Universidade de Aveiro Departamento de Biologia
2017

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Descontaminação de petróleo por comunidades microbianas bênticas

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Tese apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Doutor em Biologia, realizada sob a orientação científica da Doutora Maria Ângela Cunha, Professor Auxiliar do Departamento de Biologia da Universidade de Aveiro, do Doutor Newton Gomes, Investigador Principal do Centro de Estudos do Ambiente e do Mar (CESAM) e do Doutor Mário Simões, Professor Auxiliar do Departamento de Química da Universidade de Aveiro.

Este trabalho foi financiado pelas unidades de investigação CESAM (UID/AMB/50017/2013) e QOPNA (UID/QUI/00062/2013)

António Louvado recebeu apoio financeiro da Fundação para a Ciência e a Tecnologia (FCT) e do Fundo Social Europeu financiado por verbas nacionais e verbas do Fundo Social Europeu através do Programa Operacional Potencial Humano do QREN sob a forma de Bolsa Individual de Doutoramento (SFRH/BD/86447/2012)

Dedico este trabalho ao meu pai.

o júri

presidente

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agradecimentos

Este trabalho nunca seria possível sem a ajuda dos outros. Assim sendo, quero expressar os meus mais sinceros agradecimentos a alguns que permitir concluir com sucesso esta tese.
Aos colegas de laboratório do LEMAM e LMAA pela vossa disponibilidade, conselhos e boa disposição. Em especial, aos meus colegas no LEMAM pela ajuda na realização do ensaio no ELSS: Ao Dr. Francisco Coelho pelo incentivo dado para a realização do ensaio no ELSS e pela ajuda em todos os passos da execução do mesmo. À Dr.ª Vanessa Oliveira pela sua disponibilidade e sua partilha de experiência na análise quantitativa dos hidrocarbonetos. Ao coorientador Dr. Newton Gomes pela sua dedicação, incentivo constante e crítica construtiva durante todo processo de realização da tese. Ao coorientador Dr. Mário Simões pela sua disponibilidade, ajuda e apoio em todo trabalho realizado na análise quantitativa de hidrocarbonetos à Dr.ª Ângela Cunha pela oportunidade dada e pela ajuda disponibilizada.
Queria também agradecer ao Dr. Francisco Coelho e Dr. Newton Gomes o empréstimo do sistema de microcosmos: "Experimental Life Support System"; desenvolvidos por estes em colaboração com o Dr. Rui Miranda e financiado pela Fundação para a Ciência e Tecnologia (FCT) através do projeto Global Changes (PTDC/BIA-MIC/6473/2014).
Em último, quero agradecer aos meus pais e em especial à Raquel pelo vosso apoio constante durante estes longos 4 anos e meio.

palavras-chave

Biodegradação de hidrocarbonetos, Communidades bacterianas, Sedimentos do mar profundo, Sedimentos estuarinos, Acidificação dos oceanos, Dispersantes químicos.

resumo

Os sedimentos marinhos são um reservatório de hidrocarbonetos petrogénicos libertados naturalmente ou acidentalmente para o ambiente marinho. Nos sedimentos marinhos, os hidrocarbonetos são usados como fonte de carbono e energia por comunidades bacterianas complexas. Contudo, a eficiência de biodegradação poderá ser limitada por fatores ambientais. Este trabalho aborda o previsível impacto das condições particulares do mar profundo, da acidificação dos oceanos e da adição de dispersantes químicos nos processos de biodegradação de hidrocarbonetos em ambientes marinhos. Numa primeira fase, a função de destoxificação primária das bactérias degradadoras de hidrocarbonetos aromáticos policíclicos (HAP) nos sedimentos do mar profundo foi avaliado através de uma compilação de informação disponível na literatura científica e também através de uma análise dependente do cultivo envolvendo culturas de enriquecimento de sedimentos de vulcões de lama do mar profundo. Posteriormente, o impacto interativo da acidificação do oceano e da contaminação por hidrocarbonetos petrogénicos em comunidades bacterianas bênticas foi avaliado, em experiências de simulação multifatorial em sistema de microcosmo previamente executadas, com sedimentos subsuperficiais estuarinos. Finalmente, foi executado uma experiência multifatorial em sistema de microcosmos para avaliar o impacto da aplicação de dispersantes químicos em situações simuladas de derrame de hidrocarbonetos em sedimentos estuarinos portuários.

Os resultados obtidos, através da análise da fração cultivável, indicam que nos sedimentos do mar profundo a comunidade bacteriana degradadora de HAP é distinta da encontrada noutros sedimentos marinhos devido à predominância de bactérias relacionadas com o género *Bacillus*. Nos ensaios de microcosmos, apesar das diferenças entre os cenários testados, as comunidades bacterianas revelaram-se em geral, estáveis. Nos sedimentos subsuperficiais estuarinos, as alterações abióticas impostas foram provavelmente atenuadas pela barreira sedimentar sobrejacente e a comunidade bacteriana pareceu ser estável em termos de estrutura e atividade. Do mesmo modo, a dispersão química de hidrocarbonetos petrogénicos, apesar de aumentar a biodisponibilidade de PAH, não alterou significativamente a composição das comunidades bacteriana de sedimentos superficiais estuarinos. Possivelmente, a exposição prévia do sedimento portuário a poluição por hidrocarbonetos poderá ter condicionado a resposta da comunidade bêntica bacteriana à contaminação por petróleo.

Em conclusão, a degradação bacteriana de hidrocarbonetos é um processo ubíquo em sedimentos marinhos e as comunidades bacterianas degradadoras revelam elevada estabilidade relativamente à variação de fatores ambientais.

keywords

Oil hydrocarbon biodegradation, Bacterial community, Deep sea sediments, Estuarine sediments, Ocean acidification, Chemical dispersants.

abstract

The marine sediment compartment is a key sink for naturally and accidentally released oil hydrocarbons in the marine environment. Here, complex communities of interacting bacterial species will efficiently use oil hydrocarbons as sources of carbon and energy. However, the efficiency of the biodegradation process can be limited by some near-future scenarios. This work addresses different environmental scenarios regarding oil hydrocarbon biodegradation in marine sediments. First, the role of bacteria as primary detoxifiers of polycyclic aromatic hydrocarbons in deep-sea sediments was evaluated through the compilation of available data and through a culture-dependent analysis of enrichment cultures derived mud volcano sediments. Next, the impact of the interactive effects of ocean acidification and oil hydrocarbon contamination was further analyzed in subsurface estuarine sediments. Finally, the impact of chemically dispersed oil in estuarine port sediments is evaluated through a multi-factorial microcosm simulation.

Results show that , in deep sea mud volcano sediments, the culturable fraction of the PAH-degrading bacterial community seems distinct from other environments, with a predominance of *Bacillus*-like bacteria. In the microcosm-based assays, despite the differences between them, the overall bacterial community exhibit a reliable stability. In subsurface sediments, abiotic changes tested were possibly attenuated by the superficial sediment barrier and bacterial seem stable to environmental changes. Also, the chemical dispersion of oil, despite enhancing PAH concentration, did not impose significant alterations to the bacterial community composition at the marine sediment surface. The potential pre-exposure of the port sediment to oil hydrocarbon pollution may have preconditioned the response of the benthic bacterial communities to oil contamination.

In conclusion, oil-hydrocarbon biodegradation is ubiquitous and communities exhibit a structural stability to environmental changes**.**

Contents

List of Publications

Chapter I - Louvado A.; Gomes N.C.M.; Simões M.M.Q.; Almeida, A.; Cleary, D.F.R. and Cunha; A. Polycyclic aromatic hydrocarbons in deep sea sediments: Microbe-pollutant interactions in a remote environment. The Science of the Total Environment 2015; 526: 312-328.

Chapter III - Louvado, A.; Coelho, F.J.R.C.; Gomes, H.; Cleary, D.; Cunha,A.; Gomes,N.C.M.; Independent and interactive effects of reduced seawater pH and oil contamination on subsurface sediment bacterial communities. Manuscript submitted for revision.

Chapter IV - Louvado,A.; Coelho, F.J.R.C.; Oliveira,V.; Gomes,H.; Simões, M.M.Q.; Cunha, A.; Gomes, N.C.M.; Microcosm evaluation of the impact of oil contamination and chemical dispersant addition on bacterial communities in estuarine sediment. Manuscript submitted for revision

List of Abbreviations

AMV - Active mud volcano
ANOVA - Analysis of Variance
aPAHs - Alkylated polycyclic aromatic hydrocarbons
ARD - Aromatic ring dioxygenase
BAF - Bioaccumulation factor
BLAST - Basic local alignment search tool
CPI - Carbon preferential index
DNA - Deoxyribonucleic acid
DSS - Deep sea sediments
DSW - Deep sea water
DWH - Deepwater horizon oil spill
EC- Enrichment culture
ELSS - Experimental life support system
EPA - Environmental Protection Agency
EPS - Extracellular polymeric substances
HGT - Horizontal gene transfer
HHP - High hydrostatic pressure
HMW - High molecular weight
IARC - International Agency for Research on Cancer
IMV - Inactive mud volcano
Koc - Soil organic carbon-water partitioning coefficient
Kow - Octanol-water partitioning coefficient
LMW - Low molecular weight
LT - Low temperature
MGE - Mobile genetic elements
MV - Mud volcano
NCBI - National Center for Biotechnology Information
OA - Ocean acidification
OTU - Operational taxonomic units
PAH - Polycyclic aromatic hydrocarbons
PCO - Principal coordinates analysis
pCO_2 - CO_2 partial pressure

PCR - Polymerase chain reaction

PEEZ – Portuguese Exclusive Economic Zone

PP - Primary production

QIIME - Quantitative insights into microbial ecology

RDP - Ribosomal database project

RNA - Ribonucleic acid

rRNA - Ribosomal ribonucleic acid

RS - Reference site

TAE - Tris Acetate EDTA buffer

TEF -Toxicity equivalent factor

UNIPROT - Universal protein resource

UV - Ultraviolet

UVR - Ultraviolet radiation

List of Tables

List of Figures

Justification, Objectives and Thesis Outline

Justification, Objectives and Thesis Outline

Justification

The marine sediments and the benthic microbial communities are, respectively, an important sink and fate for oil hydrocarbons released into the environment from e.g. natural seepage, the accidental discharge of petroleum and its refined by-products and the deposition of hydrocarbonous soot. The extensive documentation of underwater seepage systems and of the most catastrophic oil spills (and the research and technological development they have sparked), has progressively enlarged our knowledge on the biodegradation of oil hydrocarbons at marine sediments. However, much is still unknown concerning the metabolic turnover of oil hydrocarbons at the marine sediments and new challenges that have or will emerge, will bring new disruptions to the system. For example, although natural seepage systems might be the most significant source of oil hydrocarbons in the ocean, a substantial fraction of the seeped oil will be degraded immediately at the sediment layer by the local microbial communities. However, the functional role of its heterotrophic members is frequently unknown and considering the extreme abiotic conditions present in these environments, the bacteria involved in oil hydrocarbon degradation here may be useful in biotechnological applications. Also, the rise of carbon dioxide emissions is projected to reach unprecedented rates, indirectly acidifying the world ocean. This phenomenon, in combination with oil hydrocarbon pollution, has previously been shown to alter the core composition of the active microbial communities at superficial estuarine sediments, potentially affecting oil hydrocarbon biodegradation. Yet, at the underlying subsurface sediments, where inhumed oil hydrocarbons can persist for decades because of a slow and thermodynamically unfavourable anaerobic biodegradation, this effect has never been demonstrated. Furthermore, chemical dispersants, although frequently used in the context of emergency responses to oil spill, are controversial since their net benefit is uncertain. Their application, in contrary to expectations, has been shown to suppress oil hydrocarbon biodegradation and increase oil hydrocarbon transfer to sediments, where its impact has never been evaluated.

Objectives

This thesis will broaden our comprehension on the effects of oil hydrocarbon contamination on sediment bacterial communities and its removal by bacterial biodegradation in marine sediments. More specifically, three main objectives are proposed:

- To determine the bacteria potentially involved in the biodegradation of polycyclic aromatic hydrocarbons at deep sea mud volcano sediments.
- To determine if the surface sediment barrier attenuates the impact of the interactive effects of oil hydrocarbon contamination and acidified seawater on subsurface benthic bacterial community composition.
- To determine the effect of chemical dispersion of oil contamination on the fate of oil hydrocarbons and on the bacterial community composition from estuarine sediments obtained from a chronically contaminated port area.

These proposed objectives will be addressed in Chapter II, Chapter III and Chapter IV, respectively.

Thesis Outline

Chapter I is composed of two parts. In the first part, a brief and informative literature review of topics that are considered essential to the understanding of the subsequent chapters is presented. In the second part, existing information regarding the role of the deep sea sediments as a potential sink for oil hydrocarbons in marine environments is summarized. Here, the interactions between recalcitrant polycyclic aromatic hydrocarbons (PAHs), deep sea sediments, and the local bacterial community are examined, with emphasis on the functional role of bacteria in the fate of these pollutants considering both current and future climate conditions.

Chapter II analyzes the cultivable fraction of the polycyclic aromatic hydrocarbon degrading bacterial community from *in vitro* enrichment cultures with added phenanthrene and chrysene as the predominant carbon sources. Results show that the culturable fractions of the enrichment cultures were dominated by *Bacillus*-like isolates. This result was unexpected since *Bacillus*-like isolates are not commonly detected as PAH-degraders in marine environments but is coherent with some studies from similarly extreme environments.

Chapter III examines the interactive and independent effects of oil hydrocarbon contamination and reduced seawater pH on the core bacterial community composition of subsurface estuarine sediments at two time points. Here, the bacterial community from the subsurface sediments involved in a previously executed multi-factorial microcosm simulation was analyzed through a 16S gene-based mass sequencing approach. Results revealed that, despite some taxa-specific alterations, the overall bacterial community was stable to the factors tested, which contrasts with indications from the most superficial sediments. It is speculated that the super-adjacent sediment barrier may function as a buffer, thus attenuating the effects of both oil hydrocarbon pollution and reduced seawater pH.

Chapter IV examines, for the first time, the potential impact of chemically-dispersed oil on the benthic bacterial community composition in estuarine superficial port sediments. A multi-factorial microcosm simulation was planned and executed to test the interactive and independent effects of oil

hydrocarbon contamination and dispersant addition. The results obtained indicate the chemical dispersion of oil increased the mass transfer of PAH to the sediment phase during the experiment and altered the relative abundance of some lesser abundant putative hydrocarbon-degrading bacteria. However, the overall bacterial community was not affected by t the factors independently and in interaction.

Chapter I

Chapter I

Bacterial degradation of hydrocarbons in marine environments

Oil hydrocarbon contamination in marine environments can be derived from both natural and anthropogenic sources. Naturally occurring seeps are responsible for an estimate of 47% of the total oil hydrocarbons released into the ocean [(Kvenvolden and Cooper 2003); Figure I-1]. However, since seep distribution may be substantially underestimated, their ultimate contribution may be higher (Kvenvolden and Cooper 2003). Despite their magnitude, a substantial fraction of oil hydrocarbons emitted are totally consumed at the seabed by the local microbial communities, which transfer the assimilated energy and carbon into the higher trophic levels, ultimately having a fertilizing effect on the local ecosystem (Coelho et al. 2016b; D'souza et al. 2016; Hovland and Thomsen 1989). Anthropogenically-derived oil hydrocarbons in marine environments are expected to increase concurrently to the global rise in oil consumption (International Energy Statistics 2015; Transportation Research Board and National Research Council 2003). Although the stricter maritime vessel regulations, approved in the last decades, have considerably reduced oil tanker related spills, new threats are emerging [e.g. riskier offshore oil extraction projects, a deteriorating infrastructure (Jernelöv 2010) and the anarchic regulation of vessels by some flag states (Miller et al. 2015)]. The recent 2010 Deepwater horizon oil spill (DWH) is an example that major marine oil spills can recur and still be catastrophic. Also, the continuous release of oil hydrocarbons through minor spills that may occur during production, transport, refining and storage of petroleum or its derived products, and through the atmospheric deposition of combusted hydrocarbonous soot is also likely to increase [(Transportation Research Board and National Research Council 2003); Figure I-1]. These sources can chronically contaminate coastal marine environments sited near high density urban and industrial areas, seaports and at the mouth of major rivers (Lipiatou et al. 1997; Telli-Karakoç et al. 2002). Here, hydrocarbon concentrations can be similar or even reach values 100 times higher (He et al. 2014; Telli-Karakoç et al. 2002) than those measured in sediments surrounding the DWH epicentre, one year after blowout (Liu et al. 2012b).

The Portuguese exclusive economic zone (PEEZ), given its central location in the north Atlantic, is susceptible to major oil hydrocarbon events. The PEEZ is both a passageway, in the Atlantic Ocean, for vessels moving north to south and east to west, and vice-versa. More importantly, the coastline of mainland Portugal is located near a vessel corridor, with intense traffic, that links the North Atlantic, North Sea and Baltic Sea to the Mediterranean Sea through the Gibraltar strait. The susceptibility of this region to oil spills is further aggravated by the rough sea conditions found during

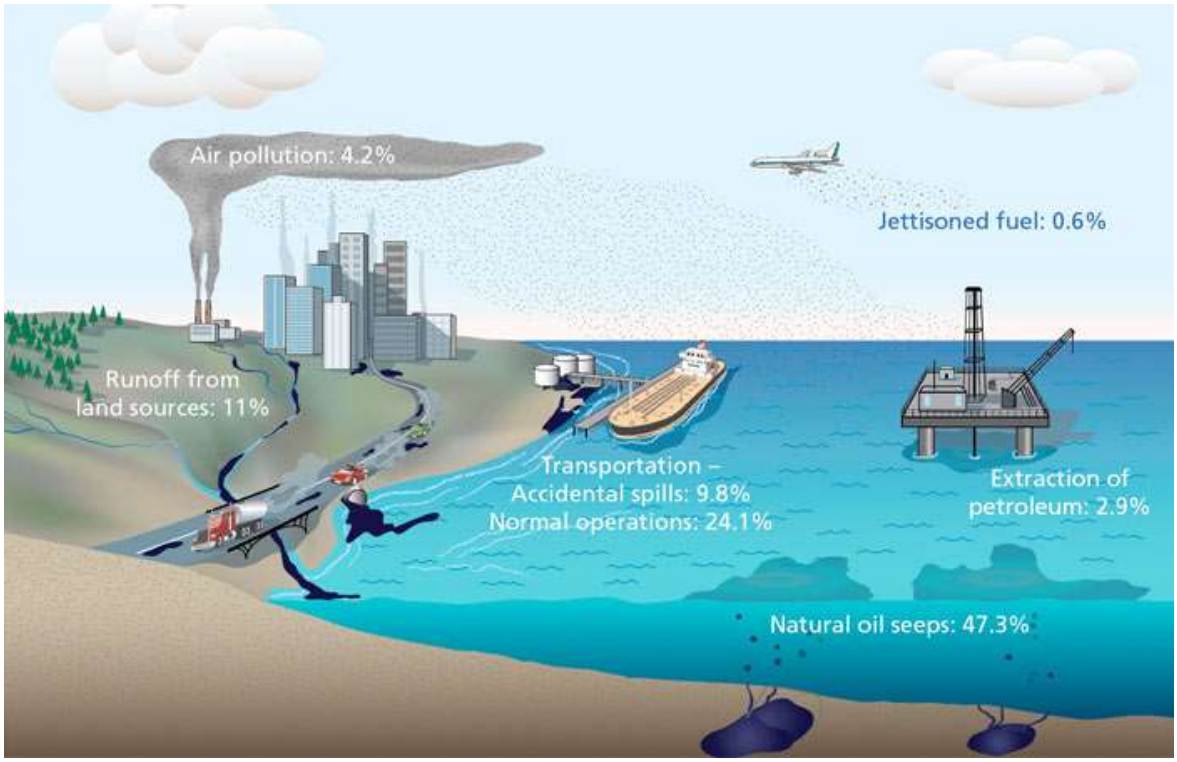

Figure I-1 - Sources of oil in the ocean. Copyright of the Woods Hole Oceanographic Institution. Used with permission. (Woods Hole Oceanographic Instituition 2011)

the winter (Otero et al. 2014). The Iberian western coast has experienced 4 of the 20 largest tanker related oil spills, namely the Jacob Maersk (1975), the Uriquiola (1976), the Aegean Sea (1992) and the most recent Prestige (2002) (ITOPF 2013).

Major oil spills, although not the main source of oil hydrocarbons in marine environments (Figure I-1), are catastrophic to local environments since they release a substantial amount of oil in a restricted location. To minimize their socio-economic and environmental impact and/or accelerate restoration, environmental and civil protection agencies employ a diverse array of oil spill response strategies. First response frequently relies on the physical containment and the quick recovery of spilled oil, by use of boomers and skimmers, respectively. These strategies are environmentally benign, but they are ineffective in harsh meteorological conditions and when spilled oil is substantial and/or outspreaded. In these situations, biodegradation by autochthonous heterotrophic microorganisms will be the most relevant removal process for oil hydrocarbons (Head et al. 2006). Nonetheless, if not intervened, the depletion of some essential metabolic factors (e.g. nutrients and oxygen) and/or the limited bioavailability of the substrate may limit and retard biodegradations full potential. The use of bioremediation strategies will attempt to enhance and accelerate this natural biodegradation by countering its limiting factors. The application of chemical dispersants is the most frequently employed

strategy (National Research Council 2005; Prince 2015). Nonetheless, its use is controversial among scientists (Kleindienst et al. 2015a; Kleindienst et al. 2016; Prince 2015; Prince et al. 2016) since its benefits may not compensate its increased environmental impact. Chemical dispersants will promote the formation and the stabilization of micron-size oil-in-water droplets that will increase oils bioavailability and buoyantly entrain the micelles in the water column, thus restraining them from reaching the seabed. However, although an increase in bioavailability will undoubtedly benefit biodegradation, it may also increase the exposure of the marine biota to the toxic constituents of oil (Barron et al. 2003; Shimada and Fujii-Kuriyama 2004; Wolfe et al. 2001). Chemically dispersed oil has been repeatedly measured to be more toxic to marine biota in general (Almeda et al. 2014; Anderson et al. 2014; Barron et al. 2003; Gardiner et al. 2013; Goodbody-Gringley et al. 2013; Özhan et al. 2014; Rico-Martínez et al. 2013) than non-dispersed oil and has, inclusively, been found to suppress the activity of hydrocarbon-degrading bacteria (Kleindienst et al. 2015b). This enhanced toxicity has been correlated with the concentration of polycyclic aromatic hydrocarbons(PAHs) in the water-accommodated fraction (Gardiner et al. 2013; Özhan et al. 2014; Radniecki et al. 2013), which are known to be enhanced by the application of chemical dispersants (Gong et al. 2014b; Zhao et al. 2016). Also, since biodegradation may instead be limited by nutrient (e.g. nitrogen, phosphorus and iron) and/or oxygen deficiencies, the unnecessary application of dispersant can further delay biodegradation by augmenting the carbon ratio in the system (Kleindienst et al. 2015a). In this situations, the exogenous supplementation of deficit nutrients (biostimulation) and oxygenation has proved to be more beneficial (Coulon et al. 2007).

Oil is a complex carbon-rich mixture of hydrophobic constituents which can be divided into four main classes, based on structural properties: aliphatics, aromatics, resins and asphaltenes. Each class has distinct physico-chemical properties that will influence their ultimate environmental fate. The constituents of each class can be progressively divided based on their volatility, solubility and biodegradability. Because of this physico-chemical heterogeneity, spilled oil will, with time, change its composition in a process known as weathering. Through weathering, most volatile and most soluble oil constituents will progressively evaporate and dissolve. Also, biodegradation will act on oil hydrocarbons differentially, with mid-range saturated linear aliphatic hydrocarbons (*n*-alkanes) being preferentially biodegraded than, for example, the PAHs and the alkylated aliphatic hydrocarbons (*iso*-alkanes) (Garrett et al. 1998). Eventually, weathering will reduce oil to a tar remnant, composed by its heaviest and most recalcitrant constituents that, by adhering to suspended particulate matter, may settle at the sediments (Gong et al. 2014a). PAHs, in particular, will be enriched in this remnant. PAHs are problematic environmental pollutants, mainly because their partial metabolism and photooxidation originates oxygenated and highly reactive metabolites (Aeppli et al. 2012; Knecht et al. 2013; Lee 2003; Toyooka and Ibuki 2007). Consequently, PAHs are considered carcinogenic, mutagenic and overall toxic to humans (IARC Working Group on the Evaluation of Carcinogenic Risks to Humans 2010) and, thus,

their concentrations in soil and water are routinely monitored and regulated by public environment and food safety agencies (Code of Federal Regulation 2015; Lerda 2010).

Despite environmentally nefarious, oil hydrocarbons are a rich source of carbon and energy for the heterotrophic microorganisms capable of degrading them. However, their degradation is limited by poor bioavailability and low chemical reactivity (Widdel and Musat 2010). Therefore, oil hydrocarbon-degrading microorganisms will need to dispend some energy to counter these limitations (Rojo 2009; Widdel and Musat 2010). For example, by producing biosurfactants , oils bioavailability will increase and the transmembrane uptake of oil hydrocarbons by microorganisms will be facilitated (Bouchez et al. 1997; Bouchez Naïtali et al. 1999; Chrzanowski et al. 2012; Noordman and Janssen 2002). In marine sediments, biosurfactants may further enhance the desorption of oil hydrocarbons from sediment particles (Mnif et al. 2014; Nakazawa et al. 2016). Also, due to the absence of a functional group, that would facilitate the enzymatic cleavage of their carbon bonds, in hydrocarbon molecule, oil hydrocarbon-degrading microorganisms are required to perform an initial metabolic endergonic reaction that would lead to the formation of an alcohol functionality (Himo 2005; Widdel and Musat 2010). This initial oxygenation involves the activity of oxygenases and requires molecular oxygen as a reactant (Seo et al. 2009). It will increase the chemical reactivity of the oil hydrocarbon (Widdel and Musat 2010), and from here a subsequent carboxyl group is enzymatically obtained and the carbon-bond can be cleaved (Seo et al. 2009). Although it involves a slight input of energy from the bacteria, this oxygenation step is crucial in oil hydrocarbon biodegradation since, in the end, the energy yield is substantially higher in comparison to that calculated for direct carboxylation (Rojo 2009; Seo et al. 2009; Widdel and Musat 2010). However, since required enzymes are not widespread among microorganisms, as in comparison to those for subsequent steps, the initial oxygenation step can be a bottleneck in oil hydrocarbon biodegradation (Rojo 2009; Seo et al. 2009).

Oil hydrocarbons, due to their hydrophobicity and consequent immiscibility with water, have the propensity to concentrate at the interfaces. Spilled oil will initially float at the air-water interface but, with time, it will accumulate at the sediment-seawater interface, horizontally through the action of waves and tides and vertically through the sinking of oil-sediment aggregates (Gong et al. 2014a; Romero et al. 2015). Simulations estimate that 65% of spilled oil in the water column will reach sediments through vertical settling (Bandara et al. 2011). Here, organic particles and clay will adhere strongly to oil hydrocarbons and entrap them. These adhesion forces between oil hydrocarbons and sediment particles will strengthen with time through a phenomenon known as aging (Kim et al. 2009) and will ultimately limit oil hydrocarbon bioavailability and, consequently, its biodegradation (Semple et al. 2003). In low energy environments (e.g. estuaries), because the organic and clay contents are higher, oil hydrocarbon pollution can be particularly problematic (Semple et al. 2003). Therefore, the sediments are considered the most sensitive areas to oil spills and their protection is prioritized during decision-making (Adler and Inbar 2007). Here, the sediment-bound hydrocarbons are quickly inhumed, by sedimentary accretion,

from a narrow superficial oxic layer to the adjacent anoxic sediments. If not resuspended, they can persist for decades (Reddy et al. 2002) because biodegradation is substantially slower in anoxic conditions (Duran and Goňi-Urriza 2010) and they are occluded from photooxidation (Kim et al. 2009).

The marine sediment surface is a biological hotspot, where the benthic microbial community is denser and more diverse that in adjacent compartments (Duran and Goňi-Urriza 2010; Paissé et al. 2008). Here, the interactive effort of a multitaxon microbial mat will synergistically enhance oil biodegradation (Coelho et al. 2016a; McGenity et al. 2012). In this heterotrophic consortium, Bacteria are of vital importance because of their versatile enzymatic capabilities (Head et al. 2006; Schmidt et al. 1998). Their possible functional replacement by Fungi has been shown to comparatively decrease *n*-alkane removal in sediments (Coelho et al. 2016a). In Bacteria, aerobic hydrocarbon biodegradation can occur through the action of generalist heterotrophic bacteria (e.g. genera *Pseudomonas* and *Bacillus*), which are capable of oxidizing other carbon substrates in the absence of oil, and/or through the action of hydrocarbonoclastic bacteria (Yakimov et al. 2007). Hydrocarbonoclastic bacteria are specialized in the biodegradation of specific classes of oil hydrocarbons [e.g. *Alcanivorax* sp. degrades saturated mid-range *n*-alkanes and *Cycloclasticus* sp. specifically degrades PAHs; (Schneiker et al. 2006; Yakimov et al. 2007; Yakimov et al. 1998)] and are more ubiquitous in marine aerobic environments than in terrestrial soils (Rojo 2009). They are sparsely present in non-contaminated environments and will respond rapidly to oil input (Yakimov et al. 2007). As crucial intervenients in the first metabolic step of aerobic hydrocarbon biodegradation (oxygenation), they will initially bloom but, gradually, as diversity rises, other heterotrophic bacteria will appear and feed on oils metabolic intermediates (Vila et al. 2010). Also, with time the predominant aerobic hydrocarbonoclastic bacteria will change concurrently with oil weathering (Dubinsky et al. 2013). The initially dominant alkane-degrading bacteria (i.e. *Alcanivorax*) are progressively replaced by PAH-degrading bacteria (e.g. *Cycloclasticus*) as the concentration of alkanes decreases (Dubinsky et al. 2013; Terrisse et al. 2017). In the end, as oil disappears, microbial communities may return to its initial pre-spill state (Yang et al. 2014).

Under anaerobic conditions, biodegradation will occur, but it will be comparatively slower than in aerobic conditions (Vitte et al. 2011; Widdel and Musat 2010). Alternative electron acceptors to molecular oxygen are abundant in sediments and microorganisms are known to efficiently use them in other metabolic pathways. Yet, in aerobic hydrocarbon biodegradation molecular oxygen is also used as a reactant in the initial activation step. In anaerobic conditions, alternative reactants (more commonly fumarate, but also water and nitrate) are used, but their energetic yields are substantially lower (Rojo 2009; Widdel and Musat 2010). For example, in hexadecane biodegradation, activation with fumarate has a potential free energy gain for the bacteria ten-fold lower than to aerobic oxygenation (Rabus et al. 2001). Anaerobic hydrocarbon biodegradation is predominantly achieved directly by sulfate-reducing bacteria in marine intertidal sediments (Davidova et al. 2006) or indirectly through syntrophic relationships with these and methanogenic archaea (McInerney et al. 2009). Other common metabolic

pathways include hydrocarbon biodegradation coupled to metal reduction [e.g. Fe(IV) and Mn(IV)] (Dorer et al. 2016). Despite its poor energetic gain, anaerobic biodegradation is still an important via for oil hydrocarbon removal in sediments, particularly in fine-grained sediments. Here, a more superficial redoxcline layer limits aerobic biodegradation to the most superficial sediments, which in oil contaminated environments can be quickly depleted of oxygen because of an increased heterotrophic activity.

In sum, the restoration of oil-impact marine environment can be achieved through the metabolic interaction of a multitaxon microbial community. However, and despite the substantial amount of research dedicated to the theme, the mechanistic details of the biodegradation of oil hydrocarbons in marine environments are still poorly understood. The functional role of specific microbial groups and the microbial dynamics inside the community, remain elusive. Although catastrophic incidents are ideal sources of new data for research, their occurrence is both undesirable and unpredictable in time and space. Therefore, validated microcosm simulations of oil spills are required to answer these surging questions. Also, climate alterations induced by global warming (e.g. ocean acidification) may alter the baseline of the microbial dynamics of oil hydrocarbon biodegradation in marine environment and should be considered when designing these experiments. In the end, a more thorough understanding of the biodegradation of oil hydrocarbons in marine environments will incite new oil response strategies and a more informative decision-making, certainly benefiting environmental protection.

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Polycyclic aromatic hydrocarbons in deep sea sediments: microbe-pollutant interactions in a remote environment

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Abstract

Recalcitrant polycyclic aromatic hydrocarbons (PAHs) released into seawater end up in the deep sea sediments (DSS). However, this compartment is often oversimplified by theoretical models. Biodegradation of PAHs in DSS is assumed to be similar to biodegradation in surface habitats, despite high hydrostatic pressures and low temperatures that should significantly limit PAHs biodegradation. Bacteria residing in the DSS (related mainly to α- and γ-proteobacteria), however, have been shown to or predicted to possess distinct genes, enzymes and metabolic pathways, indicating an adaptation of these bacterial communities to the psychro-peizophilic conditions of the DSS. This work summarizes some of the most recent research on DSS hydrocarbonoclastic populations and mechanisms of PAHs degradation and discusses the challenges posed by future high CO_2 and UV climate scenarios on biodegradation of PAHs in DSS.

Highlights

- PAHs tend to accumulate in DSS
- High hydrostatic pressure and low temperature adversely affect PAHs biodegradation in DSS
- DSS bacterial communities house distinct strains, catabolic genes, enzymes and pathways
- We review the impact of DSS bacteria on PAHs degradation under current and future climate scenarios.
- Future climate scenarios may indirectly affect PAHs biodegradation in DSS.

Keywords: Oil pollution; Abyssal sediments; biodegradation, Climate change, High hydrostatic pressure; Polycyclic aromatic hydrocarbons

Introduction

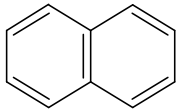
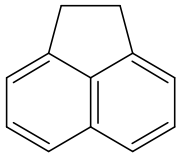
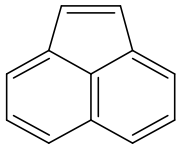
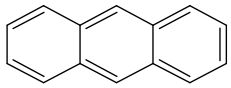
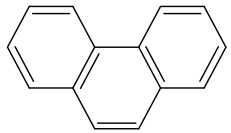
Polycyclic aromatic hydrocarbons (PAHs) are hydrophobic molecules consisting of two or more fused aromatic rings arranged in a variety of structural configurations. PAHs bioaccumulate through

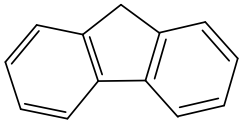
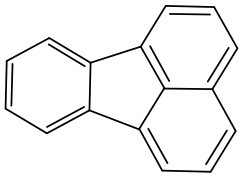
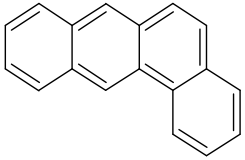
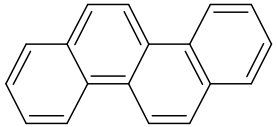
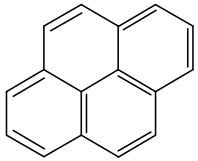
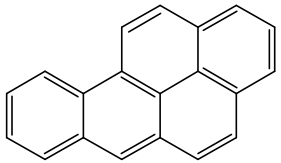
trophic webs (Clements et al. 1994; Kanaly and Harayama 2000) and exert acute toxic, mutagenic, teratogenic and carcinogenic effects on living organisms (Kanaly and Harayama 2000; Menzie et al. 1992; Neff 2002). PAHs with two and three aromatic rings are considered low molecular weight (LMW) PAHs, while compounds with four or more aromatic rings are known as high molecular weight (HMW) PAHs. In Table I-1, we listed the 16 priority PAHs as classified by the US Environmental Protection Agency (EPA) along with measured and estimated physico-chemical data. Overall, organic carbon-water partition coefficient (K_{oc}), octanol-water partition coefficient (K_{ow}) and half-life values increase with increasing numbers of rings, whereas vapour pressure decrease. An increasing number of rings reduces volatility, solubility and biodegradation rates, and increases hydrophobicity, adsorption to particulate matter, toxicity and recalcitrance (Seo et al. 2009).

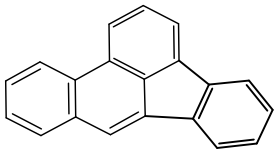
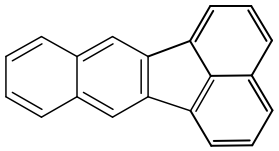
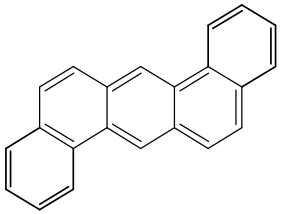
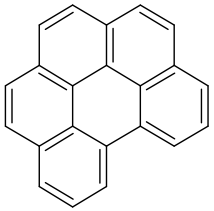
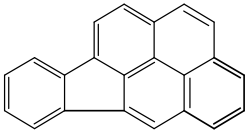
Bioavailability refers to the forms and quantity of chemicals that biota can take up during their lives and thus determines how and to what extent these chemicals enter the food chain. In the biodegradation of hydrophobic pollutants, bioavailability is important and is expressed as the relative fraction of a specific pollutant that is available to microbes. Solubility and sorption are important parameters when considering bioavailability. The first is directly correlated with bioavailability, while the second is affected by various external factors. All PAHs are hydrophobic and lipophilic, and adhere to soil particles, if available, in a seawater environment. The 'strength' of this association varies and is related, among other things, to soil properties (Semple et al. 2003). K_{oc} (Table I-1) directly reflects the sorption capacity of PAHs and increases with increasing number of rings. Sorption also increases with increasing soil organic carbon content (Wang et al. 2001) and decreasing pore size (Semple et al. 2003). In soot, a carbon-rich matrix, pyrolytic PAHs are highly sorbed and occluded, which reduces their degradation in comparison to other surfaces (Kim et al. 2009). At DSS organic carbon content is lower than at coastal marine sediments (Nagata et al. 2010) thus PAHs are more bioavailable. Over time, the rate of desorption of organic pollutants from the matrix decreases exponentially (aging). Aging functionally resumes to the shift from weaker hydrogen and van der Waals to covalent bonds. By aging, PAH-soil interaction becomes more stable and irreversible (Semple et al. 2003).

