

Development of a georeferenced library of native microbial consortia for bioremediation of oil spills

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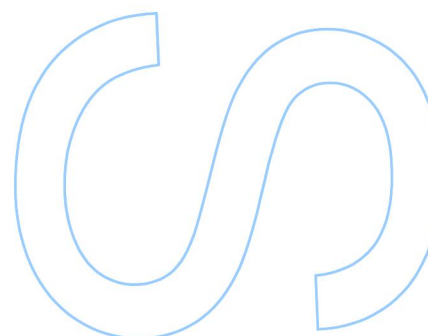
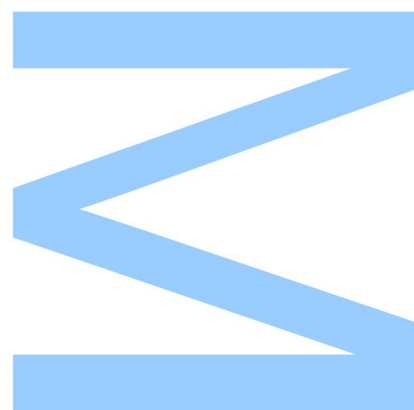
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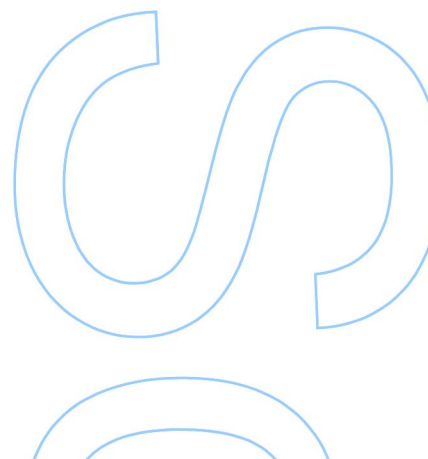
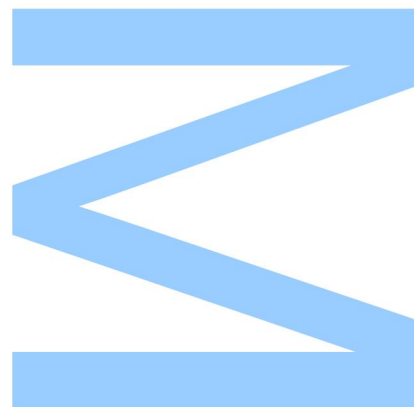




Todas as correções determinadas
pelo júri, e só essas, foram efetuadas.

O Presidente do Júri,

Porto, ____/____/____



“The fact is that no species has ever had such wholesale control over everything on earth, living or dead, as we now have. That lays upon us, whether we like it or not, an awesome responsibility. In our hands now lies not only our own future, but that of all other living creatures with whom we share the earth.”

— **David Attenborough, *Life on Earth***

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Resumo

Os derrames de petróleo são uma das maiores fontes de entrada de hidrocarbonetos nos ambientes marinhos, com consequências catastróficas que chegam a afetar todos os ecossistemas. As técnicas de remediação atuais são caras, de pouca duração e não são ecológicas, o que impede uma completa recuperação dos ecossistemas afetados por derrames de petróleo. As técnicas de biorremediação podem aumentar a atividade de microrganismos com capacidade de biodegradação, podendo tornar-se num tratamento de baixo custo capaz de acelerar a recuperação de ambientes contaminados.

O uso de consórcios microbianos nativos para a biorremediação de derrames de petróleo pode aumentar a sua eficiência. Estes microrganismos têm maior potencial para sobreviver às condições físico-químicas do sítio poluído pelo petróleo, evitando assim a introdução de organismos não-nativos. Esta tese de mestrado, integrada no projeto SpillLess (<http://spilless.ciimar.up.pt/>), tem como objetivo principal desenvolver uma biblioteca georreferenciada de consórcios microbianos nativos com capacidade para degradar compostos de petróleo.

Amostras de água do mar foram recolhidas em 29 locais georreferenciados ao longo da costa NW da Península Ibérica. Estas amostras foram expostas a petróleo durante duas semanas, em experiências de enriquecimento em microcosmos com a adição de nutrientes. As amostras marinhas recolhidas *in situ* e as amostras recolhidas após o enriquecimento com petróleo foram filtradas através de filtros Sterivex™ para caracterizar as comunidades microbianas naturais que habitam cada local georreferenciado, assim como as comunidades enriquecidas com petróleo. A identificação e caracterização das comunidades procarióticas foi realizada por sequenciação de amplificação do gene 16S rRNA na plataforma Illumina MiSeq. A previsão do perfil funcional das comunidades procarióticas foi obtido pelo programa PICRUSt. O potencial biodegradativo das comunidades microbianas, antes e depois dos enriquecimentos, foi estimado através da quantificação do número mais provável de degradadores de hidrocarbonetos.

Os resultados mostraram uma redução drástica na riqueza e diversidade procariótica após o enriquecimento com petróleo, que foi acompanhado por um aumento na abundância de microrganismos capazes de degradar hidrocarbonetos. Os consórcios microbianos nativos selecionados das distintas áreas de amostragem geográfica, com a capacidade de degradar os compostos de petróleo, mostraram um elevado grau de similaridade (Similaridade de Bray Curtis > 50%). Estas comunidades microbianas foram predominantemente compostas por *Alcanivorax* spp., *Pseudomonas* spp., *Thalassospira* spp. e por alguns géneros pertencentes à família *Flavobacteriaceae*. A previsão do perfil funcional mostrou um elevado potencial para degradar os compostos aromáticos naftaleno, PAHs, etilbenzeno, tolueno e xileno.

Este estudo relevou-se importante na seleção de consórcios microbianos nativos com capacidade de degradação de hidrocarbonetos, distribuídos ao longo da costa NW da Península Ibérica, com potencial para serem aplicados em técnicas de biorremediação para posterior implementação nas áreas estudadas. Devido à grande distribuição geográfica de microrganismos degradadores de hidrocarbonetos, o protocolo desenvolvido neste trabalho poderá vir a ser considerado no futuro para aplicação em outras regiões.

Palavras-chave: Biorremediação, derrames de petróleo, biblioteca georreferenciada, comunidades microbianas nativas; nova geração de sequenciação.

Abstract

Oil spills represent a large input of hydrocarbons in marine environments, causing catastrophic consequences that affect all ecosystems. Current remediation techniques are expensive, have a short-term effect and are not eco-friendly, therefore not allowing the complete ecological restoration of the ecosystems affected by oil spilled. Bioremediation approaches can increase the activity of microorganisms with biodegradation capacity, which can turn into a cost-effective treatment to accelerate the recovery of contaminated environments.

The use of native microbial consortia for bioremediation of oil spills can increase the bioremediation efficiency. These microorganisms have higher potential to survive to the physicochemical conditions of the oil-polluted site, also preventing the introduction of non-native organisms. This master thesis, integrated in the SpilLess project (<http://spilless.ciimar.up.pt/>), aims to develop a georeferenced library of native microbial consortia with the capability to degrade petroleum compounds.

Seawater samples were collected in 29 georeferenced sites along the NW coast of the Iberian Peninsula (IP). These samples were exposed to Arabian light crude oil for two weeks, in microcosm enrichment experiments with the addition of nutrients. The seawater samples collected *in situ*, and the samples collected after the enrichment with crude oil, were filtered through Sterivex™ filters to characterize the natural microbial communities that inhabit each georeferenced site as well as the crude oil enriched communities. Identification and characterization of the Prokaryotic communities was performed by using 16S rRNA amplicon sequencing on an illumine MiSeq platform. Predictive functional profiling of prokaryotic communities was obtained with the program PICRUST. The biodegradative potential of the microbial communities, both before and after enrichments, was estimated through the quantification of the most probable number of hydrocarbon degraders.

Results showed a drastic decrease in richness and diversity after the enrichment with crude oil, which was accompanied by an increase in abundance of microorganisms capable of degrading hydrocarbons. The selected microbial consortia with the capability to degrade petroleum compounds, native from the distinct geographic sampling areas show a high degree of similarity (Bray Curtis Similarity > 50%). These microbial communities were mainly dominated by *Alcanivorax* spp., *Pseudomonas* spp., *Thalassospira* spp. and genera of the family *Flavobacteriaceae*. Predictive functional profiling of the enriched microbial consortia showed a high potential to degrade the aromatic compounds naphthalene, PAHs, ethylbenzene, toluene and xylene.

This study provides an insight into the selection of native hydrocarbon-degrading microbial consortia, distributed along the NW coast of the IP, and their potential application

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for the future development of bioremediation techniques to be implemented in the studied areas. Giving the ubiquity of hydrocarbon-degrading microorganisms, the developed workflow can be considered for future application in other regions.

Keywords: Bioremediation; Oil spills; georeferenced library; native microbial communities, next-generation sequencing

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Abbreviations

16S rRNA	16S ribosomal ribonucleic acid
ASV	Amplicon Sequence Variants
BA	Bioaugmentation
BH	Bushnell-Hass
BS	Biostimulation
C/N/P	Ratio of carbon, nitrogen and phosphorous
CO ₂	Carbon dioxide
DNA	Deoxyribonucleic acid
DWH	Deepwater Horizon
F	Final samples (after the oil-enrichments)
HC	Hydrocarbon
i	Initial samples (before the oil-enrichments)
INT	Iodonitrotetrazolium Violet
IP	Iberian Peninsula
KEGG	Kyoto Encyclopedia of Genes and Genomes
KH ₂ PO ₄	Potassium di-hydrogen phosphate
KNO ₃	Potassium nitrate
KO	KEGG ortholog
MDS	Multidimensional scalling
ML	Maximum-likelihood
MPN	Most probable number
N	Nitrogen
NaCl	Sodium chloride
NGS	Next-generation sequencing
OHCB	Obligate hydrocarbonoclastic bacteria
OSD	Ocean sampling day
OTU	Operational Taxonomic Unit
PAH	Polycyclic aromatic compound
P	Phosphorous
PCR	Polimerase chain reaction
PICRUSt	Phylogenetic Investigation of Communities by Reconstruction of Unobserved States
QIIME	Quantitative Insights Into Microbial Ecology.
RDP	Ribosomal database project

SSU	Small subunit
W0	Week zero
W1	Week one
W2	Week two

1. Introduction

1.1. Oil Spills

In recent years, there has been a revolution regarding the use of renewable energies, however the world is still dependent on fossil fuels due to slow governmental actions, low-cost energy and clean-energy technologies not being developed enough to compete with non-renewable resources (Tollefson, 2018). The use of fossil fuels is currently under takedown, which means that the rate of energy consumption is superior to its replacement. In 35 years, it is estimated that the world's oil reserves will begin to be depleted (Shafiee & Topal, 2009)

In 2017, crude oil represented over a third of all energy consumed, being the world's most dominant fuel (BP, 2018). With the constant demand of fossil fuels for industry, plastics, agriculture, transportation, pharmaceuticals, etc., there is an increase in exploitation and transportation of this natural resource and derivatives on to the sea.

Each year, three million tons of oil are released in the sea due to leaks or accidental larger and/or smaller (<7 tons) oil spills resulted from the exploration, production, refining, transportation and storage of petroleum and its derivatives (Brooijmans et al., 2009; Das & Chandran, 2011). Whether it is from human activities or by accident, this acute form of pollution is one of the main causes of water contamination.

Intercontinental transportation of oil occurs mainly by a network of maritime routes from both tankers and barges (Siddiqui & Verma, 2018). As a result, coastal habitats are frequently affected by oil spill accidents due to an increase of frequency of passage and quantity of crude oil in near-shore areas (Figure 1.1). The Iberian Peninsula (IP) Atlantic coast is close to many trade routes due to its geographical position and is part of one of the largest oil community ports, thus being highly susceptible to oil spills (Vieites et al., 2004).

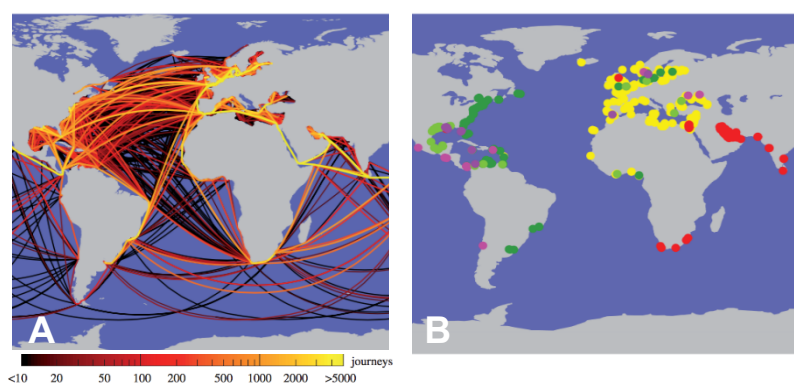


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The main source of petroleum hydrocarbons pollution in marine environments is due to chronic pollution originated from illegal cleaning of ship's bilges and leaking ships (Henkel et al., 2014). This kind of events are difficult to control and monitor which leads to an increase in hydrocarbon (HC) pollution at sea (Henkel et al., 2014). Nevertheless, oil spills still represent 10% of HC input in marine environments with hazardous consequences to the environment, having a long-term impact during many years after the spill (Ivshina et al., 2015; Kingston, 2002).

Table 1.1 Top 20 oil spills in history including amount of oil spilled (in tons), localization and year. Information gathered from CEDRE oil spills database [online: <http://wwwz.cedre.fr/en/Resources/Spills>] and ITOPF Oil Tanker Spill Statistics 2017 [<http://www.itopf.org/knowledge-resources/data-statistics/statistics/>]

Top 10	Name of the disaster	Ton	Localization	Year
1	Atlantic Empress	287 000	Off Tobago, West Indies	1979
2	ABT Summer	260 000	700 nautical miles off Angola	1991
3	Castillo de Bellver	252 000	Off Saldanha Bay, South Africa	1983
4	Amoco Cadiz	223 000	Off Brittany, France	1978
5	Haven	144 000	Genoa, Italy	1991
6	Odissey	132 000	700 Nautical miles off Nova Scotia, Canada	1988
7	Torrey Cannon	119 000	Scilly Isles, UK	1967
8	Sea Star	115 000	Gulf of Oman	1972
9	Irenes Serenade	100 000	Navarino Bay, Greece	1980
10	Urquiola	100 000	La Coruna, Spain	1976
11	Hawaiian Patriot	95 000	300 Nautical miles off Honolulu	1977
12	Independenta	95 000	Besphorus, Turkey	1979
13	Jakob Maersk	88 000	Oporto, Portugal	1975
14	Braer	85 000	Shetland Islands, UK	1993
15	Aegen Sea	74 000	La Coruna, Spain	1992
16	Sea Empress	72 000	Milford Haven, UK	1996
17	Khark's	70 000	120 Nautical miles off Atlantic coast of Morocco	1989
18	Nova	70 000	Off Kharg Island, Gulf of Iran	1985
19	Katina P	67 000	Off Maputo, Mozambique	1992
20	Prestige	63 000	Off Galicia, Spain	2002

Along the decades it was possible to witness some oil spills disasters and observe its hazardous consequences and repercussions (Table 1.1). Sometimes the biggest oil spills do not necessarily mean higher negative impacts on the environment and marine wild life. This depends on the location from where the oil spill accident occurred, the type of ecosystems affected and the mitigation techniques implemented (Teal & Howarth, 1984). For example, in

the NW coast of the Iberian Peninsula two of the most mediatic oil spill disasters occurred due to the drift of the crude-oil to coastal zones. In January of 1975, in Leixões harbour, Portugal, nearly 88 000 tons of Iranian crude oil were spilled when the tanker Jacob Maersk hit a sandbank entering the harbour, exploded and broke into three parts. One of the consequences of this disaster was a delay in the uptake of nutrients by phytoplankton, possibly due to the oil slick layer that formed in the surface and blocked UV radiation (Canelas, 1977). However, since the majority of the oil was burned, the ecosystem was able to recover months after the disaster (Wwz.cedre.fr, 2018). On the other hand, the Prestige oil tanker, that sank in 2002, off the coast of Galicia, Spain, spilled approximately 63 000 tons of crude oil into the sea. This was one the worst ecological disasters, which resulted in a substantial decrease in species richness such as insects, polychaetes, semi-terrestrial crustaceans and some species populations like the four-spot megrim, Norway lobster and also *Plesionika heterocarpus* (De la Huz et al., 2005; Sánchez et al., 2006). Also, several protected areas, natural parks and protected wetlands and marshes which are rich in ornithological biodiversity, were affected by the spill (Loureiro et al., 2006). Additionally to the costs of cleaning and recovery of the Prestige disaster, other economic sectors were also affected, like fishing and shellfish extraction which were prohibited for months (Loureiro et al., 2006).

Other oil spill disasters have also marked History for their dimension, consequences and repercussions to this day. For example, in Prince William Sound, Alaska, in 1989, the Exxon Valdez grounded the hull in a coral reef which resulted in the spilling of 37 000 tons of crude oil into the marine environment thus leading to the loss of marine and terrestrial mammals and birds (Brown, 1992). Another accident occurred from April to July 2010, where nearly 4.9 million barrels of crude oil were spilled onto the Gulf of Mexico due to the explosion and sinking of the oil rig Deepwater Horizon (DWH) (Lehr et al., 2010). The constant flow of HC lead to significant changes in structure and function in the indigenous organisms (Hazen et al., 2010; Kessler et al., 2011; Redmond & Valentine, 2011; Valentine et al., 2010) and it also affected deep-water ecosystems as seen by the presence of damage and decease coral reefs nearby (White et al., 2012).

More recently, at least two major oil spills were reported. In January 2018, on the East China Sea, the Iranian oil tanker Sanchi collided with a cargo ship, caught fire and sank (Carswell, 2018). In March 2018, an oil pipeline of the company Pertamina leaked in Borneo port city and when the oil ignited it resulted in the death of five people (Latif et al., 2018). The amount of oil spilled and the ecological impacts of these recent events are yet to be determined.

Despite the decrease of the number of oil spills throughout the years, they continue to occur, having an hazardous impact in all ecosystems (ITOPF, 2017). The high concentration

of the toxic chemicals released after an oil spill persist in the environment for years to come, which highlights the need for future prevention planning.

1.2. Petroleum composition

Crude oil is a natural resource produced over a million years ago by buried organic material or thermal decay (Varjani, 2017). It is a heterogeneous mixture of more than 17 000 complex chemical components with predominance of hydrocarbons (Brooijmans et al., 2009). After extraction, the hydrocarbons undergo through distillation to produce several products that can also be transported overseas (Varjani, 2017). Due to their complex composition, volatility, wide distribution, bioavailability and degradability, HC are one of the most persistent organic pollutants in every environment (Fuentes et al., 2014).

Hydrocarbons can be divided into four subdivisions according to their chemical behaviour: aliphatic (including alkanes, alkenes, alkynes and cycloalkanes), aromatic (which are polycyclic aromatic compounds (PAHs) and monocyclic aromatic HC, known as BTEX (benzene, toluene, ethylbenzene and xylene), resins (such as carbazoles, pyridines and quinolones) and asphaltenes (like phenols, fatty acids and ketones) (Das & Chandran, 2011; Varjani, 2017). Aliphatic compounds are the most degradable molecules, whereas aromatics have an intermediate biodegradability and have a tendency to bioaccumulate (Head et al., 2006; Wrenn & Venosa, 1996). Resins and asphaltenes are non-hydrocarbon polar components presenting the most resistance to biological degradation (Head et al., 2006; Wrenn & Venosa, 1996).

Based on the proportion of different constituents, crude oil can be classified into light, and heavy oil. Light oil is characterized by a predominance of low molecular weight saturates and aromatic hydrocarbons, whereas heavy crude oil contains higher content of PAHs, resins and asphaltenes originated from the anoxic conditions at which it was biodegraded in the petroleum reservoirs (Dupuis & Ucán-Marín, 2015; Head et al., 2006).

Petroleum can cause permanent damage to the ecosystems by affecting species' populations and communities. Acute or chronic effects can arise from direct or indirect exposure to petroleum, such as suffocation, hypothermia, developmental abnormalities. (Varjani, 2017). Some studies also detected adverse effects in microbial flora. For instances, in intact cells and isolated mitochondria, oxygen uptake was inhibited by the presence of cyclohexane (Uribe et al., 1990) and cell growth was inhibited by the presence of phenolic and quinonic naphthalene derivatives (Sikkema et al., 1995). Due to the low biodegradability of aromatic HC, resins and asphaltenes, they tend to accumulate and persist in the environment for decades (Tyagi et al., 2011). These persistent organic pollutants are highly toxic, especially

BTEX and PAHs, due to carcinogenic, teratogenic and hemotoxic compounds (Chandra et al., 2013; Varjani, 2017).

1.3. Clean-up technologies

Crude oil's composition can be modified when in contact with the hydrosphere due to weathering processes, such as evaporation, dispersion, dissolution, spreading, sinking, emulsification, photo-oxidation (limited to sunlight exposure), resurfacing, tar ball formation, microbial decomposition, absorption by biota and sedimentation (Al-Majed et al., 2012; Canelas, 1977). Therefore, in the event of an oil spill, there is an urgent need to act fast to prevent and contain the spreading of oil to larger areas and depths.

