGCAG-5' with a 5'-end GC-clamp) targeting a portion of the 16S rRNA gene that includes the hypervariable V3-V5 regions[14]. PCR reactions were performed as previously described[15]. Presence and length of PCR products were verified by electrophoresis in 1% w/v agarose gel prior to Denaturing Gradient Gel Electrophoresis (DGGE) analysis.DGGE was performed according to the method described by Muyzer et al. (1993)[14], and DGGE

bands were excised and sequenced. ARISA was performed for all samples up to Week 6 and was conducted on a standard amount of DNA on each sample by using the primer set ITSF, 5'-GTC GTA ACA AGG TAG GCC GTA-3' and ITSReub, 5'182GCC AAG GCA TCC ACC-3', as previously reported[16]. For further details on DGGE and ARISA data analyses see Supplementary Material. Nucleotide sequences of the excised bands were edited in Chromas Lite 2.01 (http://www.technelysium.com.au) and subjected to BLAST search (http://blast.ncbi.nlm.nih.gov/Blast.cgi). The partial 16S rRNA gene sequences have been deposited in the EMBL under accession numbers LK022102187LK022293, LM643751 and LM643752.

2.7 Statistical analyses

Non-parametric t-tests were performed in PAST 2.17c[17]. For ecotoxicity results, the responses of each end point were corrected for effects in controls through the Abbott's formula (http://www.astm.org/Standards/E724.htm). Differences between samples and control as well as and among samples were analyzed by one-way analysis of variance (ANOVA). When significant effects occurred, Tukeys's post-hoc test was performed. In order to study the structure of the whole bacterial community, avoiding the limitation due to the exclusive phylogenetic analyses of the cut DGGE bands, the images of the DGGE gels were converted in a numerical matrix and statistically analyzed by Principal Component Analysis (PCA) and ANOVA on the main axes.

3. RESULTS and DISCUSSION

The average number of heterotrophic bacterial CFUs per gram of sediment at Day 0, was (1±0.2)x105 . Thus, the total CFUs per treatment (1.5 kg) was estimated at 1.5x108. Hence, 1.5x109 CFUs were added to the MUCSATB, UH2C2, UTUNB and YUOW treatments from the respective consortia at Day 0, so that the initial ratio to the indigenous microbiome was 10:1.

3.1 Bioaugmentation did not improve overall degradation

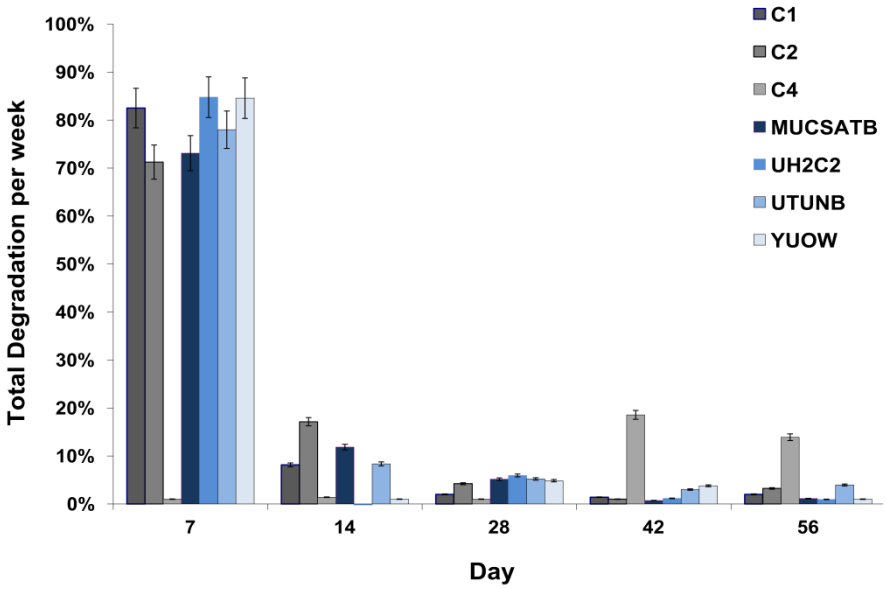

Figure 7 **Total weekly degradation of alkanes (C10-C35) at each time point (horizontal axis) for each treatment. Treatment codes are described in Table 1. Weekly degradation is defined as the percent of decrease in the concentration of the compounds at a time point n in relation to the concentration at the time point (n-1). Bars represent the standard error, derived from four technical replicates.**

GC-MS analysis results revealed a quick decrease of the saturated compounds (in total, C10-C35) within the first two experimental weeks, with 70% to 90% of the compounds being degraded by Day 7 and up to 95% by Day 56 for all treatments except C4 where degradation occurred after Day 28 (Fig. 1). Overall, the light (C10-C20) and medium chain (C20-C27) alkanes were degraded faster than the heavier (C27-C35) alkanes. After the second experimental week, the degradation of the saturated compounds decreased except from the case of C4 where a low percentage (10-20%) of degradation was observed within days 28-56.

Moreover, the aromatic components fluorene, phenanthrene and dibenzothiophene were degraded up to 50% by Day 14, and up to 90% by Day 42 (Fig. S1). The heavy polyaromatic components monitored (i.e., pyrene, fluoranthene, chrysene, benzo[a]pyrene, benzo[k]fluoranthene, benzo[b]fluoranthene, benzo[g,h,i]perylene and indeno[1.2.3216cd]pyrene) were not degraded significantly within the 56-day experimental time (results not shown). Flow cytometry results indicated that at the beginning of the treatment (Day 0), total bacterial cell numbers were higher in the bioaugmented treatments (average counts of 4.8×107 cells per g of dry sediment) than in the control samples (average counts of 2.8×107 cells per g of dry sediment) (non-parametric t-test, p=0.0108, average increase 71.4%) (Fig.2). On the contrary, the bacterial cell numbers were higher in the control treatments at the end of the first experimental week (Day 7, non-parametric t-test, p=0.01421, average counts of 4.6×107 cells per g of dry sediment in controls vs. 2.4×107 cells per g of dry sediment in bioaugmented treatments) and did not differ significantly at the end of the second experimental week (Day 14, non-parametric t-test, p=0.241) (Fig. 2). Bacterial cell counts in the non-biostimulated treatment (C4) were less than 2×107 cells per g of dry sediment at each time point. After the second experimental week, bacterial cell numbers in all treatments, except from C4, remained relatively constant until the end of the experiment (results not shown).

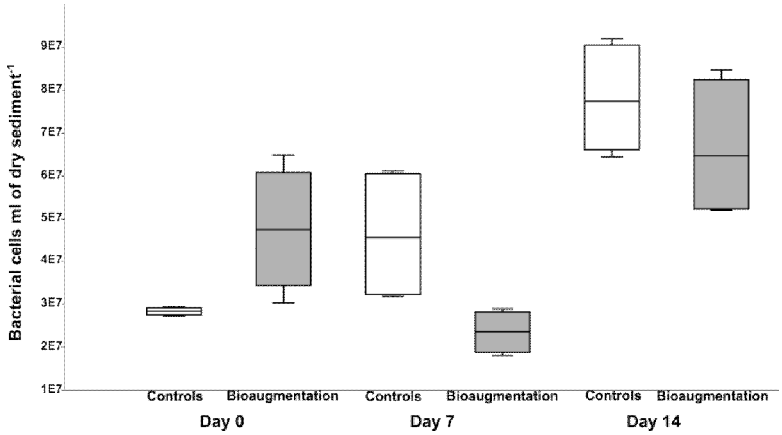

Figure 8 **Boxplots (25-75% percentiles) of total heterotrophic bacterial cells at the beginning of the experiment (Day 0) and after the first (Day 7) and second (Day 14) experimental week for treatments C1 and C2 ("Controls") and treatments UTUNB, UH2C2, MUCSATB and YUOW ("Bioaugmentation"). Bars represent the upper and lower values while the horizontal line within each box represents the median value.**

3.2 Ecotoxicity analysis

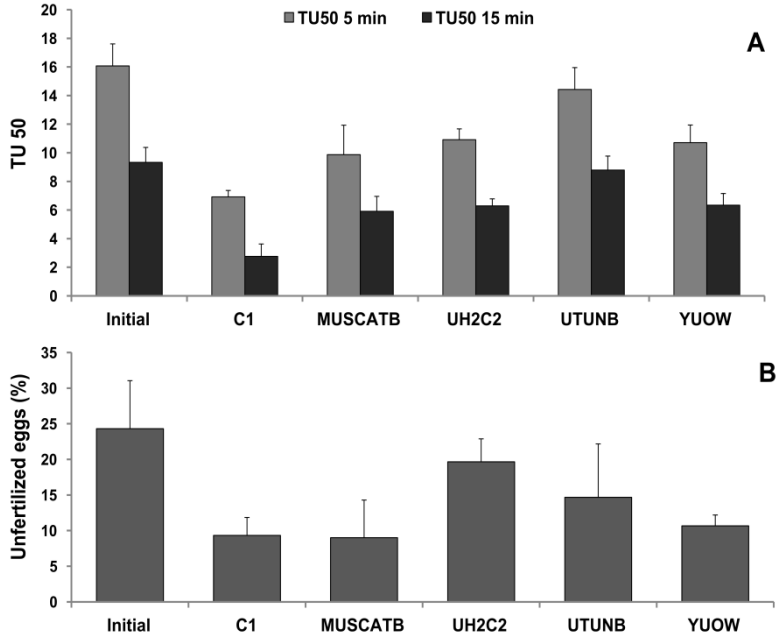

Figure 9 **Ecotoxicity assay results for the untreated sediment at the beginning of the experiment ("Initial") and for the sediment within treatments C1, MUCSATB, UH2C2, UTUNB and YUOW at the end of the experiment (experimental day 56). A: Microtox® assay, B: *P. lividus* spermiotoxicity assay. Bars represent the standard deviation.**

At the end of the incubations, results obtained through Microtox® assays showed a significant reduction of toxicity in all treatments compared to the untreated sediment (Initial) (Fig. 3A). In bioaugmented treatments, the best performance was obtained in MUSCATB, where the original toxicity of 16.08 TU50 and 9.33 TU50 was reduced, on the average, by 37.62%. The average reduction of toxicity was greater than 20% in all the bioaugmented treatments, with the only exception of UTUNB showing an average toxicity reduction of 7.98%. The samples collected from the negative control (C1) showed a greater toxicity reduction at the end of incubations compared with the bioaugmentated treatments (6.92 and 2.76 TU50 for 5 min and 15 min, respectively). The attenuation of bioluminescence was more evident after 5 min incubation than 15 min incubation in all samples. The spermiotoxicity assays on gamets of P. lividus showed a similar pattern with the Microtox® assays (Fig. 3B). The untreated (Initial) elutriates resulted in 24.31% of unfertilized eggs, whereas in treated sediments the percentage of unfertilized eggs ranged from 9.00% (MUSCATB) to 19.67% (UH2C2). Among the bioaugmented treatments, theUH2C2 displayed a significantly higher percentage of unfertilized eggs than MUSCATB and YUOW ($p<0.01$). These two treatments displayed the best performances in terms of fertilization rates among the bioaugmented ones, with an average of 9.83% of unfertilized eggs. However, the biostimulated control treatment (C1) resulted in the lowest percentage of unfertilized eggs (9.33% average value). Taken together, both assays demonstrated that toxicity at the end of the experiment in all bioaugmented treatments was lower compared to the untreated sediment; however, it was higher than in the biostimulated control C1.

