Universidade do Minho
Escola de Engenharia

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Strategies for enhancing hydrocarbons bioremediation

novembro de 2021

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Doutora Maria Alcina Pereira

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STATEMENT OF INTEGRITY

I hereby declare having conducted this academic work with integrity. I confirm that I have not used plagiarism or any form of undue use of information or falsification of results along the process leading to its elaboration.

I further declare that I have fully acknowledged the Code of Ethical Conduct of the University of Minho.

ESTRATÉGIAS PARA ESTIMULAR A BIORREMEDIAÇÃO DE HIDROCARBONETOS

RESUMO

A contaminação ambiental por hidrocarbonetos constitui um grave problema, que requer o desenvolvimento de novas soluções para proteger e restaurar os ecossistemas. Técnicas de biorremediação têm sido descritas e incentivadas como abordagens eficientes e ecológicas. Neste trabalho, diferentes estratégias foram testadas para estimular a biorremediação de hidrocarbonetos, explorando vias aeróbias e anaeróbias de degradação.

Corksorb, um biosorvente à base de cortiça utilizado em derrames de petróleo, promoveu o crescimento e a atividade de bactérias aeróbias hidrocarbonoclásticas. Na presença de corksorb, a degradação de alcanos por *Rhodococcus opacus* B4 foi mais elevada, *i.e.*, 96±1 % relativamente a 88±3 % na ausência do biosorvente. Para *Alcanivorax borkumensis* SK2, o crescimento em alcanos foi 1,5 vezes maior nos ensaios com corksorb, e 72±2 % dos alcanos foram biodegradados, enquanto que na sua ausência a degradação foi de 47±2 %. Análise trascriptómica revelou um aumento da expressão genética para rRNA e tRNA, confirmando a maior atividade metabólica da *A. borkumensis* SK2 na presença de corksorb. Imagens de microscopia eletrónica de varrimento e menor expressão de genes que codificam para a formações de pili sugerem que a fixação das células ao corksorb foi determinante para o aumento da atividade. A existência de bactérias nativas degradadoras de alcanos no corksorb foi revelada. Assim, o uso de corksorb pode induzir um efeito combinado de sorção e estímulo da biodegradação, com elevado potencial na biorremediação *in situ*.

A biodegradação anaeróbia de hidrocarbonetos é geralmente um processo lento. Nanomateriais condutores foram testados para acelerar a conversão de hidrocarbonetos a metano, nomeadamente nanotubos de carbono (CNT), CNT impregnados com 2 % de ferro (CNT@Fe) ou magnetite (MG). Quando se utilizou um sedimento ribeirinho recentemente contaminado com óleos como inóculo e fonte de carbono, o teor de hidrocarbonetos não se alterou significativamente ao longo do tempo, em todas as condições testadas, apontando para a ausência de atividade degradadora de hidrocarbonetos no sedimento. No entanto, a produção cumulativa de metano foi 10,2 e 4,5 vezes maior nos ensaios com CNT@Fe e CNT, respetivamente, quando comparada com os ensaios realizados sem nanomateriais, mostrando um efeito estimulador destes na degradação da matéria orgânica natural. Numa segunda experiência, 1-hexadeceno foi convertido a metano em quantidades *quasi*-estequiométricas, mas não houve estímulo pelos nanomateriais, possivelmente devido à incapacidade dos microrganismos para receberem/transferirem eletrões para os materiais.

Globalmente, esta tese traz informação importante sobre o potencial de aplicação de um biosorvente (Corksorb) e de nanomateriais condutores na conversão aeróbia e anaeróbia de hidrocarbonetos, com vista à biorremediação *in situ* e *ex situ* de matrizes ambientais contaminadas ou resíduos oleosos. Estudos adicionais e investimento na investigação são necessários nesta área, para projetar e desenvolver novas estratégias de estímulo da biorremediação, uma vez que a recuperação de áreas poluídas por processos naturais é geralmente difícil, e o mundo tem urgência em encontrar soluções para a descontaminação de derrames de petróleo.

Palavras-chave: Hidrocarbonetos, biorremediação, corksorb, nanomateriais condutores.

ABSTRACT

Environmental contamination with hydrocarbons is a major problem that requires attention, in order to obtain novel solutions to protect and restore ecosystems. Bioremediation techniques have been described and empower as efficient and environmental friendly approaches in remediation processes. In this research, different strategies were tested for enhancing bioremediation of hydrocarbon-polluted environments, exploiting both aerobic and anaerobic degradation pathways.

Corksorb, a cork-based biosorbent used in oil spills, was shown to promote the growth and activity of hydrocarbonoclastic bacteria. In the presence of corksorb, alkane degradation by *Rhodococcus opacus* B4 was enhanced, *i.e.* 96±1 % relatively to 88±3 % in the absence of the biosorbent. For *Alcanivorax borkumensis* SK2, growth in alkanes was 1,5 times higher in the presence of corksorb, and 71.6±1.9 % of the added alkanes were degraded, while in its absence only 47.3±1,5 % was converted. Transcriptomics analysis revealed an increased expression of rRNA and tRNA coding genes, which confirms the higher metabolic activity of *A. borkumensis* SK2 in the presence of corksorb. Scanning electron microscopy images and downregulation of pili formation coding genes, which are involved in cell mobility, suggest that cell attachment on corksorb is a determinant for the improved activity. The existence of native alkane-degrading bacteria in corksorb was revealed, which may assist *in situ* bioremediation. Hence, the use of corksorb in marine oil spills may induce a combined effect of sorption and stimulated biodegradation, with high potential for accelerating *in situ* bioremediation processes.

In anoxic environments, anaerobic hydrocarbons biodegradation is generally a slow process. Conductive nanomaterials were tested to enhance hydrocarbons conversion to methane, namely carbon nanotubes (CNT), carbon nanotubes impregnated with 2 % iron (CNT@Fe) or magnetite (MG). When river sediments recently contaminated with oil were used as inoculum and also as carbon source, total petroleum hydrocarbons did not change significantly over time in all the conditions tested, thus pointing to the absence of hydrocarbon-degrading activity in the sediments. Nevertheless, cumulative methane production was 10,2 and 4,5 times higher in the assays with CNT@Fe and CNT, respectively, than in the assays without nanomaterials, showing a stimulatory effect in the degradation of the natural organic matter. In a second experiment, 1-hexadecene was converted to methane at close-to-stoichiometric amounts, but this process was not stimulated by the nanomaterials, possibly due to the inability of the microorganisms to receive/transfer electrons to the materials.

Overall, this work gave important insights on the potential application of a biosorbent (Corksorb) and conductive nanomaterials on hydrocarbons conversion under aerobic or anaerobic conditions, for *in situ* and *ex situ* bioremediation of environmental contaminated matrixes, as well as petroleum-based oily wastes. More studies and research investments are required in these study fields to design and develop new strategies for enhanced hydrocarbons bioremediation, as the recovery of polluted areas by natural processes is generally difficult, and the world urges in finding solutions for oil spills decontamination.

Keywords: Hydrocarbons, bioremediation, corksorb, conductive nanomaterials.

"Cominciate col fare il necessario, poi ciò che è possibile e all'improvviso vi sorprenderete a fare l'impossibile."

San Francesco d'Assisi

Table of Contents

List of figures

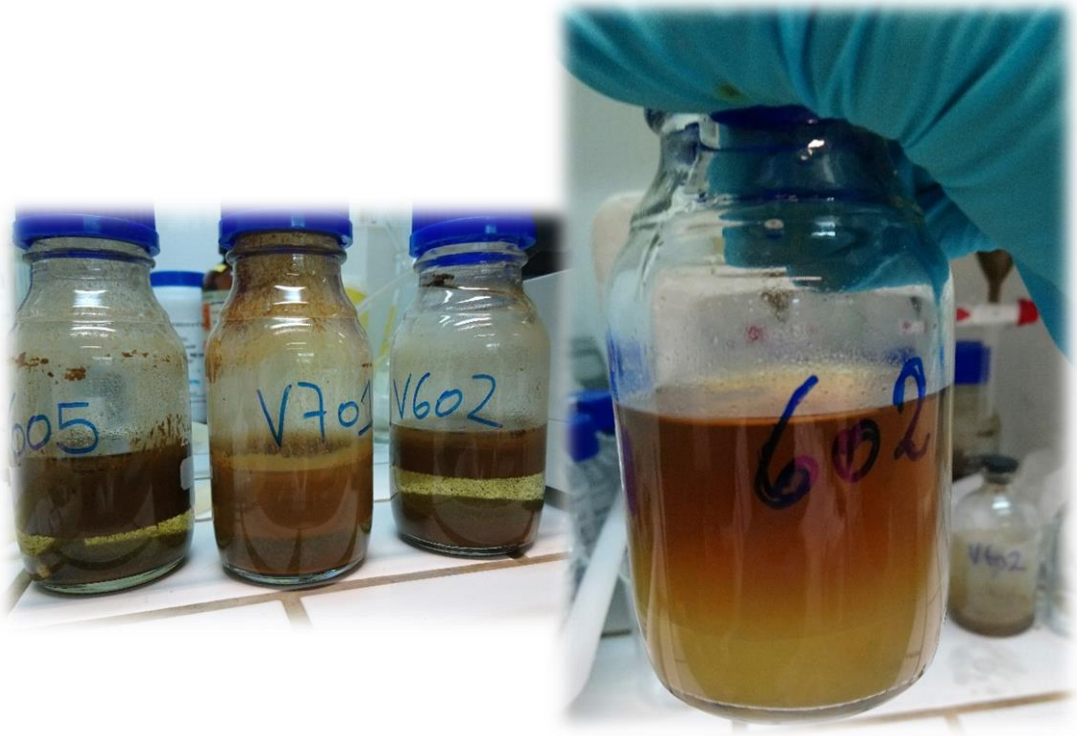
List of Tables

Abbreviations

BTEX	Benzene, toluene, ethylbenzene and xylene
C14	Tetradecane
C16	Hexadecane
C20	Eicosane
C24	Tetracosane
CNM	Conductive Nanomaterials
CNT	Carbon Nanotubes
CNT@Fe	Carbon Nanotubes impregnated with 2 % Iron
DIET	Direct Electron Interspecies
FID	Flame Ionization Detector
GC	Gas Chromatography
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
MG	Magnetite
MS	Mineral Salt
OD_{600}	Optical Density of a Sample Measured at a Wavelength of 600 nm
PBS	Phosphate Saline Solution
STP	Standard Temperatura and Pressure
TPH	Total Petroleum Hydrocarbons
TS	Total Solids
TSN	Total Suspended Nitrogen
ST	Surface Tension
VFA	Volatile Fatty Acids
VS	Volatile Solids

Chapter I

Context, Aim and Thesis Outline



1.1. General introduction

The dependence of human activities on petroleum has reached high proportions that promote a reflexion about the consequences of its application and exploitation through our living time. According to the United States Energy Information Administration (U.S. EIA [1]), oil production and consumption has been increasing worldwide, and this increase is expected to continue, with over 100 million barrels per day being extracted in 2020 from the Earth's crust [1], transported all over the world, refined and used for several kinds of Human activities such as energy, transportation, among others.

However, the recent pandemic scenario due to the COVID-19 (the disease caused by Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-COV-2)) outbreak, led governments all over the world to enforce border shutdowns, travel restrictions and quarantine obligations to population, sparking uncertainties of an imminent global economic crisis and recession, since World Health Organization declared COVID-19 outbreak, on January 30 of 2020, as a global emergency [2]. Thus, the demand [1] and consumption of petroleum, as well as the forecasts, were recently reviewed, as shown in figure 1. If in the past a cheaper oil may have acted advantageous for economies, nowadays as populations are instructed and encouraged into practicing social distancing behaviours and the working-masses have some uncertainties regarding their jobs security, oil-consumptions are directly affected [2].

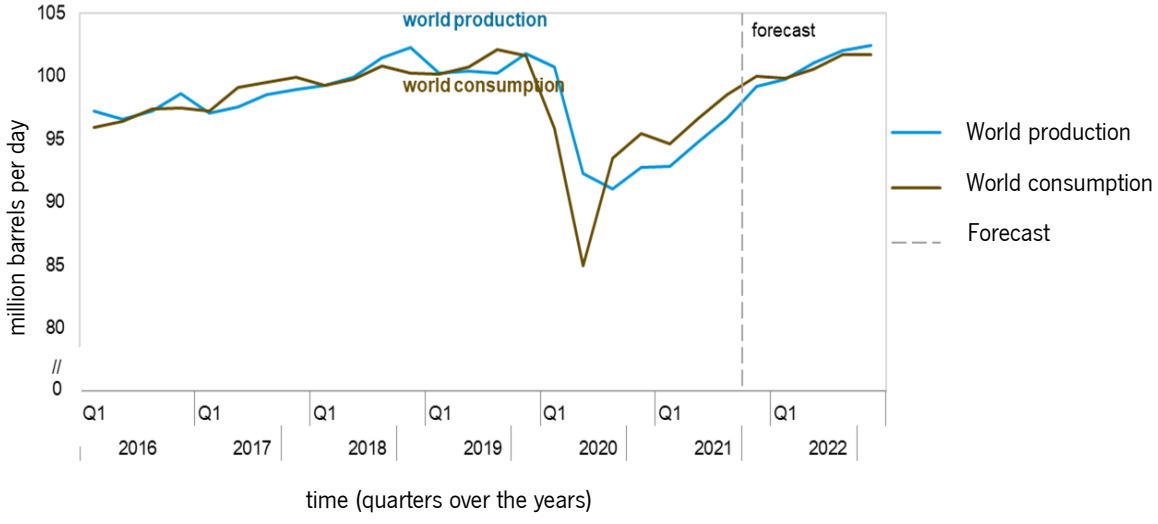


Figure 1 - World liquid fuels consumption balance, in million barrels per day, since the first quarter of 2016 to the first quarter of 2021, with forecast for the rest of 2022. Adapted from short-term energy outlook of U.S. Energy Information Administration [1].

As we can see in figure 1, it is expected that by the end of the first quarter of 2022 to petroleum production and consumption to reach pre-COVID19 values with over 100 million barrels per day.

Crude oil is mostly composed by a mixture of hydrocarbons, mainly alkanes, cycloalkanes (naphthenes) and aromatics, which are present in liquid phase both in underground geologic formations and at the surface. Crude oil refinement takes advantage of the different molecular weights, volatilities and boiling points of hydrocarbons for their separation, giving origin to intermediary and final petroleum products that later can be used for different applications.

Crude oil production, from extraction to distillation, can cause environmental contamination [3]. Exposure to crude oil-contaminated environments presents significant risks for living organisms due to the high toxicity of some components in crude oil [4]. Activities of the petroleum industry such as oil extraction, transport and refinement generate environmental impacts that cause severe damage to ecosystems, and produce significant amounts of contaminated solid and/or liquid wastes. Oil spills can be accidental or intentional discharge of petroleum hydrocarbons into the environment [5] All oil spills scenarios require attention and actions must be implemented. Over the years, accidents with oil tankers or platforms have occurred, leading to massive investment of resources to control and clean the damaged areas. Also, environmental adaptation/restoration of the contaminated ecosystems needs several years, depending on the site and biotic environment [6]. In estuarine zones, hydrocarbons are frequently detected in the sediments, due to wastewater discharges from industry, boats and ships oil leakages, among other. Studies conducted by Ribeiro and collaborators in 2013 [7] suggested that some salt march plants may promote the development of hydrocarbonoclastic bacteria in its rhizosphere, especially during flowering season, when rhizoremediation activity can be higher, conducing to natural rhizoremediation strategies in estuarine areas.

The first time in history that a society was called to present its opinion about environmental damages caused by hydrocarbon spills was after the accident with the tanker *Exxon Valdez*, which released *circa* 37000 metric tons of crude oil into the Prince William Sound, Alaska coast (United States of America), due to a rupture in the oil compartments. At that time, it was the biggest volume of oil spilled in United States of America' waters, being also a major environmental catastrophe to public nation, which leaded to the major large scale contingent-valuation application and lost passive use estimation of how much American families are willing to pay in other to prevent oil spills like *Exxon Valdez* [8]. *Circa* 4 billion U.S. dollars were use in the clean-up processes and damage repair [9]. In order to prevent the damages caused by this kind of accidents, researchers

and companies all over the world have been developing and improving remediation techniques, both *in situ* and *ex situ*, using physical, chemical and biological processes, which may allow the reduction of the negative impacts caused by oil spills in the environment, promoting a faster restoration of the contaminated area [5][10][11][12][13][14].

When compared to the physical and chemical approaches, bioremediation techniques have the advantage of facilitating the recovering of the natural environment, associated with low application costs. *In situ* bioremediation aims to enhance the natural treatment processes, which depends on the activity of several types of microorganisms with the ability to degrade crude oil, without much interference in the natural dynamics of the decontamination zone. Alternatively, bioremediation can be performed *ex situ*, in biological reactors or piles, requiring the removal and transport of the environmental contaminated matrixes to the treatment place. Hydrocarbon-degrading microbial communities may also have an important role in engineered systems, in the treatment and decontamination of wastewater or oily sludge from the petroleum industry. All these microbial processes can be done by aerobic or anaerobic microbial communities [15], with the major limitation of these processes being the time period needed for the decontamination processes [16].

The biodegradation rates, both *in situ* and *ex situ*, will depend on the amount of hydrocarbons present in the matrix and their bioavailability, although the complexity of hydrocarbons mixtures may interfere in the degradations processes, and different bacteria are needed to degrade different petroleum-compounds [16][17]. Enhancing these processes constitutes a major challenge for hydrocarbons removal from contaminated sites, establishing a valuable increment of protecting ecosystems and societies.

1.2. Aim and thesis outline

This thesis aims to explore new strategies to promote hydrocarbons biodegradation by aerobic and anaerobic microorganisms. For that, biostimulation with corksorb or nanomaterials, using hydrocarbonoclastic bacteria or complex mixed cultures, were performed, in order to promote a faster recovery of the contaminated ecosystems.

This document is organized in chapters. It begins with a general introduction of the theme (Chapter I) followed by the fundamentals of hydrocarbons bioremediation as state of the art (Chapter II). The next chapters are the experimental activities presenting three different bioremediation strategies

for three different case-scenarios of petroleum-pollution. Chapter III focus the potential of corksorb, a cork-based biosorbent, to promote the growth and hydrocarbon degradation by hydrocarbonoclastic bacteria. Chapter IV adresses the use of conductive nanomaterials to promote the bioremediation of contamined river sediments. In Chapter V, the effect of conductive ferric nanomaterials on the conversion of hydrocarbons to methane was tested. Each of these chapters was organized according to a common structure, with a general introduction, materials and methods, results and discussion and final conclusions. In Chapter VI, general conclusions and future perspectives are stated.

Chapter II

Fundamentals of Hydrocarbons (bio)Remediation

2.1. Introduction

Crude oil is a highly complex mixture of organic compounds, mainly composed by hydrocarbons (aromatics and saturated hydrocarbons), asphaltenes and resins [17]. Aliphatic hydrocarbons account for up to 50 % of the hydrocarbon fraction in crude oil [18]. Using different electron acceptors, is possible to biologically oxidize aromatic and aliphatic hydrocarbons, despite the lack of functional groups and lower reactivity of the later [17]. Hydrocarbons physical-chemical properties such as volatility and solubility in water or fat, deeply influences the behaviour of this kind of contaminants in soil, after an accidental oil spill. Further solubilization of the contaminants facilitates their transport into groundwater, where biodegradation will mainly occur. However, few studies have attempted to quantify the retention/release of this compounds in the soil matrix [19]. The low water solubility and relatively high soil-water partition coefficient (Koc) values of aliphatic and aromatic hydrocarbons suggest that mobility of petroleum hydrocarbons may be lower than previously thought. Koc values higher than 1000 represent compounds tightly bound to the organic matter in soils and are generally considered immobile; chemicals with Koc lower than 100 are considered moderately to highly mobile. Straight chain alkanes are less water soluble than monoaromatic hydrocarbons such BTEX (benzene, toluene, ethylbenzene and xylene), and present higher affinity for the organic carbon content of soil. Hence, it is likely that retention of a less soluble hydrocarbon portion of oil happens in soil, leading to oil fractioning *in situ*, however knowledge on these subjects is insufficient and entails additional progress.

Hydrocarbons can be used by either aerobic or anaerobic microorganisms as carbon and energy source [20], from gaseous to liquid or solid hydrocarbons [21]. Hydrocarbon-degrading microorganisms are generally present in most oil polluted ecosystems, where they may become the dominant microbial group [20], and hydrocarbons degradation by bacteria is reported to increase with repeated exposure to hydrocarbons [22]. Microbial communities present in oil-contaminated sites are expected to be highly complex, due to the toxic environment and the complexicity of the interactions among microorganisms and between these and the substrates. Aerobic conditions prevail in surface layers of soil, sediments and water; in deeper layers anoxia overcomes and microbial activities are driven by various alternative electron acceptors [23]. The characterization of these microbial communities is essential to understand what may be happening *in situ*, and may be applied *ex situ* for the treatment of different hydrocarbon-contaminated environmental matrixes/wastes. Microbial physiology and ecology of anaerobic hydrocarbon-

degrading communities is not deeply studied when compared to aerobic biodegradation, therefore arising the need to study better these microorganisms. Only in the late 20th century it was possible to demonstrate alkanes conversion in anoxic environments, by measurement of quantitative consumption of *n*-alkanes by sulphate-reducing bacteria [17]. Several studies have been carried on since then for hydrocarbons degradation by chlorate-, nitrate- and sulphate-reducing bacteria mainly using enrichment cultures [24]. Appling methanogenic conditions in enrichment cultures for *n*-alkanes degradations have also been studied [25][26][27][28].

2.2. Oil spills logistic processes

An oil spill, in land or water, entails several environmental issues that can reach catastrophic proportions and put in risk the subsistence of the damaged ecosystems. Therefore, the response to an oil spill must be fast and effective. The resources applied to mitigate the environmental damages are generally high, either referring to the number of human resources involved, or to the number of services, materials and machines used in this cleaning process. Usually this type of operational processes are the longest and costliest, and it is difficult to plan the waste management implied to these situations [29].

Before starting the cleaning and decontamination processes by removing all contaminated materials, it is necessary to implement worksites that will promote a better performance of the cleaning and recover of the contamination agents, allowing a better management of the land operations. This is done mainly by creating direct pathways to the contamination sites and by defining special places to recover the contamination agents, avoiding the contamination of other zones by spilled contaminant. These worksites must be equipped with all tools, reagents, human resources and machinery needed to recover, clean and transport the contaminated materials. It is extremely important to have efficient management capacity of the collected and produced residues of the recovery processes [30].

After the worksite implementation, recovery proceeds through different stages. In a first phase, it is necessary to collect the pollutant in order to limit the range and contact time of the spill. It is extremely important in this phase to collect sediments, algae, animals, contaminated water, among others, avoiding the spreading of the contaminated area. The collecting of the contaminant

materials is made manually and with the use of machinery, promoting the transportation of these materials into the worksites where they will be stored until application of the best treatment solution available. In a second phase happens the "final cleaning" where more sophisticated techniques are applied in order to promote the cleaning of the oil present in the contaminated sites, as *e.g.*, water high-pressure cleaning. In a third and last phase, it is essential to proceed to the restoration of the contaminated sites, by promoting propitious conditions that will help the contaminated sites to return to their "original state" – before the spill.

All cleaning processes must be previously defined and after rethought in every step of cleaning and treatment. The techniques applied to the treatment of the contaminated sites must always rely on local typology, nature and pollution extension, natural environment involved in the spill and the availability of resources and degree of cleaning required. In the cleaning processes, besides the residues generated by the spill itself, it is also necessary to consider the residues resulting from this cleaning process, *e.g.*, the protection clothes used by the collaborators of the cleaning services, materials used, sorbents, contention barriers, among others. All people involved in cleaning processes must be decontaminated afterwards, as well as vehicles and heavy machines. Considering the high amounts of residues produced, it is very important to plan and organize all the actions aiming its minimization and also a costs reduction [31]. The treatment applied to the residues recovered from the contamination sites and storage sites depends on several factors, related to the volume and type of hydrocarbons, always based on the legislation applicable to that type of residues and attempting to have lower application costs and less environmental impacts. It is important to reduce the volume of dangerous residues to ease the reuse, recycle or even final disposal.

Portugal has an emergency plan against hydrocarbons and other dangerous substances contamination of marine waters, ports, estuarine zones and boats navigations paths in rivers. The so-called project "Mar Limpo" aims to stablish a response mechanism to accidental oils spills, with all the logistics and institutional responsibilities required to provide a fast response in an oil spill scenario.

2.3. Remediation strategies

2.3.1. Physical processes

Physical barriers are the first approach to an oil spill in order to contain the spill, preventing its propagation. Mechanical and hand recovery, high pressure washing, sand screening, among other techniques allow the collection of different contaminated wastes from the environment, namely liquids from contaminated water, pastes and solids as contaminated sand, polluted pebbles and stones, polluted sorbents (*e.g.*, bulk, mops, pillows, sheets), polluted seaweed, polluted solid waste (including contaminated gloves and boots) and polluted fauna (all birds and mammal corpses should be counted before disposal) [32].

Application of sorbent materials for hydrocarbons removal have the advantage of allowing the transfer of the contaminants from the liquid phase to the solid (semi-solid) phase, facilitating the removal process [13]. Moreover, it can also be easily applied for hydrocarbons removal from solid surfaces, such as soil and pavements in oil refining industries or used-oils storage sites, or for the removal of oil spills from roads.

The sorbent material efficacy depends on its hydrophobic and oleophilic properties, sorption capacity (in period of time and in quantity), possibility of later recovery of the contaminant, biodegradability and reuse [13]. Biodegradability of the sorbent material may be advantageous for *ex situ* bioremediation processes, since it may function as a co-substrate, thus supporting microbial growth, and/or contribute to increase hydrocarbons' bioavailability and, consequently, their biological degradation. The choice of the sorbent material should consider, among other aspects, the place where it will be applied and its environmental conditions. For instance, if the spill occurred in marine environment or in a lake, it is necessary to choose a sorbent that preferably retains the contaminant and not the water [33]. Storage and treatment or disposal methods of the contaminated sorbent materials after its use should also be carefully planned [11].

The sorbent materials can be inorganic (mineral) or organic (synthetic or natural) [34]. Some inorganic sorbents (as perlite or fly ashes) present the lowest sorption and floatability rates (the floating capacity facilitates the removal process in aquatic environments). Thus, some organic materials are more advantageous for remediation purposes. Organic biosorbents present low application costs, availability in nature (most of them are renewable) and may have the possibility of being biodegradable after its use [34].

Recent studies performed by Amorim Cork Composites (Portugal Patent N° PT 103492 B, 2008) [35] concluded that cork granules constitute a good sorbent material, giving a new use and a new value to this material. It was shown that natural cork granules can absorb up to 5 times its weight in oil, and the regranulated cork granules (heat treated with steam at 380 °C) are capable of absorbing more than 9 times its weight in oil, in which all process is made by capillarity in only 15 seconds, with sorption capacity of 9,43 L Kg^{-1} [36]. Also, it was proved that the sorption capacity of this material is inversely proportional to its granulometry. The hydrophobicity of this material presents an increased advantage for its application in water. In most cases, the residue of this sorption process is not reused, and goes to incineration [36]. Oil recovering processes have been studied and meliorated [37][38] and represent a possible strategy for the reuse of cork granules used in oil spills. Another interesting possibility may be the anaerobic treatment of this material in bioreactors, with hydrocarbons conversion to methane and incorporation of the cork granules in the digestate, which can be used as organic fertiliser and soil conditioner.

