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Characterization and comparison of microbial communities from different tourist ports in Mediterranean Sea and evaluation of applicability of bioremediation treatments

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ABSTRACT

Marine ecosystems represent the greatest volume of the biosphere and the largest surface of the planet. They are home to an enormous diversity of animals, plants and microbes and are crucially important resource for fishing, transport, mining and recreation. Humans have been dependant on the seas and oceans for many millennia and have used the coastal waters as a prehistoric resource for food and expansion around the planet (Moore et al., 2013). Coastal region are frequently areas of great natural beauty and are used extensively for recreation and tourism as reflected in the mass migration of people to the coasts in the holiday seasons (Golyshin et al., 2003).

The Mediterranean has become one of the most demanded destinations for organized touristic routes (increasing cruising market) and "self-made" ones (boating and chartering). Despite the strict link between tourism development in coastal areas and port facilities, the sector growth has not been sustained neither by an adequate expansion nor by an adaptation of the ports.

Ports have a decisive role in the development of coastal areas and the risk of impact of infrastructures construction and maritime traffic on the coastal zone is high. Ports are part of the logistics chain and the transport networks with a decisive role in the development of coastal areas. However, the risk of impact of infrastructures construction and maritime traffic on the coastal zone is high. The problem with any port expansion is the balance between environmental and social-economic issues (sustainable development). In fact, if on one hand the development of infrastructures has to take into account environmental impact and restoration to ecological standards, on the other hand, denial of port expansion for environmental reasons may favour other competing ports.

Due to their strategic location between sea and land, ports are particularly critical environments as they can receive pollution coming from land, ships and from the port facilities themselves. Furthermore, ports are not closed systems and their pollution may impact large parts of the adjacent coastal areas. Specifically, pollution by petroleum hydrocarbons is one of the major environmental problems in ports and it is mainly associated with the heavy ship/boat traffic and the related facilities located in these areas. The ship transport in ports is powered by diesel.

The present study was carried out in the framework of the MAPMED project (Management of Port areas in the Mediterranean Sea Basin). The general objective of the project was to improve the environmental sustainability of tourist coastal areas in the Countries of the Mediterranean Sea Basin through the promotion of a long term cooperation between Institutional Authorities and the scientific community and, at a more specific level, to optimize, validate and transfer tools to guide Institutional Authorities in the sustainable management of tourist ports/harbours with regard to monitoring and reduction of hydrocarbon pollution.

Strategies for cleaning up hydrocarbon pollution are greatly affected by a variety of factors, such as type of oil, characteristics of the spill site, and occasionally political considerations. A number of approaches and technologies have been developed for controlling oil pollution in marine environments. Bioremediation has emerged as one of the most promising treatment options for oil removal since its successful application after the 1989 Exxon Valdez spill.

The general objective of this work was to evaluate and compare the applicability of different bioremediation approaches of seawaters and sediments in Mediterranean tourist ports. Particularly, it was directed to contribute to the answer to two following main questions:

- *Do different tourist ports share the same bacterial communities?*
- *Do bioremediation strategies have similar applicability in different tourist port areas?*

The specific aims of the present study were:

1. Definition and comparison of the pollution status of seawater.
2. Comparison of the bacterial communities in seawater.
3. Characterization of the dominant cultivable hydrocarbon-degrading bacteria from seawater and sediments.
4. Comparison of the effects of biostimulation treatment on bacterial communities in seawater at laboratory scale.
5. Comparison of the effect of bioaugmentation treatments on PAH degradation in sediments at laboratory scale.

Three tourist ports located across the Mediterranean Sea were selected as case study sites representative tourist harbours in the Basin: Cagliari (Sardinia, Italy) in the western part, El Kantaoui (Tunisia) in the central part, and Heraklion (Crete, Greece) in the eastern part. Beside their geographic position, port selection was also based on other different elements, such as categories of maritime traffic, port dimension and existing information on pollution. The maritime traffic inside the three tourist ports is represented by recreational boats, passenger ships and fishing vessels. In addition to the marine traffic, the major pollution sources related to the three port areas are wastewater discharges into the sea, river mouth, fuelling stations, and fishing activities.

Data demonstrated that Cagliari port was characterized by a high level of eutrophication. Coherently with the high nutrient load, the viable title of heterotrophs was one-log higher in the seawater of Cagliari port when compared with values found in the other ports. The structure of the microbial communities in seawater from Cagliari and El Kantaoui were more similar to one another than the others while Heraklion presented different community structures.

During the biostimulation treatment in seawater microcosms, comparable degradation kinetics were found for Cagliari and Heraklion sites. Furthermore, nutrient amendments in El Kantaoui microcosms resulted in a two-fold increase in the degradation rates as compared to the other two port areas. The structures of the bacterial communities of El Kantaoui were more homogenous than those from Cagliari, while Heraklion presented a moderate change in community structures during bioremediation treatment. Moreover, different community structures were found for the three sites at the end of the treatment.

The community of cultivable degraders from seawater of the Cagliari port was dominated by copiotrophic bacteria belonging to the genus *Pseudomonas* whereas the majority of the isolates from Port El Kantaoui were *Alcanivorax*. Both OHCBs and non-professional hydrocarbonoclastic bacteria were equally represented in the community from Heraklion port.

The autochthonous population from the sediments of El Kantaoui does not possess the metabolic routes necessary to metabolize BaP being the addition of an allochthonous strain a successful strategy for promoting BaP degradation. Thus, a selectivity of the bioaugmentation treatments was demonstrated both in terms of typology of PAHs and sediments.

CHAPTER 1

1. Comparison of the pollution status and bacterial communities in seawater of different Mediterranean tourism ports

1.1. INTRODUCTION

1.1.1. Petroleum hydrocarbons

Crude oils are extremely complex mixtures of organic compounds, which can be classified into four main operationally defined groups of chemicals: aliphatic (AHs) and aromatic hydrocarbons as well as and the more polar non-hydrocarbon components, resins and the asphaltenes. Light oils are typically high in saturated and aromatic hydrocarbons, with smaller proportions of resins and asphaltenes. Heavy oils are characterized by a much lower content of saturated and aromatic hydrocarbons and a higher proportion of the more polar chemicals (Zhu et al., 2001).

Diesel oils are complex combination of hydrocarbons produced by the distillation of crude oil. The carbon number of diesel hydrocarbons is between C_{11} and C_{25} and the distillation range is between 180 to 380°C. Diesel fuels predominantly contain a mixture of C_{10} - C_{19} hydrocarbons, which include approximately 64% aliphatic hydrocarbons, 1-2% olefin hydrocarbons, and 35% aromatic hydrocarbons (Air Force, 1989).

Different microorganisms (bacteria, fungi and algae) are able to degrade petroleum hydrocarbons with different kinetic rates depending on both the environmental conditions and molecular structures. In this context, bacteria are known to be ubiquitous in virtually all marine environments and they are considered as key players in oil degradation and natural autodepuration processes (Head et al., 2006). According to van Hamme et al. (2003), the susceptibility of hydrocarbons to microbial degradation is in the following order: alkanes > monoaromatics > cycloalkanes > polycyclic aromatic hydrocarbons (PAHs) > asphalthenes. *n*-alkanes are the most biodegradable petroleum hydrocarbons; however, those compounds with 5 - 10 carbon atoms are inhibitory to most hydrocarbon degraders because they can disrupt lipid membrane. On the other hand, petroleum hydrocarbons with C_{20} - C_{40} are hydrophobic solids at physiological temperatures. This probably explains their low biodegradability (Bartha and Atlas, 1977). The aromatic hydrocarbons are generally more resistant to biodegradation than saturated compounds (Focht and Westlake, 1987). Monoaromatic hydrocarbons are toxic to some microorganisms due to their solvent action on cell membranes, but they are easily biodegradable under aerobic conditions at low concentrations. PAHs with 2-4 rings are less toxic and biodegradable than monoaromatics at rates that decrease with the level of complexity. PAHs with five or more rings can only be degraded through co-metabolism, in which microorganisms fortuitously transform non-growth substrates while metabolizing simpler hydrocarbons or other primary substrates. PAHs are of particular environmental concern since several PAH members have

been identified as carcinogenic, mutagenic, and teratogenic compounds. Compared to saturates and aromatic hydrocarbons, very little is known about biodegradation of resins and asphaltenes.

1.1.2. Oil pollution in the Mediterranean by ship traffic and port activities

Marine ecosystems represent the greatest volume of the biosphere and the largest surface of the planet. They are home to an enormous diversity of animals, plants and microbes and are crucially important resource for human activities, such as fishing, transport, mining and recreation. Humans have been dependant on the seas and oceans for many millennia and have used the coastal waters as a prehistoric resource for food and expansion around the planet. (Moore et al., 2013). Coastal regions are frequently areas of great natural beauty and they are used extensively for recreation and tourism, which is reflected in the mass migration of people to the coasts in the holiday seasons (Golyshin et al., 2003). Particularly, the Mediterranean Basin is becoming one of the most popular attractions for coastal tourism, which represents a major activity for many of the 22 Mediterranean riparian countries or territories. In 2005, Mediterranean countries received 246 million international tourists, that is 30.5% of global international tourism. By 2025, national and international tourism visits would be about 637 million in Mediterranean countries, of which 312 million in the Mediterranean coastal zones alone (Blue Plan, 2008).

The Mediterranean Sea is also a crucial route for marine traffic. It is a fact that 30% of all international maritime trade by volume has its origin or destination in Mediterranean ports or passes through this semi-enclosed region (Abdulla and Linden, 2008). For the available data, UNEP/IOC 1988 considers the Mediterranean Sea to be more polluted by oil than any other sea. Ports are part of the logistics chain and of the transport networks with a decisive role in the development of coastal areas. However, the risk of impact of infrastructures construction and maritime traffic on the coastal zone is high. Due to their strategic location between sea and land, ports are particularly critical environments as they can receive pollution coming from land, ships and from the port facilities themselves. Furthermore, ports are not closed systems and their pollution may impact large parts of the adjacent coastal areas (Goulielmos, 2000). Specifically, pollution by petroleum hydrocarbons is one of the major environmental problems in tourism ports and it is mainly associated with the heavy ship/boat traffic and the related facilities located being diesel the most common fuel for ship/boat transport in ports.

1.2. AIM OF THE WORK

The aim of this work was to describe and compare the pollution status of seawater in three port areas selected as case study sites representative tourist harbours in the Mediterranean Sea. The pollution status of the selected ports was defined by determining the levels of nutrients and heterotrophic bacteria as well as the concentrations of petroleum hydrocarbons and the viable titles of hydrocarbon degraders. For their

adverse effects on biota, PAHs were the target of both chemical and microbiological analyses. Nevertheless, the group of AHs was also characterized.

1.3. MATERIALS AND METHODS

1.3.1. Study areas, samplings and physical parameters

The case study sites were the port areas of Cagliari, El Kantaoui, and Heraklion (**Table 1.1**). On the southern coast of the Island of Sardinia, the Cagliari port is located in front of the city centre. The sampling was limited to the Historic Port of Cagliari, while the industrial zone of it was excluded. The Port of El Kantaoui is a small artificial marina situated on the eastern Tunisian coast within an international tourist centre without permanent inhabitants. On the northern coast of the Island of Crete, the Port of Heraklion is situated close to the town centre.

Table 1.1. Case study ports.

Port	Coordinates	Water surface (km^2)	Maritime traffic	Station
Cagliari (Sardinia, Italy)	N39° 12.3' - E09°07.0'	2.0	Passenger ship, cruiser, fishing vessels, cargos	5
El Kantaoui (Tunisia)	N35° 53.6' - E10°35.9'	0.04	Recreational boats, little passenger ships, fishing vessels	3
Heraklion (Crete, Greece)	N35° 20.7' - E25°08.7'	0.9	Passenger ships, cruises, recreational boats, fishing vessels, cargos	5

Port selection was based on the different elements, such as categories of maritime traffic, port dimension and existing information on pollution. The maritime traffic inside the tourist ports is represented by recreational boats, passenger ships and fishing vessels. In addition to the marine traffic, the major pollution sources related to the port areas are discharge into the sea of sewage effluent via pipes, fuelling stations and fishing activities. The Cagliari port also receives the drainage water from the surrounding lagoons through an artificial channel (**Figure 1.1**).

All sampling activities in the three sites were carried out by the Hellenic Centre for Marine Research (HCMR) of Crete. A grid of sampling stations was defined on each case study site in order to describe the spatial heterogeneity of chemical and microbiological parameters. The selected sampling stations were chosen according to their characteristic position within the port area and their related use. Three sampling were carried out in 2012 during winter, spring (before tourism season), and summer (after tourism season). Samples for both chemical and microbiological parameters were labelled with a common nomenclature: the first number in sample labels refers to sampling campaign (1: winter, 2: spring at the beginning of the tourist season, 3: late summer at the end of the tourist season), the letter refers to the sampling site (C: Cagliari, E: El Kantaoui, H: Heraklion), the second number refers to the sampling station, a three letter code refers to the parameter (i.e. H: hydrocarbons; MPN: viable titles).

Sampling operations were carried out from a small boat. The exact position of each sampling station was recorded on board using a Garmin 60 CS portable GPS. The physical properties of the surface seawater (temperature, salinity, oxygen, pH) were measured at each station using a 3420 WTW multi-meter. Surface water samples (3 replicates) were collected with 5L-carboys from each station for the determination of hydrocarbons and nutrients. For microbiological parameters, a surface seawater sample was collected from each station using a 15 L collapsible carboy sterilized with 10% bleach and washed with filtered-sterilized water.

Figure 1.1. Position of sampling stations in the three selected port areas: a) Cagliari, b) El Kantaoui, c) Heraklion. The blue marks indicate the position where water samples were collected.

1.3.2. Chemical parameters

Determination of chemical parameters was performed by HCMR. Subsamples of 500 mL (3 replicates) were filtered in the field using Whatman GF/F filters. Filtered water samples and filters were stored at -20°C, transported to the laboratory and used for analysis of inorganic nutrients and particulate organic carbon (POC), respectively.

Measurements of inorganic nutrients (NO_2, NO_3, PO_4) and POC were performed according to the Strickland & Parsons (1972) method, while the Ivančić and Degobbis (1984) method was used for NH_4. The concentration of dissolved inorganic nitrogen (DIN) was calculated as the sum of the individual ions (NO_2, NO_3, NH_4).

For determination of AHs and PAHs in seawater, subsamples of 1000 mL (3 replicates) were used and the liquid-liquid extraction was carried out in the field. The sample was transferred into a cylindrical separation

funnel. A volume of 70 mL of hexane and 100 µL of a multistandard mixture (in methanol), containing the recovery standards of PAHs (Acenaphthene-D10, Chrysene-D12, Naphthalene-D8, Perylene-D12, Phenanthrene-D10 and Pyrene-D10; 1.5 ng/µL each) or AHs (1-Cl-Dodecane and 1-Cl-Octadecane; 8.3 ng/µL each) were added. The mixture was shaken for 10 min and then allowed to settle for 1 min for phase separation. The organic phase was collected and the extraction procedure repeated another two times with the same volume of solvent. The extracts were pooled together and transported to the laboratory.

At HCMR, the extracts were evaporated to 0.5 mL using a rotary vacuum evaporator. The final residue was loaded onto a glass column packed with 0.5 g of silica gel (40-63 µm particle size). Prior of use, the silica column was activated at 300 °C for 3 h and rinsed with 5 mL of 20% CH_2Cl_2 in hexane followed by 5 mL hexane. The elution of PAHs and AHs from the silica column was achieved by passing 4 mL hexane and 3 mL of 20% CH_2Cl_2 in hexane. The collected fraction was evaporated to 200 µL, transferred into a tampered glass vial, and evaporated to 20 µL using a gentle stream of nitrogen. The final extract was mixed with 20 µL of a standard solution (in toluene) containing the internal standards of PAHs (Benzo[b]fluoranthene-D12; 1 ng/µL) and AHs (1-Br-Tetracosane; 30 ng/µL). Subsequently, 1 µL was injected in splitless mode (splitless time of 1.5 min) into the GC-MS (Agilent 6890 gas chromatograph interfaced with an Agilent 6890 mass spectrometer operating under electron impact ionization mode). The target analytes were separated on a FS-Supreme-5MS capillary column (30 m, 0.25 mm i.d, 0.25 µm film thickness) using helium as carrier gas (constant velocity 35 cm/s). The temperature of the injector and transfer line was kept constant at 260 and 280°C, respectively, while the GC oven was operated under the following temperature program: from 85°C (1.35 min hold) to 130°C, at 80°C/min, to 200°C at 2.5°C/min and finally to 290°C (5 min hold) at 4°C/min (total chromatographic time: 54.9 min). The mass spectrometer was operated in Single Ion Monitoring mode and separate injection runs were performed for the detection of 39 members of PAHs and 26 members of AHs in the range C_{10}-C_{35}. After peak area integration in SIM chromatograms, the internal standard method was applied to determine the concentrations of individual PAH and AH members, as well as to calculate total concentrations for both compound groups.

1.3.3. Determination of bacterial viable titles

For each station, a seawater subsample (50 mL) was aseptically prepared in the field in a sterile vial. Subsamples were stored at 4°C, transported to the laboratory in a thermal bag at 4°C, and immediately used for enumeration of cultivable bacteria in Cagliari.

Heterotrophic and hydrocarbon-degrading bacteria were enumerated by using the 'five tube' Most Probable Number (MPN) method. Determinations were carried out in Bushnell Haas minimal medium supplemented with NaCl 20 g/L (Haines et al., 1996; Zhu et al., 2001). Tenfold serial dilutions (up to 10^{-10}) of seawater samples were prepared in sterile medium. For each sample, the selective enumeration of three different metabolic groups of cultivable aerobic bacteria was carried out by supplementing the minimal medium with

different carbon sources in separate 96-well microtiter plates in a total culture volume of 200 µL per well. MPN index and 95% confidence limits were calculated according to Alexander (1982) and data were log-transformed.

The viable title of three bacterial metabolic groups were determined: i) heterotrophs on yeast extract as model mixture of non-hydrocarburic organic compounds, ii) total hydrocarbon degraders on diesel fuel as representatives of pollution resulting from maritime transport, iii) degraders of phenanthrene, as a model of degradation of PAHs. Yeast extract solution was filter sterilized and added to the final concentration of 1 g/L. Diesel was filter sterilized and 5 µL added to each well. Phenanthrene was dissolved in hexane at a concentration of 5 mg/mL and 20 µL of the solution added to each well. The hexane was evaporated in a sterile flow cabinet.

The inoculation was performed by adding 20 µL of each 10-fold dilution (up to 10^{-10}) to five wells per plate. For each plate, five wells were used as sterile control, omitting sample inoculation. The microtiter plates were wrapped in plastic bags and incubated for 30 days at 20 ± 1°C. At the end of the incubation period, 20 µL of an iodonitrotetrazolium violet (INT, SIGMA) sterile solution (3 g/L) was added to each well and the plates were incubated for 24 h. Wells were scored as positive if a red precipitate was detected due to INT reduction and intracellular deposition of insoluble formazan.

1.3.4. On-field processing, DNA extraction and purification, T-RFLP analysis

One litre-aliquots of seawater (Denaro et al., 2005) were pre-filtered using a peristaltic pump (100 mL/min) through glass microfiber GF/A filters (nominal pore size 1.6 µm, Whatman), to screen out larger eukaryotes and particulate matter (**Figure 1.2a**). The fraction passing through the pre-filters was collected onto 0.2 µm Sterivex GP cartridges (Millipore) (**Figure 1.2b**).

a **b**

Figure 1.2. On-field processing of seawater samples for DNA extraction. a) Seawater filtration apparatus. b) Sterivex GP cartridge.

The water was completely evacuated from the filter units with a sterile syringe and the samples were stabilized by adding RNAlater® reagent (Ambion) into the filter units. Filters were stored on-site at 4 °C until shipment to the laboratory at room temperature. RNAlater® is an aqueous, non-toxic storage reagent, which

protects nucleic acids in unfrozen samples. This reagent was selected for sample stabilisation during on-field processing because Tunisia are not currently allowed to air shipment of dry ice. DNA extraction from cells collected onto Sterivex filters was carried out as described by Somerville et al. (1989) and modified by Riemann et al. (2000). DNA was further purified using the PowerClean® DNA Clean-Up Kit (MoBio), following the manufacturer's instructions. The integrity of DNA was checked on 1% agarose gels.

For the analysis of terminal restriction fragment length polymorphism (T-RFLP), the bacterial 16S rRNA genes were amplified by using the primer 27f, labelled with 6-carboxyfluorescein at the 5'end, and the primer 1492r as previously described by DeLong (1992). The PCR products from three replicate reactions were combined in order to minimize stochastic PCR bias and purified from the agarose gel. 150 ng aliquots of the purified PCR products were digested with 10 U of *Alu* I. PCR product extracted from agarose gel using the QIAquick Gel extraction kit (Qiagen) following manufacturer's instructions. Digested products were precipitated and resolved by capillary electrophoresis on an ABI310 Genetic Analyzer using LIZ500 as size standard.

1.3.5. Statistical analysis

Nonparametric statistical tests were performed as implemented by Past software (Hammer et al., 2001) for detecting the overall effects using the Kruskal-Wallis test (KW), followed by pairwise comparisons using Mann-Whitney (MW) test. The *p* values were given after a Bonferroni correction.

T-RFLP profiles were analysed by the software GeneMarker 2.4.0 (Softgenetics). Only fragments with sizes from 40 to 500 bp and fluorescence intensity >30 arbitrary units of fluorescence were used in the analysis. Data were standardized by calculating the area of each peak as a percentage of the total area. Peaks with an area less than 0.5% of the total one were reassigned as zero and the percentage values of the remaining terminal restriction fragments (T-RFs) recalculated (Rees et al., 2004). A similarity matrix was obtained from the dataset by calculating the Bray-Curtis coefficient and nonmetric Multi-Dimensional Scaling (nMDS) was used to ordinate similarity data by means of the software Primer 6. Analysis of Similarity (ANOSIM) was used to examine statistical significance between samples collected from different port areas during different sampling campaigns.

1.4. RESULTS AND DISCUSSION

1.4.1. Comparison of the pollution status in seawater

Physico-chemical and microbiological parameters in surface seawater from the three selected port areas are shown in **Figure 1.3**. The three ports did not show significant differences regarding water temperatures (KW $p>0.05$). The Cagliari port was characterised by the highest oxygen concentration at the surface water (MW $p<0.02$), whilst no significant difference was observed between El Kantaoui and Heraklion (MW $p>0.05$). The Cagliari port was also characterised by the widest variation in salinity values. Salinity was significantly lower in Cagliari than in the other two ports (MW $p<0.002$), whilst it was highest in Heraklion (MW $p<0.01$). The

inorganic nutrients and POC were all significantly higher in the seawater samples of Cagliari port (MW $p<0.05$). Particularly, the DIN reached a maximum value of 1.47 mg/L in Cagliari, whilst maximum values of 0.06 mg/L and 0.56 mg/L were determined in samples of El Kantaoui and Heraklion, respectively. Overall data demonstrated that Cagliari port is characterised by a high level of eutrophication. Coherently with the high nutrient load, the viable title of heterotrophs was one-log higher in the seawater of Cagliari port when compared with values found in the other ports (MW $p<0.002$). On the contrary, no significant difference was registered between El Kantaoui and Heraklion (MW $p>0.05$). The strict link between heterotrophs and nutrient levels (DIN, NO_2, NO_3, NH_4, PO_4) was also confirmed by a positive linear correlation between these parameters (r=0.52-0.64, $p<0.002$).

The mean concentrations of AHs in the three port areas were 3.9, 4.2, and 2.5 µg/L in Cagliari, El Kantaoui and Heraklion ports, respectively. Significantly lower concentration of AHs (MW $p<0.05$) was found in the Heraklion port as compared to El Kantaoui and Cagliari. In Heraklion, the lower mean concentrations of total PAHs and phenanthrene were also found even though differences were not significant (KW $p>0.05$). In the three port areas, the concentrations of PAHs were 50 fold lower than that of AHs (**Table 1.2**) and differences were not significant (KW $p>0.05$).

In order to evaluate the pollution status of the selected ports, the levels of PAHs were compared with concentrations measured in the Mediterranean Sea in previous studies (**Table 1.2**). The GEF (2002) reports that the PAH content in the dissolved phase in the open Mediterranean Sea was 0.4 - 0.9 ng/L, with values around 2.0 ng/L in coastal areas. In 1997, levels of PAHs in samples from the North Aegean Sea were found to be 10 - 30 ng/L. Along the Turkish coast, concentrations vary over a wider range, with high concentrations from offshore samples caused most probably from direct discharges from the ships.

The lower concentrations of AHs and PAHs in Heraklion seawater samples was also accompanied by the lower number of viable titles of diesel and phenanthrene degraders being differences significantly lower with El Kantaoui ($p<0.05$), whereas intermediate values were observed in Cagliari (MW $p>0.05$). Despite its small dimensions, the El Kantaoui port exhibits the highest level of hydrocarbon pollutants and diesel degraders in seawater.