PAHs are introduced into the marine environments sporadically through marine oil spills and continuously through urban run-off, industrial and domestic wastewater/sludge discharge, atmospheric deposition, ship ballast cleaning, offshore oil exploration and natural seepage (Guitart et al. 2007; Guitart et al. 2010; Notar et al. 2001). PAHs inputs into marine environments can be classified into two groups: petrogenic and pyrolytic (Ke et al. 2002). Petrogenic sources derive directly from petroleum and derivatives, while pyrolytic sources derive from the combustion of fossil fuels. Higher PAHs concentrations in seawater are normally associated with anthropogenic coastal activities, particularly shipping harbours (Cincinelli et al. 2001; Coelho et al. 2010; Fang et al. 2008; Wurl and Obbard 2004) and major rivers. The highly urbanized coastal waters are reported to be 1.5 and 4.5 times more enriched in dissolved and particulate PAHs, respectively, than pristine sites (Guitart et al. 2007).

Table I-1 - List and associated relevant information of PAHs labeled as priority pollutants by the US Environmental Protection Agency — EPA.

EPA 16 Priority PAHs	Conformation		Physical Chemical Parameters						Toxicology			Biodegradation	
	nº rings	Structure	Solubility mg L^{-1} [a]	$\log k_{oc}$ MCI [h]	$\log k_{oc}$ k_{ow} [i]	$\log k_{oc}$ Measured	$\log k_{ow}$ [b]	Vapour Pressure (mm Hg at 25ºC)	TEF [c]	IARC [d]	EPA [e]	Estimated Half-lives (days) [f]	Measured Half-lives (days) [g]
Naphthalene	2		31.0 [j]	3.189	2.864	2.96 [k]	3.30	8.5×10^{-2} [l]	n.d.	2B	C	5.56	n.d.
Acenaphthene	3		3.93	3.701	3.402	3.59 [k]	3.92	2.5×10^{-3} [m]	0.001	3	D	18.77	n.d.
Acenaphthylene	3		1.93	3.701	3.419	3.75 [k]	3.94	6.68×10^{-3} [m]	0.001	n.c.	D	30.7	n.d.
Anthracene	3		0.076	4.214	3.862	4.31 [k]	4.45	6.53×10^{-6} [n]	0.01	3	D	123	2.7
Phenanthrene	3		1.20	4.223	3.870	4.35 [k]	4.46	1.2×10^{-4} [m]	0.001	3	D	14.97	5

Fluorene	3		1.68-1.98	3.962	3.627	3.7[k]	4.18	6.0×10^{-4}[m]	0.001	3	D	15.14	n.d.
Fluoranthene	4		0.20-0.26	4.744	4.426	4.8[k]	5.10	9.22×10^{-6}[m]	0.001	3	D	191.4	9.2
Benzo[a] anthracene	4		0.010	5.508	4.999	5.37[k]	5.76	4.11×10^{-3}[o]	0.1	2B	B2	343.8	>182
Chrysene	4		1.5×10^{-3}	5.256	5.042	5.37[p]	5.60	6.23×10^{-9}[q]	0.010	2B	B2	343.8	n.d.
Pyrene	4		0.132	4.735	4.235	4.9[k]	4.88	4.5×10^{-6}[m]	0.001	3	D	283.4	151
Benzo[a] pyrene	5		3.8×10^{-3}	5.769	5.320	5.95[s]	6.13	5.49×10^{-9}[t]	1.0	1	B2	421.6	11

Benzo[b] fluoranthene	5		0.0012	5.778	4.495	5.82[p]	5.18	$5.0x10^{-7}$[m]	n.d.	2B	B2	284.7	n.d.
Benzo[k] fluoranthene	5		$7.6x10^{-4}$	5.769	4.235	5.6[u]	6.11	$9.7x10^{-10}$[t]	0.1	2B	B2	284.7	n.d.
Dibenzo[a,h] anthracene	5		$5.0x10^{-4}$	6.281	5.858	6.22[k]	6.75	$9.55x10^{-10}$[v]	n.d.	2A	B2	511.4	n.d.
Benzo[g,h,i] perylene	6		$2.6x10^{-4}$	6.290	5.754	6.26[p]	6.63	$1.0x10^{-10}$[w]	n.d.	3	D	517.1	n.d.
Indeno[1,2,3-cd] pyrene	6		0.062	7.333	5.815	6.26[p]	6.70	$1.25x10^{-10}$[q]	n.d.	2B	B2	349.2	n.d.

[a] (ATSDR 1995)

[b] (Paraíba et al. 2010)

[c] Toxic equivalent factor relatively to Benzo[a]pyrene (Chang et al. 2014)

[d] International Agency for Research on Cancer Classification Monographs Volume 1-111 updated 18 February 2015 (1-Carcinogenic to humans; 2A-Probably carcinogenic to humans; 2B-Possibly carcinogenic to humans; 3-Not classifiable as carcinogenic to humans; n.c.-not classified)

[e] EPA Carcinogenic Classification: A-Human carcinogenic; B1 and B2: Probable human carcinogenic; C-Possible human carcinogenic; D-Not Classifiable as to human carcinogenicity; E-Evidence of non-carcinogenicity for humans

[f] Estimation using BioHCwin software v1.01 on EPI Suite software develop by (Howard et al. 2005)

[g] (Comber et al. 2012)

[h] Estimation using KOCWIN v2.00 software on EPI Suite program by the MCI method(Meylan et al. 1992; SRC 1991)

[i] Estimation using KOCWIN v2.00 software on EPI Suite program by the kow method using kow values presented above

[j] (Bojes and Pope 2007)

[k] (Schüürmann et al. 2006)

[l] (Ambrose et al. 1975)

[m] (Sonnefeld et al. 1983)

[n] (Oja and Suuberg 1998)

[o] (Mackay and Shiu 1977)

[p] (Hawthorne et al. 2007)

[q] Estimated using Mpbpwin v1.43 software of EPI Suite program using the Grain Method(Neely and Faust 1985)

[r] (Hoyer and Peperle 1958)

[s] (Meylan et al. 1992)

[t] extrapolated (Murray et al. 1974)

[u] (HSDB 2008)

[v] extrapolated (Lei et al. 2002)

[w] (Lee et al. 1993)

Increasing energy demand will lead to an increase in the transportation, storage and use of fossil fuels thereby increasing the risk of oil spill. Remediation of oil pollution response rely on physical, biological and chemical processes. Biological methods mainly focus on enhancing natural biodegradation through the supplementation of nutrients, surfactants (biostimulation) or microorganisms (bioaugmentation). Nutrient biostimulation approaches rely on N and P supplementation in the form of inorganic salts to contaminated areas. Microbial biodegradation of oil pollutants is often limited by the low concentrations of bioavailable nitrogen in comparison to carbon. Supplementation of N and P increases microbial degradation but is only effective when these elements are limiting. This approach is more effective in soil, sand and embayments. In the open ocean, nutrient dispersion significantly reduces its effectiveness and requires continuous supplementation, which raises costs (Nikolopoulou et al. 2007). Oil degradation is also limited by the reduced oil-water interface, where biodegradation occurs. Application of surfactants reduces the interfacial tension and increases oil-water emulsification, enhancing biodegradation (Hazen et al. 2010). Bioaugmentation (addition of exogenous or endogenous bacteria) is an overlooked tool in oil spill response and has been deemed ineffective and relatively expensive (Megharaj et al. 2011). Maladaptation of the inoculated microorganisms to the new environment constitutes a major barrier to successful bioaugmentation (Churchill et al. 1995). However, the use of autochthonous strains could help to overcome this limitation and improve bioremediation efficiency (Hosokawa et al. 2009) and bioaugmentation with PAH-degrading bacteria, immediately after spill, can minimize persistent PAH afterwards (Ron and Rosenberg 2014).

The extension of oil exploration towards deeper ocean introduces novel technological challenges and environmental concerns. Therefore, in depth scientific knowledge and new technological skills need to be developed. Here we focus on the particular features of the deep sea that make it a relevant bioremediation site: the origin and fate of PAHs in deep sea sediments and the microbial players, pathways and genes involved in PAHs biodegradation.

Bacterial life in the deep sea

In the present review, deep sea sediments refer to the sea floor below 1000 m depth. This environment is considered extreme because of the challenging conditions to microbial life in terms of nutrient supply, electron donors and acceptors, and exposure to physical factors such as temperature and pressure.

The DSS environment is characterized by high pressure (10–50 MPa), low temperatures (2-3ºC, except in hydrothermal vents) and low concentration of labile organic carbon. Salinity varies between 34.3–35.1 g L^{-1} and pH between 7.5–8.0 (Nagata et al. 2010). Collectively, the data indicate that physico-chemical conditions of the deep sea environment are more stable than the ocean surface (Nagata et al. 2010). Although the abyssal plains form the major part of the deep sea, other habitats are present and

include hydrothermal vents, cold seeps, mud volcanoes, Fe(III)-Mn(IV) nodules, trenches, seamounts and canyons (Nagata et al. 2000).

Marine sediments, in general, are covered with biofilms of dense and diverse microbial communities (Chipman et al. 2010). They are efficient players in the biogeochemical cycles of carbon, nitrogen and phosphorus (Kostka et al. 2011; Shao et al. 2010; Silva et al. 2003). Life in the deep sea mainly depends on the input of organic carbon from the ocean above. Dissolved organic carbon in the superficial deep sea sediments varies from 509-1038 μmol L^{-1} in the Atlantic Ocean and 672-1529 μmol L^{-1} in the more eutrophic Arabian Sea (Lahajnar et al. 2005). Exogenous organic carbon inputs into the deep sea derive from lateral advection from slopes and shelves, diffusion and sinking. Resuspension from bottom sediments by turbulence mixing, chemosynthetic activity and hydrocarbon seeps also contributes to the microbial food web.

Organic matter is continuously degraded by microbes while sinking, leaving behind a refractory remnant (Nagata et al. 2010). Bacteria in the deep sea are adapted to degrade these recalcitrant compounds. Specific adaptations to oligotrophic environments, such as the production of bioflocculants, have been reported in deep sea bacteria and assist in nutrient and carbon sequestration (Wu et al. 2013). Deep sea microbial communities have been reported to decompose biodegradable plastics (Sekiguchi et al. 2011), hydrocarbons, PAHs (Cui et al. 2008; Shao et al. 2010; Wang et al. 2008) and polychlorinated biphenyls (Froescheis et al. 2000).

The microbial community in the bathypelagic environment has been shown to be surprisingly diverse (DeLong et al. 2006; Sogin et al. 2006). Although microbial abundance is relatively stable at 10^4-10^5 cells cm^{-3} and biomass decreases with depth (Nagata et al. 2000; Nagata et al. 2010; Sogin et al. 2006), microbial diversity has been shown to increase due to the preponderance of low abundance operational taxonomic units (OTU) also known as the "rare biosphere" (Sogin et al. 2006). The "rare biosphere" is thought to serve as a microbial seed-bank which may increase in relative abundance in response to environmental change (Agogué et al. 2011). Deep sea microbial communities contain numerous novel taxa; for example, a culture-independent analysis of an extinct hydrothermal field, revealed that only 4% of the bacterial sequences had > 94% sequence similarity to sequences from NCBI database (Nercessian et al. 2005).

A number of studies have reported the composition of DSS bacterial communities (Table I-2) However, these results should be compared with caution due to the variation in molecular techniques employed and biases in DNA extraction and PCR amplification protocols that may over- or under-represent certain taxa (Al-Awadhi et al. 2013; Hazen et al. 2013). Overall, Proteobacteria, particularly belonging to the α-, γ- and δ- classes are the most abundant taxa in molecular surveys of DSS microbial communities (Jamieson et al. 2013; Jiang et al. 2007; Li et al. 1999; Nercessian et al. 2005; Pachiadaki et al. 2011; Wu et al. 2013; Xu et al. 2005; Zeng et al. 2005). ϒ-Proteobacteria the most abundant group in the mid-Atlantic ridge's Rainbow hydrothermal vent (≈50% of total OTU) (Nercessian et al. 2005), the

Table I-2 - List of culture-independent studies of the deep sea benthic bacterial community.

Matrix	Location	Method	Main conclusions	Reference
Hydrothermal vents	Rainbow Hydrothermal vent field, Mid Atlantic Ridge (2300m)	16S rRNA clone library	Clones correspond to sequences classified as γ- (≈50%), α-(12%) and δ- Proteobacteria (6%), Planctomycetes (8%) and Bacteriodetes (6%)	(Nercessian et al. 2005)
DSS	Western Pacific "Warm Pool" (1901m)	16S rRNA clone libraries	Clones correspond to γ- (45.3%), α- (17%) and δ- (12.3%) Proteobacteria. Less represented OTU correspond to β- (4.7%) and ε- (7.5%) Proteobacteria, Cytophaga/Flavobacteria/Bacteriodetes group (5.6%), Planctomycetes (2.8%) and gram-positive bacteria (2.8%)	(Zeng et al. 2005)
	Indian Ocean sediments at a high and low Chlorophyll Sites	16S rRNA clone library	Clones correspond to γ- and α-Proteobacteria. Other less abundant clones included δ- and ε-proteobacteria (grouped) and Planctomycetes. Actinobacteria relevant at a particular site.	(Jamieson et al. 2013)
	Sea of Okhotsk (1225m) core with of clay with interlaying layers of volcanic ash.	16S rRNA clone libraries	Distinct geohydrological environments have distinct bacterial communities. In clay, green sulfur bacteria at the surface, and the candidate division OP9 in subsurface. Volcanic ashes dominated by γ-Proteobacteria (genus *Halomonas, Methylophaga* and *Pyschobacter*) and α and δ-Proteobacteria.	(Inagaki et al. 2003)
Gas Hydrates	South China Sea sediments (1508m)	16S rRNA clone library	OTU correspond to γ- Proteobacteria (82%). Less abundant clones include α-(13%) and δ-(3.2%) Proteobacteria. Bacterial community presented low diversity and high relatedness to cultured organisms. Sequences had high similarity with sequences from HC and PAH-degrading and Fe(III)-Mn(IV) and sulfate reducing isolates.	(Jiang et al. 2007)
Polymetallic nodules	South Pacific Ocean (5000-5500m)	16S rRNA clone library	OTU were similar mainly to sequences classified as α-, γ- and δ-Proteobacteria. Other less abundant clones	(Wu et al. 2013)

			included Actinobacteria, Acidobacteria and Planctomycetes.	
Mud Volcanoes	East Mediterranean Sea sediment core 0-30cm (2025)	16SrRNA clone library	All together 20 phylogenetic groups were represented in this study, Shannon–Wiener diversity index H of 1.92-4.03, that decreased from 0cm to 15cm. δ- (21.3%), γ- (22.3%) and ε-Proteobacteria (14.9%) dominated 0-5 cm samples and were substituted by division Chloroflexi (42.3%) and JS1 candidate division (15.5%) at 10 cm sample, δ-Proteobacteria (24.6%-27.1%) and JS1 candidate division (34.8%-28.8%) at 15-20 cm and δ-Proteobacteria at 25 cm (65.6%) (in part due to abundance of the *Desulfosarcina/Desulfococcus* group). Unaffiliated sequences and Chloroflexi (19.2%) were the highest at the deepest sample (30cm)	(Pachiadaki et al. 2011)

Pacific Ocean Fe(III)-Mn(IV) nodules (≈70% of clones) (Xu et al. 2005) and the Western Pacific Warm Pool sediments (≈ 45%) (Zeng et al. 2005). δ-Proteobacteria were the most abundant group in cold seeps in the Japan Trench (6200 m) (Li et al. 1999) and ε-Proteobacteria, along with γ- and α-Proteobacteria, composed the majority of sequences from some deep sea hydrothermal vent systems (Moyer et al. 1995; Moyer et al. 1998; Reysenbach et al. 2000). Other relatively abundant taxa include the Cytophaga/Flavobacteria/Bacteroidetes group (Li et al. 1999; Martín-Cuadrado et al. 2007; Nercessian et al. 2005; Xu et al. 2005; Zeng et al. 2005), Planctomycetes (DeLong et al. 2006; Jamieson et al. 2013; Martín-Cuadrado et al. 2007; Nercessian et al. 2005; Wu et al. 2013; Zeng et al. 2005), Chloroflexi (mainly affiliated with Dehalococcoidetes (Inagaki and Nakagawa 2008)) (DeLong et al. 2006; Martín-Cuadrado et al. 2007; Pachiadaki et al. 2011), Acidobacteria (Martín-Cuadrado et al. 2007; Wu et al. 2013), Firmicutes (Li et al. 1999; Martín-Cuadrado et al. 2007; Zeng et al. 2005), CFB group (Xu et al. 2005; Zeng et al. 2005), JS1 candidate division (previously joined with the OP9 candidate division) (Inagaki and Nakagawa 2008; Inagaki et al. 2006; Inagaki et al. 2003) and Actinobacteria (Jamieson et al. 2013; Wu et al. 2013). Functions such as sulfate reduction in hydrothermal vents and cold seeps have been mainly attributed to ε- and δ-Proteobacteria (Desulfococcus and Desulfosarcina) (Inagaki et al. 2002; Longnecker and Reysenbach 2001; Zeng et al. 2005), sulfur oxidation to γ- and ε-Proteobacteria (Inagaki and Nakagawa 2008), methane oxidation to members of the uncultured candidate division JS1 (Inagaki et al. 2002), γ- and α-Proteobacteria (Xu et al. 2005) and dechlorination to members of δ-Proteobacteria and Chloroflexi (Inagaki and Nakagawa 2008). Archaea are also crucial players in the chemosynthetic activity of the DSS microbial community, e.g., in the fixation of dissolved inorganic carbon (Herndl et al. 2005), methane oxidation (Havelsrud et al. 2011) and nitrification (Beman et al. 2008). Archaea have also been shown to be relatively more abundant components of DSS microbial communities (Herndl et al. 2005). The unique physico-chemical conditions of the DSS induce a selective pressure on the resident microbial community (Table I-3). There are a number of pronounced differences between microbial communities in DSS and surface waters (Konstantinidis et al. 2009). Microbial communities from the deep sea maintain higher metabolic activity under pressurized conditions than under normal atmospheric pressure, which indicates an adaptation to a piezophilic environment (Nagata et al. 2010). Furthermore, metagenomic and biochemical analysis of the microbial community has unveiled a number of distinct features. The occurrence of surface attachment and biofilm associated genes encoding for pilus, polysaccharide, and antibiotic synthesis suggest surface-attachment and biofilm organization as important lifestyles in bathypelagic microbial communities (DeLong et al. 2006). The absence of genes that encode for proteins involved in light driven DNA repair (DeLong et al. 2006), a higher metabolic diversity (Konstantinidis et al. 2009), cell membranes with a higher proportion of unsaturated fatty acids suited to sustain cold and high pressure conditions (Wang et al. 2008), enzymes with weaker intermolecular interactions favouring molecular flexibility and catalytic efficiency, and larger genomes (1.35±0.25 fold in comparison to bacteria from surface waters) with extensive intergenic regions and

Table I-3 - List of unique phenotypical features found in the DSS microbial community indicative of adaptation to this extreme marine habitat.

Features	References
Genes associated to surface attachment and biofilm lifestyle	(DeLong et al. 2006)
Absence of genes involved in light driven reactions	(DeLong et al. 2006)
Higher metabolic diversity	(Konstantinidis et al. 2009)
Higher proportion of unsaturated fatty acids in cell wall	(Wang et al. 2008)
Weaker intramolecular enzymatic interactions	(Nagata et al. 2010)
Large genomes	(Konstantinidis et al. 2009)

large numbers of rRNA genes (Konstantinidis et al. 2009; Nagata et al. 2010) are also characteristic features of bacteria from DSS.

Accumulation of PAHs in deep sea sediments

Despite its remoteness, the deep sea has been an overlooked sink for PAHs (Bouloubassi et al. 2006; Shao et al. 2010). In Table I-4 a list of studies measuring ΣPAHs in DSS is available. Overall, concentrations of ΣPAHs in DSS are lower than those measured in other marine sediments, particularly when compared to salt marshes and estuaries (Yuan et al. 2014), although in coastal shelf sediments, relatively low concentrations of ΣPAHs have been measured (Wang et al. 2014). Nevertheless, it may prove incorrect to generalize information, since the quantity and quality of PAHs pollution in DSS depends on the distance from land-based sources, anthropogenic pressure, natural seepage and hydrological circulation. The Mediterranean Sea has been relatively well-studied due to the relatively high anthropogenic impact and low water exchange (Bouloubassi et al. 2006; Mandalakis et al. 2014; Parinos et al. 2013). Here, a 1-year sediment trap at 2850 m revealed a mean daily flux of 53 ± 39 ng m^{-2} d^{-1}(Bouloubassi et al. 2006) . HMW PAHs and LMW PAHs were dominating winter and summer sampling events, respectively, indicating the main source of PAH varied with season and climate (Bouloubassi et al. 2006). An extensive survey in the Mediterranean measured ΣPAHs from 11.6-223 ng g^{-1} (mean of 63.9 ng g^{-1}); the mean ΣPhenanthrene/ΣHMWPAHs ratio was 0.45 ± 0.19 indicating that pyrolytic sources were dominant (Parinos et al. 2013). With the exception of a single dataset (Bouloubassi et al. 2006), this seems to be the general consensus of all studies in the Mediterranean Sea (Bouloubassi et al. 2012; Mandalakis et al. 2014; Tolosa et al. 1996). PAHs concentrations in the Mediterranean Sea are similar to those found in distant mid-Atlantic sub-surface sediments (455 ng g^{-1}) (Shao et al. 2010), in at Arctic Sea surface sediments (113-2504 ng g^{-1}) (Yunker et al. 2011) and the Gulf of Mexico (Soliman and Wade 2008). In remote DSS, LMW PAHs (Shao et al. 2010) and alkylated PAHs (aPAHs)

Table I-4 - List of studies measuring ΣPAHs in DSS.

Reference	Location	Depth (m)	Average ΣPAHs (ng g^{-1})	Source
(Bouloubassi et al. 2006)	West Mediterranean Sea (39ºN 6ºE)	2850	551 ± 198	HMW PAHs and LMW PAHs were dominant in winter and summer sampling times, respectively
(Bouloubassi et al. 2012)	West Mediterranean Sea/ Gulf of Lions	2854	289.3	Pyrolytic PAHs have higher relative abundance
(Tolosa et al. 1996)	Western Mediterranean Sea	>1000	pyrolytic (147-604)	HMW PAHs clearly dominant ΣPAHs. Indicating a high contribution of pyrolytic sources.
			petrogenic (24-112)	
			diagenetic (1-10)	
(Parinos et al. 2013)	East Mediterranean Sea	1018-4087	63.9	Mean Phenanthrene / ΣHMWPAHS ratio was 0.45 ± 0.19 indicating a pyrolytic source.
(Mandalakis et al. 2014)	Southern Cretan Basin	215-4392	9-60	All ratios used indicated a pyrolytic origin. Benzo[b]fluoranthene, indenol[1,2,3-cd]pyrene and Benzo[ghi]perylene had the highest abundance in all samples. LMW PAHs / HMW PAHs was at average 0.55 ± .010.
	Northern Cretan Basin		14-34	
	Levantine Basin		17-27	
(Yunker et al. 2011)	Arctic Sea extensive sampling	867-4230	113-2504	Predominance of aPAHs indicate petrogenic origin
(Shao et al. 2010)	Mid Atlantic Ocean (11°11'S 11°41'W)	3962	445	A high relative abundance of LMW PAH (Phenanthrene (222 ng g-1)) indicate petrogenic origin. Analysis did not target aPAHs
(Cui et al. 2008)	Mid Atlantic Ocean (0°8.42'N, 24°23.63'W)	3542	266	A high relative abundance of LMW PAH (Acenaphthene (89 ng g-1)) indicate petrogenic origin. Analysis did not target aPAHs
(Dong et al. 2014c)	Arctic Ocean North-South Transect		2.02 to 41.63	LMW PAHs were dominant in all but the site nearest to land. Petrogenic sources are dominant sources in sites furthest from land.
(Ohkouchi et al. 1999)	Pacific Ocean 175ºE Transect	2505-5906	0.81-60.6-	Authors assume that atmospheric deposition of pyrolytic PAHs are the main origin of PAHs in DSS, although aPAHs/PAHs ratios are between 2-6 indicative of petrogenic origin
(Yang 2000)	South China Sea	1045-2432	124.7-199.1	Despite high relative abundance of naphthalene, Phenanthrene/Anthracene ratio was always <15 indicative of pyrolytic origin.

(Soliman and Wade 2008; Yunker et al. 2011) had the highest relative abundances, indicating that petrogenic sources are the main source of PAHs, and natural seeps are the most probable explanation (Shao et al. 2010). Air-sea exchange may also be an important contributor when LMW are dominant, as explained below.

Geochemical processes such as hydrothermal activity and, especially, cold seeps, are an important source of PAHs in offshore sites such as the mid-Atlantic ridge (Cui et al. 2008; Konn et al. 2009). Here, PAHs have been reported to accumulate in surrounding sediment (Burns et al. 2010; Etkin 2009), despite natural communities degrading a significant fraction of the pollutant (Kappell et al. 2014). Natural seepage represents 47% of the oil introduced to marine environments (Kvenvolden and Cooper 2003; National Academy of Science 2002). The estimated quantity of seep oil has been reported to be between 10^8-10^9 L annually) (Kvenvolden and Cooper 2003). However, estimations are based on available data, mostly from continental margins and in oil producing regions, and omit the vastness of the ocean realm including the deep sea. Therefore, overall marine seepage is probably higher.

Of the remaining oil introduced in the marine environments, only 2.9% and 11.5% result from the production and transportation of oil, respectively. Nonetheless, major oil spills represent a major threat to marine ecosystems, especially near oil production areas and vessel corridors, because of the rapid release of high volumes of oil over a limited area and the frequency of spills near coastal regions. Even in areas with abundant natural seep sites (i.e., the Gulf of Mexico) the capacity of microbes to remove high amounts of oil has been questioned, due to limited dispersion and low oxygen concentrations at some depths (Ramirez-Llodra et al. 2011). During oil spills, physical weathering completely or partially removes the more volatile and polar hydrocarbons, namely small chain alkanes (< C14) and LMW PAHs (Ke et al. 2002), while biodegradation removes long chain alkanes and some branched alkanes (Reddy et al. 2002). Subsequently, a remnant fraction that includes HMW PAHs and aPAHs, among others, persists (González et al. 2006; Teira et al. 2007; Yamada et al. 2003) and will tend to adhere to the sediment (Bouloubassi et al. 2006). In the recent Deepwater Horizon (DWH) oil spill, HMW PAHs represented 31% of ΣPAH in the particulate phase while their relative abundance in oil was only 8% (Boehm et al. 2011). Traces of PAHs, mainly HMW and aPAHs have been found in marshes 30 years after oil spill incidents (Peacock et al. 2007; Reddy et al. 2002).

The remaining hydrocarbons that enter marine environments are derived from land run-off and atmospheric deposition. Here, rivers are important contributors of PAHs, particularly HMW PAHs with high Koc, to deep sea basins near river mouths. Rivers concentrate and transport these pollutants from their basin to the river mouth (Lipiatou et al. 1997; Scheringer et al. 2004). As an example, the sub basin of the Adriatic Sea (75 m) is enriched in PAHs derived from the agro-industrialized Po Valley hydrological system (Marini and Frapiccini 2013). When freshwater reaches the sea, the increase in salinity causes mass transfer of PAHs to the particulate phase and subsequent sedimentation (Marini and Frapiccini 2013). Sedimentation is the main process by which PAHs enter marine sediments (Lipiatou

et al. 1997). The concentration of PAHs in marine sediments is positively correlated with the concentration of particles < 15 μm (Charlesworth et al. 2002) and organic carbon (Mandalakis et al. 2014). PAHs fluxes to the deep sea are influenced by size and degree of pollution of nearby rivers and can be enhanced by natural meteorological events such as typhoons, hurricanes and floods (Lin et al. 2013). PAHs are transported to the deep sea by lateral advection from the continental shelf, mainly through submarine canyons (Bouloubassi et al. 2012). Atmospheric deposition is an important contributor of PAHs in oceans (Tsapakis et al. 2006). Atmospheric deposition consists of three processes: wet deposition and dry deposition that mainly transports PAHs with higher Koc, and air-sea gaseous exchange processes that mainly transport PAHs with higher vapour pressure (Tsapakis et al. 2006). In contrast to what was initially assumed, air-water exchange is a significant source of PAHs into oceans (mean 706 μg m^{-3} yr^{-1}) and accounts for ≈ 76% of atmospheric deposition in the Eastern Mediterranean Sea (≈200 km) (Tsapakis et al. 2006). At sites further from pollution sources, air-sea exchange represented almost the totality of PAHs in atmospheric deposition (Ma et al. 2013). A 0.7% fraction (8 μg m^{-3} yr^{-1}) of the total atmospheric deposition (929 μg m^{-3} yr^{-1}) of PAHs reached sediment traps located at 1440 m of depth and phenanthrene (associated to air-gas exchange) was the most abundant PAH (92 ng g-1) (Tsapakis et al. 2006).

PAHs are known to bioaccumulate in macrofauna in rivers, estuaries and coastal marine habitats (Bandowe et al. 2014; Li et al. 2014). In DSS, PAHs and other organic pollutants such as organochlorinated compounds (Froescheis et al. 2000) tend to bioaccumulate in deep sea organisms (Escartín and Porte 1999; Soliman and Wade 2008). At 480 m depth in the Mississippi Canyon, Gulf of Mexico (before the DWH incident), PAHs were quantified in deep sea amphipods and results revealed a mean bioaccumulation factor (BAF) of 4.37 ± 2.55, with higher values for aPAHs in comparison to the respective parent-PAHs (Soliman and Wade 2008). This was attributed to the high impact of oil exploration and natural oil seepage present in the area and reduced rates of degradation of aPAHs (Soliman and Wade 2008). BAFs were positively correlated with $\log K_{ow}$ (Soliman and Wade 2008); however, in another study using marine fish samples aPAHs were excluded from the analysis and BAF-$\log k_{ow}$ correlation was parabolic with a peak estimated at a log K_{ow} value of 5 (Li et al. 2014). Overall, aPAHs and HMW PAHs have a higher tendency to bioaccumulate, but this relationship is not always linear and may depend on the type of fauna sampled (Li et al. 2014).

The half-lives of PAHs increase in deep sea environments due to low temperature, high salinity and absence of light (Tansel et al. 2011). High salinity increases adsorption, due to the salting out effect, and consequently reduces bioavailability and increases persistence, particularly among HMW PAHs (Marini and Frapiccini 2013). Low temperature and absence of light favours PAHs persistence in deep sea environments due to lower metabolic activity of PAH-degrading organisms and, possibly, reduced solubility and absence of photodegradation (Marini and Frapiccini 2013).

Degradation of PAHs under hydrostatic high pressure (HHP) and low temperature (LT).

PAHs degradation in the deep sea is often assumed to be similar to surface waters. However, similarly to what was proposed for the ecotoxicological impact to marine macrobiota (Mestre et al. 2014), HHP and LT, may synergistically affect hydrocarbon degradation (Boesch and Rabalais 1987). HHP in DSS increases 10 MPa km^{-1} and temperature is 2-3ºC (Nagata et al. 2010). HHP is used to increase yield in biotechnological applications by enhancing oxygen rate in high-density bioreactors (Follonier et al. 2012). Nonetheless it has also been shown to reduce poly-β-hydroxybutyric acid degradation by deep sea fungi (Gonda et al. 2000). Meanwhile, LT is known to universally reduce the kinetics of biochemical reactions, and thus, to reduce PAHs degradation under aerobic conditions (Brakstad and Bonaunet 2006; Eriksson et al. 2003; Weissenfels et al. 1990) thereby leading to a build-up of dead-end metabolites (Eriksson et al. 2003). Synergistically, HHP and LT can affect PAH degradation reactions by 1) affecting cell physiology; 2) affecting catabolic enzymes and 3) affecting substrate bioavailability (Eisenmenger and Reyes-De-Corcuera 2009).

HHP and LT affect biological activity in mesophilic bacteria by reducing membrane functionality (Barria et al. 2013), and by denaturing proteins leading to a reversible inactivation (Privalov 1990). As an acclimation reaction, bacteria alter their membrane composition and synthesize protective cold-shock proteins (Barria et al. 2013). Monomeric proteins are generally well preserved by HHP, while pressure can affect the supramolecular configuration and catalytic site of polymeric enzymes and, consequently, reduce substrate affinity (Eisenmenger and Reyes-De-Corcuera 2009; Follonier et al. 2012). In dioxygenases(Seo et al. 2009), HHP can induce the release of iron from Fe-S clusters (Malone et al. 2006). PAHs degradation relies on Fe-S clusters to initially oxygenate PAHs.

Solubility is an important aspect of PAHs degradation in DSS. In DSS, LT reduces PAHs solubility differently (41.2% and 35% reduction for anthracene and pyrene, respectively, between 25ºC and 9ºC; (Bamford et al. 1999; Reza et al. 2002) and thus affects bioavailability. The overall effects on PAHs bioavailability may depend on the quality of the PAHs pool. The influence of an increase of HPP on PAHs solubility at low or ambient temperature has, to our knowledge, not been reported.

Few PAHs-degrading DSS strains have been tested under simultaneous HHP and LT conditions. Recently, the PAHs degrading strain *Sphingobium yanoikuyae* B1, isolated from a polluted stream (Gibson et al. 1973), was grown in naphthalene minimal medium at HHP ranging from 0.1-13 MPa and LT of 4ºC (Schedler et al. 2014). Results show a reduction in cell growth and naphthalene degradation at 13.9 MPa in comparison to 0.1 MPa. Bacterial growth was also shown to slightly decrease until 8.8 MPa followed by a sharp reduction in growth until 12 MPa. The final naphthalene concentration was below the detection limit from 0.1 to 12 MPa, but was 25.2% and 17.9%, respectively, at 12.5 and 13 MPa (Schedler et al. 2014). HHP reduced growth and, above 12 MPa, reduced PAHs degradation

(Schedler et al. 2014). This represented the first evidence of a reduction in PAHs degradation under DSS conditions. Future work employing DSS isolates or the DSS microbial community, without depressurization, is necessary to elucidate the fate of PAH in abyssal marine sediments.

The combined effect of LT and HHP can reduce PAHs biodegradation, since the reduction in permeability may hamper PAHs uptake; this is essential for the initial oxygenation reaction that occurs intracellularly, and the alteration of PAH-dioxygenase supramolecular conformation and/or catalytic site may inactivate essential enzymes. It is plausible that PAH-degrading bacteria are adapted to these conditions. Microbial communities from deep sea maintain higher metabolic activity under pressurized conditions than under atmospheric pressure, which indicates adaptation to a piezophilic environment (Nagata et al. 2010). This may be similar to adaptations found in psychrotrophic environments (e.g. Arctic), because similar taxa are found in both types of environment (McFarlin et al. 2014). It would be interesting to assess these psychro-piezophilic adaptations in PAHs degradation and outline their benefits for bioremediation or other biotechnological process applications.

PAH-degrading marine bacteria

PAH-degrading strains have been isolated from numerous marine environments including seawater, marine sediments, saltmarshes and estuaries. These isolates are phylogenetic distributed to phyla Proteobacteria (α, β and γ classes), Actinobacteria, Cyanobacteria, Bacteroidetes and Firmicutes. Several genes encoding for PAH-degradation, that have been identified from different bacterial species share high homology, thus suggesting that PAHs catabolic genes are transferred horizontally - [horizontal gene transfer (HGT)] - this is acquired through conjugal transfer of plasmids (Anokhina et al. 2004; Coelho et al. 2011; Jutkina et al. 2011). Genes encoding PAH-degrading enzymes are often located in IncP plasmids, that are prone to broad range HGT and insertion in DNA sequences (Dennis 2005). For example, in a microcosm experiment, the *nah* gene detected in *Shewanella oneidensis* and *Bacillus* sp. was similar to that detected in Pseudomonads from the same sample (Ben Said et al. 2008). Similar results were obtained in a microcosm seeded with sea surface samples (Coelho et al. 2011) and in saltmarsh rhizospheres (Oliveira et al. 2014a). PAH-degrading bacteria are generally found in low abundance in microbial communities from non-impacted marine environments (Yang et al. 2014).

The dynamics in the microbial community, following an acute PAH pollution event, is characterized by a rapid shift in composition, due to sudden dominance of the pioneer degrading strains present (Vila et al. 2010). As PAHs are gradually degraded, other taxa, such as *Roseobacter* (Buchan and González 2010), start to recover and feed on the PAHs intermediates as secondary consumers (Wang and Tam 2011). At the end of this process, the bacterial community has been reported to return to its original state (Kasai et al. 2001; Yang et al. 2014).