Annunciado and collaborators in 2005 [6] studied different types of vegetable fibres (*e.g.*, mixed leaves residues, sawdust, sisal (*Agave sisalana*), coir fibre (*Cocos nucifera*), sponge-gourd (*Luffa cylindrica*) and silk floss) as sorbent for oil removal. These authors demonstrated that the materials' granulometry influences oil sorption parameters, verifying that a decrease in granulometry from more than 3,35 mm to 0,85-1,70 mm increased the sorption capacity 205 %. In the same study, it was verified that silk floss, sisal and sawdust presented better sorption capacities when compared to other vegetable materials. Also, for the silk floss the rates of water adsorption varied between 2,5 % - 6,3 %, which is important when considering the viability of using these sorbent materials in water. The silk floss can absorb 85 grams of oil per gram of sorbent, a value that can be considered good, compared to other sorbents tested, some of them are commercially available (*e.g.*, peat), according to the same study. Normally, bio-based waste materials are more cost-effective and environmental friendly, because they are abundant and their biological origin does not represent high production costs. The capacity of most of the materials for allowing the recovery of the sorbed oil and reuse of the sorbent material, represents a better solution from the economic and environmental points of view. The main problem with the use of these biological materials relies on their low hydrophobicity, resulting in low sorption capacities, needing to be modified to enhance their potential in oil spill treatment [5]. Aerogels are considered ideal sorbent materials, providing high surface area and low density, which results in high sorption capacities and floatability. Nevertheless, these materials also require some transformation to introduce some hydrophobicity

[5]. Although oil removal from the environment is preferential, economic reasons may restrict their use in spill treatment [5]. Generally, if booms and skimmers do not provide the effectiveness required in rough seas, dispersants composed of surfactants could be a better option for responding to the oil spill.

2.3.2. Chemical processes

The use of chemical compounds in oil leakage are the most common strategy applied during the treatment/recovery processes in the marine environment. Although more aggressive for the environment, their efficiency and fast response contribute for minimizing the environmental damages. In this way, the use of chemical dispersants has been presented not only as "the best" solution, but also as the most efficient solution in a brief period of time, given the emergency of the situation [39].

Dispersants are easily and quickly pulverized in the water surface where the oil was spilled, promoting its dispersion in the water column in low concentrations [40]. Dispersants are composed by surfactants dissolved in solvents, having the chemical affinity to both petroleum and water (lipophilic and hydrophilic, respectively) [39]. When dispersed through the petroleum film, the molecules align in order that the lipophilic side can attach to the petroleum and the hydrophilic can be extended in the water. Therefore, it accomplishes a reduction of the interfacial tension which allows mixing of the oil in the first 5 to 10 meters of water column, in the form of droplets of 1 to 70 μm [41]. This process might not be immediate, depending on the presence of heavier hydrocarbons that difficult the chemical agents' action.

The use of chemical dispersants makes the oil droplets available for the hydrocarbon-degrading bacteria, promoting a natural *in situ* decreasing of the contamination levels. Moreover, the damage of leakage when the oil hits the shoreline are higher than in the water column, because the natural recovery is slower. In this perspective, the dissolution in the water column is preferable, for reducing the oil impact in the environment [42][43][44]. The major drawback of this technology is the potential toxicity of the dispersant compounds [44]. Nevertheless, since the 60s of last century attempts have been made to reduce this toxicity, in order to promote a less malicious action for the marine environment [44].

Nevertheless, the use of dispersants may compromise the oil degradation by bacteria, as shown in the studies conducted by Kleindienst *et al.* [45], that assessed the effects of dispersant

applications on microbial communities, since 7 million litters of dispersants Corexit (9527A and 9500A) were applied in *Deepwater Horizon* oil spill. Their studies concluded that the use of these dispersants altered the microbial community composition, favouring the dispersant-degrading bacteria from the *Colwellia* genus. These bacteria also bloomed *in situ* on Gulf of Mexico after dispersants use. Kleindienst *et al.* [45] verified that samples without dispersant and with oil addition promoted the growth of hydrocarbonoclastic bacteria from the *Marinobacter* genus. Adding dispersants did not enhance the microbial community diversity nor hydrocarbons oxidation rates. The injection of dispersant Corexit (9527A and 9500A) occurred not only by the water surface, but also in the deep sea, nearby the leakage zone [46]. The massive use of dispersants dunk oil into deep ocean, in which the degradation process dependent on anaerobic bacteria (mostly sulphate-reducing species).

2.3.3. Biological processes (bioremediation)

Bioremediation is a natural treatment process that makes use of microorganisms to degrade hazardous substances into non toxic or less toxic ones [47]. Bioremediation can occur naturally in the environment (natural attenuation) [20][48][49], however its application in case of an oil leakage generally involves the addition of nutrients, oxygen or other alternative electron acceptors, or co-substrates to stimulate the native microorganisms' proliferation *in situ*, that is, *biostimulation* [50][51][52]. Due to the high carbon content of hydrocarbons, phosphorus and nitrogen concentrations generally limit microbial growth in oil-contaminated environments, which explains the observed lag phases of 2 to 4 weeks reported to occur in the marine environment after an oil spill [53]. Thus, additional nitrogen and phosphorous supply can improve hydrocarbon biodegradation. On the other hand, when communities of non-native microorganisms (natural or engineered) are introduced into the environment, in order to speed up the natural processes of biodegradation, we are presented with a process of *bioaugmentation* [54]. In some cases, phytoremediation was applied making use of plants and algae for bioremediation processes (sometimes coupled with biostimulation). Some studies suggest that in oil-contaminated sites the combined action of plants and the microbial communities supported in rhizosphere have improved the capability for degrading hydrocarbons [7][55][56][57][58].

Bioremediation techniques have been studied and applied *in situ* in oil spill situations, both in water and on land, to promote the decontamination and damage control [47][59][60]. The predominance

of the aerobic treatment *in situ* is related to the fact that for a long time it was though that oxygen was needed for hydrocarbons' microbial activation [61][62], as only in the late 80s of the last century it was discovered and recognised the capacity of anaerobic bacteria to metabolize hydrocarbons [62]. Microorganisms such as bacteria and fungi that utilize hydrocarbons in the presence of oxygen have been found at the beginning of the 20th century [63], and the aerobic hydrocarbons degradation processes and microbial communities that do those processes are well known and studied, while the study of anaerobic processes and microbial communities involved is still very recent, from the past 30 years. New insights are constantly been generated, under sulphate-, iron- manganese- or nitrate-reducing conditions, as well as under methanogenic conditions [64]. Anaerobic bioremediation of hydrocarbons has been limited essentially to the treatment of contaminated groundwater. It is in the subsurface areas that oil biodegradation under anoxic conditions occurs primarily, mediated by sulphate-reducing bacteria [65] or other anaerobes using a variety of electrons acceptors.

The use of bioremediation techniques for oil removal from contaminated sites present numerous economic advantages, *e.g.*, the lower operational costs, and also it is considered less "invasive", that is, which brings less perturbations to the sites, than other physical-chemical methods [66]. When compared to chemical and physical approaches, biological approaches have been proved to be more cost efficient [17]. Moreover, bioremediation techniques can also be performed *ex situ*, by collecting the contaminated material from the natural site and treating it elsewhere, as in biopiles or bioreactors [67]. Also, adding a bulking agent in contaminated soil may enhance the biodegradation of hydrocarbons, since it improves the contact between oil and bacteria in the tillage [68]. Bioreactors have been known as the most effective *ex situ* soil remediation processes, as long as adequate degradation conditions can be achieved promptly [59].

2.3.4. Hydrocarbons degradation by aerobic microorganisms

Since the isolation of the first hydrocarbon-degrading bacterium in 1913 by Söhngen [69], over 79 genera of bacteria capable of using hydrocarbons as sole energy source have been identified [70] [71]. Kimes and collaborators [69] performed a compilation of studies addressing microbial communities' changes over time following the *Deepwater Horizon* oil spill, in the Gulf of Mexico ranging from coastal sediments to the water column and deep sea sediments. Microorganisms like *Oceanospirillales*, *Cycloclasticus* and *Colwellia* genera, that were not known for their capabilities of

degrading hydrocarbons, where found with significant abundance and proliferation. Mikolasch and collaborators [72] isolated microorganisms from oil contaminated soils in Kazakhstan and identified species of *Gordonia* and *Rhodococcus* as very effective in degrading crude oil components.

Marchand *et al*. [73] examined the potential for petroleum hydrocarbons biodegradation by 95 bacterial and 160 fungal strains isolated from a former petrochemical plant, where they observed that fungal isolates belonged to the group *Sordariomycetes*, and bacterial isolates belonging to the groups *Actinobacteria*, *Betaproteobacteria* and *Gammaproteobacteria* showed high potential for polyaromatic hydrocarbons degradation. Three of the strains analysed, *Rhodococcus* sp., *Trichoderma tomentosum*, and *Fusarium oxysporum*, showed excellent results by degrading all four polyaromatic hydrocarbons presented in the assays (anthracine, phenanthrine, fluorene and pyrene, with more than 10 %, 13 %, 8 %, and 17 %, respectively, degraded by these three strains). Also concluded that the concentration of polyaromatic hydrocarbons in the initial soil samples, used for microbial isolation, as well as the type of culture medium, did not had a significant impact on polyaromatic hydrocarbons degradation ability of the isolates. On the other hand, phylogenetic affiliation played a significant role on the ability of the isolates on degrading these contaminants.

In bioremediation processes, this metabolic potential of microorganisms is explored for the conversion or removal of hazardous components from the environment [74]. Under aerobic condition, the degradation of crude oil is relatively rapid and complete. In general, alkenes (hydrocarbons containing double bonds) and short-chain alkanes (hydrocarbons containing only single bonds) are the most easily degraded, followed by branched alkanes (alkanes with side chains) and then aromatics (hydrocarbons with a stable ring structure) [75]. However, degradation rates vary based on environmental parameters and decrease with hydrocarbon complexity, as oil composition depends on the source of the petroleum and age of the spill. As referred above, the primary rate-limiting factor in aerobic biodegradation is the delivery of oxygen. Oxygen availability is dependent on the ability of oxygen to move or diffuse through the environmental site as well as on the uptake rate by microorganisms.

The chemical inertness of aliphatic hydrocarbons poses an energetic and mechanistic challenge for microbial metabolism. This is particularly true for the activation and eventual cleavage of the apolar C-H bond, in which high energy barriers must be overcome. In aerobic environments, the activation of C-H bond is manly accomplished by oxygen molecules. However, in anoxic environments other mechanisms are probably involved in this activation step. The only exceptions

are the recently discovered 'intra-aerobic' anaerobes which apparently derive oxygen species from using chlorate or nitrite to employ monooxygenases for attacking the hydrocarbon bonds [76].

Studies conducted by Warr *et al*. [77] reported that hydrocarbons degradation in marine environments could be enhanced by adding mineral sorbents like clay powder, degrading not only the saturated hydrocarbons as alkanes, but also the heavier ones, as well as resins and asphaltenes. Alabresm *et al*. [78] studied the use of polyvinylpyrrolidone (PVP) coated magnetite nanoparticles and oil degrading bacteria (*Halomonas* sp., *Vibrio gazogenes* and *Marinobacter hydrocarbonoclasticus*) for enhanced oil removal at laboratory scale and concluded that the combined use of magnetite nanoparticles and oil degrading bacteria worked effectively to remove approximately 100 % of the added oil within 48 hours or less. In the presence of high oil concentrations (375 mg L^{-1}), magnetite nanoparticles could remove approximately 70 % of lower-chain alkanes (from C9 to C22) and 65 % of higher-chain (from C23 to C26) after only 1 hour, with no increment of oil removal being observed thereafter, probably due to nanoparticles saturation absorption capacities. In the assays amended with oil degrading bacteria, 80 % to 90 % removal was attained after 24 to 48 hours. Sorbent material lignite was added to soil and promoted microorganisms growth, as well as their biodegradation ability of pentachlorophenol [79]. Urea-formaldehyde (commercial sorbent) was tested in an oil spill [80] and the sorbed hydrocarbons appeared to be as biodegradable as the ones which were not sorbed, turning possible to clean up a hydrocarbon spill with the polymer and then to biodegrade the hydrocarbons.

A study performed by Pontes and collaborators [81] focused on bioremediation efficiency of buried oil in a estuarine zone, where biostimulation, bioaugmentation and natural attenuation assays were performed, by using soil columns to test hydrocarbons degradation. The bioaugmentation treatment used as inoculum an indigenous oil-degrading microbial consortium, previously stimulated in the laboratory by supplementation with an oleophilic fertilizer. The results showed that this bioaugmentation supplemented with nutritive conditions yielded better results when compared to the natural attenuation (*i.e.*, at the same period, degradation in the natural attenuation assays was approximately half of the total degraded in the bioaugmentation assays). In 60 days, the degradation of hydrocarbons reached 80 % in the bioaugmentation assays, representing a cost-effective methodology for bioremediation of underground sediments, recovering the polluted area.

2.3.5. Hydrocarbons degradation by anaerobic microorganisms

Anaerobic degradation of petroleum hydrocarbon under different redox conditions (nitrate reduction [82], iron(III) reduction [83], sulfate reduction [84] and methanogenenesis [85]) is an exergonic process which is theoretically suitable for microbial energy conservation [86]. Several anaerobic bacteria capable of hydrocarbons degradation coupled to nitrate, iron(III) or sulphate reduction have been isolated and characterized [18]. Denitrifying species can degrade a wide range of alkylbenzenes, such as toluene, *m*-xylene or ethylbenzene. Denitrifying bacteria grow relatively fast on alkylbenzene, *e.g.*, the maximum growth rate on toluene can reach 0,12 h^{-1} [87], because toluene is more water soluble and less stable (due to the aromatic structure) when compared to saturated hydrocarbons [88]. Iron-reducing species (*e.g.*, *Desulfitobacterium* and *Geobacter* species) are reported to degrade aromatic hydrocarbons [89]. Research shows that some monoaromatic hydrocarbons like toluene can be degraded rapidly under iron-reducing conditions [51][58]. However, pure bacterial cultures that can degrade saturated hydrocarbons, like alkanes, under iron-reducing conditions have not been isolated thus far [90] [91].

Since some LCFA (*e.g.*, palmitate) and hydrocarbons have similar compostions, it is interesting to observe that LCFA-rich wastewaters can be biologically converted to biogas, under anaerobic conditions [92][93][94]. However, in the presence of sulphate, methanogenesis may be inhibit, resulting in the reduction of biogas production from LCFA valorisation. Oleate and palmitate enrichments of anaerobic sludge can lead to the development of two different syntrophic communities where in the presence of sulphate, the LCFA conversion did not occurred. On the other hand sulphate-reduction of hydrogen and acetate consumers were identified, competing in methanogenesis process [95]. Under sulphate-reducing conditions, several hydrocarbons (*e.g.*, polycyclic aromatic hydrocarbons) can be biodegraded [96]. In fact, it was suggested that it may be possible to use sulphate-reduction rather than aerobic respiration as a treatment strategy for hydrocarbon-contaminated dredged sediments [96]. Other study revealed the taxonomy and metabolic potential of sulphate-reducing bacteria linked to anaerobic hexadecane and phenanthrene degradation [97].

Under anaerobic conditions, microbial communities can promote the reduction of ferric to ferrous iron [83]. This process may have its influence in the exchange of nutrients and trace metals, both in sediments and water. Ferric iron concentration in sediments normally exceeds the other electron

acceptors like sulphate, oxygen, or nitrate. Hence, ferric iron has great potential to be used as electron acceptor in the mineralization of organic compounds [98]. Previous studies suggest that iron(III)-reducing bacteria are cable of out-competing sulphate-reducing and methanogenic communities for organic matter when iron(III) is available [99]. Previous studies have indicated an incredible metabolic versatility of iron(III)-reducing bacteria, which are able to transform various organic pollutants (*e.g.*, benzene, toluene, phenol and chlorinated compounds), heavy metal or radionuclide contaminants [90][99]–[102]. Instead of serving as direct electron acceptor, (semi)conductive iron oxide minerals can also serve as electron conduits in natural soil ecosystems, that transfer electrons to distant electron acceptors [98]. Therefore, it is possible to establish conductive networks through microbial interactions with iron oxide minerals in the sediments or soil [103], which may constitute a relevant adjuvant in microbial hydrocarbon oxidation processes.
Knowledge about the microorganisms directly involved in hydrocarbons biodegradation to methane is still scarce. Up to know, bacteria from the *Syntrophaceae* family were reported as having an important role in alkanes biodegradation to methane [104], as well as the direct involvement of *Smithella* sp. [105]. The degradation of 1-hexadecene by a non-adapted methanogenic community was recently shown and was considerably enhanced by the addition of extra electron donors [106]. Syntrophic bacteria closely related to *Syntrophus* and *Smithella*, as well as hydrogenotrophic methanogens, were enriched in these 1-hexadecene-degrading cultures, showing the syntrophic nature of this conversion [106].
In fact, hydrocarbons biodegradation to methane is through to be a syntrophic process, performed by more than one group of microorganisms that interact sequentially in mixed cultures [18]. A scheme of the anaerobic degradation pathway of alkanes is represented in figure 2, referencing some of the possible microorganism involved in the processes.
The first step in alkanes biodegradation is activation of the molecule, which is described as a slow rate-limiting process. After, classical fermentation, acetogenesis and methanogenesis take place, performed by different groups of bacteria and archaea. Alkanes biodegradation under methanogenic conditions has been reported as a syntrophic process, where syntrophic bacteria convert these compounds to hydrogen and acetate, which are further used by methanogens [107]. The complete degradation is only thermodynamically feasible when the hydrogenotrophic partner is present, decreasing the hydrogen partial pressure.
The depress of oil reservoirs into certain levels may have its cause in anaerobic microbial activity. The so-called “biogenic methane”, which usually is associated with biodegraded petroleum wells,

is the outcome of microbial decomposition of the alkanes fraction in oil [27]. If in one hand the degradation of oil alkanes by methanogenic microorganisms may cause several problems in the extraction of high-value crude oil, on the other hand it has potential for valorising the extinguished oil reservoirs, through the so called MEOR – microbially enhanced oil recovery [108]. This strategy makes use of microbial communities capability in degrading heavy crude oil remaining in the capillary pores of rocks formations in oil reservoirs, to reduce its viscosity, being considered very effective, cost-efficient and eco-friendly [109].

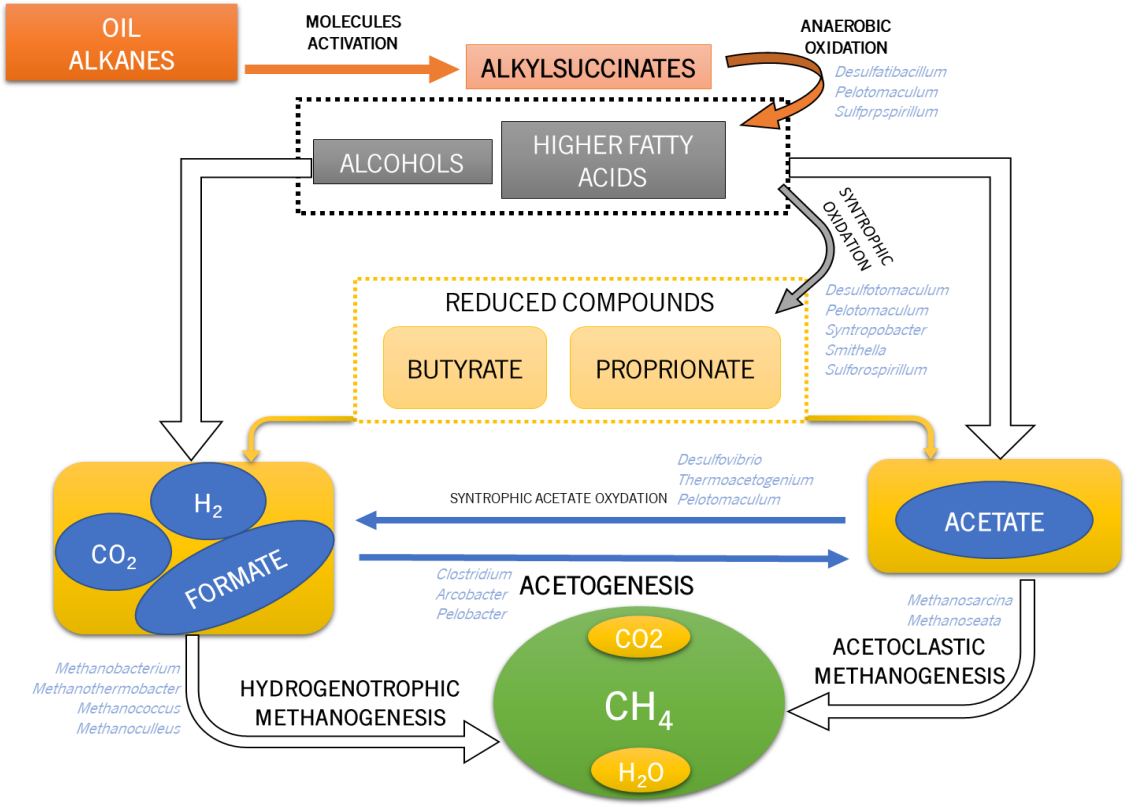


Figure 2 - Presumptive methanogenic degradation of oil, using alkanes as an example, with reference to the microorganisms possibly involved in hydrocarbons conversion to methane (adapted from [15][97]).

CHAPTER III

Corksorb Enhances Alkane Degradation by Hydrocarbonoclastic Bacteria

Martins VR, Freitas CJB, Castro AR, Silva RM, Gudiña EJ, Sequeira JC, Salvador AF, Pereira MA, Cavaleiro AJ (2021) Corksorb Enhances Alkane Degradation by Hydrocarbonoclastic Bacteria, Frontiers in Microbiology, 12(618270). doi: https://doi.org/10.3389/fmicb.2021.618270

Abstract

Biosorbent materials are effective in the removal of spilled oil from water, but their effect on hydrocarbonoclastic bacteria is not known. Here, we show that corksorb, a cork-based biosorbent, enhances growth and alkane degradation by *Rhodococcus opacus* B4 and *Alcanivorax borkumensis* SK2. *R. opacus* B4 and *A. borkumensis* SK2 degraded 96 ± 1 % and 72 ± 2 %, respectively, of a mixture of *n*-alkanes (2 g L^{-1}) in the presence of corksorb. These values represent an increase of 6 and 24 %, respectively, relative to the assays without corksorb. The biosorbent also increased the growth of *A. borkumensis* SK2 by 51 %. However, no significant changes were detected in the expression of genes involved in alkane uptake and degradation in the presence of corksorb relative to the control without the biosorbent. Nevertheless, transcriptomics analysis revealed an increased expression of rRNA and tRNA coding genes, which confirms the higher metabolic activity of *A. borkumensis* SK2 in the presence of corksorb. The effect of corksorb is not related to the release of soluble stimulating compounds, but rather to the presence of the biosorbent, which was shown to be essential. Indeed, scanning electron microscopy images and downregulation of pili formation coding genes, which are involved in cell mobility, suggest that cell attachment on corksorb is a determinant for the improved activity. Furthermore, the existence of native alkane-degrading bacteria in corksorb was revealed, which may assist *in situ* bioremediation. Hence, the use of corksorb in marine oil spills may induce a combined effect of sorption and stimulated biodegradation, with high potential for enhancing in situ bioremediation processes.

Keywords: corksorb, alkanes, *Rhodococcus opacus*, *Alcanivorax borkumensis*, growth, bioremediation, biosorbent, comparative transcriptomics

3.1. Introduction

Global oil demand averaged 108 barrels per day in 2019 [1] entailing intensive exploitation of petroleum resources and significant environmental risks. Accidental oil spills occur frequently in marine environments, causing severe damage to ecosystems and human population [110] [111] [112] Crude oil is mainly composed of hydrocarbons, in particular alkanes, that account for more than 50 % of the hydrocarbon fraction. These compounds are relatively inert, originating severe ecological problems upon their release to the environment [113] [114].

In the oceans, although the average number of large- (>700 t) and medium-sized (7–700 t) oil spills caused by tankers has been progressively decreasing [115], major accidents still occur at irregular periods [110]. Several other sources also contribute for marine oil contamination, namely, drilling wastes and produced waters resulting from both onshore and offshore activities, oil well blowouts, releases from subsea equipment and pipelines, damages on oil platforms, and operational discharges in marine ports and harbours [110] [112] [116] [117].

Considering that most of the conventional remediation technologies are environmentally unfriendly [118] and that bioremediation may not be fast enough to prevent the severe damages caused by oil spills, immediate containment and physical removal of the oil guarantee a sufficiently fast and efficient response without significant environmental disturbances. Sorption is one of the most effective remediation techniques, and sorbent materials have demonstrated good results in removing hydrocarbons from contaminated sites [5]. One interesting approach is the use of biosorbents, which are materials from natural origin that are renewable and generally biodegradable. The use of agricultural or industrial wastes as biosorbents represents an effective, ecofriendly, and low-cost alternative that complies with the circular economy approach [5] [34].

In particular, cork-based sorbents have been used for the removal of different pollutants, including hydrocarbons [119] [120], and a commercial product – Corksorb – is available for oil spill remediation [121]. Corksorb presents an absorption capacity of up to 10 times its weight in oil [119] and is obtained through thermal treatment of regranulated cork particles, which are byproducts of cork stopper production [120]. Due to its particular physical and chemical properties, cork materials present good sorbent capacity and low water permeability [120].

In situ or *ex situ* bioremediation of oil-contaminated environments/matrixes can be accomplished by hydrocarbonoclastic bacteria, which utilize the oil components as carbon and energy source for growth [122]. Some of the most representative members of this group belong to the genera

Alcanivorax and *Rhodococcus*. Members of both genera are able to produce surfactants, which is a relevant feature for hydrocarbon consumption [123] [124]. *Alcanivorax* is considered the dominant Gram-negative genus degrading aliphatic hydrocarbons in saline environments. These bacteria have been applied in the bioremediation of oil-spilled marine ecosystems [51] [125] [126] due to their capacity of using high hydrocarbon concentrations (specially alkanes up to C32) as sole carbon source [127] [128] [129]. The genus *Rhodococcus* is one of the most versatile concerning hydrocarbon degradation, being able to metabolize different types of hydrocarbons – from alkanes with C6–C36 [130] [131] to complex polycyclic aromatic compounds present in gasoline, diesel, engine, and crude oil [132] [133] [134] [135]. They can be found in different natural environments, including marine sediments and water [136] [137] [138] [139]. Due to its remarkable catabolic versatility, several works were performed using *Rhodococcus* strains in oil bioremediation strategies [140] [141] [142].