3.3 Hydrolytic activity

Sediment hydrolytic activity (Fig. S2) showed a similar trend in all treatments except from C4 where it remained minimal throughout the experiment, and C2. The highest activity was observed at the beginning of the experiment (Day 0) and then a gradual decrease was evident until Day 14. After that, the activity gradually increased until Day 28 when a second peak was observed. Finally, the activity constantly decreased until the end of the experiment. The only exception to the overall trend was recorded in the case of C2, for which the hydrolytic activity peak was observed one week before all the other treatments. The difference in the observed enzymatic activity between C2 and the bioaugmented treatments indicates that unlike in treatment C2, the added allochthonous bacteria in the bioaugmented treatments were not dead at the point of their addition to the sediment, nor did they perish immediately after that point. All the above results indicate that the remediation process was rapid in all biostimulated treatments and was not enhanced by bioaugmentation. Most of the degradation occurred within the first experimental week with minor differences among the treatments. The total bacterial populations of bioaugmented treatments were lower compared to the controls and the trend in sediment hydrolytic activity was similar among all treatments afterthe first experimental week, while sediment ecotoxicity at the end of the experiment was higher in the bioaugmented treatments compared to the biostimulated control C1. Other studies performed in polluted sediment and soil have also reported a limited effect of bioaugmentation on the removal of hydrocarbons, sediment metabolic activity and bacterial population densities [3, 18, 19]. Moreover, in the present study we observed an increased ecotoxicity of the biostimulated and bioaugmented treatments compared to the biostimulated276only control C1. The toxic compounds present in the bioaugmented treatments at the end of the experiment have to be different from the residual hydrocarbons, since their concentration was similar within all the microcosm. There is, to our knowledge, no previous report with similar findings. We hypothesize that these compounds may had been released as a result of antagonism among the allochthonous and indigenous bacteria.

3.4 Biodegradation was mostly performed by the autochthonous populations

For monitoring bacterial community structure dynamics through time and partially identify the bacteria inhabiting the sediments, DGGE and ARISA were performed on the bioaugmented treatments (YUOW, UH2C2, MUCSATB and UTUNB), the N/P biostimulated control (C1) and the control corresponding to the original sediments that were neither biostimulated nor bioaugmented (C4). The cluster analysis of ARISA profiles identified four groups of samples showing less than 40% of similarity (Fig. 4A). A first cluster comprised all the sediment samples collected at Day 0, after the addition of the selected consortia, and the C4 samples collected after the first week of treatment. Excluding those belonging to treatment C4, all the samples collected at Week 1 formed a second cluster. The third group of samples was represented by all the samples analyzed after Week 4 and Week 6 excluding treatment C4, the latter forming the fourth identified cluster. The bacterial community of the sediments collected at Week 4 and 6were only partially distinguishable within these two groups. Overall, ARISA fingerprints clearly showed that the bacterial communities clustered according to the experimental time rather than to the addition or the identity of allochthonous consortia (Fig. 4A). The only exception from the observed trend was represented by the community in the non298biostimulated treatment C4. The ARISA results implied that biostimulation, rather than bioaugmentation, significantly influenced the temporal changes in bacterial communities (Fig. 4A).

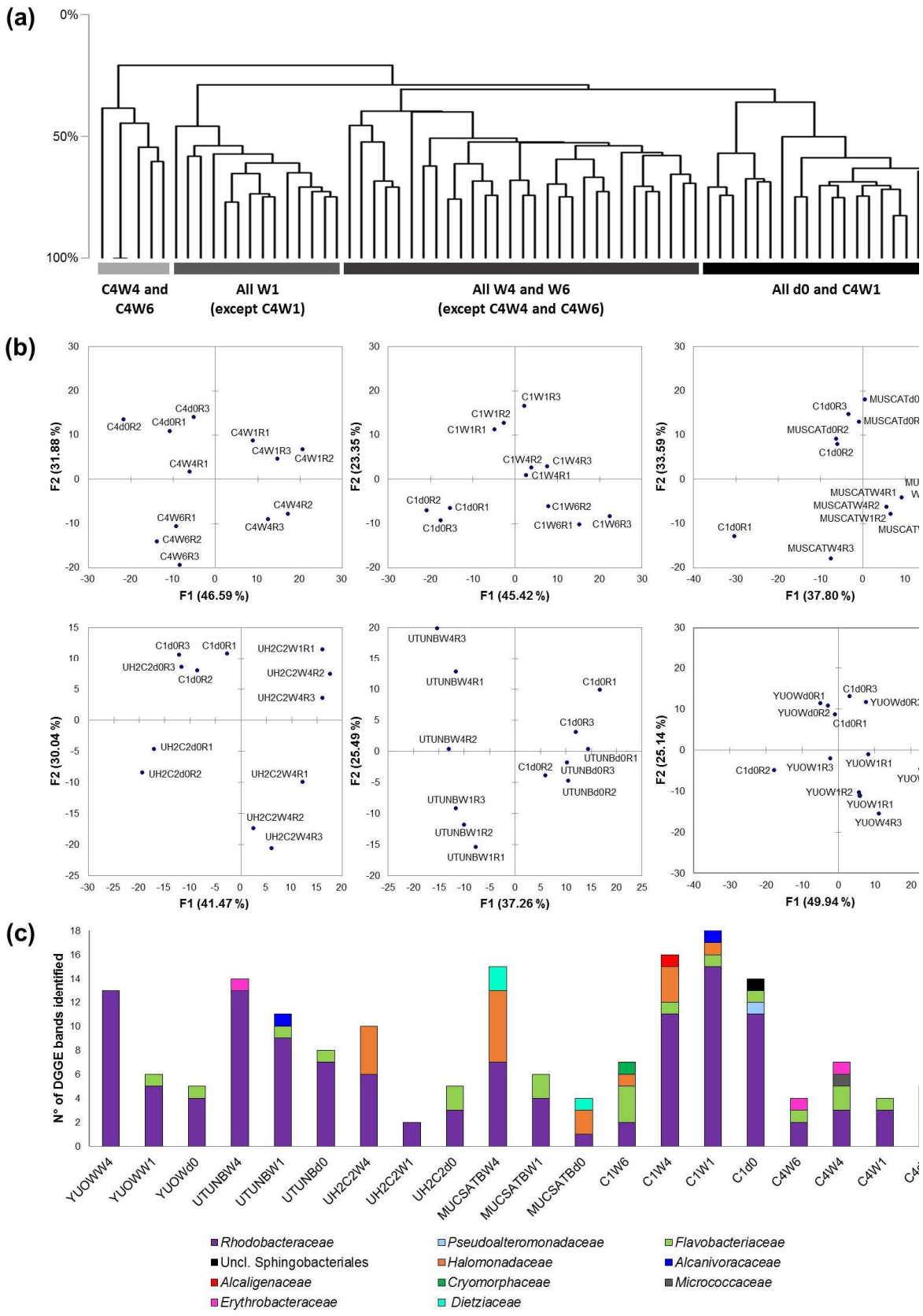

Figure 10 **Bacterial community fingerprinting results. (a) Cluster analysis based on theARISA fingerprints, (b) Principal Component Analysis based on the DGGE profiles of the16S rRNA amplified from the metagenome extracted from the sediments at different timepoints during the landfarming experiment. For each time point, the three analyzed replicatesare indicated as R1, R2 and R3, (c) Taxonomic identification of bacterial 16SrRNA sequences excised and amplified from DGGE bands.**

The DGGE analysis was performed up to Week 6 for control treatments C1 and C4 and up to Week 4 for the bioaugmented treatments. We aimed to directly compare the possible effect of the allochthonous inoculum addition on the overall structure of the bacterial microbiome, by visualizing on the same DGGE gel the profile of the inoculum before the addition, the profile of the bioaugmented sediment after the addition and that of C1 at Day zero. The PCA of the DGGE profiles of the sediments collected at each experimental time for each treatment, showed that, overall, the results obtained from the analytical replicates clustered together, demonstrating the reliability of the adopted technique (Fig. 4B). As previously observed by ARISA results, the sediments collected at Day 0 and Week 1 from the control treatment C4 were not significantly different, similarly to those collected at Week 4 and 6 (Fig. S3a; Fig. S3g). On the contrary, the DGGE profiles of the bacterial communities dwelling the biostimulated control C1 showed that the community structure significantly changed between Day 0 and Week 1 (Fig. S3b), a result confirmed by ANOVA (Fig. S3h) and shared by all the bioaugmented treatments, irrespectively of the applied consortium (Fig. S3i-n). The microbiological dataset supports the chemical and activity data, according to which the main hydrocarbon degradation activity occurred within Week 1, in correspondence to the detected change of the bacterial community composition. Moreover, ANOVA results showed that the C1 and the bioaugmented treatments were not significantly different in terms of community structure at Day 0 (Fig. S3i-n). Accordingly, the DGGE bands present in the DNA extracted from the bacterial consortia used for bioaugmentation were only rarely detected in the corresponding treated samples at Day 0 (Fig. 4C), suggesting a low capability of the selected bacteria to efficiently compete with the autochthonous bacterial populations for sediment colonization. DNA sequences were obtained from 192 DGGE bands, cut from the six DGGE gels, and their phylogenetic affiliation was identified in order to examine the bacteria inhabiting the polluted sediments at each treatment and time point (Table S1, Fig. 4C). The number of sequences retrieved for each treatment ranged between a minimal value of 17 (UH2C2) up to 55 in the case of control C1 (Fig. 4C) and the vast majority of them (122 out of 192 sequences) belong to the family Rhodobacteraceae (Table S1). The second and third most abundant taxonomic groups were Flavobacteria and Halomonadaceae, respectively. Most of the detected sequences are widely spread in marine sediments while members of well-known hydrocarbonoclastic bacteria (e.g. Alcanivorax genus) were rarely retrieved (Table S1). However, Rhodobacteraceae has been reported as key player of hydrocarbon bioremediation in beach sands impacted by the recent Deepwater Horizon Spill by Kostka et al. [20] who proposed this family as a taxonomic sentinel for the second step of oil remediation, when recalcitrant compounds are the primary oil constituents. Moreover, among the Rhodobacteraceae genera detected by DGGE in the analyzed sediments, Roseobacter comprises bacteria for which a relevant role in biodegradation has been reported [21]. Similarly the Halomonadaceae family encompasses oil-degrading bacteria [20, 22], including members of the Halomonas [23] and Cobetia [22] genera, also retrieved in the present study (Table S1). In addition, Flavobacteria, widely detected by DGGE, have been previously indicated to possibly include secondary users involved in the methane and oil degradation [24]. Taken together, the DGGE phylogenetic data suggested the involvement in hydrocarbon degradation, of a rich and diverse bacterial community comprising species that are poorly characterized compared to the generally abundant obligate hydrocarbonoclastic genera (e.g. Alcanivorax sp., Cycloclasticus sp.). We further examined the similarity in the distribution of the oil constituents that were present within each biostimulated treatment (i.e., C1, C2, UTUNB, MUCSATB, UH2C2 and YUOW) after Week 1, when most of the degradation had occurred. The comparison of the distribution of the remaining hydrocarbon compounds in the different treatments can explain if the degradation was performed by the same microorganisms. We observed that four out of the six treatments showed a similar compound distribution, with higher concentrations at the medium-chained alkanes (C22-C25, Fig. 5). On the other hand, the distribution of the compounds in treatments UH2C2 and YUOW was more uniform, without significant differences among different alkanes (Fig. 5). This observation suggests that, most probably, the added consortia in treatments UH2C2 and YUOW contributed significantly to the oil degradation, altering the distribution of the remaining compounds as compared to the rest of the treatments, but not the total amount of oil degraded. If that had occurred, then the allochthonous degraders must have performed and subsequently perished within the first week of treatment, as their populations were not detected by the end of that time. This is also supported by the detection of phylotypes related to second-step remediation by the end of the first week at all treatments. Several studies have