PAH-degrading isolates are frequently found within the α- and γ-Proteobacteria classes, although this may be related to their ease of cultivability. The genera *Cycloclasticus*, *Pseudomonas* and some genera of the *Sphingomonadaceae* family (*Novosphingomonas, Sphingomonas* and *Sphingobium*) are also frequent among marine PAH-degrading isolates (Dyksterhouse et al. 1995; Kasai et al. 2002). The genus *Cycloclasticus* is widely distributed in marine environments and has been isolated from estuarine environments (Chung and King 2001; Dyksterhouse et al. 1995; Niepceron et al. 2010), sea sediments (Cui et al. 2014) and DSS (Wang et al. 2008). *Cycloclasticus* is considered an obligate marine PAH-degrader and the chlorinated derivatives thereof (Yakimov et al. 2007). It is frequently found during the mid-succession stages of oil degradation (Yakimov et al. 2007) and in cold environments (Coulon et al. 2007; Lozada et al. 2008). The genera *Novosphingomonas*, *Sphingomonas* and *Sphingobium* (*Sphingomonadaceae*) are known to use many organic compounds including PAHs (Huang et al. 2008; Kertesz and Kawasaki 2010; Leys et al. 2005). Sphingomonads have been isolated from various marine environments including DSS. They are efficient PAH-degraders in oligotrophic environments because they possess a high-affinity uptake system (Johnsen et al. 2005). In *Pseudomonas*, PAHs degradation genes and biochemical pathways are extensively studied (Gomes et al. 2005; Suen and Gibson 1993). *Pseudomonas* spp. are ubiquitous and are described as competitive PAH-degrading bacteria in polluted terrestrial soils and sediments (Cébron et al. 2008; Daane et al. 2001), in estuarine water (Coelho et al. 2011; Niepceron et al. 2010), sediments (Oliveira et al. 2014a) and in seawater (Uad et al. 2010). Although apparently ubiquitous, Pseudomonads were absent in enriched consortia from DSS in the Pacific and Atlantic Oceans (Cui et al. 2008; Wang et al. 2008), but were found in DSS from the Arctic (Dong et al. 2014c). Pseudomonads are considered useful for biotechnological applications due to extensive data available on their activity and metabolome, ease of cultivation, stress resistance and presence of genetic mobile elements (Puchałka et al. 2008).

PAH-degrading marine bacteria have been used in bioaugmentation approaches to mitigate environmental contamination, but the results have been disappointing so far (Kadali et al. 2012). Complete mineralization tends to be slow (Wang et al. 2008), or cannot be achieved by a single strain, because of the accumulation of toxic intermediates (Festa et al. 2013; Tixier et al. 2002). Under these circumstances, mineralization is better achieved by consortia (Cui et al. 2014; Festa et al. 2013; Gallego et al. 2013; HuiJie et al. 2011; Mao et al. 2012; Nzila 2013; Vallero 2010). Bioaugmentation with bacterial consortia will have a synergistic effect because complementary PAHs degradation pathways promote cross-feeding and avoid the build-up of inhibiting metabolites (Bouchez et al. 1999). Also, the inclusion of secondary strains with, for example, high cell hydrophobicity and biosurfactant production in the consortia will enhance the efficiency of the bioaugmentation process (Pedetta et al. 2013; Sorensen et al. 2005) by increasing PAHs bioavailability (Pedetta et al. 2013) and providing essential growth factors and nutrients (Sorensen et al. 2005).

PAH-Degrading Bacteria in Deep Sea Sediments

PAH-degrading isolates from deep sea environments known to date are listed in Table I-5. Fifteen PAH-degrading strains from deep sea microbial communities were classified as novel species. Isolation efforts have focused on the 4 major ocean basins: Arctic, Atlantic, Indian and Pacific. Some reported isolates were not included in Table I-5 because they had low similarity with sequences from the NCBI database or were not classified by the authors. PAH-degrading isolates from the deep sea were mainly classified as γ- and α-Proteobacteria, which are abundant classes in deep sea environments. Representatives of the β-Proteobacteria, Actinobacteria and Flavobacterium have also been found. Other abundant taxa found in DSS, such as Chloroflexi, δ- and ε-Proteobacteria and Planctomycetes, have no reported PAH- degrading isolates. Several marine PAH-degrading genera including Cycloclasticus, *Sphingomonas/Novosphingobium/Sphingobium*, *Halomonas*, *Alcanivorax*, *Thalassophira* and *Marinobacter* have been found in DSS and are often similar to their coastal counterparts (Cui et al. 2008). Others, such as *Oceanicola*, *Parvibaculum*, *Nitratireductor* (α-Proteobacteria) and *Bowmanella* (γ-Proteobacteria), were first reported as PAH-degraders in DSS. During the DWH oil spill, the first major deep sea (≈1500 m) oil pollution event, strains classified as *Colwellia*, *Cycloclasticus* and *Pseudoalteromonas* dominated in the oil-degrading community when the relative aromatic (mono- and PAHs) content was the highest (Hazen et al. 2010).

An OTU assigned to the genus *Cycloclasticus*, from DSS of the Western Pacific Ocean (Wang et al. 2008) and Mid Atlantic Ocean Ridge (Cui et al. 2008; Shao et al. 2010), had 99-100% similarity with a strain of *Cycloclasticus spirillensus* M4-6 (AY026915) isolated from the intertidal sediments of Lowes Cove, Maine (Chung and King 2001). In addition to Cycloclasticus, other OTU were similar to the following sequence in NCBI database: *Flavobacterium frigoris* (AJ601393) (92.6%), *Novosphingobium pentaromativorans* (AF502400) (98.8%), *Halomonas alimentaria* (AY553075) (96.9%), an uncultured proteobacterium (DQ230971) (98.2%) and *Roseovarius tolerans* (Y11551) (94.7%) (Wang et al. 2008b). In the Western Pacific Ocean, a pyrene-degrading microbial consortium degraded 93.87% of pyrene in 7 weeks, with 60% removal by week 2. Overall, a reasonable amount (46%) of the sequences obtained from DGGE had low similarity (<97%) with sequences available at NCBI database (Wang et al. 2008).In the universe of the 21 genera represented, the proteobacteria phylum (α- (53.8%) γ- (38.6%) and β- classes (3.8%)) (Wang et al. 2008) was dominant.

PAH-degrading microbial consortia from two subsurface sediment samples from the mid-Atlantic ridge revealed a dominance of α- and γ-Proteobacteria using DGGE (Cui 2008; Shao 2010).In addition to the main band, associated with Cycloclasticus spirillensus M4-6, other DGGE bands had high similarity with *Alcanivorax venusti* ISO4 (98-100%), *Marinobacter alkaliphus* (100%), *M. viniformis* FB1 (99%), *Marinobacterium georgiense* (100%) and *Tistrella mobilis* (99%) (Cui et al. 2008). At a different location in the mid-Atlantic ridge OTU were assigned to the taxa *Cycloclasticus* sp. P1 (99%), *Alcanivorax borkumensis* SK2

Table I-5 - PAH-degrading isolates from the deep sea environment. (*) indicates strains that were published as novel species and (Δ) indicates the complete genome sequence. DSS- Deep Sea Sediments; DSW - Deep Sea Water.

Class	Specie	Strain	Matrix	Location	Accession nº NCBI database for partial 16S rRNA	Accession nº at NCBI database for draft or complete genome$^{\Delta}$	Accession nº at NBCI database for gene or MGE encoding enzymes crucial for PAHs degradation	Reference
α-Proteobacteria	*Celeribacter indicus*	P73T	DSS	Indian Ocean				(Lai et al. 2014)
	Erythrobacter flavus	W4-3C	DSS	Pacific Ocean	DQ649539			(Wang et al. 2008)
	Erythrobacter aquimaris	MARC2A11	DSS	Atlantic Ocean	DQ768671			(Shao et al. 2010)
	Hyphomonas jannaschiana	W6-15	DSS	Pacific Ocean	DQ649546			(Wang et al. 2008)
	Labrenzia aggregata	W6-16	DSS	Pacific Ocean	DQ649543			(Wang et al. 2008)
	Maribaculum marinum *	P38	DSW	Indian Ocean	EU819081			(Lai et al. 2009a)
	Maricaulis virginensis	MARC4M	DSS	Atlantic Ocean	DQ768642			(Shao et al. 2010)
	Martelella mediterranea	MARC4H	DSS	Atlantic Ocean	DQ768639			(Shao et al. 2010)
	Nitratiredutor pacificus *	pht-3B	DSS	Pacific Ocean	DQ659453	AMRM00000000		(Lai et al. 2012a; Lai et al. 2011c)
	Novosphingobium indicum	H25*	DSW	Indian Ocean	EF549586		EU526902 EU526901 EU526900 EU526899	(Yuan et al. 2009b)

		TVG9-II	Deep Sea hydrothermal environment	Indian Ocean	JF706227	JF710635	(Dong et al. 2011)
	Oceanibaculum indicum	P24T *	DSW	Indian Ocean	EU656113		(Lai and Shao 2012a; Lai et al. 2009c)
		MARC2P-F	DSS	Atlantic Ocean	DQ768653		(Cui et al. 2008)
	Oceanibaculum pacificus *	LMC2up-L3	Deep Sea hydrothermal environment	Pacific Ocean	FJ463255		(Dong et al. 2010)
	Oceanicola pacifus *	W11-2B	DSS	Pacific Ocean	DQ659449		(Yuan et al. 2009a)
	Parvibaculum indicum *	P31	DSW	Indian Ocean	FJ182044		(Lai et al. 2011b)
	Pseudaminobacter sp-	W11-4	DSS	Pacific Ocean	DQ649552		(Wang et al. 2008)
	Roseovarius inducus *	B108	DSW	Indian Ocean	EU742628		(Lai et al. 2011d)
	Roseovarius pacificus *	81-2	DSS	Pacific Ocean	DQ120726		(Wang et al. 2008; Wang et al. 2009)
	Sphingobium sp.	C100	DSS	Arctic Ocean		AYOY00000000	(Dong et al. 2014b)
	Stappia indica *	B106T	DSW	Indian Ocean	EU726271		(Lai et al. 2010)
	Thalassopira profundimaris *	WPO211	DSS	Pacific Ocean		AMRN00000000	(Lai and Shao 2012b)
	Thalassospira sp	DBT	DSS	Pacific Ocean	DQ649535		(Wang et al. 2008)
	Tistrella mobilis	MARC2P-R	DSS	Atlantic Ocean	DQ768659		(Cui et al. 2008)
β-proteobacteria	*Achromobacter xylosoxidans*	2MN-2	DSS	Pacific Ocean	DQ649533		(Wang et al. 2008)

γ-proteobacteria	*Alcanivorax borkumensis*	MARC4D	DSS	Atlantic Ocean	DQ768649		
	Alcanivorax deiselolei	MARC2C-S	DSS	Atlantic Ocean	DQ768647		(Cui et al. 2008)
	Alcanivorax pacificus *	W11-5	DSS	Pacific Ocean	DQ629451	AJGP00000000	(Lai et al. 2011a)
	Alcanivorax sp.	MARC2C-G	DSS	Atlantic Ocean	DQ768658		(Cui et al. 2008)
		MARC2C-P	DSS	Atlantic Ocean	DQ768644		(Cui et al. 2008)
		MARC2C-R	DSS	Atlantic Ocean	DQ768646		(Cui et al. 2008)
		521-1	DSS	Pacific Ocean	DQ659430		(Wang et al. 2008)
	Alcanivorax venustensis	MARC2C-T	DSS	Atlantic	DQ768621		(Cui et al. 2008)
	Bowmanella pacifica *	W3-3AT	DSS	Pacific Ocean	EU440951		(Lai et al. 2009b)
	Cycloclasticus sp.	P1	DSS	Pacific Ocean	NR074683	CP003230 [Δ]	(Lai et al. 2012b; Wang et al. 2008)
	Halomonas meridiana	MARC2C-B	DSS	Atlantic Ocean	DQ768623		(Cui et al. 2008)
		MARC4B	DSS	Atlantic Ocean	DQ768627		(Shao et al. 2010)
	Halomonas sp.	2MN-1	DSS	Pacific Ocean	DQ649534		(Wang et al. 2008)
	Marinobacter alkaliphilus	MARC2C-P	DSS	Atlantic Ocean	DQ768658		(Cui et al. 2008)
	Marinobacter bryozoorum	MARC2C-C	DSS	Atlantic Ocean	DQ768624		(Cui et al. 2008)
	Marinobacter hydrocarbonoclasticus	MARCAF	DSS	Atlantic Ocean	DQ768638		(Shao et al. 2010)
	Marinobacter sp.	MARC2C-K/ 521-2	DSS	Atlantic/ Pacific Ocean	DQ768641/ DQ659431		(Cui et al. 2008; Wang et al. 2008)

		MARC4V	DSS	Atlantic Ocean	DQ768635		(Shao et al. 2010)
		MARC4S	DSS	Atlantic Ocean	DQ768634		(Shao et al. 2010)
	Marinobacter vinifirmus	MARC2P-E	DSS	Atlantic Ocean	DQ768652		(Cui et al. 2008)
	Marinomonas profundimaris *	D104	DSS	Arctic Ocean		AYOZ00000000	(Bai et al. 2014; Dong et al. 2014a)
	Mesorhizobium sp.	W6-20	DSS	Pacific Ocean	DQ649544		(Wang et al. 2008)
	Pseudoalteromonas ganghwensis	MARC2C-A	DSS	Atlantic Ocean	DQ768622		(Cui et al. 2008)
Actinobacteria	*Micrococcus luteus*	W5-11	DSS	Pacific Ocean	DQ659431		(Wang et al. 2008)
	Rhodococcus sp-	TW35	DSS	Pacific Ocean	DQ462176		(Peng et al. 2008a)
Flavobacterium	*Flavobacterium sp.*	W6-14	DSS	Pacific Ocean	DQ649545		(Wang et al. 2008)

(100%), *A. venusti* ISO4 (100%), *Marinobacter salsuginis* SD-14B (100%) *Roseovarius crassostreae* CV919-312 (100%) and *Marinobacter sediminum* (94% similarity) (Shao et al. 2010).

The contribution of broad host range plasmids to PAHs biodegradation in the deep sea is still poorly understood. Genes *pheA1a* (UniProt accession nº G4WYQ4) and *pheA1b* (UniProt accession nº G4WYQ4), that encode for the α- and β- subunits of phenanthrene dioxygenase, respectively, were detected in the transposon tnp1 (JF710635.1) in strain TVG9-VII isolated from a deep sea hydrothermal vent (Dong et al. 2011). This strain was closely related to the isolate *Novosphingobium indicum* H25, also from the deep sea (Jun et al. 2008). BLAST similarity search (http://blast.ncbi.nlm.nih.gov/Blast.cgi) indicated a 99% similarity between partial sequences (query coverage - 63%) of these two genes (*pheA1a* and *pheA1b*) and those detected in the large plasmid pNL-1, which is commonly detected in sphingomonads, inclusively in *N. aromaticivorans* DSM 12444 [NCBI accession nº CP000676, isolated from subsurface sediments from Atlantic coastal plains (Balkwill 1989)]. The pNL-1 plasmid is transferred among many strains belonging to the *Sphingomonas* and *Pseudomonas* genera (Basta et al. 2005). However, this similarity may be limited to the *nah* gene, and not to the mobile genetic element itself, since the transposases of TVG9-II have no similarity with those of *N. aromativorans*. The aminoacid sequence of both genes (*pheA1a* and *pheA1b*) have a 99% sequence similarity to aromatic degrading enzymes from strains DSM 12444, N. indicum H25 and *Sphingobium sp.* C100, all isolated from deep sea waters from the Indian or Arctic Ocean, and with *Sphingobium* strains PNB and LH128 isolated from wastewater treatment plant (Roy et al. 2013b) and contaminated soil (Schuler et al. 2009).

A total of 6 strains from the deep sea environment have a draft or complete genome publicly available (Table I-5): *Sphingobium sp.* C100, *Marinomonas profundimaris* D104, *Alcanivorax pacificus* W11-5, *Thalassopira profundimaris* WPO11, *Cycloclasticus sp.* P1 and *Oceanibaculum indicum* P24. Using this public information we performed a BLAST (http://www.uniprot.org/blast/) analysis of the putative catabolic enzymes' aminoacid sequences involved in initial steps of PAHs degradation [as determined by gene annotation in bacterial.ensemble.org] (Curwen et al. 2004; Kersey et al. 2012; Potter et al. 2004). Results revealed a high similarity with sequences from UNIPROT database. Enzymes of strains *Sphingobium* C100 and *M. profundimaris* D104 had 100% similarity with enzymes from *Sphingobium* strains PNB and LH128 (Roy et al. 2013b), and from *Alteromonas sp.* SN2 (Math et al. 2012), respectively. Strain SN2 was isolated from petroleum contaminated tidal flats and its genes involved in PAHs degradation are located at genomic island GI-11 flanked by two transposases, indicative of a past HGT event (Math et al. 2012). For *Cycloclasticus sp.* P1, the aminoacid sequences of estradiol dioxygenase, α- and β- subunit dioxygenase, catechol dioxygenase and ferredoxin reductase, all had high similarity (between 99 to 100%) with sequences from *C. zancles* 7-ME, isolated from marine sediment at the site of the MT Hazen oil tanker shipwreck (78 m depth), and with sequences from *Cycloclasticus sp.* PY97M, isolated from Yellow Sea sediments [17.8 m depth; (Cui et al. 2014)]. Additionally, gene clusters encoding α- and β- subunits of dioxygenase and ferredoxin/ferredoxin reductase in the isolate *Cycloclasticus* P1 were previously reported

to have 98.8% and 99.8% similarity with equivalent genes in *Cycloclasticus sp.* A5, isolated from seawater (15m) (Kasai et al. 2002). In *T. profundimaris* WPO11, the aminoacid sequences of protein involved in PAH-degradation by gene annotation had 74-82% similarity with sequences from *T. xiamensis* M-5. M-5 was isolated from oil wastewater and both were initially reported to be similar on the basis of morphology and 16S rRNA gene similarity (98.5%) (Wang et al. 2008).

In *A. pacificus* W11-5 and *O. indicum* P24, the aminoacid sequences of proteins involved in PAH-degradation had low similarity (<51% and <58%, respectively) to database sequences. Deep sea bacteria thus contain PAH degrading genes that are similar to and markedly different from genes encountered in shallow water bacterial communities. Enzymes unique to the deep sea bacterial community can be availed for biotechnological applications and promoting DSS as a relevant habitat for bioprospection effort.

Pathways of PAH degradation in marine environments.

The fate of PAHs, after entering the marine environment, is determined by various processes, including sedimentation, volatilization, sinking, resuspension, photo- and biodegradation. Bacterial biodegradation strongly depends on chemical properties such as molecular size (number of aromatic rings) and molecule angularity (Kanaly and Harayama 2000). Generally, an increase in size and angularity leads to an increase of toxicity (Table I-1; (Banerjee et al. 1995; Kanaly and Harayama 2000), chemical reactivity and a reduction in aqueous solubility and volatility (Kanaly and Harayama 2000; Seo et al. 2009). In soil, the estimated half-lives of 3-ring phenanthrene and 5-ring benzo[a]pyrene range from 16 to 126 days and 229 to 1400 days, respectively (Peng et al. 2008b; Shuttleworth and Cerniglia 1995). In situ biodegradation of PAHs in nature also depend on environmental factors, such as nutrient and substrate bioavailability, oxygen availability, electron acceptors and temperature (Quan et al. 2009). PAHs biodegradation involves metabolic reactions catalysed by a variety of enzymes.

Generally, the initial step of PAHs catabolism is crucial and, although varying among substrates and microorganisms (Seo et al. 2009), in aerobic conditions always involves the introduction of one or two oxygen molecules in the ring structure of the PAHs with formation of a metabolite with one or two -OH radicals, in a reaction known as oxygenation, catalyzed by di- or monooxygenases (Kanaly and Harayama 2000; Seo et al. 2009). This reaction is the rate-limiting step in PAHs biodegradation and is carried out by the α- subunit of the aromatic ring dioxygenases (ARD) with a non-heme iron catalytic domain (Demanèche et al. 2004). Iron and oxygen availability are thus vital in aerobic PAHs catabolism. The diversity of genes encoding ARD among PAH-degrading bacteria from soil has been clustered into two groups associated with gram-negative and gram positive bacteria (Cébron et al. 2008). ARD of gram-negative bacteria is further clustered into various subtypes, three of which are well-defined: phnAC, phnA1 and nahAC, related to *Alcaligenes faecalis* AFK2, genus *Cycloclasticus* and genus

Pseudomonas/*Sphingomonas*, respectively (Lozada et al. 2008). In DSS, ARD sequences related to phnAC/phnA1 and nahAc subtypes are encountered in draft genomes of previously mentioned isolates.

Substrate specificity of ARD varies widely among PAHs (Peng et al. 2008b). Microbial communities potentially harbour a wide array of organisms with distinct ARD and that can shift quickly in response to PAH inputs (Kimura and Kamagata 2009; Selvakumaran et al. 2011; Wang et al. 2008). After the oxygenation step, the dihydrodiol intermediate ring structure is cleaved by intradiol- or estradiol oxygenases in between the hydroxyl groups (meta-cleavage) or before the hydroxyl groups (ortho-cleavage) yielding protocatechuate and catechol intermediates that can be further converted to tricarboxylic acid cycle intermediates (Jiménez et al. 2004; Nzila 2013; Seo et al. 2009). Frequently, PAHs from three to *n* benzenic rings form sequentially, intermediate dihydriol with *n*-1 aromatic rings until reaching 1,2-naphthalene dihydrodiol from which the pathway proceeds as described in naphthalene biodegradation (Nzila 2013). In PAHs degradation, the intermediate metabolic products and enzymes are similar to other metabolic pathways and are widespread among bacteria (de Lorenzo 2008).

There is little information on the metabolomics of PAH-degrading isolates in DSS. In *Cycloclasticus* sp. P1, some metabolites from pyrene biodegradation identified by GC-MS (Wang et al. 2008) were distinct from those previously reported (Kweon et al. 2011; Moscoso et al. 2012; Seo et al. 2009). A pathway involving an initial dioxygenation of the pyrene molecule to form a pyrene-4,5-dihydrodiol, which was further metabolized to cyclopenta(def)phenanthrone (main metabolite) was proposed. This was followed by cyclopenta(def)phenanthrone metabolization to a lactone and subsequently to a 4-phenantrenol (secondary metabolite). The subsequent pyrene biodegradation pathway would follow the most usual phenanthrene degradation pathway via 3,4-dioxygenation (Seo et al. 2009). Although many intermediates lack analytical confirmation, DSS may harbour unknown metabolic pathways since the main metabolite (cyclopenta(def)phenanthrone) has not yet been detected in pyrene degradation pathways in the available literature.

Future perspectives: PAHs biodegradation in DSS within a global change context.

Future climate scenarios have proposed an increase in partial CO_2 pressure (pCO_2), which will lead to a reduction in oceanic pH (Rhein et al. 2013). Mean pH values for ocean waters have already decreased from 8.21 to 8.10 from pre-industrial times until present and will be further reduced by 0.3-0.4 units by 2100 (Caldeira and Wickett 2003; Doney et al. 2009). Under this scenario, some biological processes in the marine environment are predicted to increase, namely the production of extracellular polymeric substances (EPS), nitrogen fixation and enzyme activity (Liu et al. 2010), while calcification in the majority of calcifying organisms will be adversely affected (Doney et al. 2009). Iron bioavailability will also be reduced, particularly in distant offshore waters, by increasing the adsorption by organic particles, (Shi et al. 2010). UV radiation (UVR) is currently higher than in pre-industrial times, but

because of the recovery of the ozone layer is expected to regress and stabilize to levels of the 1980s (and possibly to 1960s levels) in high an mid latitudes and an increase in 3% in the tropics by the end of the 21st century, but also be adversely affected by an increase in greenhouse gases and pollution (Bais et al. 2011).

To our knowledge, the impact of future climate scenarios on the biodegradation of PAHs in DSS has not been addressed in the scientific literature. Climate change is thought to synergistically exacerbate overall marine pollution, by adversely affecting the biodegradation of oil and other pollutants (Coelho et al. 2013). However, few studies have experimentally measured this impact and interlinked the compilation of direct and indirect impacts that result from the rise in greenhouse gases. The integration of the predicted effects of the interplay of climate change and pollution on the structure and activity of microbes in the water column and in marine sediments may provide the basis for a theoretical model on the fate of PAHs in DSS in future climate scenarios.

In the deep sea, the direct impacts of global warming are expected to be mild. Although future scenarios for the deep sea environment are not consensual, the rise in temperature is expected to occur at a reduced rate (0.03ºC per decade) when compared to the ocean surface (Rhein et al. 2013). However, at a regional scale, abrupt temperature shifts can occur (Danovaro et al. 2004). Also, the concentration of the carbonate ion and salinity are expected to decrease at a depth range of 1000 – 4000 m and >4000 m, respectively (Rhein et al. 2013), while pH is predicted to slightly increase (Rhein et al. 2013). However, the impact of climate change effect on surface waters will affect marine primary production (PP), which will impact deep sea ecosystems due to shifts in chemical energy derived from the sedimentation of detritus (McClain et al. 2012). Increased pCO_2 is expected to directly increase marine primary production (PP) (Riebesell et al. 2007). However, increased pCO_2 rise will also increase surface water temperatures leading to stratification that will adversely affect PP and increase the relative abundance of pico- and nanoplankton (Smith et al. 2008). Any alteration in PP will affect heterotrophic activity, organic carbon content and bacterial respiration (Joint et al. 2011) and bacterial community structure (Endo et al. 2013) in the overall ocean. Additionally, an increase in atmospheric pCO_2 will increase terrestrial PP and increase organic carbon fluxes to DSS, particularly via the major rivers. An increase in particulate matter may increase PAHs adhesion and thus reduce its bioavailability due to strong adhesion of PAHs to particles, particularly HMW PAHs (Nam et al. 1998). Also, a more prolonged and defined thermal stratification, as a result of an increase in surface water temperature, and a reduction in upwelling may reduce total PP (Chavez et al. 2011). Ocean stratification and the overall warming of seawater will also reduce oxygen by 6 to 12 μmol kg^{-1} by the year 2100 (Frölicher et al. 2009), mainly at 200 – 400 m depth and at mid-latitude, expanding the marine hypoxic and suboxic zones (Rhein et al. 2013; Stramma et al. 2010). Because oxygen is an important element in the initial steps of PAHs biodegradation, PAHs degradation may be limited by oxygen in some marine areas and depths under future climate scenarios.

Long term studies on the impact of a pCO_2 increase on bacterial processes of degradation and mineralization of organic matter are, however, lacking (Witt et al. 2011).

A mesocosm study provided evidence that the interactive effect of reduced pH and oil contamination can adversely affect the structure and functioning of sediment benthic communities in marine coastal ecosystems(Coelho et al. 2015). This study also showed that reduced seawater pH caused a significant change in the structure of the active bacterial communities, with a marked reduction in the relative abundance of the anaerobic oil-degrading and sulfate-reducing Desulfobacterales. This effect was, however, negated in the reduced seawater pH and UV-B treatment (Coelho et al. 2015). The presence of UVR can thus potentially alleviate the impact of ocean acidification on oil-degrading organisms. This may be because photooxidation converts oil compounds, including PAHs, into more water soluble metabolites that are more reactive and toxic (Mallakin et al. 2000) (namely epoxides and quinones (Petersen and Dahllöf 2007)) but are also more bioavailable (Yu 2002). This is particularly the case for recalcitrant HMW PAHs (Guieysse et al. 2004; Lehto et al. 2000). PAHs bioavailability is the most limiting factor in PAHs removal in polluted environments (Pedetta et al. 2013), thus UVR can accelerate the overall PAHs detoxification (Zhang et al. 2008). We speculate that when oil pollution originates from natural seepage and leakage or blowout of oil extraction at aphotic depths, the absence of photooxidation will lower its degradation rate under reduced pH conditions.

In addition to UVR, PAHs bioavailability, solubility and volatility will increase because of an increase in water and atmospheric temperature and therefore, the amount of HMW PAHs that reach DSS may be reduced. Additionally, changes in PAHs toxicity in the deep sea environment may occur as a result of interactions between high hydrostatic pressure and the physiological adaptations of DSS organisms (Mestre et al. 2014). If, as observed in shallow sediments, the synergy between acidification and pollution leads to a change in the structure of the active bacterial community, the interaction between changing pCO_2, UVR and temperature may have site or region specific effects on the amount, composition and rate of biodegradation of the PAHs pool in DSS.

Conclusions

Inputs of PAHs in marine environments are likely to increase and this represents a long-term environmental risk. During descent, recalcitrant compounds are little affected by biodegradation in comparison to the more labile organic carbon. Thus, the DSS can be considered a "sink" for PAHs in the marine environment. The source and quality of the PAHs pool encountered at DSS may vary between locations. DSS nearer to land have higher relative abundance of HMW PAHs derived from wet and dry deposition and from land run-off, while land-distant DSS have higher relative abundance of LMW PAHs and aPAHs that derive from gas-water exchange and underwater oil seeps. Theoretical models of PAHs biodegradation in DSS neglect the importance of the abiotic conditions, namely HPP

and LT. More realistic data on biodegradation rates of PAHs in DSS are needed to understand the ultimate fate of PAHs there. Here, we assume that, although these abiotic conditions complicate PAHs degradation, bacterial adaptation may counter this. Overall, many novel isolates have been obtained in the DSS and are mainly related to phylum Proteobacteria, particularly the α- and γ- classes. PAH-degrading isolates in DSS harbour genes and enzymes involved in initial steps of PAHs degradation, have low and high similarity to those present in the NCBI database, while others have high similarity with known genes encoded in mobile genetic elements. Thus, the DSS gene pool simultaneously harbours genes indicative of past HGT of mobile genetic elements and genes that are unreported and potentially novel.

It is still difficult to predict the role of DSS on hydrocarbon biodegradation under future climate scenarios, and field and laboratory results are urgently needed. However, from the integration of field evidence with experimental results obtained from the water column and in shallow sediments, local or region-specific effects may be expected.

Acknowledgements

A. Louvado was supported by a PhD scholarship (SFRH/BD/86447/2012) funded by FCT, Portugal (QREN-POPH - Type 4.1 – Advanced Training, subsidized by the European Social Fund and national funds MCTES). This work had financial support from CESAM research unit (PEst-C/MAR/LA0017/2013) and QOPNA research unit (PEst-C/QUI/UI0062/2013).

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Chapter II

Chapter II

Isolation and characterization of phenanthrene- and chrysene-degrading bacteria from deep sea mud volcano sediments

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Abstract

Oil hydrocarbons emitted by deep sea mud volcanoes (MV) are efficiently consumed at seabed by the local benthic microbial community. Among the oil hydrocarbons emitted are include, in small amounts of other hydrocarbons, such as polycyclic aromatic hydrocarbons (PAH). Yet, in contrary to the other hydrocarbons, the bacterial players involve in PAH turnover has never been accessed. Furthermore, these bacteria may possess interesting adaptations to the extreme abiotic conditions and thus have potential biotechnological adaptations. In this study we isolated and identified PAH-degrading bacteria from sediment samples obtained from apparently active and inactive craters of the Mikhail Ivanov mud volcano and a reference site (abyssal plain). Minimal medium containing phenanthrene and chrysene as primary sources of carbon were used to enrich and isolate PAH-degrading bacteria. Our results revealed that the isolates from all enrichment cultures were mostly composed by members of the family *Bacillaceae* (*Bacillus* and *Virgibacillus* genera) and, in most of the cases, capable of producing biosurfactants. Additionally, in the inactive crater and in the reference site, isolates from *Acinetobacter* and *Rhodococcus* genera, respectively, were obtained. Our results indicated that *Bacillus*-like bacteria may contribute for the metabolic turnover of seeped PAHs in the deep sea Mikhail Ivanov mud volcano and in surrounding environments.

Keywords: Polycyclic aromatic hydrocarbons, Cold seeps, *Virgibacillus*, *Bacillus*, Biosurfactant, Bathyal zone

Introduction

An extensive occurrence of mud volcanoes (MV) has been reported, at depths between 200 m and 5000 m, in vicinities of strike-slip fault lines at the frontier of the Eurasian and African tectonic plates in the southwest Iberian margin of the gulf of Cadiz (Hensen et al. 2015; León et al. 2012; Pinheiro et al. 2003). MV where first reported at the accretionary wedge of the Gulf of Cadiz, but during the SWIMGLO/Transflux M86/5 cruise, three new MV [Abzu, Tiamat and Mikhail Ivanov (M. Ivanov)] were discovered at the outer margins of the accretionary wedge, in the Horseshoe valley (Hensen 2014). MV are piercement structures created by compression forces (e.g. tectonic force) that propel upward, through a soft sediment layer (e.g. accretionary wedges or deltas), a pressurized fluid mostly composed of water and hydrocarbons, thus creating mud seepage (Dupré et al. 2014; Mazzini et al. 2009). This pressurized hydrocarbon flux will feed a diverse heterotrophic benthic microbial community that will transfer the assimilated carbon and energy up to the higher trophic levels, thus supporting an oasitic biological hotspot in the, usually barren, deep sea surface (Coelho et al. 2016b; Gibson et al. 2005). This microbial community has been frequently analyzed through mass-sequencing approaches (Coelho et al. 2016b; Pachiadaki et al. 2011); however, this information, although valuable, provides limited information regarding the functional role of the microorganisms present. These metabolism-microbe associations can be instead better established through culture-dependent analyses.

Underwater seepage system are spread throughout the marine sediments and the documented sites alone are estimated to represent 47% of all oil hydrocarbons emitted into the marine realm (Kvenvolden and Cooper 2003). The gaseous hydrocarbons methane, ethane, propane and butane are progressively the most predominant oil hydrocarbons expelled at MV. Yet, other hydrocarbons such as polycyclic aromatic hydrocarbons (PAHs) have been detected at sediments of MVs and other seepage phenomena (Geptner et al. 2006; Lorenson et al. 2002; Polonik et al. 2015; Wang et al. 2011) and in the tissues of sessile organisms in various cold seeps systems (Powell et al. 1999; Serafim et al. 2008; Wade et al. 1989). Curiously, in contrast to the gaseous hydrocarbons, the microbial communities involved in the degradation of PAH in MV have been largely overlooked. To our knowledge, only a few studies have reported PAH degrading isolates from hydrocarbon seepage system, all from hydrothermal vents (Dong et al. 2011; Dong et al. 2010). These bacteria may have interesting biotechnological applications given their capability to efficiently degrade xenobiotics under extreme environment conditions (Deming 1998) [e.g. low temperatures, high hydrostatic pressure and potentially high sulphide and methane concentrations (Van Gaever et al. 2006)].

PAHs comprise hydrocarbons with two or more merged benzene rings (Wang et al. 2003). They are environmentally problematic pollutants, because their partial degradation produces toxic intermediates that increase macrofauna mortality (Gardiner et al. 2013; Özhan et al. 2014) and can alter the bacterial community core composition, potentially disturbing key biogeochemical cycles (Lindgren

et al. 2014; Pietroski et al. 2015; Scott et al. 2014). In marine environments, PAH-biodegradation is initially achieved through the action of hydrocarbonoclastic bacteria (Yakimov et al. 2007). These initially latent, but will respond quickly to exogenous oil input and bloom (Yakimov et al. 2007). However, due to unique abiotic conditions present in deep sea MV, the PAH-degrading microbial communities may be distinct from those typically found in contaminated coastal areas. Bacteria from γ- and α-Proteobacterial classes, most notably the *Cycloclasticus* genus, are typically found in contaminated coastal areas and that they are important players in the process of sediment decontamination here (Louvado et al. 2015). In addition, these bacteria are usually able to produce extracellularly biosurfactants, amphiphilic molecules that will increase oil in water micellization and, consequently, enhance oils bioavailability, thus contributing to speed up the process of oil degradation (Ron and Rosenberg 2001).

Curiously, despite of all the knowledge on PAH degradation in coastal areas there is a shortage of information on the composition of PAH degrading communities in MV areas and their ability to produce biosurfactants. In this study we aimed at isolating and identifying PAH-degrading bacteria from sediment samples obtained from M. Ivanov mud volcano area (active and apparently inactive craters) and in a reference site (located in the surrounding abyssal plain of the Horseshoe valley) and their ability to produce biosurfactants was also evaluated.

Methods

Medium and chemicals

Enrichment culture (EC) medium was adapted from M9 medium (Notomista et al. 2011) containing NaH_2PO_4 (7 gL^{-1}), K_2HPO_4 (3 gL^{-1}), NH_4Cl (1 gL^{-1}), peptone (0.1 gL^{-1}), yeast extract (0.1 gL^{-1}), $MgSO_4$ (0.1365 gL^{-1}), FeNH$_4$ citrate (0.0214 gL^{-1}), $CaCl_2$ (5 mgL^{-1}), $ZnSO_4.7H_2O$ (3.5 mgL^{-1}), $MnSO_4.H_2O$ (2.8 mgL^{-1}), $Co(NO_4)_2.6H_2O$ (0.7mgL^{-1}), $CuSO_4$ (0.625mgL^{-1}), H_3BO_3 (0.15mgL^{-1}), $Na_2MoO_4.2H_2O$ (60 μgL^{-1}) and $NiCl_2.6H_2O$ (20 μgL^{-1}). M9 solid medium was prepared by adding 1.2% agarose (Fischer scientific, Hampton, NH, USA). After autoclave sterilization, filter sterilized cycloheximide solution at a final concentration of 0.1 g L^{-1} was added. Cooled enrichment culture medium was spiked with phenanthrene (98%) and chrysene (99%) at final concentrations of 100 and 5 mg L^{-1}, respectively, from 200 x concentrated acetone-dissolved stock solutions (20 g L^{-1} and 1 g L^{-1} for phenanthrene and chrysene, respectively). For solid medium, cycloheximide, phenanthrene and chrysene were added to molten solid M9 medium at ≈50ºC, and immediately poured into petri dishes. After the addition of the PAHs, the acetone was left to evaporate in a laminar-flow chamber for approximately 10 minutes. Phenanthrene, chrysene and cycloheximide were all supplied by Sigma-Aldrich (St. Louis, MO, USA).