In this work, we hypothesize that oil sorption by biosorbents can be combined with oil biodegradation by hydrocarbonoclastic bacteria, representing a novel approach for (bio)remediation of oil spills. Moreover, we hypothesize that the presence of the biosorbent may stimulate the activity of these bacteria, *e.g.*, by promoting the contact between bacteria and hydrocarbons, acting as a support for bacterial growth or by containing stimulatory compounds (*e.g.*, nutrients, cofactors) in its chemical composition, which could contribute to enhance oil spill bioremediation. The potential of corksorb to enhance growth and hydrocarbon degradation by hydrocarbonoclastic bacteria was investigated in batch assays. A mixture of *n*-alkanes, the main components of crude oil, was used as carbon and energy source. Two different bacterial strains were tested: *R. opacus* B4, a Gram-positive bacterium able to degrade both aromatic and aliphatic hydrocarbons [143] [144], and *A. borkumensis* SK2, a marine Gram-negative bacterium isolated from seawater/sediment samples that uses almost exclusively alkanes as carbon and energy source [127] [145]. Transcriptomics analysis of *A. borkumensis* SK2 growing on alkanes in the presence and absence of corksorb was also performed. Additionally, the presence of native bacteria in corksorb, capable of growing with alkanes, was investigated.

3.2. Materials and methods

3.2.1. Biosorbent

Corksorb granules with particle diameters between 0,3-1 mm were provided by Corticeira Amorim, S.G.P.S. (Portugal). The corksorb granules may contain apolar hexane-extractable compounds which can potentially cause interferences in the identification and quantification of alkanes by gas chromatography (GC). To evaluate these potential interferences, triplicate assays were prepared in 250 mL Erlenmeyer flasks, each one containing corksorb (25 mg) and distilled water (50 mL). After incubation at 30 °C in a rotatory shaker (150 rpm) for 48 h, hydrocarbons extraction and quantification were performed. The presence of apolar, hexane-extractable compounds in corksorb was confirmed (figure 3a), but the comparison of this chromatogram with the one from the alkanes' mixture used in the assays (described below – see section 3.2.4.) (figure 3b), allowed to rule out the occurrence of important interferences of these compounds in the experiments performed in this work.

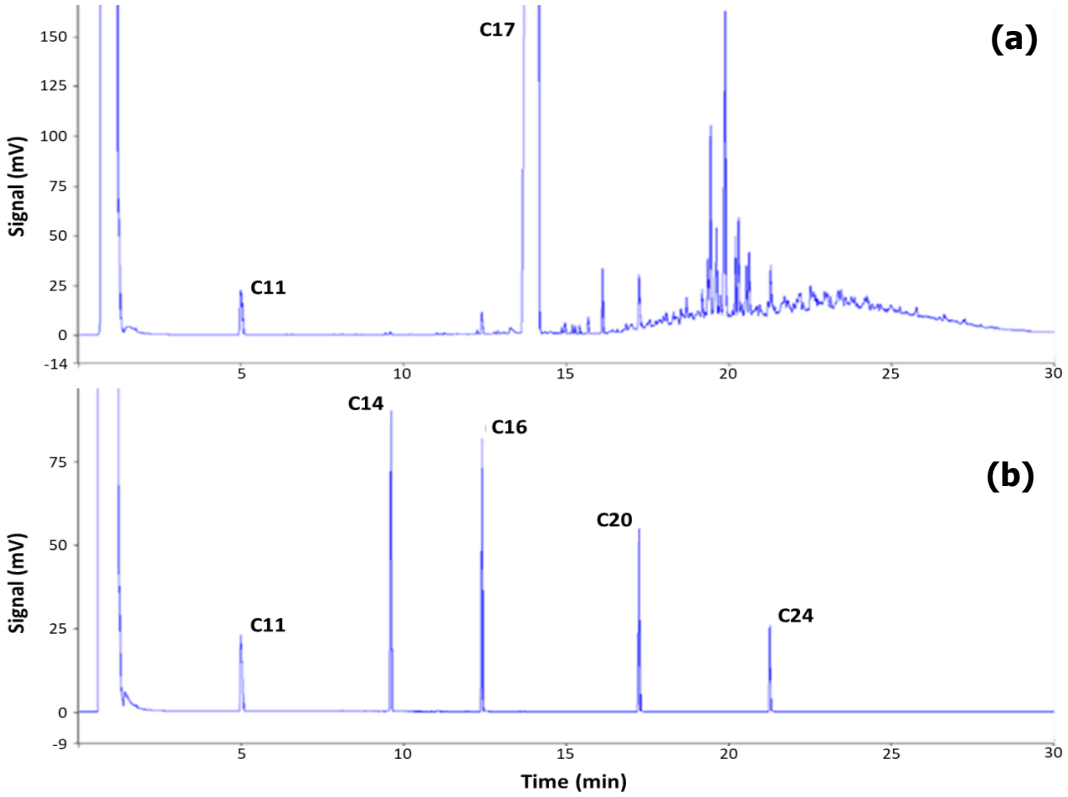


Figure 3 - Chromatogram of the compounds extracted from corksorb, 50 times concentrated relatively to the assays (a), and chromatogram of the alkanes mixture used in the assays at 100 mg L^{-1} individual concentrations, *i.e.* 5 times diluted relatively to the assays (b). Undecane (C11) was used as internal standard, and its retention time is 5 min.

3.2.2. Bacterial strains and growth conditions

Rhodococcus opacus B4 (NBRC 108011) and *Alcanivorax borkumensis* SK2 (DSM 11573^T) were purchased from the National Institute of Technology and Evaluation, Biological Resource Center, Japan (NBRC) and from the Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ, Braunschweig, Germany), respectively. Growth and maintenance of the bacterial cultures were performed using 802 medium with agar (1,5 %) for *R. opacus* B4 [144] and an artificial seawater mineral salts medium (ONR7a) with agar [104] and sodium pyruvate (10 g L^{-1}) as carbon source for *A. borkumensis* SK2. Cultures were incubated at 30 °C in a rotary shaker (120 rpm).

3.2.3. Preparation of seed cultures

R. opacus B4 was grown in solid medium for 4 days, after which a single colony was transferred to 50 mL mineral salts (MS) medium [165] supplemented with glucose (40 g L^{-1}). A single colony of *A. borkumensis* SK2, grown in solid medium for 6 days, was transferred to 50 mL of ONR7a medium with 10 g L^{-1} sodium pyruvate. Seed cultures were grown in 250 mL Erlenmeyer flasks, at 30 °C with agitation (120 rpm) until reaching the middle of the exponential growth phase, *i.e.* 48 h for *R. opacus* B4 and 45 h for *A. borkumensis* SK2 (figure 4). Growth of the seed cultures was evaluated by optical density at 600 nm (OD_{600}). Cells were harvested and washed two times with sterile phosphate buffered saline solution (PBS) by centrifugation at 13000 min^{-1} during 10 minutes at 20 °C. The pelleted cells of *R. opacus* B4 and *A. borkumensis* SK2 were then suspended in fresh MS and ONR7a medium, respectively, and used as inoculum in the assays.

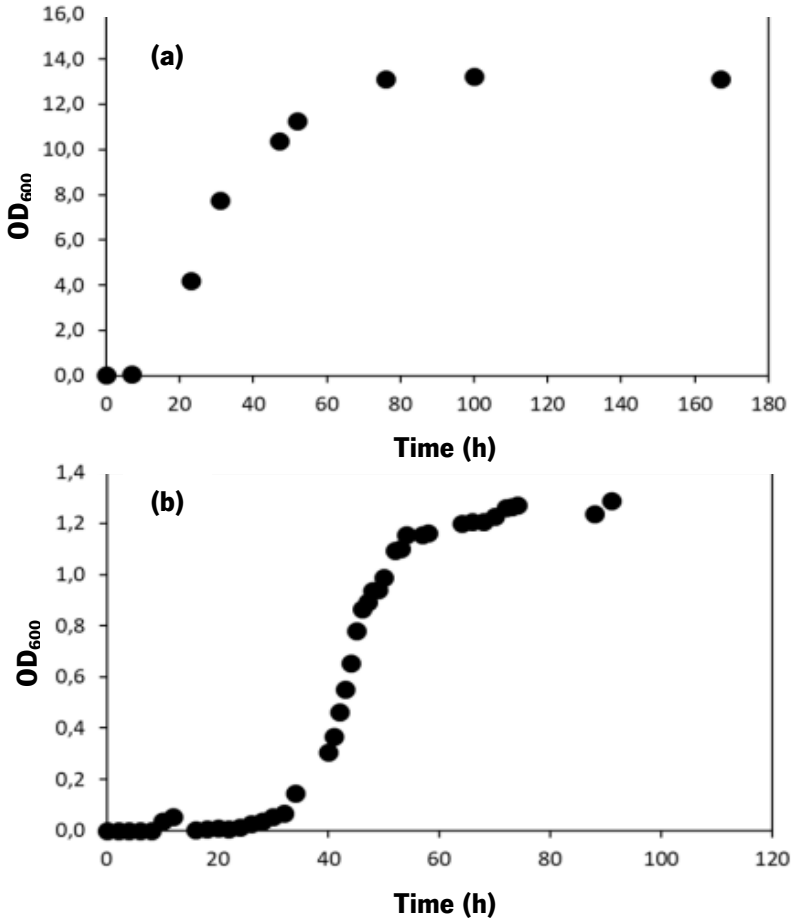


Figure 4 - Growth curves of *R. opacus* B4 in MS medium with glucose (40 g L^{-1}) (a), and *A. borkumensis* SK2 in ONR7a medium with pyruvate (10 g L^{-1}) (b).

3.2.4. Experiment 1 - Effect of corksorb on growth and alkane degradation by hydrocarbonoclastic bacteria

A mixture of *n*-alkanes was prepared in hexane, containing tetradecane (C14), hexadecane (C16), eicosane (C20) and tetracosane (C24), at individual concentrations of 50 g L^{-1}. The assays were performed in sterile 250 mL Erlenmeyer flasks, containing corksorb (25 mg), mixture of alkanes at a final total concentration of 2 g L^{-1} (corresponding to 500 mg L^{-1} of each individual alkane), sterile culture medium (50 mL) and seed culture (*R. opacus* B4 or *A. borkumensis* SK2) at an initial OD_{600} of 0,2. The mixture of alkanes was added with a glass syringe and by the flame in sterile conditions. Medium MS or ONR7a were used for *R. opacus* B4 or *A. borkumensis* SK2, respectively. These assays were designated Ab-Alk-CrkS and Ro-Alk-CrkS, as shown in table 1 and figure 5. Assays (i) without corksorb (Ab/Ro-Alk), (ii) without the mixture of alkanes (Ab/Ro-CrkS), and (iii) without inoculum (Alk-CrkS) were also prepared.

Table 1 - Summary of the experimental conditions tested. *Ro*, *Rhodococcus opacus* B4; *Ab*, *Alcanivorax borkumensis* SK2; *Alk*, mixture of alkanes; *CrkS*, corksorb. * The medium was previously in contact with corksorb for 150 h, after which it was transferred (without corksorb) to new sterile flasks where the assays were performed.

	Assay	Inoculum	Alkanes	Corksorb
Experiment 1	Ro-Alk-CrkS	✓	✓	✓
	Ro-Alk	✓	✓	×
	Ro-CrkS	✓	×	✓
	Ab-Alk-CrkS	✓	✓	✓
	Ab-Alk	✓	✓	×
	Ab-CrkS	✓	×	✓
	Alk-CrkS	×	✓	✓
Experiment 2	Ab-Alk-CrkS	✓	✓	✓
	Ab-Alk-afterCrkS	✓	✓	×(*)

All cultures were incubated at 30 °C in a rotary shaker (120 rpm) and samples were collected over time, after reaching the exponential growth phase (first sampling points at 283 h and 150 h for *R. opacus* B4 or *A. borkumensis* SK2, respectively). These values were defined based on preliminary tests carried out to evaluate the growth of the two hydrocarbonoclastic bacteria with the alkane mixture under study. For each sampling point, the total content of three replicate flasks was acidified at pH 2,0 with HCl (5 mol L^{-1}) and preserved at 4 °C until hydrocarbons analysis. The total content of three other Erlenmeyer flasks was used for bacterial growth quantification, assessed by measuring total suspended nitrogen (TSN). At the end of the experiment, samples of well-homogenized biomass were also collected from the assays containing corksorb and *A. borkumensis* SK2 (*i.e.*, Ab-Alk-CrkS and Ab-CrkS), centrifuged at 10000 *g* for 10 minutes at 4 °C, washed with PBS and stored at -20 °C, for further bacterial community analysis.

3.2.5. Experiment 2 - Assessment of potential stimulation of *A. borkumensis* SK2 by soluble compounds from corksorb

An experiment was designed to evaluate if the effect of corksorb on the growth of *A. borkumensis* SK2 was related with the release of soluble compounds to the medium. Erlenmeyer flasks containing only corksorb (25 mg) and medium ONR7a (50 mL) were incubated at room temperature for 150 h, without agitation, after which the medium was transferred with sterilized syringes to a new set of sterilized Erlenmeyer flasks. These were designated Ab-Alk-afterCrkS (table 1, figure 5), and received also *A. borkumensis* SK2 seed culture and the mixture of *n*-alkanes, following the procedures described in section 3.2.4. Control assays, which contained fresh medium ONR7a, corksorb, alkanes and *A. borkumensis* SK2 (Ab-Alk-CrkS, table 1, figure 5) were also prepared as described in section 3.2.4. TSN was measured at the end of the assay using the total content of triplicate flasks.

3.2.6. Effect of corksorb on differential gene expression and on surface tension of *A. borkumensis* SK2 cultures

Erlenmeyer flasks of 1 L (total volume), containing 250 mL ONR7a medium and 125 mg of corksorb, were inoculated with *A. borkumensis* SK2. Control assays were performed without corksorb in order to compare the gene expression and surface tension (ST) when *A. borkumensis* SK2 was growing with and without corksorb. The alkane mixture and all the other experimental details were similar to the ones described in section 3.2.4. Samples from each culture (5 mL) were collected at different time points to evaluate biosurfactant production through ST measurement. For transcriptomics analysis, cell cultures (40 mL) were harvested at the exponential growth phase (167 h) and centrifuged (10000 rpm, 10 min, 4 °C), suspended in RNA later (Thermo Fisher Scientific) and frozen at −20 °C until RNA extraction. Three independent cultures were sampled for each condition. At the same time point (t = 167 h), scanning electron microscopy (SEM) analysis was also performed.

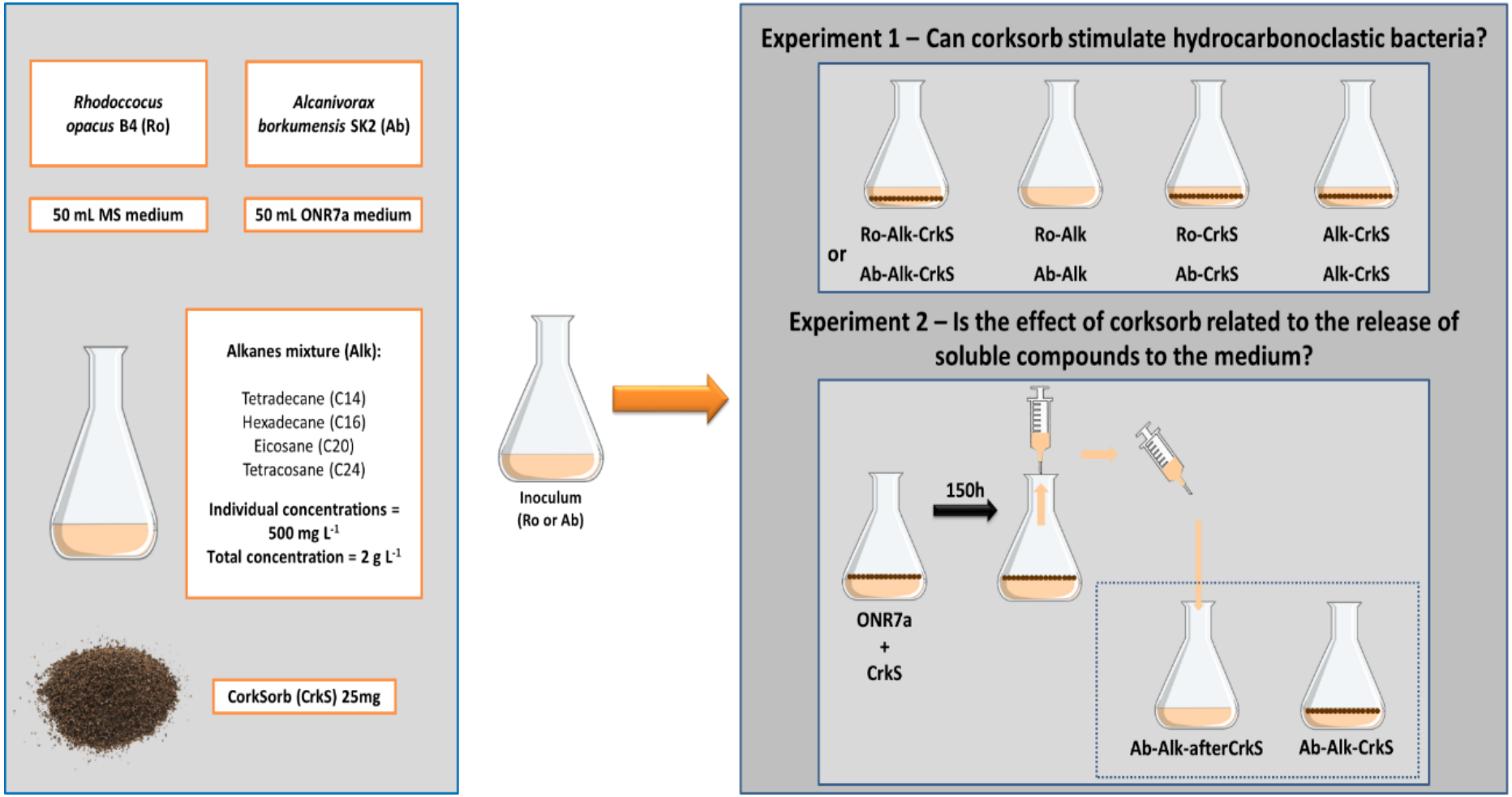


Figure 5 - Scheme of the experimental procedure applied in experiments 1 and 2.

3.2.7. Enrichment cultures

To evaluate the presence of native bacteria in corksorb and their capacity to grow using alkanes, enrichment cultures were developed. Corksorb (25 mg) was incubated in a sterile Erlenmeyer flask (250 mL volume) containing 50 mL of ONR7a medium, at room temperature without agitation for 150 h. After this period, sterile loops were used to inoculate ONR7a agar (1,5 %) medium with sodium pyruvate (10 g L^{-1}). Incubation was performed at 30 °C, 120 rpm. Growth was visually checked and two sequential transfers were made to Erlenmeyer flasks with ONR7a medium (50 mL), using the mixture of *n*-alkanes described in section 3.2.4 as carbon source at 1 g L^{-1} individual concentrations (total alkane concentration of 4 g L^{-1}). The obtained enriched culture was designated CA (2) and characterized in terms of taxonomic composition.

3.2.8. Analytical methods

OD measurements were performed with a spectrophotometer U-1500 (Hitachi, Tokyo, Japan). TSN was quantified using standard cuvette tests (Hach-Lange GmbH, Dusseldorf, Germany) and a DR 2800 spectrophotometer, after washing the samples three times with ultra-pure water by centrifugation at 10000 min^{-1} (15 minutes, 20 °C). For alkane analysis, samples were sequentially extracted three times with hexane as organic solvent using separatory funnels, as described by Castro *et al.* [144]. Heptadecane (C17) was added to the samples at a final concentration of 200 mg L^{-1}, from a stock solution prepared in hexane, as surrogate. The extracts were cleaned with Sep-Pak Florisil® cartridges (Waters, Milford, MA) and concentrated to a final volume of 1 mL in hexane, using TurboVap® LV (Biotage, Uppsala, Sweden). Alkane quantification was made by GC with a flame ionization detector (GC-MS Varian® 4000, Agilent, Santa Clara, CA) and a VF1-ms column (Agilent, 30 m x 0.025 mm, Santa Clara, CA). Helium was used as carrier gas at 1 mL min^{-1}. Detector and injector temperatures were 360 °C and 285 °C, respectively. Column temperature was maintained at 60 °C for 1 minute and then increased up to 290 °C at a temperature ramp of 8 °C min^{-1}. Undecane (C11) was used as GC internal standard, at a concentration of 380 mg L^{-1}, in hexane. ST was determined by the Ring method [146], at room temperature (25 °C), using a KRÜSS K6 Tensiometer (KRÜSS GmbH, Hamburg, Germany). All the

measurements were performed in triplicate. SEM analysis was performed using an SEM FEI Nova 200 (FEG/SEM) equipment (FEI, Hillsboro, OR, United States) at SEMAT (University of Minho, Guimarães, Portugal). Samples for SEM images were prepared and processed as described by Salvador *et al.* [147].

3.2.9. DNA extraction and amplification for bacterial community analysis

Bacterial community composition was assessed in biomass samples collected from the assays containing corksorb and *A. borkumensis* SK2 (see section 3.2.4). Total genomic DNA was extracted using a FastDNA SPIN Kit for Soil (MP Biomedicals LLC, Santa Ana, CA, United States) according to the manufacturer's instructions. DNA amplification, Illumina libraries preparation, amplicon sequencing (Illumina MiSeq, Inc., San Diego, CA, United States), and bioinformatics analysis were performed by RTL Genomics (Lubbock, TX, United States), and the methodology is detailed elsewhere [148]. The amplicon primer set used was the 28 f/388 R [149] [150], targeting the bacterial 16S rRNA gene. Nucleotide sequences were submitted to the European Nucleotide Archive (ENA) under the study number PRJEB36602.

3.2.10. Transcriptomic analyses

RNA later was removed from *A. borkumensis* cells by centrifugation, and total RNA was extracted by using FastRNA Pro™ Soil-Direct Kit (MP Biomedicals, Solon, OH, United States), in accordance with the manufacturer's instructions. RNA extracts were sent in dry ice to Eurecat (Reus, Spain), where DNAse treatment (18068-015, Invitrogen), RNA quality assessment (using the Agilent TapeStation team and the Agilent High Sensitivity RNA ScreenTape Assay), libraries preparation, and sequencing were performed. The sequencing libraries were created using the Illumina Stranded Total RNA Prep ligation with Ribo-Zero Plus (20040525, Illumina). The obtained cDNA libraries were quantified by microfluidic electrophoresis on Agilent's TapeStation equipment and the Agilent DNA High Sensitivity ScreenTape kit. The length and concentration of each sample were determined, and quantification was performed with Qubit.

Sequencing was performed in the NextSeq2000 sequencing system (Illumina), generating millions 2 × 76 pb reads per sample. Initial bioinformatics data analysis was performed by Eurecat, which included mapping against a reference genome using HISAT2 (2.2.1), annotation and quantification

of aligned reads with StringTie (2.1.4), and differential gene expression level comparison using DESeq2 R package (1.30.0). Samples were normalized by the Relative Log Expression (RLE) method, and expression levels were represented in counts per million (CPM). Genes were considered differentially expressed, upregulated or downregulated, if the difference in expression between conditions was at least two-fold, double or half expression levels, respectively, and if the adjusted p-value obtained for that gene was less than 0,05.

Additional bioinformatics analysis of *A. borkumensis* SK2 differentially expressed genes was performed to obtain extra functional information. For that purpose, initial gene IDs (locus tag) were converted to National Center for Biotechnology Information (NCBI) RefSeq protein IDs and UniProt IDs. By using the UniProt ID mapping web service, information from cross-reference databases and FASTA sequences were obtained. Protein sequences were then submitted to reCOGnizer1 that retrieved information from Clusters of Orthologous Groups of protein (COG), EuKaryotic Orthologous Groups (KOG), Conserved Domain Database (CDD), Pfam, NCBIfam, TIGRFAM, Protein Clusters, and Smart databases. FASTQ files were submitted to the European Nucleotide Archive under the study accession number PRJEB46411.

3.3. Results and Discussion

3.3.1. Experiment 1 - Effect of corksorb on growth and alkane degradation by hydrocarbonoclastic bacteria

Addition of oxygen or nutrients, such as nitrogen and phosphorous [81] [151] [152], as well as surfactants [153], have been studied as biostimulation strategies to increase the activity of hydrocarbon-degrading bacteria. Considering the unique chemical composition, structure and properties of corksorb [119] [120], we hypothesize that this biosorbent may have the potential to enhance the growth and hydrocarbons degradation by hydrocarbonoclastic bacteria, which was studied in this work.

Alkanes were chosen as model compounds, since they are the main components of crude oil and their biodegradation has an important impact on oil removal and environmental cleanup [70]. The *n*-alkanes tested are characterized by low water solubility, low vapor pressure, and high boiling point (table 2; [154]) and thus present a reduced tendency to dissolve or volatilize, generally forming a floating layer at the water surface.

In the assays performed with *R. opacus* B4, total alkane degradation reached 96 ± 1 % in the presence of corksorb, which was 6 % higher than without corksorb (90 ± 2 %) ($p < 1{,}21 \times 10^{-5}$). This bacterium was able to efficiently degrade the mixture of alkanes, particularly C14, C16, and C20, as they were no longer detected in the culture medium since the first sampling point (283 h of incubation) either in the presence or absence of corksorb. On the other hand, corksorb exerted a positive effect on C24 degradation, the alkane with the highest chain length tested. C24 concentrations averaged 73 ± 21 mg L^{-1} and 194 ± 42 mg L^{-1} in the assays with and without corksorb, respectively, from 283 h until the end of the incubations (715 h). These values correspond to a 24 % increase in C24 removal in the presence of corksorb.

Table 2 - Selected physicochemical properties of the *n*-alkanes tested [154].

Property	C14	C16	C20	C24
Molar mass (g mol^{-1})	198,4	226,5	282,6	338,7
Density (g cm^{-3})	0,756	0,760	0,762	0,760
Boiling point (°C)	253	287	281	391
Vapor pressure (Pa at 20 °C)	1,6	$1{,}9 \times 10^{-1}$	$6{,}2 \times 10^{-4}$	$5{,}4 \times 10^{-4}$
Solubility in water (mol L^{-1} at 25 °C)	$2{,}0 \times 10^{-8}$	$4{,}0 \times 10^{-9}$	$6{,}7 \times 10^{-9}$	$7{,}3 \times 10^{-9}$

Corksorb did not influence the growth of *R. opacus* B4 (table 3) possibly due to the naturally high alkane-degrading efficiency of this bacterium and to the fact that, among the alkanes tested, only C24 was more difficult to degrade. TSN concentrations of 85 ± 3 mg L^{-1} and 81 ± 6 mg L^{-1} were measured at the end of the experiment in the presence and absence of corksorb, respectively (figure 5), showing no statistical differences. In the assays with *R. opacus* B4 and corksorb (without the mixture of alkanes, Ro-CrkS), TSN concentration was always lower than 2 ± 0 mg L^{-1}.

Table 3 - Growth of *R. opacus* B4 from alkanes, expressed as total suspended nitrogen (TSN) concentration, with corksorb (Ro-Alk-CrkS) and without corksorb (Ro-Alk). Control assays without alkanes (Ro-Alk) are also shown.

Time (h)	Total Suspended Nitrogen (TSN) (mg L^{-1})		
	Ro-Alk-CrkS	**Ro-Alk**	**Ro-CrkS**
0	1,4	1,4	1,4
283	26,7 ± 5,4	41,8 ± 0,4	1,0 ± 0,2
474	58,6 ± 7,5	41,0 ± 2,5	1.2 ± 0,2
715	81,2 ± 5,8	84,5 ± 2,9	1.3 ± 0,3

The results presented are the averages and standard deviations for triplicate assays.