The effectiveness of a response to mitigate an oil spill depends on the oil type, location and the spill size. However, the costs associated with the clean-up response can also influence the type of technology used (Etkin, 2000). Current remediation techniques involve mechanical removal (skimming, removal of oil and *in situ* burn) as well as, chemical and biological approaches (Mapelli et al., 2017). Based on estimations of historical oil spills, natural mitigation, incineration and use of dispersants are three of the most cost-effective options with higher reported field effectiveness (Table 1.2; Etkin, 2000).

Table 1.2 - Marine Non-US Oil Spill Clean-up cost per ton associated with reported effectiveness of clean-up technologies based on estimations from historical oil spills. Adapted from (Etkin, 2000)

Method	US\$/ton	Reported Field Effectiveness
Manual Removal	\$23 403.45	Varies
Mechanical Containment and Recovery	\$9 611.97	10-20%
Dispersants	\$5 633.78	80-90%
<i>In-situ</i> burning	\$3 127.87	90-98%
Natural Cleansing	\$1 286.00	Up to 90% (with right conditions)

1.3.1. Mechanical techniques

One of the first responses applied in an oil spill is skimming the oil layer from the surface by boats and boomers, application of natural or synthetic sorbent materials and to dislocate the oil from the contaminated site elsewhere (Mapelli et al., 2017). This implies searching for a new place to dispose the material and also involves high risks in its transportation. However, this type of method cannot be applied in open seas due to the fact that the access of boats and humans is limited, being not feasible for large oil spills.

Incineration may be another approach implemented to help control the diffusion of an oil spill. However, besides removing only the low weight HC, it is an extremely polluting technique releasing massive amounts of volatile compounds, like CO₂ and PAHs, thus contributing to the increase of atmospheric contamination (Ivshina et al., 2015; Mapelli et al., 2017). Despite the disadvantages, *in-situ* oil burning is still one of the most used methods in oil spills. For example, in the Deepwater Horizon disaster, the amount of oil burned was superior to the amount of oil collected (Kerr, 2010)

1.3.2. Chemical techniques

Combined with mechanical techniques, the use of chemical dispersants is another method recurrently applied at the surface of an oil spill. In order to increase the availability of HC compounds to be degraded by natural microbial communities, a combination of solvents and surfactants are used to disperse the oil into droplets (Mapelli et al., 2017; Nikolopoulou & Kalogerakis, 2009). For example, for the Jacob Maersk spill, in Portugal, about 160 barrels with 200L of dispersant were used in the first spill and around 2 000 barrels after the second spill (Canelas, 1977).

The applicability of the surfactants depends on the wave action, temperature and water salinity, being ideally applied before low molecular weight HC formation (Mapelli et al., 2017). Some authors agreed that this approach proved to be effective for the Deepwater Horizon spill, where dispersants were applied at a great depth, which increased the formation and dispersion of oil droplets thus increasing microbial HC biodegradation rates (Ivshina et al., 2015). However, Kleindienst et al. (2015) simulated the environmental conditions of the DWH oil spill in the laboratory and observed that due to the toxicity of chemical dispersants, it did not stimulate microbial hydrocarbon degradation rates.

Dispersants help to break the oil into smaller droplets which can increase its solubility in water and also its bioavailability by increasing the surface area of contact between microorganisms and HC (Kleindienst et al., 2015; Salerno et al., 2018). Nevertheless, high concentrations of surfactants can result in aggregation of the oil-droplets and can also inhibit the growth of microorganisms.

Biosurfactants may become a viable alternative as a green-remediation methodology since they are produced by some bacterial strains in the presence of oil (Mapelli et al., 2017). In comparison to chemical surfactants, biosurfactants can act in environments with extreme conditions of salinity, pH and temperature, have a higher rate of biodegradability and they also present higher selectivity.

1.3.3. Biological techniques – Bioremediation as a promising tool

A more effective approach to mitigate oil spills is to transform the pollutants into less toxic substances or to completely degrade them (Sánchez-Palencia González, 2011). Bioremediation, in this context, is an approach that uses the natural capability of microorganisms to detoxify/degrade petroleum compounds. This approach ensures the mineralization of the oil and avoids the risk of recontamination with secondary contaminants as the ones produced after the use of mechanical and chemical remediation techniques (Chandra et al., 2013; Cheng et al., 2017; Fuentes et al., 2014; Marinescu et al., 2017; Tyagi et al., 2011). Since bioremediation can be helpful to ensure ecological restoration and to fasten HC degradation, this methodology can be considered to be an environmental-friendly, cost-effective and efficient tool to mitigate oil polluted sites (Almeida et al., 2013; Gouveia et al., 2018; Hosokawa et al., 2009; Pontes et al., 2013).

There are two main strategies for the use of bioremediation as a spill response tool: biostimulation (BS) and bioaugmentation (BA).

Biostimulation identifies and adjusts the factors that may limit the natural biodegradation rate of the crude oil spilled by the indigenous microorganisms, such as nutrients type and concentration, like nitrogen (N) and phosphorous (P) (Nikolopoulou & Kalogerakis, 2009). Other factors like concentration, chemical composition and physical state of the HC, levels of oxygen available, temperature, salinity, pH and water conditions can also influence the HC degrading efficiency (Leahy & Colwell, 1990).

In the last years, there has been an increase in the development of biostimulants that target directly the bacteria around the oil droplets (slow release fertilizers) and are not diluted and washed out by waves (oleophilic fertilizers) (Nikolopoulou & Kalogerakis 2009; 2018). The combination of the use of the slow release fertilizer Customblen with an oleophilic fertilizer on some shores of Prince William Sound, proved to increase the biodegradation rates of HC (Nikolopoulou & Kalogerakis, 2018).

Lack of nutrients may hinder the metabolization of HC compounds, however a decrease in bioremediation efficiency may also occur due to the lack of oil degrading microbial communities. To counteract this scenario, bioaugmentation might be implemented.

Bioaugmentation is characterized by the addition of high concentrations of specialized bacterial strains (isolated or in a consortium) to the polluted site (Leahy & Colwell, 1990; Pontes et al., 2013). This method is more efficient when applied to environments that lack sufficient microbial cells and/or when the autochthonous population lacks the metabolic routes essential to degrade petroleum components (Tyagi et al., 2011). Four possible methods are

commonly applied when using BA: by adding an autochthonous or exogenous pre-adapted pure bacterial strain or consortium, by introducing genetically modified bacteria and also by adding vectors with relevant genes for HC degradation to be transferred by conjugation to the indigenous microorganisms (Tyagi et al., 2011).

An efficient biodegradation of oil spills requires the joined action of a consortium of microorganisms due to the complex metabolic interactions and networks established among them (Dombrowski et al., 2016; Head et al., 2006). Ikuma and Gunsch (2013) proved the efficacy of horizontal transfer of remediation genes to indigenous organisms in degrading toluene in soil columns. However, when applied to real-time scenarios, the instability of mobile genetic elements associated with lower adaptation and survival skills and low ability to compete with the natural microbial communities, may compromise the process of bioremediation of HC (Hosokawa et al., 2009; Joutey et al., 2013). The best obtained results for HC removal were observed when indigenous microorganisms, pre-adapted to degrade oil compounds, were used in an oil spill accident simulation (Nikolopoulou et al., 2013). Therefore, autochthonous microorganisms can be an advantageous strategy to enhance HC degradation thus avoiding the unpredictable ecological impacts that may result from the introduction of exogenous organisms (Gouveia et al., 2018; Tyagi et al., 2011).

Commercial products of species capable of degrading petroleum compounds are currently available (Tyagi et al., 2011). Nevertheless these products need to be robust enough to be implemented in diverse environments with positive outcomes, which has not been the case, for example, for the removal of petroleum in wetlands (Simon et al., 2004).

Limitations on both bioremediation approaches, biostimulation and bioaugmentation, have led to the emerging of these two techniques combined. For instance, in soil contaminated with PAH, the combination of BS with BA allowed higher PAH dissipation rates especially for anthracene and pyrene (Hamdi et al., 2007).

1.4. PAH degrading microorganisms and their potential

Every year tons of petroleum are released into the oceans from both natural and anthropogenic sources. The fact that the world is not covered in an oil layer demonstrates the efficiency and versatility of the microorganisms' capability to degrade hydrocarbons and their evolution to use oil as a source of carbon and energy (de Lorenzo, 2006). These organisms include strains of Archae, Bacteria and Fungi (Leahy & Colwell, 1990).

The complexity of hydrocarbons groups causes a progression in biodegradability, where low-molecular weight HC are degraded first and high-molecular weight HCs secondly, (Das & Chandran, 2011; Varjani, 2017). Single bacterium strains do not have the enzymatic

capability to degrade all of the compounds present in petroleum, and thus the most powerful biodegradative potential can be achieved by the performance of microbial consortia (Joutey et al., 2013).

Petroleum degrading microorganisms can be classified into obligate hydrocarbonoclastic bacteria (OHCB), that have the ability to grow on a narrow range of HCs and HCs degradation products, and generalists, that can grow on a wider set of carbon sources (Mapelli et al., 2017; Ron & Rosenberg, 2014; Yakimov et al., 2007). Most bioremediation studies usually focus on hydrocarbonoclastic bacteria, specialists, because of their prevalence, ubiquity and significant role in the mitigation of oil contaminated sites (Mapelli et al., 2017; Yakimov et al., 2007).

In oil-contaminated environments, an enrichment transition of specialized microorganisms that degrade different HC molecules occurs, starting with an enhancement of aliphatic HC degraders, like *Alcanivorax* spp., and subsequent enrichment of aromatic HC degraders, like *Cycloclasticus* spp. as observed in beaches (Rodriguez-R et al., 2015), sediments (Païssé et al., 2010) and seawater (Kimes et al., 2014). For example, in the DWH accident, a succession of taxa occurred in the water column by first appearing *Oceanospirillales* and pseudomonads, followed by *Colwellia*, *Cycloclasticus*, organisms methylotrophs and then *Flavobacteria*, *Rhodobacteraceae* and *Alteromonadaceae* (Kimes et al., 2014; Redmond & Valentine, 2011). This ecological transition as a natural response to the oil contamination can be compared with HC composition and quantity present in the oil plume (Dubinsky et al., 2013; Hazen et al., 2010).

Altogether, 79 genera of microorganisms capable of degrading hydrocarbons were isolated from marine environments. *Acinetobacter*, *Alcanivorax*, *Cycloclasticus*, *Oleiphilus*, *Oleispira*, *Pseudomonas* and *Thalassolituus*, all belonging to the *Proteobacteria* phylum, and *Rhodococcus*, from the *Actinobacteria* phylum, are some of the best described bacterial genus capable of degrading HC (Brooijmans et al., 2009; Head et al., 2006; Ron & Rosenberg, 2014). Only two strains of the phyla *Firmicutes* and *Bacteroidetes*, that are dependent on NaCl, have been isolated. These are the alkane degrader *Planomicrobium alkanoclasticum* MAE2 and the polyhydroxyalkanoate (PAH) degrader *Yeosuana aromativorans* GW1-1, respectively (Yakimov et al., 2007). In the Arctic sea, four genera in oil-degrading communities were identified and isolated: *Marinobacter* spp., *Psychrobacter*, *Pseudoalteromonas* and *Shewanella* (Yakimov et al., 2007).

Alcanivorax spp. is a genus well-known for its capacity to feed only on hydrocarbons. In the event of an oil spill its population can grow up to 80% of the whole bacterial community. An adaptation acquired by these microorganisms is the capacity to produce exopolysaccharides that act as a biosurfactant (Brooijmans et al., 2009). This genus is an

early colonizer in oil spill events, decreasing its population after the decrease of saturated hydrocarbons in the oil-contaminated site (Head et al., 2006).

Acinetobacter spp. were found to be capable of using as sole source of carbon, *n*-alkanes of chain length C10-C40 (Das & Chandran, 2011). This genus also has the ability to enhance their attachment to the oil droplet by synthesizing emulsifiers (Rosenberg et al., 1982).

Rhodococcus erythropolis 20S-E1-c has an external hydrophobic layer that facilitates the attachment to the oil droplets, however, they possess different mechanical and dynamical properties to attach to oil droplets (Abbasnezhad et al., 2011)

Cycloclasticus spp. have been described to be capable to aerobically degrade aromatic HC, playing an important role in *in situ* biodegradation of ethane and propane (Kimes et al., 2014). Studies also suggest that this genus succeeds *Oceanospirillales* spp. due to its intrinsic capacity to degrade gaseous and aromatic petroleum HC compounds (Kimes et al., 2014).

Marinobacter spp. are marine hydrocarbon degraders metabolically more versatile than *Alcanivorax*, possessing 1500 additional genes that *Alcanivorax* spp. lacks in their ability to denitrify HCs, for example (Yakimov et al., 2007).

Table 1.3 – Some examples of genera known to have the capacity to degrade hydrocarbons.

Genus	Reference	Genus	Reference
<i>Acinetobacter</i>	(Kubota et al., 2008)	<i>Pseudomonas</i>	(Whyte et al., 1997)
<i>Alcanivorax</i>	(Yakimov et al., 1998)	<i>Pseudoalteromonas</i>	(Hedlund & Staley, 2006)
<i>Colwellia</i>	(Redmond & Valentine, 2011)	<i>Psychrobacter</i>	(Deppe et al., 2005)
<i>Cycloclasticus</i>	(Dyksterhouse et al., 1995)	<i>Rhodococcus</i>	(Finnerty, 1992)
<i>Marinobacter</i>	(Gauthier et al., 1992)	<i>Shewanella</i>	(Gerdes et al., 2005)
<i>Oleiphilus</i>	(Golyshin et al., 2002)	<i>Thalassolituus</i>	(Yakimov et al., 2004)
<i>Oleispira</i>	(Yakimov et al., 2003)	<i>Yeosuana</i>	(Kwon et al., 2006)
<i>Planomicrobium</i>	(Engelhardt et al., 2001)		

Metagenomic studies helped to detect the presence of catabolic genes involved in the metabolism pathways for petroleum hydrocarbons biodegradability. Alkane degradation genes were encountered in several genera, such as *Pseudomonas*, *Actinobacteria*, *Burkholderia*, *Acinetobacter*, *Alcanivorax*, *Oleiphilus*, *Mycobacterium*, *Rhodococcus*,

Nocardia and *Prauserella* for aerobic degradation and in *Desulfococcus*, and *Desulfatibacillum* for anaerobia degradation (Fuentes et al., 2014; Rojo, 2009).

Aromatic HCs degradation involves complex communities due to the multiple biodegradation metabolic routes necessary. The degradation of benzene, toluene, ethylbenzene and xylene can occur in *Pseudomonas*, *Rhodococcus* and *Ralstonia*. The naphthalene compound can be metabolized by *Pseudomonas*; phenanthrene can be degraded by *Pseudomonas* spp. and *Haemophilus* spp. and pyrene can be broken by *Haemophilus* and *Mycobacterium*. The highly carcinogenic benzo[a]pyrene can be metabolized by *Rhodococcus* and *Mycobacterium* (Tyagi et al., 2011). Despite that, *Arthrobacter*, *Burkholderia*, *Mycobacterium*, *Pseudomonas*, *Sphingomonas*, and *Rhodococcus* were found to be involved in the degradation of alkylaromatic compounds (Das & Chandran, 2011). In locations with low oxygen levels, the abundance of *Flavobacteria*, *Rhodobacteraceae*, and *Alteromonadaceae* increased when levels of soluble hydrocarbons, e.g. BTEX, increased (Kimes et al., 2014).

1.5. How does a prokaryotic organism mitigate oil?

In an environment contaminated by crude oil, the structure of microbial communities changes by increasing the amount of hydrocarbon-oxidizing microorganisms due to adaptation processes (Leahy & Colwell, 1990). Selective pressure, activation or depression of specific enzymes, horizontal gene transfer and mutations are some of the adaptations suffered by aquatic microorganisms (Leahy & Colwell, 1990). So, after an oil spill, the selected biodiversity is capable of using carbon from petroleum to obtain energy or to assimilate it into cell biomass (Varjani, 2017).

Bioremediation approaches depend on the biodegradation capacity of the microorganisms to remove or detoxify the pollutant resorting to the activity of enzymes (Tyagi et al., 2011). An effective bioremediation occurs when microorganisms can biodegrade contaminants to levels below the limits established by competent authorities or to harmless states to human health and to the environment (Joutey et al., 2013).

Hydrocarbon biodegradation generally goes from lower molecular weight HCs (linear alkanes) to higher ones (like cyclic alkanes or aromatic HC) starting from *n*-alkanes to branched-chain alkanes, branched alkenes, low-molecular-weight *n*-alkyl aromatics, monoaromatics, cyclic alkanes, PAHs and then asphaltenes (Tyagi et al., 2011). Therefore, the susceptibility to biodegrade an HC compound depends on its structure (Das & Chandran, 2011). Numerous prokaryotes are capable of degrading *n*-alkanes, however, as the

complexity of the HC molecular structure increases, the number of organisms capable of degrading them decreases (Varjani, 2017).

Since different organisms can degrade different petroleum compounds, a joined action of a consortium of microorganisms will be able to achieve a better bioremediation rate by degrading a larger set of HCs through the combination of the genetic machinery of more than one microorganism (Adam, 2016; Sathishkumar et al., 2008).

One limitation to HCs' biodegradation is its bioavailability due to the hydrophobic characteristics of oil. To counteract this, some bacterial strains, like *Alcanivorax* spp., can modify the cell's membrane hydrophobicity and produce and secrete extracellular enzymes, like phospholipids, lipopolysaccharides and glycolipids, that act as biosurfactants, therefore increasing the oil-surface area (Barbato et al., 2016; Nikolopoulou & Kalogerakis, 2009). Reducing the water-oil tension allows the dispersion of oil into small droplets thus increasing HC availability for organisms to degrade (Brooijmans et al., 2009; Das & Chandran, 2011).

In addition, bioremediation of HCs depends on environmental conditions like oxygen, moisture, pH, temperature and salinity (Das & Chandran, 2011; Varjani, 2017). Another major limiting factor is the amount of nutrients, especially N and P, which must be available in the environment in proportion to the carbon content available in the spilled oil. The ideal proportion of carbon, nitrogen and phosphorous used in assays of bioremediation follows the ratio of 100:10:1 (C/N/P) for optimal biodegradation rates (Almeida et al., 2013; Nikolopoulou & Kalogerakis, 2009).

Bioremediation occurs as soon as the oil is spilled and enters in contact with both air and sea surface (Ron & Rosenberg, 2014). First, low molecular weight HCs volatilize and soluble HC are metabolized by microorganisms followed by the degradation of the remaining HC by high-cell-surface hydrophobicity microorganisms (Ron & Rosenberg, 2014).

Hydrocarbons can be transformed by undergoing a series of reactions performed by the metabolic machinery present in microorganisms (Gouveia et al., 2018; Sánchez-Palencia González, 2011). There are two metabolic pathways for hydrocarbon degradation: chemotrophic aerobic and chemotrophic anaerobic (Varjani, 2017).

The aerobic pathway is considered to be the most efficient since it requires less energy to occur, facilitates the action of oxygenases and produces more energy per reaction (Fuentes et al., 2014). For alkane degradation, the substrate is activated by monooxygenases being converted to alcohol, and through the action of alcohol and aldehyde dehydrogenases, aldehydes and fatty acids are produced (Das & Chandran, 2011; Sabirova et al., 2006). Regarding cyclohexane degradation, the enzymatic steps are: alkane monooxygenases, then cyclohexanol dehydrogenase followed by cyclohexane monooxygenase and the resulting product could be finally degraded through β -oxidation (Sabirova et al., 2006). In the aromatics HC biodegradation, the molecule is activated by oxygenases, where a benzene can be

cleaved in two ways forming protocatechuates or catechols which are further converted in the tricarboxylic acid cycle (Varjani, 2017). The final result of the complete degradation of HC would be non-toxic and non-carcinogenic products like carbon dioxide, water and cell biomass (Das & Chandran, 2011; Varjani, 2017).

Regarding the anaerobic metabolism, although not much information is available, it is thought to be an important biodegrading pathway for aromatic HCs in the deeper water column and sediments (Leahy & Colwell, 1990; Mapelli et al., 2017). For example, the oxidation of BTEX compounds results in phenols or organic acids that are later transformed into long-chain volatile fatty acids resulting in methane and carbon dioxide as a final product. Alkane degradation can be activated by the addition of fumarate and catalyzed by alkyl-succinate synthases and the degradation of toluene, ethylbenzene and alkyl benzene involved oxidation into benzoyl-CoA (Mapelli et al., 2017; Varjani, 2017).

More recently, metagenomics studies conducted using high-throughput sequencing are providing important understanding on how and what are the connections between the different metabolic networks of PAHs degradation by microbial consortia. Looking at a deeper level, this breakthrough made possible to study the genomic capacity of different strains by looking at genes encoding for enzymes involved in the biodegradation of hydrocarbons has the ones described by Brooijmans et al. (2009); Fuentes et al. (2014); Mapelli et al. (2017); Ribeiro et al. (2018) and Sabirova et al. (2006).