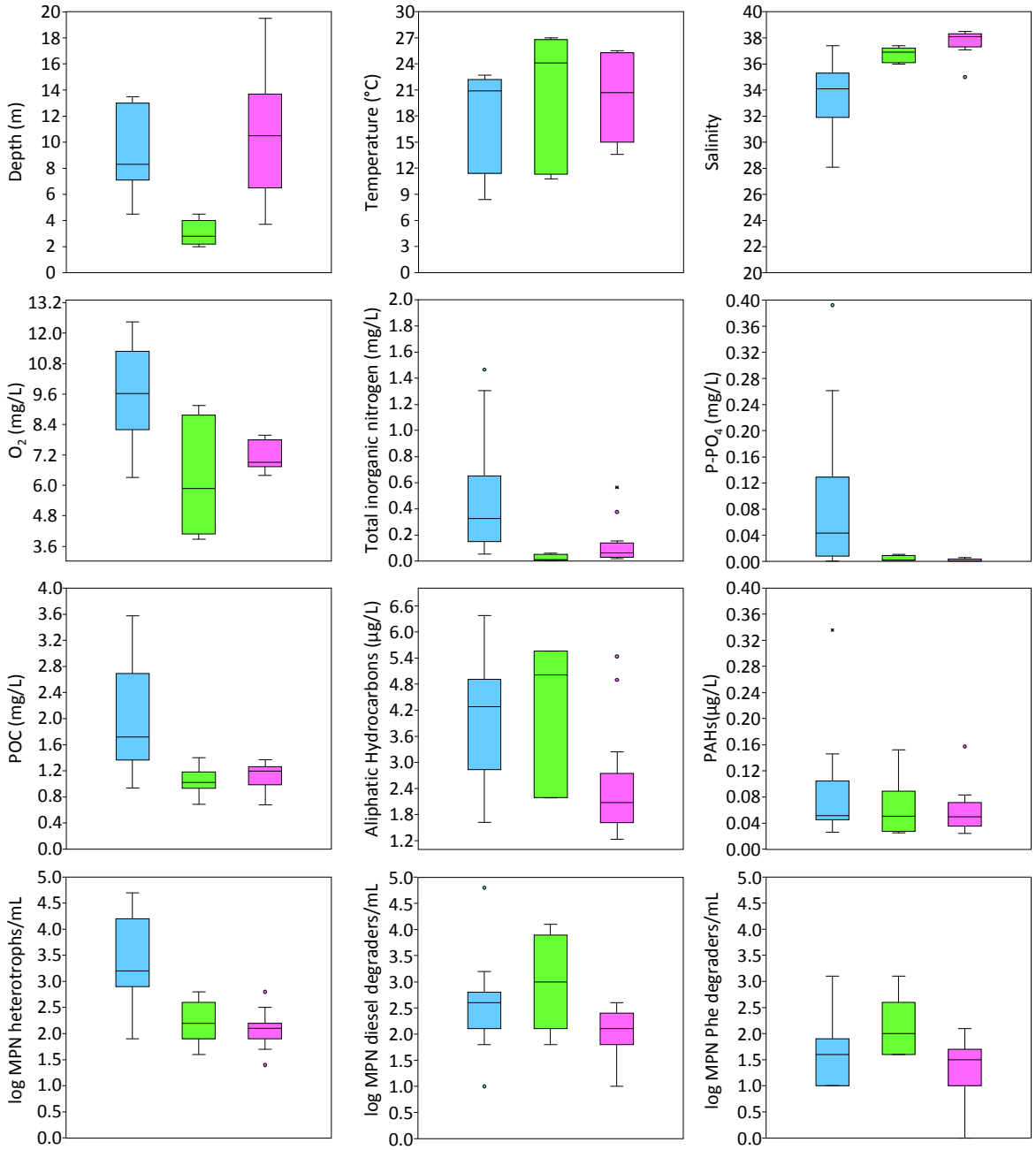

Figure 1.3 Physico-chemical and microbiological parameters in surface seawater from the ports of Cagliari (blue), El Kantaoui (green) and Heraklion (pink). Box-and-whisker plots indicate 25-75 percent quartiles (box) and the medians (horizontal line inside each box). Bars are drawn from each box to the higher and lower data within 1.5 times the interquartile range. Values outside the inner fences are shown as circles (outliers).

Table 1.2. Content of PAHs in superficial seawater from case study ports and other Mediterranean sites (GEF, 2002).

Sites	Levels of PAHs ng/L
Open Mediterranean Sea	0.4 - 0.9
Coastal areas	2.0
Izmit Bay (Marmara Sea)	0.2 - 7.4
North Aegean Sea	10 - 30
El Kantaoui	60.7
Cagliari	82.4
Heraklion	59.1
Turkish coast	20 - 40,000

1.4.2. Comparison of the bacterial communities in seawater

The application of DNA-based molecular tools has greatly enhanced the understanding of microbial diversity as culture independent techniques circumvent the limits of cultivation by determining the diversity in genes present within a given sample (Rees et al., 2004). Among different methodologies, T-RFLP analysis was chosen as fingerprinting technique to describe the structure of the prokaryotic community owing to its well-known properties. T-RFLP is a robust, reproducible, and high-throughput methodology allowing the rapid analysis of microbial community structures and monitoring of bacterial population shifts on spatial and temporal scale (Avaniss-Aghajani et al. 1994; Liu et al. 1997 ; Osborn et al., 2000). In this method, the genomic DNA is extracted from a sample and used as a template for a polymerase chain reaction, in which at least one of the primers is labelled with a fluorescent dye. Amplified products are digested with one or more restriction enzymes and the size of the T-RFs, with the fluorescent label, is determined by capillary electrophoresis. Traces, termed electropherograms, typically exhibit is a set of peaks varying in number, size and fluorescence intensity. As a whole, the profile represents the structure of the bacterial community present in the sample.

nMDS ordination of T-RFLP data obtained on seawater samples collected at the three case study sites is shown in **Figure 1.3**. A stress value lower than 0.2 was found corresponding to useful two-dimensional representation (Rees et al., 2004). The ANOSIM one way test was applied in order to perform a statistical pairwise comparison between different port areas (**Table 1.2**).

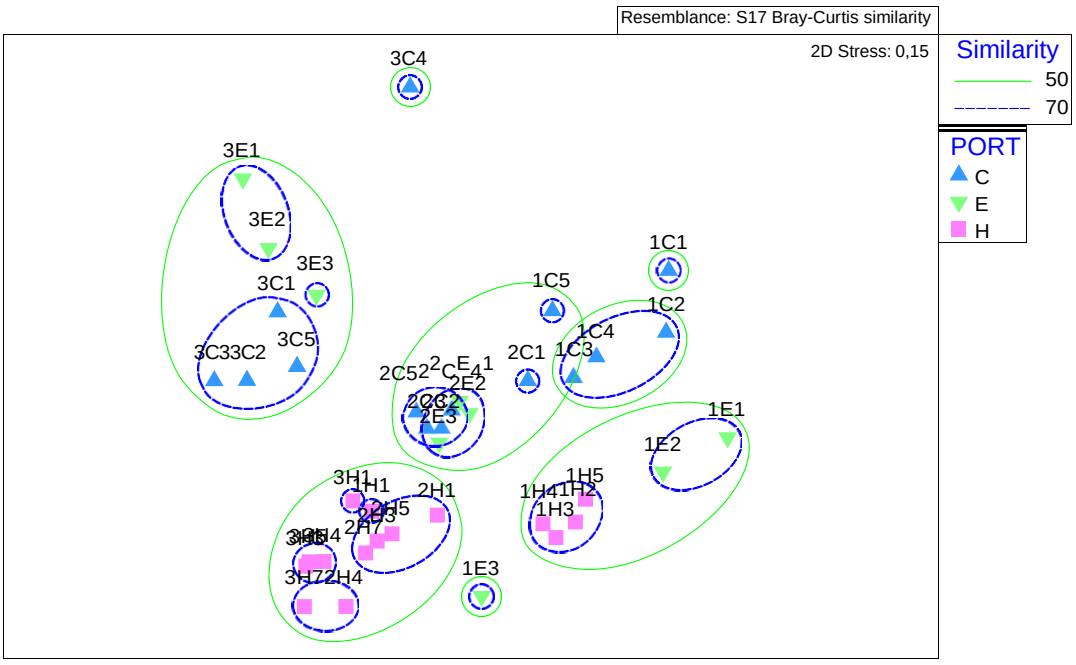

Figure 1.3 nMDS ordination based on Bray-Curtis similarities of T-RFLP profiles of bacterial communities from seawater samples collected at Cagliari (C), El Kantaoui (E), and Heraklion (H) ports.

The R-value for the Cagliari and El Kantaoui pairwise comparison was 0.112 indicating that the communities in seawater from these two ports were more similar to one another than the others (**Table 1.2**). Moreover, the difference between Cagliari and El Kantaoui was not significant ($p>0.01$) while differences between communities from Heraklion and the other two ports were significant ($p<0.01$).

Table 1.2 Analysis of similarity (ANOSIM one way R = 0.353, p= 0.0001) of the three port areas.

Groups		R-value	Significance Level *p*
Cagliari	El Kantaoui	0.112	0.0830
Cagliari	Heraklion	0.466	**0.0001**
El Kantaoui	Heraklion	0.436	**0.0003**

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CHAPTER 2

2. Comparison of the effects of biostimulation treatment on bacterial communities in seawater from different Mediterranean tourist ports

2.1. INTRODUCTION

2.1.1. Behaviour of oil in the marine environments

Human activity has led to the release of liquid petroleum hydrocarbon into the environment, causing the pollution of marine/coastal waters, shorelines and land. Marine environments suffer a greater ecological damage compared to terrestrial environments mainly as a result of the greater difficulty of controlling the pollution (Macaulay et al., 2014). All fuel oil consist of complex mixtures of aliphatic and aromatic hydrocarbons. When oil is introduced into the marine environment the composition of the original oil changes with respect to time due to a series of physical, chemical and biological processes. The physicochemical and biological processes that take place in the weathering of oil are: spreading, evaporation, dissolution, photo-oxidation, dispersion, emulsification, biodegradation (**Figure 3.1**).

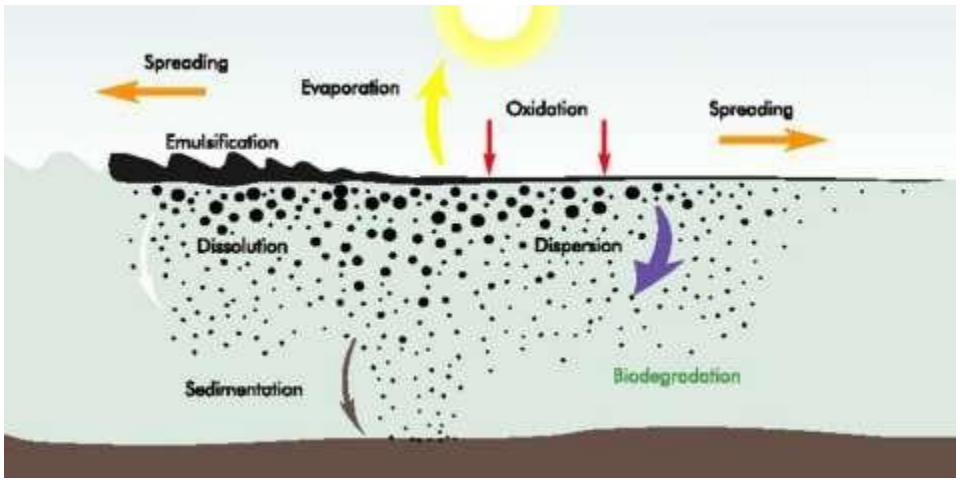

Figure 3.1 Weathering processes of oil in marine environments

Protection of oil from dispersion by wind and wave action in beaches, harbours, small lake and ponds explains the presence of high concentrations of hydrocarbons in these places and the accompanied negative effects on biodegradation. The lowest rates of degradation of diesel oil were observed in protected bays, such as ports, while the highest rates happened in the areas of greatest wave action (Rashid, 1974).

2.1.2. Microbial network involved in oil degradation

Biodegradation of hydrocarbons by natural populations of microorganisms represents one of the primary mechanisms by which petroleum hydrocarbons are eliminated from the environments. Many microbes have

evolved or acquired the ability to utilize hydrocarbons as sources of carbon and energy. Almost a century has passed since the first hydrocarbon-degrading bacteria was isolated and described and a recent list includes almost 200 bacterial, cyanobacterial, algal and fungal genera, representing more than 500 species and strains (Yakimov et al., 2007).

It should be noted that there is no single bacterial strain with the metabolic ability to degrade all the organic compounds found within crude oils and refined petroleum products. In nature, biodegradation of oil constituents typically involves a succession of microorganisms. The primary degraders are responsible for the initial attack of oil constituents. Degradation intermediates are further degraded by the secondary consumers. Microorganisms classified as non-hydrocarbon utilizers may also play an important role in the removal of petroleum from the environment (i.e. production of surface-active compounds) (**Figure 2.1**). Finally, degraders form part of an ecological network, which involves other members of the microbial communities, such as predators (Head et al., 2006).

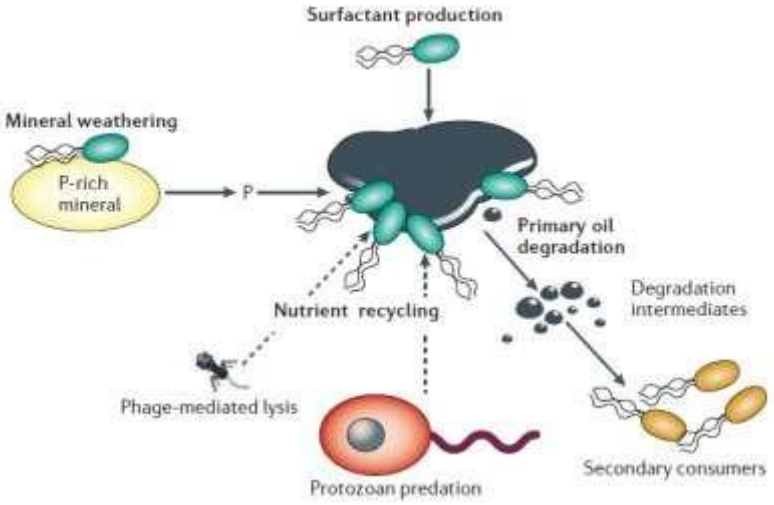

Figure 2.1. Microbial network involved in oil degradation (Head et al., 2006).

The distribution of the hydrocarbon-utilizing microorganisms is related to the historical exposure of the environment to hydrocarbons. Those environments with a recent or chronic oil contamination usually have a higher percentage of hydrocarbon degraders than unpolluted areas. In “pristine” ecosystems, hydrocarbon utilizers may make up less than 0.1% of the microbial community while they can constitute up to 100% of the viable microorganisms in oil-polluted environments (Atlas, 1981). In polluted environments, the selective pressure resulting from pollutants appeared to enrich environments with degraders. Marchal et al. (2003) have demonstrated that the degradation rates of diesel in polluted environments were generally higher than those measured in non-polluted environments. In the marine environments, bacteria are considered to be the predominant hydrocarbon degraders with a distribution range that even covers extreme cold Antarctic

and Arctic environments (Floodgate, 1984; Jordan and Payne, 1980). Hydrocarbon-degrading microorganisms usually exist in very low abundance in marine environments. However, Pollution by petroleum hydrocarbons may stimulate the growth of such organisms and cause changes in the structure of microbial communities in the contaminated area (Caruso et al., 2004). Hassanshahian et al. (2010) have shown oil contamination can induce major changes in marine microbial communities at Persian Gulf and Caspian Sea, that when the pollution occur the number of crude oil degrading bacteria increased and also inhibit some catalytic enzymes (Kohno et al., 2002).

2.1.3. Hydrocarbon bioremediation in marine environments

A number of approaches and technologies have been developed for controlling oil spills in marine shorelines and freshwater environments (Zhu et al., 2001). Strategies for cleaning up an oil spill are greatly affected by a variety of factors, such as the type of oil, the characteristics of the spill site, and occasionally political considerations. Biodegradation is a particularly important mechanism for removing the non-volatile components of oil from the environment. Various types of microorganisms that are capable of oxidizing petroleum hydrocarbons are widespread in nature. For petroleum hydrocarbons, biodegradation is the most important and preferred mechanism since it is the only natural process that results in actual reduction in the mass of petroleum hydrocarbon contamination (Zhu et al., 2001).

Bioremediation has been defined as "*the act of adding materials to contaminated environments to cause an acceleration of the natural biodegradation processes*" (OTA, 1991). This technology is based on the premise that a large percentage of oil components are readily biodegradable in nature (Atlas, 1984, 1981; Prince, 1993). The success of bioremediation depends on ability to establish and maintain conditions that favour enhanced oil biodegradation rates in the contaminated environment.

There are two main approaches to bioremediation used for oil spill clean-up: i) biostimulation, in which the growth of indigenous oil degraders is stimulated by the addition of nutrients, mainly N and P, growth-limiting cosubstrates, other amendments, such surface active compounds, and/or by alterations in environmental conditions (e.g. aeration); ii) bioaugmentation, in which selected hydrocarbon-degrading bacteria are added to supplement the autochthonous microbial communities (Zhu et al., 2001; Tyagi et al., 2011). Among bioremediation strategies, bioaugmentation is still a controversial strategy since different studies have demonstrated either enhancing or inefficient effects. The decision to implement either or both of these techniques for bioremediation largely depends on the degrading capability of the indigenous microbes and the extent of contamination of the site to be treated. Bioaugmentation technique is best suited for sites that (i) do not have sufficient microbial cells or (ii) the native population does not possess the metabolic routes necessary to metabolize the compounds under concern (Tyagi et al., 2011).

2.1.4. Biostimulation in seawater

Nutrient concentrations are variable in marine environments. When a major oil spill occurs in marine and freshwater environments the supply of carbon is dramatically increased and the availability of nitrogen and phosphorus generally becomes the limiting factor for oil degradation (Atlas, 1984; Leahy and Colwell, 1990). For the microorganisms present in marine ecosystems, spilled petroleum hydrocarbons represent a large carbon source. In theory, approximately 150 mg of nitrogen and 30 mg of phosphorus are utilized in the conversion of 1 g of hydrocarbon to cell materials (Rosenberg and Ron, 1996).

Biostimulation involves the addition of rate-limiting nutrients to accelerate the biodegradation process. Several laboratory experiments have shown that the addition of growth-limiting nutrients, namely nitrogen and phosphorus, enhances the rate of oil biodegradation. However, the optimal nutrient types and concentrations vary widely depending on the oil properties and the environmental conditions (Xia et al., 2006; Nikolopoulou et al., 2009). Biostimulation has been widely demonstrated to accelerate the degradation of alkanes (Mueller et al., 1990), BTEX (Joshi et al., 2011) and PAHs (Nikolopoulou et al., 2002; Mueller et al., 1990).

The nutrients supplied could be from organic or inorganic sources. Wrenn et al. (1994) have studied the effects of different forms of nitrogen on biodegradation of light Arabian crude oil in respirometers. In poorly buffered seawater, nitrate is a better nitrogen source than ammonia because acid production associated with ammonia metabolism may inhibit oil biodegradation. Joshi and Pandey (2011) have reported the success of organic nutrient (cow dung) application, while the success of inorganic nutrients (sodium nitrate and dihydrogen phosphate) has been recorded by Roling et al. (2002). However, high concentrations of nutrients applied to the environment may lead to eutrophication in aquatic environments (Nikolopoulou et al., 2002, Smith et al., 1998).

2.2. AIMS OF THE WORK

The aims of this chapter was to answer the following questions: "*Are the effects of bioremediation treatment in seawater comparable among different tourist port areas?*". Laboratory microcosms were used as experimental systems to determine the degradation activities of the autochthonous microbial communities in seawater during bioremediation treatments. The viable title of heterotrophs and hydrocarbon degrading bacteria, the efficiency of alkane degradation as well as the structure of bacterial communities were compared among the three selected case study sites during treatments.

2.3. MATERIALS AND METHODS

2.3.1. Design of microcosms

A laboratory microcosm is a laboratory incubation system that is designed to simulate the environmental conditions of the studied habitat. The tests were carried out in closed systems in order to allow assessment of the carbon balance at the end of incubation period (Solano-Serena et al., 1999a).

Surface seawater samples were collected from the leisure boat port sectors of Cagliari, El Kantaoui, and Heraklion on July 2015 using sterile bottles. The samples were stored at 4°C and immediately used for microcosm set up. The temperature, salinity, pH and concentration of dissolved oxygen in the surface seawater at the time of sampling are shown in **table 2.1**.

Table 2.1 Physical parameters in surface seawater during collection of seawater samples for microcosms

Port	Temperature °C	Salinity	pH	O_2 (mg/L)
Cagliari	26.4	35.5	8.15	6.33
El Kantaoui	27.6	38.0	8.05	5.13
Heraklion	24.9	38.6	8.19	6.58

Microcosms were established in sterile 100 mL dark bottles containing 30 mL of seawater (**Figure 2.2**). All microcosms were prepared by spiking seawater with diesel (1 g/L) and incubated at 25°C in a rotary shaker at 180 rpm. Triplicate microcosms were analysed at 0, 7, 15 and 30 days. At each kinetic time, 13 mL of seawater were collected for each microcosm and used for determination of microbiological parameters: viable titles of heterotrophic and hydrocarbon-degrading bacteria by MPN method (***Ref Par. 1.3.3***) and bacterial community structure. The remaining seawater in the microcosm (17mL) were used to determine the residual hydrocarbon concentration.

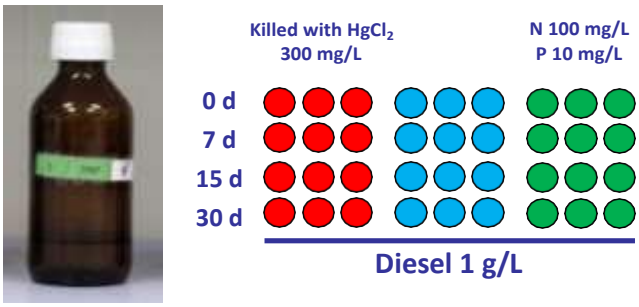

Figure 2.2 Design of bioremediation tests in seawater microcosms

Different bioremediation tests were set up: i) abiotic controls were amended with mercuric chloride (300 mg/L) to measure the contaminant lost without the participation of biological processes (DK); ii) natural

attenuation trials were carried out by adding only diesel in order to evaluate the natural ability of the autochthonous bacterial communities to degrade diesel (intrinsic bioremediation) (D); iii) biostimulation treatments were carried out on seawater supplemented with inorganic nutrients ($NaNO_3$, KH_2PO_4) with the ratio of C:N:P equal to 100:10:1 (DNP) according to Xia et al. (2006).

2.3.2. Residual hydrocarbon determination

For residual hydrocarbon determination, the extraction was carried out by adding 50 µL of o-terphenyl solution as standard (CAS: 84-15-1; 1000 µg/mL in hexane) to the total volume of the cultures. Hydrocarbons were extracted in a separatory funnel (U.S. EPA Method 3510C with minor modifications) using 5 mL of hexane. The mixture was shaken for 2 min and subsequently allowed to settle for 1-2 min for phase separation. The water phase was drained to a 50 mL-beaker and the hexane phase was collected into a 50 mL-amber bottle. The procedure was repeated four times. The hexane extracts were pooled and dried with anhydrous sodium sulphate.

Hydrocarbons were analysed by the Department of Civil-Environmental Engineering and Architecture, University of Cagliari, Italy. The concentrations in the extracts of Diesel Range Organics (DRO, C_{10} - C_{25}) were determined by an Agilent 6890N gas chromatograph with a mass spectrometer (GC-MS), using an Agilent VF-1ms capillary column (30 m x 0.25 mm i.d. x 0.25 µm film thickness). Helium was used as the carrier gas at a flow rate of 1.0 ml/min. The oven temperature program was as follows: 45°C hold for 3 min, increased to 300°C at 12°C/min and kept at 300°C for 5 min. The quantification of DRO was performed by external calibration standard (16 compounds DRO Mix, 1000 mg/L of each *n*-C_{10}-C_{25}). Phytane and pristine were also included in the analysis. The correlation coefficients for calibration curves were all higher than 0.9754. The detection limit of DRO was 0.01 mg/L.

The peak area of each compound (C_{10}-C_{25}) was normalized as respect to the injection and extraction standards and the ΣC_{10}-C_{25} was calculated. For evaluating the residual hydrocarbons in microcosms, the ratio was calculated between the ΣC_{10}-C_{25} value obtained at each kinetic time and the ΣC_{10}-C_{25} value at day 0, and expressed as percentage.

2.3.3. DNA extraction and purification for T-RFLP analysis

Water aliquots from microcosms were filtered throughout a 0.2 µm pore-size filter (Sartorius, Goettingen, Germany) and stored at -20 °C until analysis. The filters were cut into 1-2 mm under axenic conditions. DNA extraction from cells collected onto filters was carried out by Powersoil® DNA Isolation Kit (MOBIO Laboratories, Inc) following manufacturer's instructions. The structure of bacterial community was determined by T-RFLP (***Ref. Par. 1.3.4***).

2.3.4. Statistical analysis

One-way ANOVA (ANalysis Of VAriance) is a statistical procedure for testing the null hypothesis that several univariate samples (in columns) are taken from populations with the same mean. ANOVA one way and the Tukey test ($p < 0.05$) was used for comparison of means as implemented in the software PAST 1.42 (Hammer et al., 2001).

2.4. RESULTS AND DISCUSSION

2.4.1. Preliminary tests of natural attenuation

To determine the degradation activities on diesel of the autochthonous microbial communities in seawater during natural attenuation laboratory microcosms amended and abiotic control were set up for each port. After 30 days, no significant hydrocarbon degradation (ANOVA $p>0.05$) was found for the three port areas being differences between natural attenuation and abiotic control not significant (data not shown).

Abundant populations of diesel degraders were found in seawater of the three case study sites (***Ref. Chapter 1***). However, biodegradation of diesel was not observed in microcosms during natural attenuation treatment. This result suggesting the presence of limiting factors to microbial degradation in seawater when an oil spill is simulated (spiked with diesel). High concentration of hydrocarbons in water means heavy undispersed oil slicks causing a limited supply of nutrients and oxygen, and thus resulting in the slow degradation rates.

2.4.2. Comparison effect of the biostimulation treatments

In order to compare the degradation activities on diesel of the autochthonous microbial communities colonizing the seawaters of the three selected case study sites, microcosms were set up seawater with nutrient regulation reaching a C:N:P ratio of 100:10:1.

Comparable initial viable titles of diesel degraders were found from Cagliari (mean $4.0 \pm 0.4 \times 10^2$ MPN/mL) and Heraklion (mean $4.5 \pm 0.1 \times 10^2$ MPN/mL) whereas the abundance of hydrocarbon degraders in El Kantaoui was 2 log higher as compare to other harbors (**Figure 2.3**). Moreover, the abundance of heterotrophs was comparable in Cagliari (2.4×10^2 MPN/mL) and Heraklion (1.0×10^2 MPN/mL) while El Kantaoui showed a viable title 1 log higher as compare to other harbors. It is interesting to note that after 30 days, the two metabolic groups of bacteria were equally abundant (10^6 MPN/mL) showing comparable values for the three ports.