In the assays performed with *A. borkumensis* SK2, significantly lower (p < 0,04) alkane concentrations were detected in the presence of corksorb, which corresponded to significantly higher degradation of all the alkanes tested when compared to the assays without corksorb (figure 6 and table 4). Total alkane degradation was 24 % higher in the assays with corksorb relative to the assays without this biosorbent (*i.e.*, 72 ± 2 % and 47 ± 2 %, respectively; table 4). Degradation of longer-chain alkanes (C20, C24) was slower than that of C14 and C16 (figure 6), confirming the more recalcitrant nature of longer-chain alkanes, thus requiring more time to be consumed, as reported by Head *et al.* [70]. The enhancement of alkane degradation by *A. borkumensis* SK2, induced by corksorb, was related to bacterial growth. After 452 h of incubation, the bacterial biomass (as TSN) was significantly higher (p < 0,02), *i.e.*, 51 %, in the presence than in the absence of corksorb, with TSN values of 12 ± 0 mg L^{-1} and 8 ± 0 mg L^{-1}, respectively (table 5). In the control assay Alk-CrkS, no growth was observed, and the expected alkane recovery was 98 ± 3 % (based on previous experiments performed by Castro *et al.* [131]). All these results show that the higher alkane removal in the presence of corksorb is a result of alkane degradation and not an absorption phenomenon. Moreover, the results indicate that corksorb effectively stimulated *A. borkumensis* SK2 growth.

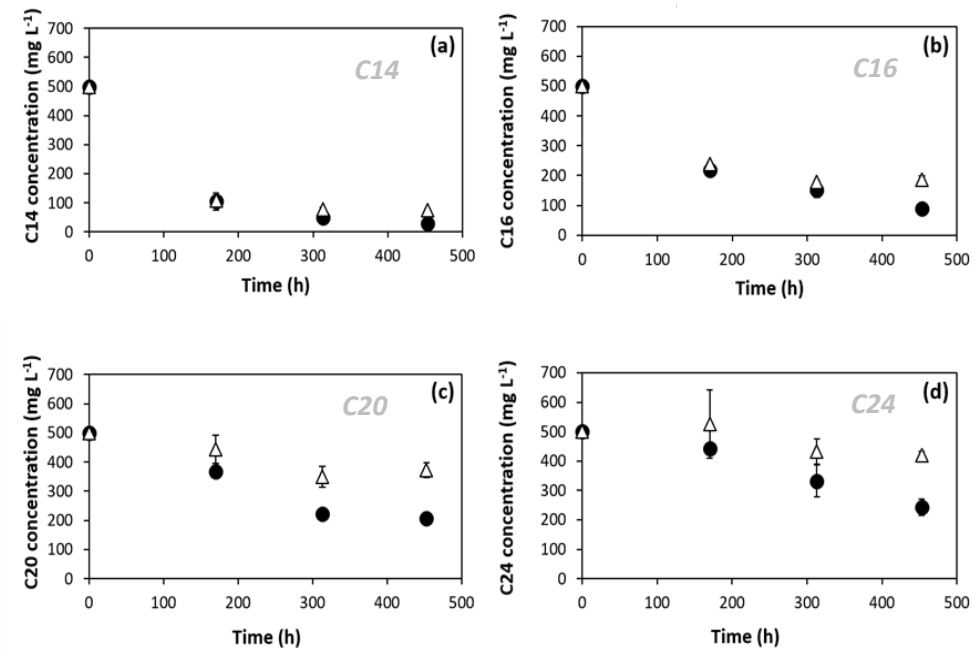


Figure 6 - Alkane degradation by *A. borkumensis* SK2 with corksorb (Ab-Alk-CrkS, •) and without corksorb (Ab-Alk, **Δ**). (a) C14, (b) C16, (c) C20 and (d) C24. The results presented are the averages and standard deviations for triplicate assays.

Table 4 - Alkane degradation (%) by *A. borkumensis* SK2 at the end of the experiment, with corksorb (Ab-Alk-CrkS) and without corksorb (Ab-Alk).

Time (h)	Total Suspended Nitrogen (TSN) (mg L^{-1})	
	Ab-Alk-CrkS	**Ab-Alk**
C14	94 ± 2	85 ± 0
C16	82 ± 2	63 ± 2
C20	59 ± 4	26 ± 5
C24	51 ± 6	16 ± 3
Total	72 ± 2	47 ± 2

The results presented are the averages and standard deviations for triplicate assays.

Table 5 - Growth of *A. borkumensis* SK2 from alkanes, expressed as total suspended nitrogen (TSN) concentration, with corksorb (Ab-Alk-CrkS) and without corksorb (Ab-Alk). Control assays without alkanes (Ab-Alk) are also shown.

Time (h)	Total Suspended Nitrogen (TSN) (mg L^{-1})		
	Ab-Alk-CrkS	**Ab-Alk**	**Ab-CrkS**
0	4,7	4,7	4,7
167	4,4 ± 0,2	8,8 ± 1,1	3,5 ± 0,03
313	11,8 ± 0,2	8,4 ± 1,2	4,3 ± 0,6
453	12,2 ± 0,4	8,1 ± 0,4	5,6 ± 0,8

The results presented are the averages and standard deviations for triplicate assays.

Bacterial communities' composition was analyzed in the assays with corksorb *and A. borkumensis* SK2 (*i.e.*, Ab-Alk-CrkS and Ab-CrkS; table 1 and figure 4). *A. borkumensis* SK2 was the dominant genus identified both in the presence and absence of alkanes (table 6), with relative abundances of 81 and 91 %, respectively, based on 16S rRNA gene sequencing. Bacteria from the genera *Curtobacterium* and *Roseomonas* were also present in the assays with alkanes (Ab-Alk-CrkS; table 5), accounting for 13 and 5 % relative abundance, respectively. When *A. borkumensis* SK2 was incubated only with corksorb (Ab-CrkS), members of the *Rhizobiales* order were detected and represented 8 % of the bacterial community, including *Pelagibacterium luteolum* (4 % relative abundance) (table 5).

The ability to perform hydrocarbon degradation has been reported in bacteria from the *Curtobacterium* and *Roseomonas* genera [155][156][157][158][159], although other strains within these genera do not have this ability, even when detected/isolated from petroleum-polluted sites sites [160][161]. In fact, the presence of bacterial strains that are not able to degrade hydrocarbons in oil contaminated environments is quite common, generally ascribed to their tolerance to hydrocarbons or to their involvement in the degradation of intermediary compounds. *P. luteolum* typestrain 1_C16_27^{T} was isolated from solid wastes of an oil shale chemical industry [162].

In the assays with corksorb, the presence of hydrocarbon degrading bacteria, or other bacteria

exhibiting tolerance to these compounds, suggest that they might have a role in the degradation of the added alkanes. Nevertheless, the high abundance of *A. borkumensis* SK2 in the microbial communities at the end of the assays points out that this bacterium was the key player in this process. Considering that *Alcanivorax* species tend to become the dominant group in bacterial communities present in marine waters contaminated with hydrocarbons [163], the use of corksorb as a biosorbent material may represent an interesting strategy for enhancing its growth and *in situ* bioremediation.

Table 6 - Bacterial community composition at the genus level based on 16S rRNA gene sequencing by Illumina MiSeq, in the assays Ab-Alk-CrkS, Ab-CrkS and in the enrichment culture CA(2).

Taxonomic identification at the genus level [1]	Relative abundance (%)			Closest cultured relatives [2]	Identity (%) [2]	Accession number
	Ab-Alk-CrkS	Ab-CrkS	CA(2)			
Alcanivorax	80.5	91.0	-	*Alcanivorax borkumensis* SK2	100.0	NR_074890.1
Curtobacterium	12.5	-	-	*Curtobacterium citreum* strain DSM 20528	100.0	NR_026156.1
Roseomonas	4.9	-	-	*Roseomonas deserti* strain M3	92.2	NR_159351.1
Pelagibacterium	-	3.8	-	*Pelagibacterium luteolum* strain 1_C16_27	100.0	NR_116053.1
Unclassified (*Rhizobiales*)	-	3.8	-	*Pelagibacterium halotolerans* B2	96.0	NR_102924.1
Ochrobactrum	-	-	48.5	*Ochrobactrum anthropi* ATCC 49188	100.0	NR_074243.1
Gordonia	-	-	43.1	*Gordonia aichiensis* strain E9028	100.0	NR_037030.1
Pseudomonas	-	-	3.7	*Pseudomonas alcaligenes* strain ATCC 14909	97.7	NR_114472.1
Microbacterium	-	-	3.3	*Microbacterium phyllosphaerae* strain P 369/06	98.7	NR_025405.1
Other [3]	2.2	1.4	1.9	-	-	-

[1] Taxonomic identification at the genus level based on 16S rRNA genes sequences of 291 bp length by Illumina MiSeq. [2] Results of sequence alignment by using BLASTN toward the RefSeq_rna database. [3] Operational taxonomic unit (OTU) with relative abundance

3.3.2. Experiment 2 - Stimulation of *A. borkumensis* SK2 is not linked with the release of soluble substances by corksorb

The observed stimulatory effect of corksorb on the growth and alkane degradation in *A. borkumensis* SK2 assays may be associated with different mechanisms, as for example the presence of compounds (either soluble or embedded in the structure of corksorb) that can act as co-factors, stimulating the growth and activity of this bacterium. Corksorb may also function as support for bacterial growth or promote the contact between the bacteria and the hydrocarbons (since these tend to float, while bacteria are generally dispersed in the medium). *A. borkumensis* SK2 is a known alkane degrader, capable of using these compounds as carbon and energy sources, being also expected to have the ability to produce surfactants. Therefore, in this work, the possible release of soluble compounds from corksorb to the medium was chosen for further studies. Growth of *A. borkumensis* SK2 was measured in experiments performed with medium that did not contain corksorb but had been previously in contact with it (Ab-Alk-afterCrkS assays, table 1), and compared with the growth in the presence of the biosorbent (Ab-Alk-CrkS assays). Statistically higher growth ($p < 0{,}006$) was achieved when corksorb was present in the medium, reaching TSN values of approximately 30±2 mg L^{-1} in the Ab-Alk-afterCrkS assays versus 24±2 mg L^{-1} in the Ab-Alk-CrkS, after 300 h of incubation (Figure 7). These results highlight that the presence of corksorb is essential, and that the release of soluble stimulatory compounds was not the main reason for the stimulation of *A. borkumensis* SK2.

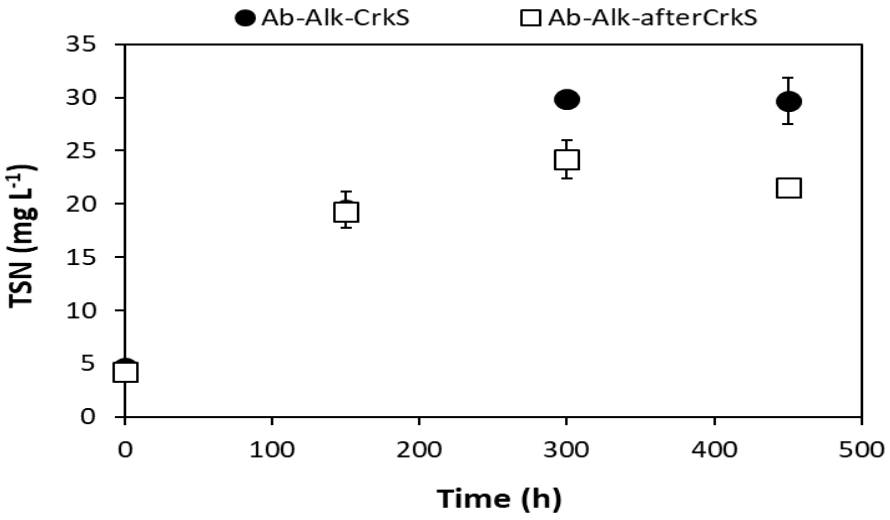


Figure 7 - Growth of *A. borkumensis* SK2, expressed as TSN concentration, over time in the presence and absence of corksorb. In the assay performed without corksorb, the medium used was previously in contact with this biosorbent for 150 h, after which it was transferred to the assay flasks.

3.3.3. Effect of corksorb on differential gene expression and on surface tension of *A. borkumensis* SK2 cultures

To further understand why *A. borkumensis* SK2 growth and alkane degradation are improved by corksorb, an additional experiment was performed. Corksorb may have a direct effect on alkane degradation or alternatively/additionally influence other cellular processes, such as biosurfactant production. Corksorb may also function as support for bacterial growth or promote the contact between the bacteria and the hydrocarbons (since these tend to float and bacteria are generally dispersed in the culture medium). Therefore, a transcriptomics analysis was performed to detect potential differences in gene expression. Particular focus was given to the genes involved in alkane degradation, biosurfactant production, and biofilm formation, which were described by Schneiker *et al.* [128]. Variations on ST were also assessed. The Illumina sequencing originated a total of 905,664,672 reads that, after alignment with the reference genome, decreased to 383,563,992. A total of 2,825 different RNA molecules were detected. The results showed 128 upregulated genes and 130 downregulated genes when *A. borkumensis* SK2 was incubated with corksorb relative to the control without corksorb.

A high number of differentially expressed genes (66) coding for hypothetical proteins were detected, but additional functional information could be obtained by performing conserved domain analysis with the reCOGnizer tool. The differentially expressed protein-coding genes could be assigned to the following COG functional categories: Cellular processes and signaling (77 genes), Information storage and processing (28 genes), Metabolism (64 genes), and Poorly characterized (28 genes). However, 61 genes, including 19 non-protein-coding genes, could not be assigned to any COG category. All the differentially expressed non-coding genes detected were found to be overexpressed in the presence of corksorb. These genes coded for rRNA or tRNA and are among the most upregulated genes detected in this experiment (table 7). For instance, these genes were 3–27 times more expressed in the presence of corksorb (table 7). These results corroborate the higher activity of *A. borkumensis* SK2 cells when growing with corksorb, as more rRNAs and tRNAs, involved in protein synthesis, were expressed. This is in agreement with the higher growth and alkane degradation by *A. borkumensis* SK2 observed in the presence of corksorb (tables 4, 5; figure 6).

Table 7 - List of upregulated genes, annotated as ribosomal RNA, transfer RNA, or related genes expressed by *Alcanivorax borkumensis* SK2 when growing in the presence of corksorb relative to the control assay without corksorb.

Gene identifier (Locus tag)	Number of times genes are upregulated	Annotation (NCBI RefSeq database)
ABO_RS14190	27	transfer-messenger RNA
ABO_RS14160	26	RNase P RNA component class A
ABO_RS02520	12	16S ribosomal RNA
ABO_RS10185	11	16S ribosomal RNA
ABO_RS01870	11	16S ribosomal RNA
ABO_RS14235	8	signal recognition particle sRNA small type
ABO_RS10180	8	23S ribosomal RNA
ABO_RS02525	7	23S ribosomal RNA
ABO_RS09450	7	tRNA-Pro
ABO_RS14330	6	6S RNA
ABO_RS01665	6	tRNA-Met
ABO_RS01885	6	23S ribossomal RNA
ABO_RS09645	6	tRNA-Met
ABO_RS07825	6	tRNA-Ala
ABO_RS07820	4	tRNA-Glu
ABO_RS06250	4	tRNA-His
ABO_RS04560	4	tRNA-Ser
ABO_RS09255	3	tRNA-Ser
ABO_RS01880	3	tRNA-Ala

Nevertheless, few genes associated with alkane degradation were differentially expressed, namely, two alkane 1- monooxygenases (locus tag ABO_RS13835 and ABO_RS00610) and three other enzymes that are involved in alkane conversion to alkanol and alkanol conversion to alkanal (a ferredoxin reductase and two alcohol dehydrogenases, locus tag: ABO_RS01020, ABO_RS13850, and ABO_RS06065, respectively), that were downregulated in the presence of corksorb. A total of 14 genes coding for different DUF domaincontaining proteins were found, eight upregulated and six downregulated. However, these are poorly characterized domains and with unknown function, and therefore, their role in the increased growth and alkane degradation of *A. borkumensis* SK2 in the presence of corksorb remains unclear. Nevertheless, one should mention that the most downregulated gene during incubation with corksorb (32 times less expressed) is an uncharacterized protein containing a DUF1656 domain. Although the function is unknown, annotation against the Pfam database indicated that some DUF1656-containing proteins are putative membrane proteins. Several genes (a total of 15) related to pili formation (most Type IV pili) were downregulated by *A. borkumensis* SK2 when corksorb was present (table 8). Pili are thin appendages in the surfaces of bacteria performing several functions, including twitching motility,

uptake of external DNA to the cell, microcolony formation, biofilm formation, and adherence [164] [165].

Probably because corksorb absorbs the hydrocarbons and offers a support for cell growth, some of the typical functions of pili were possibly less relevant for the bacteria growing with corksorb than when corksorb was not present. For instance, cells may have less necessity to move toward feeding. In this way, *A. borkumensis* SK2 population growing attached to corksorb probably shifts the energy toward cellular processes other than mobility. This hypothesis is reinforced by the SEM images obtained that show an extensive biofilm formed around corksorb granules (figures 8B–D), as well as the proximity between hydrocarbons and cells (figures 8E, F). This proximity could facilitate alkane degradation and consequently cell growth. Figure 8A shows a corksorb granule without biofilm, for comparative purposes. Another possibility for the enhanced alkane degradation by *A. borkumensis* SK2 in the presence of corksorb could be a decrease in biosurfactant production due to the proximity between bacteria and hydrocarbons promoted by corksorb. This would potentially allow the bacteria to redirect their energy toward other cellular processes, such as growth.

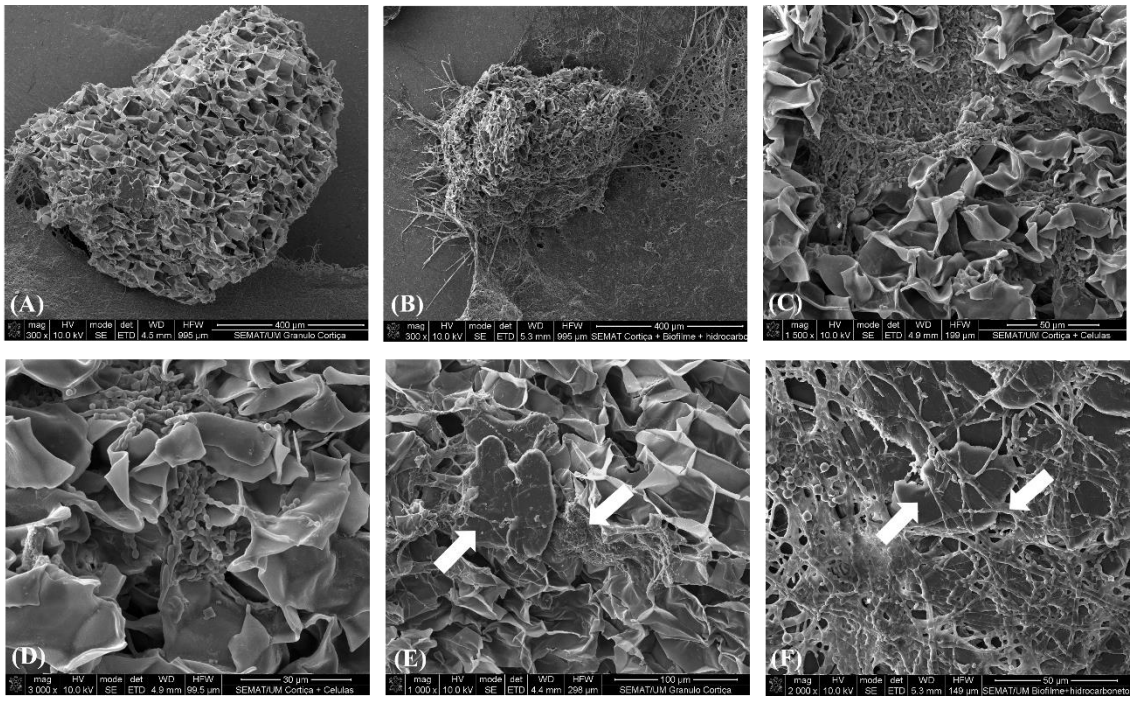


Figure 8 - Scanning electron microscopy (SEM) images of (A) corksorb granule without *A. borkumensis* SK2, 400 µm; (B) corksorb granule surrounded by *A. borkumensis* SK2 biofilm, 400 µm; (C) *A. borkumensis* SK2 biofilm attached to corksorb, 50 µm; (D) *A. borkumensis* SK2 biofilm attached to corksorb, 30 µm; (E) detail of *A. borkumensis* SK2 biofilm (white arrow on the right) attached to corksorb in the vicinity of a carbonaceous *n*-alkane plate (white arrow on the left), 100 µm; and (F) *A. borkumensis* SK2 biofilm (white arrow on the left) attached to a carbonaceous *n*-alkane plate (white arrow on the right), 50 µm.

Table 8 - List of downregulated genes related to pili formation and respective functional annotation obtained when *Alcanivorax borkumensis* SK2 was incubated in the presence of corksorb relative to the control assay without corksorb.

Gene identifier (Locus tag)	Number of times genes are downregulated	Annotation against UniProt database	Annotation against COG and TIGRFAM databases
ABO_RS02430	12	UPI00031985B5: Uncharacterized protein; GspH/FimT family pseudopilin	COG4970: Type IV pilus assembly protein FimT
ABO_RS02420	5	Q0VSD3: Pilin biogenesis related protein	COG3419: Type IV pilus assembly protein, tip-associated adhesin PilY1
ABO_RS02400	5	Q0VSD7: Pilus biogenesis protein	COG4970: Type IV pilus assembly protein FimT
ABO_RS02425	5	UPI0005A1964E: Type IV pilin protein;secretion protein	COG4968: Type IV pilus assembly protein PilE
ABO_RS11470	4	Q0VMB6: Type IV fimbrial biogenesis protein PilP	COG3168: Type IV pilus assembly protein PilP
ABO_RS11485	4	Q0VMB3: Type IV fimbrial biogenesis protein PilM	COG4972: Type IV pilus assembly protein, ATPase PilM; TIGR01175: Type IV pilus assembly protein PilM
ABO_RS11480	4	Q0VMB4: Type IV pili biogenesis protein PilN	COG3166: Type IV pilus assembly protein PilN
ABO_RS01120	3	UPI0002E0144D: Type II secretion protein F	COG4965: Flp pilus assembly protein TadB
ABO_RS02405	3	Q0VSD6: Uncharacterized protein	COG4967: Type IV pilus assembly protein PilV; TIGR02523: Type IV pilus modification protein PilV
ABO_RS03175	3	Q0VRY5: Pilin protein family, putative	COG4969: Type IV pilus assembly protein, major pilin PilA
ABO_RS02410	3	UPI0003154941: Uncharacterized protein;N-terminal cleavage protein;PilW family protein	COG4966: Type IV pilus assembly protein PilW; TIGR02532: prepilin-type N-termina
ABO_RS11475	2	UPI0015687014: Type IV pilus biogenesis protein PilO	COG3167: Type IV pilus assembly protein PilO
ABO_RS11465	2	Q0VMB7: Fimbrial assembly protein pilQ	COG4796: Type II secretory pathway, component HofQ; TIGR02515: Type IV pilus secretin (or competence protein) PilQ. Members of this family include PilQ itself, which is a component of the Type IV pilus structure, from a number of species.
ABO_RS01105	2	UPI0011D0765B: pilus assembly protein N-terminal domain-containing protein	COG4964: Flp pilus assembly protein, secretin CpaC
ABO_RS01110	2	Q0VT52: TadZ_N domain-containing protein	COG4963: Flp pilus assembly ATPase CpaE/TadZ, contains N-terminal REC/TadZ_N domain

Biosurfactants have been demonstrated to efficiently emulsify hydrocarbons in several members of oil-degrading γ-Proteobacteria, facilitating hydrocarbon bioavailability, uptake, and biodegradation [166]. The biosurfactants produced by *A. borkumensis* SK2 are mainly glycolipids [167]. Here, we investigated if the genes coding for proteins involved in the production of glycolipid surfactants were differentially expressed by *A. borkumensis* SK2 in the presence of corksorb. Proteins involved in glycolipid surfactant production include glycoside hydrolases (also called glycosidases or glycosyl hydrolases), esterases, particularly lipases and also proteases and peptidases [168]. We found three upregulated hydrolases (locus tag: ABO_RS05215, ABO_RS00720, and ABO_RS10845) and four downregulated hydrolases (locus tag: ABO_RS04970, ABO_RS05665, ABO_RS07690, and ABO_RS12680) in the presence of corksorb. Three different esterases were downregulated (ABO_RS03820, ABO_RS08725, and ABO_RS10855) and none was upregulated. Similarly, no lipases were upregulated, and one phospholipase was downregulated (locus tag: ABO_RS13795). No proteases were differentially expressed and no peptidases were upregulated, although five peptidases were found to be downregulated (locus tag: ABO_RS03130, ABO_RS03780, ABO_RS08845, ABO_RS08840, and ABO_RS05575). In addition, two glycosyl transferases (locus tag: ABO_RS04745 and ABO_RS04860) were downregulated when the culture grows with corksorb. However, none of these enzymes are specifically described as involved in surfactant production. Nevertheless, a gene coding for an outer membrane protein (locus tag: ABO_RS05955), that according to Schneiker *et al.* [128] is possibly involved in emulsifier production, was found to be downregulated when *A. borkumensis* SK2 was incubated with corksorb. These results do not allow to conclude that the genes related to biosurfactant production are differentially expressed when corksorb is present. Also, the evolution of ST values, which is a direct measurement of biosurfactant activity, showed only minor differences between the cultures grown with and without corksorb (figure 9).

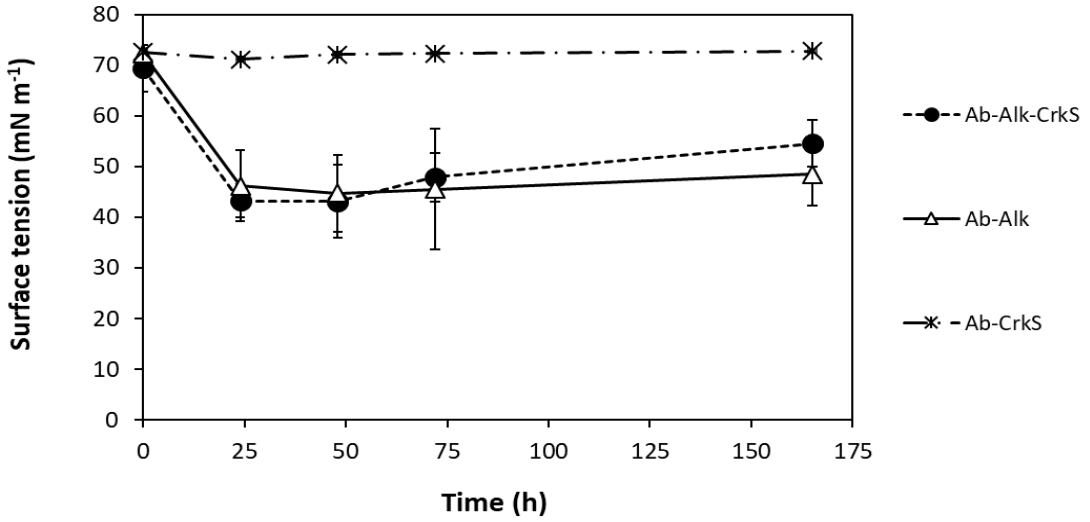


Figure 9 - Evolution of surface tension (ST) over time in cultures of *A. borkumensis* SK2 grown with alkanes in the absence (Ab-Alk, **Δ**) and presence of corksorb (Ab-Alk-CrkS, •). Control assays without alkanes (Ab-Alk, ∗) are also shown. Results represent the average of three independent experiments ± standard deviation.