1.6. Previous Studies

1.6.1. *In situ* bioremediation

In the Exxon Valdez oil spill disaster of 1989, in both Prince William Sound and Gulf of Alaska oiled shorelines, the use of biostimulation was proved for the first time to be a potential and efficient bioremediation approach (Ivshina et al., 2015). A total of 5 200 kg of phosphorus and 48 400 kg of nitrogen were applied in separated applications, from 1989-1991, using three types of fertilizer: water-soluble, solid and slow-release and oleophilic liquid fertilizer (Bragg et al., 1994). Overall, 28% of residual superficial oil mass and 12% of sub-surface oil mass were removed per year. From that point on, bioremediation approaches gained an increased interest as a potential suitable clean-up method to mitigate oil spills.

In Antarctica, a sea trial was performed using the oleophilic fertilizer Inipol EAP 22 to treat Arabian light crude oil. Despite few data provided, results appear to indicate that biodegradation of oil was stimulated (Swannell et al., 1996).

Biaugmentation approaches have also been tested in the environment, in an abandoned salt works filled with coastal seawater, in Embiez Island, France. While a

disappearance of the added microorganisms from 10 m³ basin enclosures containing oiled seawater were observed, the indigenous communities showed an adaptation to hydrocarbon degradation under identical conditions (Tagger et al., 1983).

Biotechnology combining autochthonous bioaugmentation with nutrients have also proved to be effective at a small-scale. For example, two types of cultures of *Flavobacterium* spp. and *Pseudomonas* spp., isolated from Alaska estuaries water, were applied in an open flowthrough system treated with oleophilic nutrients and flushed continuously with seawater. One lyophilized inoculum was uncoated and the other was coated with octadecane to render them oleophilic. Results indicated that uncoated inoculum was washed from the experimental system and did not enhance HC degradation whereas the addition of the oleophilic strains improved biodegradation rates (Horowitz & Atlas, 1978).

Atlas and Busdosh (1976) observed that 65% of Prudhoe Bay crude oil was removed, from floating Plexiglas cylinders containing oleophilic fertilizers and an oil-degrading strain of *Pseudomonas* sp., in the saline pond at Barrow, Alaska. In this experiment, the addition of the fertilizers alone accounted for 51% of mass loss of HC and the addition of microorganisms alone only caused 27% loss.

More recently, a study conducted at two sites affected by the Prestige oil spill demonstrated that the indigenous microbial communities had the ability to degrade petroleum hydrocarbons after being enhanced by bioremediation techniques. Gallego et al. (2006) conducted a two-year study, to monitor the natural attenuation of the residues in shore rocks and evaluate the effectiveness of bioremediation with the oleophilic fertilizer S200. Hydrocarbon depletion by natural attenuation was only effective in the first year after the spill, with total alkane degradation occurring in the first months. However, the addition of the fertilizer S200 increased the growth of hydrocarbon degrading populations which resulted in an enhancement of hydrocarbon degradation.

1.6.2. Laboratory experiments

Before the application of any standardized bioremediation technique in field trials, to remediate petroleum components in the environment, laboratory experiments must be accomplished. In this way, it will be possible to avoid increasing the hazardous consequences of oil spills and to gain knowledge about the efficiency of bioremediation approaches when applied in different environments.

After the Prestige oil spill disaster, Medina-Bellver et al. (2005) estimated the activity of natural microorganisms on oil components by the carbon isotopic ratio of the dissolved inorganic carbon in seawater samples, collected in December 2002 and February 2003,

revealing their ability to biodegrade the crude oil into inorganic carbon *in-situ*. Afterwards, in the laboratory, the presence of PAHs-degrading bacteria was confirmed by the Most Probable Number method, for samples collected at Vigo and Ferrol, which showed the highest number of aromatic degrading microorganisms for naphthalene and phenanthrene. In addition, microcosms enrichment experiments were performed to determine the possible limiting factors of the bioremediation processes at the Prestige spill areas, showing that N/P deficiency in seawater can be one of the main limiting factors for crude oil degradation (Medina-Bellver et al., 2005).

Three days after the Texas City “Y” oil Spill, on March 2014, biostimulation of indigenous microbial communities was also tested in samples collected from the Galveston Bay. Bioassays were assembled in bottles, with N and P addition, and incubated for seven days at a floating corral at Galveston boat basin. From this experiment resulted an increase in heterotrophic microbial abundance regardless of nutrient concentration and autotrophic microorganisms maintained a significantly higher abundance after nutrient addition which altogether resulted in a decrease in PAHs (Williams et al., 2017).

In an oil-contaminated cold environment, Álvarez et al. (2017) demonstrated that the use of BS approaches enhanced the removal of HC, by adding N and P on-site in a hydrocarbon contaminated soil in Antarctica.

For a pristine environment, Nikolopoulou et al. (2013) evaluated the bioremediation efficiency of autochthonous populations through the use of biostimulation-only approaches versus autochthonous bioaugmentation with biostimulation strategies. The simulation of oil spills was performed in bioreactor assays with seawater collected in the East Mediterranean Sea, spiked with crude oil and incubated for 90-days. Three BS treatments were applied: no nutrient addition, addition of KNO_3 and K_2PO_4 and addition of uric acid, lecithin and a biosurfactant and three autochthonous bioaugmentation treatments were also tested: addition of KNO_3 and K_2PO_4 + pre-adapted indigenous cultures, addition of KNO_3 and K_2PO_4 + biosurfactant + pre-adapted indigenous cultures and also addition of uric acid and lecithin + biosurfactant + pre-adapted indigenous cultures. The most effective treatments to degrade *n*-alkanes and low-molecular-weight PAHs were the autochthonous BA complemented with BS and BS of indigenous population with uric acid, lecithin and biosurfactant (Nikolopoulou et al., 2013).

More recently, with the development of metagenomics, a study conducted for cold Norwegian seawaters in microcosms experiments, at 5°C, with seawater and chemically dispersed oil, linked the efficiency of oil degradation with succession patterns and metagenome dynamics by the microbial community (Ribicic et al., 2018). Results showed clear successional patterns in both microbial communities and metagenome composition of genes coding for HC degrading enzymes, thus emphasising that a successful substrate

transformation can only be achieved by cooperation among hydrocarbon biodegrading organisms (Ribicic et al., 2018).

Some oil bioremediation studies using autochthonous microorganisms were also conducted for coastal and estuarine sediments. For instance, it was observed that microbial communities present in sediments from coastal sandy beaches, along the NW coast of Portugal and from Douro and Minho estuaries, have the potential to degrade hydrocarbons (Gouveia et al., 2018). For a beach affected by an old oil spill, Reis et al. (2014) observed that 70% of total PAHs and 85% of total aliphatic HCs were degraded meaning that the autochthonous consortia could still react to an oil spill. Almeida et al. (2013) tested three bioremediation approaches for an unimpacted beach sediment: BS, autochthonous bioaugmentation (AB) and BS with AB. The results showed that BS with AB treatment was the best approach to apply in recently impacted beaches. Simulation of oil buried in a sand column, collected from a beach affected by an oil spill 7 years before, with different nitrogen supplements showed that a change in microbial community occurred and that the indigenous bacteria were able to respond to the contaminant (Pontes et al., 2013).

1.7. Methodology used to study PAHs degrading microorganisms

Traditional methodology for assessing microbial community rely on cultivation-dependent approaches of hydrocarbon-degrading bacteria and heterotrophic bacteria. However, only less than 1% of microbial diversity is recovered from environmental samples thus underestimating the vastly biodiversity present worldwide (Fuentes et al., 2014; Hugenholtz, 2002). Moreover, this constraint prevented scientists to reproduce natural environments in synthetic/artificial media and to fully understand the real highly complex and interactive individuals of multi-assemblages (Joint et al., 2010).

In the last second half of the last century, culture-independent approaches based on molecular techniques allowed a new era of knowledge about new phylogenetic groups and metabolic routes (Fuentes et al., 2014). Assigning single-species assemblages of a certain community to a unique taxon was made possible through the use of the 16S rRNA (ribosomal RNA) gene, therefore allowing a better insight into the structure, composition and dynamics of natural microbial populations (Apprill et al., 2015; Pace, 1997). The 16S rRNA gene marker is widely used for profiling bacterial communities due to its hypervariable regions that allow the identification of phylogenetic characteristics of prokaryotes, separated by nine highly conserved regions used for primer design (Yang et al., 2016).

With the development of low-cost and high-throughput sequencing, provided by next-generation sequencing (NGS) technologies, it was possible to observe the rise of metagenomics to aid the study of functional characteristics of the microbial communities (Mapelli et al., 2017). The principles of metagenomics are to study the collective genomes recovered from environmental samples in comparison to genomes isolated from clonal cultures (Handelsman, 2004; Head et al., 2006). NGS technologies rely on massive sequencing and among the most affordable and widely used NGS platforms are the Ion Torrent (Thermo Fisher) and Illumina. The Illumina MiSeq platform can process quickly and efficiently a great amount of environmental samples, producing 1.5 Gb per day from 5 million 150 base paired-end reads (Caporaso et al., 2012). To overcome the small read length, the V3 chemistry used with the Illumina MiSeq instrument, allowed the paired-end sequencing read lengths of up to 2 x 300bp. Altogether, the Illumina sequencing platforms became the preferable approach for large-scale projects like Ocean Sampling Day (OSD) (Kopf et al., 2015) and Earth Microbiome Project (Gilbert et al., 2014).

The Illumina MiSeq technology is filled with fluorescently labelled nucleotides, with four different colours corresponding to each base, a terminator prevents the addition of several bases at the same time by DNA polymerase. Through several PCR cycles, in each location, a new base is added, emitting a fluorescent signal with the corresponding colour of the nucleic acid. After the removal of the fluorescent signal and the terminator, to prevent the contamination of the next image, a new cycle begins with the addition of the next base. The assembly of the sequences are performed by computers through analysis of each image.

Before annotating against a non-redundant reference database, the large amounts of raw sequence data generated by high-throughput DNA sequencing need to be processed.

For a first observation of the data analysed, the user-friendly automated pipeline web server SILVAngs (Quast et al., 2012) (<https://www.arb-silva.de/ngs/>) can be used. However, in this platform, chimeric sequences are not identified and removed, which in SSU rRNA (small subunit) libraries can represent about 10% of the sequences (Schloss et al., 2009). This can lead to a misrepresentation analysis of our data because those artificial sequences are considered to be novel taxa (=OTU - Operational Taxonomic Unit) leading to an overestimation in sample richness. A more accurate data analysis would be one where the user would be able to modify any steps and/or parameters.

Several bioinformatics pipelines have been developed to perform tasks previously mentioned, such as mothur (Schloss et al., 2009) and Quantitative Insights Into Microbial Ecology (QIIME) (Caporaso et al., 2010). Despite both being limited to command-line and programming skills, both programs provide documentation and tutorials comprehensive enough for non-programmer's usage. However, QIIME proved to be quicker to analyse large datasets, the most user-friendly with easier understanding of which commands to use and it

also generates output graphs and tables for better data visualization and analysis (Plummer et al., 2015).

To discriminate a set of sequences from individuals of the same taxonomic levels the method select was an OTU. These are clusters of reads differentiated by a fixed sequence dissimilarity threshold being 3% the most commonly used (Callahan et al., 2017). The two most significant definitions of OTU are based on the closed-reference and on *de novo* methods (Callahan et al., 2017). In this work, the closed-referenced OTU method was used, where reads were recruited into a corresponding OTU if they were sufficiently similar to a sequence in a referenced database (Callahan et al., 2017). Currently, a possible replacement for OTUs in being developed which is the Amplicon Sequence Variants (ASV) (Callahan et al., 2017; Thompson et al., 2017). The difference between them is that ASV distinguishes variants of sequences by a difference of one nucleotide, therefore being reproducible in future data sets, reusable across studies and not limited by incomplete reference datasets (Callahan et al., 2017; Thompson et al., 2017). Despite these advantages, ASVs' analyses takes too much time and computer memory to be performed and some pipelines, like PICRUST (Phylogenetic Investigation of Communities by Reconstruction of Unobserved States) (Langille et al., 2013), are not adapted to this atomic unit of analysis.

High curated, non-redundant, chimera-free reference databases for 16S rRNA gene sequences are an important part of metagenomics' analysis. Among them are: Greengenes (DeSantis et al., 2006), SILVA (Quast et al., 2012) and Ribosomal Database Project (RDP-II) (Cole et al., 2013). SILVA is the largest non-redundant reference database in comparison with the other two mentioned above, is constantly updated whereas, for example, Greengenes has not been updated since May 2013, and a subset of sequences from the EMBL-EBI/ENA, Greengenes and RDP-II databases are also included in the SILVA database (Balvočiūtė & Huson, 2017; Yilmaz et al., 2013).

1.8. The SpilLess Project

This work was developed within the framework of the project SpilLess – First line response to oil spills based on native microorganisms cooperation (<https://spilless.ciimar.up.pt/>). This project proposes to create a novel environmental-friendly approach to mitigate oil spills through bioremediation, by using native cooperative consortia with the potential to degrade petroleum compounds. The indigenous consortia will help to empower the bioremediation efficiency by being adapted do the contaminated environment thus increasing microorganisms' survival rates (Joutey et al., 2013).

Since the NW coast of the Iberian Peninsula is located in a hotspot of oil spill disasters, the European Atlantic area, this project was performed at a pilot-scale for this region (from Porto, Portugal, to Ferrol, Spain).

SpilLess aims to i) develop a georeferenced library of native microbial consortia for bioremediation of petroleum HC, ii) scale-up production of indigenous microbial consortia capable of degrading petroleum compounds, iii) creation of a cocktail of additives to stimulate the consortia's activity and iv) adaptation of unmanned autonomous vehicles for *in situ* release of the autochthonous microorganisms.

By combining biotechnology with robotics, SpilLess pretends to create a fast, efficient and low-cost way to remediate the hazardous impacts of an oil spill (Figure 1.2).

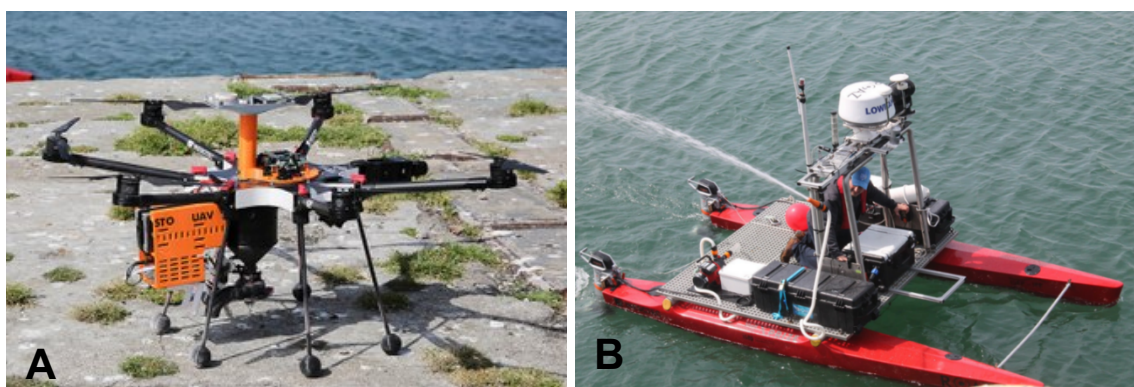


Figure 1.2 - Autonomous vehicles for in situ combat of oil spills. A - prototype of an unmanned aerial vehicle (UAVs); B - prototype of an autonomous surface vehicle (ASVs)

1.9. Aims of this Master Thesis

In this Master thesis project two questions were posed: i) are natural degrading petroleum microorganisms diversely distributed along the NW coast of the IP and ii) is it possible to successfully select them to create a native enrichment consortium with high ability to degrade petroleum compounds, for different marine georeferenced regions? Specifically, this work pursues the following four objectives outlined in the previous two hypotheses: (1) produce a georeferenced prokaryotic consortia library with the capacity to degrade petroleum compounds; (2) develop an optimal workflow to select native microbial consortia with the potential to degrade oil; (3) characterize at a taxonomic level the microbial consortia; (4) predict the functional profile for the potential oil biodegraders.

To accomplish these aims, a total of 29 samples were collected along the NW coast of the Iberian Peninsula, and for each sample was set up a microcosm enrichment experiment with petroleum as carbon source and with nutrients addition. Selected microbial consortia were identified and characterized at the taxonomic and functional level through NGS, analysed

by means of the bioinformatics pipeline of QIIME and the predictive functional profiling determined by using PICRUST.

2. Materials and methods

2.1. Study Area

The NW coast of the Iberian Peninsula has more than 53 registered oil spills incidents, most of them being caused by storms and bad weather, in autumn and winter seasons (Medina-Bellver et al., 2005; Vieites et al., 2004). In addition, there is an oil refinery in Porto, Portugal, belonging to Petrogal and another in Coruña, Spain, from REPSOL that have a constant demand of crude-oil thus increasing navigation of oil tankers in these regions (online available data: <https://www.industryabout.com/iberian-peninsula-industrial-map>). These two factors together with the intense traffic of cargo ships and oil tankers near this geographical region, makes the NW coast of the IP, a suitable area for a pilot-scale investigation of a new bioremediation technique that can be implemented in the future.

The SpilLess project aims to sample 40 sites, from both coastal and offshore locations, from three delimited areas in the NW coast of the Iberian Peninsula. Area 1 is located in the NW coast of Portugal, between the rivers Douro and Minho and Areas 2 and 3 are located in the NW coast of Spain, between Minho river and Finisterra Cape and between Finisterra Cape and Estaca de Bares cape, respectively.

For this work, a total of 22 sites within the three delimited areas and 1 from outside the areas, in Portugal, were sampled and analysed (Figure 2.1). Samples were collected from 5 beaches in Area 1 (Figure 2.1 A), 8 beaches and 5 offshore sites from Area 2 (Figure 2.1 B) and 4 beaches in Area 3 (Figure 2.1 C; Table 2.1). The site outside the limited areas was Ponta do Trovão beach, in Peniche. This sampling station was added because, in June 2017, an oil conduct of the company Plastimar ruptured and spilled four tons of oil into this area.

For each delimited Area, a seasonal sampling point was also selected to increase the temporal resolution of the study in locations near cargo ships routes: Ingleses beach in Area 1, Toralla beach in Area 2 and Caranza beach in Area 3.

The seawater sampled at each site contained the natural bacteria and was the raw material to select the native competent cooperative community with the potential to degrade petroleum compounds.

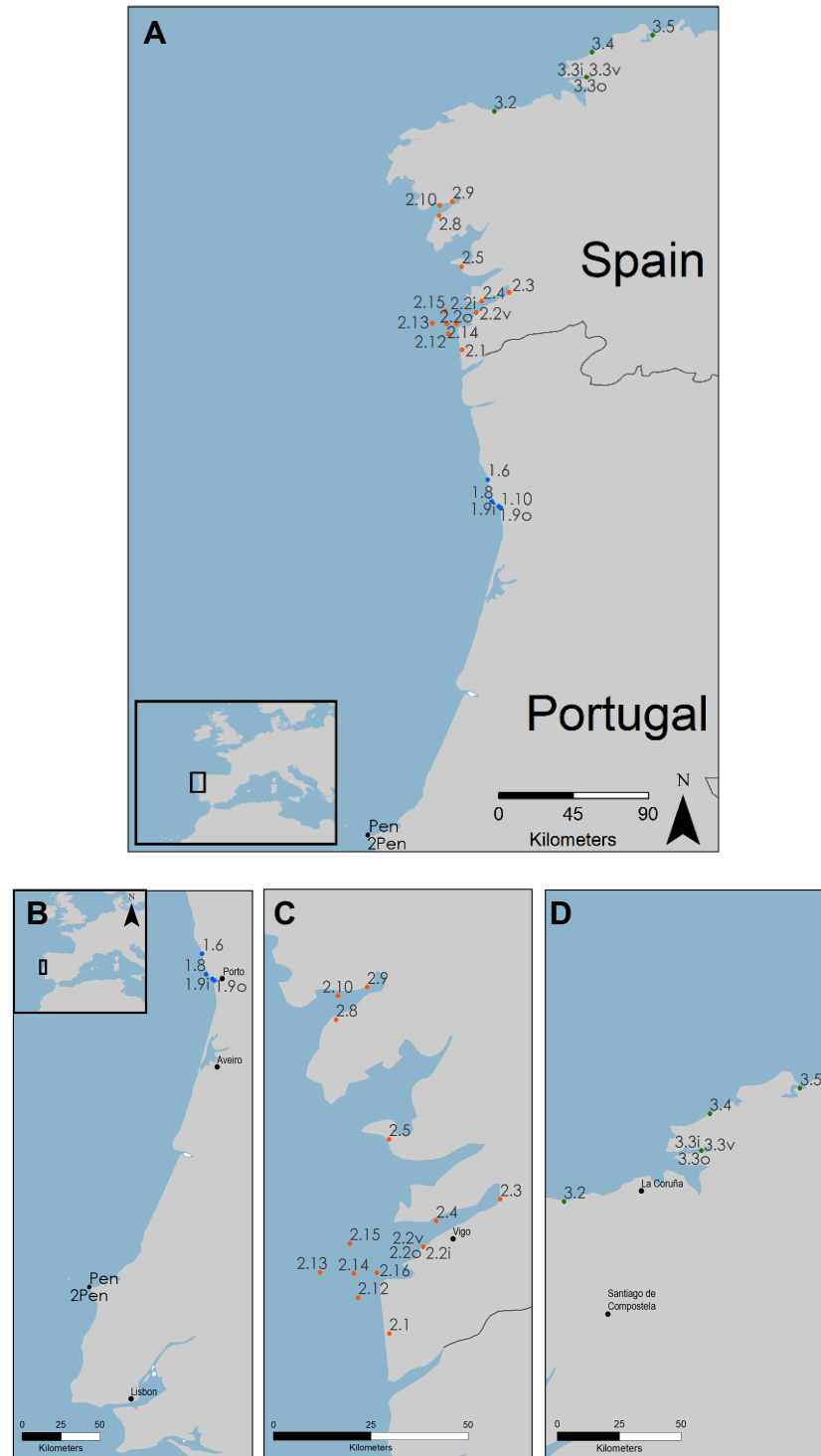


Figure 2.1 – Sites' locations along the NW coast of the Iberian Peninsula. A - Blue dots represent the sites sampled in Area 1; red dots represent the sites sampled in Area 2; green dots indicate the sites sampled in Area 3 and black dot is Ponta do Trovão, Peniche; B- Magnification of the sites of Area 1; C - Magnification of the sites of Area 2; D- Magnification of the sites of Area 3.