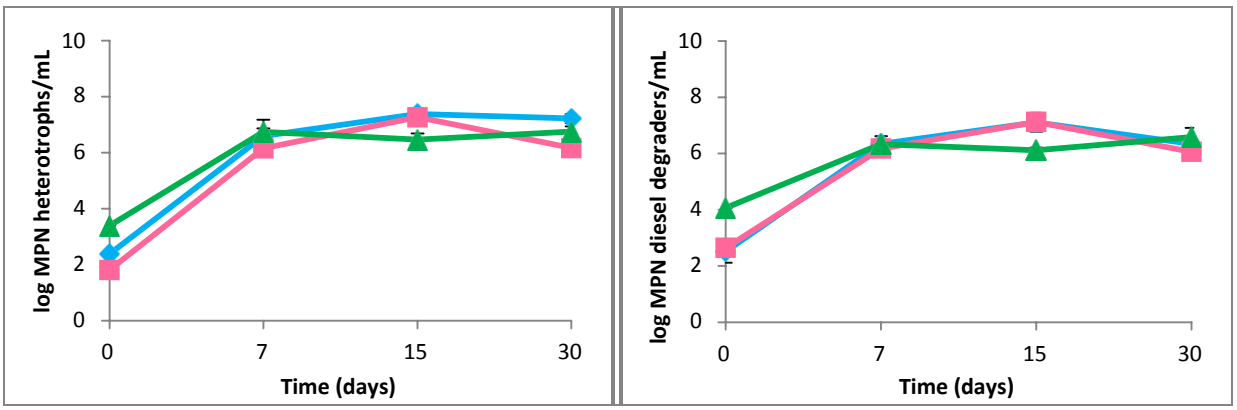

Figure 2.3 Viable titles in heterotrophs and diesel degraders in microcosms of nutrient-supplemented seawater polluted with diesel (Mean ± St.Dev, *n*=3)

Figure 2.4 shows the biodegradation kinetics in microcosms of nutrient-supplemented seawater polluted with diesel. During the biostimulation treatment, the microbial communities of El Kantaoui port removed 80% of *n*-alkanes after 15 days whereas the removal levels in tests of Cagliari and Heraklion at the same kinetic time were only 60%. After 30 days, the diesel biodegradation levels were 82%, 67%, and 72% for El Kantaoui Cagliari and Heraklion, respectively.

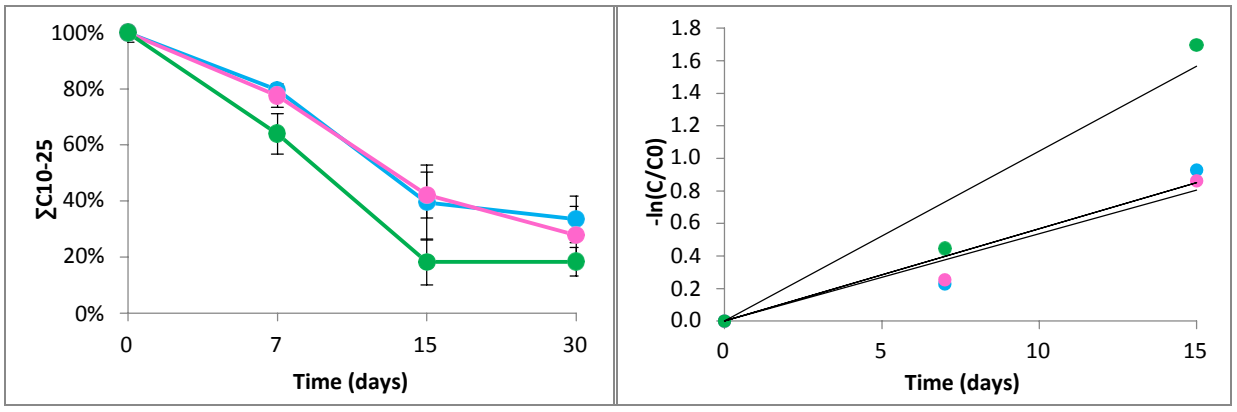

Figure 2.4 Biodegradation kinetics in microcosms of nutrient-supplemented seawater polluted with diesel (Mean ± St.Dev, *n*=3)

According to Xia et al. (2006), the biodegradation rates were estimated assuming the oil biodegradation process fits to a first order model (r=KCn, where n = 1) and plotting ln(C/C0) versus time (**Figure 2.4**): the slope of the regression line gives the value of the reaction rate coefficient K (**Table 2.2**). Data demonstrated comparable degradation kinetics for Cagliari and Heraklion sites during the biostimulation treatment. Furthermore, nutrient amendments in El Kantaoui microcosms resulted in a two-fold increase in the degradation rates as compared to the other two port areas.

Table 2.2 Parameters of the biodegradation kinetics of diesel in nutrient-supplemented seawater

Port	K	R^2
Cagliari	0.0567	0.9263
El Kantaoui	0.1044	0.9365
Heraklion	0.0537	0.9538

2.4.3. Comparison of bacterial communities during biostimulation treatment

Bacterial communities in seawater microcosms during treatment with inorganic nutrients were compared by determining their structure by T-RFLP and subsequent calculation of the similarity between profiles. **Figure 2.5** shows the result of MDS ordination, a map where the position of each sample is determined by its distance from all other points in the analysis. During treatment, the structures of the bacterial communities of El Kantaoui were more homogenous than those from Cagliari while Heraklion presented a moderate change in community structures. At the end of the treatment (30 d) different community structures were found for the three sites.

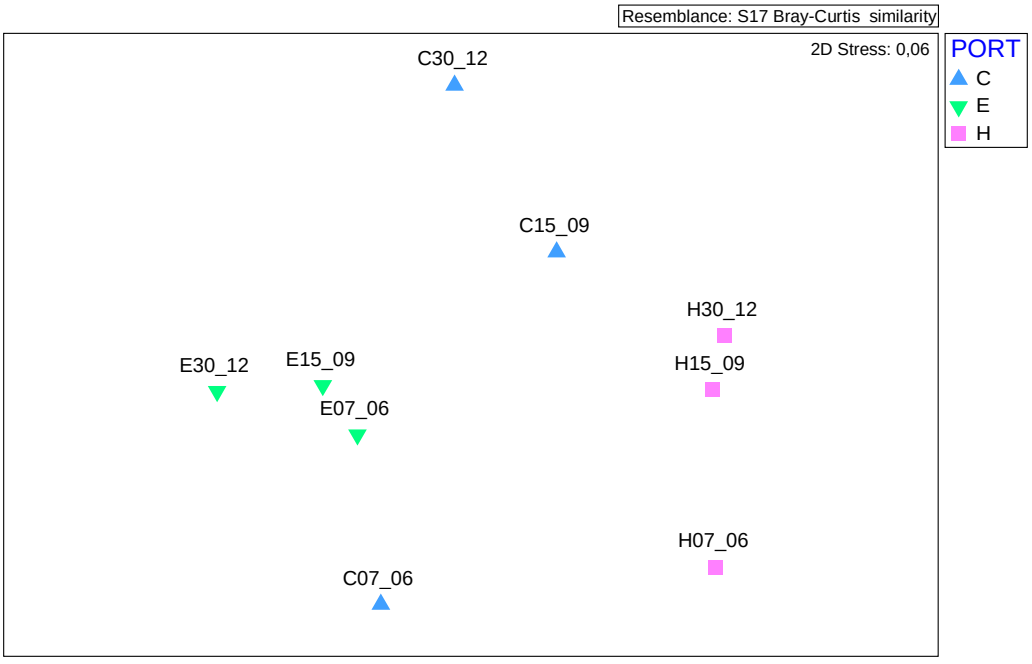

Figure 2.5 nMDS ordination based on Bray-Curtis similarities of T-RFLP profiles of bacterial communities in seawater microcosms during treatment with inorganic nutrients. Cagliari (C), El Kantaoui (E), and Heraklion (H) ports

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CHAPTER 3

3. Characterization and comparison of alkane-degrading bacteria from seawater of different Mediterranean tourism ports

3.1. INTRODUCTION

3.1.1. Bacterial aerobic degradation of alkanes

Aerobic alkane degraders use O_2 as a reactant for the activation of the alkane molecules (**Figure 3.1**). Activation of the alkane molecules requires oxygenase activity that overcomes the low chemical reactivity of the hydrocarbons by generating reactive oxygen species. In the case of *n*-alkanes containing two or more carbon atoms, aerobic degradation usually starts by the oxidation of a terminal methyl group to render a primary alcohol that is further oxidized to the corresponding aldehyde, and finally converted into fatty acid. Fatty acids are conjugated to CoA and further processed by β-oxidation to generate acetyl-CoA, which can enter the tricarboxylic acid cycle to yield carbon dioxide and energy.

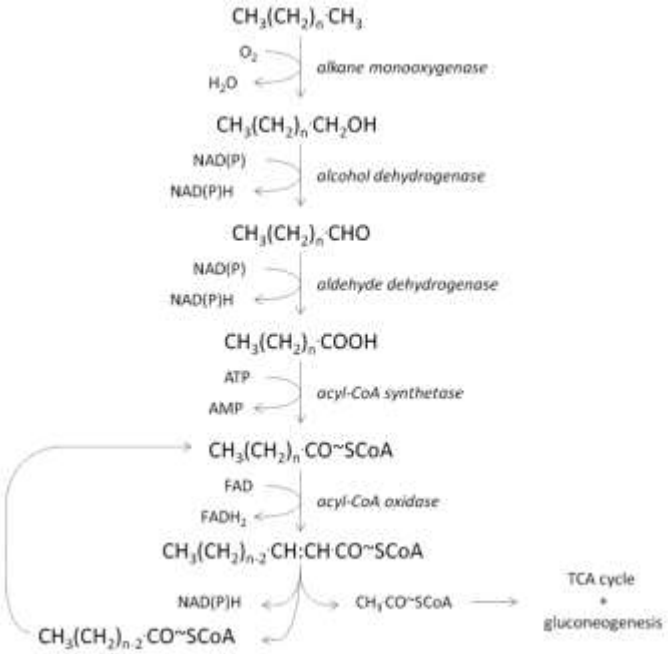

Figure 3.1. Catabolic pathway for aerobic alkane degradation (Pedrini et al., 2013).

The oxidation of alkanes have been characterized is carry out by several unrelated enzyme classes (van Beilen et al., 2003; Rojo, 2010).

A membrane-bound alkane hydroxylase was first discovered in a hexane-degrading fluorescent pseudomonad tentatively named *Pseudomonas oleovorans*, (Baptist et al., 1963; van Beilen et al., 1994), but later identified as a *P. putida* (van Beilen et al., 2001). Biochemical studies showed that the *P. putida* alkane hydroxylase system consists of an integral membrane monooxygenase (McKenna et al., 1970; van Beilen et

al., 1992), 1 or 2 rubredoxins (Peterson & Coon 1998; van Beilen et al., 2002), and a rubredoxin reductase (Ueda & Coon 1972; Eggink et al., 1990). Rubredoxin reductase transfers electrons from NADH via its cofactor FAD to rubredoxin, which transfers the electrons to the alkane hydroxylase (**Figure 3.2**). In the last years, enzyme systems related to the *P. putida* GPo1 alkane hydroxylase were cloned from bacterial strains belonging to several different genera (van Beilen et al., 2003; Rojo, 2010). While the *P. putida* GPo1 system acts on alkanes ranging from pentane to dodecane (C_5-C_{12}) (McKenna & Coon, 1970; van Beilen et al.,1994), most related membrane-bound alkane hydroxylases, belonging to the AlkB family, solely hydroxylate alkanes containing more than 10 carbon atoms (Smits et al., 2002).

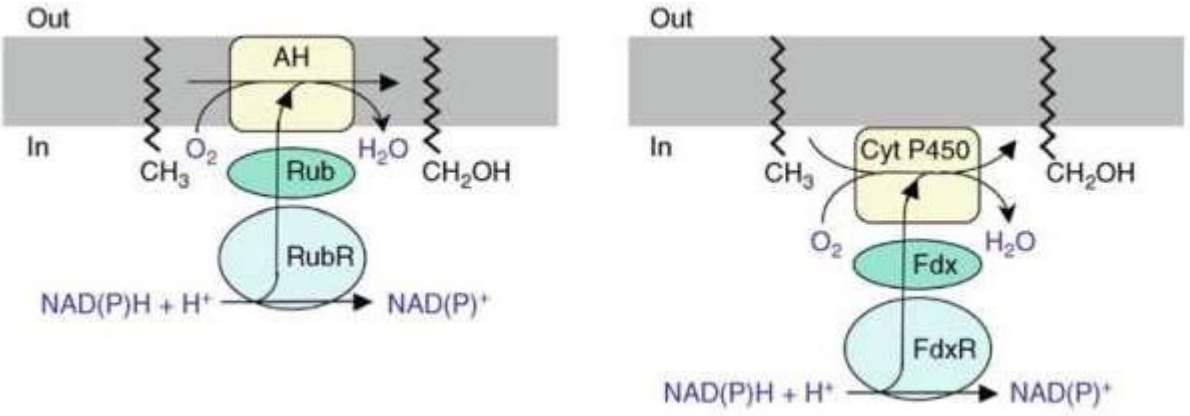

Figure 3.2. Oxidation of *n*-alkanes by alkane hydroxylases belonging to the AlkB family (left) or to the bacterial cytochrome P450 family (right). AH, membrane bound alkane hydroxylase; Rub, rubredoxin; RubR, rubredoxin reductase; Cyp P450, soluble cytochrome P450; Fdx, ferredoxin; FdxR, ferredoxin reductase. The gray bar represents the cytoplasmic membrane (Rojo, 2010).

Cytochromes P450 are heme-thiolate proteins that catalyse the oxygenation of a large number of compounds. They are ubiquitous among all kingdoms of life and can be grouped in more than 100 families on the basis of sequence similarity. Several bacterial strains that degrade C_5–C_{10} alkanes contain alkane hydroxylases that belong to a distinct family of bacterial soluble cytochrome P450 monooxygenases. The first member characterized was CYP153A1 from *Acinetobacter* sp. EB104 (Maier et al., 2001), but similar enzymes have been found in diverse strains of mycobacteria, rhodococci and proteobacteria (Sekine et al., 2006; van Beilen et al., 2005a, 2006). These cytochromes P450 require the presence of a ferredoxin and ferredoxin reductase that transfer electrons from NAD(P)H to the cytochrome. Complementation assays showed that many of these cytochrome P450 proteins can functionally substitute for P. putida GPo1 AlkB, showing that they are true alkane hydroxylases (van Beilen et al., 2006). The cytochrome P450 from *Mycobacterium* sp. HXN-1500 was purified and shown to hydroxylate C_6–C_{11} alkanes to 1-alkanols with high affinity and regioselectivity (Funhoff et al., 2006). While the GPo1 system and cytochrome P450 acts on alkanes ranging from pentane to dodecane (C_5-C_{12}) (McKenna & Coon, 1970; van Beilen et al.,1994), most related membrane-

bound alkane hydroxylases solely hydroxylate alkanes containing more than 10 carbon atoms (Smits et al., 2002).

The enzymes responsible for the oxidation of such alkanes, which are solid at room temperature, are still poorly characterized. In *Acinetobacter* sp. M1, which can grow on C_{13}–C_{44} alkanes, several alkane oxidizing enzymes have been detected. Two of them, named AlkMa and AlkMb, are related to *P. putida* GPo1 AlkB and are membrane bound (Tani et al., 2001). A third enzyme has been reported that is soluble, requires Cu^{2+}, does not receive electrons from NADH and is therefore clearly unrelated to the AlkB family of hydroxylases (Maeng et al., 1996). It has been proposed to be a dioxygenase that oxidizes C_{10}–C_{30} alkanes generating *n*-alkyl hydroperoxides that render the corresponding aldehyde. A different *Acinetobacter* strain, named DSM 17874, also contains at least three alkane-oxidizing enzymes. Two of them are AlkB paralogs similar to the AlkMa and AlkMb enzymes described above, and oxidize C_{10}–C_{20} alkanes (Throne-Holst et al., 2006). A third enzyme has been reported that oxidizes from C_{20} to $>C_{32}$ alkanes. Its gene, designated *almA*, has been identified and codes for a flavin-binding monooxygenase (Throne-Holst et al., 2007). Genes homologous to *almA* were identified in several other long-chain *n*-alkane degrading strains, including *Acinetobacter* M1. Most notably, two genes similar to *almA* were also detected in the genome of *A. borkumensis* SK2.

Branched-chain alkanes are more difficult to degrade than linear *n*-alkanes (Pirnik et al., 1974). However, several bacterial strains can degrade simple branched-chain alkanes, such as isooctane (Solano-Serena et al., 2004), or much more complex compounds, such as pristine (reviewed in Britton, 1984; Watkinson and Morgan, 1990). *Alcanivorax* sp. can also degrade branched alkanes, such as pristane and phytane, a property that seems to provide a competitive advantage in oil-contaminated seawater (Hara et al., 2003). The metabolic pathways responsible for the assimilation of branched alkanes are less well characterized than those for *n*-alkanes, and may involve an ω- or β-oxidation of the hydrocarbon molecule (Watkinson and Morgan, 1990).

Over the last years, several microorganisms, mostly belonging to the β- and δ- subclasses of proteobacteria, have also been described being able to use *n*-alkanes (except for methane) as carbon source in the absence of O_2 (Widdel et al., 2001). These microorganisms use nitrate or sulphate as electron acceptors (Heider et al., 1999). Although the growth rate is significantly slower than that of aerobic alkane degraders, the anaerobic degradation of alkanes plays an important role in the recycling of hydrocarbons in anoxic environments, such as marine sediments (Rojo, 2009).

3.1.2. Role of surface active compounds in the microbial access to hydrocarbons

The growth of microorganisms on crude oils and refined petroleum products, such as diesel, presents particular problems because petroleum hydrocarbons have a low solubility in water. The mechanisms through which the alkanes enter the cell are still mostly unknown. However, it is clear that the mechanism

may differ depending on the bacterial species considered, the molecular weight of the alkane and the physico-chemical characteristics of the environment (Wentzel et al., 2007).

Hydrocarbon metabolism is always restricted to the water/hydrocarbon interfaces since the oxygenases involved in their catabolic pathways are never extracellular but always membrane-bound enzyme (Van Hamme et al., 2003). Thus, microbial growth on hydrocarbons can be limited by the interfacial surfaces leading to a linear growth rather than exponential one. Microbial ability to biosynthesize Surface Active Compounds (SACs) is often coupled with their ability to grow on immiscible carbon sources, although many microorganisms produce amphiphilic metabolites from water soluble carbon sources (Franzetti et al., 2010).

Neu et al. (1996) divided SACs into three different classes: (i) biosurfactants are defined as low molecular weight SACs (e.g., glycolipids, lipopeptides); (ii) amphiphilic polymers are defined as high molecular weight SACs with a hydrophobic region at one end of the molecule (e.g., lipopolysaccharides, lipoteicoic acids); (iii) polyphilic polymers are defined as high molecular weight SACs with hydrophobic groups distributed across the entire polymeric molecule (e.g., hydrophobic polysaccharides, emulsan).

The low molecular weight SACs or biosurfactants lower the surface tension at the air/water interfaces and the interfacial tension at oil/water interfaces, whereas the high molecular weight SACs, also called bioemulsifiers, are more effective in stabilizing oil-in-water emulsions. The ability of different microorganisms to access hydrocarbons depends on their cell surface hydrophobicity. High cell-hydrophobicity allows them to directly contact oil drops and solid hydrocarbons while low cell hydrophobicity permits their adhesion to micelles or emulsified oils (Van Hamme et al., 2003; Bouchez Naitali, 1999).

Three different mechanisms of cell access to hydrocarbons have been postulated: (i) access to water-solubilize hydrocarbons, (ii) direct contact of cells with large oil drops, (iii) contact with pseudosolubilized or emulsified oil. The first mechanism is limited to low molecular weight hydrocarbons since the hydrocarbon solubility, dramatically, decreases with increased molecular weights. In rhodococci, cells are hydrophobic due to the presence of a hydrophobic mycolic acid layer in their cell walls and the major hydrocarbon accession mode is likely to be direct contact of hydrophobic cells with large oil drops. On the other hand, the access to hydrocarbons in *Pseudomonas* strains relays on the release in the culture broths of the extracellular surfactants, rhanmolipids, which enhance the apparent solubility of hydrocarbons. The hydrophilic surface allows *Pseudomonas* cells to interact with the hydrophilic outer layer of the hydrocarbon containing micelles. Franzetti et al. (2008) suggested that some microbial SACs play a role in changing the substrate access mode during the different growth stages on hydrocarbons.

Frequently the production of SAC is coupled with the ability to degrade oil. Alternatively, SAC-producing bacteria can function in a bacterial consortium, supplying the emulsifier for other bacteria that carry out the degradation of the hydrocarbons. Several studies tend to focus on the microorganisms that degrade the

contaminants. However, these microorganisms form part of an ecological network, which involves many direct and indirect interactions with other community members and the environment (Head et al., 2006).

3.1.3. Obligate hydrocarbonoclastic bacteria

In the marine environments, bacteria are considered to be the predominant hydrocarbon degraders with a distribution range that even covers extreme cold Antarctic and Arctic environments (Floodgate, 1984; Jordan and Payne, 1980). Interestingly, and in contrast to terrestrial hydrocarbon degraders which tend to be metabolically versatile and utilize a large range of organic substrates, their marine counterparts are mostly highly specialized obligate hydrocarbon utilizers, the so-called marine 'obligate hydrocarbonoclastic bacteria' (OHCBs). This group of hydrocarbon degraders occupy a unique trophic niche among heterotrophic bacteria participating in the global carbon cycle, in that they preferentially consume aliphatic and aromatic hydrocarbons that are relatively difficult to use by bacteria (Golyshin et al., 2003).

In the past years, several interesting marine bacteria that are specialists adapted to hydrocarbon degradation have been isolated. These bacteria use hydrocarbons almost exclusively as a carbon source, and they include *Alcanivorax* spp., *Cycloclasticus* spp., *Oleiphilus* spp., *Oleispira* spp., *Thalassolituus* spp.. Some members of the genus *Planomicrobium*. *Alcanivorax* spp., *Oleiplilus* spp., *Oleispira* spp. and *Thalassolituus* spp. use a variety of branched- and/or straight-chain saturated hydrocarbons. By contrast, *Cycloclasticus* spp. have evolved the ability to use a range of polycyclic aromatic hydrocarbons (PAHs) (Head et al., 2006; Yakimov et al., 2007).

The ubiquity of *A. borkumensis* in marine ecosystems presumably results from its capacity to grow on many saturated petroleum fraction constituents and biogenic hydrocarbons: straight-chain and branched alkanes, isoprenoids and long side-chain alkyl compounds. *Alcanivorax*-related 16S rRNA gene sequences have been retrieved from microbial communities inhabiting cold polar areas. *Alcanivorax*-like organisms have also been detected not only in marine environments but also in few terrestrial environments that share relevant properties (salinity, presence of hydrocarbons) with marine ecosystems (**Figure 3.3**).

Two other OHBC, *T. oleivorans* and *Cycloclasticus* spp., are also widely distributed, although so far they have mostly been found in the Northern hemisphere. The genera *Thalassolituus* inhabiting both marine and terrestrial environments. By contrast, the genus *Cycloclasticus* was retrieved exclusively from marine microbial communities, or PAH-supplemented enrichments thereof. In contrast to the cosmopolitan OHCBs, distribution of the psychrophilic OHCB *Oleispira antartica* is thus far limited to the colder waters found at high latitudes. *Oleiphilus messinensis,* initially isolated from harbour sediments, has also been recovered from bryozoan and dictyoceratid sponges samples in North Atlantic and equatorial Pacific oceans (Yakimov et al., 2007).

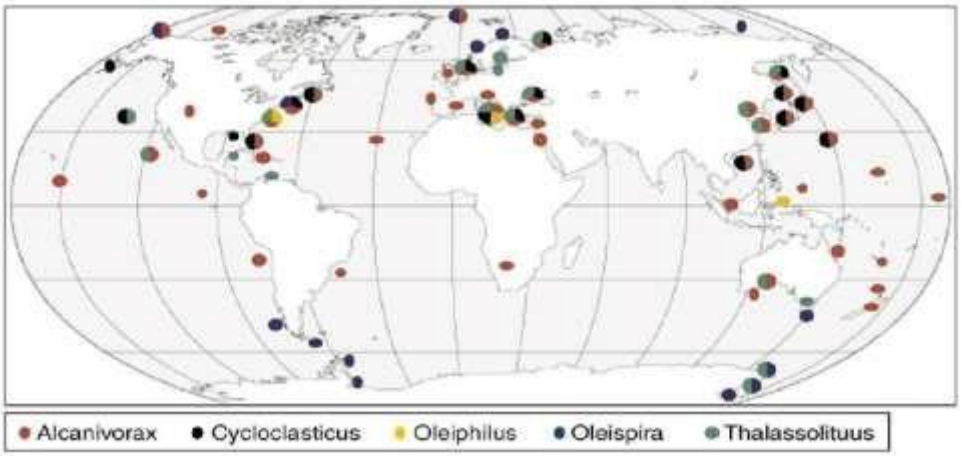

Figure 3.3. Biogeography of obligate hydrocarbonoclastic bacteria (Yakimov et al., 2007).