The efficiency of a surfactant is determined by its ability to reduce the ST (*e.g.*, an effective surfactant can lower the ST of water from 72 to 30 mN m^{-1} [169]. In our assays, the ST decreased to values of 43 ± 3 mN m^{-1} and 46 ± 7 mN m^{-1} in the presence and absence of corksorb, respectively, and these values remained approximately constant until the end of the experiment (figure 9). Transcriptomics and ST results show no evident relation between the improved activity of *A. borkumensis* SK2 in the presence of corksorb and the production of biosurfactants.

3.3.4. Enrichment of native bacteria present in corksorb

Bacterial communities' composition analysis performed with samples from the growth and biodegradation experiments (see section 3.3.1) showed the presence of native bacteria in corksorb that were capable of growing in the conditions tested. Different bacterial populations were also found in cork-processing wastewaters by del Castillo *et al.* [170], which showed that some of the most prominent bacteria corresponded to well-known phenol-degrading organisms, namely, from the genera *Ralstonia*, *Stenotrophomonas*, *Cupriavidus*, and *Lysobacter*. Mesophilic isolates belonging to the genera *Klebsiella*, *Pseudomonas*, *Stenotrophomonas*, and *Burkholderia*, as well as thermophilic isolates of the *Bacillus* genus, were also obtained in enrichment cultures developed

from cork boiling wastewater using tannic acid as a selective carbon source [171]. If corksorb is used as a biosorbent in an oil spill scenario, the native bacteria from corksorb may be able to grow and contribute to hydrocarbon bioremediation, especially considering that the relative abundance of *A. borkumensis* at the moment of the oil spill is generally low.

Microbial growth could be visually noticed in the cultures developed from corksorb using the mixture of alkanes as carbon source (figure 10). The microbial community was dominated by *Ochrobactrum* and *Gordonia* species, with 48,5 % and 43,1 % relative abundance, respectively (table 6). *Pseudomonas* and *Microbacterium* species were also present at lower relative abundances (*i.e.*, 3,7 % and 3,3 %, respectively – table 6).

Figure 10 - Growth of native bacteria from corksorb in the medium with alkanes.

Bacteria from *Ochrobactrum*, *Gordonia, Pseudomonas* and *Microbacterium* genera, that were found in the enrichment cultures, are known for their capability to degrade hydrocarbons, and their presence in hydrocarbons contaminated environments has also been reported [172][173][174][175]. However, these bacteria were not detected in the first experiment. The different conditions applied during the enrichment process, when compared to those previously used, can be the explanation for the differences observed in terms of bacterial community composition. These results reinforce the presence of native bacteria in corksorb, some of which are able to grow using hydrocarbons and thus might influence the positive effect of corksorb in alkane degradation.

3.4. Conclusions

In summary, this work shows that corksorb, currently used as oil-spill sorbent, herein contaminated with alkanes, can enhance bacterial growth and hydrocarbon biodegradation. The presence of corksorb was found to increase alkane degradation by *R. opacus* B4 and *A. borkumensis* SK2 (model hydrocarbondegrading bacteria) in 6 and 24 %, respectively, relative to the assays without corksorb. The growth of *A. borkumensis* SK2 was enhanced 1,51 times in the presence of corksorb. It was also found in this work that the positive effect of corksorb is not related to the release of soluble compounds.

The increased expression of genes coding for rRNA and tRNA (required for protein synthesis) and the decreased expression of genes involved in pili formation (associated with several cellular processes, namely, cell motility), together with biofilm formation as revealed by SEM, can possibly explain the higher growth and alkane degradation by *A. borkumensis* SK2 in the presence of corksorb. Additionally, the native bacteria present in corksorb can possibly have a role in hydrocarbon biodegradation and may assist in *in situ* bioremediation of oil-contaminated environments.

Overall, the obtained results support a novel approach with potential to improve *in situ* marine bioremediation processes by coupling hydrocarbon sorption and biodegradation by hydrocarbonoclastic bacteria. This approach will generate lower amounts of oil-contaminated biosorbent than when only remediation by physical absorption is applied, thus representing a decrease in the costs associated with the treatment and regeneration of the oily biosorbent.

Additionally, the oily biosorbent treatment can be performed *ex situ* by hydrocarbonoclastic bacteria that may convert the absorbed oil components into valuable compounds, such as lipids, biogas, or polyhydroxyalkanoates [131][176][177]

CHAPTER IV

Bioremediation of Petroleum-Contaminated Sediments: Effect of Conductive Nanomaterials

The results of this chapter were included in:

Cavaleiro AJ, Salvador AF, Martins G, Oliveira CC, Liu Y, Martins VR, Castro AR, Soares OSGP, Pereira MFRP, Pereira L, Langenhoff AAM, Pereira MA, Alves MM. (2020) Multi-walled carbon nanotubes enhance methanogenesis from diverse organic compounds in anaerobic sludge and river sediments. Applied Sciences-Basel, 10(22), 8184. doi: https://doi.org/10.3390/app10228184

Abstract

Anaerobic hydrocarbons degradation by the autochthonous microbial community present in river sediments, recently contaminated with oil was studied. In parallel, the potential of conductive nanomaterials (CNM) for enhancing anaerobic hydrocarbons degradation by this community and their conversion to methane was also investigated. The sediments were incubated in the absence or in the presence of CNM - magnetite (MG), carbon nanotubes (CNT) or carbon nanotubes impregnated with 2 % iron (CNT@Fe). All the nanomaterials were tested at a concentration of 0,5 g L^{-1}. Abiotic controls (AC) were also prepared by supplementing the microcosms with sodium azide and copper chloride. All the microcosms were incubated at room temperature, in the dark, without agitation. Methane production was measured during the experimental period. At each sampling point, pH and redox potential were immediately measured. Then, microcosms were sacrificed for total petroleum hydrocarbons (TPH) quantification, which were analyzed in the liquid and in the solid phases.

TPH values quantified over time were not significantly different in all the conditions tested (including the AC), thus pointing to the absence of hydrocarbon-degrading activity in the sediments. Nevertheless, up to 10,2 and 4,5 times higher methane production was obtained after 169 days in the assays with CNT@Fe and CNT, respectively, when compared with the assays performed in the absence of nanomaterials (W/o CNM). Assays with MG and AC showed no methane production. These results point to a stimulatory effect of CNT@Fe and CNT on the degradation of non-hydrocarbon organic compounds present in the sediments.

Keywords: hydrocarbons, sediments, conductive nanomaterials, carbon nanotubes, magnetite.

4.1 Introduction

Estuary zones have an important role as coastal transitional ecosystems between the terrestrial and marine environments, which are essential to maintain high degree of biodiversity by acting as nurseries for many different species [178]. They are susceptible to contamination since, due to their general favourable configuration, they are frequently chosen for the installation of ports and industries that pollute the area by their activities, and oil and its derivatives tend to accumulate in estuarine zones.

Complex microbial communities of anaerobic microorganisms thriving in oil contaminated environments can use hydrocarbons for growth in the subsurface, driven by the presence of alternative electron acceptors other than oxygen, such as nitrate, iron(III), sulphate and even under methanogenic conditions [12][17][64][179][180]. Autochthonous bacteria able to degrade hydrocarbons are naturally present in the environment, and also, in some ecosystems contaminated with hydrocarbons (long periods), may represent a dominant group [20]. Anaerobic hydrocarbons biodegradation is a slow process [86]. Over 100 days were necessary for the degradation of 1-hexadecene by methanogenic cultures [106][181], and longer periods of time (~600-800) have been reported for the methanogenic degradation of hexadecane [28][105]. Dim growth of hydrocarbon-degrading microorganisms is usually related with low contaminants bioavailability, which may generate some limitations to *in situ* bioremediation, as the interaction between hydrocarbons and the organic fraction of sediments may decrease the accessibility of oil compounds to microorganisms and its enzymes. Stimulating the degradation processes in the polluted areas is required to shorten the contamination period. Therefore, stimulating anaerobic biodegradation of oils in the subsurface offers a worthwhile approach for hydrocarbons bioremediation of contaminated sites. Contaminated marine sediments with polycyclic aromatic hydrocarbons can be anaerobic biostimulated with reducing of its content by 55 % [182]. Applying rhamnolipids in anaerobic hydrocarbons degradation, in contaminated sediments of an oil field, decreased the medium surface tension increasing the desorption of TPH from the sediments and consequently hydrocarbons degradation, with degradation rates in nitrate and sulphate conditions of 32.2 % and 24 %, respectively [183].

Different reports have showed that the presence of CNM improve the oxidation of diverse organic compounds such as lactate, ethanol, glucose, starch and oleic acid, at mesophilic and thermophilic temperatures [184][185][186][187]. Therefore, CNM may also be tested for improved

hydrocarbons oxidation. Some CNM, as for example magnetite (MG) or carbon nanotubes impregnated with 2 % iron (CNT@Fe), have magnetic properties that allow their recovery by applying a magnetic field, promoting its retrieval and further reuse [188]. Some CNM are present as ferric minerals, existing in nature both in soils and groundwater, and have an important role in several biogeochemical processes such as iron biomineralization [189]. MG nanoparticles are one of the most common minerals present in soils. MG nanoparticles that acted as electron acceptors were found to promote fast oxidation of ethanol (intermediary of anaerobic degradation of organic compounds) by members of the iron (III)-reducing bacteria, in paddy soil enrichments (obtained in the presence of MG nanoparticles and ethanol) and establishment of the syntrophic relationship of iron (III)-reducers with *Methanobacterium* via interspecies hydrogen transfer [190]. Even though the mechanisms behind the stimulatory effect of ferric minerals on microbial activity are not quite understood, they appear to depend on the crystallinity and conductivity of the iron(III) form [189]. Some studies have also revealed that the addition of CNM to anaerobic cultures increased the methane production from fatty acids, possibly by facilitating direct interspecies electron transfer [189][191] since CNM are though to be capable of conducting electrons between the electron donating and accepting microorganisms [184][186]. This effect may be particularly relevant in syntrophic relationships, as *e.g.*, the syntrophic cooperation between hydrocarbon degrading bacteria and methanogenic archaea in methanogenic environments [192][193][194]. As result, the hypothesis of CNM enhancing *ex situ* anaerobic bioremediation of oil-contaminated sites by stimulating syntrophic partnerships gain attention as research opportunity.

4.2. Materials and methods

4.2.1. Sediments sampling

After a recent oil spill in March of 2017 (probable origin: an used-oils deposit), contaminated sediments were collected from the middle intertidal zone of the Granja Beach (Vila Nova de Gaia, Portugal) at low tide, figure 11. Samples were collected at 2-12 cm depth, in three different spots, and transported to the laboratory at 4 °C. Then, sediment samples were combined, thoroughly homogenized and sieved through a 5 mm sieve. The homogenous sieved sediments were characterized in terms of organic matter content, total petroleum hydrocarbons (TPH), iron (II) and total iron content, and total phosphate. TPH content was measured in five replicas.

Figure 11 - Contaminated sediments in Granja beach, Vila Nova de Gaia, Northern Portugal. Samples were collected few days after the oil spill.

4.2.2. Nanomaterials

Three types of nanomaterials were used in this study: MG, carbon nanotubes (CNT) and carbon nanotubes impregnated with 2 % iron (CNT@Fe). CNT and CNT@Fe were synthesized and characterized as described by Pereira and collaborators [188]. MG (97 %) with 50-100 nm particle size was purchased at Sigma-Aldrich. All the nanomaterials were tested at a concentration of 0,5 g L^{-1}.

4.2.3. Culture medium

Basal medium was prepared as described by Stams and collaborators [195] and supplemented

with vitamins and trace nutrients. The vitamins solution was added to the medium through sterilized syringe filters (0,2 μm cellulose acetate filter, Whatman, UK). Medium salinity was 0,3 g L^{-1} and no sulphate was present in the medium. Anaerobic conditions were promoted by boiling the medium prior to distribution in the bottles, to remove the dissolved oxygen. After boiling, the medium was transferred to serum bottles, which were sealed with butyl rubber stoppers and aluminium crimp caps. The bottles were then subjected to several cycles of vacuum and pressure using a Manifold apparatus with a N_2/CO_2 (80:20 % v/v) gas mixture. Final pressure was adjusted at $1,7x10^5$ Pa at the end of the cycles. The bottles were then autoclaved at 121 °C for 20 minutes. Sterilized syringes and needles were used.

4.2.4. Bioremediation assays

Figure 12 represents an illustration of the experimental procedure. Microcosms were set-up in 220 mL glass bottles, containing 25 g of contaminated sediments, 75 mL anaerobic basal medium and 0,05 g of nanomaterial. Similar microcosms were prepared without adding the nanomaterial. In the abiotic microcosms, copper chloride (100 mg L^{-1}) and sodium azide (2 g L^{-1}) were added as inhibitors of the biological activity. Hexadecane was spiked at 0,5 g L^{-1} (50 mg) and no other carbon source was supplemented. Preparation of anaerobic microcosms required opening the bottles, containing the sterilized anaerobic medium, close to the flame to mix all the components with the medium. Then, the bottles' headspace was flushed with a mixture of N_2/CO_2 (80:20 % v/v) using a portable Bunsen burner and sterile needles and filters (0,2 μm), and pressurized to a final pressure of $1,7x10^5$ Pa. pH was measured and adjusted to 7,0-7,2 Assays were incubated at room temperature, in the dark and without agitation. No reducing agent was added.

Nitrate, sulphate, iron (II) and total iron concentrations were measured at the beginning of the experiment, as well as pH and oxidation-reduction potential (ORP). At each sampling point (32, 92, 204 and 504 days), methane concentration was first analysed in the headspace, and after, a sample was collected for immediate measurement of pH and ORP. Then, the whole content of two microcosms was sacrificed for TPH analysis. For that, the liquid and solid phases of the microcosms were separated by decantation. The liquid phase was acidified at pH 2,0 with HCl and preserved at 4 °C for future analysis. Later, from the stored samples, some were selected to

perform this analysis. The TPH in the solid phase were immediately extracted and analysed in all samples.

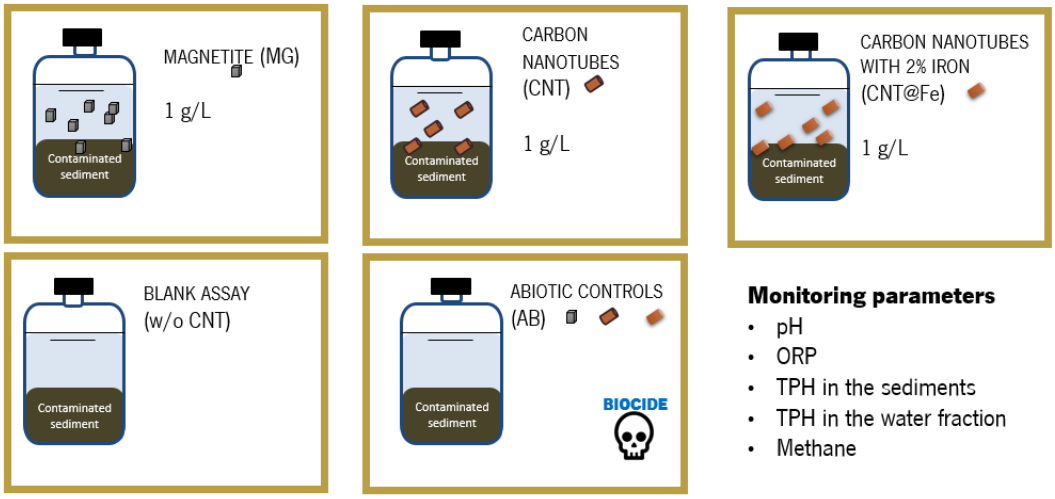


Figure 12 – Ilustration of the experimental procedure and monitoring parameters performed.

4.2.5. Analytical methods

The pH and ORP parameters were measured by potentiometry with a pH probe (JENWAY, UK) and an ORP probe (VWR, USA), respectively, in 1 mL liquid samples taken from each microcosm. As described in [196], volatile solids content was quantified by gravimetry. Total phosphate was quantified using standard test kit (Hach Lange, Düsseldorf, Germany), after digestion with HCl at 250 °C for 1 hour, as described by González and collaborators [197]. Nitrate concentration was measured by standard test kit (Hach Lange, Germany), through reaction of the sample with 2,6-dimethylphenol. Sulphate reduction was assessed through the quantification of dissolved sulphide concentrations, using LCK 653 cuvette tests from Hach Lange, based on the methylene blue method in standard methods. The Hach Lange DR2800 spectrophotometer was used in these analyses. Iron(II) quantification was performed as described in Kaser and Coates [198]. Briefly, insoluble iron(II) present in the samples was solubilized during for 1-hour contact with HCl 0,5 mol L^{-1}. Total iron(II) was then quantified spectrophotometrically at 562 nm after reaction with ferrozine (monosodium salt hydrate of 3-(2-pyridyl)-5,6-diphenyl-1,2,4-triazine-p,p'-disulfonic acid) in 50 mmol L^{-1} 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES buffer) at pH 7,5. Iron(II) standards were prepared from ferrous ethylenediammonium sulphate. Total iron content was measured in samples solubilized with HCl 0,5 mol L^{-1} for 24 hours. The acid extract was then

reacted with hydroxylamine-HCl, which reduces iron(III) to iron (II), and the ferrozine an alysis was run again.

Total petroleum hydrocarbon (TPH) were quantified in solid and liquid phases. Solid phase TPH extraction was performed as described by Siddique and collaborators [199], *i.e.*, samples were putted in contact with the solvent mixture in a closed bottle, at room temperature during 4 hours, at 180 min^{-1}, after which the organic and aqueous phases were separated. TPH in liquid samples were sequentially extracted three times using hexane in separatory funnels, based on the method described by U.S. Environmental Protection Agency [200]. A 10 g L^{-1} solution of heptadecane (C17) in hexane was prepared and used as surrogate to evaluate the extraction efficiency. The extracts were cleaned with Sep-Pak Florisil cartridges (Waters, Milford, MA)) and analysed by gas chromatography (GC Varian® star 3400 CX, USA), according with ISO 9377-2:2000 [201], using a VF-1 ms column (Agilent, USA) 30 m × 0.025 mm and a flame ionization detector (FID). Helium was used as carrier gas at 1 mL min^{-1}. Detector and injector temperature were set at 300 °C and 285 °C respectively. The column's temperature was maintained at 60 °C for 1 min and then a temperature ramp of 8 °C min^{-1} was programmed, up to 290 °C. Undecane (C11) and tetracontane (C40) stock solution was used as GC internal standard, at a concentration of 380 mg L^{-1}, in hexane.

Methane concentration in the headspace of the bottles was measured using a gas chromatograph (Shimadzu, Kyoto, Japan) equipped with a flame ionization detector (FID) and a PoraPak Q column (80/100 mesh column, 2 m x 1/8 in, 2 mm, stainless steel) with nitrogen and argon as carrier gases at 30 mL min^{-1} and 5 mL min^{-1}, respectively. The injector, column and detector temperatures were 110 °C, 35 °C and 220 °C, respectively.

4.3. Results and Discussion

Sediments were analysed in terms of total and volatile solids content, showing 874,0±3,0 g kg^{-1} and 7,9±1,0 g kg^{-1}, respectively. Therefore, these sediments presented a very low organic matter content (*i.e.*, 0,8±0,1 % in wet weight and 0,9±0,1 % in dry weight). Phosphate, iron(II) and iron(III)

concentrations in the sediments were traceless. TPH content was analysed in five replicates, showing 2,9±0,3 g kg^{-1} of sediments (dry weight). In figure 13 it is shown an example of a chromatogram of the TPH extracted from the contaminated sediments, where it is possible to notice some GC-resolved compounds, as well as an elevation of the baseline (a lump) between the 18 min and 35 min retention time. This portion of the chromatogram corresponds to an unresolved complex mixture, which has been reported for lubricant or diesel oils, and generally corresponds to compounds which are more resistant to biodegradation [18][202]. Considering the reduced number of resolved peaks, it is possible that the spill was from used/aged oils or lubricants.

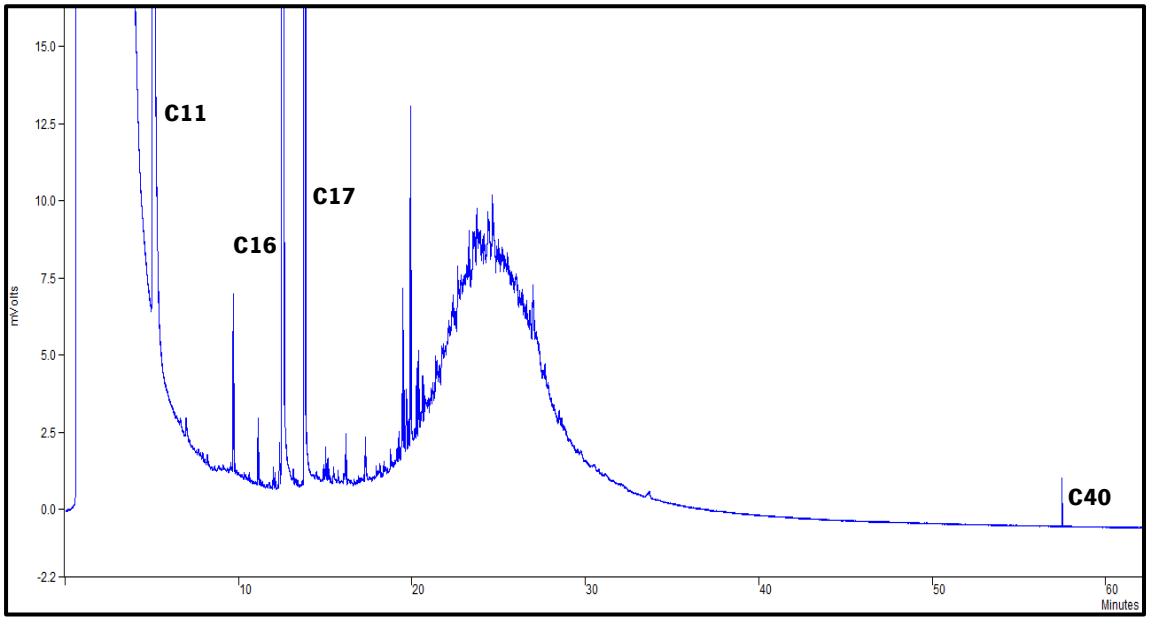


Figure 13 - Example of a GC-FID chromatogram of TPH present in the contaminated sediments. Peaks at 5 min, 14 min and 56 min correspond to undecane (C11, lower integration limit), heptadecane (C17, extraction surrogate) and tetracontane (C40, upper integration limit), respectively.

At the beginning of the experiment, pH was adjusted to around 7, which is important for methanogenic activity. At each sampling point, no relevant differences could be noticed between the biological and abiotic assays, but pH values tended to decrease slightly over time, reaching 6,7-6,9 after 204 days of incubation in all conditions (data not shown). ORP started around +100 mV and decreased to *circa* -100 to -200 mV (figure 14). Slightly higher ORP values were measured in the abiotic controls, possibly related with the absence of biological activity.

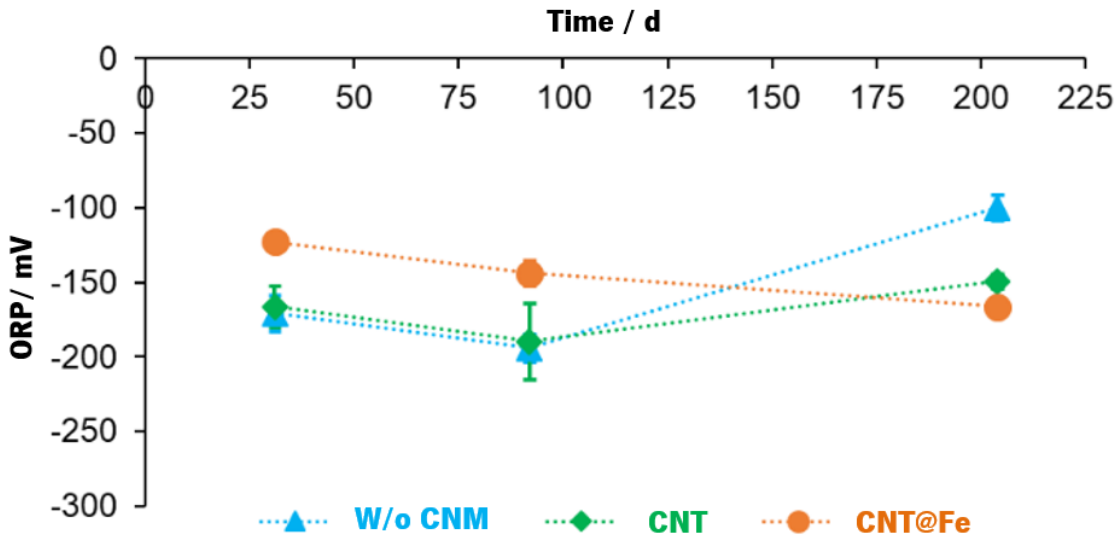


Figure 14 - ORP values measured in the biological assays in the absence of nanomaterials (W/o CNM), and in the presence of CNT or CNT@Fe.

Cumulative methane production was followed over time (figure 15). The values obtained were corrected for standard temperature and pressure (STP) conditions and expressed relatively to the working volume.

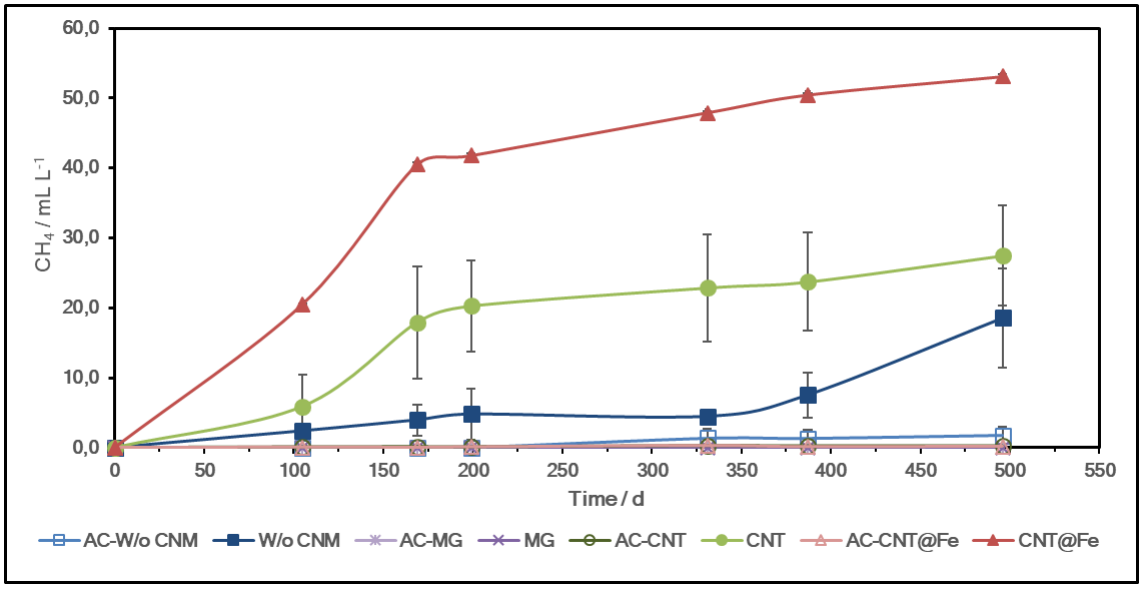


Figure 15 - Cumulative methane production (at standard temperature and pressure conditions) in the assays performed in the absence of nanomaterials (W/o CNM), and in the presence of MG, CNT or CNT@Fe. Measurements in the respective abiotic controls AC-W/o CNM, AC-MG, AC-CNT, AC-CNT@Fe) are also shown. The results presented are the averages and standard deviations for triplicate assays.