Table 2.1 - Characterization and GPS coordinates of the sites sampled for this study, along the NW coast of the Iberian Peninsula.

Area	Code	Sampling Site	Collection Date	Latitude	Longitude
N/A	Pen	Ponta do Trovão	11/07/17	39°22'13.2"N	9°23'19.4"W
	2Pen		23/08/17	39°22'13.2"N	9°23'19.4"W
1	1.6	Vila Chã	30/01/18	41°17'44.736"N	8°44'13.74"W
	1.7	Cabo do Mundo	30/01/18	41°13'10.56"N	8°42'54.23W
	1.8	Porto de Leixões	20/09/17	41°10'38.20"N	8°42'7.45"W
	1.9o	Ingleses (Autumn)	20/09/17	41°9'1.42"N	8°40'37.05"W
	1.9i	Ingleses (Winter)	30/01/18	41°9'1.42"N	8°40'37.05 W
	1.10	Cabedelo	20/09/17	41°8'30.00"N	8°39'58.38 W
2	2.1	Oia	15/02/18	42°0'3.24"N	8°52'37.931"W
	2.2v	Toralla (Summer)	06/07/17	42°12'3.6"N	8°47'57.48"W
	2.2o	Toralla (Autumn)	18/10/17	42°12'3.6"N	8°47'57.48"W
	2.2i	Toralla (Winter)	15/02/18	42°12'3.6"N	8°47'57.48"W
	2.3	Cesantes	18/10/17	42°18'39.24"N	8°37'14.16"W
	2.4	Rodeira	18/10/17	42°15'40.68"N	8°46'9.48"W
	2.5	A Lanzada	15/02/18	42°26'59.64"N	8°52'40.8"W
	2.8	Do Cruceiro	07/03/18	42°43'35.76"N	9°0'2.16"W
	2.9	Broña	07/03/18	42°48'5.76"N	8°55'44.04"W
	2.10	Reimunde	07/03/18	42°46'53.76"N	8°59'48.12"W
2	2.12	Offshore 2	23/11/17	42°5'2.605"N	8°57'0.58"W
	2.13	Offshore 1	23/11/17	42°8'32.435"N	9°2'16.289"W
	2.14	Ofshore 3	27/11/17	42°8'22.2"N	8°57'34.798"W
	2.15	Offshore 4	27/11/17	42°12'32.4"N	8°58'8.4"W
	2.16	Offshore 5	27/11/17	42°8'28.201"N	8°54'24.602"W
3	3.2	Razo	13/03/18	43°17'29.04"N	8°42'6.84"W
	3.3v	Caranza (Summer)	07/07/17	43°28'38.28"N	8°12'9"W
	3.3o	Caranza (Autumn)	09/11/17	43°28'38.28"N	8°12'9"W
	3.3i	Caranza (Winter)	13/03/18	43°28'38.28"N	8°12'9"W
	3.4	A Frouxeira	09/11/17	43°36'38.16"N	8°10'17.04"W
	3.5	Morouzos	09/11/17	43°42'10.976"N	7°50'35.336"W

2.2. Sampling Procedures

For each location, 5L of marine water were collected at the surface, at low tide, with a sterile container. The surface water was also characterized in terms of pH, salinity, temperature, conductivity and oxygen levels by using a Multi-Parameter Water Quality Sonde. The sample was then transported to the laboratory in a refrigerated ice chest. At the laboratory, the water sample was filtered through Sterivex™ filters and processed in triplicate for microcosms assembly and determination of hydrocarbon degraders abundance.

All glass and plastic materials were chemically decontaminated with 10% (v/v) HCL solution and, afterwards, washed with deionized water (conductivity $< 0.2 \mu\text{S cm}^{-1}$). The materials for microbial mass were later sterilized by autoclaving at 121°C for 20 minutes and plastic materials were sterilized by UV light for 30 minutes.

2.3. Assembly of Microcosms

The enrichment experiments were performed in order to select the autochthonous microbial consortia capable of degrading hydrocarbon compounds. The optimization of the processes regarding microcosms' assembly was based on previous experiments that proved to be effective for microbial degradation of hydrocarbons as the ones described by Almeida et al. (2013) and Nikolopoulou et al. (2013).

After the seawater collection, the microcosms were assembled in 500 mL sterilized glass flasks, in triplicate, for each site. Each flask was prepared with 150 mL of the collected sea water. Afterwards, the nutrients nitrogen and phosphorous were supplied in the form of 1.5mL of potassium di-hydrogen phosphate (KH_2PO_4) (0.8M) and 4mL of potassium nitrate (KNO_3) (1.5M), respectively. The only carbon source provided was light crude oil, supplied by an oil refinery. This was added to the flasks in a proportion of 20:0.5 (v/v), after passing through a $0.2\mu\text{m}$ sterile filter to remove any microorganisms that could contaminate our samples. The carbon from the petroleum together with the nutrients supplied formed a ratio of C/N/P of 100:10:1. In Figure 2.2 it is possible to observe a representation of the assembly for one 500mL Shott glass and the final replicates done for one sampling site.

For the preparation of the sterile solutions of nutrients, first KH_2PO_4 was dried overnight at 110°C and KNO_3 was left to dry for several hours, at 60°C , to remove water. The prepared solutions were filtered through a $0.2\mu\text{m}$ sterile filter.

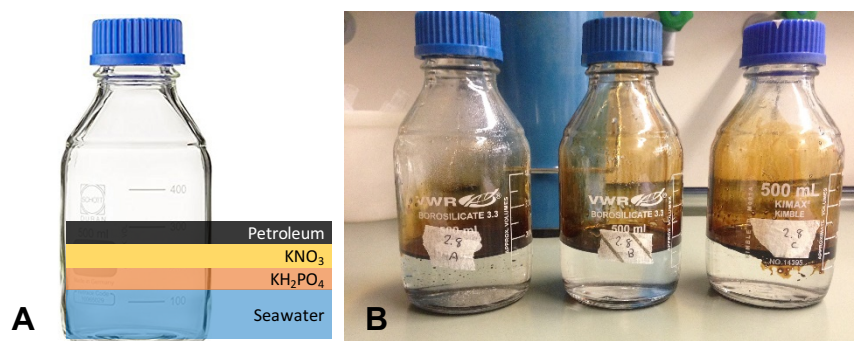


Figure 2.2 - Assembly of microcosms experiments. (A) schematic representation; (B) final assembly of a microcosms experiment for one sampling site.

The microcosms were incubated for two weeks under constant mechanical orbital agitation (100 rpm), at room temperature, in aerobic and aphotic conditions to avoid photo oxidation of the petroleum compounds (Figure 2.3).



Figure 2.3 - Incubation conditions of the microcosms experiment during two weeks.

After 7 and 15 days of incubation, 1 mL of medium from each flask was collected with sterile 1 mL syringes to determine the abundance of hydrocarbon degraders (see description of the method below). After two weeks, all three replicates were combined for further procedures.

2.4. Microbial community collection

At the beginning of the experiment (W0), the seawater collected from each sampling site was filtered, in duplicate, through Sterivex™ filters with a pore size of 0.22 µm, hydrophilic, PVDF, Durapore membrane (SVG1010RS, Merck Milipore, Portugal) with the help of 50mL sterile syringes, a PowerVac™ Manifold (Qiagen) and a vacuum pump (Figure 2.4 A), following the methodologies used in the Ocean Sampling Day event (Kopf et al., 2015). The volume filtered for each replicate was approximately 2L of seawater or the maximum volume possible in a two-hour window. Then, the remaining liquid in the filters was removed by using a syringe and the Sterivex™ was capped with the Inlet and Outlet caps, placed in sterile bags properly identified and stored in a -80°C freezer.

After two weeks of incubation (W2), the composite sample of the microcosms was also filtered with the same process described above for W0 (Figure 2.4 B). Due to the thick consistency of crude oil, the maximum volume possible was filtered (ranging from 7.0 mL to 181.5 mL), in a two-hour time frame or until the Sterivex™ units clogged.

In Table 2.2, it is possible to observe the volumes and filtration time for W0 and W2, for the replicates used in this work, for each sampling location.

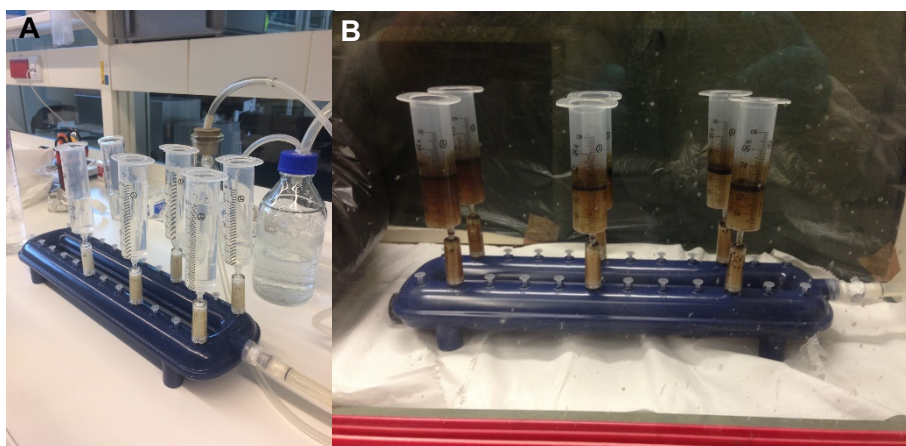


Figure 2.4 - Filtration process. A- At W0; B- At W2.

Table 2.2 - Characteristics of the filtration process for W0 and W2 of each location sampled.

Code	Sample ID	Week	Collection filter pore (μm)	Volume filtered (mL)	Filtration time (h)
Pen	Pi	W0	0.22	2000	ND
	PF	W2	0.22	27	ND
2Pen	2Pi	W0	0.22	2000	ND
	2PF	W2	0.22	13	ND
1.6	1.6i	W0	0.22	1890	≈2
	1.6F	W2	0.22	15	1.35
1.7	1.7i	W0	0.22	2000	≈ 1.73
	1.7F	W2	0.22	13	1.35
1.8	1.8i	W0	0.22	1800	ND
	1.8F	W2	0.22	40	ND
1.9o	1.9io	W0	0.22	2000	ND
	1.9Fo	W2	0.22	16	ND
1.9i	1.9ii	W0	0.22	2000	≈1.73
	1.9Fi	W2	0.22	7	1.35
1.10	1.10i	W0	0.22	1400	ND
	1.10F	W2	0.22	10	ND
2.1	2.1i	W0	0.22	2000	2.2
	2.1F	W2	0.22	17	1.5
2.2v	2.2iv	W0	0.22		ND
	2.2Fv	W2	0.22	35	ND
2.2o	2.2io	W0	0.22	1910	ND
	2.2Fo	W2	0.22	15	ND
2.2i	2.2ii	W0	0.22	2000	2.2
	2.2Fi	W2	0.22	15	1.5
2.3	2.3i	W0	0.22	2000	ND
	2.3F	W2	0.22	20	ND
2.4	2.4i	W0	0.22	1730	ND
	2.4F	W2	0.22	11	ND

Table 2.2- (Continuation)

2.5	2.5i	W0	0.22	1850	2.2
	2.5F	W2	0.22	26	1.5
2.8	2.8i	W0	0.22	2000	1.75
	2.8F	W2	0.22	13	2.2
2.9	2.9i	W0	0.22	1150	2.25
	2.9F	W2	0.22	36	2.2
2.10	2.10i	W0	0.22	2000	1.75
	2.10F	W2	0.22	46	2.2
2.12	2.12i	W0	0.22	2000	ND
	2.12F	W2	0.22	16	ND
2.13	2.13i	W0	0.22	2000	ND
	2.13F	W2	0.22	19	ND
2.14	2.14i	W0	0.22	2000	ND
	2.14F	W2	0.22	16	ND
2.15	2.15i	W0	0.22	2000	ND
	2.15F	W2	0.22	17	ND
2.16	2.16i	W0	0.22	2000	ND
	2.16F	W2	0.22	36	ND
3.2	3.2i	W0	0.22	2000	≈1.3
	3.2F	W2	0.22	60	1.5
3.3v	3.3iv	W0	0.22	1850	ND
	3.3Fv	W2	0.22	30	ND
3.3o	3.3io	W0	0.22	1890	ND
	3.3Fo	W2	0.22	16	ND
3.3i	3.3ii	W0	0.22	1330	≈2.3
	3.3Fi	W2	0.22	64	1.5
3.4	3.4i	W0	0.22	2000	ND
	3.4F	W2	0.22	16	ND
3.5	3.5i	W0	0.22	2000	ND
	3.5F	W2	0.22	13	ND

2.5. DNA extraction, DNA Quantification, PCR, Library preparation and Sequencing of 16S rDNA amplicon

After completing the initial (i) and final (F) filtrations for all sampling locations, Sterivex™ filters were defrosted at room temperature. Then, the DNA was extracted using the DNeasy® PowerWater® Sterivex Kit (QIAGEN, Inc). Isolated DNA was then quantified fluorometrically using the Qubit dsDNA HS Assay (ThermoFisher Scientific, Waltham, USA). The amplification of the V4-V5 region of the genetic marker 16S rRNA gene was performed by using the primer pair 515F (5'-GTGYCAGCMGCCGCGGTAA-3') and 926R (5'-CCGYCAATTYMTTTRAGTTT-3'), according with Earth Microbiome Project protocols (Gilbert et al., 2014). These primers were adapted from the primer pair 515F/806R, that only amplified

the V4 region, designed by Caporaso et al. (2011; 2012), and latter modified by Apprill et al. (2015) and Parada et al. (2016). The increase in amplicon length together with primers' degeneracy provides a better method to cover a broader spectrum of prokaryotic community abundances and diversity, such as the *Thaumarchae* phylum (modifications at 515F) and the SAR 11 clade of Alphaproteobacteria (modifications at 926R) (Apprill et al., 2015; Parada et al., 2016).

Sequencing of 16S rRNA gene amplicons was performed in Biocant – Biotechnology Park (Cantanhede, Portugal). Two Polymerase Chain Reaction (PCR) rounds were performed to amplify first the DNA with the specific primers and reamplified afterwards to add sequencing adapters and dual indexes. The KAPA HiFi HotStart PCR kit was used to perform the PCR reactions, which included 0.3µm of each primer and 12.5ng of template DNA in a total volume of 25µm. Conditions of PCRs involved denaturation at 95°C for 3 min followed by 25 cycles of 20s at 98°C, 30s at 50°C, 30s at 72°C with a final extension for 5 min at 72°C. The second round of PCR reactions were performed according to the manufacturer's suggestions (Illumina, 2013). At Genoinseq (Cantanhede, Portugal), the PCR products were one-step purified and normalized by using the SequalPrep 96-well plate kit (ThermoFisher Scientific, Waltham, USA) (Comeau et al., 2017), pooled and pair-end sequenced, according to the manufacturer's instructions, in the Illumina MiSeq® sequencer with the V3 chemistry (Illumina, San Diego, CA, USA).

2.6. Hydrocarbon Degraders Abundance

For this work, the Most Probable Number (MPN) procedure was adapted from Wrenn and Venosa (1996) to determine the abundance of hydrocarbon degrading bacteria. This method was performed in 96-well microtiter plates (Figure 2.5).

A volume of 180 µL of sterilized Bushnell-Hass (BH) medium supplemented with NaCl (2% v/v) was added to the wells in line A, except in A1 where 180 µL of the sample was added, and in lines C to G. Afterwards, 10µL of petroleum, which was the carbon source, were added in wells C to G after passing through a 0.2µm sterile cellulose acetate membrane filter (VWR) to certify that degradation only occurred with the bacteria present in the samples. The MPN method was done in triplicate.

Afterwards, 10-fold dilutions were done in line A after the sample was put in A1. From there, 20µL of A1 were put in A2 and so on, except for column 12 which is the control. Then, with a multichannel 200µL pipette, 20µL of line A were added to the following lines from C to G to make five replicates for each dilution. The plates were left to incubate for two weeks at room temperature.

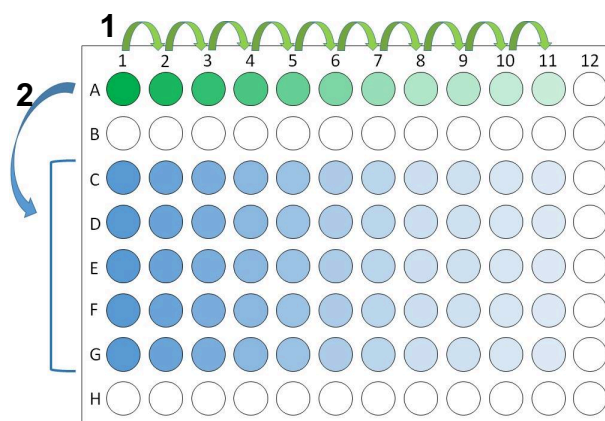


Figure 2.5 - Schematic representation of an MPN 96-well plat after 10-fold dilutions being applied (1). Afterwards, the dilutions are added in lines C-G (2)

After two weeks, 50 μ L of 3 gL⁻¹ Iodonitrotetrazolium Violet (INT) solution, sterilized by filtration through a 0.2 μ m sterile cellulose acetate membrane filter (VWR) was added to lines C, D, E, F and G. After an overnight incubation, the positive wells, with red coloration, were scored (Figure 2.6). In the presence of active respiring microorganisms, INT is reduced to formazan that precipitates with a red colour (Haines et al., 1996). The abundance of HC degrading microorganisms was then estimated using the Most Probable Number Calculator v3.1 program (Klee, 1993). The MPN method was applied in W0, after one week (W1) of incubation in the microcosms and at the end of the experiment (W2).

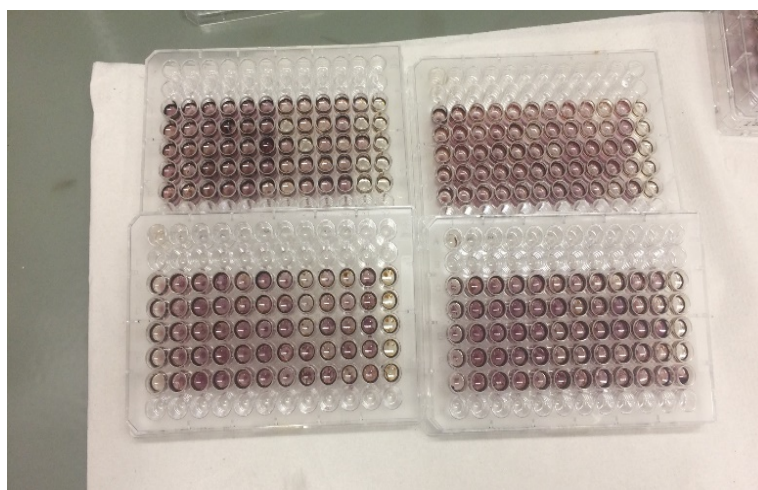


Figure 2.6 - Example of four plates where the MPN of HC degraders was estimated, after two-week incubation and INT addition.

2.7. Bioinformatic Pipelines

2.7.1. Illumina MiSeq[®] Analysis

Genoinseq (Cantanhede, Portugal) provided three files in fastq format with the sequences of the 16S rRNA gene. The first file contains the raw sequences, generated by demultiplexing the forward and reverse raw reads extracted from illumina MiSeq[®] System, by removing sequence adapters with PRINSEQ 0.20.4 (Schmieder & Edwards, 2011). In the second file the sequences were quality-filtered to remove reads with less than 100 bases and trim bases with average quality lower than Q25 in a window of 5 bases with PRINSEQ 0.20.4, thus resulting in the fastq file with filtered reads. The third fastq file contained the forward and reverse sequences, that underwent through the processing above described, merged by overlapping paired-end reads by using AdapterRemoval 2.1.5 (Schubert et al., 2016), with the default parameters.