Sampling

The M. Ivanov mud volcano is one of the three new MV discovered ≈90 km west of the limit of the accretionary wedge of the gulf of Cadiz during the SWIMGLO/Transflux M86/5 cruise (Hensen 2014; Hensen et al. 2015). Sediment samples used in the enrichment cultures were obtained from two distinct craters in the M. Ivanov mud volcano (Figure II-1): the NW crater, which was apparently inactive because no chemotrophic fauna was visually detected (M.R. Cunha, personal observation) - "inactive mud volcano" (IMV) - (35º44"41'N 10º12"18'W) and the SE crater, which was apparently active because the presence of mud breccia, gas hydrates and chemotrophic fauna was confirmed by box core sampling (M.R. Cunha, personal observation) - "active mud volcano" (AMV) - (35º44"34' N 10º12"06'W) on 05/03/2012; and from a reference site (RS - 35º42"00'N 09º57"93'W) distant from any known MV on 27/02/2012 (Hensen 2014). Cores were obtained using a box-corer for the MV samples and a TV guided multiple cores for RS samples. Final sample was composed of four 0.5-1 g of sub-samples, from the top sediment layer (0-1 cm b.s.f.) of each core, that were pooled, homogenized with 1 mL glycerol and immediately deep-frozen and stored at -80ºC, first on board and later in laboratory, until further use.

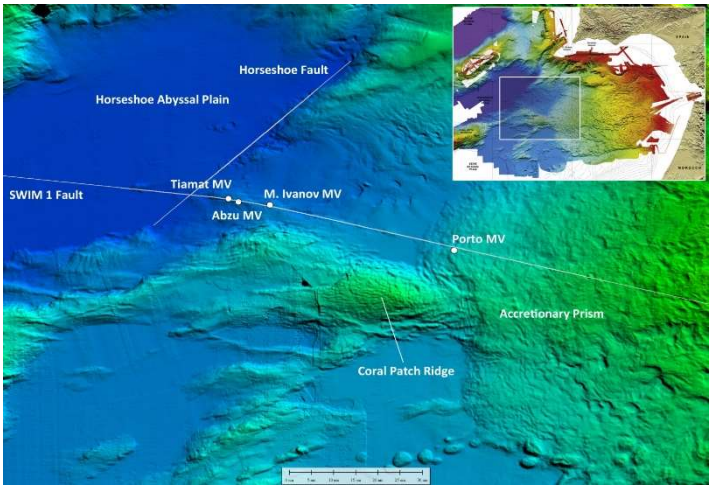

Figure II-1 - Map of Gulf of Cadiz. Sampling site (M. Ivanov MV) is located southwest of the Iberian Peninsula on the SWIM 1 fault line. Copyright belongs to GEOMAR (Helmholtz Centre for Ocean Research; Kiel, Germany).

PAH enrichment cultures

EC were obtained from each sediment sample. Ten mg of sediment inoculum was seeded in 100 mL Erlenmeyer containing 50 mL of EC and incubated at room temperature for 8 weeks. Culture medium was renewed at the second and fourth weeks. For the renewal, 0.5 mL of previous EC was inoculated into 50 mL of fresh EC medium containing the same concentration of PAH and

cycloheximide. Cultures were maintained in the dark and without agitation, to mimic deep sea conditions. For bacterial isolation, 1 mL aliquots were taken in triplicate (at day 3 and weekly, afterwards), serially diluted and spread-plated on solid M9 medium with added PAHs (Sanders 2012). Plates were incubated at 25ºC and, after visible growth (3-7 days), distinctive colonies were obtained and streaked on agar EC medium to obtain pure cultures using streak plate procedure by the quadrant method (Sanders 2012).

Genotyping and 16S RNA gene sequencing of isolates

For DNA extraction, isolates were inoculated in LB medium (Merck, USA) spiked with PAHs and grown overnight. Cells were collected from the liquid cultures and DNA was extracted through the phenol-chloroform method [adapted for bacteria (Ghosh et al. 2013)]. To exclude clones, the DNA from each isolate was PCR-amplified using BOXA1R primer (5'-CTACGGCAAGGCGACGCTGACG-3') (Versalovic et al. 1991). Extracted DNA (1 µL) was used as template in a 25 µL reaction containing 12.5 µL of Master mix (Thermo Scientific, Waltham, MA), 2.5 µL of DMSO and 2 µL of primer solution (Rademaker et al. 2000). Amplification was performed with initial denaturation at 94ºC during 5 min, followed by 40 cycles of 94ºC/1 min; 53ºC/1 min; 72ºC/8 min and ended with an final extension at 72ºc during 15 min. Electrophoresis was performed in agarose gel 1% with GelredTM (Biotium, Fremont, CA, USA), at 80 V during approximately 160 min in TAE buffer 1 x [from a 50 x stock solution (Fisher Scientific, Hampton, NH, USA); final concentration was 40 mM Tris-acetate and 1 mM EDTA], with 2 µL of DNA size marker (GeneRuler 1 kb Plus DNA Ladder, ThermoFischer Scientific, Waltham, MA, USA) that was added in the outer sockets of the agarose gel. Gels were digitalized, and bands were aligned, using the DNA size marker as reference, and analyzed in BioNumerics software (Applied Maths, Sint-Martens-Latem, Belgium). Dendrograms were constructed by applying presence/absence Jaccard index with minimum correlation of 5%. The 16S rRNA gene of representative isolates was PCR-amplified using primers 27F (5'-AGAGTTTGATCCTGGCTCAG -3') and 1492R (5'-CTACGGRTACCTTGTTACGAC-3') and 1 µL of DNA template in a 25 µL reaction containing 12.5 µL of Master mix (ThermoFischer Scientific, Waltham, MA, USA), 0.5 µL of bovine serum albumin and 0.25 µL of each primer solution. Resulting amplicon was sequenced externally at GATC-Biotech (Konstanz, Germany). The obtained 16S rRNA gene sequences were compared with those available on the NCBI database using BLAST (https://blast.ncbi.nlm.nih.gov/Blast.cgi - Accessed 17-04-2017).

Phylogenetic tree was constructed using the selected 16S rRNA sequences of bacterial isolates obtained in this study and some of their closest relatives in GenBank [http://www.ncbi.nlm.nih.gov/] (Tamura et al. 2013). All sequences were aligned using ClustalW and a phylogenetic analysis was conducted using MEGA 6 software (http://www.megasoftware.net/; version 6.06). A phylogenetic tree was constructed using the neighbour-joining method and evolutionary distances were computed using the maximum composite likelihood with discrete Gamma distribution. In the results, a bootstrap

consensus tree based on 1000 replicates is presented. The bootstrap value is shown next to each branch when it exceeds 50% and represents the percentage of replicates in which the associated taxa clustered together.

Test for biosurfactant production

Biosurfactant production was tested by the atomized oil assay (Burch et al. 2010) as described in Domingues *et al* 2012 (Domingues et al. 2012). *Escherichia coli* DH5α was used as negative control and solutions of commercial surfactants were used as positive controls: 0.008 mM Tween 80 (Merck, Darmstadt, Germany), 10 mM sodium dodecyl sulfate (SDS; BioRad, Hercules, CA, USA), 1 mM cetyl trimethylammonium bromide (CTAB; Sigma-Aldrich, St. Louis, MO,USA) and 15.4 nM surfactin (Sigma- Aldrich, USA, St. Louis, MO,USA). The negative control and the isolates were inoculated using a sterile toothpick on LB agar medium (Merck, Darmstadt, Germany) and grown overnight at 25ºC, while for positive controls 10 µL of solution were added to agar and air-dried. Afterwards, an airbrush (model BD-128P, Fengda, United Kingdom) was used to nebulise the liquid paraffin (Merck, Darmstadt, Germany) over the colonized solid medium. The presence of biosurfactants was detected by the presence of a halo surrounding the colony visible under indirect light.

Results and discussion

A total of 39 bacterial strains were isolated from all enrichment cultures and, through BOXA1R genotyping analysis, these were reduced to 14 representative isolates. The partial 16S sequencing results of these isolates was compared with sequences available on the NCBI database and their closest phylogenetic relatives are listed in Table II-1. The phylogenetic relations between isolates and some of their closest relatives is presented in Figure II-2 and phylogenetic classification by RDP classifier (https://rdp.cme.msu.edu/classifier) is listed in Table II-2. The majority of the isolates (nine) were retrieved from the enrichment cultures using IMV samples and all except um were affiliated with the family *Bacillaceae*. Only one isolate was retrieved from the AMV enrichment culture and its closest representative was *B. baekryungensis* T10-3M (Na et al. 2011). Unfortunately, geochemical data that could determine the degree of seepage activity at each crater were not available and their classification as active or inactive was based solely on the visual presence or absence of gas hydrates and meiofauna (M.R. Cunha, personal observation). However, previous mass-sequencing data revealed a significant higher abundance of the methane-oxidizing order Methylococcales (Redmond et al. 2010) in the AMV sediments, comparatively to sediment from the IMV and the RS (Coelho et al. 2016b), therefore supporting this visually-based classification. From the RS samples 4 strains belonging to *Bacillus* spp. (2 isolates), *Virgibacillus* spp. and *Rhodococcus* spp, were isolated.

Table II-1 - Similarity results using BLAST on NCBI database (https://blast.ncbi.nlm.nih.gov/Blast.cgi - Accessed 17-04-2017) and biosurfactant production of all representative isolates with valid DNA sequences. BLAST was done excluding uncultured/environmental clones. (Phe - phenanthrene; Chry-chrysene; IMV-inactive mud volcano; AMV- active mud volcano; RS- reference site)

Representative isolate	Biosurfactant Production	Sediments	Selective culture	NCBI Accession nº	NCBI closest and most relevant sequence			Reference
					Identity	NCBI Accession nº	Similarity	
CE_34^{a}	Yes	IMV	Phe/Chry	KY941916	*Bacillus paralicheniformis* KJ-16	KY694465	99%	(Dunlap et al. 2016)
PD_13^{rab}	No	RS	Phe/Chry	KY941913	*Virgibacillus halotolerans* WS 4627	NR108860	99%	(Seiler and Wenning 2013)
CE_14^{a}	Yes	IMV	Phe/Chry	KY941922	*Bacillus* sp. KT70	KJ734004	99%	(Yu et al. 2014)
CD_21^{a}	Yes	RS	Chry	KY941914	*Bacillus licheniformis* RSP-09	JX036281	99%	(Li and Yu 2012)
PE_11^{db}	No	IMV	Phe	KY941921	*Bacillus* sp. KP3	AB638888	99%	(Baig et al. 2012)
CD_31^{d}	No	RS	Chry	KY941919	*Bacillus aquimaris* TF-12	NR025241	99%	(Yoon et al. 2003)
PE_14^{b}	No	IMV	Phe	KY941918	*Bacillus aquimaris* TF-12	NR025241	99%	(Yoon et al. 2003)
PE_13^{rab}	Yes	IMV	Phe	KY941923	*Bacillus* sp. KT122	KJ733979	99%	(Yu et al. 2014)
PE_14^{a}	Yes	IMV	Phe	KY941917	*Bacillus* sp. KT122	KJ733979	99%	(Yu et al. 2014)
PE_11^{dc}	Yes	IMV	Phe	KY941912	*Bacillus subtilis* subsp. spizizenii MA-8	KY454691	99%	not published
CA_14^{aa}	No	AMV	Chry	KY941924	*Bacillus baekryungensis* T10-3M	AB617547	99%	(Na et al. 2011)
CE_21^{a}	No	IMV	Chry	KY941915	*Bacillus flexus* SL21	JN645852	99%	(Sahay et al. 2012)
D_18^{a}	No	RS	Phe	KY941920	*Rhodococcus rhodochrous* AK40	EU004419	99%	(Táncsics et al. 2008)
					Rhodococcus sp. KL88	FJ555285	99%	(Veeranagouda et al. 2009)
CE_11^{a}	Yes	IMV	Chry	KY941925	*Acinetobacer iwoffi* KAR20	KR054982	99%	(Kõiv et al. 2015)

The majority of representative isolates (11 out of 14) had a 16S rRNA amplicon sequence highly similar to those of low G+C gram-positive spore-forming bacteria from the *Bacillaceae* family (*Bacillus* and *Virgibacillus* genera), whereas the remaining two were affiliated to the gram-positive actinobacterial *Rhodococcus* and γ-proteobacterial *Acinetobacter* genera, respectively (Table II-2). The 16S rRNA amplicon sequence from these isolates were highly similar (>99%) with those retrieved from both terrestrial (Baig et al. 2012; Yu et al. 2014) and marine environments (Yoon et al. 2003) (Table II-1). Some of these sequences were related with heavy metal tolerance (Yu et al. 2014) and halophilic environments (Li and Yu 2012; Na et al. 2011; Sahay et al. 2012). Only isolate $CE_1 1^a$ had a 99% similarity with some *Rhodococcus* strains potentially involved in the biodegradation of mono-aromatic hydrocarbons (Táncsics et al. 2008; Veeranagouda et al. 2009). All isolates had high similarity (>99%) with sequences in the database.

Table II-2 - Identification and characterization of all representative isolates with valid DNA sequences

Representative isolate	RDP Classification			
	Family		Genus	
CE34a	*Bacillaceae*	100%	*Bacillus*	100%
$PD_1 3r^{ab}$	*Bacillaceae*	100%	*Virgibacillus*	100%
$CE_1 4^a$	*Bacillaceae*	100%	*Bacillus*	100%
$CD_2 1^{ab}$	*Bacillaceae*	100%	*Bacillus*	100%
$PE_1 1^{db}$	*Bacillaceae*	100%	*Bacillus*	100%
$CD_3 1^d$	*Bacillaceae*	100%	*Bacillus*	100%
$PE_1 4^b$	*Bacillaceae*	100%	*Bacillus*	100%
$PE_2 3r^{ab}$	*Bacillaceae*	100%	*Bacillus*	100%
$PE_1 4^a$	*Bacillaceae*	100%	*Bacillus*	100%
$PE_1 1^{dc}$	*Bacillaceae*	100%	*Bacillus*	100%
$CA_1 4^{aa}$	*Bacillaceae*	100%	*Bacillus*	100%
$CE_2 1^a$	*Bacillaceae*	100%	*Bacillus*	100%
$D_1 8^a$	*Corynebacterineae*	100%	*Rhodococcus*	100%
$CE_1 1^a$	*Moraxellaceae*	100%	*Acinetobacter*	100%

Previously reported mass-sequencing data of the 16S rRNA gene revealed that the bacterial community in the IMV, AMV and RS samples (and in other MV discovered) has a low percentage of Firmicutes (Coelho et al. 2016b). Firmicutes, Bacilli and *Bacillaceae* were not among the most abundant phyla, classes and orders in any of the MV samples and reference site (Coelho et al. 2016b). This is coherent with other marine mud volcano sediments (Pachiadaki et al. 2011) and with other deep sea sediments, which are normally dominated by the Proteobacteria phylum (Li et al. 1999). However, high throughput 16S rRNA gene analyses are speculated to under-represent endospore-forming bacteria from Firmicutes, possibly because of the difficulties in disrupting the hardy outer cortex of the endospores by

many traditional DNA extraction methods (Filippidou et al. 2015). Also the abundance of PAH-degrading bacteria may be niche-specific, since PAHs, although detected (Geptner et al. 2006; Lorenson et al. 2002; Polonik et al. 2015; Wang et al. 2011), are extremely less concentrated in MV sediments in comparison to the C1-C4 gaseous hydrocarbons (Lein et al. 1999; Niemann et al. 2006). However among the culturable fraction, bacteria from the phyla Firmicutes are recurrently isolated from deep sea environments (da Silva et al. 2013; Lu et al. 2001), inclusively *in situ* (Gärtner et al. 2011).

PAH-degrading bacteria affiliated with the *Bacillaceae* family have rarely been isolated from deep sea sediments (Louvado et al. 2015) and the same applies to other gram-positive bacterial groups [exception include a two actinobacterial strains (Peng et al. 2008a; Wang et al. 2008)]. In both terrestrial and marine environments, gram positive PAH-degrading bacteria are more commonly found among the high G+C Actinobacteria phylum (Vila et al. 2015) and known encoding genes of the PAH-dioxygenase enzyme in gram positive bacteria seem limited to actinobacterial strains from terrestrial environments (Cébron et al. 2008; Ding et al. 2010; Iwai et al. 2011). Although infrequent, some exceptions have been reported in marine environments, as members of the *Bacillaceae* family have been previously isolated from PAHs enrichment cultures using various mesophilic marine sediments as inoculum (Daane et al. 2002; Lin and Cai 2008; Marcos et al. 2009; Zhuang et al. 2003). For example, in a culture independent analysis of anaerobic sediment, the Firmicutes phylum (mainly represented by the Peptostrepococceae family of the Clostridiales order) was a predominant bacterial group in the oil amended microcosm (Sherry et al. 2013). However, PAH-degrading isolates obtained from deep-sea sediments are normally affiliated to taxa from the Proteobacteria phylum (Cui et al. 2008; Shao et al. 2010; Wang et al. 2008), possibly because efforts to isolate PAH-degrading bacteria here have been mostly focused on the abyssal plains (Louvado et al. 2015).

Bacillus-like isolates with PAH-degrading capacity may possibly be more frequently detected in environments with potentially extreme abiotic conditions. In terrestrial environments, *Bacillus*-like bacteria have been repeatedly reported as a key xenobiotic degrader under thermophilic and alkaliphilic conditions (Annweiler et al. 2000; Margesin and Schinner 2001). Coherently, in marine extreme environments, the *Bacillus* genus comprised the majority of the cultivable heterotrophic bacterial community at a deep sea brine lake [a geological phenomenon frequently associated with mud volcanism (Dupré et al. 2014)] (Sass et al. 2008) and at an alkaline serpentinizing fluid seepage site (Meyer-Dombard et al. 2014). The *Bacillaceae* family was relevant in the culture-independent analysis of deep sea cold seep-associated pockmark (Cambon-Bonavita et al. 2009). Furthermore, the *Bacillaceae* family was detected as the predominant taxa in overall bacterial community of an anaerobic EC, with petroleum as it sole carbon source, using the same IMV samples from this study, and a relevant family in similar anaerobic EC using AMV. sediments (Domingues et al. 2016). Gram-positive bacteria, potentially because of its stronger outer peptidoglycan cell wall, thrive in extreme and highly variable abiotic conditions (Zhuang et al. 2003). Yet, from the RS samples *Bacillus* spp. isolates were also retrieved, revealing that *Bacillus*-like

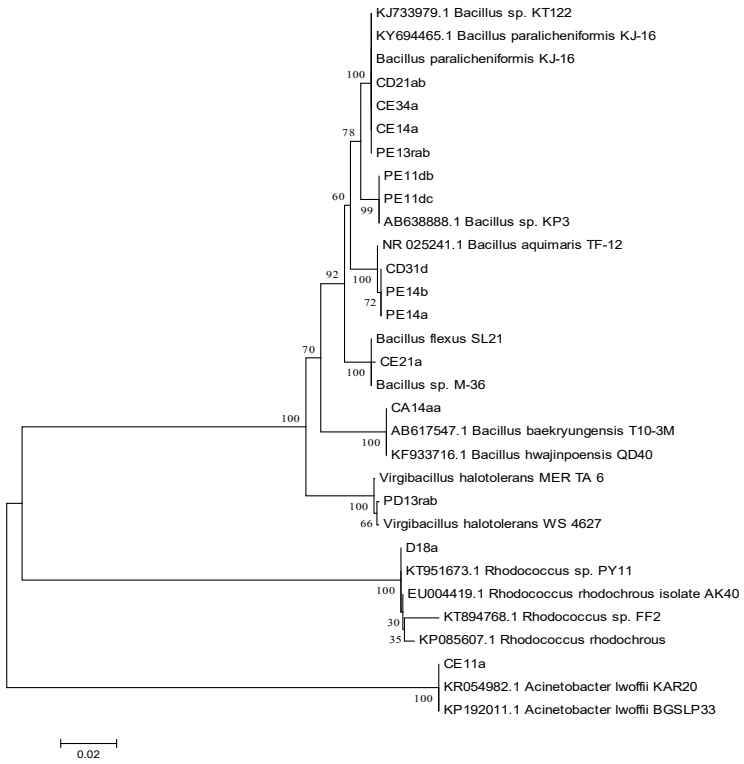

Figure II-2 - Phylogenetic relations of the 16S rRNA gene sequences of bacterial isolates obtained in this study and some of their closest relatives in GenBank [http://www.ncbi.nlm.nih.gov/] (Tamura et al. 2013). All sequences were aligned using ClustalW and a phylogenetic analysis was conducted using MEGA 6 software (http://www.megasoftware.net/; version 6.06). A phylogenetic tree was constructed using the neighbor-joining method and evolutionary distances were computed using the maximum composite likelihood with discrete Gamma distribution and a bootstrap consensus tree based on 1000 replicates is presented. The bootstrap value is shown next to each branch when it exceed 50% and represents the percentage of replicates in which the associated taxa clustered together.

bacteria are important PAH-degraders in these stable deep sea abyssal plains or that the RS was influenced by potential and undiscovered natural oil seepage sites in its surroundings

Biosurfactant production by hydrocarbon-degrading bacterial communities is a strategic adaptation to cope with the low aqueous solubility and consequent bioavailability of oil hydrocarbons. In this study, biosurfactant production (Figure II-3) was detected by the atomized oil assay in 5 representative isolates: 4 were affiliated to the *Bacillus* genus and one to the *Acinetobacter* genus (Table II-2). Both genera are known to comprise biosurfactant-producing strains (Ceresa et al. 2016; Dong et al. 2016; Patowary et al. 2015). Biosurfactant-producing *Bacillus* spp usually produce lipo-cyclicpeptides (e.g. surfactin) (Ceresa et al. 2016), while *Acinetobacter* spp. produce glycolipid biosurfactants (Dong et al.

2016). Additionally, three isolates described here [PD_13^{rab} (LF1), CE_21^{a} (LF3) and CE_34^{a} (LF5)] were previously reported to

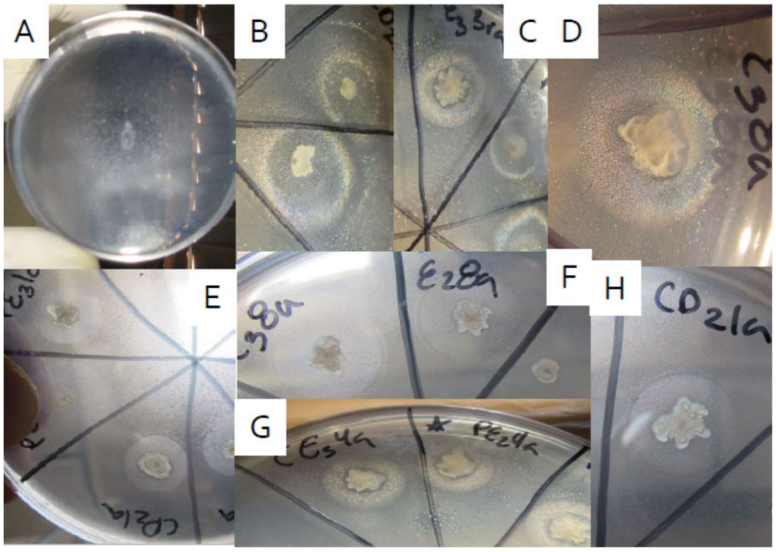

Figure II-3 - Results from the atomized oil assay used for the detection of biosurfactant production among isolates. A - Negative control (*Escherichia coli* DH5α); B to H - Examples of some positive result.

efficiently degrade the endocrine-disrupting compound 17β-estradiol (E2) and its subsequent metabolic intermediate (E1) (Fernández et al. 2016). These results demonstrate a versatile metabolism of xenobiotic pollutants and highlight the biotechnological potential of these isolates.

Our study showed that *Bacillus*-like species may potentially be involved in PAH biodegradation in deep sea environments. However, although the analysis of the culturable fraction of a particular bacterial community may establish direct functional roles inside the consortium, it may only represent a minor fraction of the microorganism's present. Many bacteria may have been excluded in both the enrichment culture and in the isolation procedures because of several factors such as inappropriate culture medium composition, incapacity to grow on solid medium, not ideal *in vitro* abiotic conditions and strong interdependencies between organisms (Zhang et al. 2017).

Conclusions

Isolates attributed to the *Bacillaceae* family dominated the culturable fraction of the PAH-degrading bacterial community from the enrichment cultures established using deep sea MV sediments. This dominance was unexpected since, to the best of our knowledge, PAH-degrading gram-

positive bacteria are rarely isolated from the deep sea environment. The potential biotechnological application in the biodegradation of xenobiotics is promising.

Acknowledgements and Funding

The M86/5 cruise was funded by the German Research Foundation (TransFlux grant) with additional support by the Portuguese Foundation for Science and Technology (FCT) with the SWIMGLO project (FCT PTDC/MAR/100522/2008), and FLOWS (EU-COST, ES 1301). We thank the Captain and crew, as well as the scientific and technical parties onboard the R/V Meteor during the M86/5 cruise for excellent support at sea. A. Louvado was supported by a PhD scholarship (SFRH/BD/86447/2012) funded by FCT, Portugal (QREN-POPH - Type 4.1 – Advanced Training, subsidized by the European Social Fund and national funds MCTES). This work had financial support from CESAM research unit (PEst-C/MAR/LA0017/2013) and QOPNA research unit (PEst-C/QUI/UI0062/2013).

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Chapter III

Chapter III

Independent and interactive effects of reduced seawater pH and oil contamination on subsurface sediment bacterial communities

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Abstract

Ocean acidification may exacerbate the environmental impact of oil hydrocarbon pollution by disrupting the core composition of the superficial (0-1 cm) benthic bacterial communities. However, the biogeochemical characteristics of subsurface sediments may act as a potential buffer to environmental changes. In this study, we used a microcosm experimental approach to access the independent and interactive effects of reduced seawater pH and oil contamination on the composition of subsurface benthic bacterial communities, at two time points, by 16S rRNA gene based high-throughput sequencing. An in-depth taxa-specific variance analysis revealed that the independent effects of reduced seawater pH and oil contamination were significant predictors of changes in the relative abundance of some specific bacterial groups (e.g. Bacillales, Clostridiales, Rhizobiales and *Desulfobulbaceae*). However, overall the results indicate that the independent and interactive effects of reduced pH and oil contamination had no significant impact on the subsurface bacterial communities. This study provides evidence that, despite previous indications, bacterial communities inhabiting subsurface sediment may be less susceptible to the effects of oil contamination in a scenario of reduced seawater pH.

Keywords: Oil spill, Sulfate-reducing bacteria, *Desulfobulbaceae*, Ocean acidification, Climate change, Subsurface sediments

Introduction

Carbon dioxide partial pressure (pCO_2) in the atmosphere has increased from 280 to 404 ppm between the preindustrial era and October 2016 (Dlugokencky and Tans 2016; Indermuhle et al. 1999). This rise was caused by fossil fuel consumption and its rate has no parallel with past events (IPCC 2013).

The oceans have attenuated this rise by absorbing 30% of the CO_2 emitted, but at a cost (Canadell et al. 2007). In seawater, an increase in pCO_2 alters the balance of inorganic carbon species and, consequently, reduces seawater pH in a process known as ocean acidification (OA) (Doney et al. 2009). It is estimated that, in average, pH levels in ocean surface water will drop between 0.3 to 0.4 units (Hartin 2016; IPCC 2013), depending on models, and by the end of this century, and up to 0.7 units by 2300 (Hartin 2016).

The total impact of OA on marine microbial communities is difficult to predict, mainly due to the multiple environmental and biological factors involved. An increase in pCO_2 is certain to have a negative impact on all calcifying biota (Doney et al. 2009) and has the potential to augment photosynthesis, thus indirectly increasing nitrogen fixation and additional CO_2 absorption (Riebesell et al. 2007). OA may alter the speciation, solubility, organic complexation and, consequently, the bioavailability of essential and/or toxic trace metals (Hoffmann et al. 2012). This may affect lithotrophic metabolism (Kirk et al. 2016) and, subsequently, anaerobic hydrocarbon biodegradation (Zeng et al. 2015), which relies on nitrate, sulfate, iron(II) or CO_2, among others, as electron acceptors (Wentzel et al. 2007; Widdel and Musat 2010). An increase in seawater pCO_2 may differently affect these electron acceptors, increasing (e.g. CO_2) or decreasing [i.e. NO_4^{2-}, SO_4^{2-} and Fe(II)] their bioavailability, and, thus, potentially altering the predominant metabolism in the microbial community (Kirk et al. 2016; Millero 2009). OA may also reduce burrowing, and expand the suboxic and anoxic zones of sediments (Laverock et al. 2013), where hydrocarbon biodegradation is substantially less efficient (Widdel and Musat 2010), but essential (McGenity 2014). Here, the lack of disturbance and the high adhesion forces of the predominant clay/mud sediments physically entrap and occlude buried hydrocarbons from initial oxygenation and photolysis (Louvado et al. 2015; McGenity 2014), promoting their persistence (Peterson et al. 2003).

A previous study showed that the interaction between reduced seawater pH and oil contamination significantly altered the active microbial community at the uppermost sediment layer (≈ 0-1cm b.s.f.) (Coelho et al. 2016a; Coelho et al. 2015). The interaction between these two factors significantly reduced the relative abundance of anaerobic hydrocarbon-degrading sulfate reducing bacteria (Order Desulfobacterales and Desulforomonadales) (Coelho et al. 2015). This reduction was later found to be coupled with a reduction in specific archaeal groups and an increase in putative hydrocarbonoclastic fungal OTUs (Coelho et al. 2016a). However, the implications of the potential effects of reduced seawater pH and oil contamination on microbial communities inhabiting subsurface-sediment anaerobic layers remains unknown. We suspect that the impact of OA on microbial communities and their functions will be attenuated in sediments (Braeckman et al. 2014), particularly at the subsurface sediments (Dashfield et al. 2008; Widdicombe et al. 2009), by the buffering capacity of sediments constituents (Ben-Yaakov 1973; Pertusatti and Prado 2007).

Here, we used a 16S rRNA gene based high-throughput sequencing approach to study the independent and interactive effect of reduced seawater pH and oil contamination on the structure of

subsurface sediment bacterial communities under controlled microcosm conditions. Taking into account the potential buffer effect of subsurface sediments against environmental changes, we hypothesized that bacterial communities colonizing this habitat will be less susceptible to the effects of oil contamination in a climate change scenario of reduced seawater pH.

Methods

Microcosm experiment

This study is based on a microcosm experiment that was previously reported (Coelho et al. 2015). The microcosm platform, named experimental life support system (ELSS) is a flow-through system and has been previously validated to study the effects of climate change scenarios on benthic marine communities (Coelho et al. 2013). It was originally used to investigate the independent and interactive effects of ocean acidification, ultraviolet radiation and oil contamination in estuarine and coastal marine benthic communities [bacteria, an epibenthic gastropod (*Peringia ulvae*) and a benthic polychaete (*Hediste diversicolor*)]. The ELSS maintains individual microcosms at a controlled temperature, by submersion in water bath, while allowing the simulation of a diurnal light and tidal cycle, and the manipulation of pH. Full details regarding microcosm architecture and validation can be found in Coelho et al. (2013).

In this study, we specifically focused on bacteria, the subsurface sediment layer (≈ 5 cm b.f.s.) and the independent and interactive effect of the factors: oil hydrocarbon contamination and seawater pH. Each factor had two levels: normal and reduced pH; with and without oil. In total, four treatments were tested: a) 'Cont' (normal seawater pH without oil contamination); b) 'OnpH' (reduced seawater pH without oil contamination); c) 'OnOi' (normal seawater pH with oil contamination); d) 'pHOi' (reduced seawater pH with oil contamination). Each treatment was replicated in four independent microcosms randomly arranged. Seawater pH was reduced by bubbling CO_2 and automatically stabilized by a V2 Pressure Regulator Pro coupled to V2 Control pH Controller (TMC Iberia, Lisbon, Portugal) (Gattuso and Lavigne 2009). The average synthetic seawater pH was 7.62 ± 0.11 in microcosms supplied with acidified seawater and 7.94 ± 0.10 in the control microcosms. This difference falls within the 0.3–0.4 pH decrease modelled for global sea surface for the year 2100 (Caldeira and Wickett 2003). The simulation of an oil spill was done by pouring crude oil (0.5% v/v) into randomly selected treatments during five consecutive high tides. Detailed information regarding the experiment can be found in Supplementary Files.

Sampling and DNA Extraction

Composite sediment samples were collected, at the end of the high tide, seven (time point 7 - T7) and twenty-one (time point 21 - T21) days after the simulated oil spill. Four mini-cores (ø 1 cm) per microcosm were retrieved from the sediment and a ≈1 cm layer at approximately 5 cm b.s.f. was sliced, collected and pooled. Samples were stored immediately at -80ºC. DNA was extracted using E.Z.N.A Soil DNA Kit (Omega Bio-tek, Norcross, GA, USA) following manufacturer recommendations.

Illumina MiSeq sequencing

The 16S rRNA gene V4 region was PCR amplified using primers 515 (5´-GTGCCAGCMGCCGCGGTAA-3´) and 806 (5´-GGACTACHVGGGTWTCTAAT-3´) with barcode on the forward primer in a 30 cycle PCR using the HotStarTaq Plus Master Mix Kit (Qiagen, USA) under the following conditions: 94°C for 3 minutes, followed by 28 cycles of 94°C for 30 seconds, 53°C for 40 seconds and 72°C for 1 minute, after which a final elongation step at 72°C was performed during 5 minutes. After amplification, PCR products were checked in 2% agarose gel. Pooled samples were purified using calibrated Ampure XP beads. Library preparation and sequencing were performed externally (MR. DNA, Shallowater, TX, USA) on a MiSeq sequencing platform following standard Illumina protocols.

Data analysis

Sequencing analysis was performed using the Quantitative Insights Into Microbial Ecology (QIIME) software package (http://qiime.org; Accessed 01 January 2014) according to published recommendations and following previously described methods (Cleary et al. 2015; Kuczynski et al. 2011) with the exception of the operational taxonomic unit (OTU) picking step, where the UPARSE (Edgar 2013) clustering method and chimera check was used. The most recent Greengenes database (ftp://greengenes.microbio.me/greengenes_release/gg_13_5/gg_13_8_otus.tar.gz) was used for taxonomic assignment. Detailed information regarding the data analysis is available in Supplementary Information. Sequences are available at NCBI sequence read Archive (study accession nº PRJNA391281).

Statistical analysis

The OTU tables were uploaded to R software (version 3.1.1; http://www.r-project.org/) for removal of unassigned and singleton OTUs, chloroplast and mitochondrial sequences, statistical computing and graphic generation. A self-written function (Gomes et al. 2010) was used to estimate total rarefied OTU richness for each treatment and timepoint. Variation in composition among treatments

was assessed using principal coordinates analysis (PCO). The PCO was generated using the cmdscale() function in the R base package in vegan (Oksanen et al. 2012). Prior to the PCO, the raw data was log(x+1) transformed and used to produce a distance matrix with the Bray-Curtis index with the vegdist() function in vegan. Also in vegan, the adonis() function for permutational multivariate analysis of variance (PERMANOVA) was used to test for significant variations in composition among factors (Oksanen et al. 2012). In the adonis analysis, the Bray–Curtis distance matrix of OTU composition was the response variable with factors oil and pH (oil*pH) as the independent variables. The number of permutations was set at 999. To test variation in OTU composition between time points a repeated measure permutational analysis of variance was performed with the adonis() function in the vegan package (Oksanen et al. 2012). We also preformed, for all relevant taxa (phylum to family with a mean relative abundance ≥0.1 %) at each time point, a taxon-specific analysis, in which the significance of their compositional variation, between treatments, was accessed through an ANOVA. A linear regression distribution was created using the glm() function in the stats package with the OTU matrix as the response variable and oil, pH and the interaction between these two factors (oil*pH) as independent variables. We limited our analysis by establishing a threshold (mean relative abundance ≥0.1 %) to all levels. The relative abundance was calculated for each time point as a percentage of the total number of sequences in each sample using the prop.table() function in base package and the mean relative abundance was obtain through the arithmetic mean of these percentiles for each bacterial group in samples and treatments. Next, a two-way ANOVA test was applied to the models, using the anova() function in the stats package in R with the F test [adapted from (Roy et al. 2013a)]. The most significant (p-value ≤ 0.05) taxa were plotted using the boxplot() function from the graphics package.