Higher methane production was attained in the assays with CNT@Fe and CNT, reaching 40,5±5,8 mL L^{-1} and 18,0±8,0 mL L^{-1}, respectively, after 169 days (figure 15). In comparison with the assays without nanomaterials, at the same time period (4,0±2,2 mL L^{-1}), these value represented an

increment of methane concentration up to 10,2 times and 4,5 times, respectively. Methanogenic activity in the assays W/o CNM occurred mainly after 330 days of incubation (figure 15). These results suggest that conductive nanomaterials such as CNT@Fe and CNT may enhance anaerobic microbial activity in general, ultimately improving methane production, or may have a direct stimulatory effect over the methanogens. In fact, CNT were shown to enhance methane production in pure cultures of hydrogenotrophic methanogens [147] . MG seems to have inhibited the methanogenic activity, since in these assays almost no methane was produced, despite the fact that positive effects of (semi)conductive iron oxide minerals in methane production was previously reported for the conversion of butyrate or a mixture of acetate and ethanol to methane by soil enrichments [184] [203].

TPH analysis was performed in the solid and liquid phase, and the mass values thus obtained were used to calculate the total mass of TPH present in each microcosms. Similar analysis and calculations were made for the spiked hexadecane. All these values are presented in table 9 and table 10 and show the occurrence of variations in the TPH or hexadecane values retrieved from the solid phase. The possibility of hydrocarbons adsorption by the nanomaterials does not seem relevant, since the low amount of nanomaterials used in these experiments should not be sufficient to justify these fluctuations in the measured values, and these were also observed in the assays W/o CNM. Despite these changes, in most situations the total mass of hexadecane or TPH in the microcosms (correspondent to the sum of the values obtained in the solid and liquid phases) stayed relatively constant along the time and for all the conditions studied, revealing some phase exchange dynamics in the microcosms. Overall, total hexadecane and TPH averaged 67,4±8,4 mg and 259,8±46,1 mg, respectively. Both biotic and abiotic assays demonstrate a similar behaviour, suggesting the absence of hydrocarbons biodegradation.

This hypothesis is supported by the methane production on the microcosms as the stoichiometric conversion of hydrocarbons (estimated using hexadecane as model compound, equation 1) did not have its correspondence on methane production, suggesting instead the conversion to methane of some more biodegradable organic matter present in the sediments. In fact, from the stoichiometry of the reaction, 1,225 mL of methane at STP conditions could be expected per mg of hexadecane. Considering that total hexadecane and total TPH values measured in all the microcosms varied between 56-86 mg and 157-341 mg, respectively, cumulative methane production (at STP) between 678-1041 mL L^{-1} and 1900-4126 mL L^{-1} (expressed relatively to the working volume) could be expected from these two groups of hydrocarbons, respectively. These values are much higher

than the measured ones. On the other hand, the estimated amounts of organic matter in the microcosms (which is generally easier to biodegrade), could potentially generate 690 mL L^{-1} of methane (at STP).

$$C_{16}H_{34} + 11{,}25\ H_2O \rightarrow 3{,}75\ HCO_3^- + 12{,}25\ CH4 + 3{,}75\ H^+ \quad \text{(Equation 1)}$$

As discussed before, the chromatogram of figure 13 suggests that TPH were probably from aged oils or lubricants, most possibly composed by compounds difficult to biodegrade. On the other hand, hexadecane is a very stable compound, difficult to activate. Nevertheless, the main reason for the lack of biological degradation probably relies on the absence of a microbial community with the capacity for hydrocarbons degradation. Samples for microbial community analysis were preserved over the experiment, which may contribute to validate this hypothesis.

Table 9 - TPH mass retrieved from solid and liquid phase, for all the conditions studied. The sum of TPH mass retrieved from solid and liquid phases is also shown when extractions from both phases were performed.

	TPH mass (mg)													
	T1 – 32 days				T2 – 92 days				T3 – 204 days				T4 – 504 days	
SAMPLE/REPLICA	solid	Average	Liquid	TOTAL	solid	average	liquid	TOTAL	solid	average	liquid	TOTAL	solid	average
NA 1	207,3	225,2±17,9	-	-	87,4	77,4±10,0	-	-	145,3	138,6±6,7	-	-	61,0	61,7±0,7
NA 2	243,0		26,8	269,9	67,4		-	-	131,9		138,4	270,3	62,5	
AC NA 1	213,6	241,8±28,2	-	-	93,2	92,5±0,7	-	-	138,9	141,4±2,6	-	-	44,6	44,6
AC NA 2	270,0		-	-	91,9		-	-	144,0		-	-	-	
CNT 1	199,0	212,8±13,8	-	-	145,8	149,9±4,1	124,2	270,0	131,1	124,9±6,2	-	-	22,0	37,1±15,1
CNT 2	226,6		114,1	340,7	154,0		-	-	118,7		38,0	156,7	52,3	
AC CNT 1	203,2	195,3±7,8	-	-	118,5	117,3±1,2	98,6	217,1	134,4	136,6±2,2	-	-	57,5	57,5
AC CNT 2	187,5		-	-	116,1		-	-	138,8		-	-	-	
MG 1	220,8	209,6±11,1	31,7	252,5	67,4	69,1±1,7	-	-	182,4	189,8±7,4	96,1	278,5	68,2	69,3±1,1
MG 2	198,5		-	-	70,9		-	-	197,2		-	-	70,4	
AC MG 1	194,1	216,3±22,1	-	-	50,7	67,7±17,1	-	-	156,6	166,0±9,4	-	-	54,6	54,6
AC MG 2	238,4		-	-	84,8		-	-	175,4		-	-	-	
CNT@Fe 1	189,8	196,9±7,0	-	-	135,3	138,3±3,1	-	-	127,1	136,0±8,9	134,0	261,1	53,7	56,7±3,1
CNT@Fe 2	203,9		20,0	223,9	141,4		116,6	257,9	145,0		101,1	246,1	59,8	
AC CNT@Fe 1	217,8	212,8±4,9	122,0	339,8	203,9	166,4±37,5	-	-	100,2	123,4±23,2	-	-	45,9	45,9
AC CNT@Fe 2	207,9		-	-	128,9		124,2	253,1	146,5		-	-	-	

Table 10 - Hexadecane mass retrieved from solid and liquid phase, for all the conditions studied. The sum of hexadecane mass retrieved from solid and liquid phases is also shown when extractions from both phases were performed.

	Hexadecane mass (mg)													
	T1 – 32 days				T2 – 92 days				T3 – 204 days				T4 – 504 days	
SAMPLE/REPLICA	solid	Average	liquid	TOTAL	solid	average	liquid	TOTAL	solid	average	liquid	TOTAL	solid	average
NA 1	38,3	39,2±0,9			75,5	75,2±0,3			46,3	50,6±4,3			40,2	35,4±4,8
NA 2	40,0		27,1	67,1	74,9				55,0		2,4	57,3	30,6	
AC NA 1	29,2	34,9±5,7			68,8	71,1±2,4			62,9	61,8±1,1			32,8	32,8
AC NA 2	40,6				73,5				60,7					
CNT 1	59,9	63,6±3,7			56,1	66,6±10,6	0,3	56,4	66,5	64,1±2,3			54,7	46,7±8,1
CNT 2	67,4		0,8	68,2	77,2				61,8		11,3	73,2	38,6	
AC CNT 1	61,8	54,2±7,6			68,5	69,5±1,0	0,6	69,1	49,6	56,6±7,0			44,0	44,0
AC CNT 2	46,6				70,5				63,5					
MG 1	46,8	39,8±7,0	12,0	58,8	69,5	66,4±3,1			75,7	81,0±5,3			54,2	46,4±7,8
MG 2	32,7				63,4				86,3				38,6	
AC MG 1	56,4	52,3±4,1			69,5	69,7±0,2			71,6	72,2±0,6			41,5	41,5
AC MG 2	48,3				69,9				72,8					
CNT@Fe 1	48,1	52,3±4,2			60,1	61,9±1,8			78,4	82,0±3,6	1,2	79,6	44,9	49,4±4,5
CNT@Fe 2	56,5		8,1	64,6	63,7		0,4	64,1	85,6		0,2	85,8	53,9	
AC CNT@Fe 1	67,1	59,3±7,8	0,9	68,0	72,6	67,8±4,8			63,4	68,7±5,3			45,6	45,6
AC CNT@Fe 2	51,6				63,0		1,4	64,5	74,0					

4.4 Conclusions

Recent contaminated sites by hydrocarbons are a major problem that requires bold approaches to reduce or avoid the toxicity to the environment. *Ex situ* anaerobic bioremediation of these oil-contaminated matrixes may combine their treatment with methane production. Nevertheless, these are generally slow processes and thus, in this work, CNM addition was tested as a strategy to enhance hydrocarbons conversion to methane.

TPH and hexadecane mass values, retrieved from the solid or liquid phases of the microcosms, presented slight variations over the time. However, the total mass of these compounds (*i.e.*, the sum of the values obtained from the solid and liquid phases) did not showed important changes for all conditions tested, both in biological assays and abiotic controls, which point to the absence of hydrocarbons degradation.

Nevertheless, assays amended with CNT@Fe reached a cumulative methane production of 40,5±5,8 mL L^{-1} after 169 days of incubation, which was 10,2 times higher when compared with the assays W/o CNM. In the presence of CNT, cumulative methane production reached 18,0±8,0 mL L^{-1} at the same time point, which represented an increase of 4,5 times relatively and W/o CNM assays. Methanogenic activity in the assays W/o CNM occurred mainly after 330 days of incubation. MG seems to have inhibited the methane production by this microbial community.

These results show that CNT@Fe and CNT stimulated the methanogenic activity of the autochthonous microbial community and thus it would be an interesting approach to combined these nanomaterials with bioaugmentation with bacterial strains capable of performing hydrocarbons biodegradation, boosting *ex situ* anaerobic treatment of these contaminated matrixes coupled to value recover as biogas.

CHAPTER V

Microbial Conversion of Oily Wastes to Methane: Effect of Conductive Ferric Nanomaterials

Martins VR, Martins G, Castro AR, Soares OSGP, Pereira MFR, Pereira L, Alves MM, Cavaleiro AJ (2019) Microbial conversion of oily wastes to methane: effect of ferric nanomaterials. In Wastes: Solutions, Treatments and Opportunities III. C. Vilarinho, F. Castro, M. Gonçalves and A.L. Fernando (Eds.), CRC Press, Taylor & Francis Group, London, UK., 339-345.

Abstract

Petroleum-based oily wastes are generated by the oil industry and can be treated/valorized by anaerobic microbial conversion to methane. However, this process is generally slow. Conductive nanomaterials were reported to accelerate the interspecies electron transfer in anaerobic communities and therefore their addition to anaerobic processes treating hydrocarbons may also be advantageous. In this work, two conductive ferric nanomaterials (magnetite and carbon nanotubes impregnated with 2 % iron) were tested in microcosms amended with hexadecane and 1-hexadecene. Assays were also made with palmitate, acetate and H_2/CO_2, which are intermediates of hydrocarbons biodegradation. Apart from hexadecane, methane was produced at close-to-stoichiometric amounts for each of the substrates tested. Methane production rates were similar with and without the nanomaterials, possibly due to the inability of the microorganisms to receive/transfer electrons to the materials in this microbial community, suggesting that electron transfer occurred indirectly via soluble electron shuttles (*e.g.*, hydrogen or formate).

5.1. Introduction

The intense activity of the oil industry generates substantial amounts of petroleum-based oily wastes [204][205]. The management of these wastes is a concerning issue in the oil and gas sector, currently restricted by stringent regulations. Anaerobic treatment of these oily wastes is an attractive option since it can couple organic treatment with the recovery of bioenergy through methane production. Hydrocarbons biodegradation to methane is performed by complex microbial communities, where different groups of microorganisms interact in a series of metabolic steps that culminate in methane production. After hydrocarbons activation, these compounds are converted into smaller molecules such as short-chain fatty acids, alcohols or hydrogen by fermentative bacteria [206]. Further degradation of these intermediates to methane is restricted by thermodynamics, and only becomes feasible when hydrogenotrophic methanogens are present, decreasing the hydrogen partial pressure [206]. Therefore, a close syntrophic relationship between bacteria and methanogenic archaea is essential [206]. The overall syntrophic reactions yield extremely low Gibbs free energy, and thus methanogenic hydrocarbon degradation typically proceeds at very low rates [206].

Iron oxide nanomaterials exist in many forms in nature, *e.g.*, magnetite (Fe_3O_4), maghemite (γ-Fe_2O_3), and hematite (α-Fe_2O_3) are the most common forms. Iron oxide nanomaterials presents low toxicity, chemical inertness and biocompatibility, which shows an enormous potential in combination with biotechnology [207]. Iron oxide nanomaterials have wide application in wastewater treatment. Magnetism is a unique physical property which could influence the property of the pollutants in the water [208]. Magnetism reduce the cost on the recycling of materials. Iron oxide nanomaterials are used as nanosorbents for heavy metals and organic compounds [209][210]. Iron oxide nanomaterials coated with carbon materials integrate the abilities from carbon nanomaterials and iron oxide nanomaterials. The modification and chemical treatment can enhance the ability of iron oxide NMs, like absorption capacity, biocompatibility. It has been applied on extracting of trace polycyclic aromatic hydrocarbons and reduction of industrial dye [210][189].

Conductive ferric minerals have been reported to accelerate the syntrophic conversion to methane of diverse substrates, such as volatile fatty acids (acetate, propionate and butyrate) and benzoate (an intermediate of benzene degradation), as reviewed recently by Martins and collaborators [189]. However, the role of these materials on syntrophic hydrocarbons biodegradation to methane has never been studied. The mechanisms underlying the stimulating effects of ferric minerals are not clearly

understood and appear to depend on the crystallinity and conductivity of the iron(III) form. Several authors suggested that conductive ferric minerals may stimulate direct interspecies electron transfer (DIET) in syntrophic processes, by conducting the electrons between electron donating and electron accepting microorganisms [184][186]. A similar effect was also described for some conductive carbon materials, namely granular activated carbon, biochar and carbon cloth [189], but recently Salvador and collaborators [147] showed that conductive multi-walled carbon nanotubes (CNT) can directly stimulate methanogens when grown in pure culture, which may constitute an alternative or complementary mechanism of methanogenesis stimulation. Preliminary results in our research group also showed higher methane production rates in pure cultures of methanogens amended with magnetic CNT@Fe (CNT impregnated with 2 % of iron).

The potential of two different conductive ferric nanomaterials (magnetite (MG) and CNT@Fe) to accelerate hydrocarbons biodegradation to methane was investigated in this work. The two nanomaterials chosen present magnetic properties, which facilitate their recovery in waste treatment processes by applying a magnetic field [188]. No toxic effects of these materials to the environment have been reported.

5.2. Materials and methods

5.2.1. Conductive ferric nanomaterials

MG (97 %, Sigma-Aldrich, 50-100 nm particle size) and CNT@Fe were tested in this study. The CNT@Fe were synthesized at the University of Porto, and their characterization was published by Pereira and collaborators [188].

5.2.2. Effect of conductive ferric nanomaterials on anaerobic hydrocarbons degradation

Anaerobic microcosms were prepared in 120 mL glass bottles containing the nanomaterial (MG or CNT@Fe, 1 g L^{-1}) and anaerobic bicarbonate buffered mineral salt medium (50 mL, as described by Paulo and collaborators [106]). Sludge from a real treatment plant performing *ex situ* bioremediation of hydrocarbon-contaminated groundwater was mixed with granular sludge from the sludge digester of

a brewery wastewater treatment plant (1:3 v/v), and used as inoculum (5 g) at a final volatile solids (VS) content of 5 g L^{-1}, in the microcosms. The presence of hexane-extractable hydrocarbons in the sludge from the real treatment plant was confirmed by GC analysis (figure 16), corresponding to a total petroleum hydrocarbons (TPH) content of 0,08±0,03 g g^{-1} (wet weight). The bottles were sealed with *Viton* rubber stoppers and aluminium crimp caps, and the headspace was flushed with N_2/CO_2 (80:20 % v/v), at a final pressure of 1,7x10^5 Pa. Before incubation, the medium was reduced with $Na_2S.9H_2O$ (0,8 mmol L^{-1}).

Hexadecane (99 %, Sigma-Aldrich, 1 mmol L^{-1}) or 1-hexadecene (99 %, Sigma-Aldrich, 1 mmol L^{-1}) were added as model hydrocarbons. Assays amended with sodium palmitate (≥ 98,5 %, Fluka, 1 mmol L^{-1}), H_2/CO_2 (80:20 % v/v, 1,7x10^5 Pa final pressure) or acetate (99 %, Sigma-Aldrich, 20 mmol L^{-1}) were also performed. In the assays with sodium palmitate, after 54 days of incubation the added substrate was completely degraded, and thus a second addition of this electron donor was performed. For that, the headspace of the bottles was flushed and pressurized with N_2/CO_2 (80:20 % v/v, 1,7x10^5 Pa) under sterile conditions before new substrate addition. Control assays without nanomaterials and blank assays without any added substrate were also prepared. All tests were performed in triplicate. The bottles were incubated upside down at 37 °C, with shaking (120 min^{-1}) in the dark. Methane production was measured along the time; volatile fatty acids (VFA) and hydrocarbons concentrations were quantified at the end of the experiments.

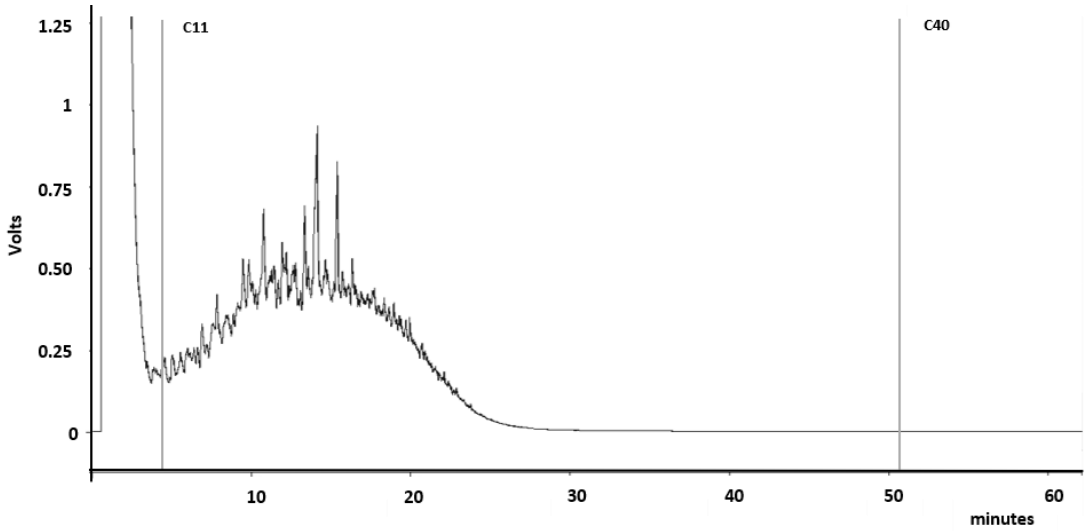


Figure 16 - GC chromatogram of TPH extracted from 20 g of sludge obtained from a groundwater treatment plant performing *ex situ* bioremediation. Retention time for undecane (C11) and tetracontane (C40) are 5 and 50,2 minutes, respectively.

5.2.3. Analytical methods

Methane concentration in bottles headspace was measured using a gas chromatograph (Shimadzu, Kyoto, Japan) equipped with a flame ionization detector (FID) and a PoraPak Q column (80/100 mesh column, 2 m x 1/8 in, 2 mm, stainless steel) with nitrogen and argon as carrier gases at 30 mL min^{-1} and 5 mL min^{-1}, respectively. The injector, column and detector temperatures were 110 °C, 35 °C and 220 °C, respectively. VFA were quantified in liquid samples after centrifugation and filtration by high-performance liquid chromatography (HPLC Jasco equipment) with UV detection, as described by Paulo and collaborators [106]. Hydrocarbons were analysed in the liquid and solid phases of the microcosms, which were separated by decantation. The liquid samples were sequentially extracted three times with hexane using separatory funnels, as described by Castro and collaborators [211]. The solid samples were shaken with hexane for 4 hours at 120 rotations per minute in Schott flasks [199]. All the extracts were cleaned using Sep-Pak Florisil® cartridges (Waters, Milford, MA) and evaporated in TurboVap® LV (Biotage, Uppsala, Sweden). Hydrocarbons were quantified in a gas chromatograph with a FID, as detailed in Paulo and collaborators [106].

5.3. Results and Discussion

Anaerobic conversion of hydrocarbons to methane has been described as a syntrophic process. Therefore, the presence of an active methanogenic community is essential for ex-tensive biodegradation of these compounds. The methanogenic activity of the inoculum used in this study was evaluated in microcosms supplemented with acetate (figure 17a) or with a mixture of H_2/CO_2 (figure 17b), either in the presence or absence of the conductive ferric nanomaterials.

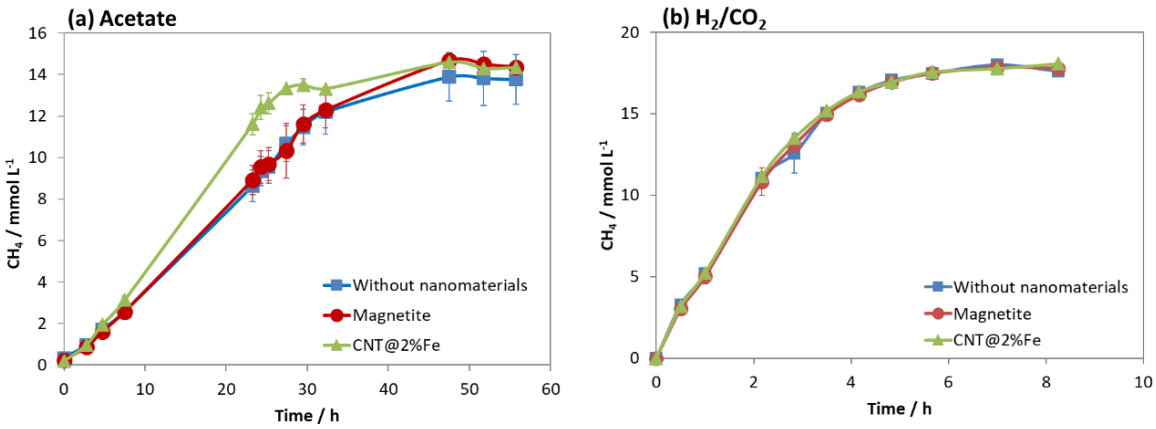


Figure 17 - Cumulative methane production in the assays amended with acetate (a) or H_2/CO_2 (b). The results presented are the averages and standard deviations for triplicate assays.

In the absence of nanomaterials, the cumulative methane production stabilized after 35-45 hours and after 5 hours of incubation in the assays supplemented with acetate and H_2/CO_2, respectively (figure 17a and 17b), corresponding to a methanogenic activity of 9,2±0,6 and 125.2±19 mmol L^{-1} day^{-1}. The addition of CNT@Fe accelerated acetoclastic methanogenesis up to 13,7±0,4 mmol L^{-1} day^{-1}, representing a 1,5 times faster methane production comparatively to the assays with magnetite or in the absence of the materials. The addition of the nanomaterials did not stimulate the methane production from H_2/CO_2 (Figure 17b), possibly due to the good initial activity of the hydrogenotrophic methanogenic community.

High methane production (16±2 mmol L^{-1}) was measured in the blank assays during the first 19 days of incubation (figures 18 and 19), being similar either in the presence or in the absence of nanomaterials. As such, average values were calculated with all the blanks (figures 18 and 19). The residual substrate was composed by hydrocarbons (figure 16) and probably by other more easily biodegradable compounds. After this period, methane production proceeded at a much slower rate, probably deriving from more recalcitrant compounds or from the mineralization of dead cells (endogenous respiration).

In the assays with palmitate (figure 18), the cumulative methane production measured after 19 days (discounting the methane produced in the blanks in the same time period) accounted for the degradation of 64-95 % of the added substrate, considering the stoichiometry of palmitate conversion to methane (equation 2).

$$C_{16}H_{31}O_2^- + 7\ H_2O + H^+ \rightarrow 11{,}5\ CH_4 + 4{,}5\ CO_2 \qquad (2)$$

Moreover, more than 50 % of the palmitate had been degraded to methane already after 8 days of incubation. These results show that the microbial community present was capable of effectively degrading LCFA and performing efficiently the necessary syntrophic relationships between the LCFA-degrading bacteria and the methanogens. Considering that the presence of residual substrate could influence the observed methane production rates, a second palmitate addition (marked with the vertical dotted line in figure 18) was made, that confirmed the previous observations. No VFA were detected in the medium before the second substrate addition, neither at the end of the incubations. This efficient conversion of intermediates to methane suggests an efficient electron transfer and is probably the reason why the addition of the nanomaterials did not improve the methane production from palmitate (figure 18).

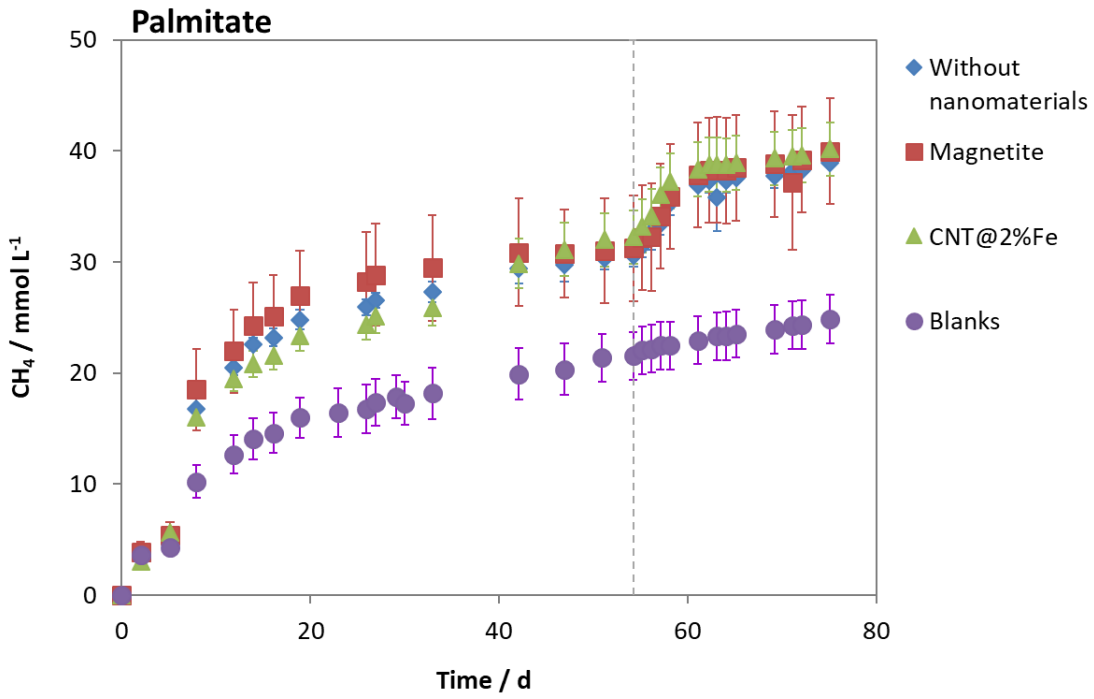


Figure 18 - Cumulative methane production in the assays amended with palmitate and in the blank assays. For the blanks, the values shown represent the average of all the assays performed (in the presence and in the absence of nanomaterials). The vertical dotted line represents the moment of second palmitate addition.