2.7.2. Microbial Community Analysis with SILVAngs

The microbial communities' structure was assessed by using the SILVAngs 1.3 analysis pipeline (Quast et al., 2012). This automatic software pipeline provides a service to analyse data of amplicon reads of rRNA gene from massive parallel high-throughput NGS technologies. The workflow provided by SILVAngs divides into five modules: alignment, quality management, de-replication by identifying identical sequences, clustering and classification of OTU's.

Before importing the files to SILVAngs, the merged fastq files provided by Genoinseq were converted to fasta format in the open-source web-based platform named Galaxy v1.1.1, using the program FASTQ to FASTA converter from FASTX - toolkit (Blankenberg et al., 2010; Gordon & Hannon, 2010). Afterwards, all reads from each file were aligned by SINA v1.2.10 (Pruesse et al., 2012) and quality controlled (Quast et al., 2012). A percentage of 8.69 of all sequences were rejected based on the default parameters for the alignment score, the alignment identity, length, ambiguous bases and number of homopolymers. Later, the sequences were de-replicated where identical reads were identified and the unique reads, with 98% of sequence identity, were clustered in OTUs. Only the longest read of each OTU was retained as the reference read and classified. Both de-replication and clustering were performed by cd-hit-est v3.1.2 (<http://www.bioinformatics.org/cd-hit>) (Li & Godzik, 2006). The classification of each representative sequence of each OTU was done by a local nucleotide BLAST search, using blastn version 2.2.30+ (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>), against the non-redundant version of the SILVA SSU Ref dataset (release 132; <http://www.arb->

silva.de). Sequences that did not have any BLAST hits or BLAST hits that did not exceed 93% of identity were unclassified and assigned to the group “No Relative” in SILVAngs fingerprint and Krona charts (Ondov et al., 2011).

2.7.3. Microbial Community Analysis with QIIME

The pipeline QIIME2 was used to pre-process, merge, filter by quality, assign taxonomy, reconstruct phylogenies and explore diversity patterns of the 16S rRNA gene sequenced amplicons (<https://qiime2.org>) (Caporaso et al., 2010).

Since the raw data provided by Genoinseq (Cantanhede, Portugal) was already demultiplexed, the first step taken in QIIME2 pipeline was to remove the forward and reverse primers of each amplicon by using cutadapt (Martin, 2011). Default parameters were used with exception of the error rate in the paired region which was of 0.3 for forward sequences and 0.2 for reverse sequences. This criterion was chosen to allow mismatch and indels errors in the degenerated primers in order to avoid discarding too many sequences. Afterwards, a quality control of the high throughput fastq data was assessed through graphics and plots by using the java-based software FastQC, version 0.11.7 (Andrews, 2010). This step was important to assure that the raw data had no problems or biases originated in both the sequencer and the starting library material. Then, paired-end reads were joined with vsearch (Rognes et al., 2016). This was followed by quality filtering bases on the Q scores, using the default settings. Then the vsearch was again used to de-replicate the sequences followed by *de novo* chimera checking to identify artificial sequences. Chimeras and borderline chimeras were then excluded from further analysis. Afterwards, sequences were clustered into OTUs at 97% of similarity with the closed-referenced method in QIIME2 using the vsearch algorithm. This method compares each OTU representative sequence against the Silva reference database version 13.2 (available at <https://www.arb-silva.de/download/archive/qiime>) (Yilmaz et al., 2013). Sequences that did not have high similarity with any reference sequence in the database were discarded and excluded from downstream analyses.

After obtaining the representative sequences for each OTU and the OTU table with the frequency of occurrence of each OTU sequence, it was possible to assign the taxonomy. To do so, the Naïve Bayes classifier was used due to the training accuracy of this classifier being improved by the hypervariable region targeted by the primers used in this study.

From the taxonomic OTU table it was possible to proceed to the alpha and beta diversity analysis. Since some of these diversity metrics are based on phylogenetic methods, it was built a maximum-likelihood (ML) tree with the representative sequences of OTUs (Price et al., 2010). First a multiple sequence alignment was performed with the MAFFT algorithm

(Kato & Standley, 2013) plugin, followed by the elimination of the highly variable positions, using the default parameters, to avoid overestimate distances. The ML tree was built by using FastTree (Price et al., 2010) and rooted based on the midpoint rooting method. Then the core diversity analysis plugin was performed using the default parameters with the exception of the sampling depth. In order to avoid bias estimations due to the different number of sequences in each sample, the rarefaction approach was used and all data was subsampled randomly at 18 665 number of reads (which corresponds to the sample with the lowest number of reads present). To assess if the sequencing effort was enough to catch all the diversity present in all samples, it was calculated the alpha diversity metric for the rarefaction curves of observed number of OTUs. In this step, the default parameters were used except for maximum rarefaction depth which was of 18 665.

2.7.4. Predictive Functional Profiling (PICRUST)

The prediction of the functional composition of the metagenomes present in our data was accomplished by using PICRUST (Langille et al., 2013). Since this computational approach also requires an OTU table, the same steps as the ones described above, in section 2.7.3, were performed with the exception of the database used. For this analysis the representative sequence of each OTU was compared against the Greengenes reference database version 13.5 (DeSantis et al., 2006), because the pre-calculated ML-tree used was built with sequences from this database.

After attaining the OTU table it was possible to normalize our data by 16S rRNA gene copy numbers and to profile the metagenomes using the normalized OTU table. This final output allowed to categorize our data to estimate the composite metagenome, by combining KEGG orthologs (KOs) by families of metabolisms and to predict the presence of single KOs from the KEGG database (<https://www.genome.jp/kegg/kegg1.html>) (Kanehisa & Goto, 2000; Kanehisa et al., 2015; Kanehisa et al., 2016).

The KOs analysed in this work, to predict the functional profile of the native microbial consortia to assess their potential to degrade aromatic compounds, were selected from i) 7 described metabolic pathways (Carbazole degradation (M00544); Degradation of aromatic compounds (ko01220); Ethylbenzene degradation (ko00642); Naphthalene degradation (map00626); Polycyclic Aromatic Hydrocarbon degradation (ko00624), Toluene degradation (map00623), Xylene degradation (map00622) and ii) other petroleum compounds degradation pathways described in the literature (Ribeiro et al., 2018; Sabirova et al., 2006).

2.8. Data and Statistical Analysis

Bacterial richness (or total number of species) and Shannon diversity indexes were estimated for the absolute abundance of OTUs, using PRIMER 6 software (Gorley, 2006). These indexes allowed to observe the ecological description of the microbial communities among samples.

Two heatmaps were generated using R software (Team, 2014) and the package ORCA (Hočevár & Demšar, 2016). With this analysis, the percentile of the absolute abundance of OTUs and KOs present in microbial communities, in relation to the sampled sites for before and after enrichments was observed.

To evaluate the structure of the microbial communities present in before and after oil-enrichments, a multidimensional scaling (MDS) ordination plot was performed using default parameters, to generate a configuration plot based on percent similarity, in PRIMER 6 software (Gorley, 2006). Three MDS plots were created for each different matrix used (absolute number of OTUs and absolute abundance of KOs). First a plot was created for all microbial communities and, afterwards, two different plots were created to analyse the structure of the microbial communities present before and after the oil-enrichment experiments.

In order to evaluate similarities between all microbial communities, an analysis of similarities (ANOSIM) was performed using PRIMER 6 software (Gorley, 2006). The ANOSIM is a permutation-based hypothesis statistical test, that allows to test for differences between groups (multivariate) of samples from different experimental treatments or factors. For the two different matrix analysed in this work (absolute abundance of OTUs and KOs) was performed a one-way ANOSIM analysis for all microbial communities and a two-way crossed ANOSIM analysis for natural communities and another for communities enriched by petroleum.

All statistical tests were performed using the commercial software STATISTICA, version 12, StatSoft, Inc. (2013). For species richness and diversity, significant ($p < 0.05$) differences among groups of samples were evaluated through the non-parametric test Kruskal-Wallis.

3. Results

In this chapter it is presented results from five main topics. First a comparison of the output of the two pipelines used to analysed the 16S rRNA gene sequences was accomplished. Then shifts on the microbial diversity and richness between natural and enrichment samples were assessed by analysis of rarefaction curves and Shannon diversity. Afterwards, both natural and enriched samples were characterized at the highest (phylum) and lowest (genus) taxonomic levels, followed by the prediction of functional genes present in five metabolic pathways of aromatic compounds' degradation. This information allowed to produce a georeferenced library of native microbial consortia with the capability to degrade petroleum compounds for each georeferenced sampled site. Finally, the potential of the native communities to biodegrade petroleum compounds in natural and through the enrichment experiment was evaluated by the MPN method.

3.1. QIIME and SILVAngs outputs comparison

Analysis of data from NGS was performed and processed using the software SILVAngs and also the QIIME bioinformatic pipeline. Sequencing analysis yielded 4163070 raw sequences, which after initial filtering/chimeric detection, decreased in number to 3278517 (SILVAngs) and 2503238 (QIIME). Overall, the number of available sequences, for all samples, was lower in QIIME (Table 3.1), which is due to the identification and discard of chimeras thus providing more reliable outcomes (Caporaso et al., 2010). The sequences were clustered into 37755 and 98547 OTUs, respectively, with SILVAngs and QIIME (Figure 3.1 A and B). While SILVAngs picks the longest sequence as representative of an OTU, QIIME considers the most abundant one (Coninck & Nieuwerburgh, 2017). Since assigning taxonomy to one OTU is performed by searching for the most similar sequences, QIIME provides a more accurate analysis. In addition, the number of sequences unassigned in QIIME varied between 0 and 0.003%, whereas in SILVAngs the values ranged between 0.119 and 9.01%. Therefore, the results obtained in QIIME will be further analysed in this chapter.

Table 3.1 - Number of available sequences after quality filtering, per pipeline, in initial (i) and final (F) samples.

	SILVAngs	QIIME		SILVAngs	QIIME
1.6i	36375	25067	1.6F	36524	28232
1.7i	60023	44778	1.7F	45172	38509
1.8i	90208	67224	1.8F	49451	35386
1.9io	48609	32076	1.9Fo	77033	59810
1.9ii	68433	52674	1.9Fi	46010	38389
1.10i	64687	46832	1.10F	85802	64406
Pi	51745	34152	PF	39060	29209
2Pi	77583	48544	2PF	63017	43747
2.1i	85790	64432	2.1F	22377	18665
2.2iv	51743	35182	2.2Fv	49447	32931
2.2io	52467	34934	2.2Fo	49143	32751
2.2ii	64107	42414	2.2Fi	25901	21860
2.3i	69106	52885	2.3F	51488	41311
2.4i	52998	40319	2.4F	53779	42756
2.5i	65458	50688	2.5F	39565	28595
2.8i	65574	48656	2.8F	37780	28517
2.9i	57066	45027	2.9F	60497	43786
2.10i	43068	33893	2.10F	46802	35449
2.12i	71010	59136	2.12F	39829	31632
2.13i	67581	56552	2.13F	29193	24162
2.14i	91160	78870	2.14F	36772	30962
2.15i	96155	82849	2.15F	43878	36261
2.16i	62322	53945	2.16F	45911	36200
3.2i	70872	59192	3.2F	49223	39073
3.3io	45975	30656	3.3Fo	51569	40202
3.3iv	68220	52744	3.3Fv	35069	25200
3.3ii	86583	64448	3.3Fi	67476	48885
3.4i	45946	34889	3.4F	48948	41223
3.5i	98247	78150	3.5F	42690	33921

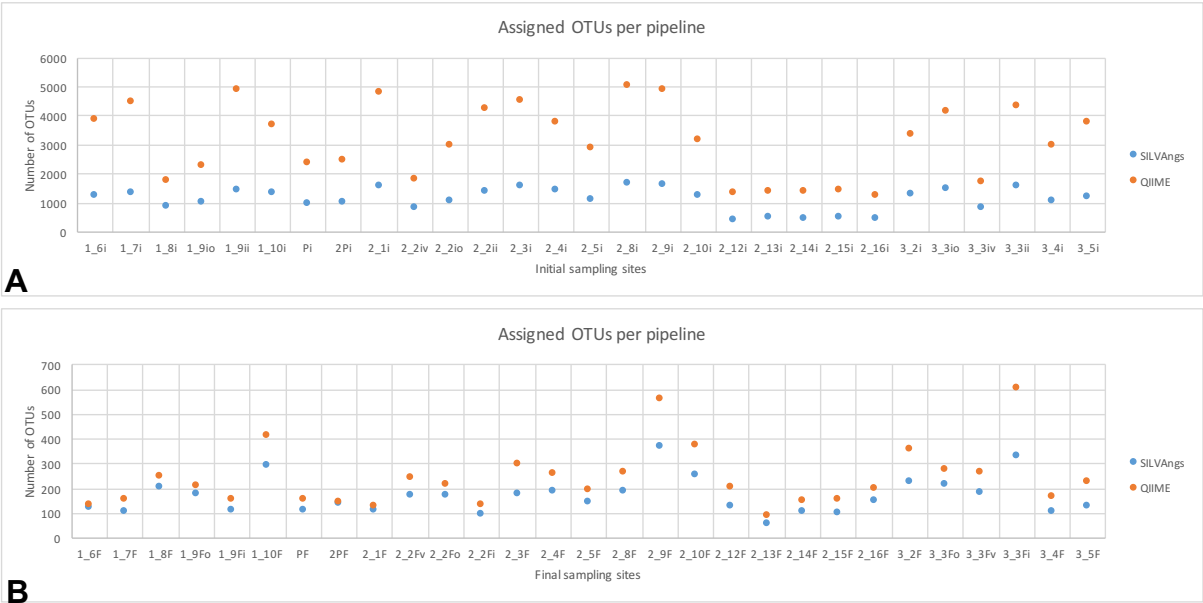


Figure 3.1 - Graphical comparison of OTU attributions per sampling site, for each pipeline. A- Natural sampling sites, B- Oil-enriched sampling sites

3.2. Shifts on prokaryotic diversity

An analysis of the alpha diversity was performed to understand the microbial diversity and structure by relating the number of OTUs with the number of sequences obtained by NGS analysis. Through rarefaction curves (Figure 3.2) it was possible to observe that the sequencing effort was not enough to capture the entire diversity present, in most natural samples, as the curves have not reached a plateau.

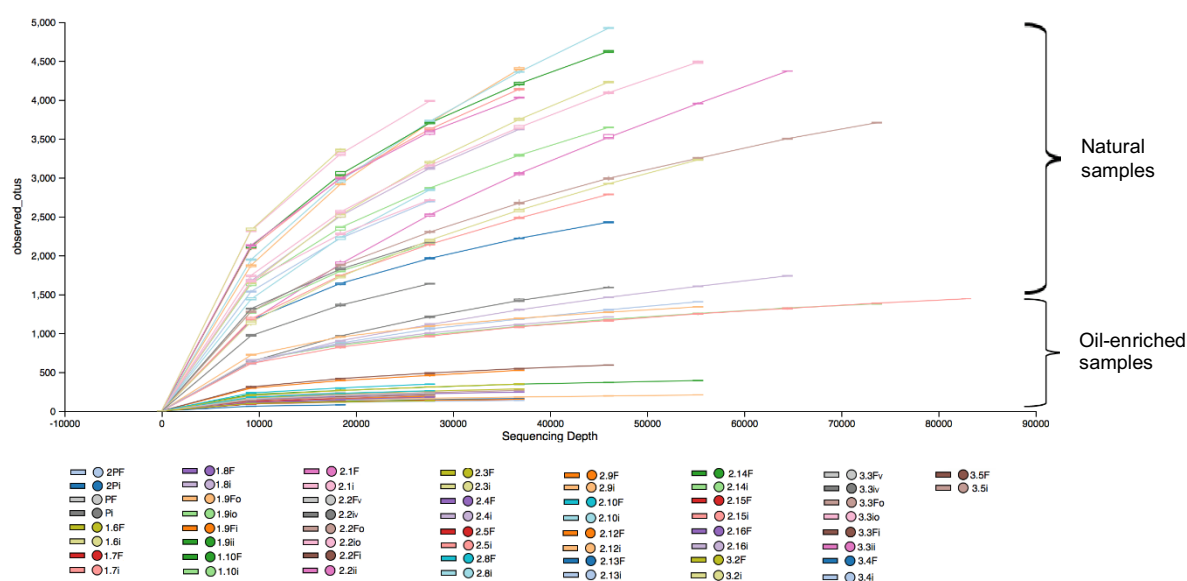


Figure 3.2 - Rarefaction curves of alpha diversity for each natural community and after crude-oil enrichment community, for each sampling site.

Rarefaction curves showed a clear decrease in the biodiversity of natural microbial communities after two-weeks of enrichment with crude oil, which was confirmed by the lower richness (Figure 3.3 A) and Shannon diversity indexes (Figure 3.3 B). In fact, independently of the area where the samples were collected, there was always a significant ($p < 0.05$) decrease in richness (approximately 15 times) and in Shannon diversity (around 3 times) after exposure to crude oil. In addition, in Area 2, the offshore natural samples were significantly ($p < 0.05$) less rich and diverse than coastal natural samples.

In stations where samples were collected in different seasons (summer, autumn and winter), it was possible to observe differences in the prokaryotic communities with season that was reflexed in different oil-enriched communities. The higher richness' and diversity index values of natural communities were observed in winter samples with the exception of site 3.3, where species diversity was higher in autumn. Regarding oil-enriched communities, the winter samples presented lower richness' values when compared to other seasons, with the exception of sample 3.3 where the highest richness was observed in the winter (Figure 3.3 A).

In terms of diversity, the sample 1.9 from autumn enrichments presented higher values than winter enriched samples, whereas in the sample 2.2 from summer was observed the highest value in diversity in comparison to autumn and winter samples. Finally, enriched communities from station 3.3 in winter were more diverse in comparison to summer and autumn samples (Figure 3.3 B).

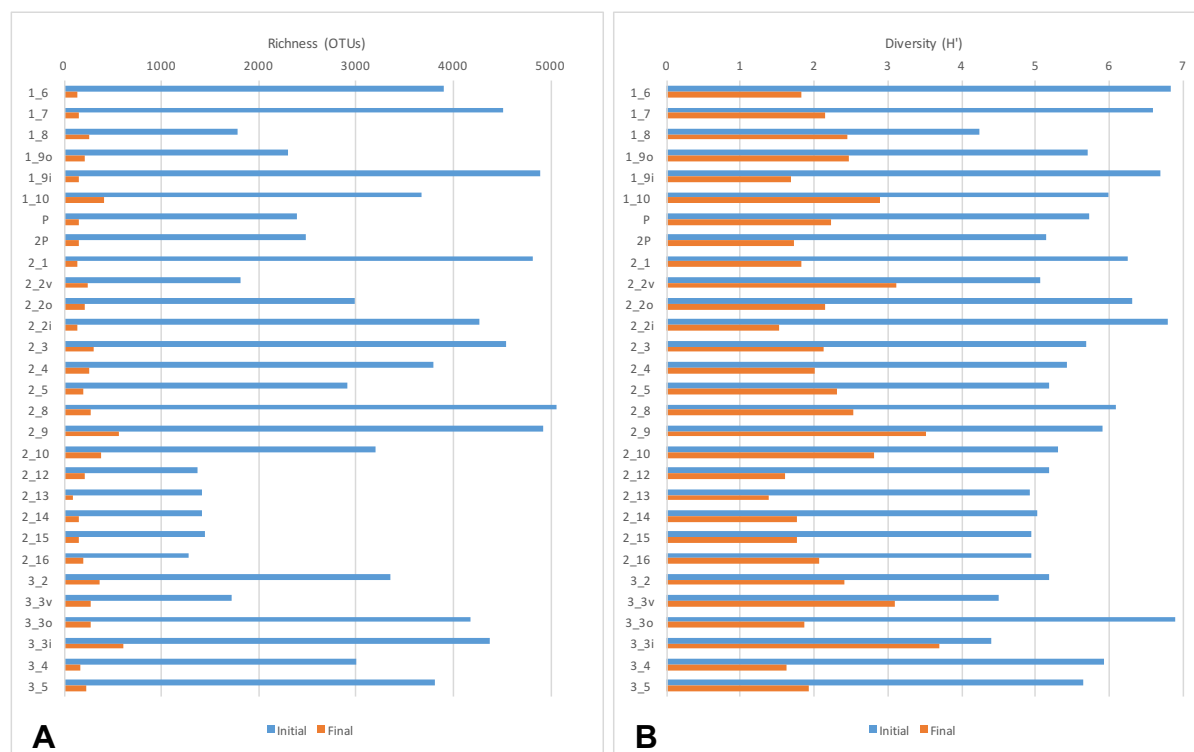


Figure 3.3 - Different alpha diversity metrics for each 29 sampled sites in natural (initial) and after crude-oil enrichment (final) samples. A- Observed species/OTUs, B- Species diversity given by Shannon index.

3.3. Descriptive analysis of taxonomic profiles – Who's there?

3.3.1. Distribution of the most relevant phyla in natural and crude oil enrichment samples

Taxonomic characterization, at the highest taxon, of the natural communities (before crude-oil enrichments) showed that these communities presented higher relative abundances for the phyla *Proteobacteria* (between approximately 31-51%), *Bacteroidetes* ($\approx$ 12-50.5%), *Cyanobacteria* ($\approx$ 1-32%), *Thaumarchaeota* ($\approx$ 0.02-14%) and *Actinobacteria* ($\approx$ 1-11%) (Figure 3.4 A).