Few studies have revealed unusual and interesting features of the marine OHCB lifestyle. Organisms analysed so far exhibit features typical of oligotrophic bacteria. Studies on *C. oligotrophus* have shown that the cytoplasm of this small bacterium is very dilute, with a dry mass per cell 7-8 times lower than that of *E. coli,* while the DNA content is up to 14% of the dry weight, contrasting with 2% for *E. coli* (Button et al., 1998). Only few rRNA operons in the genome (1–3), few cytoplasmic proteins (not more than 300), and a small genome size (3-4 Mbp) are characteristic properties of OHCBs. A single strain can use only few hydrocarbons as sources of carbon and energy, and, as a rule, only one or two low molecular weight organic acids, such as acetate and pyruvate. Sugars and amino acids cannot usually be metabolised by OHCB. The number of membrane proteins in OHCB cells is about half that of other heterotrophic bacteria, such as *E. coli* and *Pseudomonads*, which may partly explain the narrow substrate spectrum. Moreover, genomic analysis of the *A. borkumensis* strain SK2 has revealed a large repertoire of genetic determinants for the uptake of mineral nutrients that are limiting in marine environments, particularly following a sudden input in oil, which leads to severe imbalances in carbon/nitrogen and carbon/phosphorus ratio. (Yakimov et al., 2007).

In the genome of *A. borkumensis* strain SK2, multiple systems for hydrocarbon catabolism, namely two alkane hydroxylase systems AlkB1 and AlkB2 and three P450 cytochromes, have been found (Schneiker et al., 2006 - van Beilen et al.,2004 – 2005, Hara et al.,2004 – Kubota et al.,2005). Both alkane hydroxylase systems are located close to the origin of replication of the chromosome, which provides a high gene dosage and presumably high expression levels of these catabolic systems. Proteomic profiling suggested that both AlkB systems and all three cytochromes P450(a-c) participate in catabolism of saturated hydrocarbons (Sabirova et al.,2005). Quantitative real-time transcriptional analysis showed that the P450(b) and P450(c) genes, which encode identical polypeptide sequences, were expressed only in the presence of alkane, whereas P450(a) was also expressed in cells growing on pyruvate. Similar expression profiles of the *alkB1*, *alkB2* and cytochromes P450 (a,b,c) genes were observed in exponential cultures growing on *n*-tetradecane.

Interestingly, a strong induction of the cytochrome P450(a) gene was found in phytane-grown cells, but no transcription of *alkB1*. Such a differential expression of these two genes may be useful in the application of *A. borkumensis* gene transcription profiling as a possible bio-indicator of oil pollution (Schneiker et al.,2006).

The *alkSB1GHJ* gene clusters are found in a number of bacteria, e.g. in the genome-sequenced obligate marine hydrocarbon-degrading *Marinobacter aquaeolei* (strain VT8) and the ubiquitous alpha-proteobacterium *Oceanocaulis alexandrii* (strain HTCC2263). Comparative sequence analysis of *alkS/alkB1* genomic regions revealed that the genetic organization in these bacteria and *A. borkumensis* is more similar than that found in soil pseudomonads (Smits et al.,2002). As it has been shown previously for a number of alkane-degrading microorganisms, the entire *alkSB1GHJ* gene cluster is a prominent region of alien origin, typically characterized by a significantly lower G+C content than the rest of the genome [2004]. This is also the case for these three marine bacteria: the G+C content of *alkSB1GHJ* in *O. alexandrii* is 7.8% lower than the average, in *A. borkumensis* -6.8%, in *M. aquaeolei* -7.4% and -4.1% (two distinct *alkB* clusters). The bracketing of the *alkSB1GHJ* clusters of *M. aquaeolei* and *O. alexandrii* with putative transposase genes is consistent with an earlier observation that gene clusters for alkane degradation can be transferred among bacteria via mobile genetic elements (Smits et al., 2002).

A. borkumensis forms stable emulsions of hydrocarbon in water and produces biosurfactants (Passeri et al., 1992). These biosurfactants are anionic glucolipids carrying four fatty acids of varying chain lengths (Abraham et al., 1998). This glucose-lipid exists in two forms, a glycine-containing precursor form, linked to cell surface, and a glycine-lacking form that is released from the cell and is free in the surrounding medium. The presence of the biosurfactant precursor on the cell surface increases the hydrophobicity of the cell and its affinity for oil droplets suspended in the water phase. On the other hand, the extracellular form promotes the formation of oil-water micelles emulsions, and thereby increases oil bioavailability. This suggests that the glucose–lipid biosurfactant may be a key component of the success of *Alcanivorax* in oil-polluted marine systems.

The genetic organization of the glucolipid biosynthesis remains unclear in *A. borkumensis* SK2, but genome annotation revealed candidate genes potentially involved in biosurfactant production. ABO_1783 and ABO_2215 encode glycosyltransferases, exhibiting significant homology with RhlB from *Pseudomonas aeruginosa* (Ochsner et al.,1994) and glycosyltransferase protein family (Syutsuboet et al., 2001), respectively. These gene products possibly provide the sugar moiety of the glucolipids, yielding glucose lipid surfactants. *A. borkumensis* SK2 also expresses an OprF/OmpA protein encoded by ABO_0822, which is upregulated when grown on alkane (Dutta et al., 2001). OmpA proteins, the active constituents of the biosurfactant Alasan, have been demonstrated to efficiently emulsify hydrocarbons (Toren et al., 2002) and to be produced by a number of oil-degrading γ-Proteobacteria (Ron & Rosenberg, 2001). *A. borkumensis* SK2 harbours determinants of OprG/OmpW (ABO_1922) and OmpH (ABO_1152), which are also possibly involved

in emulsifier production. Qiao and Shao (2010) have identified proline lipid produced by *A. dieselolei* B-5. This strain is the first-reported lipopeptide producer in the genus *Alcanivorax*.

3.2. AIMS OF THE WORK

The aims of the present study were to isolate, characterize and compare the dominant hydrocarbon-degrading bacteria from seawater of three different tourist ports in the Mediterranean Sea selected as case study sites: Cagliari (Sardinia, Italy) El Kantaoui (Tunisia) and Heraklion (Greece). The catabolic abilities of the selected strains were defined on diesel as a model mixture of petroleum hydrocarbons representatives of pollution resulting from maritime transport. Moreover, the ability of the isolates to produce surface-active compounds, biosurfactants and bioemulsifiers, were also evaluated.

3.3. MATERIALS AND METHODS

3.3.1. Medium carbon sources

n-dodecane 99% and *n*-hexadecane 99% were obtained from Sigma-Aldrich (Steinheim, Germany) whereas diesel were obtained from Kuwait petroleum Italia spa. The carbon sources were filter-sterilized using solvent-resistant cellulose filters (0.2 mm pore size).

3.3.2. Strain selection and isolation in pure culture

The isolation procedure was carried out from MPN cultures with diesel as unique carbon and energy source obtained from samples of superficial seawaters collected in the three port areas during the seasonal campaigns performed in 2012 (***Ref. Chapter 1***).

In order to characterize the dominant bacterial hydrocarbon-degraders, aliquots of the most dilute MPN cultures exhibiting growth were streaked onto solid ONR7a supplemented with diesel. ONR7a is an artificial seawater mineral salts medium based on the ionic composition of seawater commonly used for cultivation of hydrocarbonoclastic bacteria (Dyksterhouse et al., 1995). ONR7a medium composition was the following (per litre): NaCl 22.79 g, Na_2SO_4 3.98 g, $C_7H_{17}NO_7S$ 1.3 g, KCl 0.72 g, NH_4Cl 0.27 g, $Na_2HPO_4 \cdot 7\ H_2O$ 89 mg, NaBr 83 mg, $NaHCO_3$ 31 mg, H_3BO_3 27 mg, NaF 2.6 mg, $MgCl_2 \cdot 6\ H_2O$ 11.18 g, $CaCl_2 \cdot 2\ H_2O$ 1.46 g, $SrCl_2 \cdot 6\ H_2O$ 24 mg, $FeCl_2 \cdot 4\ H_2O$ 2.0 mg (Dyksterhouse et al., 1995). The carbon source was offered as vapour phase by placing a filter disc with 80 mg of diesel in the lid of each plate and wrapping the plates in Parafilm. Cultures were incubated for 20 days at 25°C. Colonies with different morphologies were isolated by repeat streaking on the same medium. Once culture purity was established, the isolates were grown onto Marine agar supplemented with pyruvate (10 g/L) and stored at -20°C. The letter of the strain label refers to the sampling site (C: Cagliari, E: El Kantaoui, H: Heraklion).

3.3.3. 16S rRNA genes sequencing and phylogenetic analysis

Bacterial cells (50 mg/mL) were lysed by heating in a microwave oven for 40 s at 800 W and the cell lysates used as template in PCR reaction without further purification. The amplification of the 16S rRNA genes and ARDRA were performed as previously described by Tamburini et al. (2003) using the universal bacterial primers P0 and P6. The amplified products were purified with the QiaQuick PCR purification Kit (Qiagen, Hilden, Germany). DNA sequencing was performed on an ABI Prism 310 Genetic Analyzer.

The isolates were assigned at genus level by comparing their sequences with the RDP database (Wang et al. 2007). Moreover, sequences of isolates and selected type strains were used to construct phylogenetic trees by applying the neighbour-joining algorithm as implemented in the software MEGA 5.1 (Tamura et al., 2011). The evolutionary distance matrix was generated by Kimura-2-parameter (Kimura, 1980). The robustness of the phylogeny was tested by bootstrap analysis with 1,000 replicates. We are currently depositing the 16S rRNA sequences obtained in this work in the GenBank.

3.3.4. Growth and SAC production on hydrocarbons

3.3.4.1. Inoculum preparation

The isolates were grown onto solid Marine Agar supplemented with pyruvate (10 g/L) for 3 days at 25°C. Liquid cultures were prepared in Marine Broth and incubated for 3 days at 25°C in a rotary shaker at 180 rpm. The cells were centrifuged, washed twice in ONR7a and suspended in the same medium.

3.3.4.2. Growth test in multiwell

The cultures were prepared in 96-well plate (Sarstedt, Nümbrecht, Germany) containing 180 µL of ONR7a medium. Bacterial cells were inoculated to an initial optical density at 600 nm (OD_{600}) of 0.050. After 10 days at 25°C, bacterial growth was evaluated spectrophotometrically with an automatic plate reader (Sunrise Tecan, Grodig/Salzburg, Austria) at OD_{630} nm. For each isolate, eight replicate cultures were prepared supplementing the medium with 5 µL/well of diesel. In all multiwells, the eight wells of the last column were not inoculated (sterile control). For each isolate and the sterile control, the average values (n=8) of OD_{630} were calculated. The ΔOD_{630} was calculated by subtraction the average value of the sterile control from the average value of each strain.

Cultures were also set up in separate multiwells inoculating each strain in ONR7a without any additional carbon source (control culture w/o C). In the control cultures w/o C, an increase in the cell density lower than five fold as compared to the inoculum (ΔOD_{630} = 0.010) was obtained for all tested strains.

3.3.4.3. Growth test on solid medium

The bacterial suspensions were diluted at OD_{600} of 0.5 and 5 µL of the suspension were spotted onto plates of ONR7a solidified with agar and supplemented with pure *n*-alkanes (C_{12}, C_{16}) or diesel as vapour phase. Cultures were also prepared on solid ONR7a without any addition of carbon source as control culture w/o C as negative control to eliminate agar-utilizing bacteria and autotrophs (**Figure 3.4**). Cultures were incubated for 10 days at 25°C. Growth was evaluated by visual inspection comparing cultures of each strain with hydrocarbons with the relative control culture w/o C. Two replicate tests were carried out for each strain.

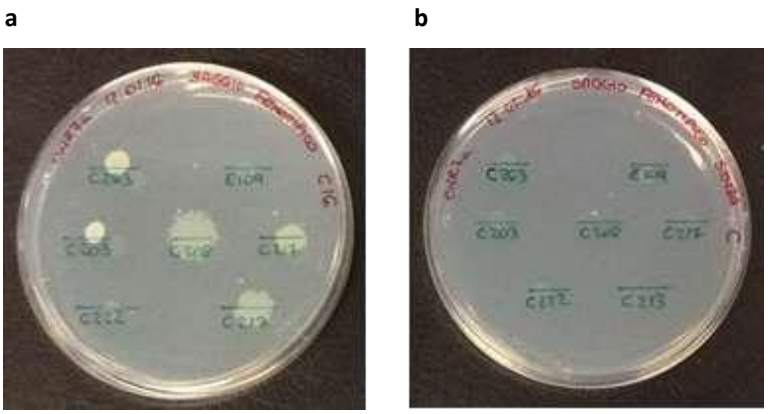

Figure 3.4 Growth test on solid medium. **a)** ONR7a + *n*-C_{16}**, b)** ONR7a without carbon source

3.3.4.4. Growth test in batch culture in flask

Batch cultures were prepared in 100-mL Erlenmeyer flasks with 30 mL ONR7a medium. The cultures were inoculated to an initial OD_{600} of 0.050 and supplemented with diesel at an initial concentration of 20.0 g/L (w/v). Cultures were incubated in a rotary shaker at 180 rpm for 7 days at 25°C. Bacterial growth was evaluated spectrophotometrically at OD_{600} nm. Two replicate tests were carried out for each strain.

3.3.4.5. Determination of SAC production

SAC production was evaluated on batch cultures exhibiting a 5-fold or higher increase in OD_{600}. The surface tension was determined on whole cultures by the Du Noüy ring method using a 3S tensiometer (GBX, Romans sur Isere, France, **Figure 3.5**). The sterile ONR7a medium (74.73 mN/m) and MilliQ water (73.55 mN/m) were used as negative controls. All measurements were performed at least three times for each culture.

Figure 3.5 Determination of SAC activities by Du Nouy ring method

The emulsification assay was performed on whole cultures as previously described (Cooper and Goldenberg, 1987). A volume of 3 mL of culture was vortexed while the same amount of diesel was added drop by drop over 30 s in a glass graduated tubes. Then, the tube was vortexed for additional 120 s. The mixture was allowed to settle for 24h (**Figure 3.6**). The emulsification activity (E24%) is given as percentage of middle emulsion phase normalized to the total volume. The sterile ONR7a medium (0%) and MilliQ water (0%) were used as negative controls. All determinations were performed at least in duplicate for each culture.

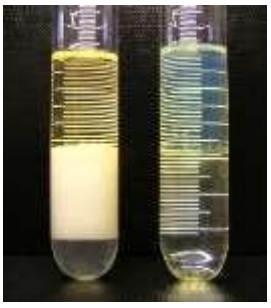

Figure 3.6 Emulsification assay

3.3.5. Hydrocarbon degradation test

Batch cultures were prepared in 100-mL Erlenmeyer flasks with 20 mL ONR7a medium. The cultures were inoculated to an initial OD_{600} nm of 0.050 and supplemented with diesel at an initial concentration of 1.0 g/L (w/v). Cultures were incubated in a rotary shaker at 180 rpm for 7 days at 25°C. Sterile controls were also prepared with ONR7a medium supplemented with diesel (1 g/L) without the addition of the inoculum.

After incubation, the residual hydrocarbons in cultures were extracted by liquid-liquid extraction and the removal of *n*-alkanes, phytane and pristine determined by GS/MS (***Ref. Par. 2.3.2***). GC/MS analysis was performed by the Department of Civil-Environmental Engineering and Architecture, University of Cagliari, Italy. For each strain, the ratio was calculated by dividing the normalized area of each compound in the culture by the normalized area of the same compound in the undegraded sterile control and express as

percentage. The percentage removal was calculated as 100% - percentage residual of each compound. Two replicate tests were carried out for each strain.

3.4. RESULTS AND DISCUSSION

3.4.1. Definition of the selection strategy of hydrocarbon-degrading bacteria

The aim of the present phase of the work was to develop a high-throughput screening strategy of potential hydrocarbon-degrading bacteria. In order to analyse a great number of strains, the protocol must be rapid, cheap and reproducible, thus reducing time and costs of the screening phase.

The main analytical methods usually used to test the ability of a strain to degrade pure hydrocarbons or complex mixtures are gas chromatography and high-pressure liquid chromatography after the extraction of the residual hydrocarbons by liquid-liquid extraction method. Though accurate, these techniques are time-consuming, complex and expensive. In literature, the majority of the authors have used growth tests on solid medium or liquid batch cultures at flask scale for the selection of the candidate degraders. In order to reduce time, costs and space and to improve the standardization as compared to the classical methods, a growth test at 96-well multiwell was evaluated as screening protocol.

A subset of the isolates obtained from the three case study sites were grown in batch culture on diesel (1 g/L) and evaluated for their ability to degrade the fraction of *n*-alkane by GC-MS (**Table 3.1**).

Table 3.1. Tests of hydrocarbon degradation, growth on hydrocarbons and SAC production of isolates from the three case study sites.

Strain ID	Genus	GS/MS Removal (%) each compound C_{10}-C_{25}	Growth on hydrocarbons					SACs	
			Agar	Agar	Agar	Flask OD_{600}	MW ΔOD_{630}	TS mN/m	E24 %
			Diesel	*n*-C12	*n*-C16	Diesel	Diesel	Diesel	Diesel
E101B	*Alcanivorax*	>25	+	+	+	0.540	**0.073**	39.39	4
C203	*Pseudomonas*	>25	+	+	+	1.187	**0.239**	41.30	0
H16	*Oleibacter*	>25	+	+	+	0.884	**0.323**	40.95	17
H14	*Marinobacter*	>25	+	+	+	0.651	**0.348**	26.47	0
C264	*Pseudoalteromonas*	<10	-	-	-	0.147	0.021	51.43	0
E162	nd	<10	-	-	-	0.107	0.033	56.22	0
E109	*Pseudoalteromonas*	<10	-	-	-	0.250	0.037	54.63	0
E208	*Vibrio*	<10	-	-	-	0.503	**0.285**	32.20	0
H26	*Pseudoalteromonas*	≤25	-	-	-	0.293	**0.237**	38.65	0
C239	*Pseudomonas*	≤25	-	-	-	0.742	**0.222**	38.23	0
C206	*Pseudomonas*	<25	-	-	-	0.561	**0.369**	41.04	0
H25	*nd*	<10	-	-	-	0.278	**0.114**	46.00	0

Bold: ΔOD_{630} in mutiwell test higher than 0.050.

Among tested strains, four ones were found to reduce the initial concentrations of one or more analytes to a quantity lower than 75%. These strains were thus considered as alkane degraders. All the other strains

showed a removal lower than 25% for all the analytes and thus they were considered as non-alkane degraders.

All the strains of the subset were further characterized for their ability to grow on hydrocarbons on solid medium and in liquid cultures. Results are presented in **Table 3.1**. All the strains identified as alkane degraders by GC-MS were also positive to the growth test on solid medium while all the non-degraders were found to be negative.

At multiwell scale, the threshold ΔOD_{630} value identifying the candidate degraders was fixed at the lowest value of ΔOD_{630} obtained on diesel for the strains identified as alkane degraders by GC-MS. The strain *Alcanivorax* sp. E101B showed the lowest ΔOD_{630} value (0.073) among degraders. Thus, the threshold value was fixed at 0.050 corresponding to a fivefold increase as compared to the initial inoculum concentration (ΔOD_{630} = 0.010).

Among the non-alkane degraders, three strains were also found to have a ΔOD_{630} lower than 0.050 at multiwell scale (<0.250 at flask scale) while all the others presented values higher than the threshold. All these strains also reduced the superficial tension to values lower than 40 mN/m; thus, they can be considered as biosurfactant producers according to Cooper (1986) suggesting that the turbidity measured in the culture should be attributed to the oil pseudosolubilization instead of by the increase in cell density. All these strains also exhibited OD_{600} values at flask scale comparable to alkane degraders. On the contrary, the non-alkane degraders which were also found to have a ΔOD_{630} lower than 0.050 at multiwell scale were not biosurfactant producers (ST > 40 mN/m).

Overall results demonstrated that the growth test at multiwell scale with a threshold values set at 0.050 can be used for the preliminary screening of candidate alkane degraders and/or biosurfactant producers.

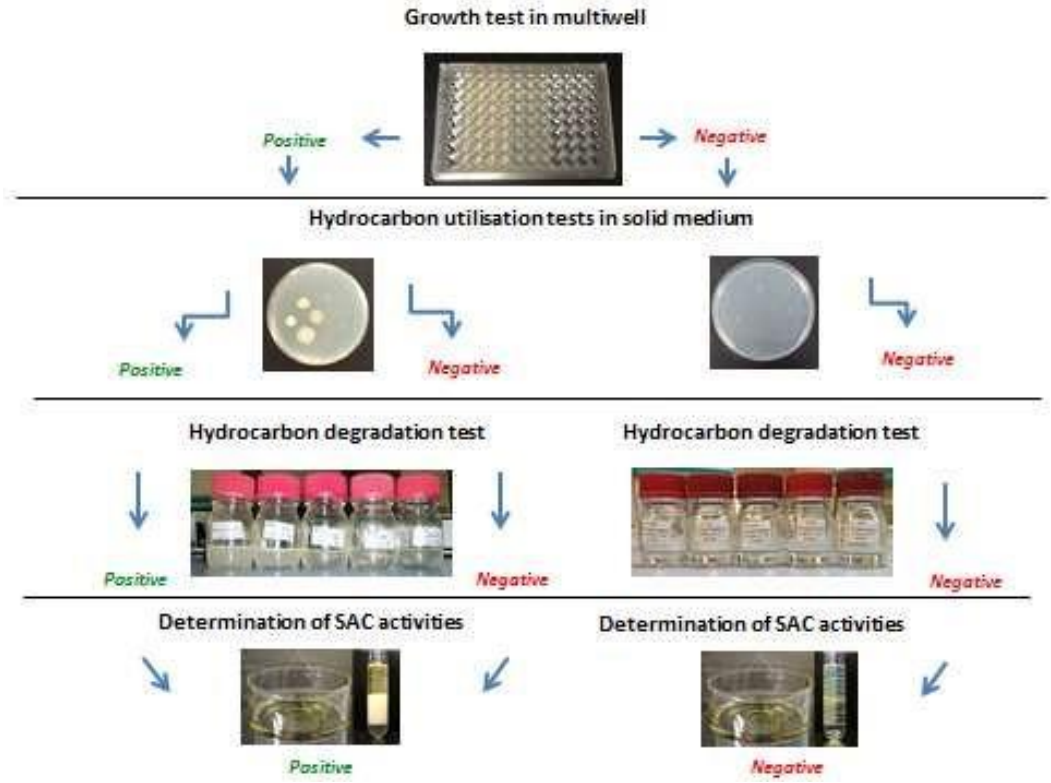

Figure 3.7 Strategy used for the selection of the hydrocarbon-degrading bacteria

3.4.2. Selection and taxonomic affiliation of alkane degraders

A total of 76 isolates were obtained from MPN cultures with diesel as selective carbon and energy source, including 25 isolates from superficial seawater samples of Cagliari Port, 27 isolates from El Kantaoui Port, and 24 isolates from Heraklion Port. Among them, 58 strains were demonstrated to be able to efficiently grow with diesel in the multiwell test including 24 isolates from Cagliari, 16 isolates from El Kantaoui, 18 isolates from Heraklion. In solid cultures, 35 strains efficiently grew on *n*-C12, *n*-C16, and diesel and can be considered as candidate degraders based on the previously defined selection strategy (**Figure 3.7**). Thus, the degradation percentages by GC-MS of linear (C_{10}-C_{25}) and branched (phytane and pristine) alkanes and the production of biosurfactants and bioemulsifiers by the selected strains were evaluated in batch cultures on diesel and results are shown in **Table 3.2**. All the selected strains were demonstrated to efficiently degrade alkanes and produced biosurfactants while none of the selected strains were found to produce bioemulsifiers.

All the alkane degraders were further characterized by 16S rRNA gene sequence analysis. The strains were successfully attributed to six different bacterial genera. Only the isolate C202 from the Cagliari port was affiliated to the phylum Actinobacteria and assigned to the genus *Mycobacterium* whereas all the other isolates from the three sites were attributed to the division γ-proteobacteria. The isolates from El Kantaoui and Heraklion were affiliated to three and four different genera, respectively. All the other isolates from Cagliari were assigned to the genus *Pseudomonas*. Two *Pseudomonas* strains were also found in El Kantaoui.

The majority of the isolates from El Kantaoui were OHCBs, highly specialized obligate hydrocarbon utilizers, belonging to the genus *Alcanivorax*. OHCBs belonging to the genera *Alcanivorax* were also isolated from Heraklion. Strains belonging to the genus *Marinobacter*, a well-known taxon for hydrocarbon degradation in marine environments, were found both in El Kantaoui and Heraklion. Alkane degrader affiliated to the genera *Oleibacter* and *Pseudoalteromonas* was isolated from seawater of the Heraklion port. Members of this genus *Pseudoalteromonas* have been recently recognised as marine "non-professional hydrocarbonoclastic bacteria" capable of degrading PAHs (Hedlund & Staley, 2006; Hassanshahian & Cappello, 2013).

In literature, medium length *n*-alkanes (C_{12}–C_{18}) have been demonstrated to be degraded to a greater extent than short (C_9–C_{11}) and long chains (C_{19}–C_{25}) alkanes because short chain *n*-alkanes are toxic to bacteria while long-chain *n*-alkanes are solid and their low water solubility limits mass transfer to the aqueous phase resulting in slow degradation rates. The majority of the isolates from superficial seawaters of the three port areas were able to degrade the whole spectrum of n-alkanes. Even if different strains showed different efficiencies, each strains showed comparable removal levels of short, medium and long chains alkanes. A minority of the strains were also isolated from the three ports, which were found to be specialized for the degradation of the short chain *n*-alkanes. Interestingly, *Pseudomonas* spp. strains played this functional role is both in Cagliari (C222, C265) and El Kantaoui (E201, E204).

As far as branched alkanes degradation are concerned, members of the genus *Alcanivorax* were found both in El Kantaoui and Heraklion while only the *Pseudomonas* sp. strain C203 from Cagliari was able to degrade both phytane and pristine. Branched alkanes are less readily degrade in comparison to *n*-alkanes. Methyl branching increases the resistance to microbial attach because fewer alkanes degraders can overcome the blockage of beta-oxidation (Zhu et al., 2001). Several authors have described the ability of *Alcanivorax* strains to degrade isoprenoid molecules (Yakimov et al., 2007). The two *Alcanivorax* sp. isolates (H02, H03) from Heraklion, phylogenetically closely related to *A. borkumensis*^{T}, efficiently degraded phytane and pristine (**Figure 3.8**). The isolates belonging to the genus *Alcanivorax* isolated from El Kantaoui showed the highest sequence similarity with the type strains of the species *A. borkumensis*^{T} (E100), *A. hongdengensis*^{T} (E101A, E101B, E212, E214), and *A. jadensis*^{T} (E213). Among them, only the strains E212 showed degradation percentages of branched alkanes higher than 50%.