Results and discussion

Overall bacterial community analysis

The sequencing effort generated ≈1.9x10^6 sequences that, after quality screening, were clustered in 26 237 OTUs. Rarefied richness (Figure S III-1) had no asymptote for in any sample, which indicates that the true richness is higher than reported here. These OTUs were assigned to 67 phyla (95.88% of all OTUs), 163 classes (89.80%), 233 orders (67.89%) and 281 families (38.78%). Overall, the bacterial community of the anaerobic sediment fraction was dominated by Proteobacteria (mean relative abundance 53.49 ± 4.29%), followed by Chloroflexi (10.62 ± 4.54%), Bacteriodetes (8.94 ± 2.57%), Actinobacteria (6.82 ± 2.61%), Planctomycetes (3.97 ± 0.99%), Acidobacteria (3.79 ± 0.38%), Gemmatimonadetes (1.53 ± 0.19%), candidate division WS3 (1.12 ± .0.26%), Cladithrix (1.03 ± 0.24%) and Spirochaetes (1.03 ± 0.41%). The occurrence of Proteobacteria in the subsurface sediment layer was lower than in the superficial sediment (RNA-based study) (Coelho et al. 2015). In the superficial sediment, there was a considerable increase in the relative abundance of this phylum in the end of the

microcosm system validation (95.74%) when compared with the sampling site (74.03%) (Coelho et al. 2013). In the subsurface layer, however, the relative abundance of this phylum was relatively stable throughout the experiment (T7 - 53.69 ± 3.86%; T21 - 53.28 ± 4.81 %). However, representativeness of the Proteobacteria phylum is similar with previous studies at the Ria de Aveiro water system (Coelho et al. 2013; Gomes et al. 2013; Oliveira et al. 2014b). In our dataset, the Proteobacteria phylum was mainly represented by classes δ- (24.03 ± 4.35%)and γ-(22.70 ± 4.86%) proteobacteria. The predominance of deltaproteobacterial OTU is mainly attributed to the sulfate-reducing bacterial OTU from the Desulfobacterales order (15.13 ± 3.95%) which normally dominates the bacterial community in anoxic barren wetland environments (Gomes et al. 2013).

Table III-1 Output results from the Adonis statistical test for factors independently and in interaction.

	Timepoint 7			Timepoint 21		
	$F_{1,15}$	R^2	*p*-value	$F_{1,15}$	R^2	*p*-value
pH	0.302	0.021	0.793	0.625	0.046	0.549
Oil	1.383	0.098	0.225	0.768	0.056	0.486
pH and Oil	0.375	0.027	0.726	0.316	0.023	0.799

The PCO analysis (Figure III-1) revealed no clear separation among treatments, thus suggesting that the independent and interactive effect of seawater pH and oil contamination had no major effect on the overall structure of the subsurface bacterial communities. This was further confirmed by the adonis statistical test (Table III-1). The repeated measures adonis analysis showed that time was not a significant predictor of overall bacterial OTU composition (repeated measures adonis, $F_{1,31}$=0.472, R^2=0.016, P=0.543), indicating that bacterial community structure was stable throughout the experiment. The biogeochemical characteristics of subsurface sediments may have attenuated the effects of the tested factors. However, despite the lack of major changes in the subsurface communities, a group specific analysis for each time point revealed that four phyla, seven classes, twelve orders and ten families responded (ANOVA *p*-value < 0.05) to the independent and/or interactive effects of reduced seawater pH and oil contamination. These taxa are plotted for T7 in Figure III-2 and for T21 in Figure III-3.

Independent effects of oil hydrocarbon contamination

Overall, the independent effect of oil contamination did not significantly alter the subsurface bacterial community composition at any of the time points (Table III-1). Despite this, the group-specific analysis detected significant variation (ANOVA *p*-value < 0.05) in the relative abundance of some bacterial groups by oil at T7 (Figure III-2) and T21 (Figure III-3). Some taxa previously associated with oil hydrocarbon degradation increased in relative abundance in response to oil amendment. For example,

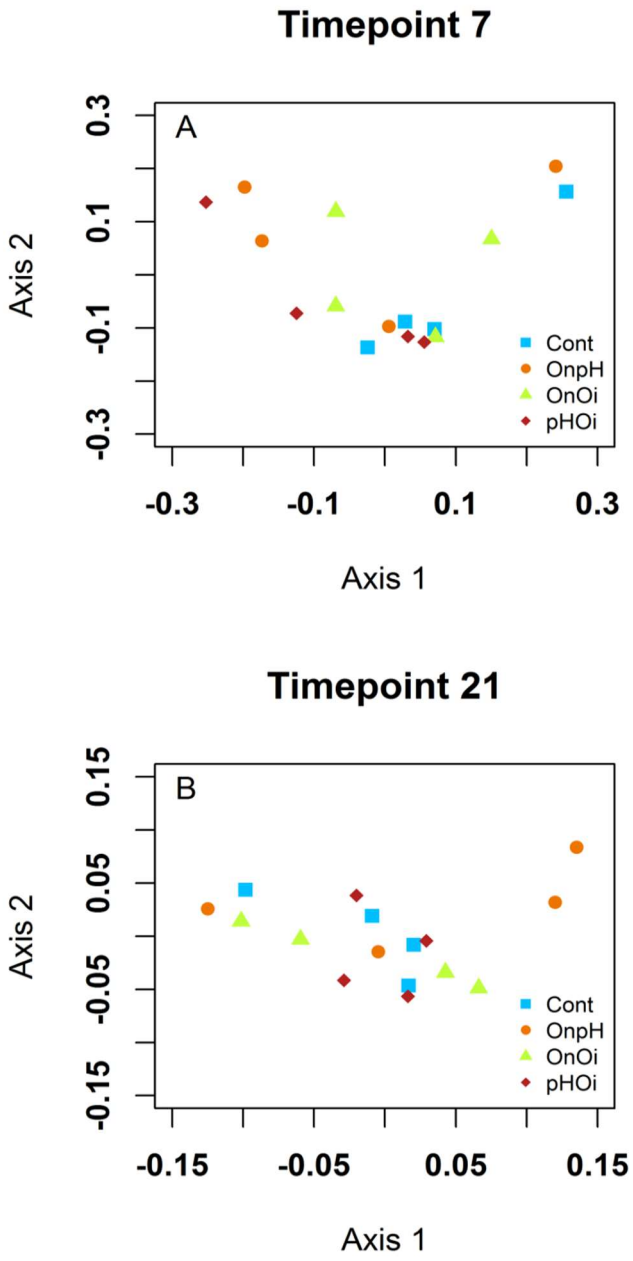

Figure III-1 - Principal components analysis (PCO) of bacterial operational taxonomic units (OTU) 7 days (A) and 21 days (B) after oil contamination. The PCO was generated using the cmdscale() function in the R base package and wascores() function in vegan. Prior to the PCO, the raw data was log10(x+1)-transformed and used to produce a distance matrix based on the Bray-Curtis distance with the vegdist() function in vegan (Oksanen 2012). The first two explanatory axes are shown. Cont: control with no treatment; OnpH: reduced pH; OnOi: contaminated with oil; pHOi: reduced pH and contaminated with oil. Sequence read (from MiSeq Illumina) was assigned to OTU with QIIME software (http://qiime.org) using the most recent Greengenes database

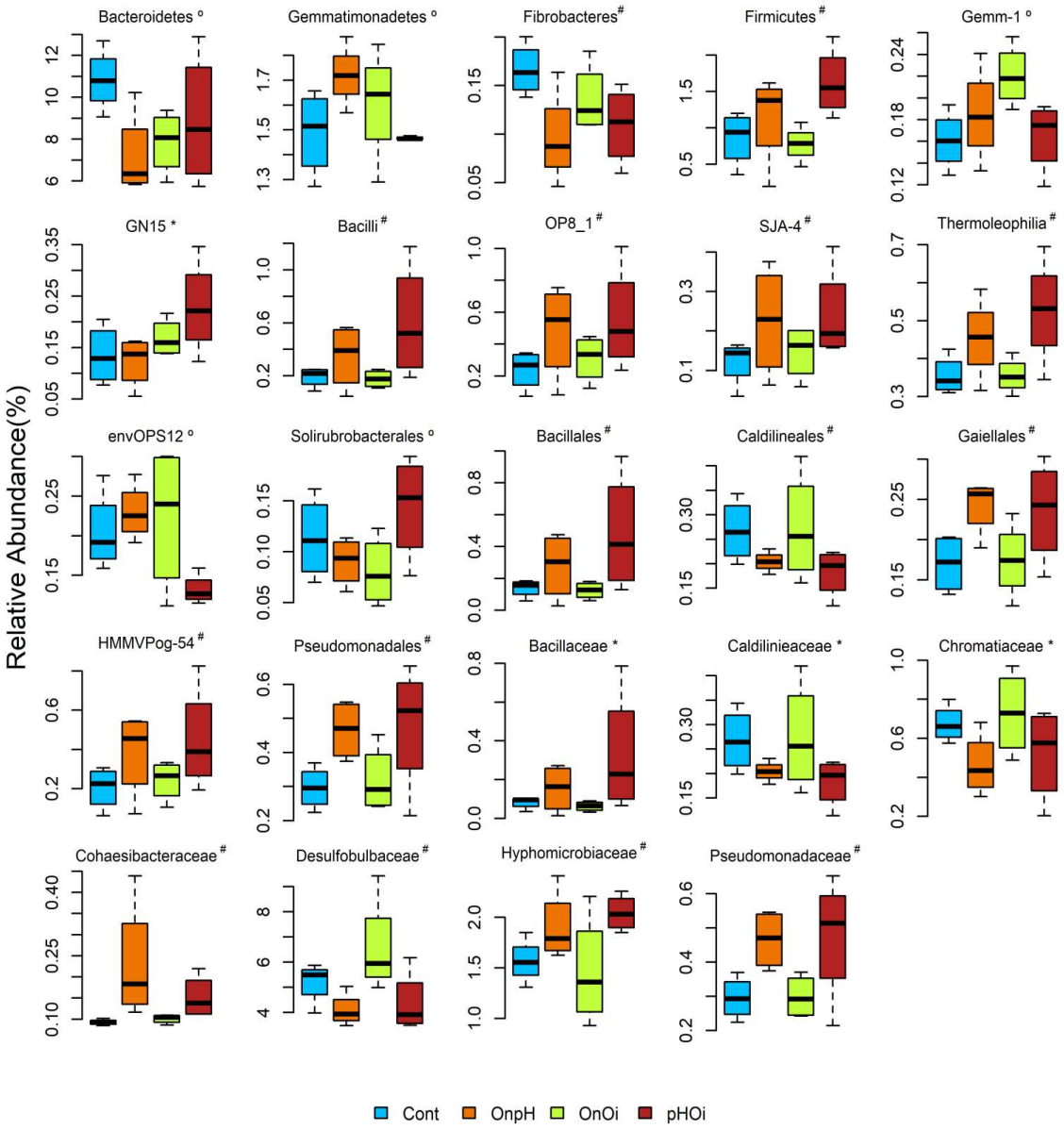

Figure III-2 - Boxplot with the relative abundances of OTU classified to bacterial groups (phylum, class, order and family) interaction at time point 7. These bacterial groups were statistically significant (Two-way ANOVA p-value<0.05) to the factors reduced seawater pH (#) and oil contamination (*) independently and to their interaction (º). Cont: control with no treatment; OnpH: reduced pH; OnOi: contaminated with oil; pHOi: reduced pH and contaminated with oil. Plot was generated using the barplot() function in the graphics package using OTU relative abundance matrix. Sequence read (from MiSeq Illumina) were assigned to OTU with QIIME software (http://qiime.org) using the most recent Greengenes database (ftp://greengenes.microbio.me/greengenes_release/gg_13_5/gg_13_8_otus.tar.gz). In the ANOVA, each factor independently and the interaction had 1 degree of freedom

order Sphingomonadales includes well known aerobic hydrocarbon degrading bacteria (Kim and Kwon 2010). However, order Sphingomonadales represent at most an average of 0.16 ± 0.05% of the bacterial community. The lack of oxygen in the subsurface sediment layer probably limited its proliferation. On the contrary, putative anaerobic hydrocarbon-degrading bacteria belonging to the *Clostridiaceae*, *Hyphomicrobiaceae* and *Phyllobacteriaceae* families (and their respective orders) were more abundant and had a significant response to oil contamination at T21 (Figure III-3). Order Clostridiales are frequently detected in anoxic oil contaminated matrixes, including wetland sediments (Sherry et al. 2013) and anaerobic oxidation has been found to be coupled with nitrate (van der Zaan et al. 2012), thiosulfate (Sherry et al. 2013) and iron (Lentini et al. 2012) reduction. The relative abundance of Clostridiales was previously found to increase with depth in oil contaminated microcosm sediments (Sanni et al. 2015). *Hyphomicrobiaceae* and *Phyllobacteriaceae* families include known oil degrading bacteria and have been frequently detected in anoxic oil-contaminated matrixes (Fahrenfeld et al. 2014; Jin et al. 2013; Oren and Xu 2014; Pan et al. 2014; Wu et al. 2009). Representative sequences from our most abundant *Hyphomicrobiaceae* OTUs were compared with sequences available on NCBI database and had 99%sequence similarity to the 16S sequences of bacterial strains retrieved from anoxic, hydrocarbon- and sulfate-rich (2mM) port sediments (OTU 12 and 17377) .

The number of groups affected by oil and their representativeness increased substantially at T21 (Figure III-3). This late response is probably explained by a delayed migration of oil hydrocarbons through the sediments. However, since hydrocarbon concentration was not measured at sampling depth, this can only be speculated. In estuarine environments, the fine-grained sediments form a compact sediment barrier that substantially slows oil migration (Cheong and Okada 2001). Furthermore, in our microcosms, the sediment cores were always submerged and pore saturation may have further impeded oil permeation (Oliveira and Nicolodi 2017). The sediment barrier may have also reduced the quantity of hydrocarbons that reached the subsurface sediments, attenuating its overall impact. For example, in a similar oil spill microcosm simulation, oil hydrocarbons were percolated from the surface layer to the subjacent layer, but, their concentrations at the subadjacent layer were considerably lower (≈10-fold) than those measured initially at the surface layer (Nakazawa et al. 2016). This attenuation in concentrations of oil hydrocarbons may be explained by an efficient biodegradation in overlying sediments (Nakazawa et al. 2016).

Independent effects of reduced seawater pH

Acidified seawater did not change the overall bacterial community composition (Figure III-1 and Table III-1). The absence of geochemical data at sampling depth limits our interpretation of this results. Sediment pH profiles are complex and naturally variable, and their response to high-CO_2 systems will be greatly influenced by sediments physico-chemical properties (Queirós et al. 2015).The dissolution

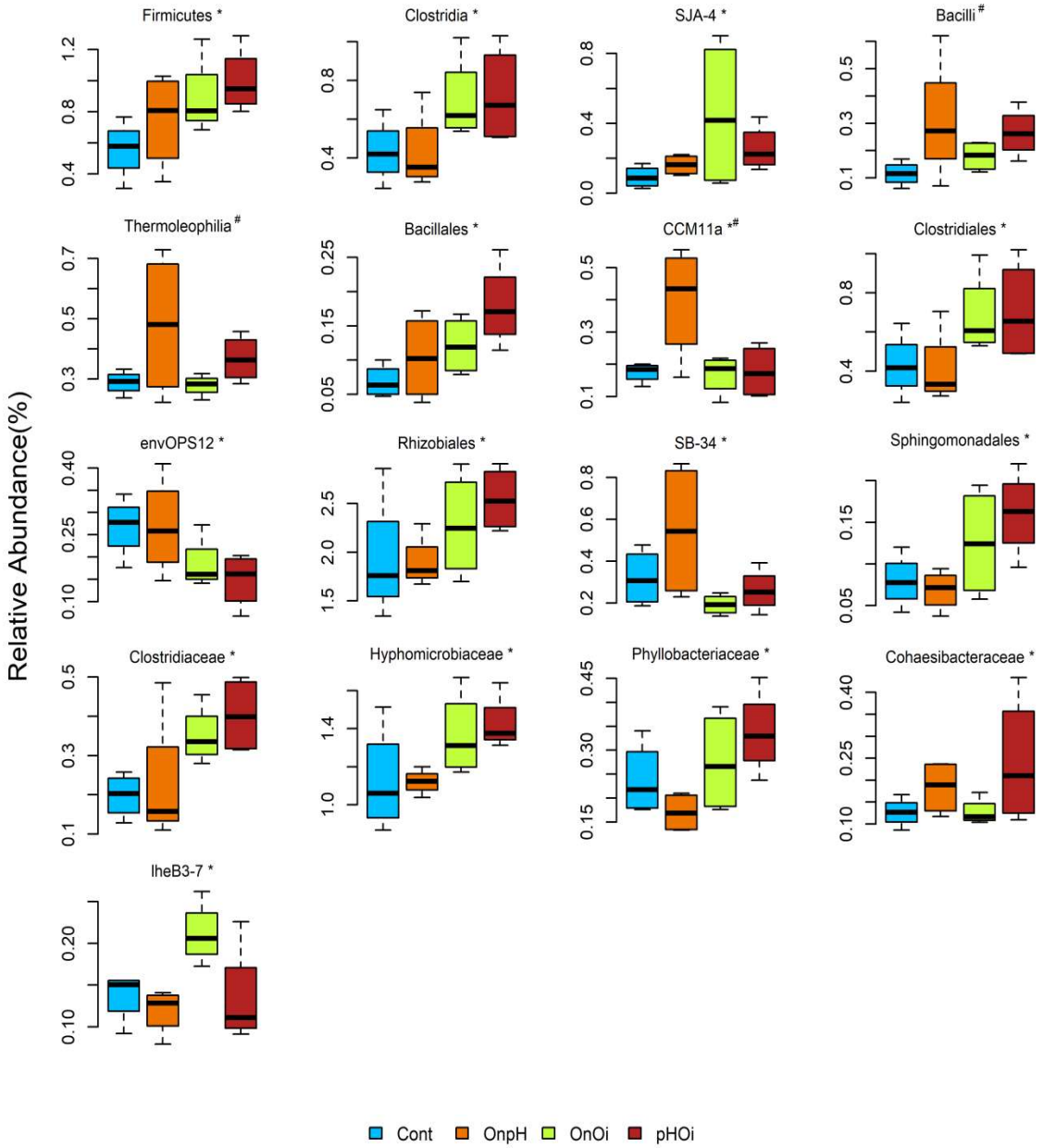

Figure III-3 - Boxplot with the relative abundances of OTU classified to bacterial groups (phylum, class, order and family) interaction at time point 21. These bacterial groups were statistically significant (Two-way ANOVA p-value<0.05) to the factors reduced seawater pH (#) and oil contamination (*) independently. Cont: control with no treatment; OnpH: reduced pH; OnOi: contaminated with oil; pHOi: reduced pH and contaminated with oil. Plot was generated using the barplot() function in the graphics package using OTU relative abundance matrix. Sequence read (from MiSeq Illumina) were assigned to OTU with QIIME software (http://qiime.org) using the most recent Greengenes database (ftp://greengenes.microbio.me/greengenes_release/ gg_13_5/gg_13_8_otus.tar.gz). In the ANOVA, each factor independently and the interaction had 1 degree of freedom

of carbonate salts (Ben-Yaakov 1973; Morse et al. 2006), the absorption of [H^+] ions by humic acids (Pertusatti and Prado 2007) and the high levels of pCO_2 and [H^+] derived from organic matter mineralization (Soetaert et al. 2007; Zhu et al. 2006) all contribute to the sediment buffer capacity, which may have stabilized porewater pH during their transit. Previous studies have indicated that porewater pH decreases in the most superficial sediments (2-3 mm)but this acidification is progressively attenuated in deeper sediments (Dashfield et al. 2008; Widdicombe et al. 2009).

Despite this, at both time points the group specific analysis of sub-surface sediment communities revealed that some bacterial groups responded to the reduction in seawater pH (Figure III-2, Table S III-1 and Table S III-2). This compositional stability of the overall bacterial community and the some significant group-specific variations are consistent with previous studies (Kerfahi et al. 2014; Queirós et al. 2015; Raulf et al. 2015; Roy et al. 2013a; Tait et al. 2013). Among the bacterial groups that had a significant response to reduced seawater pH is included class Bacilli, which had a significant higher relative abundance in the reduced seawater pH treatments at both T7 (Figure III-2 - Cont: 0.19± 0.08%; OnpH: 0.35 ± 0.25%; pHOi: 0.6 ± 0.44%; OnOi: 0.18 ± 0.07%) and T21 (Figure III-3- Cont: 0.12 ± 0.04%; OnpH: 0.31 ± 0.23%; OnOi: 0.18 ± 0.06%; pHOi: 0.27 ± 0.09%). Although statistically significant, the relative abundance of class Bacilli had a high variation among replicates in reduced pH treatments. Nonetheless, this trend is consistent with other studies, as bacteria associated to the phylum Firmicutes tend to increase in abundance in reduced pH conditions (Chauhan et al. 2015; Meron et al. 2011; Morrow et al. 2015). For example, the relative abundance of the order Bacillales, in the bacterial community associated to the coral *Acropora eurystoma*, increased when pH was artificially reduced from 8.2 to 7.3 (Meron et al. 2011). Also, at an underwater CO_2 vent (low pH marine environment), order Bacillales relative abundance was the highest when pH was the lowest and it decreased along the pH gradient created by the vent (from pH 6.7 to 8.0) (Chauhan et al. 2015)

Acidified seawater also caused a significant decrease ($F_{1,16}$=6.991; P=0.018) of the relative abundance of the Family *Desulfobulbaceae*, but only at T7 (OnpH: 4.1±0.67; pHOi: 4.37±1.24; Cont: 5.21 ± 0.84; OnOi: 6.58 ± 1.95). These results are coherent with results obtained in the top layer (Coelho et al. 2015) and a negative correlation between reduced seawater conditions and these taxa may be possible. *Desulfobulbaceae* are obligate sulfate reducing bacteria (Kuever et al. 2015) frequently involved in syntrophic oxidation of methane along with the anaerobic methanotrophic archaea (Green-Saxena et al. 2014). In this syntrophy, bacteria from the *Desulfobulbaceae* family characteristically use nitrate as preferential nutrient source (Green-Saxena et al. 2014). Reduced seawater pH may decrease nitrate concentrations, since it is known to decrease ammonia oxidation (Beman et al. 2011), thus possibly explaining the significant lower abundance of family *Desulfobulbaceae* in acidified seawater treatments.

Interactive effects of reduced seawater pH and oil pollution

The interactive effects of reduced seawater pH and oil contamination were characterized by a significant reduction in the relative abundance of five taxa at T7 (Figure III-2, Table S III-3 and Table S III-4) at T7. Responsive groups include some notably abundant taxa [e.g. phylum Bacteroidetes (average relative abundance: 8.69 ± 2.42%) and Gemmatimonadetes (1.57 ± 0.18%)]. Bacteroidetes are heterotrophic bacteria characteristically involved in degradation of recalcitrant polymeric organic matter (Fernandez-Gomez et al. 2013), particularly polysaccharides. The catabolism of polysaccharides may be indirectly stimulated in CO_2-acidified seawater by an increased activity of their catabolic enzymes (Piontek et al. 2010). Thus, reduced pH conditions and an increase in carbon inputs could explain the higher relative abundance of phylum Bacteroidetes in pHOi (8.89 ± 3.21%) when compared to OnpH (7.19 ± 2.06%) and OnOi (7.87 ± 1.52%). The Gemmatimonadetes phylum is divided into five non-curated groups in the SILVA database (Hanada and Sekiguchi 2014) and in our results only the first group (Gemm-1) had a significant response to the interactive effect of reduced seawater pH and oil contamination (Figure III-2). Gemmatimonadetes are speculated to be characteristically involved in phosphate removal through polyphosphate intracellular accumulation (Zhang et al. 2003). Nonetheless, Gemmatimonadetes have been detected in hydrocarbon contaminated coastal sediments, including mangrove sediments (Gomes et al. 2008; Rosano-Hernández et al. 2012), which may explain their slight increase in OnOi (1.61 ± 0.23%) in comparison to Cont (1.49 ± 0.17%). The reason for their relative increase in OnpH (1.72 ± 0.13%) at T7 and the decrease in pHOi (1.47 ± 0.01%), in comparison to OnOi and OnpH treatments, remain elusive and, since these differences are small, these variations may have no ecological significance at all. At T21 there was no similar pattern in any of the taxa.

The previous published results obtained from superficial sediments showed that the interactive effects of oil contamination and reduced seawater pH had a significant effect on the relative abundance of active members of the order Desulfobacterales (Coelho et al. 2016a; Coelho et al. 2015). In the present study, the interaction between these two factors was not a significant driver of the relative abundance of this taxon (Figure S III-2). The biogeochemical characteristics of the sediment barrier could have attenuated the effects of the factors tested.

Conclusions

Previous results indicated that the interaction between oil hydrocarbon contamination and reduced seawater pH may have a significant effect on the composition of the active microbial communities in surface sediments (Coelho et al. 2016a; Coelho et al. 2015). Here, we now show that, although the group specific analysis indicated some significant taxa-specific responses, namely a clear effect of low seawater pH on *Desulfobulbaceae* family after 7 days and the appearance of several putative anaerobic hydrocarbon-degrading bacteria after 21 days, the overall bacterial community did not

significantly change. Neither the independent nor the interactive effect of reduced seawater pH and hydrocarbon contamination had a significant compositional impact on the overall structure of the bacterial communities inhabiting the subsurface sediment. It is possible that the initial oil hydrocarbon biodegradation in the surface layer combined with its low oil permeability and sediment chemical buffering capacity may attenuated the effects of oil contamination and low pH on subsurface sediment bacterial communities. Overall, this study provided evidences that bacterial communities inhabiting subsurface sediment may be less susceptible to the independent and interactive effects of reduced seawater pH and oil contamination in a scenario of ocean acidification.

Funding

This work was supported by the Foundation for Science and Technology at the Portuguese Ministry of Education and Science [UID/AMB/50017 for research unit CESAM; SFRH/BD/86447/2012 for AL and SFRH/BPD/92366/2013 for FJRC], which is co-funded by national funds and FEDER within the PT2020 Partnership Agreement and Compete 2020. The ELSS construction was financed by European Funds through COMPETE (FCOMP-01-0124-FEDER-008657) and by National Funds through the Portuguese Science Foundation (FCT) within project PTDC/AAC-CLI/107916/2008

Conflict of interests

Authors declare no conflict of interests.

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Supplementary information

Microcosm experiment

Sediment cores (approximately 13 cm high) were collected on the 24 May 2011 at the Ria de Aveiro (40°37'32"N 8°44'10"W), an estuarine coastal lagoon, transferred directly into individual microcosm (glass tanks of 25 cm in height, 28 cm in length and 12.4 cm in width, and with a headspace volume of approximately 3L) and immediately placed in the ELSS. A semidiurnal tidal regime was implemented with individual microcosms filled and emptied in approximately 1 min, during which about 50% of the water volume (1.5L) was renewed (flow-through non-recirculated). A 14-hour diurnal light cycle (average day length in summer months at Portuguese latitudes) was simulated with light intensity varying from 50% to 100% of the total fluorescence tube intensity. A water bath ensured that sediment temperature was maintained constant at 19 ºC. Synthetic seawater was prepared by dissolving a commercially available salt mixture (Tropic Marin Pro Reef salt – Tropic Marine, Germany) into freshwater purified by a four-stage reverse osmosis unit (Aqua-win RO-6080) at 32 ppt as measured by a refractometer. Salinity and temperature were defined to correspond to conditions recorded at the sampling location and were kept constant (Coelho et al. 2013). Before the experimental spiking with oil, the sediment was stabilized during 21 days and seawater pH was gradually adjusted in external glass reservoirs to the desired test conditions. The pH was monitored daily at the end of each low tide with a calibrated pH meter (Orion StarTM portable pH meter, Thermo Fisher Scientific Inc., Waltham, MA, USA).

Data analysis

In QIIME, .fasta and .qual files were used as input for the split_libraries.py script. Default arguments were used except for the minimum sequence length, which was set at 218 bps after removal of forward primers and barcodes; reverse primers were removed using the 'truncate only' argument and a sliding window test of quality scores was enabled with a value of 50 as suggested in the QIIME description for the script. OTUs were selected using UPARSE with usearch7 (Edgar 2013). Chimera checking was performed using the UCHIME algorithm. First reads were filtered with the -fastq_filter command and the following arguments -fastq_trunclen 250, -fastq_maxee 0.5 and -fastq_truncqual 15. Sequences were then dereplicated and sorted using the -derep_fulllength and - sortbysize commands. OTU clustering was performed using the -cluster_otus command (cut-off threshold at 97%). An additional chimera check was subsequently applied using the -uchime_ref command with the gold.fa database (http://drive5.com/uchime/gold.fa). In QIIME, representative sequences were selected using the pick_rep_set.py script in QIIME using the 'most_abundant' method. Reference sequences of OTUs were assigned taxonomies using default arguments in the assign_taxonomy.py script in QIIME with the

rdp method (Wang et al. 2007). In the assign_taxonomy.py function, the most recent Greengenes database (ftp://greengenes.microbio.me/greengenes_release/gg_13_5/gg_13_8_otus.tar.gz) was used for OTU picking and taxonomic assignment. The make_otu_table.py script was used to produce an OTU by sample table containing the abundance and taxonomic assignment of bacterial OTUs.

Supplementary files

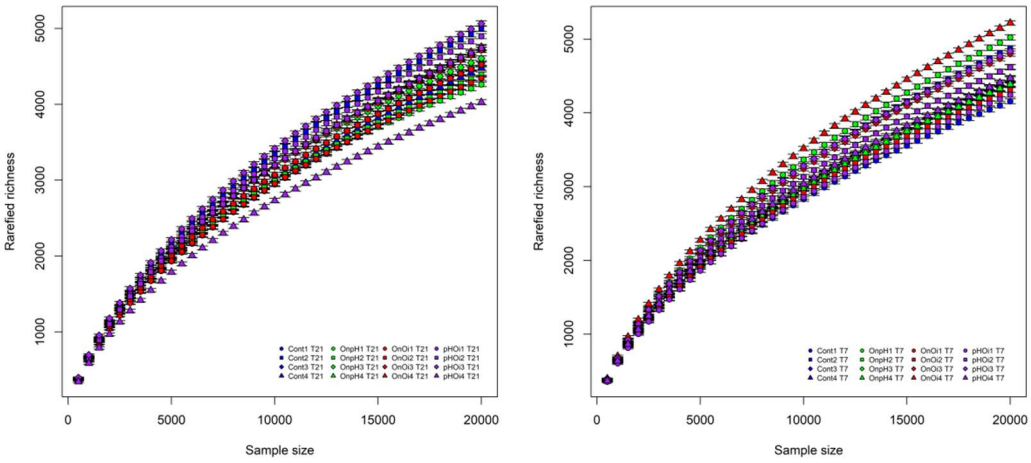

Figure S III-1 - Rarefaction curve plots for each sample. Calculated using a self-written function (Gomes et al. 2010)

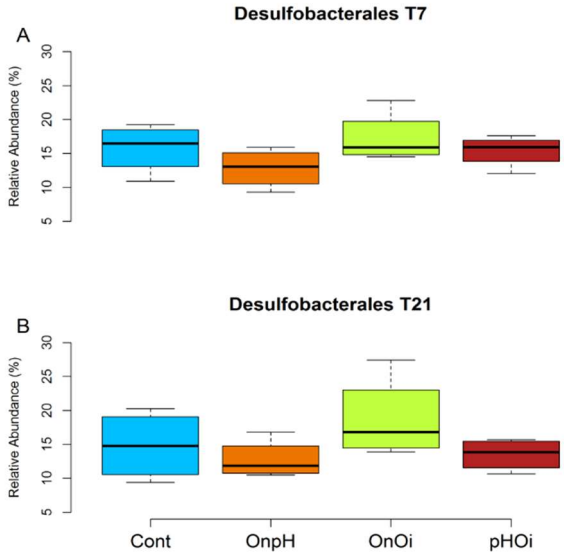

Figure S III-2 - Boxplot with the relative abundances of OTU classified to the Desulfobacterales order at 7 days (A) and 21 days (B) after oil contamination.

Table S III-1 List of statistically significant (ANOVA p-value ≤0.05) phylum, classes and orders with mean relative abundance >0.1% for time point 7. Each factor independently and the interaction had 1 degree of freedom.

		p-value			F-value	Mean Relative abundance (%)
		pH	Oil	pH and Oil		
Phylum	Fibrobacteres	0.039	-	-	5.349	0.13 ± 0.04
	Firmicutes	0.026	-	-	6.413	1.10 ± 0.53
	Bacteroidetes	-	-	0.047	4.925	8.69 ± 2.42
	Gemmatimonadetes	-	-	0.028	6.197	1.57 ± 0.18
Class	GN15	-	0.041	-	5.231	0.16 ± 0.07
	Bacilli	0.021	-	-	7.014	0.33 ± 0.29
	OP8_1	0.042	-	-	5.164	0.4 ± 0.26
	SJA-4	0.05	-	-	4.771	0.18 ± 0.11
	Thermoleophilia	0.016	-	-	7.77	0.42 ± 0.11
	Gemm-1	-	-	0.041	5.237	0.18 ± 0.04
Order	envOPS12	-	-	0.031	5.975	0.20 ± 0.06
	Solirubrobacterales	-	-	0.039	5.341	0.11 ± 0.04
	Bacillales	0.021	-	-	7.019	0.26 ± 0.25
	Caldilineales	0.023	-	-	6.809	0.23 ± 0.07
	Gaiellales	0.021	-	-	7.0	0.205 ± 0.055
	HMMVPog-54	0.046	-	-	4.949	0.32 ± 0.20
	Pseudomonadales	0.019	-	-	7.381	0.39 ± 0.14

Table S III-2 List of statistically significant (ANOVA p-value ≤0.05) families with mean relative abundance ≥0.1% for time point 7. Each factor independently and the interaction had 1 degree of freedom.

	p-value		F-value	Mean Relative Abundance (%)
	pH	Oil		
Bacillaceae	0.03318	-	5.7866	0.156 ± 0.190
Caldilineaceae	0.02283	-	6.809	0.232 ± 0.075
Chromatiaceae	0.03797	-	5.4359	0.597 ± 0.202
Cohaesibacteraceae	0.00991	-	9.3610	0.144 ± 0.089
Desulfobulbaceae	0.01816	-	7.4709	5.063 ± 1.515
Hyphomicrobiaceae	0.0293	-	6.1185	1.744 ± 0.401
Pseudomonadaceae	0.01232	-	8.6578	0.383 ± 0.134

Table S III-3 - List of statistically significant (ANOVA *p*-value ≤0.05) phylum, classes and orders with mean relative abundance ≥0.1% for time point 21. Each factor independently and the interaction had 1 degree of freedom.

		p-value		F-value	Mean Relative abundance (%)
		pH	Oil		
Phylum	Firmicutes	-	0.049	4.794	0.8 ± 0.28
Class	Clostridia	-	0.026	6.49	0.57 ± 0.24
	SJA-4	-	0.03	6.072	0.24 ± 0.25
	Bacilli	0.034	-	5.715	0.22 ± 0.14
	Thermoleophilia	0.043	-	5.147	0.35 ± 0.14
Order	Bacillales	-	0.023	6.768	0.12 ± 0.06
	CCM11a	0.032	-	5.953	0.23 ± 0.14
		-	0.039	5.384	
	Clostridiales	-	0.026	6.49	0.56 ± 0.24
	envOPS12	-	0.02	7.261	0.22 ± 0.09
	Rhizobiales	-	0.029	6.111	2.16 ± 0.51
	SB-34	-	0.031	5.985	0.33 ± 0.22
	Sphingomonadales	-	0.007	10.558	0.11 ± 0.06

Table S III-4 - List of statistically significant (ANOVA *p*-value ≤0.05) families with mean relative abundance ≥0.05% for time point 21. Each factor independently and the interaction had 1 degree of freedom.

	p-value		F-value	Mean Relative Abundance (%)
	pH	Oil		
Clostridiaceae	-	0.014	8.32	0.29 ± 0.13
Hyphomicrobiaceae	-	0.007	10.581	1.26 ± 0.23
Phyllobacteriaceae	-	0.018	7.551	0.25 ± 0.1
Cohaesibacteraceae	0.011	-	9.031	0.17 ± 0.09
lheB3-7	0.031	-	5.958	0.15 ± 0.05

Chapter IV

Chapter IV

Microcosm evaluation of the impact of oil contamination and chemical dispersant addition on bacterial communities in estuarine port sediment

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Abstract

The use of chemical dispersants in oil spill response is controversial, particularly in near-shore environments (e.g. ports). Their application will certainly increase the bioavailability of oil, which may enhance microbial biodegradation. However, it may also increase the mass transfer of constituents of oil to the sediment surface, where their long-term environmental impact is severer. In this study, we aimed at investigating the effects of oil contamination and chemical dispersant addition on the fate of oil hydrocarbons [polycyclic aromatic hydrocarbons (PAH) and alkanes] in a estuarine port sediment. For that a microcosms experimental setup was used to evaluate the impact of chemical oil dispersants on the structure of the benthic bacterial communities from a port environment by a 16S rRNA gene based high-throughput sequencing approach. In addition, the effect of oil-dispersant on putative functional traits was evaluated. The results revealed that chemically dispersed oil, while having similar alkane concentration, seems to enhance PAHs accumulation in sediments and altered the relative abundance of some less abundant bacterial groups. Nonetheless, overall no significant effect was detected on the bacterial community composition at the uppermost sediment layer, which was stable to the independent and interactive effects of oil contamination and dispersant addition. In sum, chemical dispersants enhance the mass transfer of PAH to the sediment surface but do not impose a significant shift to the composition of the overall bacterial communities from port environments.