After the two-week microcosms enrichments, the selected indigenous prokaryotic consortia were composed predominately by *Proteobacteria* (approximately between 56-99%) and *Bacteroidetes* (≈ 0.2 -44%) (Figure 3.4 B).

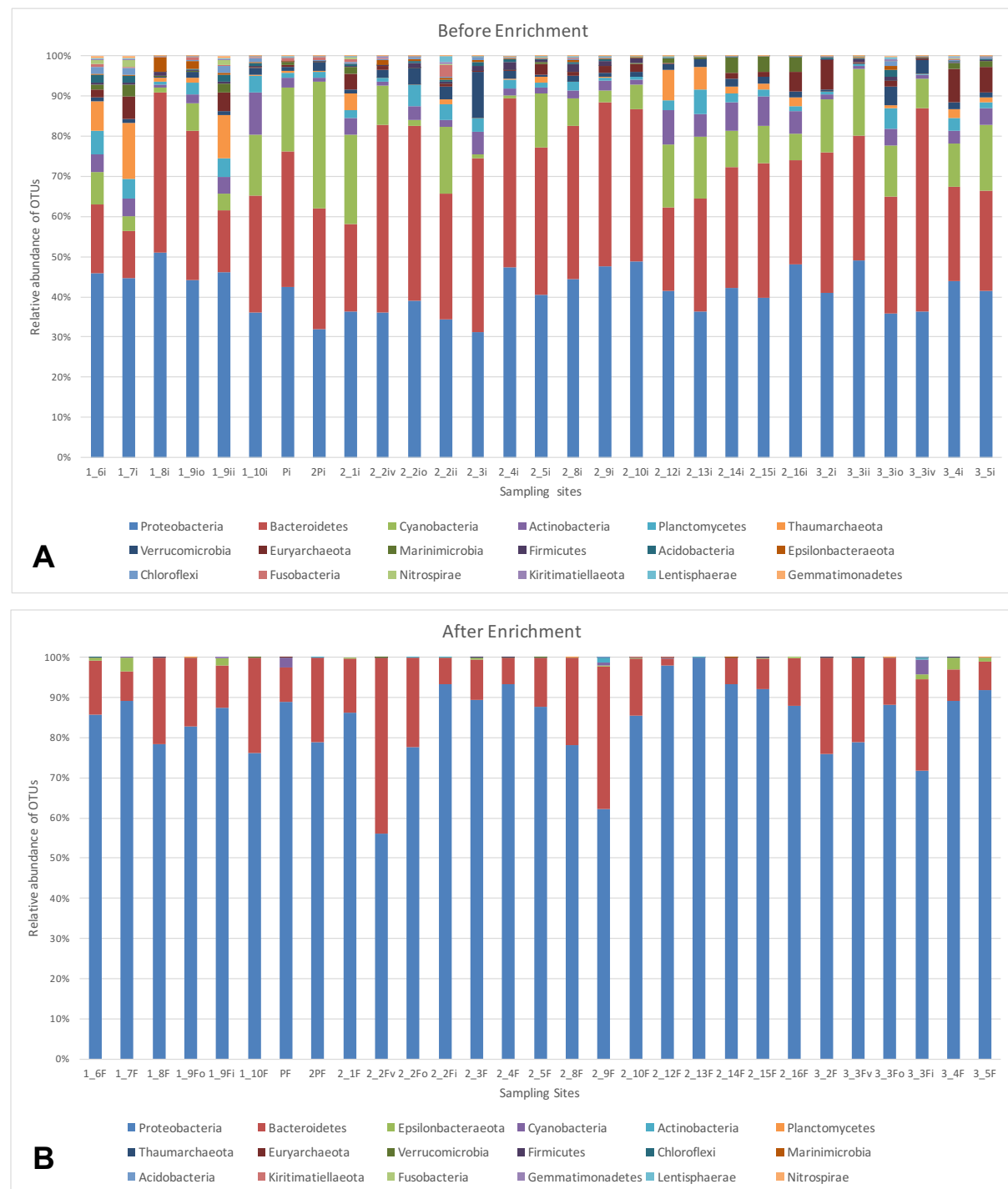


Figure 3.4 - Taxonomic profile of the relative abundances of OTUs of natural prokaryotic communities and after crude-oil enrichment communities, for 29 sampled sites, at the highest taxonomic level, Phylum. (Phyla with relative abundances below 0.5% were not considered). A- Distribution of phyla in natural communities B- Distribution of phyla in after crude-oil enrichment communities.

3.3.2. Distribution of the most relevant prokaryotic groups at the lower taxonomic level in natural and crude oil enrichment samples

The analysis of the distribution of the lower taxonomic level groups in all samples, was performed for relative abundances of OTUs above 9% in one or more stations, which resulted in a Top 20 of the most relevant lower taxonomic groups in natural and crude oil enrichment samples.

Analysing the lowest taxonomic level, it is possible to observe that native communities were composed by a higher abundance of bacteria belonging to the *Flavobacteriacium* family (≈ 1.2 -23%), the order SAR11 (≈ 0.2 -17%), the *Rhodobacteraceae* family (1-20%), the NS5 marine group (≈ 0.8 -13%), the class of *Oxyphotobacteria* (≈ 0.2 -12%) and the genus *Amylibacter* (≈ 0.02 -10%) (Figure 3.5 A).

After crude-oil enrichments, the natural microbial communities showed an overall outweigh of the genera *Alcanivorax* (varying between 13-72%), with the exception of three samples (1.10; 2.9 and 3.3i) where its relative abundance varied between approximately 0.3 and 5%. Other genera also had an overall increase in abundance such as *Thalassospira* (between ≈ 0.6 -19%), *Pseudomonas* (≈ 0.08 -41%), specially in 2.9, 3.3i and 3.3v samples with relative abundances of, 26%, 41% and 31%, respectively, and bacteria from *Flavobacteriaceae* family (≈ 0 -18%) (Figure 3.5 B).

Regarding seasonal sampling sites, it was observed some variation between natural microbial communities (Figure 3.5 A). In station 1.9 during autumn, the native communities were mainly composed by genera from the family *Flavobacteriaceae* and the family *Rhodobacteraceae* whereas in the winter, the community was mainly composed by genera from the class *Alphaproteobacteria*. In the sampling point 2.2, the family *Flavobacteraceae* was present in the three seasons. However, communities' composition also shifted from a predominance in NS5 marine group and *Planktomarina*, in the summer, to genera from the family *Rhodobacteraceae* in the autumn, and in the winter there was a dominance of genera from the class *Oxyphotobacteria*. In station 3.3, the family *Flavobacteriaceae* was highly present in summer and autumn while the class *Oxyphotobacteria* was abundant in the autumn and winter samples.

After crude-oil enrichments, the communities also shifted according to the different seasons (Figure 3.5 B). For the sampling point 1.9, in the autumn there was a predominance of *Alcanivorax* spp., *Thalassospira* spp., *Acinetobacter* spp. and *Pseudomonas* spp. whereas

in the winter, the community was mainly composed by *Alcanivorax* spp. Station 2.2 during summer was dominated by *Coralibacter* spp. *Marinobacter* spp., *Thalassospira* spp. and *Alcanivorax* spp., whereas in autumn there was a predominance of *Alcanivorax* spp., *Flavobacterium* spp. and *Pseudomonas* spp. and in the winter it was observed an increase in *Alcanivorax* spp. and *Thalassospira* spp. For station 3.3 in the summer, the communities were dominated by *Alcanivorax* spp. and *Pseudomonas* spp., in the autumn *Alcanivorax* spp. was the main hydrocarbon-degrader and in the winter there was a dominance of *Pseudomonas* spp. and *Aequorivita* spp.

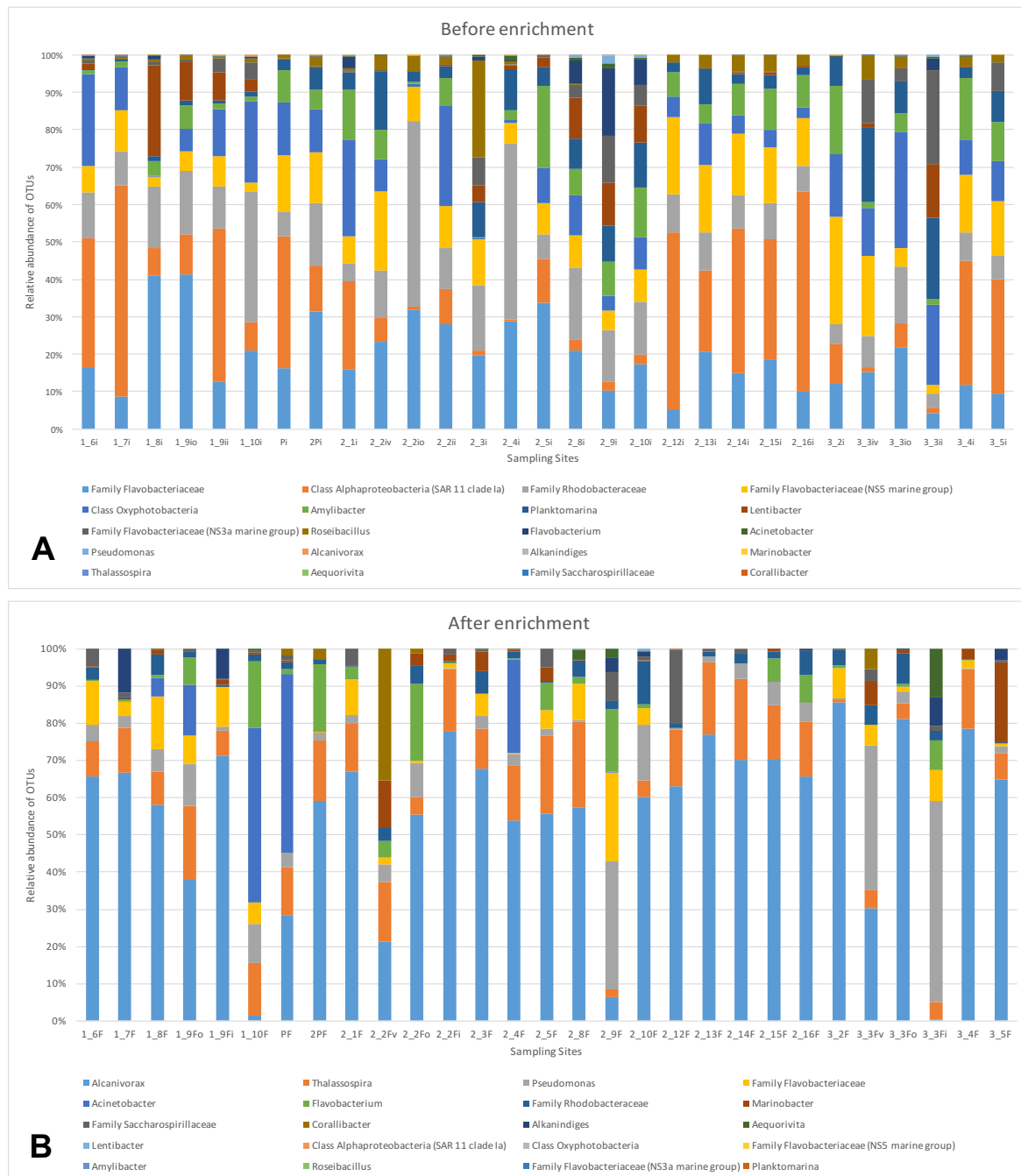


Figure 3.5 - Taxonomic profile of relative abundances of OTUs for natural prokaryotic communities and after crude-oil enrichment communities, for 29 sampled sites, at the lowest taxonomic level, Genus. (Genera with relative abundances below 9% were not considered). A- Distribution of genera in natural microbial communities. B- Distribution of genera after the oil enrichment communities

3.4. Multivariate analysis of taxonomic profiles – who's there?

Multivariate analysis was applied to the lower taxonomic level OTU table generated through QIIME pipeline to analyse if some conditions or environmental variables could significantly influence the taxonomic profiles. Here, the factors analysed were natural and after

enrichment communities (respectively Initial and Final), location of the sampled sites (Coast and Offshore) and geographic area from where samples were collected Areas 1, 2, 3).

Hierarchical cluster analysis of the absolute 16S rDNA sequences abundance of the top 20 taxa at lower taxonomic level, was performed (Figure 3.6). Natural and crude oil enrichment samples are grouped in two different clusters, the top right cluster includes all natural samples and the top left cluster include samples amended with crude oil (Figure 3.6). Also, the different top 20 taxonomy groups are grouped into two major clusters according to their abundance within natural and enriched samples. It is possible to observe that many groups of bacteria that were abundant in native prokaryotic communities decreased after oil-exposure (mainly NS5 and NS3 marine groups, *Planktomarina*, *Roseibacillus*, *Oxyphotobacteria*, *Lentibacter*, *Amylibacter*, SAR 11; Figure 3.6). However, the inverse was observed for after enriched microbial communities, where the most abundant bacteria groups presented in these samples (*Alcanivorax*, *Thalassospira*, *Marinobacter*, *Saccharospirillaceae*, *Flavobacterium* and *Pseudomonas*), had low abundance in the non-enriched natural communities (Figure 3.6).

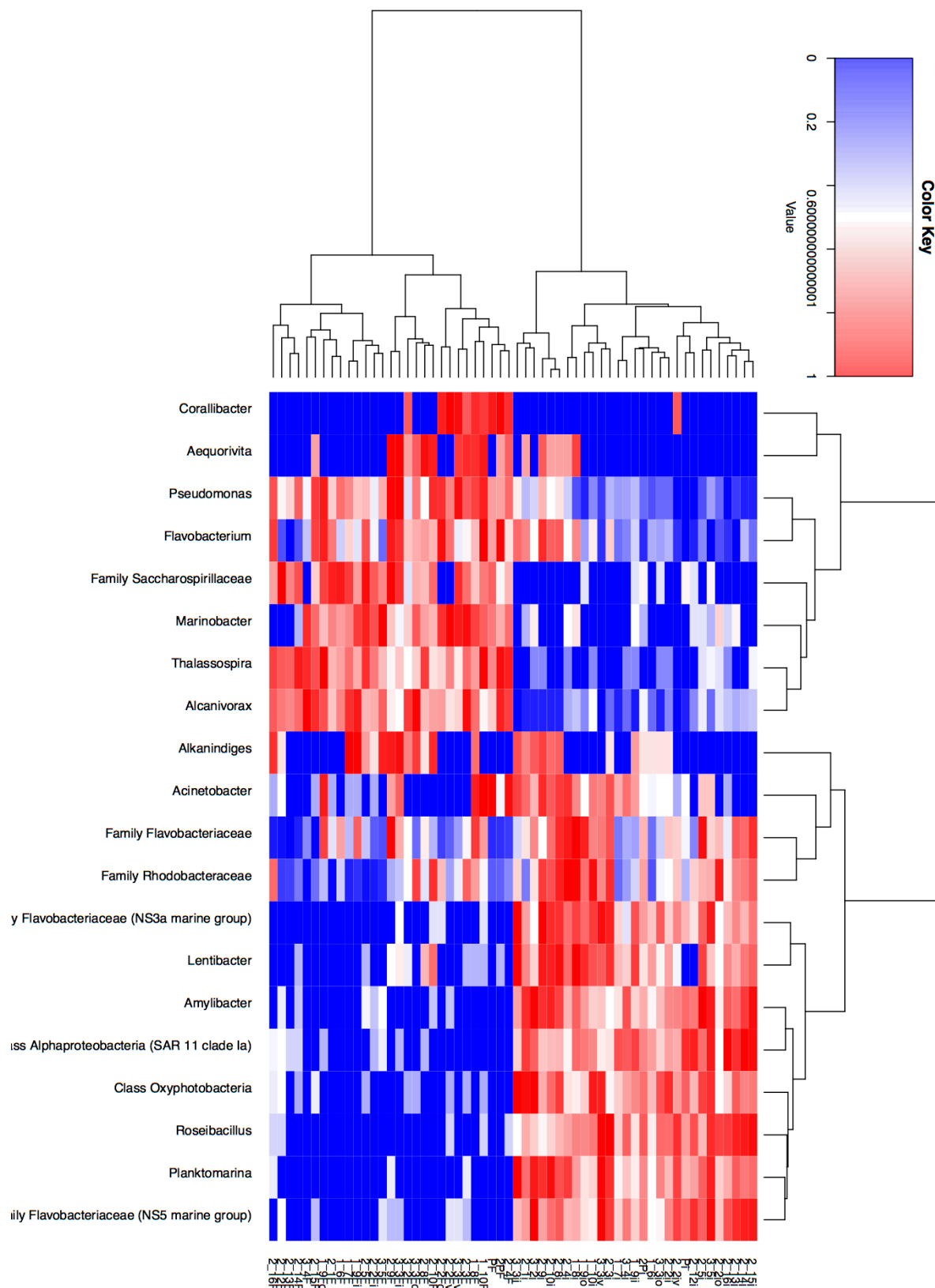


Figure 3.6 - Heatmap of the percentile of the absolute sequences abundance of taxa at the lower taxonomic level in natural and crude oil enrichment samples arranged by hierarchical clustering (using Ward minimum variance criterion). Lateral dendrogram depicts clustering of top 20 identified lower taxonomic groups by co-occurrence. The lower taxonomic level 16S rDNA OTU table generated by QIIME was used as the input table.

Multidimensional (MDS) ordination analysis (Figure 3.7) was performed based on the lower taxonomic OUT table produced by QIIME pipeline. This analysis gives a visual representation of community patterns responding to different environmental or experimental variables. A clear distinction was observed between natural and enriched samples, sharing only 5% of between-group similarity (Figure 3.7 A). Nonetheless, coast and offshore areas seem to have no effect in establishing differences in community pattern between samples (Figure 3.7 A). These results were confirmed by the one-way ANOSIM test that showed only significant differences ($p < 0.05$) between natural communities and crude oil enrichment samples (Table 3.2). However, a two-way crossed analysis showed that there were no significant differences between coastal and offshore for the natural samples and for the enriched samples (Table 3.2).

After observing that only one factor was discriminant, samples were divided and two analyses were performed, one including only samples from natural communities and the other including only crude oil enriched communities (Figure 3.7 B). For the natural communities, the two-way crossed ANOSIM analysis showed that the variables coast and offshore displayed a high significant impact in differentiating prokaryotic communities (Table 3.2). Although, the different geographic areas did not have a significant impact on prokaryotic structure differentiation among samples (Table 3.2).

Regarding the crude oil enrichment samples, it was possible to observe that there were no significant differences between prokaryotic communities from different georeferenced areas or between coastal and offshore samples (Figure 3.7 C), which was also confirmed by a two-way crossed analysis (Table 3.2).

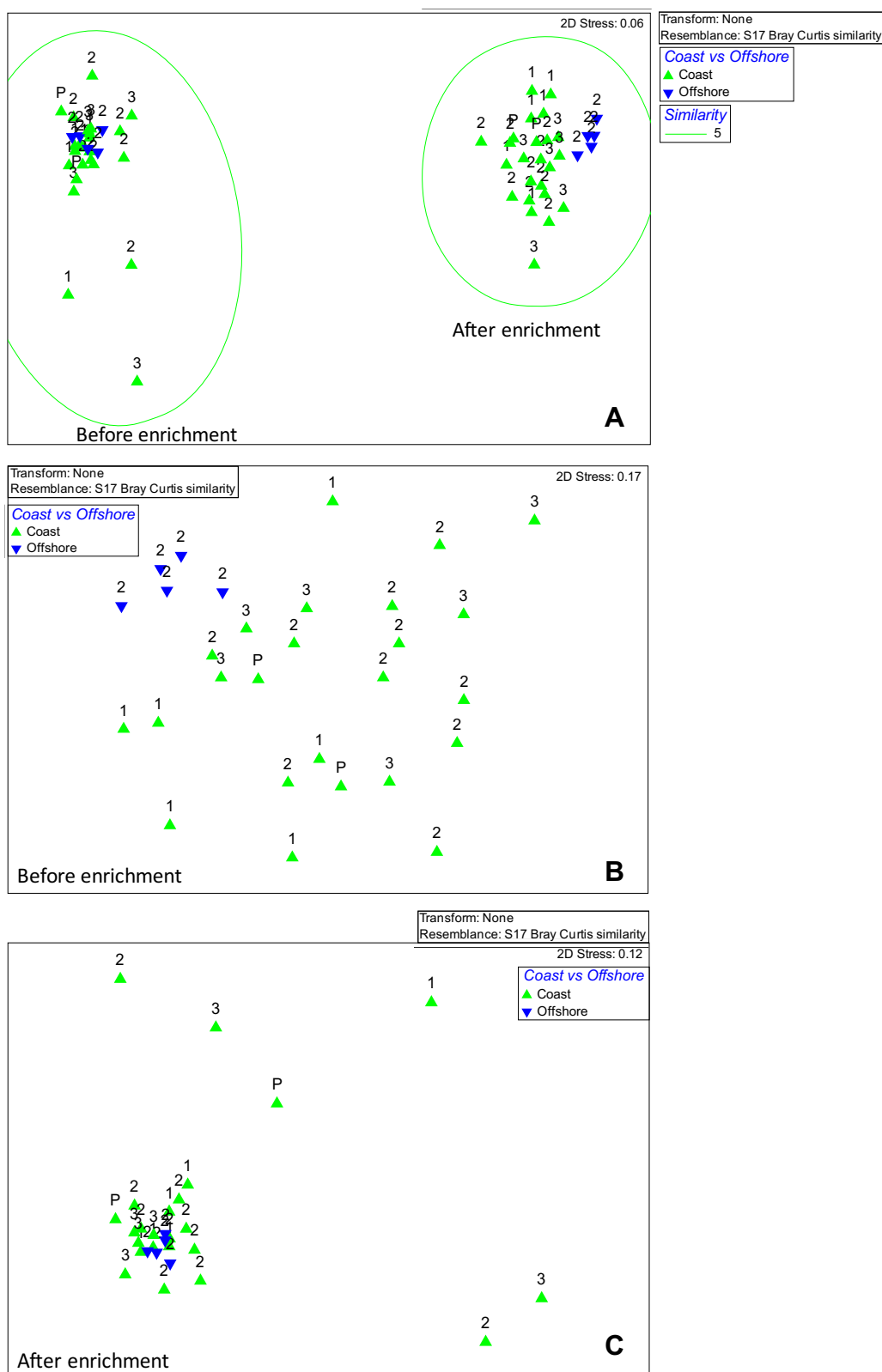


Figure 3.7 - Multidimensional (MDS) ordination for all samples sites (29) based on absolute abundance of OTUs in relation to three factors: natural (initial) and oil-enriched (final) samples, coast vs offshore and area (1, 2, 3 and Peniche (P)). A- MDS analysis for both natural and oil-enriched communities, B- MDS analysis for natural samples, C- MDS analysis for after oil-enriched samples.