Overall, results demonstrated the alkane degraders from the three case study sites are endowed with similar metabolic properties even if the community of cultivable degraders from seawater of the Cagliari port is dominated by copiotrophic bacteria belonging to the genus *Pseudomonas* whereas the majority of the isolates from Port El Kantaoui were *Alcanivorax* exhibiting features typical of oligotrophic bacteria (Yakimov et al., 2007; Schneiker et al., 2006). Both OHCBs and non-professional hydrocarbonoclastic bacteria are equally represented in the community from Heraklion port. The success of copiotrophic bacteria in the

Cagliari site can be attributed to the high levels of inorganic nutrients as compared to the other port areas (***Ref. Chapter 1***).

In the characterization and comparison of the pollution status of the three selected case study sites, a most-probable-number procedure was used to selectively enumerate heterotroph and hydrocarbon-degrading bacteria (***Ref. Chapter 1***). Heterotrophic bacteria use organic compounds as a source of energy and carbon. Many heterotrophic bacteria utilize sugar, alcohol, and organic acids. Heterotrophs were enumerated on yeast extract, a complex mixture of non-hydrocarburic organic compounds while hydrocarbon-degrading bacteria were enumerated on diesel.

Hydrocarbon-degrading bacteria are specialized heterotrophic bacteria able to use hydrocarbons as a carbon source. Among them, "*no specialized hydrocarbon degrading bacteria*" are able to degrade not only hydrocarbons but also utilized a wide range of (non-hydrocarburic) organic compounds while OHCBs are specialist for hydrocarbon degradation. Thus, the first group of hydrocarbon-degrading bacteria were enumerated also on yeast extract while OHCBs are exclusively enumerated on diesel. It is interesting that heterotrophs were only 25% of diesel degraders in seawaters from Cagliari. In Heraklion, comparable viable titles of heterotrophs and diesel degraders were found in seawater samples. El Kantaoui showed the viable titles of diesel degraders in seawaters 10-fold higher than the viable titles of heterotrophs suggesting the populations of diesel degraders were dominated by OHBCs. The isolation procedure confirmed that seawaters from El Kantaoui are colonized by obligated hydrocarbon degraders. On the contrary, the community of degraders from Cagliari is composed by "*no professional hydrocarbon degraders*" whereas both groups of hydrocarbon degraders were found in Heraklion.

Table 3.2. Degradation percentages of *n*- and *iso*-alkanes in diesel by the strains isolated from seawater of the ports of Cagliari (C), El Kantaoui (E), and Heraklion (H).

Strain ID	Genus	Type strains Best match	TS [a] mN/m	Removal (%) alkanes	100%-76%	75%-51%	50%-26%	25%-1.0%												
				Short length		Medium length							Long length							Branched	
				C_{10}	C_{11}	C_{12}	C_{13}	C_{14}	C_{15}	C_{16}	C_{17}	C_{18}	C_{19}	C_{20}	C_{21}	C_{22}	C_{23}	C_{24}	C_{25}	Pristane	Phytane
C202	*Mycobacterium*	*austroafricanum*	40.19	99	99	97	80	51	28	18	13	10	8	8	11	8	12	10	15	1	0
C208	*Pseudomonas*	*guineae*	42.54	48	47	49	57	53	49	57	55	49	43	44	46	49	55	56	67	0	0
C218	*Pseudomonas*	*guineae*	48.68	60	63	63	65	64	60	61	59	57	53	51	50	50	49	50	49	24	19
C201B	*Pseudomonas*	*oleovorans*	42.69	25	21	28	35	25	30	37	34	27	18	19	22	25	30	28	38	0	0
C213	*Pseudomonas*	*oleovorans*	40.68	56	53	57	64	52	53	62	60	51	38	35	43	45	49	53	76	0	0
C203	*Pseudomonas*	*oleovorans*	41.30	78	88	95	97	97	94	95	96	94	92	90	92	94	93	93	97	55	49
C222	*Pseudomonas*	*pachastrellae*	30.59	85	48	17	4	0	1	1	0	0	1	2	4	8	10	19	29	0	0
C265	*Pseudomonas*	*pachastrellae*	38.83	94	63	22	2	0	0	0	0	0	0	0	0	3	5	12	23	0	0
C204B	*Pseudomonas*	*peli*	35.23	65	64	77	88	82	77	75	79	72	66	60	62	66	64	61	54	0	0
C207A	*Pseudomonas*	*peli*	27.49	49	47	60	89	70	66	72	71	63	55	49	51	55	51	53	43	0	0
C209	*Pseudomonas*	*peli*	29.91	34	20	24	34	30	30	35	33	28	23	20	19	21	20	19	18	8	10
C211	*Pseudomonas*	*peli*	31.11	69	58	60	68	64	59	67	65	60	56	53	53	56	52	51	55	9	12
C212	*Pseudomonas*	*peli*	39.59	52	53	58	62	59	56	61	61	57	48	51	54	55	61	61	73	0	0
C214	*Pseudomonas*	*peli*	31.91	50	35	36	42	40	38	43	42	39	34	33	33	34	33	31	32	7	10
C217	*Pseudomonas*	*peli*	35.74	38	29	34	59	42	39	46	41	36	27	21	23	24	24	23	23	0	0
C220	*Pseudomonas*	*peli*	27.23	61	66	65	90	85	81	87	87	83	81	71	73	81	77	74	88	0	0
C229	*Pseudomonas*	*peli*	28.51	75	67	66	67	58	38	29	24	20	19	7	0	0	0	0	0	0	0
C263	*Pseudomonas*	*peli*	30.05	31	32	37	45	44	41	47	45	42	37	31	31	33	34	32	31	0	1
E100	*Alcanivorax*	*borkumensis*	51.78	75	72	68	66	64	61	60	60	54	51	48	46	47	51	53	53	25	17
E101A	*Alcanivorax*	*hongdengensis*	42.06	98	97	90	89	77	71	69	70	69	67	65	64	69	62	60	62	40	44
E101B	*Alcanivorax*	*hongdengensis*	39.39	96	93	88	88	82	77	77	76	74	72	70	69	68	67	65	66	42	45
E212	*Alcanivorax*	*hongdengensis*	55.09	96	95	93	92	91	91	92	91	89	86	87	87	89	90	96	98	93	62
E214	*Alcanivorax*	*hongdengensis*	54.26	88	86	84	83	81	81	82	81	79	72	79	75	80	82	81	95	0	14
E213	*Alcanivorax*	*jadensis*	46.49	96	96	96	91	83	81	83	84	84	83	81	79	79	73	66	46	52	60
E114	*In progress*		37.05	95	97	95	97	98	93	97	100	91	93	84	84	83	84	85	82	4	0
E152	*Marinobacter*	*litoralis*	41.32	41	12	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
E201	*Pseudomonas*	*pachastrellae*	37.99	43	15	4	4	4	1	0	0	0	1	0	0	0	0	3	4	5	0
E204	*Pseudomonas*	*pachastrellae*	32.35	47	26	16	13	12	9	5	7	7	7	6	6	5	6	9	9	13	7
H02	*Alcanivorax*	*borkumensis*	39.69	99	98	98	99	96	98	95	93	96	93	89	87	89	90	92	88	70	67
H03	*Alcanivorax*	*borkumensis*	38.29	99	99	99	99	98	99	99	98	92	86	82	81	82	83	84	85	80	75
H01	*Marinobacter*	*mobilis*	26.42	46	35	37	43	44	43	36	41	39	38	36	33	37	34	32	26	23	8
H14	*Marinobacter*	*xestospongiae*	26.47	47	41	47	55	55	53	54	53	51	50	49	49	52	50	49	46	27	23
H57	*Marinobacter*	*xestospongiae*	30.01	43	35	39	44	45	44	44	42	40	37	37	35	38	34	32	27	20	11
H16	*Oleibacter*	*marinus*	40.95	86	98	98	99	99	96	98	100	100	96	95	97	98	97	98	97	5	2
H19	*Pseudoalteromonas*	*distincta*	48.01	41	46	47	31	18	13	9	11	10	11	12	12	13	14	17	22	11	13

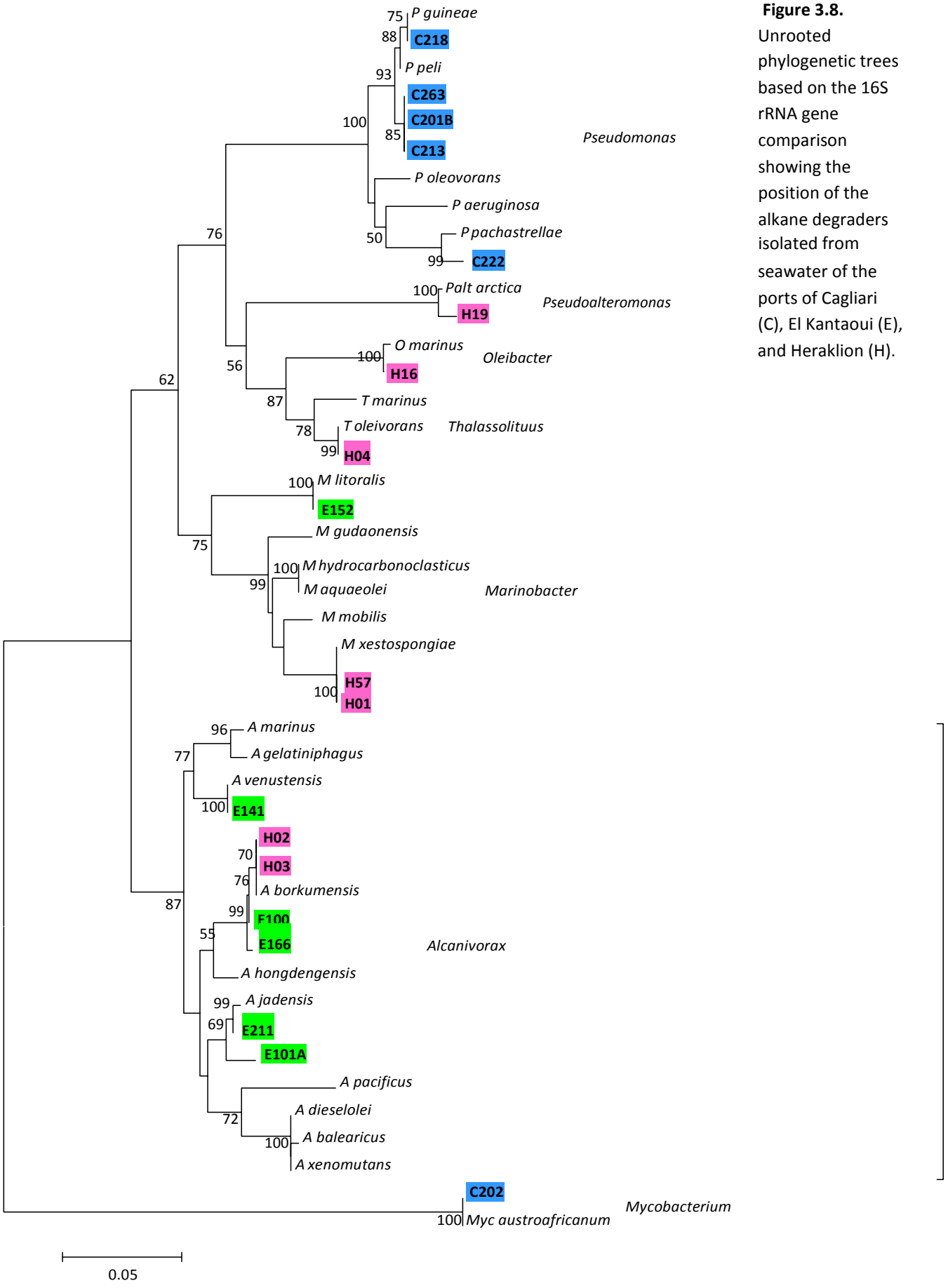

Figure 3.8. Unrooted phylogenetic trees based on the 16S rRNA gene comparison showing the position of the alkane degraders isolated from seawater of the ports of Cagliari (C), El Kantaoui (E), and Heraklion (H).

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CHAPTER 4

4. Comparison of the effect of bioaugmentation treatments on PAH degradation in sediments from different Mediterranean tourist ports

4.1. INTRODUCTION

4.1.1. Sediment contamination by polycyclic aromatic hydrocarbons in ports

Although significant progress has been achieved in the reduction of the marine pollution, thanks also to more stringent regulations, port areas still pose major concern due to the presence of toxic pollutants and their harmful effects on the marine ecosystem and human health. Even in the best maintained ports, due to usual leakages of fuel and lubricating oils from vessel and dock filling stations, seawater and sediments, within and adjacent to ports, are contaminated by high levels of petroleum hydrocarbons.

Among petroleum hydrocarbons, polycyclic aromatic hydrocarbons (PAHs) pose a major risk for human health since some PAHs have been found to be carcinogenic, teratogenic and mutagenic (Arcos et al., 1975). Due to their adverse effects on the biosphere, US EPA included 16 PAHs in a priority pollutant list (Wilson et al., 1993; De Luca et al., 2005).

In the aquatic environments, PAH accumulation rate greatly exceeds natural degradation rate due to their low volatility, recalcitrance to biological degradation, low solubility, and high hydrophobicity. Their hydrophobic nature promotes the tight sorption of PAHs to sediment particles (Lu et al., 2011). Because of their affinity to organic matter, PAHs easily adsorb onto suspended particulate and deposit on sea bottom. Once in sediments, they can persist until they are degraded, solubilised, bioaccumulated or removed with sediment dredging. PAHs persistence in the environment depends on a wide variety of factors, such as chemical structure, concentration, dispersion, bioavailability, and toxicity (Alexander 1995; Hughes et al.,1997). In addition, environmental factors, such as sediment type and structure, pH, temperature, oxygen, nutrients, determine the extent of biological degradation (Sutherland et al., 1995).

A large volume of sediments needs to be dredged every year from ports in order to maintain an adequate shipping depth. Dredging operations imply the removal of sediments, their transport and relocation. During this process, large amounts of pollutants are remobilized from dredged sediments, greatly enhancing their bioavailability and toxic effects on aquatic organisms. The risk of dredging operations in port areas should thus be carefully monitored during and after the end of the operations (Bocchetti et al., 2008). The subsequent sediment decontamination is a complex technical issue, requiring the choice among different treatment alternatives.

4.1.2. Bacterial aerobic degradation of polycyclic aromatic hydrocarbons

PAHs include 70 natural and anthropogenic organic compounds, composed of fused aromatic rings, ranging between two and seven. Different bacteria, fungi and algae are able to degrade PAHs with different kinetic rates depending on both the environmental conditions and molecular structures (i.e. molecular weight, number of rings, pattern of ring linkage).

Various studies have demonstrated an important relationship between PAH environmental persistence and the number of benzene rings, correlating environmental biodegradation rates and molecule size. Low molecular weight (LMW) PAHs (2-3 rings) are more soluble and degradable than the high molecular weight (HMW) PAHs. In soils and sediments, the half-life of phenanthrene (3 rings) ranged from 16 to 126 days, while the half-life of benzo[a]pyrene (BaP, 5 rings) ranged from 229 to more than 1400 days (Shuttleworth et al., 1995). Angularity is another factor resulting in an increase in hydrophobicity and stability of PAH molecules (Kanaly et al., 2000).

PAH degradation occurs both under aerobic or anaerobic conditions, even though the anaerobic degradation is considerably slower than the aerobic one. The initial step in the aerobic catabolism of PAHs by bacteria occurs via oxidation of the aromatic compound to a dihydrodiol, which undergoes an ortho or a meta cleavage of the aromatic ring, leading to central intermediates, such as protocatechuates and chatecols. The central intermediates are further oxidized by the tricarboxylic acid cycle (Van der Meer et al., 1992).

The degradation of LMW PAHs by a wide bacterial diversity is extensively documented. Moreover, a variety of bacteria has been demonstrated to degrade 4-ring HMW PAHs, such as fluoranthene and pyrene, in pure culture and their biodegradation pathways have been proposed (Kanaly et al., 2000). Degradation of BaP has been extensively studied since this 5-ring HMW PAH is one of the most potent carcinogenic PAHs. Co-metabolism is a fundamental for BaP biodegradation, which requires additional carbon sources for energy and growth as well as an inducer to maintain or express PAH-degrading enzymes. Kanaly et al., (2000) reported the 40% degradation of BaP, after an incubation period of 100 days, when bacteria were fed with a hydrocarbon co-substrate. LMW PAHs are usually used as co-substrates to degrade HMW PAHs (Lu et al., 2011).

When oxygen is absent hydrocarbon degradation under sulphate-reducing conditions seems to be the most important process for hydrocarbon removal in marine sediments being sulphate abundant in marine environments [20]. On the contrary, other electron acceptors, such as nitrate or ferric ions, are typically scarce in marine environments limiting other anaerobic degradation processes (Chang et al., 2003; Mcnally et al., 1998).

4.1.3. Bioremediation in sediments

The success of bioremediation depends on ability to establish and maintain conditions that favour enhanced oil biodegradation rates in the contaminated environment (Zhu et al., 2001). Bioremediation of PAHs is based on stimulating the autochthonous microbial community by the addition of nutrients or other additives, such as surfactants, (biostimulation) and/or the inoculation of selected hydrocarbon-degrading microorganisms (bioaugmentation).

Laboratory and field studies have extensively demonstrated that nutrient addition increases biodegradation rate in marine sediments as compared to natural attenuation (Zhu et al., 2001). The C:N:P ratio required for the optimal PAH biodegradation varies between 100:15:3 and 120:10:1 (Bamforth et al., 2005). Sorption to solid phase can reduce contaminant bioavailability to microorganisms. Thus, PAH desorption from sediments is an important factor affecting the rate and extent of bioremediation. Surfactants may enhance this process by decreasing the capillary forces in sediment matrix (Bury et al., 1993) or increasing PAH solubility in water. Moreover, some surfactants can be substrates for microbial growth. Among chemically synthesised surfactants, non-ionic surfactants are preferred as amendment for bioremediation thanks to their lower toxicity as compared to anionic and cationic ones (Lu et al., 2011). Different studies have demonstrated contradictory effects of surfactants on PAH degradation showing both an inhibition (Prak et al., 2002; Randazzo et al., 2001; Zhao et al., 2005) and an enhancement of microbial degradation (Aronstein et al., 1991; Laha et al., 1991; Tiehm et al., 1994). Recently, phytogenic surfactants have been proposed as an alternative to chemically synthesised compounds thanks to their higher environmental compatibility even though these amendments have mainly found application in bioremediation of soils and terrestrial sediments (Bustamante et al., 2012). Moreover, microbial surface-active compounds endowed with surfactant and/or emulsifier activity have been found applications in soil or sediment remediation (Mulligan et al., 2005; Franzetti et al., 2010).

The rationale of the bioaugmentation technology is the low density of degraders in the contaminated matrix and/or the need of faster degradation kinetics (Thomson et al., 2005). In many cases, the simultaneous addition of nutrients is essential to improve the degradation rate (Venosa et al., 1992).

4.2. AIMS OF THE WORK

In ports, the sediment decontamination after dredging operations is a complex technical issue, requiring the choice among different treatment alternatives. Therefore, processes allowing for further utilization or for safe final disposal of decontaminated sediments are receiving renewed attention by authorities, technicians and researchers. The aim of the present study was the selection of bacterial strains able to degrade PAHs of different tourist ports and the evaluation of their PAH removal efficiency during bioaugmentation treatments in sediment slurry batch tests. Particularly, the autochthonous and allochthonous bioaugmentation were compared.

4.3. MATERIALS AND METHODS

4.3.1. Polycyclic aromatic hydrocarbons

Phenanthrene 99% (Phe), Fluoranthene 99% (FlA), Pyrene 99% (Pyr), Fluorene 99% (Flu), and benzo[a]pyrene ≥96% (BaP) were purchased from Sigma-Aldrich (Steinheim, Germany).

4.3.2. Strain selection and isolation in pure culture

The isolation procedure was carried out from MPN cultures with Phe as selective carbon and energy source obtained from samples of superficial sediments collected in the port areas of Cagliari and El Kantaoui during the seasonal campaigns performed in 2012. In order to characterize the dominant bacterial PAH-degraders, aliquots of the most dilute MPN wells exhibiting growth were streaked onto solid ONR7a (Ref. Par. 3.3.2) supplemented with Phe. The carbon source was solidify in the medium at the final concentration of 5 mg/L. Cultures were incubated for 30 days at 20°C. Colonies with different morphologies were isolated by repeat streaking on the same medium. Once culture purity was established, the isolates were grown onto Marine agar supplemented with pyruvate (10 g/L) and stored at -20°C. The letter of the strain label refers to the sampling site (C: Cagliari, E: El Kantaoui).

4.3.3. Growth on polycyclic aromatic hydrocarbons

4.3.3.1. Inoculum preparation

The isolates were grown onto solid Marine Agar supplemented with pyruvate (10 g/L) for 3 days at 25°C. Liquid cultures were prepared in Marine Broth and incubated for 3 days at 25°C in a rotary shaker at 180 rpm. The cells were centrifuged, washed twice in ONR7a and suspended in the same medium.

4.3.3.2. Growth test in solid medium

The bacterial suspensions were diluted at OD_{600} nm of 0.5 and 5 µL of the suspension were spotted onto plates of ONR7a solidified with agar supplemented with each pure PAH. The substrates were added to the plates as a solution in acetone at the final concentration of 5 mg/mL for Phe and 1 mg/mL from Flu, Pyr and FluA. Cultures were also prepared on solid ONR7a without any addition of carbon source as control culture w/o C as negative control to eliminate agar-utilizing bacteria and autotrophs. Cultures were incubated for 30 days at 25°C. Growth was evaluated by visual inspection comparing cultures of each strain with PAHs with the relative control culture without hydrocarbon addition. Two replicate tests were carried out for each strain.

4.3.4. Sequencing and phylogenetic analysis

Bacterial cells (50 mg/mL) were lysed by heating in a microwave oven for 40 s at 800 W and the cell lysates used as template in PCR reaction without further purification. The amplification of the 16S rRNA genes and ARDRA were performed as previously described by Tamburini et al. (2003) using the universal bacterial

primers P0 and P6. The amplified products were purified with the QiaQuick PCR purification Kit (Qiagen, Hilden, Germany). DNA sequencing was performed on an ABI Prism 310 Genetic Analyzer.

The isolates were assigned at genus level by comparing their sequences with the RDP database (Wang et al. 2007). Moreover, sequences of isolates and selected type strains were used to construct phylogenetic trees by applying the neighbour-joining algorithm as implemented in the software MEGA 5.1 (Tamura et al., 2011). The evolutionary distance matrix was generated by Kimura-2-parameter (Kimura, 1980). The robustness of the phylogeny was tested by bootstrap analysis with 1,000 replicates. We are currently depositing the 16S rRNA sequences obtained in this work in the GenBank.

4.3.5. Anaerobic microcosms

Sediment samples were collected from the port of Cagliari and El Kantaoui on July 2012. Sediments were contaminated by adding a stock solution of Phe in acetone (5 g/L) at the final concentration of 100 mg/kg. Anaerobic microcosms of sediments were seated after an overnight incubation under nitrogen flux in order to evaporate acetone and let the PAH adsorbed onto sediments as previously described by Bach et al. (2005) with minor modifications (**Figure 4.1**). Sediments (450 mL) were packed into 500 mL bottles. Synthetic seawater (SSW) was flushed with nitrogen gas and added to cover the top 1-1.5 cm of sediments. The headspace was filled with nitrogen gas. SSW composition was the following (per litre): NaCl 44.0 g, $MgCl_2 \cdot 6H_2O$ 19.4 g, Na_2SO_4 7.4 g, $CaCl_2$ 2 g, KCl 1.3 g, $NaHCO_3$ 0.4 g, H_3BO_3 0.046 g. For each port, an abiotic control was also prepared by adding glutaraldehyde (5% v/v). Microcosms were incubated at 20 °C in the dark without shaking. At each sampling time, the overlying water was siphoned off and sediments were sampled with sterile blunt 50 ml pipettes for Phe analysis. The sediments in the bottle were then topped with the same water again. Samples were collected at fixed kinetic times (0, 15, 60, 120 and days) and centrifuged at 4,000 rpm for 10 minutes.

Figure 4.1 Contamination of sediments with phenanthrene under nitrogen flux.

4.3.6. Aeriated microcosms

Sediments were sieved in order to eliminate particles greater than 2 mm diameter and stored at 4°C. Sediments were spiked with a stock solution of Flu, Phe, FlA, Pyr and BaP in acetone (200 mg total PAHs/kg dry sediments) reaching a concentration of 40 mg/kg dry sediments for each compound.

The slurry was prepared by mixing sediments and SSW reaching 10% dried solids. Aerobic batch tests were performed in duplicate in 500 mL bottles with mechanical stirring at 120 rpm. Air was provided for 24 h with an aerator equipped with a porous stone. Thereafter, the porous stone was removed and the air was provided in the headspace in order to avoid volatilization. Oxygen concentration, measured with a WTW oxi 197i probe, was maintained above 2 mg/L. Samples were collected at fixed kinetic times (0, 1, 3, 5, 7 and 10 days) and centrifuged at 4,000 rpm for 10 minutes.

The following treatments were performed: i) abiotic controls amended with glutaraldehyde (5% v/v) and PAH losses resulted negligible (data not shown); ii) natural attenuation trials by adding only PAHs without amendments or bacterial biomass addition; iii) biostimulation with nutrient regulation reaching a C:N:P ratio of 100:10:1 (considering the C provided with PAHs) (N,P); iv) bioaugmentation by inoculating the selected bacterial strains at an initial concentration of 1 x 10^5 cell/mL and nutrients (C:N:P ratio of 100:10:1).