demonstrated the high degradation potential of the indigenous communities inhabiting polluted sediments and soils, when provided with additional nutrients and after regular tilling or other means of aeration [1, 3, 19, 25], while some others have suggested that the efficiency of bioaugmentation depends on the phylogenetic relation among the applied and indigenous consortia [5] and the initial community richness of the impacted habitat, with richer communities receiving limited benefits from bioaugmentation [11]. The strains used for bioaugmentation in this study showed a limited ability to colonize the sediment, a trait probably related to both the origin of the allochthonous consortia, that were previously isolated from different oil contaminated sediments collected from North Africa (Morocco, Tunisia, Egypt) and Red Sea (Aqaba, Jordan) shorelines, hence adapted to different environmental conditions, and to the high initial richness of the autochthonous community.

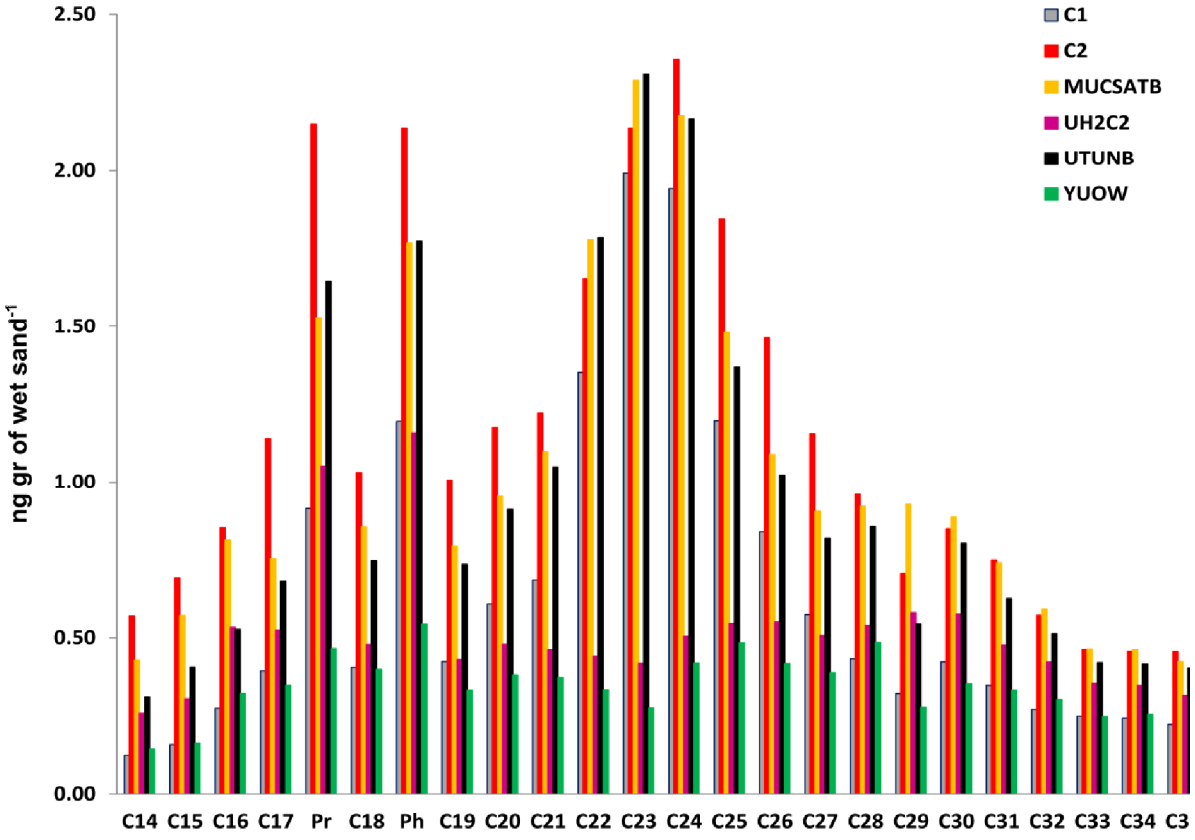

Figure 11 **The distribution of the remaining alkanes at the end of the first experimental week for each treatment. Pr: pristane, Ph: phytane.**

3.5 Engineering ramifications

The presented experimental results and the reached conclusions have significant ramifications in the management of oil-contaminated sediments. If the sediment originates from an area of chronic pollution or with intermittent / continuous seepage of even small quantities of petroleum hydrocarbons, the indigenous populations with suitable biostimulation should suffice for the ex situ treatment by landfarming. It should be noted that in such treatments, biostimulation is essentially mandatory as the N & P present within the sediments is not sufficient; it cannot be replenished by the sea as it occurs in the in situ treatment of sediments where oxygen is the primary limiting substrate [26]. The above approach can readily be adopted for polluted sites across the Mediterranean Sea, as recent studies have reported the presence of rich hydrocarbon degrading communities in the water column of even seemingly pristine areas in this region (see references in [27]).

4. CONCLUSIONS

Our results indicate that biodegradation was mostly performed by the autochthonous degraders, while bioaugmentation did not enhance the remediation process. The limited added effect of bioaugmentation likely reflects the inability of the applied consortia to effectively colonize the sediment. That, in turn, could be attributed to the origin of the consortia and to the presence of a rich autochthonous community at the contaminated site. According to our data, when environmental conditions are optimized using an efficient ex-situ treatment such as landfarming combined with biostimulation, the indigenous microbiome at a polluted site will likely outperform any allochthonous consortium.

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Chapter

IX

GENERAL CONCLUSION AND FUTURE PERSPECTIVES

The Mediterranean Sea is a delicate environment constantly exposed to high load of hydrocarbon pollution, which has consequences exasperated by its physical characteristics that do not allow for a rapid pollutant dispersion. Despite the efforts undertaken since 1975 by the countries surrounding the Mediterranean basin, several problems concerning the remediation of hydrocarbon polluted environments are still present due to the fact that the strategies nowadays available to achieve this purpose are not completely effective. Therefore, the need for innovative technologies, cheaper and safer than conventional methods, arose and bioremediation strategies can be the answer. Despite its great potentiality, routine application of bioremediation to clean-up hydrocarbon polluted marine areas is still far. To improve bioremediation strategies, more adapted and more efficient microorganisms are indeed needed. The Mediterranean Sea, hosting both a very high microbial diversity (Coll et al, 2010) and many chronically polluted areas (UNEP/WHO, 1999) could be in this context a great resource, as source of novel and useful microorganisms, possibly exploitable for site-tailored bioremediation strategies.

During this PhD thesis, the bacterial diversity and biogeography of the whole Mediterranean Sea with a particular focus on oil contaminated niches, were explored with the aim to uncover the great diversity of bacterial population and correlating it with geographical and environmental characteristics. The composition of the bacterial communities described across the water columns at different sampling stations has been significantly correlated on their geographical position and depth, but also on temperature and salinity. Furthermore, the analysis of the total bacterial populations inhabiting two chronically highly polluted areas, - namely El-Max district (Alexandria, Egypt) and the Ancona harbor (Northern Adriatic Sea, Italy) – demonstrated the influence of different pollutants in the selection of peculiar bacterial assemblages. In both the cases the concentration of hydrocarbons and heavy metals was among the major driver of the composition of the sediments dwelling bacterial communities at these sites.

A huge collection of bacterial strains was also established from the different Mediterranean sites, comprising more than 250 hydrocarbonoclastic isolates. In spite of the observed high diversity of bacterial communities inhabiting the water of the Mediterranean Sea, the identification of the isolated hydrocarbonoclastic strains revealed a low diversity level, being largely dominated by the *Alcanivorax* and *Marinobacter* genera. These are well known ubiquitous marine hydrocarbonoclastic bacteria, reported as the first populations blooming immediately after marine oil spills and responsible for the degradation of aliphatic hydrocarbons (Head et al, 2006).