Table 3.2 - ANOSIM test based on the abundance of OTUs present in both natural and oil-enriched microbial communities, to compare three different factors: natural and oil-enriched samples (initial vs final), location of the samples (coast vs offshore) and area (1, 2, 3 and Peniche).

	Statistic Value (R)	Significant level (%)
• Global samples		
<i>One-way Analysis</i>		
Initial vs Final	0.999	0.1
Coast vs Offshore	0.094	3.4
Area	0.006	38.6
<i>Two-way Crossed Analysis</i>		
Initial vs Final	0.998	0.1
Coast vs Offshore	-0.014	52.9
• Samples before enrichment		
<i>Two-way Crossed Analysis</i>		
Area	0.286	0.2
Coast vs Offshore	0.648	0.1
• Samples after enrichment		
<i>Two-way Crossed Analysis</i>		
Area	0.031	32.4
Coast vs Offshore	-0.097	70.4

3.5. Prediction of functional profiling – What's their potential?

3.5.1. Evaluation of the presence of HC degradation metabolisms in the microbial consortia

The PICRUSt bioinformatics pipeline was ran against the KEGG database to predict the functional profiles of the metagenomes at the metabolic level. Microbial communities present in natural and crude oil enriched samples were compared in terms of the total predicted abundance of genes involved in five petroleum hydrocarbon degradation metabolisms (Figure 3.8).

In natural samples, the most represented HC-degradation metabolisms were naphthalene degradation, ranging from 34-38%, followed by toluene degradation with 25-33%. PAHs degradation varying between 20-25%, ethylbenzene degradation from 9-13% and xylene degradation from 2-6% (Figure 3.8 A).

After the enrichment experiments, no significant ($p > 0.05$) differences were observed, but it was observed a tendency for a decrease in the abundance of genes involved in toluene (21-25%), PAHs (15-20%) and xylene (0.09-6%) degradation and an increase in the abundance of genes involved in naphthalene (41-45%) and ethylbenzene (13-17%) degradation (Figure 3.8 B).

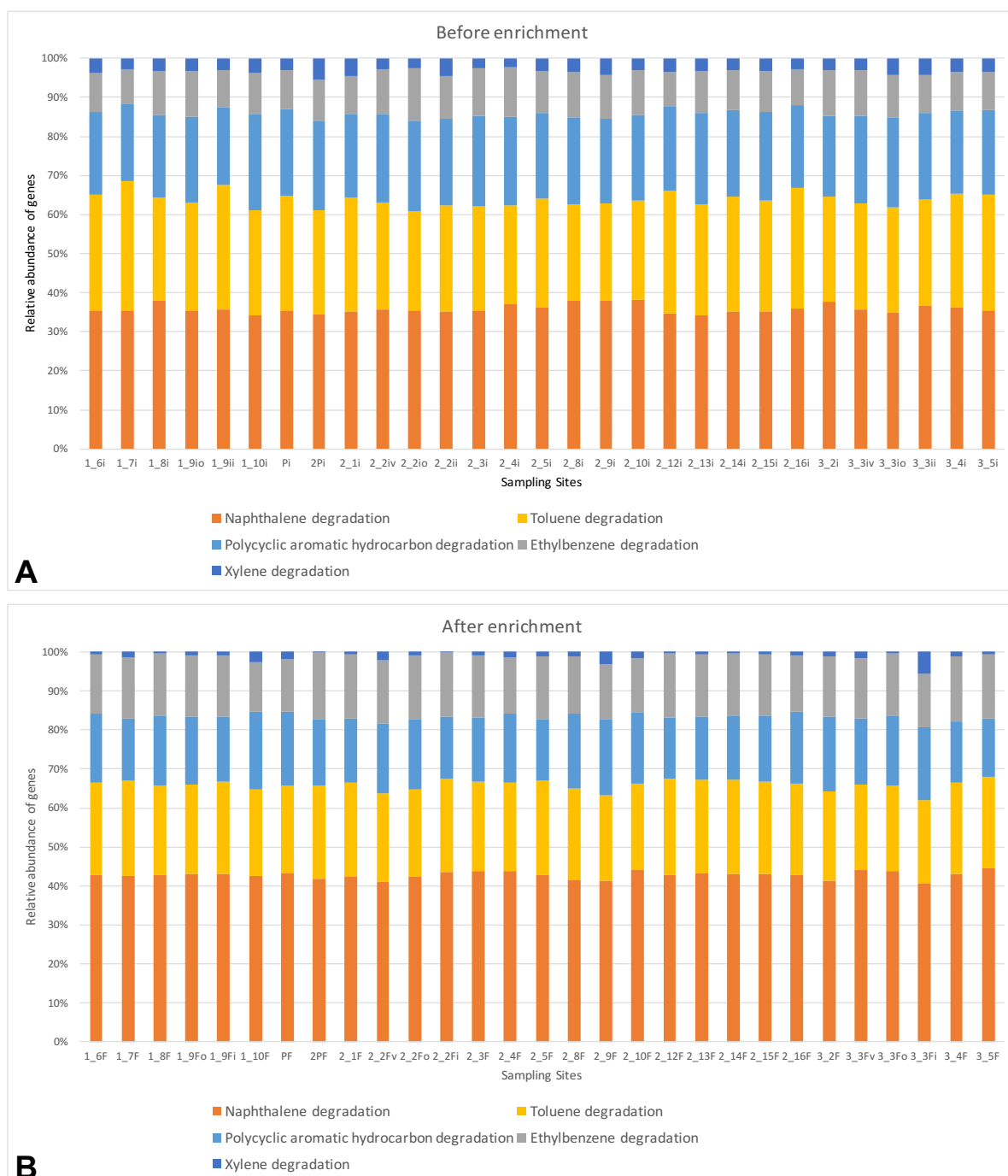


Figure 3.8 - Predicted relative abundance of genes involved in five petroleum hydrocarbon degradation metabolisms present in the natural microbial communities (A) and in oil-enriched communities (B), for the 29 sampled sites.

3.5.2. Predicted distribution of genes regarding metabolisms

A total of 89 genes known to belong to five aromatic compounds degradation were obtained after a search in KEGG database (online available information at <https://www.genome.jp/kegg/>). In addition some genes were also obtained from the previous

studies of Ribeiro et al. (2018) and Sabirova et al. (2006). These genes are involved in one or more hydrocarbon degrading metabolic pathways but can also play a role in metabolisms non-related to petroleum HCs degradation, such as Glycolysis / Gluconeogenesis, fatty acid degradation, and ascorbate and aldarate metabolism. From the genes retrieved for this analysis, 23 are involved in the xylene degradation, 18 in PAHs degradation, another 18 in toluene degradation and 13 are involved in the degradation of ethylbenzene (Table 3.3).

Table 3.3 - KOs involved in aromatic compounds degradation obtained after PICRUST analysis against KEGG database.

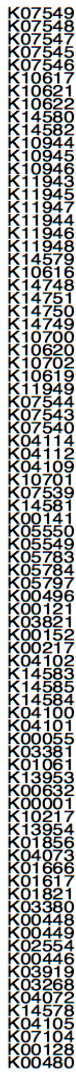
	K00001	K00055	K00121	K00128	K00141	K00152	K00217	K00446	K00448	K00449	K00480	K00496	K00632	K01061	K01617	K01666	K01821	K01856	K01860	K02554	K03268	K03380	K03381	K03821	K03919	K04072	K04073	K04100	K04101	K04102	K04105	K04109	K04112	K04114	K05549	K05550	K05783	K05784	K05797	K07104	K07539	K07540	K07543	K07544	K07545
Naphthalene																																													
PAH																																													
Xylene																																													
Toluene																																													
Ethylbenzene																																													
Aromatic compounds																																													
Others																																													

	K07546	K07547	K07548	K07549	K07550	K10216	K10217	K10616	K10617	K10618	K10619	K10620	K10621	K10622	K10623	K10700	K10701	K10702	K10944	K10945	K10946	K11943	K11944	K11945	K11946	K11947	K11948	K11949	K13953	K13954	K14578	K14579	K14580	K14581	K14582	K14583	K14584	K14585	K14746	K14747	K14748	K14749	K14750	K14751	
Naphthalene																																													
PAH																																													
Xylene																																													
Toluene																																													
Ethylbenzene																																													
Aromatic compounds																																													
Others																																													

Hierarchical cluster analysis at the level of predicted KOs involved in aromatic compounds metabolisms, showed a clear differentiation between natural and crude oil enrichment samples (Figure 3.9). In addition, the predicted genes that disappear after the crude oil enrichment are grouped in a distinct cluster from the genes that outnumbered after the incubation with crude oil (Figure 3.9).

Most of the KOs involved in the oil-enriched communities were present in the initial native microbial communities but after the crude-oil incubations sharply decreased in their abundance, while some of the KOs involved in toluene degradation metabolisms remained with low abundances before and after the oil-enrichment experiments. Nevertheless, there was a clear increase in abundancy of some KOs present in the selected microbial consortia, such as K00121, K00152, K14583, K14584, K14585 (involved in the degradation of naphthalene), K00217, K05797 (involved in the degradation of toluene), K05549, K05550, K05783, K05784 (present in the metabolisms of xylene degradation), K00141 (present in the degradation metabolic pathways of both toluene and xylene), K04102 (intervenes in the degradation of PAHs) , K14581 (present in the degradation metabolisms of naphthalene,

PAHs and ethylenzene), K00496 (involved in alkane degradation (Sabirova et al., 2006)) and K03821 (expressed protein in the growth of *A. borkumensis* SK2 (Sabirova et al., 2006)).



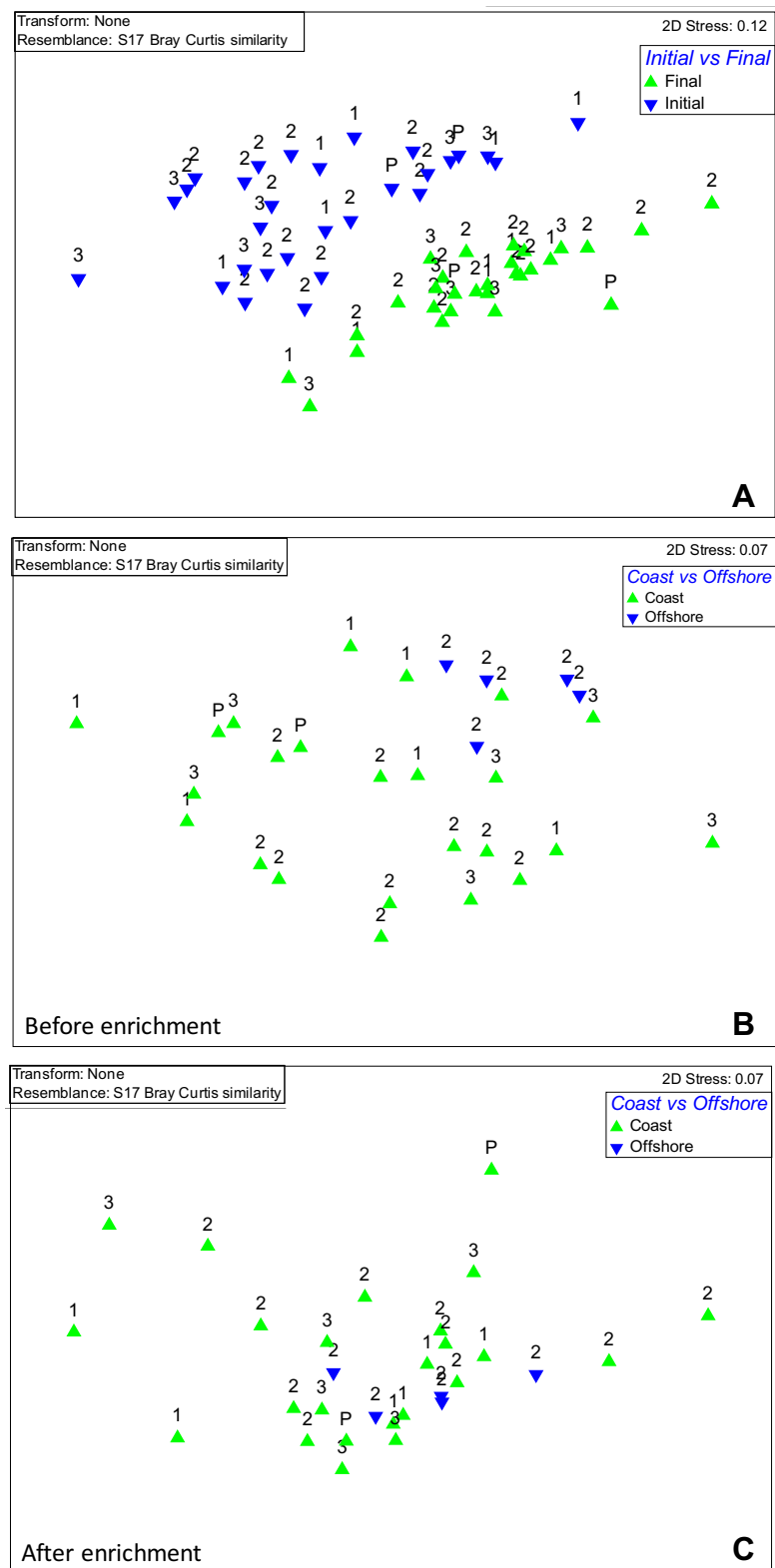


Figure 3.10 - Multidimensional (MDS) ordination for all samples sites (29) based on absolute abundance of KOs in relation to three factors: natural (initial) and oil-enriched (final) samples, coast vs offshore and area (1, 2, 3 and Peniche (P)). A- MDS analysis for both natural and oil-enriched communities, B- MDS analysis for natural samples, C- MDS analysis for after oil-enriched samples.

Analysis of the MDS ordination, showed significant differences in the predicted abundance of the KOs analysed between native and enriched samples (Figure 3.10; Table 3.4).

Individual MDS analysis for natural and crude oil enrichment samples show no differentiation between geographic areas and coastal vs offshore in the predicted relative occurrence of the selected KOs involved in hydrocarbon degradation (Figure 3.10). A two-way crossed analysis confirmed these observations with the exception of the significant differences observed between coast and offshore natural samples (Table 3.4). Despite being statistically significant ($p < 0.05$), the statistical value for this case is extremely low therefore having little to none influence in discriminating the communities.

Table 3.4 - ANOSIM test based on the abundance of selected KOs known to intervene in some hydrocarbon-degrading metabolisms, present in both natural and oil-enriched microbial communities, to compare three different factors: natural and oil-enriched samples (Initial vs Final), location of the samples (Coast vs Offshore) and area (1, 2, 3 and P).

	Statistic Value (R)	Significant level (%)
• Global samples		
<i>One-way Analysis</i>		
Initial vs Final	0.521	0.1
Coast vs Offshore	0.045	26.9
Area	-0.006	51.6
• Samples before enrichment		
<i>Two-way Crossed Analysis</i>		
Area	0.13	6.9
Coast vs Offshore	0.466	0.3
• Samples after enrichment		
<i>Two-way Crossed Analysis</i>		
Area	-0.038	61.6
Coast vs Offshore	-0.159	93.6

3.6. Abundance of hydrocarbons degraders by the Most Probable Number method

The potential of each native microbial consortium to degrade petroleum hydrocarbons was tested and evaluated by MPN method. This method was also applied after one (W1) and two (W2) weeks of crude oil enrichments to monitor the response of the prokaryotic communities during microcosms' incubations.

Overall, there was an increase in abundance of hydrocarbon-degrading microorganisms throughout the enrichment period for all sampled sites (Figure 3.11). Results indicate that both native and enriched microbial communities had the ability to degrade petroleum HCs (Figure 3.11). In addition, it was possible to observe that despite the natural communities in the sampling point 1.6, were below the detection limit, after the crude-oil enrichments, the abundance of hydrocarbon-degraders microorganisms sharply increased.

For some sampling sites, MPN estimations are missing due to positive wells in the control column, which invalidated results' interpretation, or after INT reaction the results were too heterogeneous to be consider for MPN calculation. Nonetheless, for Peniche sampling point it was possible to observe that the natural communities had the capacity to degrade petroleum hydrocarbons.

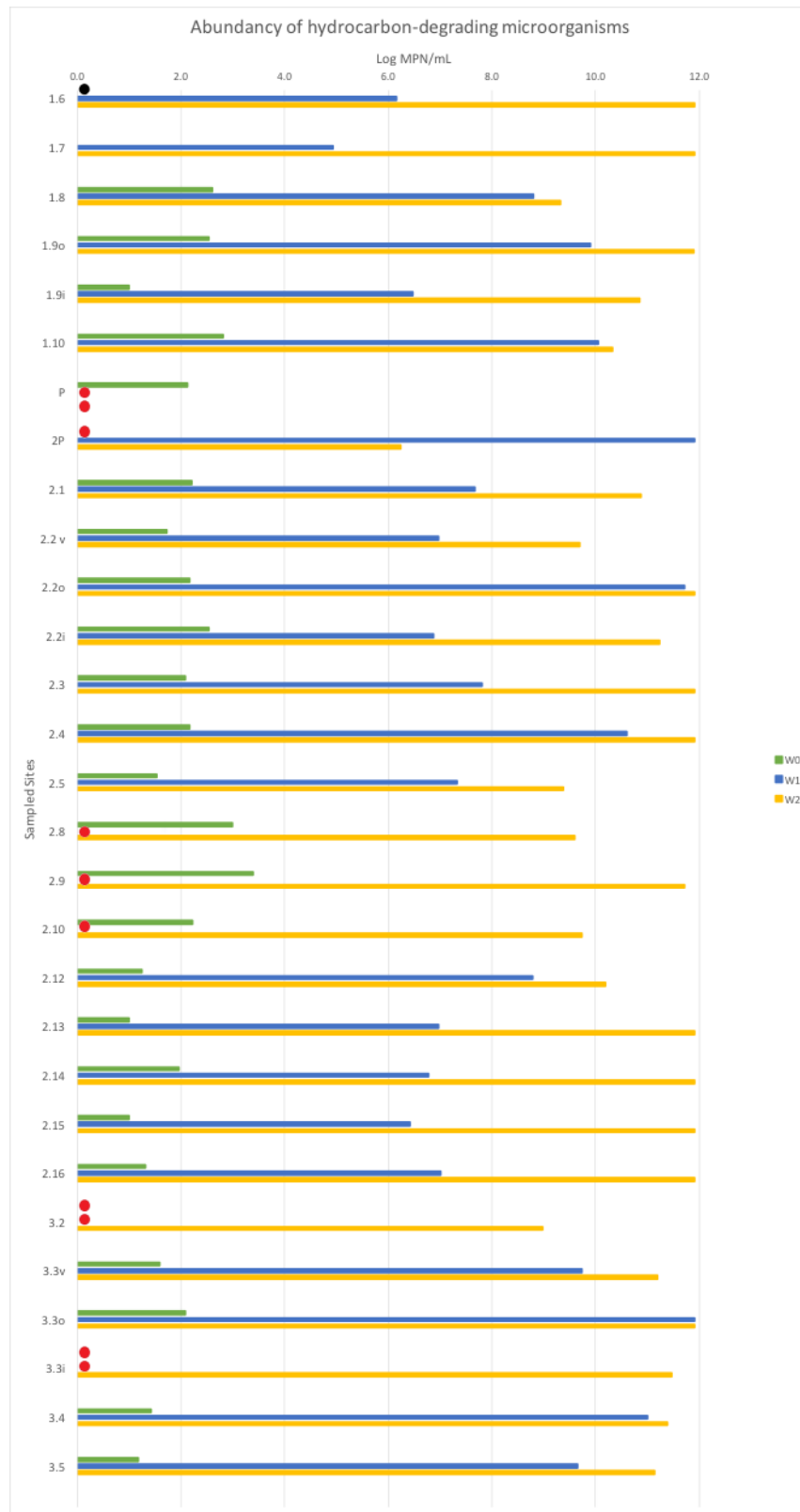


Figure 3.11 - Abundance of hydrocarbon-degrading microorganisms for each sampled site (29), along the two-week enrichment experiments with crude-oil, (mean values, n=3,) evaluated by the MPN method. ● - values below the detection limit (<10 MPN/mL); ● - unavailable information

4. Discussion

Oil spills disasters and current remediation methods have devastating consequences on the environment and marine wild-life (Al-Majed et al., 2012). In the last years, bioremediation techniques have been widely studied as an eco-friendly, cost-effective and innovative approach to mitigate oil spills (Mapelli et al., 2017). The use of native microorganisms capable of degrading petroleum HCs ensures the ecological restoration and mineralization of the oil, without additional contamination or introduction of invasive species (Hosokawa et al., 2009). The NW coast of the IP is a hotspot of maritime traffic and oil spills accidents (Vieites et al., 2004), thus making this geographical region a suitable area for a pilot-scale investigation for a potential new bioremediation technique implementation to mitigate oil spills.