Keywords: *Alcanivoraceae*, Surfactants, PAHs, Dispersant, *Helicobacteraceae*, Oil spill

Introduction

Chemical dispersants are used to mitigate the environmental impact of oil spills (National Research Council 2005; Prince 2015; Walker 2017). Non-dispersed oil will agglomerate into large surface slicks and oil droplets, thus reducing the oil-water interface to a minimum (National Research Council 2005). It is at this interface that bacterial biodegradation preferentially occurs (Rosenberg and Rosenberg 1981). Therefore, if requirements of other potentially limiting factors (e.g. nutrients and oxygen) are fulfilled, this minimal interface will drastically reduce oil bioavailability and, consequently, limit oil hydrocarbon biodegradation. To counter this, chemical dispersants in combination with wave action will synergistically promote the formation and stabilization of micron-size oil-in-water micelles (National Research Council 2005). This will drastically increase the oil-water interface and may potentiate oil bioremediation. However, the use of chemical dispersants is a subject of debate among scientists (Kleindienst et al. 2015a; Kleindienst et al. 2016; Prince et al. 2016). Experimental results can be contradictory, with biodegradation being enhanced (Brakstad et al. 2014; Ferguson et al. 2017), inhibited (Foght and Westlake 1982; Kleindienst et al. 2015b) or not significantly altered (Rahsepar et al. 2016) by the addition of a dispersant. In the intervening time, chemical dispersants may increase the reach and exposure of to the toxic constituents of oil (Adams et al. 2014; Carls et al. 2008; Redman et al. 2017), of which polycyclic aromatic hydrocarbons (PAHs) are particularly problematic (Barron et al. 2003; Shimada and Fujii-Kuriyama 2004). In addition, ecotoxicological studies have repeatedly shown that chemical dispersion increases oils acute toxicity to aquatic organisms (Almeda et al. 2014; Anderson et al. 2014; Barron et al. 2003; Gardiner et al. 2013; Goodbody-Gringley et al. 2013; Özhan et al. 2014; Rico-Martínez et al. 2013), with toxicological effects being frequently correlated with PAHs concentration (Gardiner et al. 2013; Özhan et al. 2014; Radniecki et al. 2013).

In efficient oil dispersion interventions, oil-dispersant micelles are expected to be buoyantly entrained in the water column and, consequently, restrained from reaching the seabed and shorelines (Beyer et al. 2016; Lee et al. 2012). Furthermore, the micellization of oil by chemical dispersants is thought to reduce oil "stickiness" and, consequently, reduce oil-sediment interactions (Lessard and DeMarco 2000). Yet, *in vitro* simulations revealed that by reducing micelle size (Khelifa et al. 2008), dispersant addition may promote the adhesion of oil to suspended particulate matter, thus facilitating mass transfer to the marine sediments (Cai et al. 2017; Gong et al. 2014b; Khelifa et al. 2008). Even in the deep sea sediments, dispersant-enhanced mass transfer of oil hydrocarbons was found to occur in the aftermath of the Deepwater Horizon oil spill (Romero et al. 2015).

The ideal environmental fate of oil hydrocarbons is their complete oxidation. This is normally achieved through the metabolic association of a multitaxon microbial community (Coelho et al. 2016a;

Coelho et al. 2016b). Bacteria, due to their versatile metabolic features, participate directly (McGenity et al. 2012; Yakimov et al. 2007), and indirectly [e.g. nitrogen cycling (Musat et al. 2006; Toccalino et al. 1993) and biosurfactant synthesis (Ali Khan et al. 2017)] in this metabolic network. However, chemically dispersed oil may disrupt this network (Kleindienst et al. 2015a) by inhibiting some bacterial groups [e.g. key alkane-degrading bacteria (Hamdan and Fulmer 2011; Kleindienst et al. 2015b) and ammonia-oxidizing bacteria (Radniecki et al. 2013)]. Structural changes in the composition of bacterial communities are known to affect microbial processes (Kleindienst et al. 2015a) and, consequently, these can delay or preclude a full environment restoration.

The sedimentation of oil hydrocarbons is undesirable in oil response strategies since the strong adhesion forces between oil and sediment particles decrease oil hydrocarbon bioavailability and this may consequently perpetuate the associated environmental impact (Lee 2002). Also, with time, oil hydrocarbons will be inhumed in anoxic sediments where oil hydrocarbon biodegradation is slower (McGenity 2014). Consequently, the use of dispersants in shallow near shore waters (e.g. ports) is strongly regulated or banned due to these concerns, despite their impact and fate have not been fully elucidated (National Research Council 2005). However, the response of the benthic bacterial community in port environments may differ from that of other less contaminated sites. Ports are particularly prone to oil spills and port activities expose surrounding benthic ecosystems to continuous xenobiotic pollution from the discharge bulk cargo, bilge and ballast water, soot emissions and water contamination by oil-derived fuels and lubricants and anti-fouling paints (Darbra et al. 2009). Furthermore, port sediments can be adversely affected by anthropogenic activities from nearby industrialized and urbanized regions (Gomes et al. 2013). Therefore, the composition of the benthic bacterial communities and their catabolic gene pool at sites subject to chronic xenobiotic pollution is distinct that of less contaminated sites (Gomes et al. 2007; Gomes et al. 2008; Gomes et al. 2013; Tavares et al. 2016). Also, in response to oil contamination events, the compositional changes in the benthic bacterial community from chronically contaminated sites is not profound, yet their metabolic response is rapid and the oil hydrocarbon biodegradation rate is faster than in pristine sites (Païssé et al. 2010). Thus, when determining the net benefit of chemical dispersant addition in port environments it should taken in consideration the response of this precondition bacterial community.

In this study, we aimed at investigating the effects of oil contamination and chemical dispersant addition on the fate of oil hydrocarbons in estuarine port sediments and evaluate their impact on the structure of the benthic bacterial communities. In addition, the effect of oil-dispersant interaction on putative functional traits was evaluated.

Methods

Microcosm Experiment

A microcosm simulation was conducted in an experimental life support system (ELSS) that was previously validated to test the effects of reduced seawater pH and UV radiation on the benthic biosphere (Coelho et al. 2013). This flow-through system allows the control of light intensity, tide regime and temperature. The ELSS supports up to 32 microcosms in four independent modules, which allows the testing of a total of eight different treatments (assuming four replicates each). More information regarding the architecture of the microcosms apparatus can be found in Coelho *et al.* 2013 (Coelho et al. 2013).

Sediment cores were collected at low neap tide on the 23th of May 2016 at a branch of the Ria de Aveiro (40°38'38"N 8°44'53"W). The Ria de Aveiro is a shallow estuarine coastal lagoon with a mean depth of ≈1 m, but with a deeper central channel that accesses the port infrastructures. It is influenced by freshwater, through the various rivers that drain there, and by the ocean with which it communicates through a single artificial channel. The sampling site was a barren intertidal mudflat, briefly exposed during low neap tide, located in between various shipping harbours and adjacent to a fuel storage facility (Figure IV-1). Sediment cores (≈13 cm high) were collected intact [following guidelines (Cravo-Laureau et al. 2017)], immediately placed into individual glass microcosms (glass tanks of 25 cm in height, 28 cm in length and 12.4 cm in width, and with a headspace volume of approximately 3 L) and transferred directly to the ELSS. At the ELSS, the 16 sediment cores were distributed equally among the four independent modules. A semidiurnal tidal regime filled and emptied the microcosms in approximately 1 minute and renewed around 50% of the total water volume. The ELSS water flow was continuous. A 14-hour diurnal light cycle was programmed with light intensity varying between 50% and 100%. The temperature of the sediment cores was maintained at 19 ºC by immersion in a refrigerated water bath (Seachill TR10, Teco S.R.L., Italy). Synthetic seawater was prepared by dissolving a commercial salt mixture (Tropic Marin Pro Reef salt – Tropic Marine, Germany) in freshwater purified by a four-stage reverse osmosis unit (Aqua-win RO-6080 - Aqua-win, Taiwan) and salinity was adjusted to ≈32 ppt as measured through a refractometer (V² Refractometer - Tropic Marine, Germany). Photoperiod (14 h), salinity (32.6± 1.5 ppt) and temperature (19 ± 1.5 ºC) were defined to simulate the conditions recorded at the sampling location and were kept constant (Coelho et al. 2015).

A factorial experiment was designed using 16 microcosms with two factors: Oil and Dispersant, each with two conditions (with and without). Four experimental treatments were defined: "Cont" (control, without oil and without dispersant), "OnDisp" (without oil and with dispersant), "OnOi" (with oil and without dispersant) and "OiDisp" (with oil and with dispersant). A stabilization period of 19 days was performed before experimental oil contamination to allow the biological communities in sediment cores to acclimatize to microcosm conditions. The experiment began when the surface waters of

respective microcosms were contaminated. The contamination was performed immediately following a simulated high tide, during 5 consecutive tide cycles. Previously, oil was allowed to evaporate in an aluminium tray, in a fume hood at ambient temperature, for 24 hours. This step was carry out because dispersant application guidelines advocate a window of opportunity of 1-2 days, since a too early application will disperse the most volatile hydrocarbons (Aurand and Coelho 2005) and a too late application will not disperse oil efficiently (National Research Council 2005). Also, in real scenarios, environmental protection response is lagged due to logistics and decision-making. This weathering process reduced oil weight by 12.53% (w/w). Next, to assure that all dispersant was used to create the oil:dispersant in water emulsions, water from the system, oil and dispersant were mixed at a water:oil:dispersant ratio of 25:7.5:0.375 in a 50 mL plastic falcon tube. The same was done with artificial seawater and oil (25:7.5) for the OnOi treatment and with artificial seawater and dispersant (25:0.375) for the OnDisp treatment. These mixtures were mechanically shaken (Rocker-Shaker MR-12; Bioscan, Riga, Latvia) at 70 rpm at a 10º angle for one hour and immediately transferred to the respective microcosms. The oil:dispersant ratio applied was set at 20:1 in accordance to manufacturer's guidelines and Maritime Safety Agency recommendations (ITOPF 2014). Also, at this ratio the smallest micelle droplet size is achieved (Khelifa et al. 2008). Arabian Light Crude oil was provided by the PETROGAL refinery (Matosinhos, Portugal) and the chemical dispersant Slickgone NS was provided by DASIC International Lda (Hampshire, United Kingdom). Since this experiment objectively focuses on the impact of chemically dispersed oil and not that of the chemical dispersant solely, this dispersant was intentionally chosen because of its lower direct toxicity to fish (juvenile sea bass) in comparison to other commercialized chemical dispersants (Dussauze et al. 2015).

Sampling and DNA extraction

For DNA extraction and hydrocarbon quantification, four replicates of sediment mini-cores (of ø 1.0 cm and ø 1.5 cm, respectively) were extracted per microcosms 21 days after oil contamination (T21). Additional cores were extracted 1 day after oil contamination (T1) and at sampling site (Env) for hydrocarbon analysis only. The top centimetre of each core was sliced and replicates were pooled into 2 mL plastic microtubes for DNA extraction and into aluminium cartridges for hydrocarbons analysis. All samples were immediately stored at -80 ºC until further use. DNA extraction was performed using FastDNA™ Spin kit (MP Biomedicals, USA) for 0.5 g of sediment following instructions of the manufacturer. DNA quality was confirmed by PCR amplification, Nanodrop and Qubit quantification before shipping for analysis.

Illumina MiSeq sequencing

The V4 region of the 16S rRNA gene was amplified by PCR (94ºC for 3 minutes, 28 cycles of 94ºC for 30s, 53ºC for 40s and 72ºC for 60s, and 72ºC for 5 minutes) using primers 515F (5´-GTGCCAGCMGCCGCGGTAA-3´) and 806R (5´-GGACTACHVGGGTWTCTAAT-3´), with barcode on the forward primer, and the HotStarTaq Plus Master Mix Kit (Qiagen, USA). After amplification, PCR products were checked in 2% agarose gel. Pooled samples were purified using calibrated Ampure XP beads. Library preparation and sequencing were performed at MR DNA

Figure IV-1- Sampling site (A) was barren mudflat briefly exposed during neap. It is located in Ria de Aveiro system in the central Atlantic coast of the Iberian peninsula (★) adjacent to fuel deposits (B) and a commercial shipping harbour (C)

(www.mrdnalab.com, Shallowater, TX, USA) on a MiSeq sequencing platform following standard Illumina protocols (Illumina, San Diego, CA, USA).

Hydrocarbons quantification

Microcosm and environmental sediment samples were extracted using a soxhlet apparatus following EPA Method 3540C (Protection and Agency 1996). Approximately 4 g of previously lyophilized sediment was extracted with 150 mL of dichloromethane for 24 h in a fume hood, maintaining the condensers circulating water at around 4 ºC. Before extraction, all sediments were spiked with 25 µL of a solution of deuterated PAHs (acenaphthene-d_{10}, chrysene-d_{12}, naphthalene-d_8, perylene-d_{12}, 1,4-dichlorobenzene-d_4 and phenanthrene-d_{10} corresponding to a final concentration of 0.25 µgL^{-1} each) and deuterated alkanes (tetracosane-d_{50} and hexadecane-d_{34} at a final concentration of 0.52 mM each) as surrogates to determine extraction efficiency. The extract was evaporated in a rotary evaporator at 35ºC to ≈10 mL, afterwards additional 10 mL of hexane were added and again reduced at 60ºC to ≈2-4 mL. When required, extracts were ice-cooled to minimize volatilization of oil hydrocarbons. Extracts were cleaned by filtering through a deactivated alumina:silica gel column (2:1) topped with anhydrous $NaSO_4$. The column was sequentially eluted with 15 mL of hexane and 30 mL of hexane:dichloromethane (1:1), and all fractions were collected. The eluate was reduced to ≈1 mL in a rotary evaporator at 60ºC and to ≈200 µL under a gentle N_2 flow. Immediately before injection in the chromatograph, each sample was spiked with 25 µL of hexamethylbenzene (final concentration of 1.5 µM) and 1,2-dibromobenzene (0.52 mM) as internal standards for PAHs and alkanes, respectively. Analytic separation and detection was conducted on a Gas Chromatograph Mass Spectrometer (GC-MS Shimadzu QP2010 Ultra) coupled to an AOC 20i autosampler (Shimadzu, Japan) and with the electron impact ionization (EI) at 70 eV. Injector and transfer-line temperatures were both set a 350 ºC, while the ion source was set at 230ºC. For PAHs, samples were injected in the splitless mode and the selected ion monitoring (SIM) mode was used for the detection of all 16 EPA priority PAHs. Alkanes samples were injected in split mode and detection was done in full scanning mode from m/z 20 to m/z 500. For both, 1 µL of sample was injected automatically with a 10 µL glass syringe into a VF-5ms column (30 m x 0.25 mm with 0.25 µm film thickness; Agilent Technologies, Santa Clara, CA, USA). Helium was used as the carrier gas and the linear velocity was set at 40 cm s^{-1}. The oven temperature conditions were set at 60 ºC for 1 min, followed firstly by a rise to 200 ºC at a 10 ºC per minute rate, then at 5 ºC per minute until 300 ºC where it stabilized for 8 min. All the analytes were quantified through calibration curves of analyte/internal standard created using a range of concentrations of certified analytical standards. Certified internal standards, deuterated alkanes (tetracosane-d_{50} and hexadecane-d_{34}) and deuterated PAHs solution were all supplied by Sigma-Aldrich (St. Louis, MO, USA). All the deuterated surrogates were recovered with an efficiency > 70% and the standard deviation was between 10-15%.

Data analysis

High-throughput sequencing data was analyzed using the Quantitative Insights Into Microbial Ecology (QIIME) software package (http://qiime.org; Accessed 01 January 2014) according to published recommendations and following previously described methods (Cleary et al. 2015; Coelho et al. 2016a; Kuczynski et al. 2011) with the exception of the operational taxonomic unit (OTU) picking step, where the UPARSE (Edgar 2013) clustering method and chimera check were used. In QIIME, .fasta and .qual files were used as input for the split_libraries.py script. Default arguments were used except for the minimum sequence length, which was set at 218 bps after removal of forward primers and barcodes; reverse primers were removed using the 'truncate only' argument and a sliding window test of quality scores was enabled with a value of 50 as suggested in the QIIME description for the script. OTUs were selected using UPARSE with usearch7 (Edgar 2013). Chimera checking was performed using the UCHIME algorithm. First reads were filtered with the -fastq_filter command and the following arguments -fastq_trunclen 250, -fastq_maxee 0.5 and -fastq_truncqual 15. Sequences were then dereplicated and sorted using the -derep_fulllength and - sortbysize commands. OTU clustering was performed using the -cluster_otus command (cut-off threshold at 97%). An additional chimera check was subsequently applied using the -uchime_ref command with the gold.fa database (http://drive5.com/uchime/gold.fa). In QIIME, representative sequences were selected using the pick_rep_set.py script in QIIME using the 'most_abundant' method. Reference sequences of OTU were assigned taxonomies using default arguments in the assign_taxonomy.py script in QIIME with the RDP method (Wang et al. 2007). In the assign_taxonomy.py function, the most recent Greengenes database (ftp://greengenes.microbio.me/greengenes_release/gg_13_5/gg_13_8_otus.tar.gz) was used for OTU picking and taxonomic assignment. The make_otu_table.py script was used to produce an OTU by sample table containing the abundance and taxonomic assignment of bacterial OTUs. Sequences are available at NCBI ShortRead Archive (BioProject nº PRJNA392217 and BioSample nº SAMN07285933-SAMN07285948).

Statistical analysis

The OTU tables were uploaded to R software (version 3.1.1; http://www.r-project.org/) for removal of unassigned and singleton OTU, chloroplast and mitochondrial sequences, statistical computing and graphic generation. A self-written function (Gomes et al. 2010) was used to estimate total rarefied OTU richness for each sample. The top 5 most abundant phyla, classes, orders and families (excluding the "Unclassified" group) were determined by the overall mean relative abundance, calculated from the 16 datasets using the prop.table() function. Their relative abundance was plotted for each treatment using the boxplot() function in the graphics package. Compositional differences among treatments was assessed using principal coordinates analysis (PCO). The PCO was generated using the

cmdscale() function in the R base package. Prior to the PCO, the raw data was log(x+1) transformed and used to produce a distance matrix with the Bray-Curtis index with the vegdist() function in the vegan package (Oksanen et al. 2012). An Adonis analysis was performed using the adonis() function in vegan to test the significance of these compositional differences to factors tested. The log(x+1) transformed Bray-Curtis distance matrix was used as the response variable, oil and dispersant as factors and the number of permutations was set at 999. We also analyzed the differences in relative abundance for all taxa (phylum to family) between treatments to the independent and interactive effects of oil contamination and chemical dispersant addition, through a two-way ANOVA using the F-test [adapted from (Roy et al. 2013a)]. Beforehand, the scope of the analysis was set to the most relevant taxa by establishing a threshold (overall mean relative abundance ≥0.1 % all levels). ANOVA was computed by the anova() function (stats package) applied to a linear regression model produced individually for each taxa. The linear regression distribution was created by the glm() function (stats package) for each taxa using relative abundance as response variable and a presence/absence matrix of oil and dispersant as factors. An interaction term (*) was included to study the interaction between the two factors (oil*dispersant). The most significant (p-value < 0.05) taxa were plotted using the boxplot() function from the graphics package.

PAHs and alkanes concentrations in the samples were calculated from the corresponding calibration curves and then standardized for the weight of sediment analyzed (µg g^{-1}). Concentrations were plotted using boxplot() function from the graphics package in R. The independent and interactive effects of oil and dispersant addition were tested for significance (p-value ≤ 0.05) through an ANOVA F-test using the glm() and anova() functions from the stats package similarly as aforementioned above. However, input data for hydrocarbon quantification table was previously normalized for each hydrocarbon using this formula: $H - \frac{H_{min}}{H_{max} - H_{min}}$; where H is the hydrocarbon concentration and H_{max} and H_{min} is, respectively, the maximum and minimum hydrocarbon concentration in dataset. Variations in alkanes and PAHs concentrations between time points was measured for each microcosm as the percentile proportional difference between final and initial concentrations using this formula: $\frac{(H_f - H_i)}{H_i}$; in which H_f and H_i are, respectively, the final and initial hydrocarbon concentrations of all individual quantified hydrocarbons. These percentile differences were plotted using the boxplot() function the graphics package. Significant differences in hydrocarbon concentrations (p-value≤ 0.05) between time points were determined for each hydrocarbon and treated individually using glm() and anova() functions from the vegan package with oil hydrocarbon concentrations at T1 and T21 as data and time as factor.

Carbon preferential index (CPI) was calculated for data from sampling site (ENV) using previously described equation (Marzi et al. 1993). A CPI > 1 is indicative that hydrocarbons are of petrogenic origin while a CPI < 1 indicates that they are of biogenic origin (Pietrogrande et al. 2009). The biodegradation was confirmed by comparing the variation of the C_{18}/phytane ratio between

timepoints (Jones et al. 2008), assuming that linear alkanes are preferentially biodegraded, in comparison to branched alkanes (Hasinger et al. 2012; Peters et al. 2005), and that both have a similar volatilization rate (Esquinas et al. 2017). C_{18}/phytane mean variation was calculated only for oil contaminated treatments (OnOi and OiDisp). Pearson correlations between the relative abundance of the most relevant classes, order, families and OTU, and hydrocarbon concentrations (μg g^{-1}) were computed using rcorr() function from the Hmisc package and plotted using the corrplot() function from corrplot package (Wei and Simko 2016). Hydrocarbons concentrations were fit onto the PCO ordination using the envfit() function in the vegan package with permutations set to 999 and all other arguments set at default. Only significant (*p*-value <0.05) ordinations where plotted.

Results

Quantification of oil hydrocarbons

Concentrations for the 25 saturated linear alkanes quantified [from C12 (dodecane) to C36 (hexatriacontane) are plotted in Figure IV-2 for T1 and Env samples and in Figure IV-3 for T21 samples, while the statistical parameters obtained through ANOVA (F-value and *p*-value) are summarized in Table S IV-1 and Table S IV-2 for T1 and T21, respectively. As expected, almost all alkanes had a significant higher concentration in oil contaminated microcosms (OnOi and OiDisp) at T1, with the exception for the two lightest alkanes, C_{12} (which had a significant lower concentration) and C_{13}. With time, concentrations decreased and at T21 only alkanes C_{15} to C_{21} had a significant higher concentration in oil-contaminated microcosm sediments. The chemical dispersion of oil only increased the concentration of some alkanes (C_{13} and C_{33}-C_{36}) at T1; and of C_{36} at T21 samples. Alkanes C24 to C29 had a significant higher concentration in treatments with oil amendment (Cont and OnDisp). This reflects abnormally high measurements in some replicates and was interpreted as an artefact of the experimental procedure. The concentrations of the 16 PAHs quantified are plotted alongside with ANOVA statistical parameters (F-value and *p*-value) in Figure IV-4 for T1 and Env samples and in Figure IV-5 for T21 samples. The results indicate that the chemical dispersion of oil significantly increased the concentration of almost all PAHs at T1 [the exceptions are naphthalene, benzo(a)pyrene and benzo(b)fluoranthene], while at T21, only phenanthrene and benzo(b)fluoranthene had a significant higher concentration in OiDisp. Contrary to the other PAHs, the concentration of naphthalene had a significant reduction in oil contaminated microcosms at T1. At Env, mean ∑alkane and ∑PAH concentration were 30.345 ± 13.943 μg g^{-1} and 0.518 ± 0.04 μg g^{-1}, respectively, and the carbon preference index mean alkane concentration was 1.049 which is indicative that hydrocarbons present are of petrogenic origin.

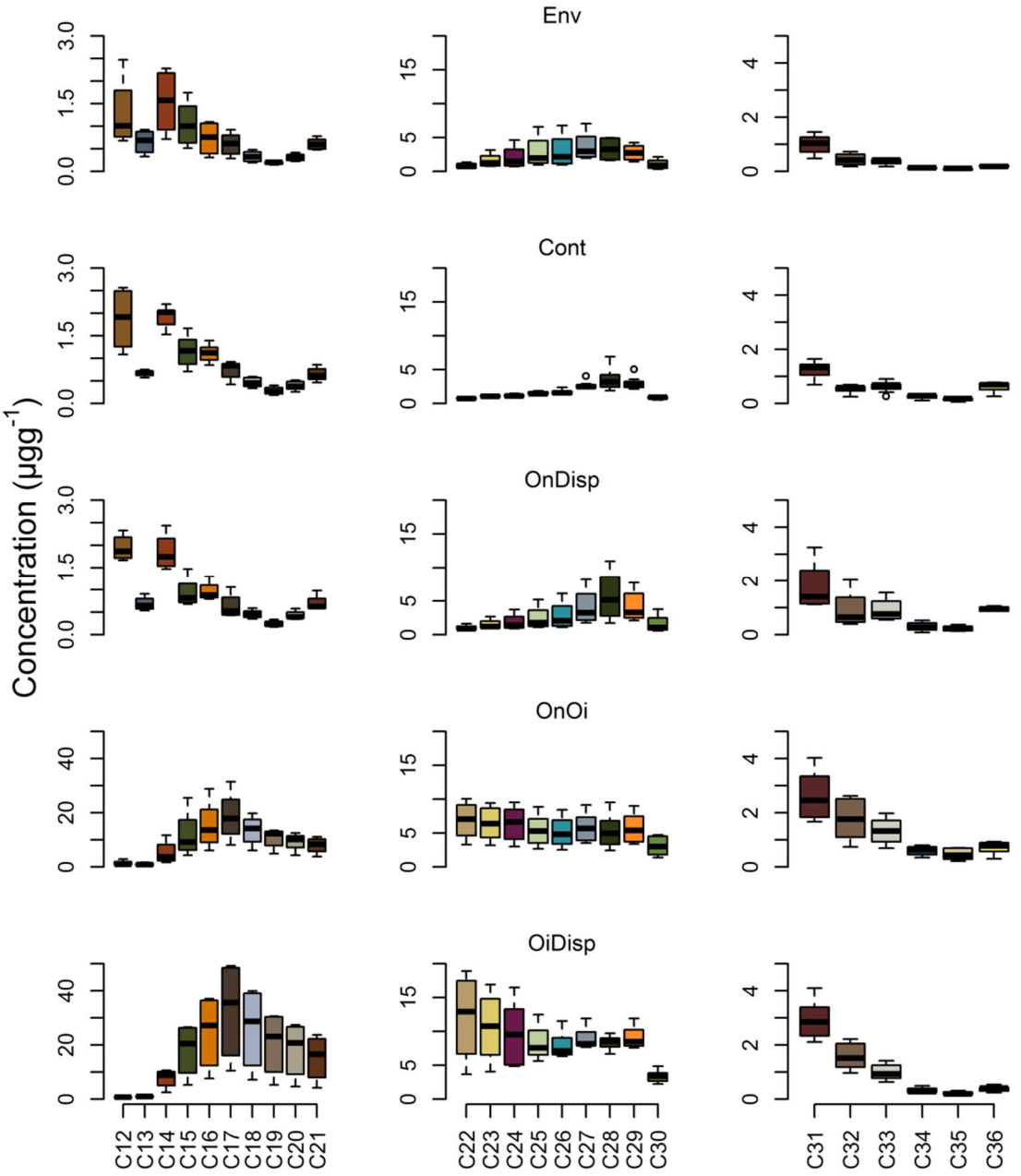

Figure IV-2 - Boxplot of concentrations (µg g-1) of linear alkanes from C12 (dodecane) to C36 (hexatriacontane) in sediments at T1. "Env" (sampling site), "Cont" (without oil and without dispersant), "OnDisp" (without oil and with dispersant), "OnOi" (with oil and without dispersant) and "OiDisp" (with oil and with dispersant).

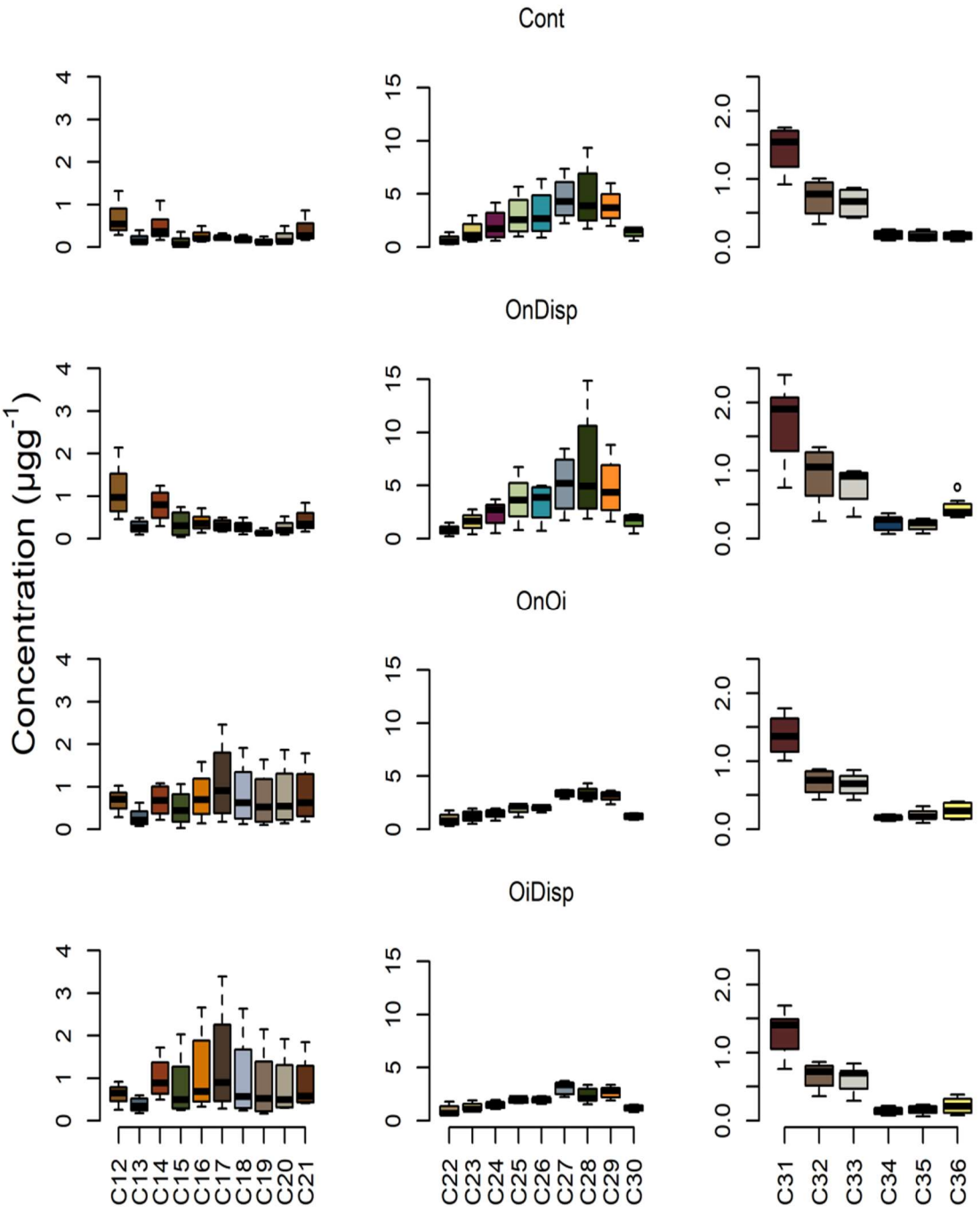

Figure IV-3 - Boxplot of concentrations (µg g^{-1}) of linear alkanes from C_{12} (dodecane) to C_{36} (hexatriacontane) in sediments at T21. "Cont" (without oil and without dispersant), "OnDisp" (without oil and with dispersant), "OnOi" (with oil and without dispersant) and "OiDisp" (with oil and with dispersant)

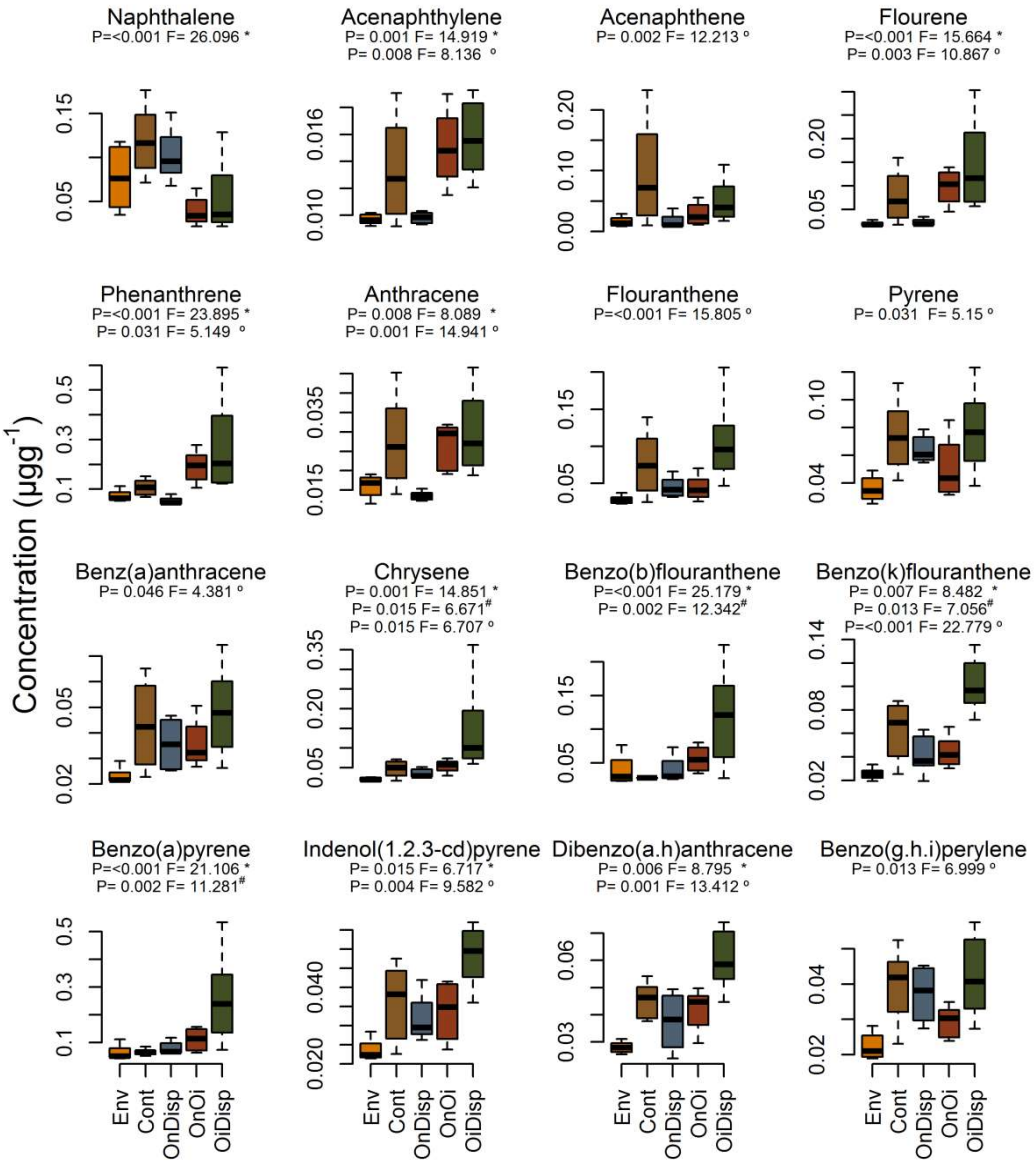

Figure IV-4 - Boxplot of concentrations (µg g^{-1}) of the 16 PAHs labelled as priority pollutants by US-EPA in sediments from all replicates of the four treatments (Cont, OnDisp, OnOi and OiDisp) at timepoint 1 and from sampling site (Env). PAHs that a significant increase or decrease (≤0.05) in response to oil addition, dispersant application or the interaction of both are marked ("*" - for oil addition, "#" -for dispersant and "○" - for the interaction. "Env" (sampling site), "Cont" (without oil and without dispersant), "OnDisp" (without oil and with dispersant), "OnOi" (with oil and without dispersant) and "OiDisp" (with oil and with dispersant). In the ANOVA, each factor independently and the interaction had 1 degree of freedom.