The bioremediation potential of these isolates was evaluated through both cultivation-dependent and molecular analyses. More than 170 strains affiliated to the genus *Alcanivorax* isolated from sites located in the whole Mediterranean Sea, from both waters and sediments were analyzed in more detail. This sub-collection comprised four bacterial species: *A. borkumensis*, *A. jadensis*, *A. dieselolei* and *A. venustensis*, which diversity was analyzed through molecular fingerprinting techniques, highlighting the occurrence of geographical divergences also for those strains belonging to the same species. The functional diversity of the strains was investigated through molecular fingerprinting applied on the *alkB* gene, encoding for an alkane monooxigenase, involved in the first step of hydrocarbons degradation (van Beilen et al., 2003). Its investigation within the *Alcanivorax* collection confirmed the existence of a high degree of geographical divergence, suggesting a potentially high metabolic diversity that could be exploited for site-tailored bioremediation interventions. The cultivation approaches adopted to enrich and isolate hydrocarbonoclastic bacteria from chronically polluted area, like the Ancona harbor, permitted, moreover, to evaluate the influence of different hydrocarbon pollutants (crude oil, diesel oil and naphthalene) used as single carbon source in the selection of specific marine bacteria populations. The results obtained taking advantage of Denaturing Gradient Gel Electrophoresis (DGGE) fingerprinting showed that under laboratory conditions the supply of different hydrocarbon compounds led to the selection of different, and specialized, bacterial

communities highlighting that the nature of the contamination is potentially able to stimulate the growth of bacterial populations with specific degrading potential.
The awful accident of Deepwater Horizon occurred on 2010 in the Gulf of Mexico, and the subsequent huge oil spill occurred at a depth of 1500 meters, unveiled the need to get more insight on the bioremediation processes specifically occurring at high depth. Most of the actual knowledge we have about oil degrading microbes is in fact based upon studies conducted at ambient pressure, and there is a lack of knowledge about the expression profiles of degrading bacteria at high depth. With the aim of contributing to cover this gap, the capability of four strains belonging to different hydrocarbonoclastic species (*A. borkumensis*, *A. jadensis*, *A. dieselolei* and *M. hydrocarbonoclasticus*) to adapt, grow and degrade hydrocarbons under different pressure conditions – setup to simulate different depths –was evaluated. This investigation pinpointed that the tested bacteria can survive at high hydrostatic pressures, even though both their growth and degradation capability were mostly inhibited as the hydrostatic pressure increased. The evidences obtained in the ambit of this study on the strains' physiological activities will be coupled with transcriptomic data in the future to individuate those genetic pathways involved in the mechanisms of adaptation and degradation active at high depth.
In the last years a new bioaugmentation strategy named autochthonous bioaugmentation (ABA) has been proposed (Nikolopoulou et al., 2013). ABA consists in the addition of autochthonous bacteria, previously enriched under laboratory conditions, to the polluted environment to be treated. This strategy allows to bypass the issues due to the overall scarce adaptability of allochthonous strains to new natural environments. To accomplish with the aim of ABA it is necessary to depict the structure of microbial communities present in the polluted environments and to be able to enrich and cultivate the bacteria involved in the pollutant's remediation. In this perspective a sub-collection of hydrocarbon degrading bacterial strains isolated from El-Max district and Ancona harbor sediments was screened for the potential exploitation in ABA strategies, by testing i) the degradation of different hydrocarbons pollutants (xylene, octane, pyrene, dibenzothiophene, phenantrene, naphthalene and crude oil), ii) the presence of genes involved in heavy metal resistance, iii) the production of biosurfactants molecules and iv) biofilm formation. The ability to tolerate heavy metals and metalloids is an important feature, since these pollutants are components of crude oil and can be found often as co-contaminants in environments subjected to oil spills (Abha & Singh, 2012). The ability to produce biosurfactants and to form biofilm can provide competitive advantages to marine hydrocarbonoclastic bacteria, increasing the bioavailability of hydrocarbon molecules. About half of the analyzed strains were proved to be good candidates for ABA, since they demonstrate both the capability to degrade several hydrocarbon molecules and to posses genes for heavy metals resistance. Furthermore, several bacteria in the analyzed collection were able to produce biosurfactants and to adhere to hydrocarbons substrates, while few could also grow as biofilm.
Within *ex situ* bioremediation strategies, landfarming is one of the most efficient and it is widely applied. The degradation of pollutants in landfarming set-up can be achieved with both biostimulation and bioaugmentantion, which specific contribute to remediation efficacy needs still to be investigated. During this thesis, a landfarming experiment was performed on the chronically polluted sediments collected at Elefsina bay (Greece). Different allochtonous consortia and a fertilizing solution were provided to the sediments and both oil degradation and the changes in the structure of sediment bacterial community were monitored, aiming to infer which strategy was the most efficient in improving the removal of hydrocarbons pollutants. The addition of the allochtonous consortia did not result in significant differences in terms of pollutants degradation. Moreover, the addition of allochtonous bacteria did not affect the indigenous bacterial community, since the inoculated bacteria quickly perished. These results indicated that biodegradation was mostly performed by the autochthonous populations while bioaugmentation, in contrast to biostimulation, did not enhance the remediation process. The optimization of the occurring environmental conditions is therefore a fundamental step to improve bioremediation of hydrocarbon polluted environments to achieve an effective autochthonous microbial resource management approach.
Overall, the result presented in this thesis confirmed the Mediterranean Sea as a very promising reservoir of microorganisms displaying potentiality in oil bioremediation processes, even though further studies still need to be undertaken to optimize and apply this valuable technology in the field.

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Appendix

SUPPORTING INFORMATION

Allochthonous bioaugmentation in *ex-situ* treatment of crude oil Polluted sediments in the presence of an effective degrading Indigenous microbiome

1. Origin and isolation of hydrocarbon-degrading consortia

Consortium MUCSATB was isolated by enrichment of sediment collected in June 2011 from El-Max Bay, Egypt (31° 8'52.62"N, 29°50'16.20"E) in the Mediterranean Sea. The water depth was 1.5 m. Enrichment was performed in sterile artificial sea water (ASW) medium (g L-1: NaCl, 27; MgSO4.7H2O, 6.6; MgCl2.2H2O, 6.5; CaCl2.2H2O, 1.5; KNO3, 1.0; NaHCO3. 0.04; Tris-HCl buffer (1.0 M, pH 7.0), 20 ml; chelated ion solution 1.0 ml composed of: (FeCl3.4H2O, 240 mg 100 ml-1, EDTA, 14.6 g 100 ml-1) and Trace metal solution, 1.0 ml - trace metal solution composition was as following per mg 100 ml-1: H3BO3, 60; MnCL2.4H2O, 40; (NH4)Mo7O24.4H2O, 37; CuCl2.2H2O, 4; ZnCl2, 4; CoCl2.6H2O, 1.5). with the addition of 1% crude oil as the sole carbon source. The enrichment was performed by inoculating 100 ml ASW broth amended with 1 % crude oil with 1 g of sediment. The flasks were incubated at 30 oC in a rotary shaker. Grown culture was collected after two successive reinoculations performed weekly. Consortium UH2C2 was isolated from hydrocarbon contaminated sediment samples at Morocco in February 2012 from Mar Chica lagoon (35°08'52"N, 2°50'53"W) on nutrient agar plates (BioRad). Consortium UTUNB was formed by equally mixing eight different strains that had been previously isolated from hydrocarbon contaminated sediment and rock biofilm samples at Bizerte, Tunisia (37°16'08.9"N, 9°53'20.1"E) on Bushnell Hass Mineral Salts (BHMS) medium (g L-1: KH2PO4, 1; K2HPO4, 0.2; MgSO4.7H2O, 0.2; CaCl2, 0.02; NH4NO3, 1; and 2 droplets of FeCl3 60%) and on artificial seawater mineral (MMC) medium (g L-1: NaCl, 24; MgSO4·7H2O, 9.0; NH4NO3, 1; KCl, 0.7; K2HPO4, 0.2; 10 ml trace element stock solution 100× concentrate and 10 ml Ferrous stock 100× concentrate). The pH was adjusted to 7.4 and the two media were supplemented with 1% crude oil as the sole carbon source. Consortium YUOW was isolated from hydrocarbon contaminated sediment adjacent to an oil refinery unit at Aqaba Gulf, Jordan, (29°22'97.4"N, 34°57'84.1"E) through enrichment in Stanier's minimal medium [1] supplemented with 1% w/v crude oil. Upon arrival to the lab, all consortia were acclimated by incubation for two weeks at sterile sediment slurry spiked with crude oil at a final concentration of 0.5% w/w.

2. Soxhlet solid-liquid extraction

Soxhlet extraction, i.e. a form of solvent extraction of solid samples commonly known as solid–liquid extraction, was used for TPH extraction from the sediment. Sediment samples (~15 gr) were transferred in Whatman extraction thimbles after the addition of an appropriate amount of Na2SO4 (~30 gr) to absorb the moisture content, and were spiked with 200 µl of a 200 ppm surrogate standard solution containing a-androstane and antrhracene_d10 standards (Absolute Standards). The extraction thimbles carrying the samples were placed in the thimble-holder of the soxhlet apparatus that during operation was gradually filled with condensated fresh solvent (DCM) from a distillation flask. When the liquid reached the overflow level, a siphon aspirated the solute of the thimble-holder and unloaded it back into the distillation flask, carrying the extracted analytes into the bulk liquid. This operation was repeated until complete extraction was achieved (overnight). The extracts were concentrated using a rotary evaporator to remove the DCM solvent, weighted, and loaded into the SPE columns.

3. Solid Phase Extraxtion

The Bond Elute Tph, SPE columns (Agilent Technologies) were pre-conditioned with 2 ml n-hexane (SupraSolvÒ, Merck). The Soxhlet extracted samples were loaded onto the column with the washings (using the minimum amount of n-hexane), the saturated components were eluted with 4 ml n-hexane (SupraSolvÒ , Merck), and the aromatic hydrocarbons were eluted with 4 ml DCM (SupraSolvÒ, Merck). The saturated and aromatic hydrocarbons fractions were collected, then dried under a nitrogen stream and finally diluted in 1 ml n-hexane and DCM respectively. Five µl of the internal standard (IS) -a 200ppm in n-hexane mixture of deuterated components (d8-naphthalene, d10-phenanthrene d12-chrysene, d12-perylene,

Absolute Standards)-was added in the samples, and the mixture was analyzed by gas chromatography/mass spectrometry (GC-MS).

4. Gas chromatography-mass spectroscopy (GC-MS) analysis

The GC-MS analysis was performed using an Agilent GC-MS HP 7890/5975C system, with an Agilent HP-5 5% phenyl methyl siloxane column (30 cm x 250μm x 0.25 μm) (Agilent Technologies). The initial oven temperature was set at 60 °C, followed by a temperature ramp of 6 °C/min up to 300 °C. The samples (1 μl) were injected through a split-splitless injector (pulsed-splitless mode, at 250 °C) diluted (1/200) in ultra-pure hexane (SupraSolv , Merck). The carrier gas used was helium. The transfer line, MS source and quantrupole temperatures were set at 280 oC, 230 oC and 150 oC respectively. The analysis was carriedout in Single Ion Detection (SIM) mode. The system was quantitatively calibrated using a hydrocarbon mixture containing n-C10_n-C35 normal alkanes and the 16 EPA priority aromatic hydrocarbons (naphthalene, phenanthrene, anthracene, fluorene, dibenzothiophene, fluoranthene, pyrene, chrysene, benzo(b)fluoranthene, benzo(k)fluoranthene, benzo(e)pyrene, benzo(a)pyrene, perylene, indeno(g,h,i)pyrene, dibenzo(a,h)anthracene, benzo(1,2,3-cd)perylene) and dibenzothiophene) (Absolute Standards). A mixture of deuterated components (d8-naphthalene, d10-phenanthrene d12 chrysene, d12-perylene) (Absolute Standards) was used as internal standard (IS) spiked in the samples (1 ppm). The saturated hydrocarbons fraction were injected diluted in 1 ml ultra-pure n-C6, and the aromatic components fraction diluted in 1ml ultra-pure (DCM), while both were spiked with the IS to 1ppm concentration (5 μl of a 200 ppm IS stock solution). The aliphatic (C10-C35), and the aromatic hydrocarbon components were quantified against the internal standard using an assumed response factor of 1 and taking into account the recoveries measured from the surrogate standard (e.g., a surrogate standard recovery of 90% was used to correct the analyte concentrations measured by multiplying with 1.11), and the concentration of the 17a21b_hopane that accounts for the degradation from all other possible degradation paths except the biodegradation.