4.3.7. Residual PAH determination

The solid phase was dried by adding a portion of 1:1 (w/w) of diatomaceous earth. The sample was then extracted with accelerated solvent extraction (ASE 150 Dionex) and the extracts analysed with high-pressure liquid chromatography (HPLC P680 dionex). Samples were also analysed with an Agilent GC/5975BMSD, equipped with an Agilent 7863B autosampler, to evaluate the potential presence of intermediates deriving from the PAH degradation. Chemical analysis were carried out by the Department of Civil-Environmental Engineering and Architecture, University of Cagliari, Italy.

4.4. RESULTS AND DISCUSSION

4.4.1. Characterization of Pseudoalteromonas spp. strains for emulsifier production and degradation of PAHs

Among the strains identified as non-alkane degraders and biosurfactant producers by the preliminary screening (Ref. Chapter 3), a total of eight *Pseudoalteromonas* spp. strains was isolated from seawaters of the three case study sites (**Figure 4.2**). This taxon was the only common genus isolated from the three studied ecosystems. Hedlund and Staley (2006) isolated and characterized different *Pseudoalteromonas* spp. with divergent PAH catabolic properties. Moreover, many *Pseudoalteromonas* species produce biologically active metabolites, such as exopolysaccharides (EPS). PAHs are poorly soluble and generally less amenable to biodegradation compared to their aliphatic counterparts. To circumvent limitations in hydrocarbon bioavailability, some microorganisms produce biosurfactants or bioemulsifiers (e.g. amphiphilic EPS) to

increase the bioavailability of these compounds for biodegradation. EPS produced by marine bacteria plays a crucial role in the removal of pollutant and toxic elements from contaminated environments. Their emulsification and surface-active properties makes them applicable for utilization in bioremediation.

The selection of the proper bacterial strains or consortium has been recognised as a key step in the implementation of this technology for sediment *ex situ* treatments (Tyagi et al., 2011). The initial screening/selection step should be based on the metabolic potential of the microorganism and also on essential features that enable the cells to be functionally active and persistent under the desired environmental conditions. Thomson et al. (2005) have suggest an bioaugmentation approach, which would probably increase success rates, in terms of persistence and activity, would be to base the initial selection step on a priori knowledge of the ubiquity, population dynamics and spatial and temporal distribution of microbial communities in sampled habitats. It seems logical that a strain, derived from a population that is temporally and spatially prevalent in a specific type of habitat, is more likely to persist as an inoculum when reintroduced, than one that is transient or even alien to such a habitat. Once abundant populations have been identified, the second phase of the selection procedure should then be to identify strains which can degrade the target contaminant.

Based on the aforementioned considerations, two strains, belonging to the genus *Pseudoalteromonas,* were selected from strain collection obtained from seawaters for bioaugmentation tests. The strain H05 from Heraklion seawaters was able to degrade Flu and FluA and to efficiently produce bioemulsifiers, which stabilize oil-water emulsion, in ONR7a supplemented with pyruvate (10 g/L). The strain E151 from El Kantaoui seawaters was able to grow on Flu, Phe, FlA, Pyr on solid medium while it does not produce emulsifiers (**Table 4.1**).

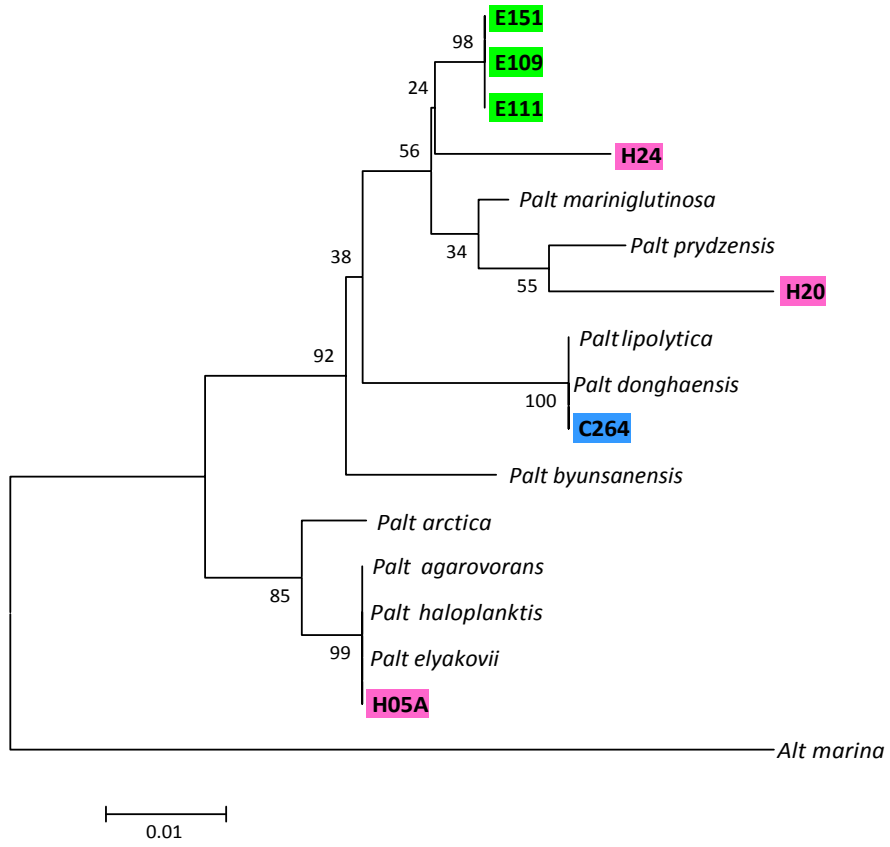

Figure 4.2 Unrooted phylogenetic trees based on the 16S rRNA gene comparison showing the position of the alkane degraders isolated from seawater of the ports of Cagliari (C), El Kantaoui (E), and Heraklion (H).

Table 4.1 Bacterial strains selected for growth on PAHs from seawater and sediments of the ports of Cagliari (C), El Kantaoui (E), and Heraklion (H).

Strain ID	MPN Carbon source	Genus	3-ring PAHs		4-ring PAHs		Bioaugmentation test
			Flu	Phe	FluA	Pyr	
C264	Diesel	*Pseudoalteromonas*	nd	nd	nd	nd	
E151	Diesel	*Pseudoalteromonas*	+	+	+	+	+
H05A	Diesel	*Pseudoalteromonas*	+	-	+	-	+
C280	Phe	*Paracoccus*	+	+	+	+	
C286	Phe	*Amaricoccus*	+	+	+	+	+
C285B	Phe	*Mycobacterium*	+	+	+	+	+
C283	Phe	*Mycobacterium*	+	-	+	+	
C289	Phe	In progress	+	+	+	+	
E160	Phe	*Arthrobacter*	+	+	+	+	
E168	Phe	*Micrococcus*	+	+	-	+	
E163	Phe	*Aurantimonas*	+	+	+	+	
E162	Phe	In progress	+	+	+	+	
E165	Phe	In progress	+	+	+	+	

4.4.2. Selection of bacterial strains on PAHs from sediments and taxonomic characterization

Comparable viable titles of Phe-degraders was found in superficial sediment samples from El Kantaoui port ($1.6 \pm 2.3\ 10^{02}$ MPN/g wet weight) and Cagliari port ($2.4 \pm 3.3\ 10^{02}$ MPN/g wet weight). From MPN cultures with Phe, a total of 20 isolates were selected for the ability to growth with Phe as selective carbon and energy source, including 12 isolates from Cagliari and eight isolates from El Kantaoui (**Table 4.1**). Two bacterial strains isolated from Cagliari sediments were selected as inoculum for bioaugmentation tests based on their taxonomic position and catabolic properties: *Amaricoccus* sp. C286 and *Mycobacterium* sp. C285B. Members of the genus *Amaricoccus* have been isolated from activated sludge biomass whereas they have not been previously described as PAH degraders. Mycobacteria were often selected through enrichment isolation methods for their ability to degrade 4-ring and 5-ring PAHs suggesting that degradation of high-molecular-weight PAHs may be a common trait within this group of bacteria (Mc Lellan et al., 2002).

4.4.3. Bioaugmentation by selected autochthonous and allochthonous strains of port sediments

Bioaugmentation tests were carried out in aerated sediment slurry batch tests. Among different treatment technologies of PAH contaminated sediments, the slurry phase system promotes the effective contact between the contaminant and the microflora. It has been demonstrated that the sediment content in the slurry significantly alters the distribution of target compounds between water and solid phase, particularly in systems with different mass transfer properties (Venkata et al., 2006). Moreover, slurry has been shown to enhance the initial rates and the overall extent of mineralization (White et al, 1999; Doick et al., 2003; Robles-González et al., 2008). Chiavola et al. (2001) have found a degradation of 80% of 3-ring and 4-ring PAHs, for an inlet total PAHs of 70 mg/kg dry weight of sediments, with a cycle length of 7 days in a sludge system sequencing batch reactor.

In both sediments, aerated treatment without the addition of amendments or bacterial biomass resulted in a complete degradation of Flu and Phe after 3-5 d, while degradation was slower for Pyr and FlA (**Figure 4.3**). FlA was not completely removed after 10 d, while no degradation of BaP was observed.

In both sediments, the biostimulation treatment resulted in removal rates of Flu comparable to the aerated control without nutrients while those of Phe, FluA, and Pyr were accelerated by the addition of inorganic nutrients being inorganic nutrients are limiting factors to microbial degradation of these PAHs. Moreover, the treatment stimulated the removal of BaP in sediments from the Cagliari port, determining a 40% degradation of BaP, while any improvement was observed in El Kantaoui sediments as compared to the control without nutrients. Thus, the autochthonous microbial communities of both sediments were able to degrade all testes PAHs except for BaP in sediments, which is not degraded by the autochthonous community from El Kantaoui under tested conditions.

Since all the bioaugmentation treatments were performed by adjusting the regulating levels of nitrogen and phosphorous, the effect of selected strains was evaluated as compared to the biostimulation treatments. Bioaugmentation treatment with the strains *Mycobacterium* sp. C285B isolated from sediments of the Cagliari port determined slower removal rates of 3-ring and 4-ring PAHs in El Kantaoui sediments. A less pronounced slowing down in the degradation of 4-ring PAHs was observed for Cagliari while the removal rates of 3-ring PAHs were comparable with the control with nutrients. The bioaugmentation with the strain C285B resulted in the removal of 60% of BaP in El Kantaoui sediments. Tyagi et al. (2011) have highlighted the effectiveness of bioaugmentation treatments when the autochthonous populations of degraders is unable to degrade the target compound. Our data suggest that the autochthonous population from the sediments of El Kantaoui does not possess the metabolic routes necessary to metabolize BaP being the addition of an allochthonous strain a successful strategy for promoting BaP degradation. On the contrary, any stimulation was observed in sediments from Cagliari being the BaP removal efficiency (40%) comparable to that of the autochthonous community. The addition of *Amaricoccus* sp. strain C286 isolated from the sediments of Cagliari resulted in the highest removal rates of FlA and Pyr as compared to all the other treatments in both sediments. In this case, comparable results were obtained by treating the sediments with an autochthonous strain in Cagliari and an allochthonous strain in El Kantaoui. The other tested strains gave results comparable to those with only biostimulation. Thus, a selectivity of the bioaugmentation treatments was demonstrated both in terms of typology of PAHs and sediments.

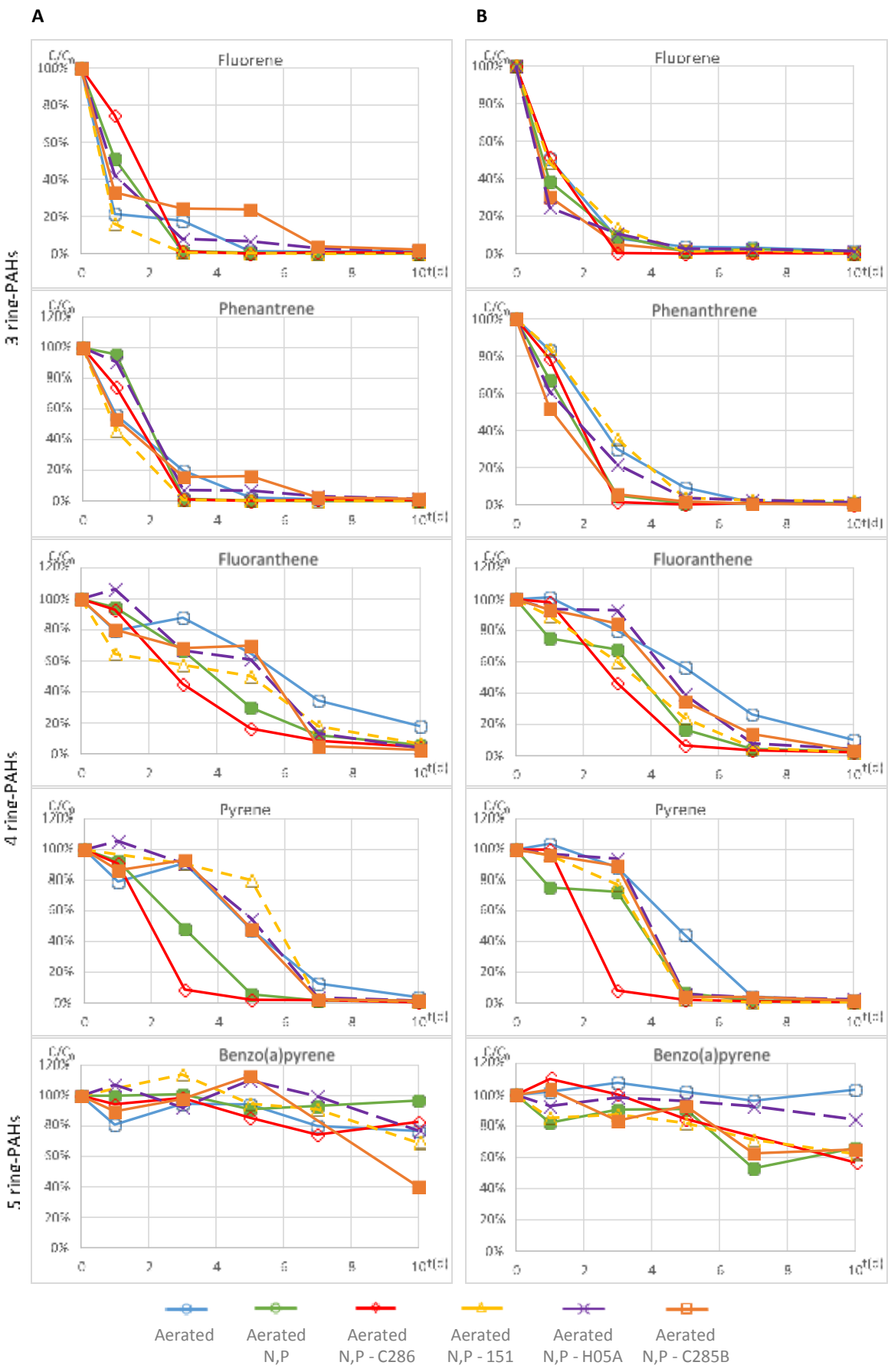

Figure 4.3 Degradation profiles of PAHs in tests conducted on sediments of El Kantaoui (a) and Cagliari (b) (Mean, *n*=2)

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4.6. DISSEMINATION

Erby G., Tamburini E, Ruggeri C., **Bullita E.**, Carucci A. (**2015**) Bioaugmentation tests for PAH bioremediation in mediterranean tourist port sediments N. Kalogerakis (Technical University of Crete), F. Fava (University of Bologna), E. Manousaki (Technical University of Crete). VI European Bioremediation Conference 2015 (Chania, Crete, Greece / June 29 – July 2, 2015). **ISBN:978-960-8475-23-6**

Ruggeri C., La Colla P., **Bullita E.**, Sergi S., Vitali F., Mastromei G., Tamburini E. (**2014**) Characterization of sulphate reducing bacteria communities in sediments from tourist ports in the Mediterranean Sea Basin. Poster at 16th European Congress on Biotechnology (ECB16) to be held in Edinburgh, Scotland 13-16 July 2014

Vitali F., Senatore G., Indorato C., Mastromei G., Csalone E., La Colla P., **Bullita E.,** Ruggeri C., Sergi S., Tamburini E. (**2013**) Fingerprinting characterization of prokaryotic communities in sediments from three tourism ports In the Mediterranean area. Proceeding of the second international conference on microbial diversity Microbial interactions in complex ecosystems, MD2013, 23-25 October 2013, Torino, Italy. Published by: SIMTREA Società Italiana di Microbiologia Agraria-Alimentare e Ambientale, San Casciano Val di Pesa, Firenze, Italia. **ISBN 978-88-908636-5-3**

Biella R., **Bullita E.**, Carucci A., Cappai G., Erby G., Ruggeri C., Sergi S., Tamburini E. (**2013**) Evaluation of PHA degradation by autochthonous microbial communities in seawater and sediments of different port areas. RemTech2013 Remediation Technologies, Gestione e bonifica dei Sedimenti, Ferrara, 18-20 Settembre, DEA Edizioni, pp. 85-92 (su CD) **ISBN 978-88-904428-7-2**

Ruggeri C., La Colla P., **Bullita E.**, Sergi S., Vitali F., Senatore G., Casalone E., Mastromei G., Tamburini E. (**2013**) Characterization of bacterioplankton communities in tourist ports in the Mediterranean Sea basin. Poster at FEMS 2013 5th Congress of European Microbiologists. Leibzig, Germany, 21-25 July 2013. No. 2768.

Vitali F., Senatore G., Indorato C., Mastromei G., Casalone E., La Colla P**., Bullita E.**, Ruggeri C., Sergi S., Tamburini E. (**2013**) Fingerprinting characterization of prokaryotic communities in sediments from three tourist ports in the Mediterranean area. Poster at FEMS 2013 5th Congress of European Microbiologists. Leibzig, Germany, 21-25 July 2013. No 2754

GENERAL CONCLUSION

Marine environments suffer a greater ecological damage compared to terrestrial environments mainly as a result of the greater difficulty of controlling the pollution (Macaulay et al., 2014). For this reason, it is important to know the potential degradation activities of the autochthonous microbial communities in marine environments. It is also important to understand if the same bioremediation treatments are applicable in different sites.

In this thesis, the microbial communities from different Mediterranean tourist ports were characterized, their potential degradation activities were determined and the applicability of bioremediation technologies was evaluated at different sites.

Data demonstrated that Cagliari port was characterized by a high level of eutrophication. Coherently with the high nutrient load, the viable title of heterotrophs was one-log higher in the seawater of Cagliari port when compared with values found in the other ports. The structure of the microbial communities in seawater from Cagliari and El Kantaoui were more similar to one another than the others while Heraklion presented different community structures.

During the biostimulation treatment in seawater microcosms, comparable degradation kinetics were found for Cagliari and Heraklion sites. Furthermore, nutrient amendments in El Kantaoui microcosms resulted in a two-fold increase in the degradation rates as compared to the other two port areas. The structures of the bacterial communities of El Kantaoui were more homogenous than those from Cagliari, while Heraklion presented a moderate change in community structures during bioremediation treatment. Moreover, different community structures were found for the three sites at the end of the treatment.

The community of cultivable degraders from seawater of the Cagliari port was dominated by copiotrophic bacteria belonging to the genus *Pseudomonas* whereas the majority of the isolates from Port El Kantaoui were *Alcanivorax*. Both OHCBs and non-professional hydrocarbonoclastic bacteria were equally represented in the community from Heraklion port.

The autochthonous population from the sediments of El Kantaoui does not possess the metabolic routes necessary to metabolize BaP being the addition of an allochthonous strain a successful strategy for promoting BaP degradation. Thus, a selectivity of the bioaugmentation treatments was demonstrated both in terms of typology of PAHs and sediments.

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Isolation, anticancer, antihelminthic, and antiviral (HIV) activity of acylphloroglucinols, and regioselective synthesis of empetrifranzinans from *Hypericum riparium*

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ABSTRACT

From the ethno-medicinally used leaves of *Hypericum riparium* we isolated a new tricyclic acylphloroglucinol (**1**), a new tetracyclic acylphloroglucinol (**2**), and a new prenylated bicyclic acylphloroglucinol (**3**) together with four known prenylated (**4-7**) and three known tetracyclic acylphloroglucinol derivatives (**8-10**). Structure elucidation was based on UV, IR, $[\alpha]^{25}_{D,}$ 1D- and 2D-NMR experiments. Furthermore, empetrifranzinans A (**8**) and C (**9**) were synthesized regioselectively in only two steps. The isolated compounds were evaluated for their cytotoxicity against PC-3 and HT-29 cancer cell lines, antibacterial, and anthelmintic activities. They were also tested in cell-based assays for cytotoxicity against MT-4 cells and for anti-HIV activity in infected MT-4 cells.

1. Introduction

Plants of the genus *Hypericum* (Hypericaceae) are used worldwide as traditional medicine against a multitude of diseases. In China, *Hypericum sampsonii* Hance is used for the treatment of disorders such as backache, burns, diarrhea, snakebite, and swellings.[1] *H. laricifolium* Juss., with the common name "Romerillo" has been used in traditional Ecuadorian medicine as a diuretic and for provoking menstruation.[2] In the folk medicine of Papua New Guinea, leaves of *H. papuanum* Ridl. are applied to treat sores.[3] In Cameroonian traditional medicine, *Hypericum* plants are multipurpose remedies commonly used for the treatment of tumors, skin infections, epilepsy, infertility, venereal diseases, gastrointestinal disorders, intestinal worms, and viral diseases.[4,5,6] In Turkey, *H. empetrifolium* Willd. is used against kidney stones and gastric ulcers, in Greece as an anthelmintic and diuretic.[7] *Hypericum perforatum* L. (St. John's wort), the most popular species of the genus *Hypericum*, is well known in Europe and North America for the treatment of mild to moderate depression.[8,9] It is believed that this multitude of biological effects occur through the action of various active compounds present in the plant.[10] Due to the great scientific interest and economic value associated with *H. perforatum*, many studies together with other plants of the same genus have been carried out throughout the world.[8,11] The most common secondary metabolites found within the genus *Hypericum* include phloroglucinols,[7,8,11,12] xanthones,[13] naphtodianthrones,[14] flavonoids,[8,9] and coumarins.[15] Acylphloroglucinols, of which hyperforin from *H. perforatum* is an outstanding example, are among the most relevant bioactive compounds isolated from *Hypericum*.[16,17] Their structures and biological activities have attracted wide attention in medicinal and synthetic chemistry fields since the isolation of hyperforin in 1975.[17] They possess, among others, cytotoxic,[17] antidepressant,[10] antibacterial,[12] and anti-inflammatory activities.[7] The phloroglucinol core of these compounds is often substituted by prenyl or geranyl moieties that are susceptible to cyclization and oxidation processes, affording bi-[11,18] or tricyclic[11] derivatives as well as complex caged compounds.[19]

Previously we have reported dimeric coumarins and flavonoids from the stem bark of *H. riparium*.[6] As part of this continuing effort to study and compare the metabolomes of different *Hypericum* species[6,7,8] and to find potential lead compounds, we have selected the leaves of *Hypericum riparium* A.Chev. (synonym of *H. roeperianum* Schimp. ex. A.Rich according to The Plant List, http://www.theplantlist.org/, Hypericaceae) for chemical investigations, which led to the isolation of acylphloroglucinols **1-10**. The biological activities exhibited by the $CHCl_3$ extract of *H. riparium* as well as its chemical and chemotaxonomic significance fostered our effort in the isolation, structure elucidation, and more detailed study of specific biological activities of compounds **1-10** from *H. riparium* as well as the regioselective synthesis of compounds **8** and **9**.