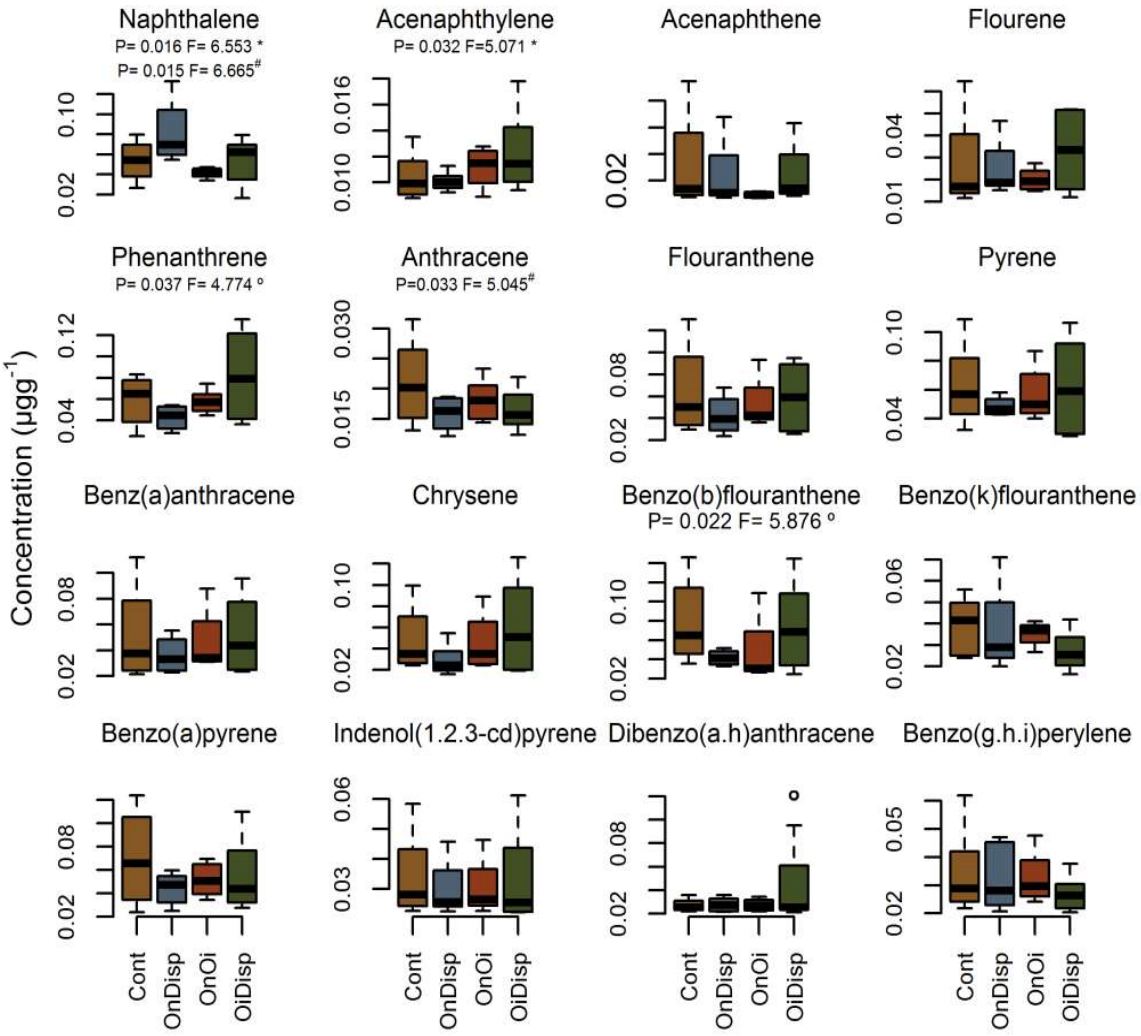

Figure IV-5 - Boxplot of concentrations (µg g^{-1}) of the 16 PAHs labelled as priority pollutants by US-EPA in sediments from all replicates of the four treatments (Cont, OnDisp, OnOi and OiDisp) at timepoint 21. PAHs that had a significant increase or decrease (≤0.05) in response to oil addition, dispersant application or the interaction of both are marked ("*" - for oil addition, "#" -for dispersant and "○" - for the interaction)."Cont" (without oil and without dispersant), "OnDisp" (without oil and with dispersant), "OnOi" (with oil and without dispersant) and "OiDisp" (with oil and with dispersant). In the ANOVA, each factor independently and the interaction had 1 degree of freedom

The variation in hydrocarbons concentration between T1 and T21 in oil contaminated treatments (OnOi and OiDisp) was measured for alkanes and PAHs and the results are plotted in Figure IV-6 alongside mean hydrocarbons concentrations (µg g^{-1}). In general, results show that the concentration of alkanes decreased considerably with time in oil contaminated treatments, with steeper decrease in concentration in OiDisp. In OnOi (Figure IV-6A) the concentration of alkanes decreased on average ≈90% for C_{15}-C_{19}, >80% for C_{20}-C_{22} and >70% for C_{14}, C_{23} and C_{24}, while in OiDisp C_{15}-C_{22}

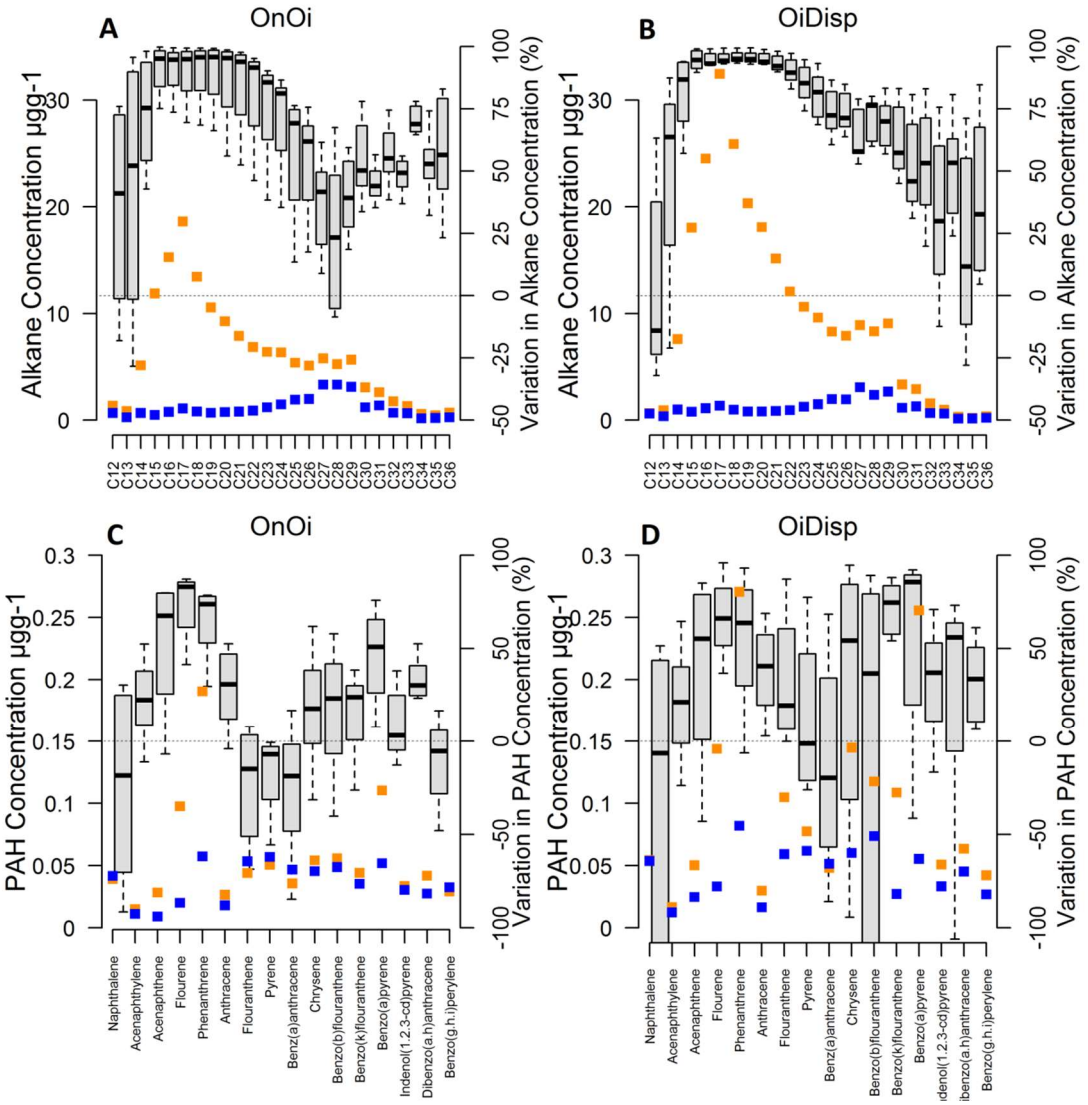

Figure IV-6 - Boxplot of percentile variation of hydrocarbon concentration in treatments OnOi (A and C) and OiDisp (B and D) for linear alkanes C12 (dodecane) to C36 (hexatriacontane) (A and B) and the 16 PAHs labeled as priority pollutants by US-EPA (C and D) between T1 and T21 (boxplot), and their mean concentration in sediments (µg g-1) at T1 (■)and at T21 (■).

decreased >90% and linear alkanes C_{14}, C_{23} and C_{24} >80% (Figure IV-6B). Nonetheless, the percent removal was still >50% for the most abundant PAHs in petroleum [phenanthrene, fluorene and chrysene]. The influence of biodegradation in the overall hydrocarbons removal was determined by comparing the C_{18}/phytane ratio between timepoints T1 and T21 (Jones et al. 2008). This ratio decreased on average >50% in both treatments (OnOi and OiDisp), indicating that biodegradation occurred (Snape et al. 2006). The mean C_{18}/phytane percent in OnOi decreased 55.842 ± 15.694 %, which was slightly more than in OiDisp (58.577 ± 34.611 %). However, the difference between treatments was not significant (one-way ANOVA; R_2=1.9209; F=0.0206; P=0.8906). PCO ordination of the OTU

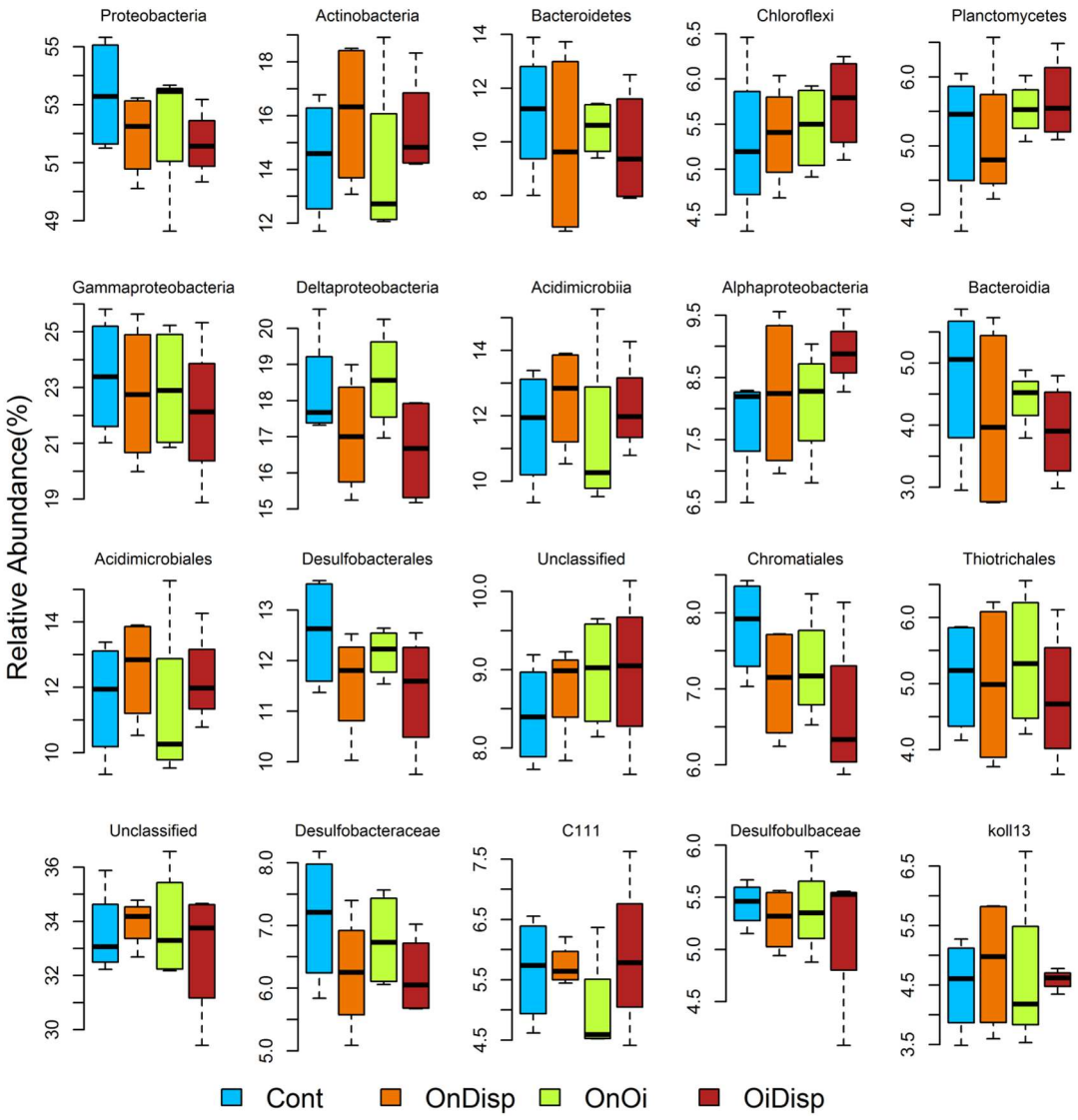

Figure IV-7 - Boxplot with the relative abundances of the five most abundant bacterial groups at T21 at the phylum, class, order and family level, respectively, in overall dataset: "Cont" (without oil and without dispersant), "OnDisp" (without oil and with dispersant), "OnOi" (with oil and without dispersant) and "OiDisp" (with oil and with dispersant).

composition-based Bray-Curtis distance matrix was conjunctively plotted with statistically significant hydrocarbon ordinations in Figure S IV-1. Here, a clear separation of C14-C19 alkanes and PAHs [acenaphthylene, phenanthrene, chrysene and dibenzo(a,h)anthracene] occurred in the lower right quadrant of the plot. These hydrocarbons seem more associated with some OnOi and OiDisp replicates, although not all.

Overall composition of bacterial communities

The 16S rRNA gene high-throughput sequencing resulted in a dataset composed of 749081 sequences distributed among 20680 OTUs. Rarefied richness (Figure S IV-2) had no asymptote for any sample, which indicates that true richness is higher than reported here. The relative abundance of the five most abundant phyla, classes, orders and families (excluding the "unclassified" bacterial group) were plotted in Figure IV-7. Sediment bacterial communities were dominated by the Proteobacteria phylum (mean relative abundance 52.32 ± 1.77%). Within Proteobacteria, classes Gammaproteobacteria (22.82 ± 2.22%) and Deltaproteobacteria (17.64 ± 1.6%) were the most abundant. At the order level, Acidimicrobiales and Desulfobacterales were the most abundant orders (11.94 ± 1.81% and 11.91 ± 1.03%, respectively).

PCO ordination and adonis statistical test of the samples (Figure IV-8 and Table IV-1) revealed that neither oil, nor dispersant, nor the interaction of both were significant predictors of the overall bacterial community. Although these was no significant change in the overall structure of the bacterial community, a taxon-specific analysis indicated that for the relative abundance of five less abundant (all had a mean relative abundance below 1%) bacterial groups a significant variation occurred (ANOVA F-test p-value ≤ 0.05) between treatments in response to the factor (independently and/or in interaction). These bacterial groups are plotted alongside ANOVA parameters in Figure IV-9. The bacterial groups that had a positive and significant response to oil addition included the Oceanospirillales order (mean relative abundance = 0.22 ± 0.20%) and its subtaxon Alcanivoraceae (0.16 ± 0.19%). For the interaction of both factors, order Legionellales (0.29 ± 0.16%) and family Phyllobacteriaceae (0.62 ± 0.07%) increased in relative abundance, whereas candidate division SBR10131 (0.13 ± 0.04%) decreased.

Table IV-1- Output results from the Adonis statistical test on bacterial OTU compositional differences at T21 for factors independently and in interaction.

Factors	$F_{1,15}$	R^2	p-value
Oil	0.95746	0.06441	0.593
Dispersant	0.99831	0.06716	0.397
Oil and Dispersant	0.90845	0.06112	0.902

Correlation analysis

Pearson correlation indicated that several taxa were correlated with hydrocarbon concentration (Figure S IV-3 and Figure S IV-4 for alkanes and PAHs, respectively). Pearson correlations compare the relationship of two datasets. In this study, we consider hydrocarbon concentration as an independent variable and relative abundance as an dependent variable. Thus, any strong correlation between both

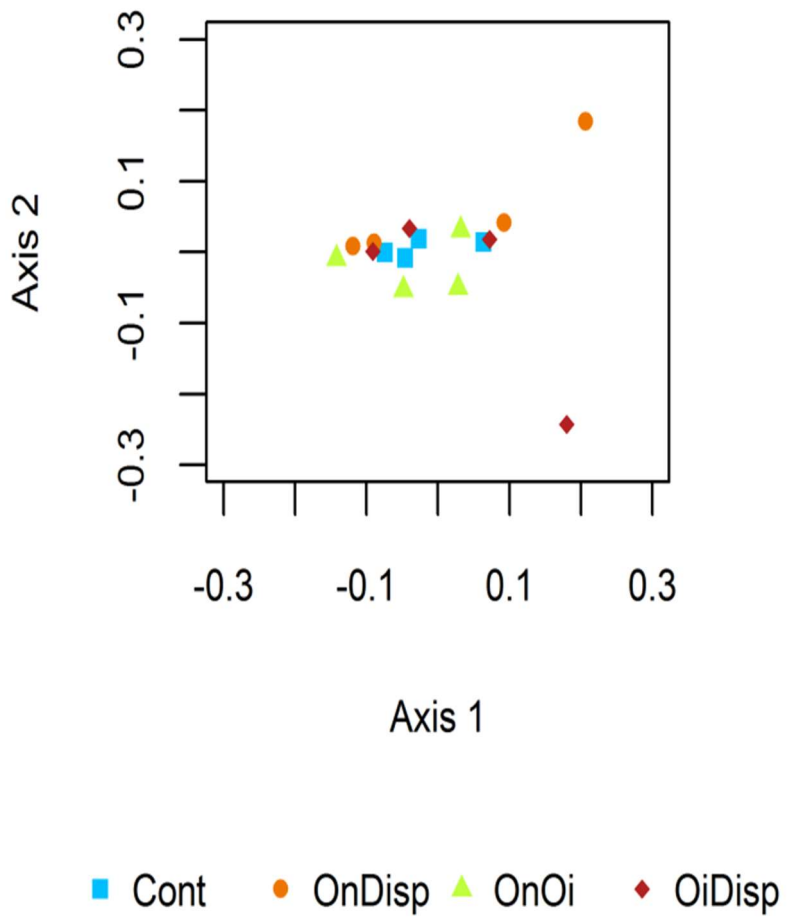

Figure IV-8 - Principal components analysis (PCO) of bacterial operational taxonomic units (OTU) at T21. The PCO was generated using the cmdscale() function in the R base package and wascores() function in vegan. Prior to the PCO, the raw data was $\log_{10}(x+1)$-transformed and used to produce a distance matrix based on the Bray-Curtis distance with the vegdist() function in vegan (Oksanen et al. 2012). The first two explanatory axes are shown. "Cont" (without oil and without dispersant), "OnDisp" (without oil and with dispersant), "OnOi" (with oil and without dispersant) and "OiDisp" (with oil and with dispersant). Sequence read (from MiSeq Illumina) were assigned to OTU with QIIME software (http://qiime.org) using the most recent Greengenes database (ftp://greengenes.microbio.me/greengenes_release/gg_13_5/gg_13_8_otus.tar.gz).

datasets may indicate a response from the bacterial taxon to hydrocarbon concentrations. A positive correlation would indicate that the bacterial taxon thrive or at least was not inhibit by higher hydrocarbon concentrations and a negative correlation indicate that bacteria was inhibited and at least did not benefit from by higher hydrocarbon concentration. Notable bacterial groups that had a positive correlation with hydrocarbon concentration include the *Alcanivoraceae* family (Oceanospirillales order) which was correlated with linear alkanes C_{16} to C_{20} and *Helicobacteraceae* family (ε-Proteobacteria class, Campylobacterales order) which was correlated with phenanthrene, fluoranthene, pyrene and

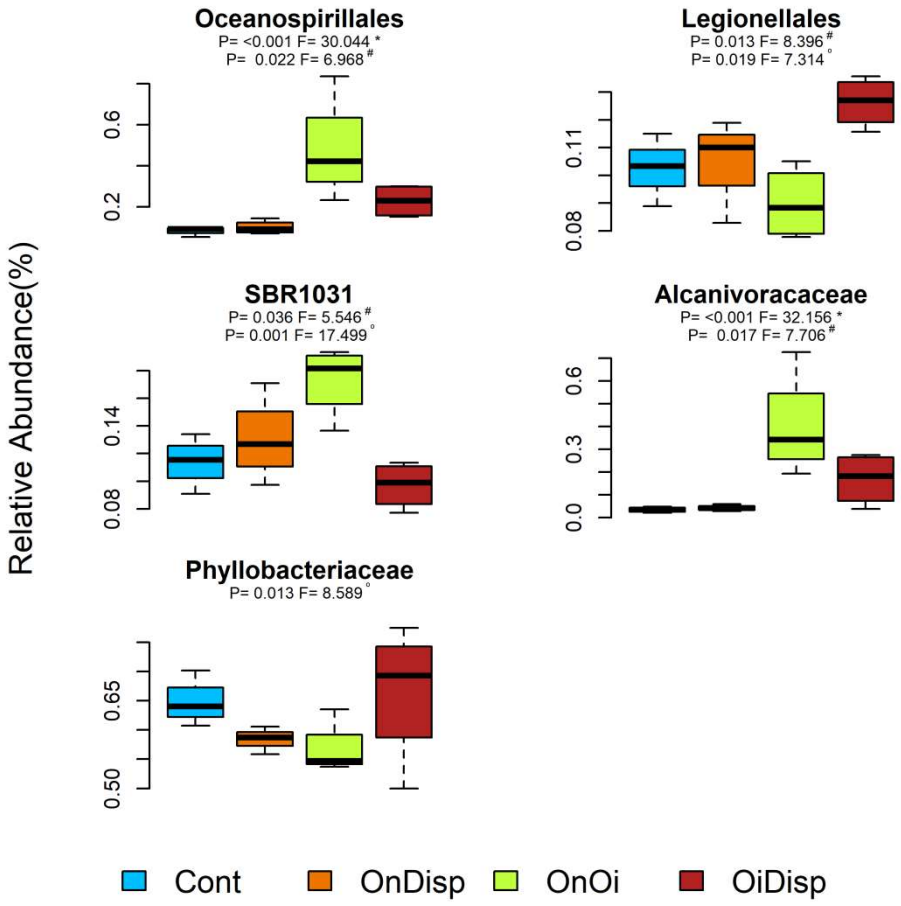

Figure IV-9 - Boxplot of the relative abundance of significant taxon at T21 in each datasets for the four treatments (Cont" (without oil and without dispersant), "OnDisp" (without oil and with dispersant), "OnOi" (with oil and without dispersant) and "OiDisp" (with oil and with dispersant)). Significant variations (p-value < 0.05) were determined through a two-way ANOVA of the linear regression of each dataset using functions anova() and glm() from the stats package in R. Significant increase or decrease (≤0.05) in response to oil addition, dispersant application or the interaction of both are marked with "*" for oil addition, "#" for dispersant and "°" - for the interaction. In the ANOVA, each factor independently and the interaction had 1 degree of freedom

benzo[b]fluoranthene. Those that had a negative correlation include candidate divisions OM190 (correlated with phenanthrene, pyrene and benzo[b]fluoranthene), it subtaxa agg27 [correlated with fluoranthene, pyrene, benzo(a)anthracene, chrysene and benzo[b]fluoranthene] and family *Ignavibacteriaceae* [correlated with fluoranthene, pyrene and benzo(a)anthracene]. Pearson correlation between the 50 most abundant OTUs and hydrocarbons concentrations was also calculated (Figure S IV-5). The information regarding their relative abundance and closest relative sequence obtained with NCBI-Blast is presented in Table S IV-3. The most notable correlations were the positive correlation between OTUs 2, 12879 and 2076 (belonging to the *Helicobacteraceae* family) and various PAHs (OTU_2

with acenaphthene, fluorene and phenanthrene and OTU_12879 and OTU_2076 with acenaphthene, fluorene, phenanthrene, fluoranthene and pyrene).

Discussion

Effect of chemical dispersant on the fate of oil hydrocarbons

Chemical dispersants, by buoyantly entraining micelles in water column, are thought to reduce the accumulation of oil at the sediment surface (Prince 2015). However, in *in vitro* simulations, chemical dispersants have been repeatedly found to facilitate the mass transfer of the micellized hydrocarbons to the sediment surface, especially PAHs (Cai et al. 2017; Gong et al. 2014b; Khelifa et al. 2008). Our results indicate that the chemical dispersion of oil did not significantly reduce nor enhance the concentration of the most abundant hydrocarbons (C_{14}-C_{22} linear alkanes) at the sediment surface. However, an increase in the concentration was observed for almost all PAHs at T1, and for phenanthrene and benzo[b]fluoranthene at T21. These results are consistent with those from previous experimental simulations that indicated that chemical dispersants enhance PAHs concentration in sediments (Gong et al. 2014b; Khelifa et al. 2008; Zhao et al. 2016). The linear alkanes had significant higher concentration at T1, in particular for C_{14} to C_{25} alkanes, which are the most abundant hydrocarbons of weathered Arabian light crude oil (Wang et al. 2003). At T21, this trend continued but the difference was attenuated and limited to alkanes C_{15} to C_{21}.

Variation in hydrocarbon concentration between timepoint was overall higher for linear alkanes than for PAHs, mainly because biodegradation acts preferentially on the alkanes (Brakstad et al. 2014; Huesemann et al. 2004; Zhao et al. 2016), whereas PAHs are intrinsically more recalcitrant to biodegradation (Huesemann et al. 2004). A >50% decrease in the mean C_{18}/phytane ratio confirms that biodegradation was the major fate for the linear alkanes. This decrease was slightly higher in OiDisp than in OnOi, but this difference between treatments was not significant. Thus, although the application of chemical dispersants seem to enhance alkanes removal, probably by increasing in the physical desorption and percolation of alkane these hydrocarbons are ressuspended into the water column and subsequently purged out of the microcosms (Brakstad et al. 2014; Zhao et al. 2016).

Independent and interactive effects of oil contamination and chemical dispersant on the composition of bacterial communities and its putative functional traits

The dataset was overall dominated by proteobacterial OTUs, among which γ- and δ-Proteobacteria were the predominant classes. This is in line with previous studies at the Ria de Aveiro water system (Coelho et al. 2013; Gomes et al. 2013; Oliveira et al. 2014b) and other wetland sediment samples (Mahmoudi et al. 2015; Rietl et al. 2016). Deltaproteobacterial OTU predominance is mainly

attributed to the sulfate-reducing bacteria from the Desulfobacterales order. This bacterial group frequently dominates the bacterial community in anoxic barren wetland environments (Gomes et al. 2013). Equally abundant was the order Acidimicrobiales, that although not frequently dominant in marine sediments, has been reported as well expressive in the Ria de Aveiro samples (Gomes et al. 2013; Oliveira et al. 2014b).

PCO ordination and adonis statistical test of compositional OTU tables indicate that neither factor nor their interaction are significant predictors of bacterial community composition at T21. This contrasts with results from a closed pelagic microcosm simulation, where chemically dispersed oil, in comparison to physically dispersed oil, was found to significantly alter the composition of the particle-associated bacterial community (Kleindienst et al. 2015b). However, our results are in accordance with to those from sediment slurry incubations in arctic conditions, which showed that, despite chemical dispersants benefiting oil hydrocarbon biodegradation, the bacterial community was similar in oil-contaminated treatments with and without chemical dispersants (Ferguson et al. 2017).

Curiously, for oil contamination alone no significant change occurred in the core composition of the bacterial community. The bacterial community of oil contaminated microcosms may have been re-establishing its initial structure since hydrocarbons concentrations were substantially lower at T21. However, it is also possible that, since the sampling site was located in the proximity of a major fuel depository and commercial port, chronic exposure to oil hydrocarbon pollution preconditioned the bacterial community (Païssé et al. 2010). Mean ∑alkane and ∑PAH concentration at sampling site are considerably higher than in many pristine sites (Louvado et al. 2015; Martins et al. 2004), yet in comparison to other port sediments, they are of the same magnitude or higher (De Luca et al. 2004; Yakimov et al. 2005), but also considerably lower than others (Bajt 2012; Tavares et al. 2016). Also, mean ∑PAH concentration (all PAH analyzed excluding acenaphthene and acenaphthylene) in Env was approximately 10x lower than in comparison with reference sediment NIST SRM 1941b (Wise et al. 2004). Additionally, Env samples had CPI >1, which is indicative of hydrocarbons of petrogenic origin (Pietrogrande et al. 2009). Chronic oil pollution has been previously recognized to stabilize the composition of bacterial communities in relation to future oil contamination, not only due to a higher abundance of latent hydrocarbonoclastic bacteria and generalist oil-degrading heterotrophic bacteria, but also inducing a higher resistance of other bacteria to hydrocarbon toxicity (Païssé et al. 2010). In this dataset, the majority of the 50 most abundant OTU have a high similarity with sequences retrieved from hydrocarbon-contaminated environments. Although it has been demonstrated that, in chronically polluted sediments, additional oil input does not significantly change the composition of the bacterial community, added hydrocarbons were found to be degraded quickly (Païssé et al. 2010). This contrasts with more pristine sites accidentally contaminated with oil, where bacterial community changes more drastically (Yakimov et al. 2004) and oil hydrocarbon biodegradation is slower (Bargiela et al. 2015).

Despite the above-mentioned stability, a group-specific analysis revealed that the relative abundance of five bacterial taxa had a significant change in response to the independent and/or interactive effect of oil hydrocarbon contamination and dispersant addition. Almost all of these bacterial groups may be potentially involved in hydrocarbon and/or xenobiotic degradation. In example, members of the *Alcanivoraceae* family, which are considered obligate oil-degraders (Yakimov et al. 2007) and their abundance is tightly coupled to the presence of oil hydrocarbons, more specifically, to the presence of short to medium linear alkanes (Joye et al. 2014), had significant higher abundance in oil contaminated treatments. Coherently, this family also had a positive correlation with the concentration of linear alkanes C_{16} to C_{20}.

The interactive effects of oil and dispersant addition had a significant reduction in the relative abundance of candidate division SBR1031. This bacterial group has never been described as oil hydrocarbon degrading bacterial group, yet it has been occasionally detected in hydrocarbon rich matrixes, namely in hydrocarbon-contaminated marine environments (An et al. 2004) and in sediments near a terrestrial gas seep (Neubeck et al. 2017). Also, members of these bacterial group are potentially involved in acetogenesis through the reduction of ethanol (Xia et al. 2016) and thus may have a role in the anaerobic degradation of oil hydrocarbons. In this work, their relative abundance had a significant increase in OnOi, but in OiDisp it was significantly lower. The addition of chemical dispersants, by significantly increasing PAHs bioavailability, could have impacted the relative abundance of candidate division SBR1031. However, candidate division SBR1031 did not have any negative correlation with PAH concentrations. Possibly, dispersant addition could have increased the concentration of other not quantified, but similarly toxic, pollutant. In contrast, order Legionellales and family *Phyllobacteriaceae* had significant higher relative abundance in OiDisp. Both have been directly or indirectly linked in oil hydrocarbon biodegradation (Bacosa et al. 2015; Lai et al. 2011c).

Spearman correlation analysis between hydrocarbons concentration and bacteria abundance revealed some interesting interactions for some bacterial groups (e.g. candidate divisions OM190, its subtaxon agg27 and order Ignavibacteriales) that are intrinsically involved in the nitrogen cycle (Gonzalez-Martinez et al. 2015; Mardanov et al. 2016; Schmid et al. 2000; Zhang et al. 2015). These groups had a negative correlation with the concentration of 4- and 5- ring PAHs. The OM190/agg24 bacterial is characteristically involved in nitrification (Schmid et al. 2000), whereas order Ignavibacteriales has been linked to various metabolic pathways in the nitrogen cycle (e.g. nitrification (Mardanov et al. 2016), denitrification (Liu et al. 2012a; Zhang et al. 2015), and anammox (Gonzalez-Martinez et al. 2015)). PAHs addition has been reported to have a negative impact on nitrification (Lindgren et al. 2014), denitrification (Pietroski et al. 2015) and the nitrogen cycle in general (Scott et al. 2014), therefore justifying the significant and negative correlation between these taxa and PAHs concentration. Meanwhile, the *Helicobacteraceae* family (E-protebacteria:Campylobacterales; mean relative abundance: 3.325±1.333%), had a positive correlation with phenanthrene, fluoranthene, pyrene and

benzo[b]fluoranthene. Genera from this family (e.g. *Sulfurimonas* and *Sulfuricurvum*) are frequent in marine sediments amended with 3- and 4-ring PAHs (Obi et al. 2017; Stauffert et al. 2014). Thus, a strong and significant correlation with PAHs concentrations may indicate that the *Helicobacteraceae* family may include putative PAH-degrading bacteria.

Conclusions

This study showed that chemically dispersed oil did not cause significant change to the overall composition of the benthic bacterial community from port sediments. The chronic oil pollution at sampling site may have preconditioned the response of benthic bacterial communities to additional oil input. However, and despite the aforementioned stability, some changes were observed among putative hydrocarbon-degrading bacteria. Also, the overall impact on oil hydrocarbon removal is ambiguous since despite alkane concentrations having decreased substantially with time, the concentration of phenanthrene and benzo(b)fluoranthene in sediments was significantly enhanced by dispersant addition 21 days after oil contamination. In this study, it was concluded that the addition of a chemical dispersant had no effects on the overall composition of port sediment bacterial communities after oil contamination and did not contribute to improve the process of oil hydrocarbons removal. Yet, an in-depth analysis of the effects of chemical dispersant addition on ultimate fate of the sediment bonded oil hydrocarbons, and their turnover rate, is require to determine to the net benefit of chemical dispersant use in oil spill response in port areas.

Funding

This work was supported by the Foundation for Science and Technology at the Portuguese Ministry of Education and Science [UID/AMB/50017 for CESAM research unit; PEst-C/QUI/UI0062/2013 for QOPNA research unit; SFRH/BD/86447/2012 for AL, SFRH/BPD/92366/2013 for FJRC and SFRH/BPD/101919/2014 for VO], which is co-funded by national funds and FEDER within the PT2020 Partnership Agreement and Compete 2020. The ELSS construction was financed by European Funds through COMPETE (FCOMP-01-0124-FEDER-008657) and by National Funds through the Portuguese Science Foundation (FCT) within project PTDC/AAC-CLI/107916/2008

Acknowledgements

Authors would also acknowledge the support of fellow colleagues: Ana Cecília Pires, Marina Ferreira and Letícia Duarte during the execution of microcosm experiment and sampling events; additionally, Cátia Venâncio and Victor Galhano for their support during the sampling events.

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Supplementary files

Table S IV-1 - Results from statistical test ANOVA (*p*-value and F-value) for linear saturated alkanes from C_{12} to C_{36} at T1. Significant results (*p*-value < 0.05) are in bold. Each factor independently and the interaction had 1 degree of freedom.

Alkanes		Factors					
		Oil		Dispersant		Oil and Dispersant	
		p-value	F-value	*p*-value	F-value	*p*-value	F-value
C12	Dodecane	**<0.001**	21.102	0.121	2.555	**0.015**	6.713
C13	Tridecane	0.058	3.922	0.754	0.1	0.886	0.021
C14	Tetradecane	**<0.001**	32.029	0.148	2.212	0.347	0.917
C15	Pentadecane	**<0.001**	67.7	0.088	3.123	0.443	0.605
C16	Hexadecane	**<0.001**	75.58	**0.037**	4.794	0.452	0.582
C17	Heptadecane	**<0.001**	71.394	**0.015**	6.777	0.5	0.467
C18	Octadecane	**<0.001**	89.032	**0.004**	10.012	0.555	0.358
C19	Nonadecane	**<0.001**	113.904	**0.002**	11.387	0.585	0.306
C20	Icosane	**<0.001**	116.92	**0.001**	12.586	0.562	0.344
C21	Henicosane	**<0.001**	111.053	**0.002**	12.037	0.424	0.659
C22	Docosane	**<0.001**	105.982	**0.003**	10.201	0.625	0.244
C23	Tricosane	**<0.001**	95.112	**0.005**	9.531	0.659	0.199
C24	Tetracosane	**<0.001**	73.303	**0.016**	6.572	0.991	<0.001
C25	Pentacosane	**<0.001**	60.021	**0.005**	9.087	0.972	0.001
C26	Hexacosane	**<0.001**	45.771	**0.005**	9.485	0.856	0.034
C27	Heptacosane	**<0.001**	33.898	**0.002**	11.068	0.908	0.013
C28	Octacosane	**0.021**	6.024	**0.006**	8.801	0.983	<0.001
C29	Nonacosane	**<0.001**	31.924	**0.002**	11.777	0.561	0.345
C30	Triacontane	**<0.001**	29.114	0.196	1.755	0.183	1.862
C31	Hentriacontane	**<0.001**	23.183	0.12	2.579	0.339	0.946
C32	Dotriacontane	**<0.001**	24.466	0.596	0.288	0.07	3.552
C33	Tritriacontane	**0.004**	10.12	0.864	0.03	**0.018**	6.298
C34	Tetratriacontane	**0.001**	14.488	**0.016**	6.617	**0.01**	7.579
C35	Pentatriacontane	**0.001**	15.132	**0.019**	6.162	**0.001**	14.057
C36	Hexatriacontane	**<0.001**	17.839	1	<0.001	**<0.001**	35.631

Table S IV-2 - Results from statistical test ANOVA (*p*-value and F-value) for linear saturated alkanes from C_{12} to C_{36} at T21. Significant results (*p*-value < 0.05) are in bold. Each factor independently and the interaction had 1 degree of freedom.