5. Bacterial colony forming unit (CFU) estimation

Bacterial CFU estimation at the beginning of the experiment was performed on Zobell Marine Agar plates (HiMedia, Mumbai, India). One g of sediment was diluted in 50 ml of autoclaved PBS solution (0.138 M NaCl, 0.0027 M KCl, pH 7.4, Sigma-Aldrich, U.S.A.) and was sonicated for 10 min. at 42 kHz in a Branson 2510 sonicator (Branson, U.S.A.). Spreading was performed in triplicates using 100 μl of the resulting solution. Colonies were counted after overnight incubation at 30 °C. The same procedure (except from the sonication stage) was followed for total bacterial CFU estimation of each consortium per ml of liquid culture before adding it to the sediment at the beginning of the experiment.

6. Flow cytometry analysis

Fixed sediment (5 gr per sample) was firstly diluted in Tween 20 (Sigma Aldrich, 0.5% final concentration) and sodium pyrophosphate (Sigma Aldrich, 1 g l-1 final concentration). Afterwards, vigorous and manual shaking and then sonication for 3 min. followed. Sonicated sediment samples were centrifuged at 800 g for 1 min. Approximately one ml of the resulted supernatant was kept for later flow cytometric analysis. Analysis was performed in a FACS Calibur flow cytometer (Beckton Dickinson) equipped with an air-cooled laser at 488 nm and standard filters. Samples were diluted 50x, 80x or 100x in previously autoclaved and 0.2 μm-filtered Tris-EDTA buffer solution (TE, 10 mM Tris and 1 mM EDTA, pH=8.0) and run at medium or high speed for 1 min. The exact flow rate of high and medium speed performance of the instrument was measured on a daily basis. Staining was conducted with SYBR Green I nucleic acid dye (Molecular Probes) in a final dilution 4x10-4 of the stock solution, followed by incubation for 15 min. in the dark at room temperature. One-μmdiameter fluorescent beads (Polysciences) were added in the stained sample immediately before analysis as internal standard. Bacterial abundance was then calculated using the acquired cell counts and the respective flow rate. All flow cytometry data were gathered and processed with the Cell Quest Pro software.

7. Sediment total hydrolytic activity

One gr of sediment (in triplicates) was incubated in 50 ml sodium phosphate buffer 60 mM , pH 7.6, for 3 h at 37 °C in the dark under horizontal agitation at 150 rpm. Two ml of acetone were added after the incubation to terminate the FDA hydrolysis and the solution was centrifuged at 8000 g for 5 minutes. The solution was then filtered through filter paper (Schleicher & Schuell, Dassel, Germany) and the filtrate's absorbance was measured at 490 nm on a UV mini 1240 (Shimatzu, Duisburg, Germany). All measurements were performed in triplicates. Controls were performed following the same procedure, but using 1 g of autoclaved sediment instead. The fluorescein standard curve (R2 = 0.996) was made using solutions of 0.03, 0.1, 0.3 and 0.5 mg fluorescein in 52.5 ml total volume (50 ml sodium phosphate buffer 60 mM, 2.5 ml acetone). Results were expressed as µg fluorescein g-1 soil 3 h-1. The associated graph was performed using Microsoft Excel.

8. Sediment ecotoxicity

Sediment elutriates were prepared by mechanically shaking (through a multi-wrist shaker, 500 rpm) 60 g sediment in 240 ml of filtered sea water (pore size 0.2 µm) collected in a pristine marine area (ratio 1:4 dw/v). After a 1 h mixing period, samples were allowed to settle for 24 h, then supernatant was filtered (pore size 0.45 µm) and used for bioassays within 24 h. For Microtox® tests, lyophilized Vibrio fischeri bacteria (Lot Number 13A4014) and Microtox® reagents were obtained from Modern Water Inc. New Castle, DE 19720 USA. Microtox testing was performed according to the standard procedure recommended by the manufacturer. The reconstituted bacteria were exposed to sediment elutriates using the 90% Basic Test [2]. Nine different dilutions were tested. The decrease of bioluminescence was used to calculate the elutriate dilution (in %) resulting in a 50% decrease in light output after 5 and 15 min of exposure (EC50). In order to ensure the validity of tests, a basic test for Phenol (reference toxicant) was run. Results were expressed as Toxic Units 50 (TU50, dimensionless), calculated as 100/EC50. For P. lividus egg fertilization assay, specimens were collected from a pristine coastal area and carried in laboratory under controlled conditions (temperature 18 °C, salinity 37‰, pH 8.00-8.20 and dissolved oxygen >90%). The specimens were induced to spawn by injection of 1 ml of KCl 0.5 M. Gametes were then screened under microscope, in order to select optimal males and females for the execution of tests. The sperm pool was dry-stored at 4 °C while the eggs were gently washed in filtered seawater and stored at 18 °C. All tests on sea urchin gametes were conducted within 1 h from collection. Dry sperm was diluted in sediment elutriates at a ratio of 1:1000 and incubated for 60 min. at 18 °C. At the end of the incubation time, a diluted sperm aliquot was added to untreated eggs in filtered sea water (sperm-egg ratio of 15000:1). The gametes were then transferred in 10 ml multiwell dishes and incubated for 30 min at 18°C, in the dark, in order to allow fecundations [3]. After incubation, the solutions were fixed by adding 1 ml of 40% formalin. The percentage of the fecunded eggs were estimated by microscope observations, dedicated to the detection of fecundation membranes on 200 eggs per replicate (3 replicates were observed for each sample). Filtered sea water was used as negative control. A positive control was prepared using Copper nitrate at increasing concentrations (24, 48, 60, 72 µg l-1). Tests were considered valid if the percentage of fertilization in the negative control was =80%, and if the values obtained in the positive control fell into the range reported in Arizzi Novelli et al (2002) [3]. Results are expressed as % of unfertilized eggs.

9. DGGE and ARISA

For DGGE, PCR products (~150 ng) were loaded in a 0.5 mm polyacrylamide gel (7% [w/v] acrylamide-bisacrylamide, 37.5:1) containing 40 to 60% urea-formamide denaturing gradient (100% corresponds to 7M urea and 40% [v/v] formamide). The gels were run for 15 h at 60 °C by applying a constant voltage of 90 V in 1X Tris-acetate-EDTA (TAE) buffer. After electrophoresis, the gels were stained for 30 min in 1X TAE buffer containing 1X SYBR Green (Molecular Probes, Leiden, the Netherlands) according to manufacturer's instructions and rinsed twice for 10 min with distilled water. Gels images were captured using a Gel Doc 2000 apparatus (Bio-Rad, Milan, Italy). The band patterns of DGGE gels were analysed using Image J software (available for free download at http://rsb.info.nih.gov/ij/) and Microsoft Excel XLSTAT software (Addinsoft Inc.,New York, NY, USA) as previously described [4]. DGGE bands were excided from the gels with a sterile scalpel and eluted in 50 µl of sterile Milli-Q water at 37 °C for 4 h.

Subsequently, 8 µl of eluted DNA was amplified by PCR using primers 357F and 907R as described in the main text. Positive amplifications were partially sequenced by Macrogen Inc., Korea (http://www.macrogen.com/) using the primer 357F. ARISA fragments were separated using the ABI3730XL genetic analyzer applying the internal standard 1200-LIZ (Macrogen, Korea). The output peak matrix was transferred to Microsoft Excel for the following data elaboration and statistical analysis, realized according to the procedure previously described elsewere [5].

10. Supplementary tables

Table S1 Taxonomic identification of bacterial 16SrRNA sequences excised and amplified from DGGE bands indicated on Figure S3. The sample name consists of the sample code, followed by the experimental time of origin and the replicate number.