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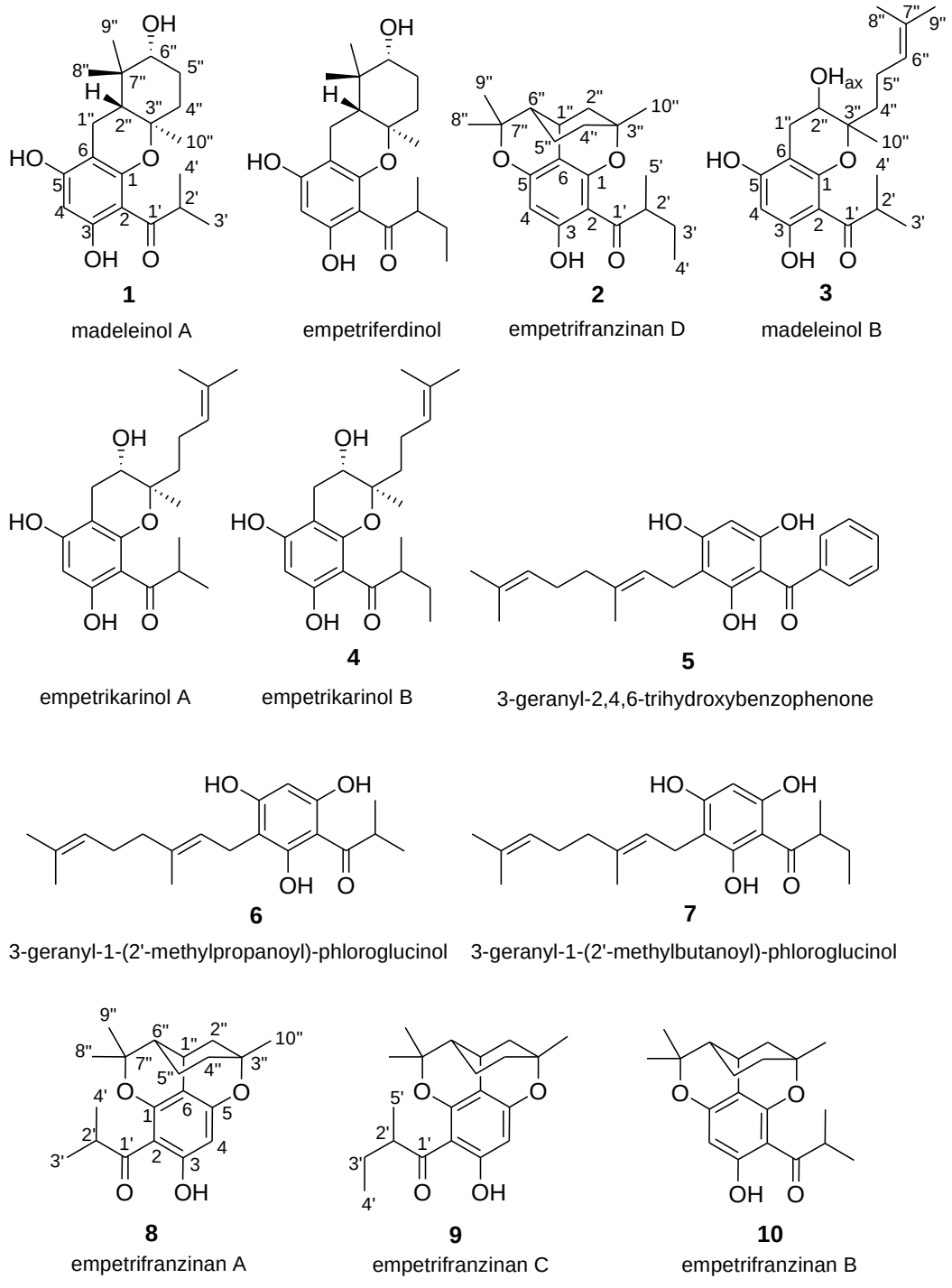


Figure 1. Structures and trivial names of compounds **1**-**10**, empetriferdinol and empetrikarinol A

2. Material and methods

2.1. General experimental procedures

Acetonitrile (HPLC grade, LiChrosolv) was obtained from Merck KGaA, Germany; double distillated water was used for HPLC analysis. HPLC was performed on a VARIAN PrepStar instrument equipped with a VARIAN ProStar PDA detector. Column chromatography was run on silica gel (Merck, 63-200 and 40-63 µm) and Sephadex LH-20 (Fluka), while TLC was performed on precoated silica gel F_{254} plates (Merck). Spots were visualized with a UV lamp at 254 and 366 nm, or by spraying with vanillin-H_2SO_4-MeOH followed by heating at 100 °C. UV spectra were obtained on a JASCO V-560 UV/VIS spectrophotometer. IR (ATR) spectra were recorded using a Thermo Nicolet 5700 FT-IR spectrometer, in MeOH or $CHCl_3$. Optical rotation $[\alpha]^{25}_D$ was measured using a JASCO P-2000 digital polarimeter. ^1H and ^{13}C NMR spectra were recorded on an Agilent DD2 400 NMR spectrometer at 400 and 100 MHz and on an Agilent VNMRS 600 NMR spectrometer at 600 and 150 MHz, respectively. 2D NMR spectra were recorded on an Agilent VNMRS 600 NMR spectrometer using standard pulse sequences implemented in Agilent VNRMJ software. The ^1H chemical shifts are referenced to internal TMS (δ 0.0); ^{13}C chemical shifts are referenced to internal $CDCl_3$ (δ77.0) and CD_3OD (δ 49.0), respectively.

The low resolution electrospray (ESI) mass spectra were performed on a SCIEX API-3200 instrument (Applied Biosystems, Concord, Ontario, Canada) combined with a HTC-XT autosampler (CTC Analytics, Zwingen, Switzerland). The samples were introduced via autosampler and loop injection.

The negative ion high resolution ESI mass spectrum of compound **3** was obtained from an Orbitrap Elite mass spectrometer (Thermofisher Scientific, Bremen, Germany) equipped with an HESI electrospray ion source (spray voltage 4 kV; capillary temperature 275 °C, source heater temperature 40 °C; FTMS resolution 60.000). Nitrogen was used as sheath gas. The sample solutions were introduced continuously via a 500 µl Hamilton syringe pump with a flow rate of 5 µl/min. The data were evaluated by the Xcalibur software 2.7 SP1.

The negative ion high resolution ESI mass spectra of compounds **1**-**2** and **4**-**10** were obtained from a Bruker Apex III Fourier transform ion cyclotron resonance (FT-ICR) mass spectrometer (BrukerDaltonics, Billerica, USA) equipped with an Infinity™ cell, a 7.0 Tesla superconducting magnet (Bruker, Karlsruhe, Germany), an RF-only hexapole ion guide and an external electrospray ion source (Agilent, off axis spray). Nitrogen was used as drying gas at 150 °C. The sample solutions were introduced continuously via a syringe pump with a flow rate of 120 µl/h. The data was acquired with 512k data points, zero filled to 2048k by averaging 16 scans and evaluated using the Bruker XMASS software (Version 7.0.8).

2.2. Plant material

The leaves of *Hypericum riparium* A.Chev. were collected in August 2011 at Mount Bamboutos (Mbouda) in the West Region of Cameroon. The plant was identified by Mr. Nana Victor, a botanist at the National Herbarium of Cameroon where a voucher specimen (No. 33796/HNC) is deposited. *H. riparium* A.Chev is presently treated as synonym of *Hypericum roeperianum* Schimp. ex A.Rich.

2.3. Extraction and isolation

The air-dried leaves (400 g) of *Hypericum riparium* were sequentially extracted with $CHCl_3$ (3 x 5 l) and MeOH (3 x 2 l) for one day under shaking to give the respective extracts, namely the $CHCl_3$ (23.74 g) and MeOH (6.10 g) extracts, after evaporation under reduced pressure. The MeOH extract from the leaves of *H. riparium* used for UPLC-PDA analysis, anti-bacterial and anti-HIV assays, was obtained using an extraction procedure previously described for *Hypericum* species.[8]

The $CHCl_3$ extract (23.48 g) from the leaves was subjected to silica gel column chromatography, eluted with step gradients of *n*-hexane-EtOAc and EtOAc-MeOH. Fractions were collected using a fraction collector (Retriever II). They were combined on the basis of their TLC profiles into 6 main fractions (Fr. 1A-Fr. 6A): Fr. 1A (170.8 mg), Fr. 2A (12.36 g), Fr. 3A (8.66 g), Fr. 4A (1.76 g), Fr. 5A (1.9 g), Fr. 6A (81.6 mg). Fr. 2A (12.36 g) obtained from *n*-hexane-EtOAc (90:10) was chromatographed on Sephadex LH-20 column (69 x 3 cm), eluted with CH_2Cl_2-MeOH (1:1), to give eight sub-fractions (Fr.$2A_1$-Fr. $2A_8$).

Fr. $2A_3$ (1.71 g) was subjected to column chromatography over silica gel eluted with *n*-hexane containing increasing amounts of EtOAc (from 100:0 to 50:50 with 5% increment) and 100% MeOH to yield 13 fractions (Fr. $2A_{3a}$-Fr. $2A_{3m}$). A portion of Fr. $2A_{3k}$ (79 mg), obtained from *n*-hexane-EtOAc (80:20), was purified through a RP18 column (5 µm, 120 x 2 mm, flow rate 17 mL/min, detection 210 nm) by semi-preparative HPLC on a Varian PrepStar eluted with H_2O-MeCN (10→100% MeCN 0-20 min, 100→10% MeCN 20-25 min, 10% MeCN 27-30 min) to afford compounds **6** (30 mg, t_R15.17 min) and **7** (34.2 mg, t_R 15.65 min). An initial approach to separate Fr. $2A_{3c}$, a mixture of citran acylphloroglucinols plus other impurities as revealed by the ^1H-NMR profile, by CC or HPLC as described above failed. Fr. $2A_{3c}$ (193 mg) was then first separated by silica preparative thin layer chromatography (PTLC) and eluted with a mixture of toluene-EtOAc-HOAc (98:2:0.5), to afford two fractions after extraction and evaporation: Fr. $2A_{3c1}$ (R_f = 0.32) and Fr. $2A_{3c2}$ (R_f = 0.5). Fr. $2A_{3c1}$ (115.6 mg) was suspended in MeCN and filtered. The resulting filtrate (53 mg) was further suspended in MeCN and filtered through a Chromabond C18 ec (Macherey-Nagel, 1 mL/100 mg), and finally purified through a RP18 column (5 µm, 120 x 2 mm, flow rate 17 mL/min, detection 210 nm) by semi-preparative HPLC on a Varian PrepStar eluted with H_2O-MeCN (30→100% MeCN 0-20 min, 100→30% MeCN 20-25 min, 30% MeCN 27-30 min) to yield **2** (2.3 mg, t_R 16.03 min), **8** (4.6 mg, t_R 14.51 min), **9** (6.3 mg, t_R 15.76 min), and **10** (4.5 mg, t_R 15.11 min).

Fr. 4A (1.76 g), obtained from *n*-hexane-EtOAc (90:10 and 50:50) and EtOAc-MeOH (100:0), was further chromatographed on Sephadex LH-20 column (69 x 3 cm), eluted with CH_2Cl_2-MeOH (1:1), to give five fractions (Fr. $4A_1$-Fr. $4A_5$). Fr. $4A_3$ (407 mg) was dissolved in MeCN and filtered through a Chromabond C18 ec (1 mL/100 mg). A portion of the resulting filtrate was purified using RP18 HPLC (5 µm, 120 x 2 mm, flow rate 17 mL/min, detection 210 nm) on a Varian PrepStar instrument and eluted with H_2O-MeCN (30→100% MeCN 0-20 min, 100→30% MeCN 20-25 min, 30% MeCN 27-30 min) to give **1** (1.9 mg, t_R 8.04 min), **4** (2 mg, t_R 12.29 min), **5** (2.7 mg, t_R 13.30 min), and also **6** (3 mg, t_R13.77 min) and **7** (3 mg, t_R 14.63 min). The remaining portion (67.5 mg) of the filtrate was purified using RP18 HPLC (10 µm, 250 x 10 mm, flow rate 8.9 mL/min, detection 210 nm) on the same instrument as above and also eluted with H_2O-MeCN (30→100% MeCN 0-20 min, 100→30% MeCN 20-25 min, 30% MeCN 27-30 min) to afford **3** (2.9 mg, t_R 13.82 min).

2.3.1. Madeleinol A (**1**), 1-((2R*,4aR*,9aR*)-2,6,8-trihydroxy-1,1,4a-trimethyl-2,3,4,4a,9,9a-hexahydro-1H-xanthen-5-yl)-2-methylpropan-1-one: Yellow oil; $[\alpha]^{25}_{D}$ -5.6 (*c* 0.35, MeOH); UV (MeOH), λ_{max} (log ε): 292 (2.06) nm; IR (ATR) υ_{max} (cm^{-1}): 3277, 2965, 2935, 2870, 1603, 1504, 1419, 1380, 1233, 1127, 1022, 824, 752, 696, 666; ^{1}H NMR data see Table 1; ^{13}C NMR data see Table 1; negative ion ESI-FTICR-MS: $[M-H]^-$ at *m/z* 347.1859 (calcd. for $C_{20}H_{27}O_5^-$, 347.1864).

2.3.2. Empetrifranzinan D (**2**), 1-(1,9-epoxy-3-hydroxy-6,6,9-trimethyl-6a,7,8,9,10,10a-hexahydro-6*H*-benzo[*c*]chromene-2-yl)-2-methylbutan-1-one: White yellowish amorphous compound; $[\alpha]^{25}_{D}$ +28.7 (*c* 0.27, $CHCl_3$); UV (MeOH), λ_{max} (log ε): 294 (2.30) nm; IR (ATR) υ_{max} (cm^{-1}): 2967, 2924, 2873, 1614, 1577, 1454, 1368, 1230, 1124, 924, 851, 831, 793, 757; ^{1}H NMR data see Table 1;^{13}C NMR data see Table 1; negative ion ESI-FTICR-MS: $[M-H]^-$at *m/z* 343.1916 (calcd. for $C_{21}H_{27}O_4^-$, 343.1915).

2.3.3. Madeleinol B (**3**), 1-[3,5,7-trihydroxy-2-methyl-2-(4-methylpent-3-enyl)chroman-8-yl]-2-methylpropan-1-one: Yellow oil; $[\alpha]^{25}_{D}$ +6.8 (*c* 0.10, MeOH); UV (MeOH),λ_{max} (log ε): 291 (1.77) nm; IR (ATR) υ_{max} (cm^{-1}): 3306, 2966, 2928, 2873, 1614, 1511, 1422, 1380, 1234, 1141, 1094, 1048, 997, 827, 756, 698;^{1}H NMR data see Table 1;^{13}C NMR data see Table 1; negative ion ESI-FTMS: $[M-H]^-$ at *m/z* 347.1857 (calcd. for $C_{20}H_{27}O_5^-$, 347.1864).

2.4. Synthesis

Synthesis of 1-(2,4,6-trihydroxyphenyl)-2-methylpropanone (**12**): Anhydrous phloroglucinol (**11**,10.0 g, 79.30 mmol) was suspended in nitrobenzene (80 mL). 40 mL of carbon disulfide (40 mL, CS_2) was added at room temperature under stirring. $AlCl_3$ (48.35 g, 362.61 mmol) was added in two portions. The reaction mixture was stirred at room temperature for 30 min to give a pale brownish solution. Isobutyl chloride (10 mL) was added and the reaction mixture was heated at 65 °C for 21 h to give a dark mixture, which was poured onto an ice-water bath. Concentrated HCl was added until pH 1-2 was reached, and the aqueous phase (400 mL) was filtered and extracted with chloroform (200 mL x 3). The chloroform of the extract was evaporated using a rotavap and the resulting residue was lyophilized to give a crude product (6.36 g), which was chromatographed through a silica gel column, eluted with *n*-hexane: EtOAc (25:75 and 40:60) to give compound **12** as yellow oil (4.89 g, 24.9 mmol, 31%, R_f = 0.39 in *n*-hexane: EtOAc (75:25)). ^{1}H NMR (CD_3OD) δ: 5.86 (*s*, 2H), 4.96 (*brs*, 2 x OH), 4.01 (*sept*, *J* = 6.6 Hz, 1H), 1.16 (*d*, *J* = 6.6 Hz, 6H). ^{13}C NMR δ: 211.7, 165.7, 104.6, 95.8, 39.8, 19.6. Positive ion ESI-FTMS: $[M+H]^+$ at *m/z* 197.0805 (calcd. for $C_{10}H_{13}O_4^+$, 197.0808).

Synthesis of 1-(2,4,6-trihydroxyphenyl)-2-methylbutanone (**13**): Anhydrous phloroglucinol (5.23 g, 41.47 mmol) was suspended in nitrobenzene (40 mL). 40 mL of carbon disulfide (40 mL, CS_2) was added at room temperature under stirring. $AlCl_3$ (22.67 g, 170.02 mmol) was added in two portions. The reaction mixture was stirred at room temperature for 30 min to give a pale brownish solution. 2-Methylbutyryl chloride (5g, 41.47 mmol) was added and the reaction mixture heated at 65 °C for 21 h to give a dark mixture, which was poured onto an ice-water bath (400 mL). Concentrated HCl was added to reach pH 1-2. The aqueous phase was filtered and successively extracted with chloroform (250 mL x 3) and ethyl acetate (250 mL x 3). These unified extracts were freed from solvent using a rotavap and the residue was chromatographed through a silica gel column (*n*-hexane: EtOAc 70:30) to give compound **13** as yellow oil (2.32 g, 11.04 mmol, 27%, R_f = 0.42 in *n*-hexane:EtOAc (70:30)). ^{1}H NMR (CD_3OD) δ: 12.22 (*brs*, 1H), 5.91 (*s*, 2H), 5.17 (*brs*, 2 x OH), 3.91 (*sext*, *J* = 6.6 Hz, 1H), 1.84 (*m*, 1H), 1.40 (*m*, 1H), 1.16 (*d*, *J* = 6.6 Hz, 3H), 0.93 (*t*, *J* = 7.45 Hz, 3H). ^{13}C NMR δ: 211.6, 165.40, 165.36, 115.2, 95.9, 46.5, 27.9, 16.9, 12.2. Positive ion ESI-FTMS: $[M-H]^-$ at *m/z* 209.0818 (calcd. for $C_{11}H_{13}O_4^-$, 209.0819).

Synthesis of empetrifranzinan A (**8**): To 196 mg (1 mmol) of **12** and citral (183 mg, 1.2 mmol) in DMF (10 mL) was added ethylene diamine diacetate (EDDA, 0.036 g, 0.2 mmol) at room temperature. The reaction mixture was heated at 100 °C for 10 h. After completion of the reaction as indicated by ESI-MS and TLC, the reaction mixture was cooled to room temperature. Water (30 mL) was added and the mixture was extracted with ethyl acetate (30 mL x 3). The combined organic extracts were freed from volatiles in a rotavap in *vacuo*, and the crude product (yellowish brownish oil, 397 mg) was purified by column chromatography on silica gel, eluted with *n*-hexane:EtOAc (95:5) to afford **8** as yellow compound (163.4 mg, 0.5 mmol, 50 %, R_f = 0.31 in *n*-hexane:EtOAc (95:5)). ^{1}H and ^{13}C NMR see Table S1; positive ion HRESI-FTMS: $[M+H]^+$ at *m/z* 331.1905 (calcd. for $C_{20}H_{27}O_4^+$, 331.1904).

Synthesis of empetrifranzinan C (**9**): To 210.23 mg (1 mmol) of compound **13** and citral (183 mg, 1.2 mmol) in DMF (10 mL) was added ethylene diamine diacetate (EDDA, 0.036 g, 0.2 mmol) at room temperature. The reaction mixture was heated at 100 °C for 10 h. After completion of the reaction as indicated by ESI-MS and TLC, the reaction mixture was cooled to room temperature. Water (30 mL) was added and the mixture was extracted with ethyl acetate (50 mL x 3). The combined organic extracts were freed from solvent in a rotavap in *vacuo*, and the crude product (yellow oil, 431.8 mg) was purified by column chromatography on silica gel, eluted with *n*-hexane:EtOAc (95:5) to afford compound **9** as yellow oil (173.9 mg, 0.5 mmol, 50%, Rf = 0.33 in *n*-hexane:EtOAc (95:5)). ^{1}H and ^{13}C NMR see Table S2; positive ion HRESI-FTMS: $[M+H]^+$ at *m/z* 345.2067 (calcd. for $C_{21}H_{29}O_4^+$, 345.2060).

2.5. Biological assays

2.5.1. Cells and viruses

Cell lines were purchased from American Type Culture Collection (ATCC). The absence of mycoplasma contamination was checked periodically by the Hoechst staining method.

Cell lines supporting the multiplication HIV-1 virus were CD4+ human T-cells containing an integrated HTLV-1 genome (MT-4). IIIB laboratory strain of HIV-1, were obtained from the supernatant of the persistently infected H9/IIIB cells (NIH 1983).

2.5.2. Cytotoxicity assays

The human prostate cancer cell line PC-3 and the colon cancer cell line HT-29 were maintained in RPMI 1640 medium supplemented with 10% fetal bovine serum, 1% L-alanyl-L-glutamine (200 mM) and 1,6% hepes (1 M). ca. 5 x 10^2 PC-3 cells and ca. 1.5 x 10^3 HT-29 cells were seeded overnight into 96-well plates and exposed to a serial dilution of each compound (10 µM and 10 nM) and extract (50 and 0.50 µg/mL) for three days. Cytotoxicity was determined utilizing modified XTT method (0.25 mg/mL XTT, 6.5 µM PMS) as described by Scudiere *et al.*[20]

Exponentially growing MT-4 cells were seeded at an initial density of 1 x 10^5 cells/mL in 96-well plates in RPMI-1640 medium, supplemented with 10% fetal bovine serum (FBS), 100 units/mL penicillin G and 100 µg/mL streptomycin. Cell cultures were then incubated at 37 °C in a humidified, 5% CO_2

atmosphere, in the absence or presence of serial dilutions of test compounds. Cell viability was determined after 96 hrs at 37 °C by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) method.[21]

2.5.3. Antibacterial assays

These assays were performed as described by the European Committee on Antimicrobial Susceptibility testing. [22]

2.5.4. Anthelmintic assays

This assay was performed as described by Thomsen *et al*.[23]

2.5.5. Anti-HIV-1 assay

Activity against HIV-1 was based on the inhibition of virus-induced cytopathogenicity in MT-4 cell acutely infected with a multiplicity of infection (m.o.i.) of 0.01. Briefly, 50 µL of RPMI containing $1x10^4$ MT-4 cells was added to each well of flat-bottom microtitre trays, containing 50 µL of RPMI without or with serial dilutions of test compounds. Then, 20 µL of a HIV-1 suspension containing 100 $CCID_{50}$ were added. After a 4-day incubation at 37 °C, cell viability was determined by the MTT method.[21]

2.5.6. Linear regression analysis

The extent of cell growth/viability and viral multiplication at each drug concentration tested was expressed as a percentage of the untreated controls. Concentrations resulting in 50% inhibition (CC_{50} or EC_{50}) were determined by linear regression analysis.

3. Results and discussion

Column chromatography on silica gel and Sephadex LH-20 as well as semi-preparative HPLC led to the isolation of ten acylphloroglucinol derivatives (**1-10**) from the $CHCl_3$ extract of the leaves of *H. riparium* including one new tricyclic acylphloroglucinol (**1**), one new tretracyclic acylphloroglucinol (**2**), and one new prenylated bicyclic acylphloroglucinol (**3**) along with four known prenylated (**4-7**) and three known tetracyclic acylphloroglucinol derivatives (**8-10**). Their structures were established on the basis of spectroscopic evidences and comparison with the literature data. As acylphloroglucinol derivatives show characteristic deshielded proton signals around 9-14 ppm in the NMR spectrum, the isolation of compounds **1-10** was guided by ^{1}H-NMR metabolite profiles of fractions. The geranylated phloroglucinol derivatives **6** and **7** were isolated as the major constituents from the leaves of *H. riparium*. These two compounds highly dominate the UPLC-PDA and ^{1}H NMR metabolite profiles (Figures S1, S2, and S23; see supporting information) of the crude MeOH extract of the leaves of *H. riparium*. MeOH was identified as the best solvent for extracting the whole metabolome of the plant.

Compound **1** was obtained as an optically active yellow oil, $[\alpha]^{25}_D$ -5.6 (*c* 0.35, MeOH). Its molecular formula was established as $C_{20}H_{28}O_5$ by means of HR-ESI-FTICR-MS (347.1859, $[M-H]^-$), indicating 7 double bond equivalents. The IR spectrum indicates the presence of hydroxyl (3277 cm^{-1}) and carbonyl (1602 cm^{-1}) groups. The ^{13}C NMR (Table 1) spectrum of compound **1** exhibits 20 signals, which were sorted by HSQC and DEPT into eight quaternary, four methine, three methylene, and five methyl carbons. The analysis of ^{1}H NMR and ^{13}C NMR (Table 1) suggests an acylphloroglucinol derivative.[11,24] The ^{1}H NMR spectrum of compound **1** reveals signals typical of a substituted (2-methylpropanoyl)phloroglucinol nucleus,[11,24] exhibiting a highly deshielded hydrogen-bonded singlet at δ 13.83 (1H, *s*, 3-OH), a broad signal accounting for one hydroxyl group at δ 5.86 (1H, *bs*, 5-OH), one aromatic proton at δ 5.95 (1H, *s*, H-4), one methine septet at δ 3.77 (1H, *sept*, *J* = 7.0 Hz, H-2'), and two methyl groups at δ 1.15 (6H, *d*, *J* = 7.0 Hz, H-3'/H-4'). Additionally, many signals are observed between 3.46-0.91 ppm, including signals of one oxygenated methine at δ 3.46 (1H, *dd*, *J* = 11.4, 4.0 Hz, H-6"), one methine at δ 1.63 (1H, *m*, H-2"), two geminal protons at δ 2.68 (1H, *dd*, *J* = 16.2, 5.3 Hz, H-1") and δ 2.36 (1H, *dd*, *J* = 16.2, 13.2 Hz, H-1"), and three singlet methyl groups (δ 1.13, C-8";δ 0.91, C-9"; δ 1.28, C-10"). The carbon signals corresponding to the acylphloroglucinol moiety include one carbonyl group at (δ 210.4, C-1'), three deshielded oxygenated aromatic carbons (δ 165.2, C-3; δ 160.0, C-5; δ 156.0, C-1), two quaternary aromatic carbons (δ 105.4, C-2; δ 100.6, C-6), and one aromatic methine (δ 95.6, C-4). An inspection of the ^{1}H NMR and ^{13}C NMR spectra and degree of unsaturation of compound **1** suggests a tricyclic ring system because no additional double bond signal to the benzene ring is observed. Moreover, a characteristic signal of one oxygenated quaternary aliphatic carbon is observed in the ^{13}C NMR spectrum at δ 78.1 (C-3"). The ^{1}H and ^{13}C NMR signals of compound **1** resemble those reported for empetriferdinol [$C_{21}H_{30}O_5$, *m/z* 362, $[\alpha]^{25}_D$ +12 (*c* 0.1, MeOH)],[25] except for the signals of the acyl side-chain at C-2. The difference of 14 mass units in compound **1** is attributed to the fact that a 2-methylbutanoyl moiety is absent in **1** and is, instead, replaced by a 2-methylpropanoyl group. ROESY, COSY, HSQC, and HMBC experiments allowed for the determination of the tricyclic structure of **1**. Key observations in the HMBC spectrum were the correlation of Me-10" (δ 1.28) to C-3" (δ 78.1), C-2" (δ 45.8), and C-4" (δ 35.7) and the correlation of H-2" (δ 1.63) to C-6 (δ 100.6), C-10" (δ 19.7), C-7" (δ 38.4), C-6" (δ 78.0), C-1" (δ 17.3), C-8" (δ 27.2), C-9" (δ 14.2). Pertinent correlations are observed from H-1" (δ 2.36 and δ 2.68) to C-7", C-5, C-6, C-2", C-3", and C-1; from H-6" to C-8" and C-9"; from H-4"(δ 1.82 and δ 2.08) to C-6", C-5", C-10" and C-2"; from H-9" to C-6", C-2", C-7", and C-8"; from H-4 to C-2, C-3, C-5, C-6 as well as from H-3' to C-1', C-2', and C-4'. The COSY spectrum (Fig. 2) reveals correlations between H-6" and H-5", H-5" and H-4", H-1" and H-2", H-2' and H-4' as well as H-2' and H-3'. ROESY interactions between H-6" and H-2", between H-6" and Me-8" as well as between Me-10" and Me-9" indicate the relative configuration of compound **1**. Compound **1,** which is reported herein for the first time, was therefore characterized to be 1-[(2R*,4aR*,9aR*)-2,6,8-trihydroxy-1,1,4a-trimethyl-2,3,4,4a,9,9a-hexahydro-1*H*-xanthen-5-yl]-2-methylpropan-1-one, trivially named madeleinol A (**1**). Madeleinol is a dedication to the principal investigator's mother named Madeleine.