Alkanes		Oil		Dispersant		Oil and Dispersant	
		p-value	F-value	*p*-value	F-value	*p*-value	F-value
C12	Dodecane	0.097	2.95	0.165	2.032	0.11	2.723
C13	Tridecane	0.122	2.547	0.106	2.787	0.678	0.176
C14	Tetradecane	0.14	2.308	0.029	5.3	0.785	0.076
C15	Pentadecane	**0.007**	8.487	0.076	3.396	0.469	0.54
C16	Hexadecane	**0.001**	14.844	0.162	2.063	0.949	0.004
C17	Heptadecane	**<0.001**	21.266	0.418	0.677	0.857	0.033
C18	Octadecane	**<0.001**	16.987	0.417	0.679	0.772	0.085
C19	Nonadecane	**<0.001**	21.13	0.622	0.249	0.894	0.018
C20	Icosane	**0.001**	13.631	0.803	0.063	0.882	0.023
C21	Henicosane	**0.013**	7.12	0.768	0.088	0.956	0.003
C22	Docosane	0.327	0.997	0.564	0.341	0.665	0.191
C23	Tricosane	0.308	1.08	0.645	0.216	0.774	0.084
C24	Tetracosane	0.036	4.876	0.618	0.254	0.724	0.127
C25	Pentacosane	**0.008**	8.3	0.417	0.678	0.569	0.332
C26	Hexacosane	**0.009**	7.963	0.845	0.039	0.812	0.057
C27	Heptacosane	**0.008**	8.117	0.762	0.093	0.484	0.503
C28	Octacosane	**0.009**	7.895	0.621	0.25	0.169	1.995
C29	Nonacosane	**0.013**	6.991	0.635	0.23	0.218	1.589
C30	Triacontane	0.059	3.867	0.49	0.489	0.362	0.858
C31	Hentriacontane	0.109	2.746	0.542	0.381	0.246	1.404
C32	Dotriacontane	0.134	2.378	0.357	0.876	0.255	1.35
C33	Tritriacontane	0.28	1.211	0.587	0.302	0.27	1.266
C34	Tetratriacontane	0.056	3.974	0.497	0.473	0.112	2.69
C35	Pentatriacontane	0.986	<0.001	0.981	0.001	0.102	2.86
C36	Hexatriacontane	0.151	2.182	**0.007**	8.458	**<0.001**	15.817

Table S IV-3 - List of the 50 most abundant OTU across the dataset. The table includes OTU affiliation from the class to the genus level, mean relative abundance (MRA %) and most similar nucleotide sequence(s) available in NCBI database, its similarity (SIM), source and, if available, reference(s).

Class	OTU	Order	Family	Genus	MRA (%)	Closest relative	NCBI Accession nº	SIM	Source	Ref.
Acidimicrobiia	1	Acidimicrobiales	C111	Unclassified	2.71 ± 0.49	Uncultured bacterium clone 7A_11-040	KY190764	99%	Hydrocarbon polluted soil Antarctica	
	13	Acidimicrobiales	koll13	Unclassified	1.58 ± 0.27	Uncultured bacterium clone JS5_258	KR825069	99%	Marine Sediments	
	4	Acidimicrobiales	C111	Unclassified	1.29 ± 0.25	Uncultured actinobacterium clone c-65	GQ144953	99%	N.D.	
	19	Acidimicrobiales	C111	Unclassified	0.6 ± 0.115	Uncultured actinobacterium clone JBS_5A309	EU702790	99%	Marine Sediments	
	37	Acidimicrobiales	koll13	Unclassified	0.47 ± 0.16	Uncultured actinobacterium clone A11	GQ249475	99%	Marine Sediments	
	425	Acidimicrobiales	koll13	Unclassified	0.43 ± 0.12	Uncultured bacterium clone N-37	HQ703834	99%	Marine Sediment	
	11680	Acidimicrobiales	C111	Unclassified	0.38 ± 0.05	Uncultured Actinobacteridae bacterium clone DY40-191	KC018164	99%	Marine Sediments	
	14756	Acidimicrobiales	koll13	Unclassified	0.3 ± 0.07	Uncultured actinobacterium clone TRAN-014	JF344430	99%	Oil contaminated Marine Sediments	(Acosta-González et al. 2013)
Actinobacteria	50	Actinomycetales	*Pseudonocardiaceae*	Unclassified	0.51 ± 0.09	Uncultured actinobacterium clone D37	JX011125	99%	Marine Environment	
Alphaproteobacteria	6	Rhizobiales	*Hyphomicrobiaceae*	Unclassified	0.89 ± 0.2	Uncultured prokaryote clone 762487	KT974436	99%	Marine rock surface	(Couradeau et al. 2017)
	17228	Rhizobiales	*Hyphomicrobiaceae*	Unclassified	0.55 ± 0.12	Uncultured bacterium clone Zeebrugge_B56	HM598599	99%	Oil contaminated Marine Sediments	(Siegert et al. 2011)
	618	Rhodobacterales	*Rhodobacteraceae*	*Phaeobacter*	0.48 ± 0.07	Uncultured bacterium clone Woods-Hole_a5207	KF798960	99%	Ascidian Gut	(Dishaw et al. 2014)
	21	Rhizobiales	Unclassified	Unclassified	0.35 ± 0.07	Uncultured bacterium clone N4-30	FJ786111	99%	Aquaculture marine sediments	
	805	Rhodobacterales	*Rhodobacteraceae*	*Roseobacter*	0.34 ± 0.05	Uncultured bacterium clone H1 BAL T270d	JF774639	99%	Oil contaminated Marine Sediments	(Stauffert et al. 2013)
Anaerolineae	15	GCA004	Unclassified	Unclassified	0.46 ± 0.07	Uncultured bacterium clone TfC20L34	EU362300	99%	Enrichment cultures of Marine Sediments with perchloroethene	(Kittelmann and Friedrich 2008)
	71	GCA004	Unclassified	Unclassified	0.41 ± 0.09	Uncultured bacterium clone Zeebrugge_B36	HM598581	99%	Oil contaminated Marine Sediments	(Siegert et al. 2011)

Bacteroidia	24	Bacteroidales	Unclassified	Unclassified	0.66 ± 0.23	Uncultured bacterium clone MK903D_B8	AB831401	99%	Deep Sea Mud Volcano	(Pachiadaki et al. 2011)
						Uncultured bacterium clone AMSMV-20-B23	HQ588572		Deep Sea Methane Seep	(Aoki et al. 2014)
Cytophagia	20	Cytophagales	*Flammeovirgaceae*	Unclassified	0.45 ± 0.08	Uncultured Bacteroidetes bacterium clone C6_138	KP016581	99%	Oil and Heavy metal contaminated Marine sediments	
Deltaproteobacteria	10	Desulfobacterales	*Desulfobulbaceae*	Unclassified	1.24 ± 0.16	Uncultured bacterium clone JS2_171	KR825042	99%	Marine Sediments	
	14	Desulfuromonadales	*Desulfuromonadaceae*	Unclassified	1.07 ± 0.27	Uncultured bacterium clone TfC20L23	EU362292	99%	Enrichment cultures of Marine Sediments with perchloroethene	(Kittelmann and Friedrich 2008)
	9	Desulfobacterales	*Desulfobulbaceae*	Unclassified	0.71 ± 0.12	Uncultured bacterium clone Woods-Hole_a5919	KF799444	99%	Ascidian Gut	(Dishaw et al. 2014)
	40	Myxococcales	Unclassified	Unclassified	0.53 ± 0.16	Uncultured delta proteobacterium clone 80.	AM882648	99%	Oil contaminated Marine Sediments	(Paissé et al. 2008)
	18	Desulfobacterales	*Desulfobacteraceae*	*Desulfococcus*	0.52 ± 0.11	Uncultured delta proteobacterium clone FII-AN139	JQ580080	99%	Oil contaminated Marine Sediments	(Acosta-González et al. 2013)
	39	Desulfobacterales	*Desulfobacteraceae*	*Desulfosarcina*	0.39 ± 0.09	Uncultured delta proteobacterium clone 70	AM882640	99%	Oil contaminated Marine Sediments	(Paissé et al. 2008)
	72	Desulfobacterales	*Desulfobacteraceae*	*Desulfococcus*	0.34 ± 0.065	Uncultured bacterium clone SB4AB21	HQ271757	99%	Salt Marsh Sediments	(Martiny et al. 2011)
Ellin6529	23	Unclassified	Unclassified	Unclassified	0.38 ± 0.06	Uncultured Chloroflexi bacterium clone A02B18	KT731752	99%	Marine Seawater	
	1336	Unclassified	Unclassified	Unclassified	0.3 ± 0.05	Uncultured bacterium clone 1NSeds_G05	GQ412873	99%	Sponge Symbiont	(Garren et al. 2009)
Epsilonproteobacteria	2	Campylobacterales	*Helicobacteraceae*	Unclassified	1.5 ± 0.66	Uncultured bacterium clone H3	HQ848030	99%	Marine Sediments	
	12879	Campylobacterales	*Helicobacteraceae*	Unclassified	0.79 ± 0.28	Uncultured *Sulfurovum* sp. clone ZLL-A38	JF806782	99%	Marine Sediment	
	2076	Campylobacterales	*Helicobacteraceae*	Unclassified	0.49 ± 0.25	Bacterium enrichment culture clone PAH63-P10	KJ409516	99%	Oil contaminated Marine Sediments	(Stauffert et al. 2014)
Flavobacteriia	8	Flavobacteriales	*Flavobacteriaceae*	*Lutimonas*	0.77 ± 0.23	*Aestuariicola saemankumensis* str SMK-142	NR044441	99%	Estuarine Sediments	(Yoon et al. 2008)
	19436	Flavobacteriales	*Flavobacteriaceae*	*Lutimonas*	0.77 ± 0.19	Uncultured Bacteroidetes bacterium clone A24	GQ249488	99%	Marine Sediments	
Gammaproteobacteria	11	Thiohalorhabdales	Unclassified	Unclassified	1.17 ± 0.29	Uncultured bacterium clone M33	KR077747	99%	Marine Sediments	

	3	Chromatiales	Unclassified	Unclassified	1.12 ± 0.21	Uncultured gamma proteobacterium clone PI_4z2c	AY580826	99%	Seawater	(Acinas et al. 2004)
	62	Thiotrichales	*Piscirickettsiaceae*	Unclassified	0.9 ± 0.29	Uncultured *Pseudomonas* sp. clone NdAmb158	FJ753064	99%	Sediments surrounding polychaete burrows	(Pischedda et al. 2011)
	5	Thiotrichales	*Piscirickettsiaceae*	Unclassified	0.9 ± 0.16	Uncultured bacterium clone JS2_33	KR824990	99%	Marine Sediments	
	12	Alteromonadales	OM60	Unclassified	0.56 ± 0.08	Uncultured bacterium clone M68	KR077745	99%	Marine Sediments	
	79	[Marinicellales]	[Marinicellaceae]	Unclassified	0.52 ± 0.09	Uncultured gamma proteobacterium clone SIMO-2236	AY711602	99%	Salt Marsh Sediments	
	10743	Alteromonadales	OM60	Unclassified	0.46 ± 0.06	Uncultured bacterium clone PropaneSIP5-6-05	GU584647	99%	Marine Hydrocarbon Seep	(Redmond et al. 2010)
	30	Chromatiales	Unclassified	Unclassified	0.45 ± 0.05	Uncultured bacterium clone Zeebrugge_B25	HM598574	99%	Oil contaminated Marine Sediments	(Siegert et al. 2011)
	22	Thiotrichales	*Thiotrichaceae*	Unclassified	0.44 ± 0.13	Uncultured gamma proteobacterium clone ARTE12_227	GU230346	99%	Estuary Sediments	
	17	Alteromonadales	OM60	Unclassified	0.42 ± 0.05	Uncultured bacterium clone PAH2startSedimOxic_M12_6_D11	KJ615848	99%	Oil contaminated Fe-Mn concretions and Marine sediment	
	283	Thiotrichales	*Piscirickettsiaceae*	Unclassified	0.38 ± 0.1	Uncultured gamma proteobacterium clone 57	AM882568	99%	Oil contaminated Marine Sediments	(Paissé et al. 2008)
	138	Chromatiales	Unclassified	Unclassified	0.38± 0.05	Uncultured gamma proteobacterium clone 56b.	HE804020	99%	Marine Sediments	
	38	Alteromonadales	*Alteromonadaceae*	ZD0117	0.34 ± 0.24	Uncultured bacterium clone PropaneSIP20-4-23	GU584788	99%	Marine Hydrocarbon Seep	(Redmond et al. 2010)
	58	[Marinicellales]	[Marinicellaceae]	Unclassified	0.35 ± 0.05	Uncultured bacterium clone 1b_5	HE803943	99%	Marine Sediments	
	17282	Thiohalorhabdales	Unclassified	Unclassified	0.33 ± 0.06	Uncultured bacterium clone Milano-WF1B-20	AY592863	99%	Marine Cold Seep	(Heijs et al. 2005)
	25	[Marinicellales]	[Marinicellaceae]	Unclassified	0.29 ± 0.05	Uncultured Chromatiales bacterium clone 16B_205.	AM501729	99%	Marine Sediments	(Borin et al. 2009)
	7321	Chromatiales	Unclassified	Unclassified	0.3 ± 0.06	Uncultured bacterium clone AMSMV-5-B14	HQ588443	99%	Mud Volcano Sediments	(Pachiadaki et al. 2011)
OS-K	26	Unclassified	Unclassified	Unclassified	0.36 ± 0.06	Uncultured bacterium clone Tokyo.16S.Bac.30	AB530230	99%	Port Marine Sediments	(Elsaied et al. 2011)

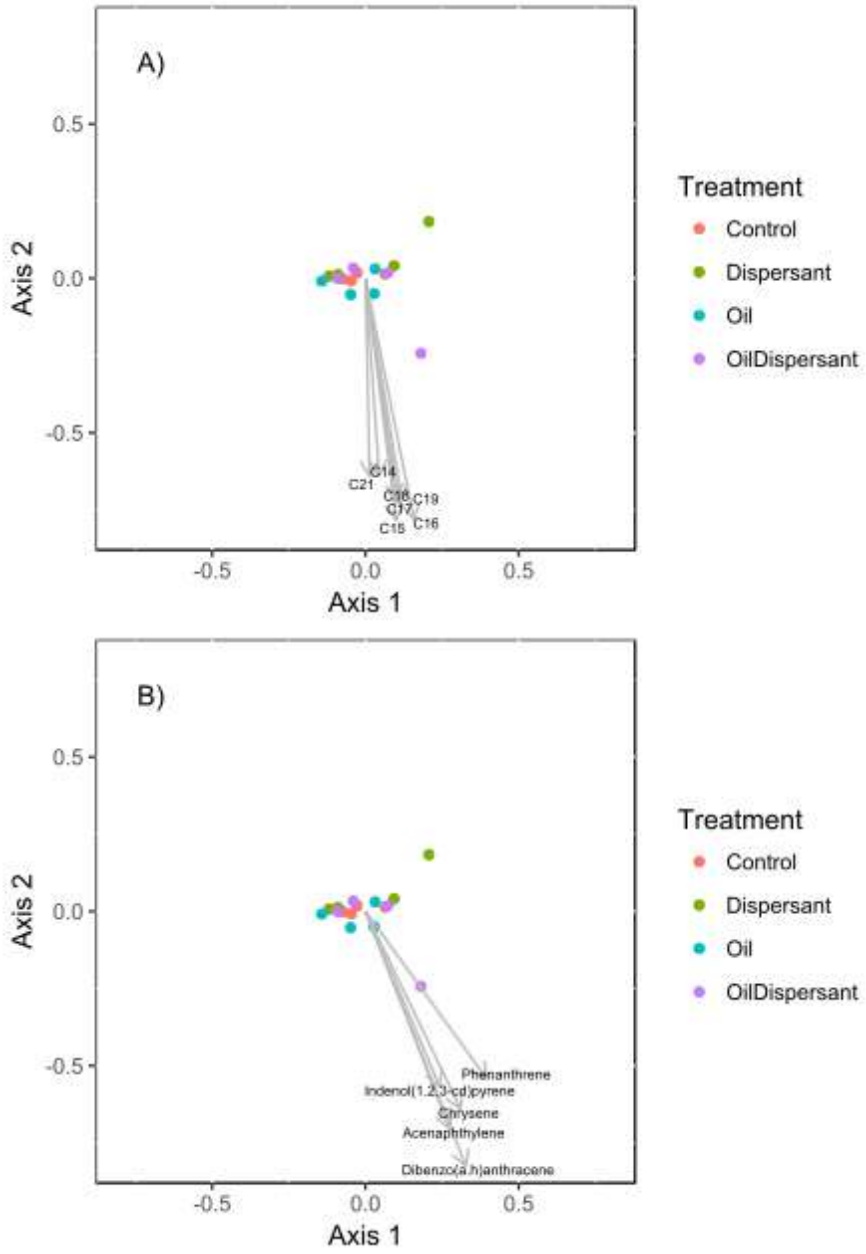

Figure S IV-1 - PCO ordination of samples, with significant (*p*-0.05) linear alkanes (A) and PAHs (B) fitted using *envfit*().

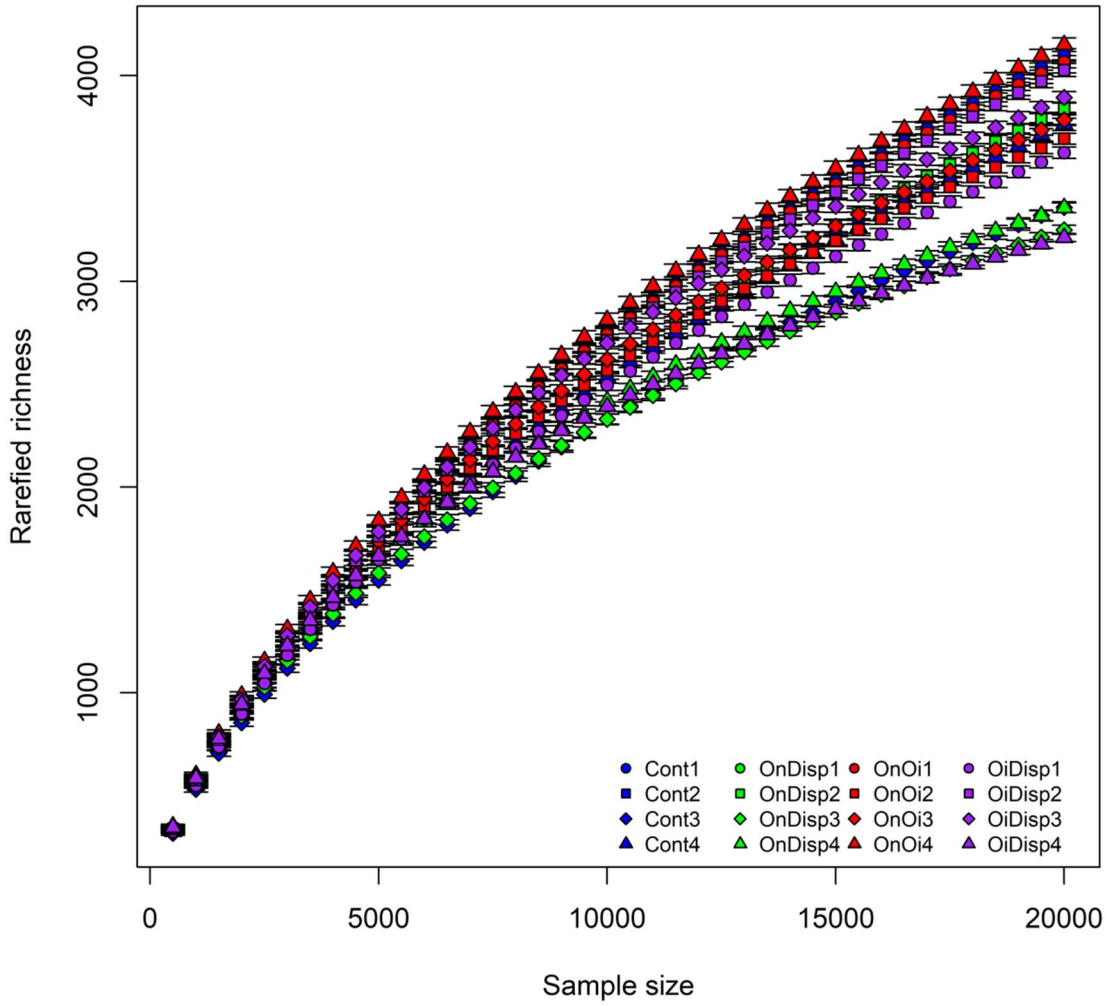

Figure S IV-2 - Rarefaction curve from each sample. Calculated using a self-written function (Gomes 2010).

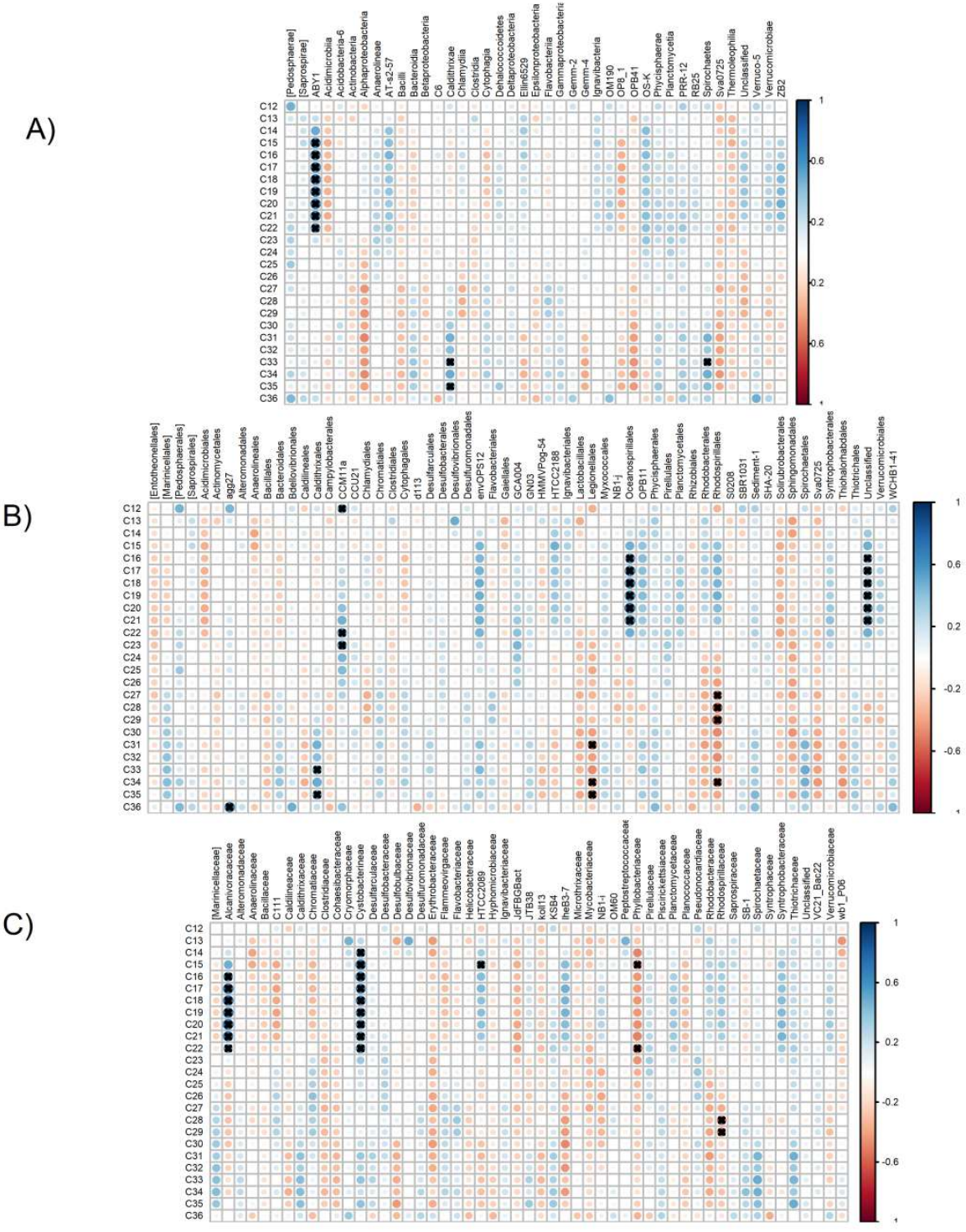

Figure S IV-3 - Correlation analysis between the relative abundance of most relevant bacterial groups [threshold mean relative abundance < 0.1%; classes (A), orders (B) and families (C)], and alkane concentrations from all replicates. Correlations and correlation significance was calculated using rcorr() function from the {Hmisc} package . Statistically significant correlations (p-value < 0.05) are marked with an "X".

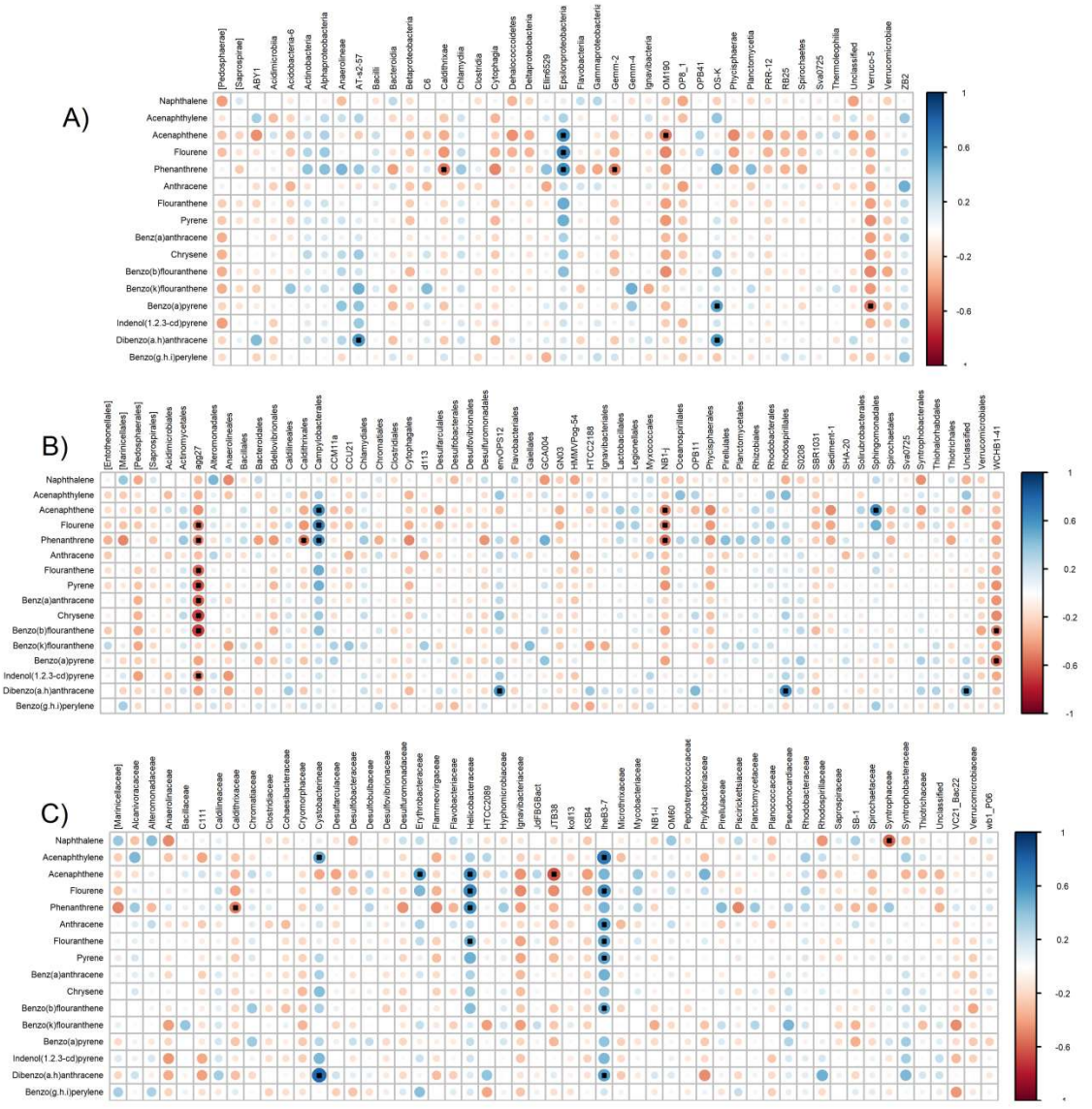

Figure S IV-4 - Correlation analysis between the relative abundance of most relevant bacterial groups [threshold mean relative abundance < 0.1%; classes (A), orders (B) and families (C)] and PAHs concentrations from all replicates. Correlations and correlation significance was calculated using rcorr() function from the {Hmisc} package. Statistically significant correlations (*p*-value < 0.05) are marked with an "X".

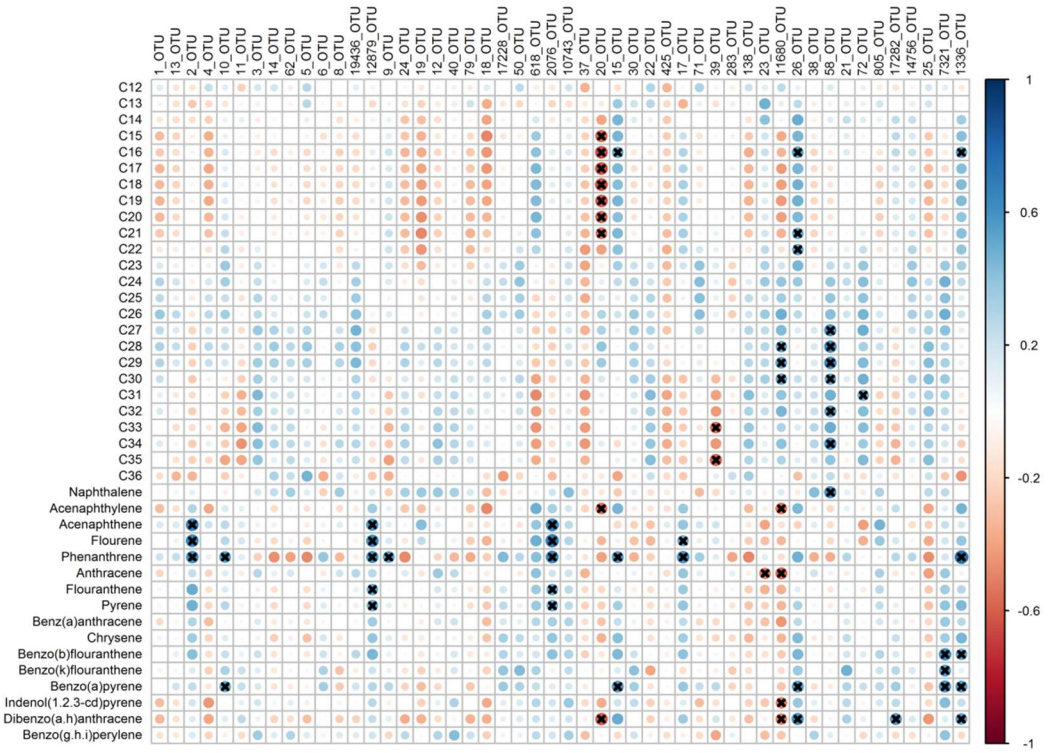

Figure S IV-5 - Correlation analysis between the relative abundance matrix of the 50 most abundant OTUs and alkanes and PAHs concentrations from all replicates. Correlations and correlation significance was calculated using rcorr() function from the {Hmisc} package. Statistically significant correlations (p-value < 0.05) are marked with an "X".

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Conclusion and Final Remarks

Conclusion and Final Remarks

The marine sediment compartment is an important sink and filter for oil hydrocarbons released by accidental and natural sources. Cold seeps linked to subsurface oil reservoirs (e.g. mud volcanoes) will trickle oil through this sediment barrier, where a dense heterotrophic microbial community will actively degrade it, creating a local biological hotspot. Yet despite biodegradation occurring under these challenging abiotic conditions, the bacterial players involved are poorly understood. Also, by adhering to the sinking sediment particles, accidentally released oil hydrocarbons will settle at the sediment surface, and, with time, it will be inhumed into the adjacent anaerobic layers. There, since biodegradation is substantially less efficient, oil may persist for long periods. Thus, although, aerobic and anaerobic biodegradation processes are determinant for oil decontamination, the fate of oil hydrocarbons will be heavily influenced by the efficiency of the initial aerobic biodegradation by the microbial communities inhabiting the surface sediment layer. However, some present and future scenarios (e.g. deep sea conditions, ocean acidification and the application of chemical dispersants) may impose changes to this bacterial-driven metabolic turnover. Yet the biogeochemical properties of sediments and the adaptation of some benthic bacterial community may attenuate their impact. In this thesis, the bacterial response to these scenarios was inferred by field data and/or was experimentally analyzed. In general, the results obtained indicate that the sediment bacterial communities can show a remarkable resilience to oil hydrocarbon contamination.

The deep sea sediment surface is an overlooked sink for released oil hydrocarbons. The mass transfer of oils most recalcitrant compounds (e.g. PAH) to the deep sea sediment surface may occur through the vertical settling of oil-sediment aggregates. Once settled, they will be available to the local bacterial community, which is speculated to have an opportunistic life strategy as an adaptation to the deep sea oligotrophic environment. PAH biodegradation at the DSS could be inhibited by abiotic conditions such as low temperature and hydrostatic high pressure, but the adaptation of local bacterial communities to these abiotic extremities may counter this. Also, occasional underwater hydrocarbon seeps can be found throughout deep sea surface. These seeps may potentially release, annually, an amount of oil hydrocarbons substantially higher than that released by accidental oil spills. Mud volcanoes are included among these geological formations. At the mud volcanoes sediments, a diverse and dense ecosystem occurs and is sustained by the heterotrophic microbial community that actively scavenges these seeped oil hydrocarbons. Using samples from the deep sea mud volcanoes (inactive and active craters) and from a reference site (abyssal plains) from the gulf of Cadiz, the PAH-degrading bacterial community was enriched, by using a culture medium with added phenanthrene and chrysene as main carbon sources, and isolates were obtained. In general, the isolates were dominated by *Bacillus*-like

bacteria. This predominance was unexpected when considering that the *Bacillaceae* family is, normally, poorly represented in the PAH-degrading community of marine environments in general. Nonetheless, these results are coherent with other studies from other cold seep-related environments and reveal that in these environment, the *Bacillaceae* family may be involved in PAH biodegradation at the deep sea.

In contrast to current deep sea abiotic conditions, the overall impact on bacterial processes of the predicted abiotic conditions of the future ocean are harder to assert and may require their simulation under controlled microcosm conditions. Ocean acidification, an indirect consequence of global warming, is known to alter key biogeochemical cycles, consequently impacting marine ecosystems. In previous microcosm simulations, the interactive effect of seawater acidification and oil contamination was shown to alter the composition of the core active bacterial community and reduce the abundance of active members of the anaerobic oil-degrading Desulfobacterales order at the superficial sediments. In this thesis, using samples from the same microcosm simulations, we conclude that this effect does not seem to extend to the subsurface sediments throughout the experiment. Although a taxon-specific analysis revealed that, although the relative abundance of some bacterial groups changed in response to the independent and/or interactive effects of reduced seawater pH and oil contamination, these were not expressed in the overall bacterial community inhabiting the subsurface sediments (≈5 cm), which was stable to the abiotic changes imposed. It is proposed that both seawater acidification and oil contamination were attenuated by the sub-adjacent sediment layer that may have functioned as a barrier and buffer.

Oil spills, although responsible for a small fraction of all oil discharged into the ocean, can have catastrophic effect at regional scale. To mitigate this effect, response strategies are employed, often involving the use of chemical dispersants. However, their use is controversial because the potential benefits may not compensate potential drawbacks. Chemical dispersants will increase oil bioavailability, which may enhance hydrocarbon biodegradation but may also increase the exposure of marine biota to toxic constituents and degradation products. Also, the use of dispersants may increase the mass transfer of oil hydrocarbons to the sediment. Although some studies have addressed the fate of chemically dispersed oil, until now none had tested their potential impact on benthic bacterial communities in coastal port areas.

Here, a multi-factorial microcosm simulation was performed to evaluate the interactive and independent effect of oil and dispersant addition on the bacterial community composition of intact sediment cores and on hydrocarbon concentration at the sediments superficial layer. The results revealed that chemical dispersion of oil did have a significant effect on the concentration of *n*-alkane in the sediments. However, chemical dispersants significantly increased the mass transfer of PAHs to the sediments. and altered the relative abundance of some putative hydrocarbon-degrading bacterial groups. However, despite the changes detected in the relative abundance of putative oil hydrocarbon degraders, the overall composition of the bacterial community was stable to the tested experimental the

independent and interactive effects of the oil contamination and dispersant addition. The previous exposure of the sediment bacterial communities to chronic oil hydrocarbon pollution in the port area may have preconditioned the bacterial communities to oil contamination.

Overall, this report broadens our understanding of bacteria-oil interactions at the sediments. Nonetheless, several questions persist. A microcosm simulation of an oil contamination event under deep sea conditions, although logistically challenging, would provide new insights on the microbial community response in field conditions. In chapter III, the microcosm experiment showed that *n*-alkane removal was enhanced in microcosms contaminated by chemically dispersed oil. However, the ultimate fate of this hydrocarbons is uncertain. Oil hydrocarbons may have moved from the sediments to the water column by desorption and purged out from the microcosms during the simulated low tides or moved to deeper layers of the sediment by percolation. This is relevant to the prediction of the overall effects since the dispersants may facilitate the transfer of oil hydrocarbon into subsurface sediments, where anaerobic conditions will substantially stall biodegradation and potentially perpetuate the presence of oil in the environment.

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