BAND	SAMPLE	CLOSEST RELATIVE; ACCESSION NUMBER	%	CLOSEST DESCRIBED SPECIES; ACCESSION NUMBER
131-UT	C1d0R1	Phaeobacter sp.; KC295369	99	Phaeobacter arcticus; KF193971
132-UT	C1d0R1	Rhodobacteraceae bacterium; DQ486500	99	Phaeobacter gallaeciensis; JX890287
35-C1	C1d0R1	Unc. bacterium; JQ347384	99	Roseobacter denitrificans; KF444162
61-C1	C1d0R1	Unc. bacterium; HQ010558	99	Sulfitobacter dubius; HQ908665
127-UT	C1d0R2	Unc. proteobacterium; JQ218665	99	Pseudoalteromonas mariniglutinosa; AB257337
11-C1	C1d0R2	Unc. Persicivirga sp.; HM140662	98	Sulfitobacter dubius; KC534303
34-C1	C1d0R2	Unc. bacterium; JQ178992	98	Antarctobacter heliothermus; NR_026406
47-C1	C1d0R2	Unc. marine organism; HM485043	97	Lewinella nigricans; EU371936
89-Y	C1d0-R2	Unc. bacterium; HE979608	99	Olleya aquimaris; NR_104531
61-Y	C1d0-R2	Thalassobius sp.; JX304651	99	Roseobacter denitrificans; KF928340
63-Y	C1d0-R3	Unc. proteobacterium; JF980892	99	Phaeobacter gallaeciensis; JX890287
9-C1	C1d0R3	Bacterium RCC; HQ871846	99	Staleya guttiformis; AB607871
10-C1	C1d0R3	Unc. bacterium; JQ387175	98	Loktanella salsilacus; AB681825
59-C1	C1d0R3	Unc. Roseobacter sp.; JX531103	99	Sulfitobacter pontiacus; KC534156
16-C1	C1W1R1	Unc. organism; JN535174	100	Roseovarius tolerans; DQ915626
36-C1	C1W1R1	Unc. bacterium; JQ732008	99	Roseovarius nubinhibens; DQ915625
38-C1	C1W1R1	Unc. Roseobacter sp.; JX531218	99	Sulfitobacter dubius; KC534303
43-C1	C1W1R1	Unc. Roseobacter sp.; JX531218	100	Sulfitobacter dubius; KC534303
4-C1	C1W1R2	Cobetia sp.; KF560356	100	Cobetia amphilecti; KF470999
17-C1	C1W1R2	Unc. bacterium; JN874186	99	Ruegeria lacuscaerulensis; HQ908705
37-C1	C1W1R2	Phaeobacter sp.; KC250896	100	Leisingera methylohalidivorans; CP006773
39-C1	C1W1R2	Unc. Roseobacter sp.; JX531403	100	Sulfitobacter dubius; HQ908665
40-C1	C1W1R2	Unc. bacterium; JX022656	99	Roseovarius pacificus; KC593284
42-C1	C1W1R2	Sulfitobacter sp.; FR821224	99	Staleya guttiformis; AB607871
48-C1	C1W1R2	Unc. Roseobacter sp.; JX531103	100	Sulfitobacter pontiacus; KC534156
14-C1	C1W1R2	Thalassospira profundimaris; KC420685	100	Thalassospira profundimaris; KC420685
76-C1	C1W1R3	Unc. Flavobacteriaceae sp.; JX529623	99	Mesonia algae; AB681172
49-C1	C1W1R3	Unc. Alcanivorax sp.; JX531430	96	Alcanivorax borkumensis; FJ218422
13-C1	C1W1R3	Roseobacter sp.; EU374898	100	Ruegeria atlantica; KF009868
15-C1	C1W1R3	Unc. marine bacterium; KF185876	98	Phaeobacter caeruleus; KC176242
41-C1	C1W1R3	Sulfitobacter sp.; KC689803	99	Sulfitobacter guttiformis; JQ675546
44-C1	C1W1R3	Unc. Roseobacter sp.; JX531103	100	Sulfitobacter pontiacus; KC534156
6-C1	C1W4R1	Burkholderiaceae bacterium; AB376637	97	Pigmentiphaga daeguensis; HQ848138
29-C1	C1W4R1	Unc. Alphaproteobacterium; GU565588	99	Huaishuia halophila; FJ436725
31-C1	C1W4R1	Unc. bacterium; JX022656	99	Roseovarius pacificus; KC593284
54-C1	C1W4R1	Unc. bacterium; KC527503	99	Poseidonocella sedimentorum; AB576006

BAND	SAMPLE	CLOSEST RELATIVE; ACCESSION NUMBER	%	CLOSEST DESCRIBED SPECIES; ACCESSION NUMBER
2-C1	C1W4R2	Burkholderia sp.; GQ176352	100	Halomonas neptunia; KC354707
3-C1	C1W4R2	Unc. bacterium; KC307472	100	Halomonas sulfidaeris; JX860259
18-C1	C1W4R2	Unc. Roseobacter sp.; EU374898	99	Ruegeria pelagia; EU694390
19-C1	C1W4R2	Unc. Roseobacter sp.; EU374898	99	Ruegeria pelagia; EU694390
20-C1	C1W4R2	Unc. Roseobacter sp.; EU374898	100	Ruegeria atlantica; KF009868
23-C1	C1W4R2	Unc. marine bacterium; GU235354	100	Citreicella aestuarii; FJ230833
65-C1	C1W4R3	Unc. Flavobacteriaceae sp.; JX529623	100	Mesonia algae; AB681172
1-C1	C1W4R3	Halomonas sp.; FR695479	100	Halomonas alimentaria; FJ161295
21-C1	C1W4R3	Roseobacter sp.; EU374898	100	Ruegeria atlantica; KF009868
22-C1	C1W4R3	Unc. organism; JN535174	100	Roseovarius tolerans; DQ915626
27-C1	C1W4R3	Unc. bacterium; KF799896	99	Leisingera methylohalidivorans; CP006773
28-C1	C1W4R3	Rhodobacteraceae bacterium; FN811289	99	Marinovum algicola; AB636140
66-C1	C1W6R1	Unc. Flavobacteriaceae; JX529623	100	Mesonia algae; AB681172
5-C1	C1W6R1	Halomonas sp.; KF669533	99	Halomonas glaciei; HQ623152
24-C1	C1W6R1	Unc. organism; JN535174	100	Roseovarius tolerans; DQ915626
63-C1	C1W6R2	Galbibacter marinus; NR_108285	97	Galbibacter marinus; NR_108285
67-C1	C1W6R2	Unc. bacterium; JX864195	99	Salegentibacter salinarum; NR_044312
64-C1	C1W6R3	Unc. bacterium; HM468038	99	Brumimicrobium glaciale; NR_025255
52-C1	C1W6R3	Unc. Rhodobacteraceae; DQ870525	100	Ruegeria atlantica; KF009868
36-C4	C4d0R1	Gaetbulibacter sp.; JX235670	97	Flavivirga jejuensis; HM475139
8-C4	C4d0R1	Bacterium CSR-61; KJ018064	98	Tateyamaria pelophila; AJ968651
27-C4	C4d0R1	Rhodobacteraceae bacterium; HE962517	100	Roseobacter denitrificans; KC703232
20-C4	C4d0R3	Unc. Actinobacteridae; KF182999	99	Arthrobacter equi; KF387690
35-C4	C4d0R3	Gaetbulibacter sp.; JX235670	98	Bizionia paragorgiae; HQ424860
1-C4	C4W1R1	Roseobacter denitrificans; KF928340	99	Roseobacter denitrificans; KF928340
32-C4	C4W1R2	Unc. bacterium; KF624160	99	Mesoflavibacter zeaxanthinifaciens; JF800672
5-C4	C4W1R2	Unc. proteobacterium; JF980892	99	Roseovarius crassostreae; NR_041731
2-C4	C4W1R3	Unc. Alphaproteobacterium; AY663902	98	Maritimibacter alkaliphilus; AB681686
14-C4	C4W4R2	Rhodobacteraceae bacterium; AM990872	98	Roseobacter denitrificans; KF928340
19-C4	C4W4R3	Arthrobacter sp.; KF999724	98	Arthrobacter equi; KF387690
31-C4	C4W4R3	Unc. bacterium; KF624160	99	Mesoflavibacter zeaxanthinifaciens; JF800672
34-C4	C4W4R3	Unc. bacterium; FJ716921	99	Lutibacter aestuarii; NR_108995
12-C4	C4W4R3	Unc. bacterium; KF886113	98	Sagittula stellata; KC534315
13-C4	C4W4R3	Unc. bacterium; JX022656	99	Roseovarius pacificus; KC593284
11-C4	C4W4R3	Unc. bacterium; KF886121	99	Erythrobacter vulgaris; KC462903
18-C4	C4W6R1	Bacterium BW4PhS17; KC012900	98	Erythrobacter vulgaris; KC462903
15-C4	C4W6R2	Roseobacter sp.; EU374898	100	Ruegeria atlantica; KF009868
33-C4	C4W6R3	Unc. bacterium; FJ716921	98	Lutibacter aestuarii; NR_108995
17-C4	C4W6R3	Unc. bacterium; JX022656	100	Roseovarius pacificus; KC593284
27-M	MUCSATBd0R1	Dietzia maris; KF410336	99	Dietzia maris; KF410336
21-M	MUCSATBd0R1	Cobetia amphilecti; KF470999	95	Cobetia amphilecti; KF470999

BAND	SAMPLE	CLOSEST RELATIVE; ACCESSION NUMBER	%	CLOSEST DESCRIBED SPECIES; ACCESSION NUMBER
23-M	MUCSATBd0R1	Unc. bacterium; GU062055	100	Paracoccus kamogawaensis ; AB275604
20-M	MUCSATBd0R3	Cobetia amphilecti ; KF470999	97	Cobetia amphilecti ; KF470999
32-M	MUCSATBW1R1	Citreicella sp.; AB266065	99	Citreicella aestuarii ; FJ230833
37-M	MUCSATBW1R2	Salegentibacter sp. ; EU823291	99	Mesonia algae; AB681172
29-M	MUCSATBW1R2	Unc. bacterium; JN874186	98	Ruegeria atlantica; KF009868
38-M	MUCSATBW1R3	Mesonia algae; AB681172	99	Mesonia algae; AB681172
11-M	MUCSATBW1R3	Paracoccus sp. ; AB362258	99	Paracoccus aminophilus; AB681111
12-M	MUCSATBW1R3	Rhodovulum sp. ; FJ161259	99	Paracoccus siganidrum ; JX398976
24-M	MUCSATBW4R1	Dietzia maris; KC747472	99	Dietzia maris; KC747472
13-M	MUCSATBW4R1	Halomonas sulfidaeris; JX860259	100	Halomonas sulfidaeris; JX860259
14-M	MUCSATBW4R1	Halomonas titanicae ; KC420683	100	Halomonas titanicae ; KC420683
25-M	MUCSATBW4R2	Dietzia maris; KF410336	100	Dietzia maris; KF410336
05-M	MUCSATBW4R2	Unc. bacterium; KF708966	100	Halomonas sulfidaeris; JX860259
06-M	MUCSATBW4R2	Halomonas boliviensis; JX262399	100	Halomonas boliviensis; JX262399
09-M	MUCSATBW4R2	Roseovarius sp. ; JF417975	100	Roseovarius tolerans; DQ915626
10-M	MUCSATBW4R2	Ruegeria sp. ; KC429860	99	Ruegeria atlantica; KF009877
17-M	MUCSATBW4R2	Roseobacter sp.; FJ440959	99	Roseobacter denitrificans ; NR_102909
04-M	MUCSATBW4R3	Halomonas sulfidaeris; JX860259	100	Halomonas sulfidaeris; JX860259
08-M	MUCSATBW4R3	Halomonas titanicae ; KC420683	100	Halomonas titanicae ; KC420683
01-M	MUCSATBW4R3	Paracoccus sp. ; AB362258	99	Paracoccus marinus ; AB727958
02-M	MUCSATBW4R3	Unc. Alphaproteobacterium; DQ861239	99	Ruegeria atlantica; KF009868
03-M	MUCSATBW4R3	Roseovarius sp. ; JF417975	99	Roseovarius tolerans; DQ915626
16-M	MUCSATBW4R3	Unc. bacterium; HQ010558	99	Sulfitobacter dubius; HQ908665
98-U	UH2C2d0R2	Gaetbulibacter sp.; JX235670	97	Olleya marilimosa; KC756871
99-U	UH2C2d0R2	Gaetbulibacter sp.; JX235670	97	Olleya marilimosa; KC756871
77-U	UH2C2d0R2	Unc. bacterium; GU584806	99	Roseovarius crassostreae; NR_041731
71-U	UH2C2d0R3	Unc. proteobacterium; JF980892	99	Phaeobacter inhibens; KC176241
76-U	UH2C2d0R3	Unc. bacterium; JQ215495	98	Roseovarius pelophilus; EU939692
92-U	UH2C2W1R1	Phaeobacter sp.; KC250896	99	Tateyamaria pelophila; AJ968651
67-U	UH2C2W1R2	Ruegeria atlantica; KF009868	99	Ruegeria atlantica; KF009868
79-U	UH2C2W4R1	Unc. bacterium; HM468021	99	Halomonas alimentaria; HM583971
69-U	UH2C2W4R2	Halomonas sp. ; JN112011	97	Halomonas variabilis; AM945682
65-U	UH2C2W4R2	Ruegeria atlantica; KF009868	99	Ruegeria atlantica; KF009868
72-U	UH2C2W4R2	Unc. Roseobacter sp.; JX531403	100	Sulfitobacter dubius; HQ908665
68-U	UH2C2W4R3	Unc. Halomonas sp. ; JX529169	99	Halomonas boliviensis; JX860249
64-U	UH2C2W4R3	Tropicibacter sp.; KC534265	99	Ruegeria atlantica; KF009868
78-U	UH2C2W4R3	Halomonas sp.; DQ344858	99	Halomonas meridiana; KC842224
66-U	UH2C2W4R3	Ruegeria atlantica; KF009868	99	Ruegeria atlantica; KF009868
70-U	UH2C2W4R3	Unc. bacterium; HQ010558	100	Sulfitobacter dubius; HQ908665
75-U	UH2C2W4R3	Unc. bacterium; FJ973602	99	Poseidonocella sedimentorum; AB576006
120-UT	UTUNBd0R1	Unc. Bacteroidetes; JQ579754	99	Muriicola jejuensis; EU443206
99-UT	UTUNBd0R1	Unc. bacterium; JX984078	99	Sulfitobacter pontiacus; KC534156