In the assays amended with 1-hexadecene, the cumulative methane production measured in the first 80 days of incubation was similar or slightly lower than the values recorded in the blanks (figure 19). This suggests that the added hydrocarbon was not being degraded and even slightly inhibited the degradation of the residual substrate. After that and until day 163 of incubation (*i.e.*, in 84 days) methane was produced in accumulated amounts that match the theoretical value expected from the degradation of the added 1-hexadecene (equation 3).

$$C_{16}H_{32} + 8H_2O \rightarrow 12\ CH_4 + 4CO_2 \qquad (3)$$

The almost complete 1-hexadecene biodegradation was confirmed by GC analysis, which showed the absence of this compound in the liquid and solid phases of all the assays, with the exception of the solid phase of CNT@Fe assays, where it could be detected in low amounts (figure 20). Moreover, no VFA could be detected in the medium at the end of the experiments.

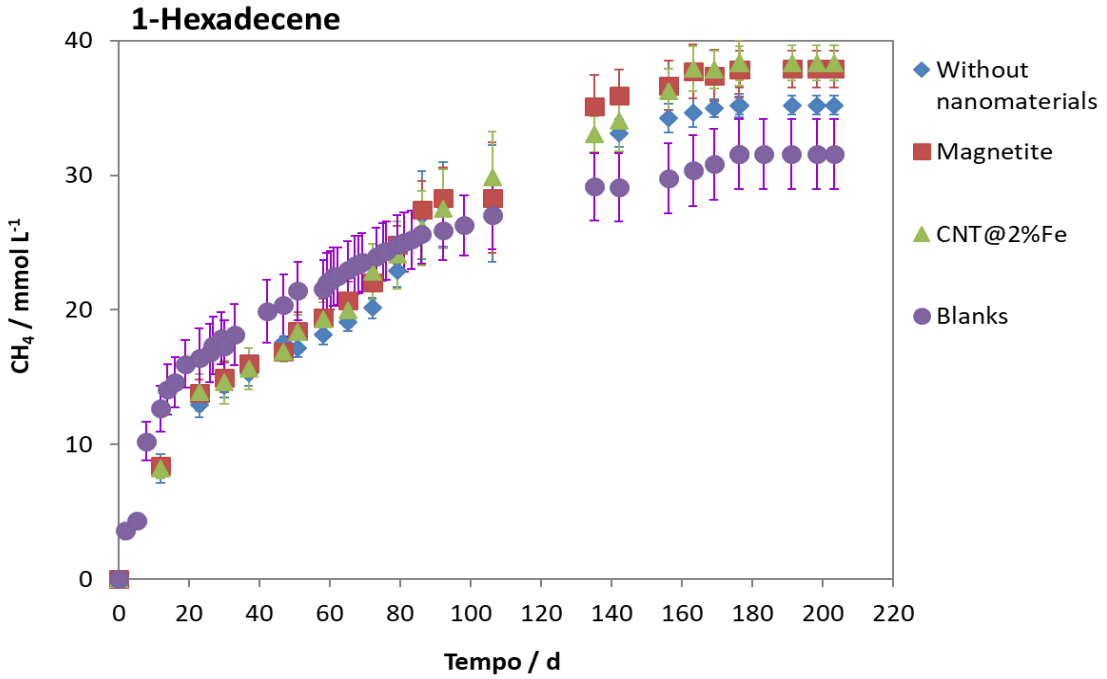


Figure 19 - Cumulative methane production in the assays amended with 1-hexadecene and in the blank assays. For the blanks, the values shown represent the average of all the assays performed (in the presence and in the absence of nanomaterials).

These are interesting results, since methanogenic degradation of 1-hexadecene is reported as a slow process, generally requiring more than 100 days for the complete conversion of 1 to 2 mmol L^{-1} to methane [106][181]. Again, the observed absence of stimulatory effect of the nanomaterials on the methanogenesis can possibly be due to the occurrence of indirect interspecies electron transfer via soluble electron shuttles (*e.g.*, hydrogen or formate). Moreover, the microorganisms present in the community may not be able to transfer/receive the electrons to/from the materials. Not all microorganisms are able to perform DIET, and the research available suggests that this may be the case for most of the anaerobic microorganisms. In fact, DIET was only clearly demonstrated in co-

cultures of *Geobacter metallireducens* with *Methanosaeta harundinacea* or *Methanosarcina barkeri*, and frequently *Geobacter* sp. (the most well know electroactive microorganisms) are not detected in improved methane production driven systems [189]. In addition, experimental evidences point out for the inability of some *Syntrophomonas* [147] and *Pelobacter* species [212] to participate in DIET.

In the assays with hexadecane, methane production was similar in all the experiments, including in the blanks, which suggests the absence of hexadecane biodegradation. This is probably related with the fact that hexadecane is highly stable and inert, and other authors have reported very long incubation periods (*e.g.*, 810 days, [28]) as necessary for the conversion of hexadecane to methane in batch assays similar to the ones performed in this experiment.

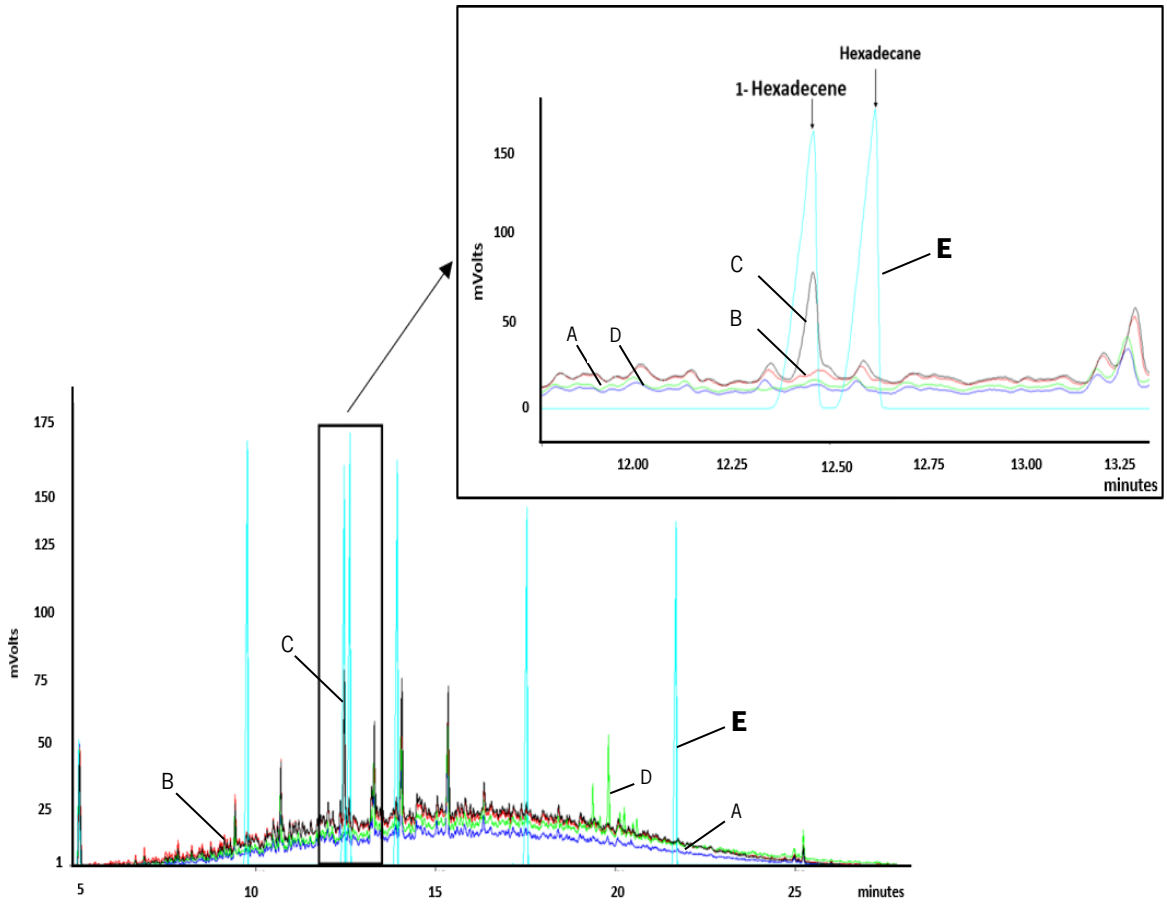


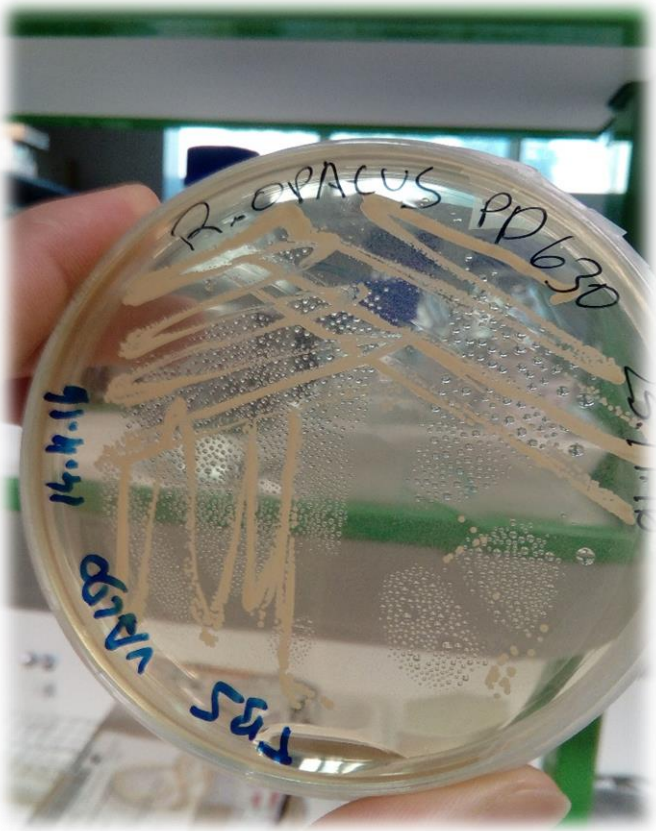
Figure 20 - GC chromatograms of TPH extracted from the solid phase of the microcosms amended with 1-hexadecene, at the end of the assays: without nanomaterials (A), with magnetite (B), with CNT@2%Fe (C) and blank (D). Retention time for undecane (C11) and tetracontane (C40) are 5 and 50,2 minutes, respectively. The chromatogram of an alkanes' mixture, containing 1-hexadecene and hexadecane at 100 mg L^{-1} each, was also included, for comparison (E).

5.4 Conclusions

The methanogenic community studied exhibited good syntrophic and methanogenic activity, being capable of performing palmitate and 1-hexadecene conversion to methane in less than 13 and 84 days, respectively. A stimulating effect of the nanomaterials on the metabolism of this microbial community was not observed, possibly due to the occurrence of indirect interspecies electron transfer via soluble electron shuttles. A more detailed knowledge on the mechanisms underlying the effect of conductive material on methanogenesis, and of the microorganisms involved in these processes, is essential for the development of new strategies targeting the conversion of oily wastes to methane.

CHAPTER VI

Final Conclusions and Future Perspectives



Environmental contamination by hydrocarbons is a major concern for today's societies as oil spills cause significant damages into ecosystems affecting not only wildlife, but also mankind, with the release of toxic compounds that can affect people's health. Hydrocarbons high energetic content can be exploited by several microorganisms, either aerobic or anaerobic, which are capable of degrading a variety of hydrocarbon-contaminated matrixes, wastes and wastewaters, with positive contribution for both in situ and ex situ decontamination processes.

Aerobic and anaerobic hydrocarbons biodegradation were exploited in this research, and the results obtained showed that aerobic hydrocarbons degradation by *A. borkumensis* SK2 and *R. opacus* B4 can be enhanced by using corksorb. Moreover, autochthonous bacteria present in this material may also have a role in hydrocarbons biodegradation. As different bacteria were found in corksorb assays it would be interesting to know how and where they are located through corksorb structure. Understanding the effects of corksorb in complex bacterial communities, namely on the autochthonous communities present in the environment, it is also important to improve bioremediation of hydrocarbons contaminated sites. Another interesting approach would be to inoculate the corksorb with hydrocarbonoclastic bacteria before applying it to the environment, working not only as biosorbent but also as bioaugmentation facilitator. Understand how corksorb and hydrocarbonoclastic bacteria interact when a complex mixture of hydrocarbons is the contaminant agent also constitute another challenging research opportunity. It would be also interesting to understand if and how corksorb could change microbial growth and hydrocarbons degradation in oil-contaminated soils, where phytoremediation is already in progress, as cork is a natural product and increases soil porosity. Corksorb autochthonous bacteria may augment the rhizosphere, helping these microbial communities in degrading hydrocarbons, enhancing in situ bioremediation. In addition, the application of this biosorbent in the operation of biological reactors treating hydrocarbon contaminated matrixes might as well improve the performance of these treatment systems and promote *ex situ* bioremediation of contaminated sites.

Regarding anaerobic hydrocarbons degradation, CNM were tested as a possible strategy to improve and accelerate these biological reactions. These materials were not able to enhance hydrocarbons conversion to methane, neither by an autochthonous microbial community in hydrocarbon-contaminated sediments, nor by sludge from wastewater treatment. Nevertheless, it is relevant to note that the presence of the nanomaterials did not induce any toxic effect on the methanogenic communities studied. Even more, in the experiments with the recently contaminated sediments, CNT@Fe and CNT stimulated the methanogenic activity of the autochthonous microbial community, an interesting effect that deserves more studies. The relevance of using CNM to stimulate the

methanogenic activity of mixed communities, and in particular of hydrocarbon-degrading communities, is largely dependent on the composition of the community itself. Therefore, detailed knowledge on the microorganisms involved in these processes is essential to assist on decision-making, as well as for the development of new strategies targeting the conversion of oily wastes to methane. It is important to investigate the reuse of biosorbents/nanomaterials in treatment/bioremediation processes of hydrocarbons, coupled with an economic viability study, which will allow to have an important notion of the costs implied at real scale applications.

Further work on hydrocarbons biodegradation processes is required, as knowledge on aerobic or anaerobic mixed communities, as well as on microbial interactions, in oily-wastes treatment still needs to be improved. For that, the use of bioreactors could represent an important advancement e.g., bioreactors operation with petroleum-contaminated wastes. Facultative bacteria may assist in biosurfactants production as well as in the first steps of hydrocarbon activation and biodegradation and so, its incorporation/stimulation in biological processes may be advantageous. Methane production from hydrocarbon-contaminated wastes allows the retrieval of the energy value contained in oily-wastes, and thus is worth to be explored, contributing to promote the restoration of affected areas by oil contamination, combined with waste treatment and value recovery as methane, in *ex situ* bioremediation processes.

The development of mathematical models may also contribute for the improvement of bioremediation strategies, working as tools for anticipating hydrocarbons removal, mobility and biodegradability in soils, sediments and groundwater after an oil spill. Mathematical models can also be applied for predicting the effectiveness of adding CNM to ex situ bioremediation systems, or other bioremediation strategies. Meta-omics analysis may also be an improvement in bioremediation strategies as diverse communities need to be study in order to understand which microorganisms are present and who is doing what, following the pathways of hydrocarbons degradation, constructing a microbial network.

It is important to design and develop new strategies for enhance hydrocarbons bioremediation, as the recovery of polluted areas by natural processes is generally difficult and slow, as the world urges to find solutions for oil spills decontamination and treatment for the contaminated residues generated by those.

References

[1] "U.S. Energy Administration Information" 2020. [Online]. Available: https://www.eia.gov/outlooks/steo/report/global_oil.php. [Accessed: 15-Jul-2020].

[2] M. Nicola, Zaid Alsafi, Catrin Sohrabi, Ahmed Kerwan, Ahmed Al-Jabir, Christos Iosifidis, Maliha Agha and Riaz Aghaf, "The socio-economic implications of the coronavirus pandemic (COVID-19): A review" *Int. J. Surg.*, vol. 78, pp. 185–193, 2020.

[3] Laurence Mansuy, and R. Paul Philp, J. Allen, L. Mansuy, R. P. Philp, and J. Allen, "Source identification of oil spills based on the isotopic composition of individual components in weathered oil samples" *Environ. Sci. Technol.*, vol. 31, no. 12, pp. 3417–3425, 1997.

[4] C. H. Chaîneau, C. Yepremian, J. F. Vidalie, J. Ducreux, and D. Ballerini, "Bioremediation of a crude oil-polluted soil: Biodegradation, leaching and toxicity assessments" *Water. Air. Soil Pollut.*, vol. 144, no. 1–4, pp. 419–440, 2003.

[5] S. Kalliola and B. Doshi, "A review of bio-based materials for oil spill treatment" vol. 135, pp. 262–277, 2018.

[6] T. R. Annunciado, T. H. D. Sydenstricker, and S. C. Amico, "Experimental investigation of various vegetable fibers as sorbent materials for oil spills" *Mar. Pollut. Bull.*, vol. 50, no. 11, pp. 1340–6, 2005.

[7] H. Ribeiro, C. M. R. Almeida, A. P. Mucha, and A. A. Bordalo, "Influence of different salt marsh plants on hydrocarbon degrading microorganisms abundance throughout a phenological cycle" *Int. J. Phytoremediation*, vol. 15, no. 8, pp. 715–728, 2013.

[8] R. T. Carson and R. C. Mitchell, "Contingent valuation and lost passive use : Damages from the Exxon Valdez oil spill" pp. 257–286, 2003.

[9] W. E. Wilhelm, "A Strategic , Area-wide Contingency Planning Model for Oil Spill Cleanup Operations with Application Demonstrated to the Galveston Bay Area" vol. 27, no. 4.

[10] E. H. Owens, Chapter 24 - Shoreline Countermeasures, *Oil Spill Science and Technology*, Mervin Fingas, Gulf Professional Publishing, Pages 907-921, 2011.

[11] G. Guidi, M. Sliskovic, A. C. Violante, and L. Vukic, "Best available techniques (BATs) for oil spill response in the Mediterranean Sea: calm sea and presence of economic activities" *Environ. Sci. Pollut. Res.*, vol. 23, no. 2, pp. 1944–1953, 2016.

[12] S. Z. YANG *et al.*, "Bioremediation of Oil Spills in Cold Environments: A Review" *Pedosphere*, vol. 19, no. 3, pp. 371–381, 2009.

[13] O. K. Karakasi and A. Moutsatsou, "Surface modification of high calcium fly ash for its application in oil spill clean up" *Fuel*, vol. 89, no. 12, pp. 3966–3970, 2010.

[14] E. A. Ivanova, E. S. Lobakova, R. K. Idiatulov, T. N. Shapiro, and D. A. Sandzhieva, "Biocomposite Materials for Purification of Aqueous Media Contaminated with Hydrocarbons" vol. 59, no. 4, pp. 420–426, 2019.

[15] Y. Ji, G. Mao, Y. Wang, and M. Bartlam, "Structural insights into diversity and *n*-alkane biodegradation mechanisms of alkane hydroxylases" *Front. Microbiol.*, vol. 4, p. 58, 2013.

[16] I. N. Sierra-García, J. C. Alvarez, S. P. De Vasconcellos, A. P. De Souza, E. V. Dos Santos Neto, and V. M. De Oliveira, "New hydrocarbon degradation pathways in the microbial metagenome from brazilian petroleum reservoirs" *PLoS One*, vol. 9, no. 2, p. e90087, 2014.

[17] S. M. Mbadinga, L. Y. Wang, L. Zhou, J. F. Liu, J. D. Gu, and B. Z. Mu, "Microbial communities involved in anaerobic degradation of alkanes" *Int. Biodeterior. Biodegrad.*, vol. 65, no. 1, pp. 1–13, 2011.

[18] W. Weisman, "Analysis of petroleum hydrocarbons in environmental media", vol. 1. Amherst Scientific Publishers, 1998.
[19] I. M. Head, "Bioremediation: Towards a credible technology" *Microbiology*, vol. 144, no. 3, pp. 599–608, 1998.
[20] R. M. M. Atlas, "Microbial degradation of petroleum hydrocarbons: an environmental perspective" *Microbiol. Rev.*, vol. 45, no. 1, pp. 180–209, 1981.
[21] C. E. Zobell, "On hydrocarbons'," *SCripps Inst. Oceanogr. Univ. Calif.*, no. 295, pp. 1–49, 1946.
[22] P. F. Greenwood, S. Wibrow, S. J. George, and M. Tibbett, "Hydrocarbon biodegradation and soil microbial community response to repeated oil exposure" *Org. Geochem.*, vol. 40, no. 3, pp. 293–300, 2009.
[23] A. Brune, P. Frenzel, and H. Cypionka, "Life at the oxic-anoxic interface: Microbial activities and adaptations" *FEMS Microbiol. Rev.*, vol. 24, no. 5, pp. 691–710, 2000.
[24] C. Cravo-Laureau, V. Grossi, D. Raphel, R. Matheron, and A. Hirschler-Réa, "Anaerobic *n*-alkane metabolism by a sulfate-reducing bacterium, *Desulfatibacillum aliphaticivorans* strain CV2803T" *Appl. Environ. Microbiol.*, vol. 71, no. 7, pp. 3458–3467, 2005.
[25] V. Grossi, C. Cravo-Laureau, R. Guyoneaud, A. Ranchou-Peyruse, and A. Hirschler-Réa, "Metabolism of *n*-alkanes and *n*-alkenes by anaerobic bacteria: a summary" *Org. Geochem.*, vol. 39, no. 8, pp. 1197–1203, 2008.
[26] A. V Callaghan *et al.*, "Diversity of benzyl-and alkylsuccinate synthase genes in hydrocarbon-impacted environments and enrichment cultures" *Environ. Sci. Technol.*, vol. 44, no. 19, pp. 7287–7294, 2010.
[27] D. M. Jones *et al.*, "Crude-oil biodegradation via methanogenesis in subsurface petroleum reservoirs" *Nature*, vol. 451, no. 7175, pp. 176–180, 2008.
[28] K. Zengler, H. H. Richnow, R. Rosselló-Mora, W. Michaelis, and F. Widdel, "Methane formation from long-chain alkanes by anaerobic microorganisms" *Nature*, vol. 401, no. 6750, pp. 266–269, 1999.
[29] Regional Marine Pollution Emergency Response Centre for the Mediterranean Sea (REMPEC), "Guidelines on Oil Spill Waste Management 2010" 2010.
[30] A. M. Caunhye, X. Nie, and S. Pokharel, "Socio-Economic Planning Sciences Optimization models in emergency logistics : A literature review" *Socioecon. Plann. Sci.*, vol. 46, no. 1, pp. 4–13, 2012.
[31] L. Palinkas, M. Downs, J. Petterson, and J. Russell, "Social, cultural, and psychological impacts of the Exxon Valdez oil spill" *Hum. Organ.*, vol. 52, no. 1, pp. 1–13, 1993.
[32] R. and E. on A. W. P. Cedre, the Centre of Documentation, "Guidance on Waste Management during a shoreline pollution incident" *Waste Manag.*, no. December, 2011.
[33] S. S. Banerjee, M. V. Joshi, and R. V. Jayaram, "Treatment of oil spill by sorption technique using fatty acid grafted sawdust" *Chemosphere*, vol. 64, no. 6, pp. 1026–1031, 2006.
[34] C. Cojocaru, M. Macoveanu, and I. Cretescu, "Peat-based sorbents for the removal of oil spills from water surface: Application of artificial neural network modeling" *Colloids Surfaces A Physicochem. Eng. Asp.*, vol. 384, no. 1–3, pp. 675–684, 2011.
[35] "Instituto Nacional de Propriedade Industrial" 2020. [Online]. Available: https://servicosonline.inpi.justica.gov.pt/pesquisas/GetSintesePDF?nord=1990152 [Accessed: 20-October-2020].
[36] A. J. Cavaleiro, T. M. Neves, A. P. Guedes, and M. M. Alves, "Anaerobic co-digestion of cork based oil sorbent and cow manure or sludge" pp. 43–48, 2015.
[37] A. M. A. Pintor *et al.*, "Oil desorption and recovery from cork sorbents" *J. Environ. Chem. Eng.*, vol. 3, no. 4, pp. 2917–2923, 2015.

[38] P. S. S. Porto, R. S. Souza, J. F. J. R. Pesqueira, R. A. R. Boaventura, and V. J. P. Vilar, "Mineral oil recovery from cork granules by a mechanical compression method: Compression cycles analysis" *J. Clean. Prod.*, vol. 147, pp. 442–450, 2017.

[39] R. R. Lessard and G. DeMarco, "The significance of oil spill dispersants" *Spill Sci. Technol. Bull.*, vol. 6, no. 1, pp. 59–68, 2000.

[40] S. Kleindienst, J. H. Paul, and S. B. Joye, "Using dispersants after oil spills: impacts on the composition and activity of microbial communities" *Nat Rev Micro*, vol. 13, no. 6, pp. 388–396, 2015.

[41] E. B. Kujawinski, M. C. Kido Soule, D. L. Valentine, A. K. Boysen, K. Longnecker, and M. C. Redmond, "Fate of dispersants associated with the Deepwater Horizon oil spill" *Environ. Sci. Technol.*, vol. 45, no. 4, pp. 1298–1306, 2011.

[42] A. Aparna, "Effect of Addition of Biosurfactant Produced by *Pseudomonas* sps. on Biodegradation of Crude Oil" vol. 6, pp. 71–75, 2011.

[43] E. Olkowska and J. Namie, "Talanta Analytical procedures for the determination of surfactants in environmental samples" vol. 88, pp. 1–13, 2012.

[44] R. D. C. F. S. Silva, D. G. Almeida, R. D. Rufino, and J. M. Luna, "Applications of Biosurfactants in the Petroleum Industry and the Remediation of Oil Spills" pp. 12523–12542, 2014.

[45] S. Kleindienst, M. Seidel, K. Ziervogel, S. Grim, K. Loftis, S. Harrison, S. Y. Malkin, M. J. Perkins, J. Field, M. L. Sogin, T. Dittmar, U. Passow, P.M. Medeiros and S. B. Joye, "Chemical dispersants can suppress the activity of natural oil-degrading microorganisms," vol. 112, no. 48, 2015.