In the present work, the distribution and selection of native hydrocarbon-degrading microorganisms in georeferenced sites, along the NW coast of the IP, were investigated. This allowed developing a georeferenced library of native microbial consortia with the capability to degrade petroleum compounds, along the NW coast of the Iberian Peninsula. The georeferenced library was characterized at two taxonomic levels, phylum and genus, and the functional genomic profile was predicted for five metabolic pathways of aromatic compounds' degradation.

Rarefaction curves showed that the biodiversity present in natural microbial communities decreased due to the oil-enrichment experiments. This was also observed for the estimations of both richness and Shannon diversity indexes. Since petroleum is composed by recalcitrant and highly toxic compounds (Varjani, 2017), the crude-oil may have induced a selective pressure onto the natural microbial communities and only a small fraction of microorganisms capable of surviving and/or degrading petroleum HCs were able to adapt. These results were also observed in a study conducted after the Deepwater Horizon oil spill, where a significant decrease in diversity was observed in oil-plume communities when comparing with non-oil plume communities, at depth (Redmond & Valentine, 2011). Ribicic et al. (2018) also obtained a decrease in species richness and Shannon diversity indexes, in microcosms experiments incubated with oil and dispersant for 64 days with natural seawater, collected at a Norwegian fjord, oil and dispersant.

In this work, a decrease in both species richness and diversity of offshore natural microbial communities was observed, when compared to coastal natural communities. The seawater samples of the georeferenced coastal sites were collected in intertidal zones, which are niches rich in nutrients, minerals and organic matter. These components provide the right conditions for biological communities to thrive and develop at intertidal zones, thus resulting

in an increase in species diversity, abundance and biomass (Walag & Canencia, 2016). This might explain the highest richness and diversity estimations observed for natural coastal communities. On the other hand, offshore or shelf environments have limited nutrient concentrations, such as nitrogen and phosphorous, that are needed for the development and growth of microorganisms (Nikolopoulou et al., 2013). The nutrient limitation associated with predation of microorganisms are two of the most important factors that can limit the increase in biodiversity and biomass of natural microbial offshore communities (Azam et al., 1983), which may result in lower richness and diversity estimations in comparison to coastal sites, as observed in the present study.

At the highest taxonomic level, there was a decrease in the number of different phyla from natural microbial communities to oil-enriched communities. After the two-week of microcosm experiments, the native microbial communities were mainly composed by the phylum *Proteobacteria*, with a relative abundance ranging between 56% to 99%, followed by *Bacteroidetes* with a relative abundance between 0.2 and 44%. The results obtained in this thesis, regarding phyla, seem to indicate that a selection of native microbial consortia capable of degrading petroleum hydrocarbons occurred. During enrichments the microcosm flasks were kept under aerobic conditions, as in previous studies that reported that during aerobic biodegradation of HCs, the majority of oil-degrading bacteria are composed by alpha-, beta- and gamma- proteobacteria (Kimes et al., 2014). Also, a study proving the potential for HC degradation by marine microbial communities present in seawater from cold environments, observed a dominance of members of the phyla *Proteobacteria* and *Bacteroidetes* in the oil-enriched microbial communities after 28 days of microcosm experiments, at both 0 and 5°C (Brakstad & Bonaunet, 2006). In addition, for a coastal salt marsh affected by the Deepwater Horizon spill, an increase in relative abundance of *Proteobacteria*, *Bacteroidetes* and *Actinobacteria* was also found in oil-contaminated sediments and once HCs were below the detection limit, their abundance decreased (Beazley et al., 2012).

Since this work is focused on prokaryotic communities, which is composed by the Bacteria and Archaea kingdoms, it is important to mention that results also showed a decrease in relative abundance of two phyla belonging to the kingdom Archaea: *Euryarchaeota* and *Thaumarchaeota*, from natural microbial communities (ranging, respectively, from 0.004-8.2% and 0.01-13.5%) to oil-enriched communities (both with relative abundances between 0-0.2%). Generally, Archaea relative abundance increases by depth in marine environments, so their role in HC degradation in aerobic marine environments is not significant (Brakstad & Bonaunet, 2006; Redmond & Valentine, 2011). Since the seawater samples, for this study, were collected at surface and the oil-enrichment experiments were performed under aerobic conditions, the results obtained seem to be in agreement with the little to none impact of Archaea in the biodegradation of HC occurring in the microcosms. In addition, the research

made by Redmond and Valentine (2011), observed the presence of *Euryarchaeota* and *Thaumarchaeota* in six deep water samples collected at different times, after the DWH accident, which further denotes the importance of Archaea for anaerobic biodegradation of HC.

Ever since the Deepwater Horizon spill disaster, in the Gulf of Mexico, in 2010, there was an increase in metagenomics studies regarding the capability to degrade petroleum compounds by the natural microbial communities (Hazen et al., 2010; Kleindienst et al., 2015 and Dombrowski et al., 2016). The relation between shifts in microbial communities with the capacity to degrade multiple classes of hydrocarbons was proved by the use of ^{13}C -labeled HCs and isolation methodologies (Gutierrez et al., 2013). Regarding the succession of the indigenous microbial communities in the plumes of the DWH, Dubinsky et al. (2013) described that first, an increase in abundance of *Oceanospirillales* and *Pseudomonas* occurred, followed by the dominance of *Colwellia*, *Pseudoalteromonas*, *Cycloclasticus* and *Thalassomonas*. These results were also in agreement with what has been observed in previous studies (Hazen et al. 2010; Valentine et al. 2010; Redmond and Valentine 2011 and Mason et al. 2012). However, this response to an oil spill portrays the scenario for a depth of over 1000m, where high pressure is a factor that must be taken into account because it can inhibit the growth of certain OHCB, such as *Alcanivorax borkumensis* as demonstrated by Scoma et al. (2016).

Regarding the relative abundance at the lowest taxonomic level, oil-enriched microbial communities presented lower number of genera than native microbial consortia. These results are in agreement with the lowest numbers observed for both species richness and relative abundance of phyla for oil-enriched communities. It is also possible to see that the selection of native microbial consortia with the capability to degrade petroleum compounds was successfully obtained, as shown by the increase in abundance of known hydrocarbon-degraders microorganisms. *Alcanivorax* spp., like *A. borkumensis*, is one of the most studied strain that can use aliphatic hydrocarbons and short-chain fatty acids as a principal carbon source for growth and can also produce biosurfactants (Yakimov et al., 1998). *Thalassospira* spp. has the capability to biodegrade several PAHs such as naphthalene, phenanthrene and fluorine (Kodama et al., 2008). *Pseudomonas* spp., such as *P. aeruginosa* DQ8, was found to degrade alkanes and PAHs, like, for example, fluorine, phenanthrene, pyrene and dibenzothiophene (Zhang et al., 2011). The genera *Flavobacterium* and *Corallibacter*, belonging to the *Flavobacteriaceae* Family, have the capability to degrade fluorobenzene, phenol and chlorinated hydrocarbons (Kaplan & Kitts, 2004). A study conducted in microcosms experiments with coastal waters spiked with paraffinic petroleum for 12 days, under aerobic conditions, showed an efficiency in biodegradation of the crude-oil of 57%, by *Flavobacterium* spp. (Atlas & Bartha, 1972). However, the potential to bioremediate HCs by the genus *Corallibacter* is still under evaluated. Regarding *Acinobacter* spp. it was observed

that these organisms can produce biosurfactants compounds and that they also harbour genes encoding the enzymatic mechanisms required for *n*-alkane oxidation (Chen et al., 2012; Di Cello et al., 1997). For example, *Acinetobacter venetianus* was capable of using fuel-oil and *n*-alkanes (C_{10} , C_{14} and C_{20}) as a unique carbon source (Di Cello et al., 1997). *Marinobacter* genus, like *M. hydrocarbonoclasticus*, has the potential to degrade a large variety of aliphatic and aromatic HCs and it can also produce biosurfactans (Gauthier et al., 1992).

Other OHCB were not enhanced by the oil-enrichment experiments performed in the present study. However, they were present in some natural microbial communities, from areas 1, 2 and 3, with relative abundances ranging approximately from 0-0.2% for *Cycloclasticus* spp., 0-0.3% for *Oleispira* and 0-0.015% for *Oleiphilus*. These findings showed the potential of natural microorganisms communities, along the NW coast of the IP, to degrade petroleum hydrocarbons but our microcosms enrichments failed in enhancing their abundance. For example some restraints in enhancing the abundance of *Cycloclasticus* spp. might have been due to the fact that bacteria from this genus are specialized in degrading more complex HC (Kasai et al., 2002). Since the enrichment experiments were only performed for two weeks, maybe the biodegradation rate of petroleum compounds did not reach the HC more difficult to degrade and the lack of specific substrate inhibited its growth. Regarding the lack of increase in *Oleispira*, after crude-oil enrichment experiments, might be due to the room temperature at which the microcosms were exposed (20°C), since an increase of organisms from this genus was only observed for microcosms maintained at 4°C (Coulon et al., 2007).

A predisposition of differentiation between natural microbial communities collected at intertidal zones (coast) and offshore locations was observed in this work, regarding the distribution of the most relevant prokaryotic genera. The differences between these two types of habitat, as mentioned above, can have influence in creating a distinction pattern between microbial communities. Nevertheless, the geographical locations of the offshore sampling sites collected for this work are highly influenced by two rias, Ria of Vigo and Ria of Pontevedra. These type of water masses have an estuarine behaviour, which results in an export of high content in organic matter and nutrients to nearby shelf areas (Bode & Varela, 1998; Pérez et al., 1992). This constant influx of organic content and nutrients to offshore locations can promote the growth of some microorganisms that may be adapted to thrive in different physiochemical conditions, such as temperature, salinity and pH. The type of habitat associated with the discharge of organic matter and nutrients from the two rias, can be a probable cause to explain why the two different types of sampling locations (coastal and offshore) do not present a clearly distinct pattern among the natural microbial consortia.

In this study, the influence of seasonality could not be assessed for all stations but only for the sampling sites that were collected during two or three consecutive seasons. Here it

was possible to observe a tendency for a shift in composition in both natural and oil-enriched communities. These results may be due the establishment of northerly winds, from June to September, that allow for a cold deep nutrient rich water mass, known as Eastern North Atlantic Central Water, to reach the surface creating a seasonal thermocline throughout the Atlantic coast of the IP, which can result in an increase in biodiversity (Alvarez et al., 2013; Cordeiro et al., 2018). Afterwards, a relaxation period occurs, where coastal temperatures became warmer and lower in salinity (Cordeiro et al., 2018). This period also corresponds to lower light hours therefore affecting the cycles and growth of microbial communities. Interestingly enough, the months with cyclic variations described above were not in agreement with the results observed for species richness and diversity, in both natural and oil-enriched communities, where, overall, higher estimations occurred in winter and autumn samples. Several factors may be at display such as ships' discharges due to the close location to ports, or maybe due to the effect of climate change, which can be slowly shifting the duration of the four seasons and, also, might cause moderate differences in seawater temperature thus influencing the population growth and ecosystem processes (Hoegh-Guldberg & Bruno, 2010).

The functional profiling of natural and crude-oil enriched microbial communities showed that both had the potential to biodegrade some aromatic compounds, such as naphthalene, toluene, PAHs, ethylbenzene and xylene, based on KOs abundance known to encode enzymatic mechanisms involved in the degradation metabolisms of these HCs. Moreover, there was a clear increase in abundance of some KOs present in the selected microbial consortia, including some that are involved in the degradation of alkanes, PAHs, naphthalene, toluene, xylene or ethylbenzene. This goes in agreement with the increase in abundance of certain microorganisms with the necessary metabolic pathways to degrade different aromatic compounds obtained in this work, as described above. The correlation between the abundance of certain KOs with the presence of different microorganisms that are known to degrade different types of HC, was observed in a study comprising 16S rRNA gene NGS data obtained during experiments on different environments polluted with HC (Mukherjee et al., 2017). In different environments, several microorganisms contributed to the abundance of the same functional genes due to microbial functional redundancy. For example, in DWH samples, the abundance of the gene K00496, which encode the protein alkane-1-monooxygenase, was attributed to the occurrence of *Alteromonadaceae*, although in taiga environments *Acetobacteraceae*, *Mycobacteriaceae*, *Nocardiaceae* and *Rhodospirillaceae* were the main contributors for the occurrence of this gene and in sandy cores samples the most responsible group for the occurrence of this gene was *Propionibacteriaceae*. Also, the abundance of the genes K04100-01 involved in the degradation of PAH, was mainly related with the occurrence of *Alteromonadaceae* in DWH samples, *Comamonadaceae* and

Bradyrhizobiaceae in taiga samples and *Comamonadaceae* and *Methylobacteriaceae* for oil sand core samples (Mukherjee et al., 2017).

In the present study, it was also observed a significant decrease in the abundance of some of the KOs present in natural microbial communities, during the enrichment with crude oil. Since some of these KOs are also found in other biological metabolic pathways, the abundance of several KOs observed in the natural microbial communities may portray the metabolic mechanisms involved in basic cell functions. Therefore, the decrease in their abundance, after petroleum enrichments communities, can be a response of the natural microbial communities to the stress induced by the enrichment conditions, which resulted in the selection of native microorganisms with the potential to degrade petroleum compounds. Moreover, despite the lack of information regarding the metabolic pathways of aliphatic degradation, these results are in agreement with the previous knowledge about the biodegradability of hydrocarbons, which starts first with *n*-alkanes, followed by branched-chain alkanes, branched alkenes, monoaromatics (such as toluene, xylene and ethylbenzene), cyclic alkanes, PAHs and then asphaltenes (Tyagi et al., 2011). Notwithstanding the lack of information regarding the composition of the petroleum used in these experiments, the fact that it was a light crude-oil may indicate a higher content in aliphatic compounds and lower content of aromatic compounds. Therefore, the decrease in abundance of KOs involved in the five aromatic compounds degradation may indicate that, during the 15 days of the enrichment experiments, the main class of hydrocarbons being biodegraded were aliphatic compounds due to their higher bioavailability.

Medina-Bellver et al. (2005) evaluated the presence of indigenous microorganisms capable of degrading some PAHs compounds and the saturated alkane undecane, in seawater from Vigo and Ferrol, Spain. Their results showed highest abundances of undecane, naphthalene and phenanthrene degraders for heavily and partially contaminated samples, while non-contaminated sites presented lower amounts of degraders. Since this study also includes stations from Vigo and Ferrol, it is possible to infer that the selected microbial communities could also have the potential to degrade some aliphatic and aromatic compounds.

The hypothesis described above goes in agreement with the results from a previous study (Almeida et al., 2013) that described that, after a two-week enrichment experiment, with Arabian light crude-oil and addition of nutrients, it was possible to observe a considerable amount of degradation of *n*-alkanes. However, the total petroleum hydrocarbons concentration was still high which might indicate that more complex hydrocarbons might have been present in the samples (Almeida et al., 2013).

In this work, the hydrocarbon-degrading microbial abundance was estimated by the MPN method, for both natural and oil-enriched microbial communities, showing that all

sampled sites present capacity to degrade petroleum hydrocarbons. Despite the low abundances obtained (ranging between $0-2.47 \times 10^3$ MPN/mL), natural microbial communities had hydrocarbon degrading microorganisms in their composition, so it was expected that by the end of the crude-oil enrichments their abundance would increase, as it was registered. This further proves that the workflow developed for this work successfully selected native microbial consortia with the capability to degrade hydrocarbons.

All statistical analysis performed, in this study, indicated that the different sampled areas present no significant differences, neither in terms of the natural microbial communities nor in terms of the selected microbial consortia. This outcome was not unexpected because the NW coast of the Iberian Peninsula is an oceanographic location well characterized by its strong currents and winds. This region is under the influence of the oceanographic Iberian Poleward current that flows over the continental slope (Cordeiro et al., 2018), which might explain the relative homogeneity observed among the communities collected along the NW coast of the IP.

It is important to mention that the present work has some limitations. Regarding the observed difference between offshore and coastal site, only five offshore sites were collected and only in the Area 2, so it is not possible to extrapolate the conclusions to the entire region. Regarding the workflow developed for the selection of native microbial consortia with the capability to degrade hydrocarbons, this was not able to enrich some microorganisms known to use hydrocarbons as a sole source of energy, such as *Cycloclasticus*, *Oleispira* and *Oleiphilus*, that were present in the natural microbial communities. In addition, the lack of predicted metabolic pathways for aliphatic degradation did not allow obtaining the potential “big picture” regarding hydrocarbon-degradation. Moreover, the PICRUST pipeline only provided the first 15039 KOs therefore, the KOs analyzed in this work do not translate the complete functional profiling of the microbial communities and their full potential to degrade other petroleum compounds. Despite the development of an accessory pipeline to obtain the remaining KOs (information available online: http://picrust.github.io/picrust/tutorials/genome_prediction.html#genome-prediction-tutorial), this was not yet automatized and it would imply a great computational effort.

When oil spills disasters occur, the indigenous microbial communities undergo through a succession of different strains of microorganisms, according to the bioavailability of different hydrocarbons in the environment. After the oil spill, the native communities have the capacity to return to composition similar to those initially present before the pollution event (Dubinsky et al., 2013). Therefore, the use of native microbial consortia to bioremediate an oil spill will help to accelerate the process of selection and succession, that already naturally happens as a response to the stress induced by the contaminant, and afterwards the microbial

communities will have the capacity to restore to its pre-oil composition due to the high resilience capacity of microbial communities.

Overall, the combined action of different microorganisms capable of degrading petroleum compounds is the best approach to mitigate oil spills, since different organisms can biodegrade different classes of hydrocarbons (Varjani, 2017). The aim of using native microbial consortia capable of degrading petroleum compounds is to avoid the introduction of exogenous microorganisms and to increase the bioremediation efficiency by using organisms already adapted to the environmental conditions of the oil-contaminated site.

This master thesis work is included in the project SpilLess – First line response to oil spills based on native microorganisms' cooperation (<https://spilless.ciimar.up.pt/>) that aims to develop a novel, fast and efficient approach to mitigate oil spills by using native microbial cooperative consortia with the capability to degrade petroleum compounds to increase bioremediation efficiency. This work demonstrates that microorganisms with the capability to degrade petroleum hydrocarbons are widely distributed along the NW coast of the Iberian Peninsula and it was possible to successfully select them to create native microbial consortia with the potential to degrade petroleum compounds. The georeferenced library of native microbial consortia developed in this work will provide an advantageous addition to produce efficient and well-adapted consortia of microbial hydrocarbon degraders specifically designed to be applied in a particular site contaminated by an oil spill.

5. Conclusion and future perspectives

Native microbial communities with the capacity to biodegrade petroleum compounds are widely distributed along the NW coast of the Iberian Peninsula. The intrinsic capacity of these communities to use hydrocarbons as a source of carbon and energy can be enhanced by bioremediation approaches.

With this work, a georeferenced library of autochthonous microbial consortia with the potential to biodegrade petroleum compounds was developed. The selected native microbial consortia were mainly composed by *Alcanivorax* spp., *Thalassospira* spp., *Pseudomonas* spp. and by genera of the family *Flavobacteriaceae*. The functional profiling indicated that these selected consortia have the potential to degrade the aromatic compounds naphthalene, ethylbenzene, PAHs, toluene and xylene.

Towards the development of an efficient hydrocarbon-degrading consortium, a shotgun metagenomics analysis should be further applied to fully understand which genes are present in the native microbial communities and select microorganisms with higher bioremediation efficiency.

These consortia should further be tested at oil spill simulations in mesocosm experiments, to prove the bioremediation efficiency of this approach and to allow the transference of this technology to be implemented by the authorities responsible for the remediation of environments impacted by an oil spill.

The application of indigenous microbial consortia as a bioremediation approach will provide an eco-friendly, efficient and economic technique to mitigate oil spill disasters, that will also allow to restore the ecosystems' functions.

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