BAND	SAMPLE	CLOSEST RELATIVE; ACCESSION NUMBER	%	CLOSEST DESCRIBED SPECIES; ACCESSION NUMBER
101-UT	UTUNBd0R1	Unc. bacterium; KC631560	99	Thalassobius aestuarii; AY442178
139-UT	UTUNBd0R1	Unc. bacterium; KF465362	99	Roseovarius litoreus; NR_109594
140-UT	UTUNBd0R1	Unc. bacterium; KF465362	99	Roseovarius litoreus; NR_109594
105-UT	UTUNBd0R2	Unc. Alphaproteobacterium; JN393000	97	Sulfitobacter dubius; KC534303
100-UT	UTUNBd0R3	Unc. marine bacterium; GU576966	99	Marinovum algicola; AB636140
138-UT	UTUNBd0R3	Unc. bacterium; JF272150	99	Poseidonocella sedimentorum; AB576006
130-UT	UTUNBW1R1	Sagittula sp.; HQ871860	98	Marinovum algicola; AB636140
119-UT	UTUNBW1R2	Unc. Flavobacteriaceae; JX529623	100	Mesonia algae; AB681172
92-UT	UTUNBW1R2	Unc. bacterium; HE979667	100	Alcanivorax borkumensis; KC565664
97-UT	UTUNBW1R2	Unc. Roseobacter sp.; JX531403	99	Sulfitobacter dubius; HQ908665
111-UT	UTUNBW1R2	Unc. marine bacterium; GU576966	99	Marinovum algicola; AB636140
123-UT	UTUNBW1R2	Unc. Roseobacter sp.; JX531103	100	Sulfitobacter pontiacus; KC534156
88-UT	UTUNBW1R3	Ruegeria atlantica; KF009868	99	Ruegeria atlantica; KF009868
95-UT	UTUNBW1R3	Unc. Roseobacter sp.; JX531218	99	Sulfitobacter dubius; KC534303
96-UT	UTUNBW1R3	Unc. Roseobacter sp.; JX531403	99	Sulfitobacter dubius; HQ908665
98-UT	UTUNBW1R3	Unc. Roseobacter sp.; JX531103	100	Sulfitobacter pontiacus; KC534156
110-UT	UTUNBW1R3	Marine sponge bacterium; EU346575	98	Tateyamaria pelophila; AJ968651
121-UT	UTUNBW4R2	Unc. bacterium; KC631507	98	Erythrobacter citreus; KF009863
93-UT	UTUNBW4R1	Unc. Rhodobacteraceae; DQ870525	98	Ruegeria atlantica; KF009868
94-UT	UTUNBW4R1	Roseobacter sp.; EU374898	100	Ruegeria atlantica; KF009868
107-UT	UTUNBW4R1	Poseidonocella sedimentorum; AB576006	99	Poseidonocella sedimentorum; AB576006
115-UT	UTUNBW4R1	Unc. Roseobacter sp.; JX531103	99	Sulfitobacter pontiacus; KC534156
124-UT	UTUNBW4R1	Roseovarius sp.; KC295345	99	Roseovarius tolerans; DQ915626
108-UT	UTUNBW4R2	Unc. Rhodobacteraceae; HQ857621	99	Roseovarius crassostreae; NR_041731
116-UT	UTUNBW4R2	Unc. bacterium; JN813894	99	Sulfitobacter guttiformis; JQ675546
118-UT	UTUNBW4R2	Marine sponge bacterium; EU346575	99	Tateyamaria pelophila; AJ968651
128-UT	UTUNBW4R2	Unc. Rhodobacteraceae; DQ870525	100	Ruegeria atlantica; KF009868
129-UT	UTUNBW4R2	Unc. bacterium; KF465362	98	Roseovarius litoreus; NR_109594
82-UT	UTUNBW4R3	Roseobacter sp.; EU374898	99	Phaeobacter caeruleus; KC176242
109-UT	UTUNBW4R3	Tropicibacter sp.; KC534265	99	Roseovarius litoreus; NR_109594
117-UT	UTUNBW4R3	Rhodobacteraceae bacterium; FN811289	99	Marinovum algicola; AB636140
37-Y	YUOWd0-R1	Unc. bacterium; JQ214051	98	Staleya guttiformis; AB607871
60-Y	YUOWd0-R1	Unc. Roseobacter sp.; JX531103	100	Sulfitobacter pontiacus; KC534156
80-Y	YUOWd0-R2	Unc. bacterium; HE979608	98	Olleya aquimaris; NR_104531
38-Y	YUOWd0-R2	Bacterium CSR-61; KJ018064	99	Tateyamaria pelophila; AJ968651
40-Y	YUOWd0-R3	Unc. Alphaproteobacterium; GU061167	97	Sulfitobacter mediterraneus; DQ915636
81-Y	YUOWW1-R1	Bizionia sp.; JX119046	100	Bizionia paragorgiae; HQ538734
71-Y	YUOWW1-R1	Unc. bacterium; JN874265	98	Sulfitobacter dubius; KC534303
73-Y	YUOWW1-R1	Unc. marine bacterium; FJ825845	99	Sulfitobacter pontiacus; KC534156
43-Y	YUOWW1-R2	Unc. bacterium; KF726963	99	Sulfitobacter pontiacus; KC534156
72-Y	YUOWW1-R2	Unc. Roseobacter sp.; JX525553	98	Sulfitobacter pontiacus; KC534156
47-Y	YUOWW1-R3	Roseobacter denitrificans; KF928340	99	Roseobacter denitrificans; KF928340

BAND	SAMPLE	CLOSEST RELATIVE; ACCESSION NUMBER	%	CLOSEST DESCRIBED SPECIES; ACCESSION NUMBER
46-Y	YUOWW4-R1	Unc. Roseobacter sp.; EU374898	99	Phaeobacter caeruleus; KC176242
50-Y	YUOWW4-R1	Unc. bacterium; KF465362	99	Mameliella alba; NR_108225
59-Y	YUOWW4-R1	Unc. Roseobacter sp.; JX531103	100	Sulfitobacter pontiacus; KC534156
44-Y	YUOWW4-R2	Sagittula stellata; HG315014	97	Sagittula stellata; HG315014
51-Y	YUOWW4-R2	Unc. bacterium; KF465362	99	Roseovarius litoreus; KF465362
55-Y	YUOWW4-R2	Sulfitobacter sp.; HG423271	99	Sulfitobacter dubius; KC534303
56-Y	YUOWW4-R2	Unc. Roseobacter sp.; JX531403	99	Sulfitobacter dubius; HQ908665
57-Y	YUOWW4-R2	Unc. Roseobacter sp.; JX531403	100	Sulfitobacter dubius; HQ908665
58-Y	YUOWW4-R2	Unc. Roseobacter sp.; JX531103	99	Sulfitobacter pontiacus; KC534156
45-Y	YUOWW4-R3	Roseobacter sp.; EU374898	99	Ruegeria atlantica; KF009868
52-Y	YUOWW4-R3	Marinovum algicola; AB636140	99	Marinovum algicola; AB636140
53-Y	YUOWW4-R3	Unc. bacterium; JQ214051	99	Roseovarius crassostreae; NR_041731
75-Y	YUOWW4-R3	Roseobacter denitrificans; KF928340	98	Roseobacter denitrificans; KF928340
82-U	UH2C2 consortium	Unc. bacterium; EF622426	98	Microbacterium trichothecenolyticum; KC429636
83-U	UH2C2 consortium	Microbacterium immunditiarum; DQ119293	99	Microbacterium immunditiarum; DQ119293
81-U	UH2C2 consortium	Gordonia terrae; KF410339	100	Gordonia terrae; KF410339
85-U	UH2C2 consortium	Bacillus cereus; HM055972	99	Bacillus cereus; HM055972
84-U	UH2C2 consortium	Bacillus sp.; JF496917	100	Bacillus thuringiensis; JN089372
80-U	UH2C2 consortium	Microbacterium immunditiarum; DQ119293	99	Microbacterium immunditiarum; DQ119293
83-UT	UTUN consortium	Alcanivorax sp.; KF317786	100	Alcanivorax balearicus; KC900374
90-UT	UTUN consortium	Acinetobacter sp.; JF343127	99	Acinetobacter venetianus; AB859738
85-UT	UTUN consortium	Achromobacter xylosoxidans; JQ282778	100	Achromobacter xylosoxidans; JQ282778
86-UT	UTUN consortium	Achromobacter sp.; HG324050	100	Achromobacter xylosoxidans; JQ900538
87-UT	UTUN consortium	Achromobacter sp.; HG324050	99	Achromobacter xylosoxidans; JQ282778
84-UT	UTUN consortium	Alcanivorax sp.; JX239756	100	Alcanivorax balearicus; KC900374
91-UT	UTUN consortium	Acinetobacter sp.; JF343127	98	Acinetobacter venetianus; AB859738
89-UT	UTUN consortium	Ochrobactrum sp.; KF737379	99	Ochrobactrum pituitosum; KF580852
133-UT	UTUN consortium	Bacterium SKUKMB1016; FJ644574	99	Marinovum algicola; AB636140
15-M	MUCSAT consortium	Dietzia maris; KC747472	99	Dietzia maris; KC747472
26-M	MUCSAT consortium	Dietzia maris; KF410336	100	Dietzia maris; KF410336
30-M	MUCSAT consortium	Unc. bacterium; GU062055	100	Paracoccus kamogawaensis ; AB275604
64-Y	YUOW consortium	Sphingobium fuliginis; KJ472812	99	Sphingobium fuliginis; KJ472812
65-Y	YUOW consortium	Unc. Alphaproteobacterium; JQ919520	96	Parvibaculum lavamentivorans; NR_074262