Compound **2** was isolated as a slightly yellowish amorphous and optically active substance, $[\alpha]^{25}_D$ +28.7 (*c* 0.27, $CHCl_3$). The HR-ESI-FTICR-MS indicates a quasi-molecular ion peak at *m/z* 343.1916 ($[M-H]^-$) consistent with the molecular formula $C_{21}H_{28}O_4$, corresponding to 8 degrees of unsaturation. The ^{1}H (Table 1) and ^{13}C NMR (Table 1) data of compound **2** are indicative of an acylphloroglucinol derivative.[11,24,25,26] The ^{1}H NMR exhibits one signal of an aromatic methine singlet (δ 6.03, H-4) and duplicate signals of one highly deshielded hydrogen-bonded singlet (δ 13.87, 3-OH),[12,25] one methine sextet (δ 3.66, *J* = 7.5 Hz, H-2'), one methyl doublet (δ 1.16, *J* = 7.5 Hz, H-5'), one methyl triplet (δ 0.96, *J* = 7.5 Hz, H-4'), and two methine multiplets (δ 1.84, H-3'A;δ 1.39, H-3'B). These observations are in agreement with the presence of a substituted (2-methylbutanoyl)phloroglucinol derivative.[12,25] The ^{13}C NMR spectrum (Table 1) of compound **2** displays signals of 21 carbon atoms, which were sorted by DEPT and HSQC experiments into eight quaternary, four methine, four methylene, and five methyl

carbons. The carbon signals corresponding to the acylphloroglucinol moiety include one carbonyl group (δ 209.7, C-1') three deshielded (oxygenated) aromatic carbons (δ 158.2, C-1; δ 165.8, C-3; δ 162.8, C-5), two quaternary aromatic carbons (δ 106.0, C-2; δ 106.6, C-6), and one aromatic methine (δ 98.7, C-4).[12,25] The ^{1}H and ^{13}C NMR data of compound **2** are similar to those of petiolin K[24] and empetrifranzinan A-C (Fig. 1)[25] recently reported in the literature. In these interesting naturally occurring tetracyclic compounds, a menthane moiety is linked to the acylphloroglucinol core via one -C-C- and two -C-O-C- bridges, forming a citran moiety.[24,25] Signals of a menthane skeleton are observed in the ^{1}H NMR spectrum (Table 1) of **2** as one methine broad singlet (δ 2.83, H-1") and six methine multiplets (δ 2.20, H-2"A; δ 1.88, H-2"B; δ 1.84, H-4"A; δ 1.49, H-4"B; δ 1.33, H-5"A; δ 0.88, H-5"B).[25] In addition, the ^{1}H NMR spectrum reveals three methyl groups as singlets (δ 1.09, H-8";δ 1.53, H-9";δ 1.44, H-10"). The HMBC spectrum (Fig. 2) of **2** reveals important correlations from H-1" to C-1, C-5, C-6, C-2", C-3", C-5", C-6", and C-7" in good agreement with the presence of one -C-C- bridge in the structure of **2** and enables the linkage of the 4 rings. The signals of two oxygenated aliphatic carbon atoms (δ 76.5, C-3" and δ 84.9, C-7") in the ^{13}C NMR spectrum and some important HMBC correlations, including from Me-8" to C-6", C-7", and C-9" as well as from Me-10" to C-2", C-3", and C-4", corroborate the presence of two –C-O-C- bridges in the molecule. The COSY spectrum shows correlations between H-2' and H-3'A/B; H-2' and Me-5', H-3'A/B and Me-4', H-1" and H-2"A/B, H-1" and H-6", H-4"A/B and H-5"A/B, as well as between H-5"A/B and H-6". The 1D and 2D-NMR data of **2** as well as the key ROESY correlation observed between Me-10" and Me-4'/Me-5' tells us that it is a new derivative of empetrifranzinan B (**10**, $C_{20}H_{26}O_4$, *m/z* 330) .[25] The difference of 14 mass units in compound **2** agrees with the absence of a 2-methylpropanoyl group, replaced by a 2-methylbutanoyl group in the structure of **2**. Given all the spectroscopic data above, compound **2** was then characterized as 1-(1,9-epoxy-3-hydroxy-6,6,9-trimethyl-6a,7,8,9,10,10a-hexahydro-6*H*-benzo[*c*]chromene-2-yl)-2-methylbutan-1-one, which is a new acylphloroglucinol derivative and named empetrifranzinan D (**2**), based on similar compounds empetrifranzinans A-C (**8-10**).

Table 1. ^{13}C NMR (δ) and ^{1}H NMR data [δ, *multiplicity*, *J* (Hz)] for compounds **1-3** ($CDCl_3$)

	1[a]		**2**[a]		**3**[b]	
Position	^{13}C	^{1}H	^{13}C	^{1}H	^{13}C	^{1}H
1	156.0		158.2		155.7	
2	105.4		106.0		105.4	
3	165.2		165.8		165.7	
4	95.6	5.95 *s*	98.4	6.03 *s*	96.3	5.97 *s*
5	160.0		162.8		159.8	
6	100.6		106.6		97.9	
1'	210.4		209.7		210.4	
2'	39.3	3.77 *sept* (7.0)	45.4	3.66 *sext* (7.5)	39.4	3.85 *sept* (6.6)
3'	19.3	1.15 *d* (7.0)	26.8	1.39 *m*	19.2	1.18 *d* (6.6)
				1.84 *m*		
4'	19.5	1.15 *d* (7.0)	12.0	0.96 *t* (7.5)	19.7	1.18 *d* (6.6)
5'			17.0	1.16 *d* (7.5)		
1''	17.3	2.36 *dd* (16.2, 13.2)	27.5	2.83 *bs*	25.2	2.60 *dd* (16.5, 6.6)
		2.68 *dd* (16.2, 5.3)				2.89 *dd* (16.5, 5.5)
2''	45.8	1.63 *m*	34.8	1.88 *m*	66.4	3.95 *dd* (6.6,5.5)
				2.20 *m*		
3''	78.1		76.5		80.7	
4''	37.5	1.82 *dd* (12.7, 4.0)	37.5	1.49 *m*	37.5	1.71 *m*
		2.08 *dt* (12.7, 3.5)		2.84 *m*		1.77 *m*
5''	28.1	1.65 *m*	22.0	0.88 *m*	22.0	2.15 *m*
		1.90 *m*		1.33 *m*		
6''	78.0	3.46 *dd* (11.4, 4.0)	46.0	2.04 *ddd* (7.5, 5.3, 2.2)	123.4	5.09 *t* (7.0)
7''	38.4		84.9		132.6	
8''	27.2	1.13 *s*	24.2	1.03 *s*	25.7	1.69 *s*
9''	14.2	0.91 *s*	29.6	1.53 *s*	17.6	1.60 *s*
10''	19.7	1.28 *s*	28.8	1.44 *s*	19.0	1.38 *s*
3-OH		13.83 *s*		13.87 *s*		13.81 *s*
5-OH		5.86 *bs*				5.43 *s*

[a] ^{13}C NMR (100 MHz), ^{1}H NMR (400 MHz)
[b] ^{13}C NMR (150 MHz), ^{1}H NMR (600 MHz)

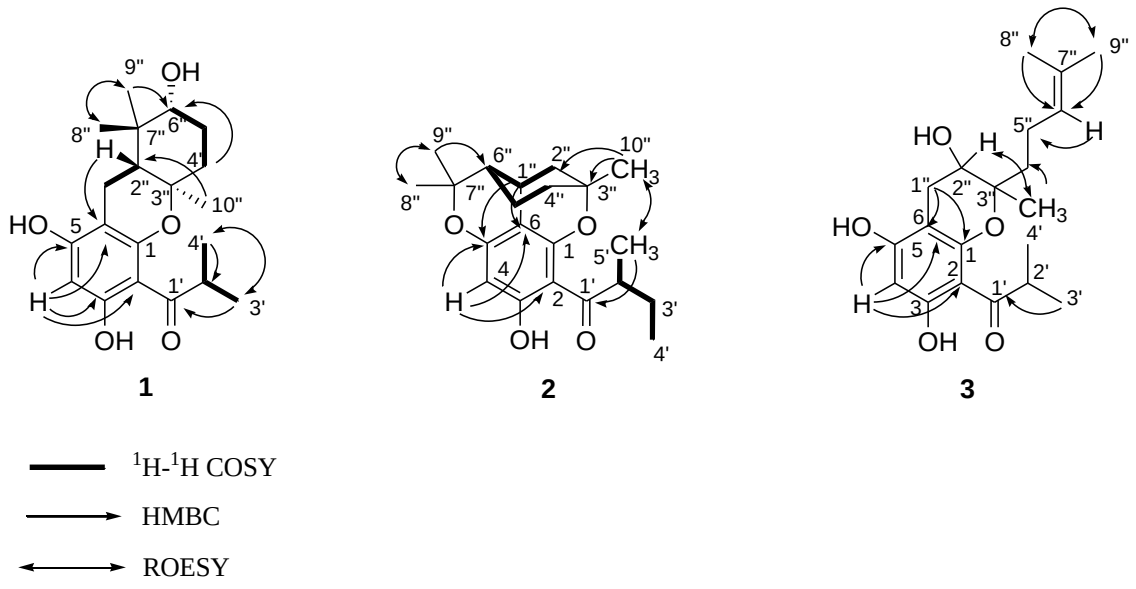


Figure 2. Selected 2D NMR correlations for compounds **1-3**

The structures of the polycyclic compounds **8, 9,** and **10** (Fig. 1) were similarly elucidated by comparing their spectroscopic (including 2D NMR) and physicochemical data with those disclosed in the literature, and they were respectively identified to be empetrifranzinan A (**8**), empetrifranzinan C (**9**), and empetrifranzinan B (**10**), recently reported as antiproliferative constitutents of *H. empetrifolium*.[25] Acylphloroglucinols containing a citran moiety in their structures, with a menthane substructure C- and O-connected to the aromatic ring, have previously been reported from Guttiferae, isolated from the genera *Hypericum* and *Clusia*.[24,25]

In order to provide additional evidence for the proposed regiomeric structures (**2**, **8**, **9**, and **10**) alongside 2D NMR spectroscopic data and to obtain more material for our biological activity testing, we performed the total and regioselective synthesis of compounds **8** and **9** in two efficient steps (Scheme 1). The key synthetic route used provided only one regiomer each (**8** and **9**) with 50 % yield. The synthetic strategy involved Friedel-Craft acylation of phloroglucinol followed by an ethylenediamine diacetate-catalyzed (EDDA) cyclization by a domino aldol-type/electrocyclization/H-shift/hetero-Diels-Alder reaction of acylphloroglucinols and citral or *trans,trans*-farnesal.[27] This reaction was previously applied for the regioselective synthesis of acylphloroglucinols bearing citrans, like the petiolin D regioisomer.[27] The spectroscopic data (Tables S1 and S2, see suppl.) of synthetic compounds **8** and **9** are identical to those of empetrifranzinan A (**8**) and empetrifranzinan C (**9**) isolated from *H. riparium* (this paper) and also previously reported from *H. empetrifolium*.[25]

Compound **3** was isolated as an optically active yellow oil, $[\alpha]^{25}_D$ +6.8 (*c* 0.10, MeOH). Its molecular formula was determined to be $C_{20}H_{28}O_5$ from its HR-ESI-FTMS, which shows the base peak at *m/z* 347.1857 ($[M-H]^-$), consistent with 7 degrees of unsaturation. The IR spectrum shows the presence of hydroxyl (3306 cm^{-1}) and carbonyl (1614 cm^{-1}) groups. As in case of **1**, the ^{1}H (Table 1) and ^{13}C NMR (Table 1) data of compound **3** are in agreement with the presence of a (2-methylpropanoyl)-phloroglucinol moiety in the molecule.[24,28] Additional signals of a prenyl chain,[25,26] including one methine triplet (δ 5.09, C-6"), one methylene multiplet (δ 2.15, C-5"), and two methyl singlets (δ 1.69, C-9"; δ 1.60, C-8") are revealed in the ^{1}H NMR spectrum. This is supported by HMBC correlations (Fig. 2) from Me-8" to C-6", C-7", and C-9" as well as from Me-9" to C-6", C-7", and C-8". The molecular formula and 1D and 2D NMR data of compound **3** are similar to those reported for empetrikarinol A,[25] except the ROESY spectrum which shows in **3** a key interaction between H-2" (δ 3.95, *dd* 6.6, 5.5 Hz) and Me-10" (δ 1.38) in accordance with an equatorial position of H-2''. Furthermore, empetrikarinol A shows an optical rotation of $[\alpha]^{21}_D$ -24 (*c* 0.1, MeOH),[25] whereas for **3** a value of $[\alpha]^{25}_D$ +6.8 (*c* 0.1, MeOH) was determined. Therefore, compound **3** is presumably a stereoisomer of empetrikarinol A, which is reported herein for the first time and named madeleinol B (**3**).

The structures of remaining known phloroglucinol derivatives were assigned by comparing their spectroscopic and physical data with those previously reported in the literature. These compounds were identified as empetrikarinol B (**4**) isolated from *H. empetrifolium*,[25] 3-geranyl-2,4,6-trihydroxybenzophenone (**5**) isolated from *Tovomita krukovii* (Guttiferae),[29] 3-geranyl-1-(2'-methylpropanoyl)-phloroglucinol (**6**),[7] and 3-geranyl-1-(2'-methylbutanoyl)-phloroglucinol (**7**).[7] Compound **6** and **7** were reported as constituents of *H. empetrifolium*[7,12] and *H. punctatum*.[30]

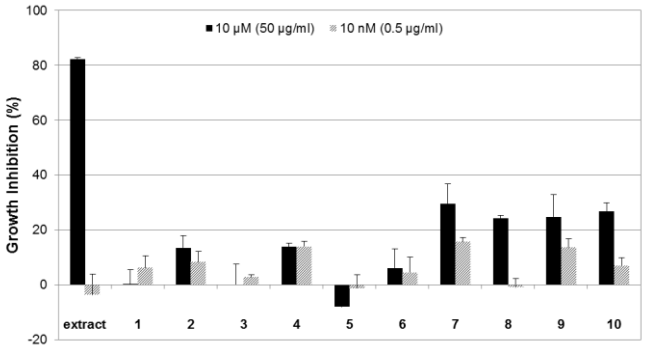


Figure 3. Cytotoxic activities of the $CHCl_3$ extract (leaves) and compounds **1-10** against the human prostate cancer cell line PC-3. The results are expressed as percentage of inhibition ± SD. Compounds were tested at two different concentrations of 10 µM and 10 nM while extracts were tested at 50 µg/mL and 0.5 µg/mL. Digitonin at 125 µM was used as positive control. 0.05% DMSO was used as negative control.

Compounds **1-10** were tested in a cell-based assay against the human immunodeficiency virus type-1 (HIV-1), using efavirenz as the reference inhibitor. Cytotoxicity against MT-4 cells was evaluated in parallel with the antiviral activity. As reported in Table 2, none of the isolated compounds shows significant anti-HIV activity.

Compounds **1-10** were also tested for antibacterial activity against representative human pathogenic Gram negative (*Escherichia coli)* and Gram positive (*Staphylococcus aureus*, and *Enterococcus faecalis*) bacteria. Ciprofloxacin was used as reference compound. None of the tested compounds shows significant inhibitory activity (MIC > 1 mg/L).

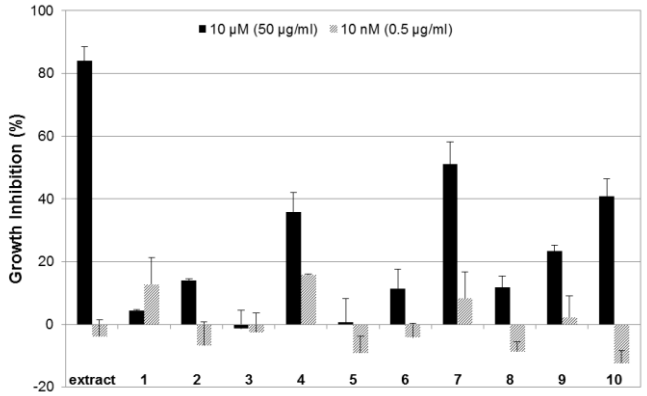


Figure 4. Cytotoxic activities of the $CHCl_3$ extract (leaves) and compounds **1-10** against the human colon cancer cell line HT-29. The results are expressed as percentage of inhibition ± SD. Compounds were tested at two different concentrations of 10 µM and 10 nM while extracts were tested at 50 µg/mL and 0.5 µg/mL. Digitonin at 125 µM was used as positive control. 0.05% DMSO was used as negative control.

The $CHCl_3$ extract as well as compounds **1-10** were screened for cytotoxicity against HT-29 and PC-3 cancer cell lines. The $CHCl_3$ extract (from the leaves) exhibits cytotoxic activities indicated by a growth inhibition of the cell lines of 84 and 82%, respectively, at a concentration of 50 µg/mL. No inhibition was observed at 0.5 µg/mL. As shown in figure 3, the isolated compounds **7-10** show weak cytotoxic effects (growth inhibition of 20-40%) against PC-3 at a tested concentration of 10 µM while compounds **1-6** are inactive (less than 20% of growth inhibition). They were all inactive (inhibition of less than 20%) at 10 nM. Compounds **4, 7, 9-10** show weak cytotoxic activities (growth inhibition of 20-51 %) against the cancer cell line HT-29 (Fig. 4). Compounds **1-10** were found not active at 10 nM. Compound **7**, one of the two major constituents (**6** and **7**) of the leaves, shows the highest cytotoxic effects among the tested acylphloroglucinols (Fig. 3 and 4). An initial explanation or presumption of the difference of activity observed between the prenylated compounds **6-7** and **3-4** may be the length of the acyl chain as (2-methylbutanoyl)phloroglucinols are more potent than (2-methylpropanoyl)phloroglucinols in the present study. However, a contrary effect is observed between the two pairs of citran acylphloroglucinols **8-9** and **2**, **10**. Another activity-relevant feature is the prenyl side chain; the loss of a prenyl side chain can decrease the activity. For instance, the tricyclic acyphloroglucinol **1**, which is derived biosynthetically from prenylated phloroglucinols, does not cause any growth inhibition of the cell lines. Cytotoxic and antiproliferative phloroglucinols, including hyperforin, have been already reported from the genus *Hypericum*.[17,25,28]

Table 2. Cytotoxicity and antiviral activity of compounds (µM) and MeOH extract (µg/mL) obtained from *H. riparium* leaves (**1-10**) against HIV-1_{IIIB}.[a]

Hypericum riparium	MT-4 CC_{50} (µM)[b]	HIV-1_{IIIB} EC_{50} (µM)[c]
Leaves extract	11.0 µg/mL	> 11.0 µg/mL
1	41.0	> 41.0
2	> 100	> 100
3	84.0	> 84.0
4	45.0	> 45.0
5	69.0	> 69.0
6	> 100	> 100
7	26.7	>26.7
8	18.7	> 18.7
9	45.0	> 45.0
10	> 100	> 100
Efavirenz	40.0	0.002

[a] Data represent mean values for three independent determinations. Variation among duplicate samples (SD) was less than 15%.

[b] Compound concentration (µM) required to reduce the viability of mock-infected MT-4 cells by 50%, as determined by the MTT method. Efavirenz is the reference drug. For extracts: µg/mL.

[c] Compound concentration (µM) required to achieve 50% protection of MT-4 cells from the HIV-1 induced cytopathogeneticy, as determined by the MTT method. For extracts: µg/mL.

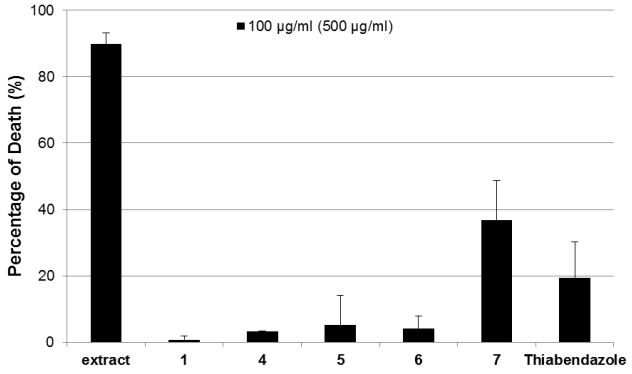


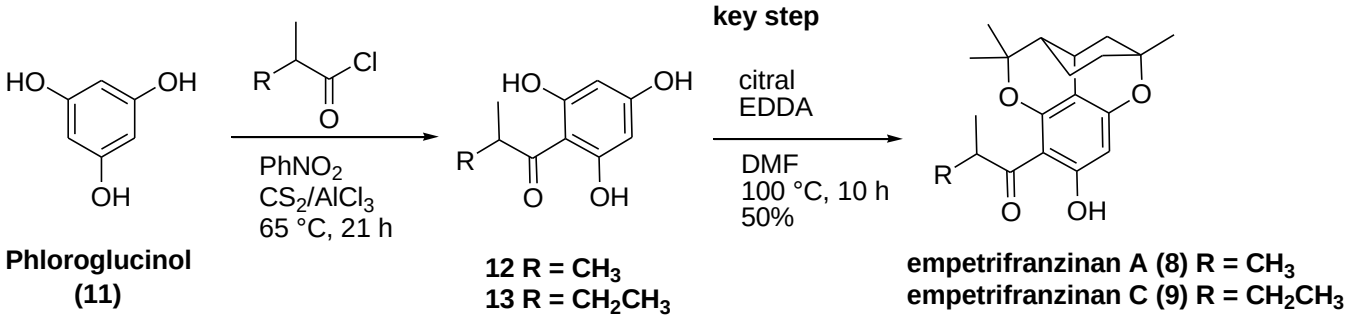
Figure 5. Anthelmintic activities of the $CHCl_3$ extract (dried leaves, 500 µg/mL) and compounds **1, 4-7** (100 µg/mL) against *Caenorhabditis elegans*. The results are expressed as percentage of death worms ± SD. 2% DMSO (3 ± 3 %) was used as negative control while ivermectin 10 µg/mL (99 ± 1 %) was used as positive control. Thiabendazole is a reference anthelmintic drug.

Some natural phloroglucinol derivatives, including aspidin and desaspidin from *Dryopteris filix-mas* (Dryopteridaceae) as well as kosins from *Hagenia abyssinica* (Rosaceae), have also been reported to show anthelmintic properties.[23,31,32] One of the objectives of the United Nations Millennium Development Goals is to halt or reverse the incidence of infections caused by neglected tropical diseases like helminths,[33] as billions of people suffer from helminthic diseases worldwide resulting in many thousands of death annually.[34] Although some effective anthelmintic drugs are available, recent treatment failures have occurred apparently due to the development of genetic resistance in nematodes.[35] Thus, there is a need for new and inexpensive drugs able to act longer before the resistance sets in. As described by Thomsen *et al.*[23,35] the non-parasitic nematode *Caenorhabditis elegans* can be used as a model organism for inexpensive and rapid initial screening for the detection of compounds active against parasitic helminths. Preliminary anthelmintic activities of the $CHCl_3$ extract from the leaves of *H. riparium* and compounds **1**, **4-7** against the model organism *Caenorhabditis elegans* were determined in a modified microtiter plate assay by enumeration of living and dead nematodes using a microscope.[23] At a test

concentration of 100 µg/mL, compound **7** shows a nematode death percentage of 37 ± 12% (Fig. 5) while the reference drug thiabendazole exhibits a percentage of death of 19 ± 11%. Compounds **1, 4**-**6** are inactive. They show a percentage of death ranging from 0.7 to 5.1 %. All the tested compounds are inactive at 12.5 µg/mL (results not shown). The known compound **7**, one of the two major constituents of the extract, may be responsible for the observed anthelmintic activity of the $CHCl_3$ extract. It hints to a potential anthelmintic lead structure, which may be structurally modified on its acyl side chain in order to investigate its structure activity relationship. An initial observation of the structures of **6** and **7** and their anthelmintic activities allows us to speculate that longer acyl side chains may give more active compounds. However, such alterations also enhance the lipophilicity and thus the bioavailability. Thus, it is yet unclear if the improved activities are based on better target binding or on better bioavailability.

4. Conclusions

In summary, we have isolated and identified new constituents of *H. riparium* for the first time from this plant, namely compounds **1-10**, which include some very minor compounds and three new natural products. Two acylphloroglucinols (**8** and **9**) bearing citran moieties were successfully synthetized in only two steps. Their anthelmintic (**1**, **4**-**7**), cytotoxic (**1**-**10**) and anti HIV (**1**-**10**) activities have been evaluated. Compounds **4**, **7**, **9**-**10** exhibit weak cytotoxic effects against cancer cell lines. The crude $CHCl_3$ extract and compound **7** show anthelmintic activity against *Caenorhabditis elegans*. This may explain the use of *Hypericum* species in Cameroonian traditional medicine against intestinal worm infections. Even though this is a preliminary screening, the force of our study lies on the ability of Hypericaceae natural products to provide yet unexplored structures with the potential for anthelmintic, cytotoxic or anti-HIV activity. Also, acylphloroglucinols have significance as chemotaxonomic markers in *Hypericum* species. Based on this and similar studies on *Hypericum*, it will be interesting to evaluate the activity profiles of more species and compare them by established metabolomics approaches for chemotaxonomic significance as well as for differences or specificities in bio-markers and bioactivities.



Scheme 1. Regioselective synthesis of acylphloroglucinol derivatives **8** and **9**

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