11. Supplementary figures

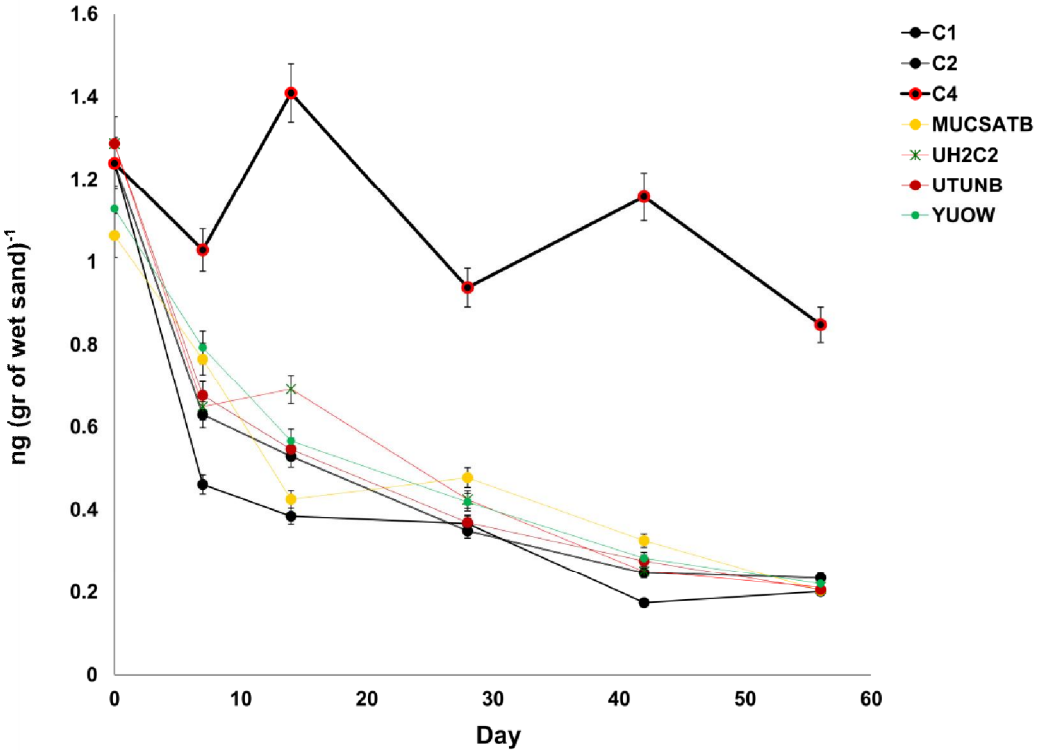

Figure S1 The average of the sum of the concentrations of the aromatic components fluorene, phenanthrene and dibenzothiophene throughout the experiment. Treatment codes are described in Table 1. Error bars represent one standard deviation, derived from four technical replicates.

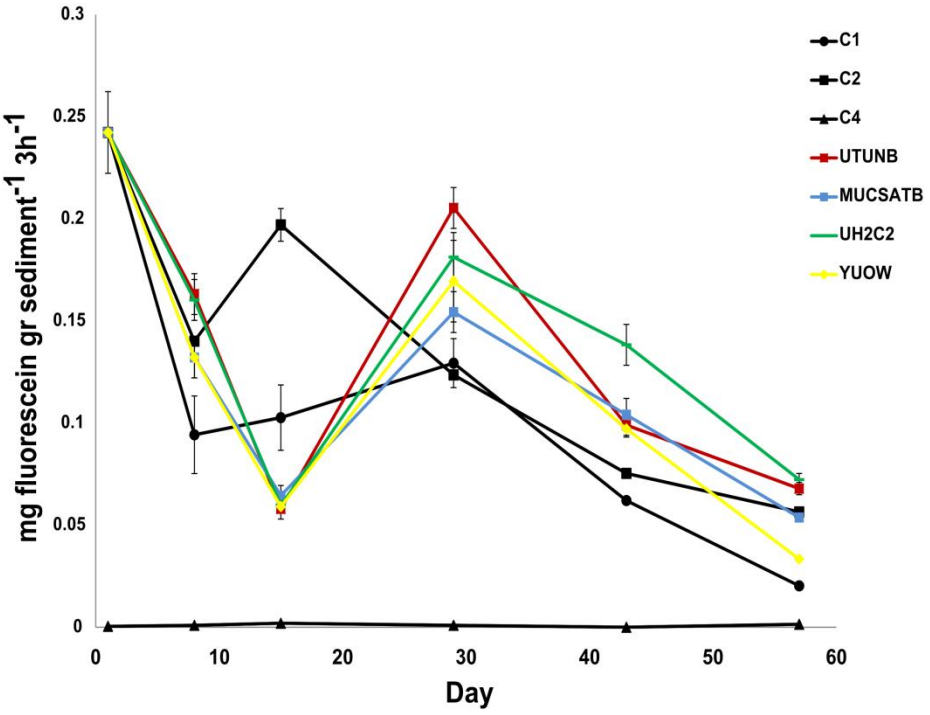

Figure S2 Sediment hydrolytic activity throughout the experiment. Control treatments (C1, C2 and C4) are represented with black lines while each bioaugmented treatment with a different colour. The bars represent one standard deviation, derived from three technical replicates. Treatment codes are described in Table 1.

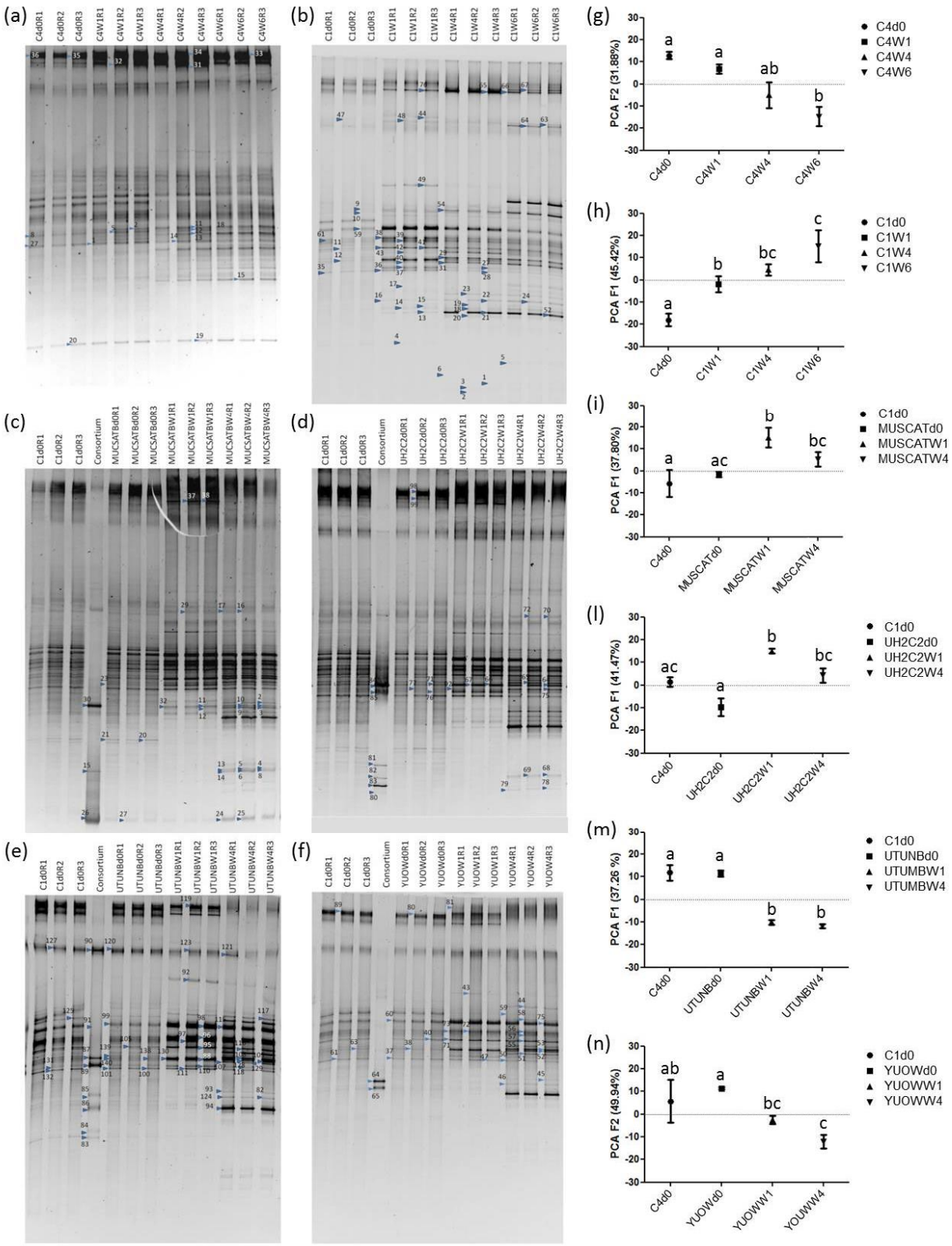

Figure S3 (a-f) DGGE analysis performed of the 16S rRNA on the metagenome extracted from the sediments at different time points during the landfarming experiment realized on control (a-b) and bioaugmented (c-f) treatments. For each time point, the three analyzed replicates are indicated as R1, R2 and R3. Taxonomic identification of bacterial 16S rRNA gene sequences retrieved from DGGE bands indicated in Figure S3a-f is reported in Table S1. (g-n) ANOVA analysis was performed on the average values of the line plot score along axis 1 (h,i,l,m) or axis 2 (g, n) of PCA analysis in order to assess the degree of similarities among sediment dwelling bacterial communities over experimental time. Different letters (a, b, and c), shown at the top of the scatter plots in the graph, indicate a statistical significance at $P \leq 0.05$ according to ANOVA analysis.

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