[46] M. Wilson, L. Graham, C. Hale, E. Maung-Douglass, S. Sempier, and L. Swann, "Perisistence, fate, and effectiveness of dispersants used during the Deepwater Horizon oil spill" *Sea Grant Programs Gulf Mex.*, 2016.

[47] EPA, "Introduction To *in situ* Bioremediation of Groundwater," 2013.

[48] G. F. Da Cruz and A. J. Marsaioli, "Processos naturais de biodegradação do petróleo em reservatórios" *Quim. Nova*, vol. 35, no. 8, pp. 1628–1634, 2012.

[49] S. Mana Capelli, J. P. Busalmen, and S. R. De Sánchez, "Hydrocarbon bioremediation of a mineral-base contaminated waste from crude oil extraction by indigenous bacteria" *Int. Biodeterior. Biodegrad.*, vol. 47, no. 4, pp. 233–238, 2001.

[50] J. Komlos, R. K. Kukkadapu, J. M. Zachara, and P. R. Jaffé, "Biostimulation of iron reduction and subsequent oxidation of sediment containing Fe-silicates and Fe-oxides: Effect of redox cycling on Fe(III) bioreduction" *Water Res.*, vol. 41, no. 13, pp. 2996–3004, 2007.

[51] M. Hassanshahian, G. Emtiazi, G. Caruso, and S. Cappello, "Bioremediation (bioaugmentation / biostimulation) trials of oil polluted seawater : A mesocosm simulation study" *Mar. Environ. Res.*, vol. 95, pp. 28–38, 2014.

[52] J. B. Müller *et al.*, "Combined iron and sulfate reduction biostimulation as a novel approach to enhance BTEX and PAH source-zone biodegradation in biodiesel blend-contaminated groundwater" *J. Hazard. Mater.*, vol. 326, pp. 229–236, 2017.

[53] J. J. Cooney, "The fate of petroleum pollutants in freshwater ecosystems" 1984.

[54] A. A. Al-Majed, A. R. Adebayo, and M. E. Hossain, "A sustainable approach to controlling oil spills" *J. Environ. Manage.*, vol. 113, pp. 213–227, 2012.

[55] S. Hwa, H. Heewook, and R. Jaisoo, "Rhizoremediation of diesel-contaminated soil using the plant growth-promoting rhizobacterium *Gordonia* sp . S2RP-17" pp. 593–601, 2011.

[56] P. J. Harvey *et al.*, "Phytoremediation of Polyaromatic Hydrocarbons, Anilines and Phenols" vol. 9, no. 1, pp. 29–47, 2002.

[57] X.-D. Huang, Y. El-Alawi, D. M. Penrose, B. R. Glick, and B. M. Greenberg, "A multi-process phytoremediation system for removal of polycyclic aromatic hydrocarbons from contaminated

soils" *Environ. Pollut.*, vol. 130, no. 3, pp. 465–476, 2004.
[58] F. Hussain *et al.*, "Combined application of biochar, compost, and bacterial consortia with Italian ryegrass enhanced phytoremediation of petroleum hydrocarbon contaminated soil" *Environ. Exp. Bot.*, vol. 153, no. May, pp. 80–88, 2018.
[59] S. C. Wilson and K. C. Jones, "Bioremediation of soil contaminated with polynuclear aromatic hydrocarbons (PAHs): A review" *Environ. Pollut.*, vol. 81, no. 3, pp. 229–249, 1993.
[60] S. Harayama, Y. Kasai, and A. Hara, "Microbial communities in oil-contaminated seawater" *Curr. Opin. Biotechnol.*, vol. 15, no. 3, pp. 205–214, 2004.
[61] N. Das and P. Chandran, "Microbial degradation of petroleum hydrocarbon contaminants: an overview" *Biotechnol. Res. Int.*, vol. 2011, 2010.
[62] J. Heider, A. M. Spormann, H. R. Beller, and F. Widdel, "Anaerobic bacterial metabolism of hydrocarbons" *FEMS Microbiol. Rev.*, vol. 22, no. 5, pp. 459–473, 1998.
[63] F. Widdel and R. Rabus, "Anaerobic biodegradation of saturated and aromatic hydrocarbons" *Curr. Opin. Biotechnol.*, vol. 12, no. 3, pp. 259–276, 2001.
[64] J. Foght, "Anaerobic biodegradation of aromatic hydrocarbons: Pathways and prospects" *J. Mol. Microbiol. Biotechnol.*, vol. 15, no. 2–3, pp. 93–120, 2008.
[65] A. I. Okoh, "Biodegradation alternative in the cleanup of petroleum hydrocarbon pollutants" *Biotechnol. Mol. Biol.*, vol. 1, no. June, pp. 38–50, 2006.
[66] N. Das, P. Chandran, N. Das, and P. Chandran, "Microbial Degradation of Petroleum Hydrocarbon Contaminants: An Overview," *Biotechnol. Res. Int.*, vol. 2011, pp. 1–13, 2011.
[67] L. C. Ferna, "A field trial for an ex-situ bioremediation of a drilling mud-polluted site" vol. 66, pp. 1595–1600, 2007.
[68] N. Vasudevan and P. Rajaram, "Bioremediation of oil sludge-contaminated soil" vol. 26, pp. 409–411, 2001.
[69] N. E. Kimes, A. V. Callaghan, J. M. Suflita, and P. J. Morris, "Microbial transformation of the deepwater horizon oil spill-past, present, and future perspectives" *Front. Microbiol.*, vol. 5, no. NOV, pp. 1–11, 2014.
[70] I. M. Head, D. M. Jones, and W. F. M. Röling, "Marine microorganisms make a meal of oil" vol. 4, no. March, pp. 173–182, 2006.
[71] R. M. Atlas, D. J. Grimes,T. C. Hazen, J. Spain, J. M. Suflita, A. Reid, "Microbes & Oil Spills" *Environ. Prot.*, pp. 1–13, 2011.
[72] A. Mikolasch, A. Reinhard, A. Alimbetova, A. Omirbekova, L. Pasler, P. Schumann, J. Kabisch, T. Mukasheva, F. Schauer, "From oil spills to barley growth - oil-degrading soil bacteria and their promoting effects" *J. Basic Microbiol.*, vol. 56, no. 11, pp. 1252–1273, 2016.
[73] C. Marchand, M. St-Arnaud, W. Hogland, T. H. Bell, and M. Hijri, "Petroleum biodegradation capacity of bacteria and fungi isolated from petroleum-contaminated soil" *Int. Biodeterior. Biodegrad.*, vol. 116, pp. 48–57, 2017.
[74] S. Kasai, Y., Kishira, H., Sasaki, T., Syutsubo, K., Watanabe, K., Harayama, "Predominant growth of *Alcanivorax* strains in oil contaminated and nutrient supplemented sea water" *Environ. Microbiol.*, vol. 4, no. 3, pp. 141-147., 2002.
[75] J. Xue, Y. Yu, Y. Bai, L. Wang, and Y. Wu, "Marine Oil-Degrading Microorganisms and Biodegradation Process of Petroleum Hydrocarbon in Marine Environments: A Review" *Curr. Microbiol.*, vol. 71, no. 2, pp. 220–228, Aug. 2015.
[76] C. Chayabutra and L. K. Ju, "Degradation of *n*-hexadecane and its metabolites by *Pseudomonas aeruginosa* under microaerobic and anaerobic denitrifying conditions" *Appl. Environ. Microbiol.*, vol. 66, no. 2, pp. 493–498, 2000.
[77] L. N. Warr, J. N. Perdrial, M. C. Lett, A. Heinrich-Salmeron, M. Khodja, "Clay mineral-enhanced bioremediation of marine oil pollution" *Appl. Clay Sci.*, vol. 46, no. 4, pp. 337–345, 2009.

[78] A. Alabresm, Y. P. Chen, A. W. Decho, and J. Lead, "A novel method for the synergistic remediation of oil-water mixtures using nanoparticles and oil-degrading bacteria" *Sci. Total Environ.*, vol. 630, pp. 1292–1297, 2018.

[79] Z. Sejáková, K. Dercová, and L. Tóthová, "Biodegradation and ecotoxicity of soil contaminated by pentachlorophenol applying bioaugmentation and addition of sorbents" *World J. Microbiol. Biotechnol.*, vol. 25, no. 2, pp. 243–252, 2009.

[80] W. W. Mohn, "Indirect bioremediation: Biodegradation of hydrocarbons on a commercial sorbent" *Biodegradation*, vol. 8, no. 1, pp. 15–19, 1997.

[81] J. Pontes, A. P. Mucha, H. Santos, I. Reis, A. Bordalo, M. C. Basto, A. Bernabeu, C. M. R. Almeida, "Potential of bioremediation for buried oil removal in beaches after an oil spill" *Mar. Pollut. Bull.*, vol. 76, no. 1–2, pp. 258–265, 2013.

[82] R. J. Schocher, B. Seyfried, F. Vazquez, and J. Zeyer, "Anaerobic degradation of toluene by pure cultures of denitrifying bacteria" *Arch. Microbiol.*, vol. 157, no. 1, pp. 7–12, 1991.

[83] D. R. Lovley, J. C. Woodward, and F. H. Chapelle, "Stimulated Anoxic Biodegradation of Aromatic-Hydrocarbons Using Fe(III) Ligands" *Nature*, vol. 370, no. 6485, pp. 128–131, 1994.

[84] P. Morgan, S. T. Lewis, and R. J. Watkinson, "Biodegradation of benzene, toluene, ethylbenzene and xylenes in gas-condensate-contaminated groundwater," *Environ. Pollut.*, vol. 82, no. 2, pp. 181–190, 1993.

[85] E. A. Edwards and D. Grbicgalic, "Anaerobic degradation of toluene and *o*-xylene by methanogenic consortium" *Appl. Environ. Microbiol.*, vol. 60, no. 1, pp. 313–322, 1994.

[86] A. M. Spormann and F. Widdel, "Metabolism of alkylbenzenes, alkanes, and other hydrocarbons in anaerobic bacteria" *Biodegradation*, vol. 11, no. 2–3, pp. 85–105, 2000.

[87] T. Biegert, G. Fuchs, and J. Heider, "Evidence that anaerobic oxidation of toluene in the denitrifying bacterium T*hauera aromatica* is initiated by formation of benzylsuccinate from toluene and fumarate" *Eur J Biochem*, vol. 238, no. 3, pp. 661–668, 1996.

[88] H. R. Beller, D. Grbic-Galic, and M. Reinhard, "Microbial degradation of toluene under sulfate-reducing conditions and the influence of iron o the process" *Appl. Environ. Microbiol.*, vol. 58, no. 3, pp. 786–793, 1992.

[89] U. Kunapuli, M. K. Jahn, T. Lueders, R. Geyer, H. J. Heipieper, and R. U. Meckenstock, "*Desulfitobacterium aromaticivorans* sp. nov. and *Geobacter toluenoxydans* sp. nov., iron-reducing bacteria capable of anaerobic degradation of monoaromatic hydrocarbons" *Int. J. Syst. Evol. Microbiol.*, vol. 60, no. 3, pp. 686–695, 2010.

[90] D. R. Lovley, M. J. Baedecker, D. J. Lonergan, I. M. Cozzarelli, E. J. P. Phillips, and D. I. Siegel, "Oxidation of aromatic contaminants coupled to microbioal iron reduction" *Nature*, vol. 239, no. 6222, pp. 297–299,1989.

[91] J. D. Coates, R. T. Anderson, J. C. Woodward, E. J. P. Phillips, and D. R. Lovley, "Anaerobic hydrocarbon degradation in petroleum-contaminated harbor sediments under sulfate-reducing and artificially imposed iron-reducing conditions" *Environ. Sci. Technol.*, vol. 30, no. 9, pp. 2784–2789, 1996.

[92] M. M. Alves *et al.*, "Waste lipids to energy: How to optimize methane production from long-chain fatty acids (LCFA)" *Microb. Biotechnol.*, vol. 2, no. 5, pp. 538–550, 2009.

[93] S. A. Silva, A. J. Cavaleiro, M. A. Pereira, A. J. M. Stams, M. M. Alves, and D. Z. Sousa, "Long-term acclimation of anaerobic sludges for high-rate methanogenesis from LCFA" *Biomass and Bioenergy*, vol. 67, pp. 297–303, 2014.

[94] M. A. Pereira, O. C. Pires, M. Mota, and M. M. Alves, "Anaerobic degradation of oleic acid by suspended and granular sludge: identification of palmitic acid as a key intermediate" *Water Sci. Technol. a J. Int. Assoc. Water Pollut. Res.*, vol. 45, no. 10, pp. 139–144, 2002.

[95] D. Z. Sousa, J. I. Alves, M. M. Alves, H. Smidt, and A. J. M. Stams, "Effect of sulfate on

methanogenic communities that degrade unsaturated and saturated long-chain fatty acids (LCFA)" *Environ. Microbiol.*, vol. 11, no. 1, pp. 68–80, 2009.

[96] J. D. Coates, J. Woodward, J. Allen, P. Philp, and D. R. Lovley, "Anaerobic degradation of polycyclic aromatic hydrocarbons and alkanes in petroleum-contaminated marine harbor sediments" *Appl. Environ. Microbiol.*, vol. 63, no. 9, pp. 3589–3593, 1997.

[97] B. Shin, M. Kim, K. Zengler, K. Chin, W. A. Overholt, L. M. Gieg, K. T. Konstantinidis, J. E. Kostka, "Anaerobic degradation of hexadecane and phenanthrene coupled to sulfate reduction by enriched consortia from northern Gulf of Mexico seafloor sediment" *Sci. Rep.*, vol. 9, no. 1, pp. 1–13, 2019.

[98] K. A. Weber, L. A. Achenbach, and J. D. Coates, "Microorganisms pumping iron: anaerobic microbial iron oxidation and reduction" *Nat. Rev.*, vol. 4, no. iii, pp. 752–764, 2006.

[99] D. R. Lovley and E. J. P. Phillips, "Organic Matter Mineralization with Reduction of Ferric Iron in Anaerobic Sediments" *Appl. Envir. Microbiol.*, vol. 51, no. 4, pp. 683–689, 1986.

[100] D. E. Holmes, C. Risso, J. A. Smith, and D. R. Lovley, "Anaerobic oxidation of benzene by the hyperthermophilic archaeon *Ferroglobus placidus*" *Appl. Environ. Microbiol.*, vol. 77, no. 17, pp. 5926–5933, 2011.

[101] D. R. Lovley and D. J. Lonergan, "Anaerobic oxidation of toluene, phenol, and *p*-cresol by the disssimilatory iron-reducing organism, GS-15" *Appl. Environ. Microbiol.*, vol. 56, no. 6, pp. 1858–1864, 1990.

[102] R. T. Anderson, J. N. Rooney-Varga, C. V. Gaw, and D. R. Lovley, "Anaerobic benzene oxidation in the Fe(III) reduction zone of petroleum- contaminated aquifers" *Environ. Sci. Technol.*, vol. 32, no. 9, pp. 1222–1229, 1998.

[103] S. Kato, R. Nakamura, F. Kai, K. Watanabe, and K. Hashimoto, "Respiratory interactions of soil bacteria with (semi)conductive iron-oxide minerals" *Environ. Microbiol.*, vol. 12, no. 12, pp. 3114–3123, 2010.

[104] S. E. Dyksterhouse, J. P. Gray, R. P. Herwig, J. C. a N. Lara, and J. T. Staley, "Hydrocarbon-Degrading Bacterium from Marine Sediments" *Cultures*, vol. 45, no. 206, pp. 116–123, 1995.

[105] M. Embree, H. Nagarajan, N. Movahedi, H. Chitsaz, and K. Zengler, "Single-cell genome and metatranscriptome sequencing reveal metabolic interactions of an alkane-degrading methanogenic community" vol. 8, no. 4, pp. 757–767, 2013.

[106] A. M. S. Paulo, A. F. Salvador, J. I. Alves, R. Castro, A. A. M. Langenhoff, A. J. M. Stams, A. J. Cavaleiro, "Enhancement of methane production from 1-hexadecene by additional electron donors" 2018.

[107] B. Kang, K. Honda, T. Aki, T. Omasa, and H. Ohtake, "Simultaneous thermogravimetry and differential thermal analysis for comparing burning characteristics between oleaginous and non-oleaginous microorganisms" *Thermochim. Acta*, vol. 517, no. 1–2, pp. 115–120, 2011.

[108] I. Lazar, I. G. Petrisor, and T. F. Yen, "Microbial enhanced oil recovery (MEOR)" *Pet. Sci. Technol.*, vol. 25, no. 11, pp. 1353–1366, 2007.

[109] B. Shibulal, S. N. Al-Bahry, Y. M. Al-Wahaibi, A. E. Elshafie, A. S. Al-Bemani, and S. J. Joshi, "Microbial enhanced heavy oil recovery by the aid of inhabitant spore-forming bacteria: An insight review" *Sci. World J.*, vol. 2014, 2014.

[110] V. Tornero and G. Hanke, "Chemical contaminants entering the marine environment from sea-based sources : A review with a focus on European seas" *MPB*, vol. 112, no. 1–2, pp. 17–38, 2016.

[111] A. Cruz, A. J. Cavaleiro, A. M. S. Paulo, A. Louvado, M. M. Alves, A. Almeida, Â. Cunha, "Microbial Remediation of Organometals and Oil Hydrocarbons in the Marine Environment" 2017, pp. 41–66.

[112] M. Daghio, F. Aulenta, E. Vaiopoulou, A. Franzetti, J. B. A. Arends, A. Sherry, A. Suárez-Suárez,

Ian M. Head, G. Bestetti, K. Rabaey, "Electrobioremediation of oil spills" *Water Res.*, vol. 114, pp. 351–370, 2017.
[113] W. H. Weisman, Analysis of Petroleum Hydrocarbons in Environmental Media, vol. 1. 1998.
[114] S. Singh and B. Kumari, "Microbial degradation of alkanes" *Microb. Degrad. xenobiotic Ed. by Singh, S.N.*, 2011.
[115] ITOPF (International Tanker Owners Pollution Federation Limited), "Oil tanker spill statistics 2019," 2020. [Online]. Available: http://www.itopf.org/knowledge-resources/data-statistics/statistics/. [Accessed: 14-Jan-2020].
[116] A. A. Miola, V. Paccagnan, A. Massarutto, and A. Perujo, External costs of Transportation Case study : maritime transport.
[117] M. H. Van Liedekerke, E. Commission, G. Prokop, S. Rabl-berger, and M. G. Kibblewhite, Progress in the management of Contaminated Sites in Europe, no. December. 2014.
[118] G. Lofrano and G. Libralato, *Nanotechnologies for Environmental Remediation*, SPRINGER INTERNATIONAL PU.2017.
[119] A. M. A. Pintor *et al.*, "Use of cork powder and granules for the adsorption of pollutants: a review" *Water Res.*, vol. 46, no. 10, pp. 3152–66, 2012.
[120] A. M. A. Pintor *et al.*, "Textural and surface characterization of cork-based sorbents for the removal of oil from water," *Ind. Eng. Chem. Res.*, vol. 52, no. 46, pp. 16427–16435, 2013.
[121] S. P. A. S. E. Martins and R. L. G. Reis, "Meio de absorção/adsorção à base de derivados de cortiça para absorção/adsorção de óleos" Patent No. PT103492 (B), 2007.
[122] B. A. McKew, F. Coulon, A. M. Osborn, K. N. Timmis, and T. J. McGenity, "Determining the identity and roles of oil-metabolizing marine bacteria from the Thames estuary, UK" *Environ. Microbiol.*, vol. 9, no. 1, pp. 165–176, 2007.
[123] P. Rapp and L. H. E. Gabriel-Jürgens, "Degradation of alkanes and highly chlorinated benzenes, and production of biosurfactants, by a psychrophilic *Rhodococcus* sp. and genetic characterization of its chlorobenzene dioxygenase" *Microbiology*, vol. 149, no. 10, pp. 2879–2890, 2003.
[124] Y. C. Liu, L. Li, Y. Wu, W. Tian, L. Zhang, L. Xu, Q. Shen, B. Shen, "Isolation of an alkane-degrading *Alcanivorax* sp. strain 2B5 and cloning of the alkB gene" *Bioresour. Technol.*, vol. 101, no. 1, pp. 310–316, 2010.
[125] N. Dashti, N. Ali, M. Eliyas, M. Khanafer, N. A. Sorkhoh, and S. S. Radwan, "Most hydrocarbonoclastic bacteria in the total environment are diazotrophic, which highlights their value in the bioremediation of hydrocarbon contaminants" *Microbes Environ.*, vol. 30, no. 1, pp. 70–75, 2015.
[126] X. Gao, W. Gao, Z. Cui, B. Han, P. Yang, C. Sun, L. Zheng, "Biodiversity and degradation potential of oil-degrading bacteria isolated from deep-sea sediments of South Mid-Atlantic Ridge" *Marine Pollution Bulletin*, vol. 97, no. 1–2. pp. 373–380, 2015.
[127] M. M. Yakimov, P. N. Golyshin, S. Lang, E. R. B. Moore, W. Abraham, H. Lünsdorf and K. N. Timmis, "*Alcanivorax borkurnensis* gen. now, sp. nov., a new, hydrocarbon-degrading and surfactant-producing marine bacterium" *Int. J. Syst. Bacteriol.*, vol. 48, no. 1 998, pp. 339–348, 1998.
[128] S. Schneiker, V. Santos, [...], P. N Golyshin, "Genome sequence of the ubiquitous hydrocarbon-degrading marine bacterium *Alcanivorax borkumensis*" *Nat. Biotechnol.*, vol. 24, no. 8, pp. 997–1004, 2006.
[129] S. Al Kharusi, R. M. M. Abed, and S. Dobretsov, "EDTA addition enhances bacterial respiration activities and hydrocarbon degradation in bioaugmented and non-bioaugmented oil-contaminated desert soils" *Chemosphere*, vol. 147, pp. 279–286, 2016.
[130] A. Wentzel, T. E. Ellingsen, H. K. Kotlar, S. B. Zotchev, and M. Throne-Holst, "Bacterial

metabolism of long-chain *n*-alkanes," *Appl. Microbiol. Biotechnol.*, vol. 76, no. 6, pp. 1209–1221, 2007.

[131] A. R. Castro, M. Guimarães, J. V Oliveira, and M. A. Pereira, "Science of the Total Environment Production of added value bacterial lipids through valorisation of hydrocarbon-contaminated cork waste" *Sci. Total Environ.*, vol. 605–606, pp. 677–682, 2017.

[132] D. Koma, Y. Sakashita, K. Kubota, Y. Fujii, F. Hasumi, S. Chung, M. Kubo, "Degradation of car engine base oil by *Rhodococcus* sp. NDKK48 and *Gordonia* sp. NDKY76A" *Biosci. Biotechnol. Biochem.*, vol. 67, no. 7, pp. 1590–1593, 2003.

[133] M. Binazadeh, I. A. Karimi, and Z. Li, "Fast biodegradation of long chain *n*-alkanes and crude oil at high concentrations with *Rhodococcus* sp. Moj-3449" *Enzyme Microb. Technol.*, vol. 45, no. 3, pp. 195–202, 2009.

[134] X. Song, Y. Xu, G. Li, Y. Zhang, T. Huang, and Z. Hu, "Isolation, characterization of *Rhodococcus* sp. P14 capable of degrading high-molecular-weight polycyclic aromatic hydrocarbons and aliphatic hydrocarbons," *Mar. Pollut. Bull.*, vol. 62, no. 10, pp. 2122–2128, 2011.

[135] M. D. Auffret, E. Yergeau, D. Labbé, F. Fayolle-Guichard, and C. W. Greer, "Importance of *Rhodococcus* strains in a bacterial consortium degrading a mixture of hydrocarbons, gasoline, and diesel oil additives revealed by metatranscriptomic analysis" *Appl. Microbiol. Biotechnol.*, vol. 99, no. 5, pp. 2419–2430, 2015.

[136] A. K. Bej, D. Saul, and J. Aislabie, "Cold-tolerant alkane-degrading *Rhodococcus* species from Antarctica" *Polar Biol.*, vol. 23, no. 2, pp. 100–105, 2000.

[137] R. Margesin and F. Schinner, "Biodegradation and bioremediation of hydrocarbons in extreme environments" *Appl. Microbiol. Biotechnol.*, vol. 56, no. 5–6, pp. 650–663, 2001.

[138] H. M. Alvarez, R. A. Silva, P. A. Haro, A. F. Alvarez, J. D. Perez, and M. S. Villalba, "From basic research to application: Use of *Rhodococcus* spp. for environmental biotechnology in semiarid Patagonia, Argentina" *J. Biotechnol.*, vol. 131, no. 2 Supplement, pp. S243–S244, 2007.

[139] M. Mahjoubi, A. Jaouani, A. Guesmi, S. B. Amor, A. Jouini, H. Cherif, A. Najjari, A. Boudabous, N. Koubaa, A. Cherif "Hydrocarbonoclastic bacteria isolated from petroleum contaminated sites In Tunisia: Isolation, identification and characterization of the biotechnological potential" *N. Biotechnol.*, vol. 30, no. 6, pp. 723–733, 2013.

[140] B. Gargouri, F. Karray, N. Mhiri, F. Aloui, and S. Sayadi, "Application of a continuously stirred tank bioreactor (CSTR) for bioremediation of hydrocarbon-rich industrial wastewater effluents" *J. Hazard. Mater.*, vol. 189, no. 1–2, pp. 427–434, 2011.

[141] C. C. C. R. De Carvalho, "Adaptation of *Rhodococcus erythropolis* cells for growth and bioremediation under extreme conditions" *Res. Microbiol.*, vol. 163, no. 2, pp. 125–136, 2012.

[142] F. Suja, F. Rahim, M. R. Taha, N. Hambali, M. R. Razali, A. Khalid, A. Hamzah, "Effects of local microbial bioaugmentation and biostimulation on the bioremediation of total petroleum hydrocarbons (TPH) in crude oil contaminated soil based on laboratory and field observations" *Int. Biodeterior. Biodegrad.*, vol. 90, pp. 115–122, 2014.

[143] K.-S. Na, A. Kuroda, N. Takiguchi, T. Ikeda, H. Ohtake, and J. Kato, "Isolation and characterization of benzene-tolerant *Rhodococcus opacus* strains" *J. Biosci. Bioeng.*, vol. 99, no. 4, pp. 378–82, 2005.

[144] A. R. Castro, I. Rocha, M. M. Alves, and M. A. Pereira, "*Rhodococcus opacus* B4: a promising bacterium for production of biofuels and biobased chemicals" *AMB Express*, vol. 6, no. 1, p. 35, 2016.

[145] S. H. Baik, "Biotransformation of medium-chain alkanes using recombinant P450 monooxygenase from *Alcanivorax borkumensis* SK2 expressed in *Escherichia coli*" *Korean J. Chem. Eng.*, vol. 27, no. 3, pp. 905–909, 2010.

[146] L. R. Rodrigues, J. A. Teixeira, H. C. van der Mei, and R. Oliveira, "Physicochemical and

functional characterization of a biosurfactant produced by *Lactococcus* lactis 53. *Colloids Surf. B Biointerfaces* 49, 79-86, 2006

[147] A. F. Salvador, G. Martins, M. Melle-Franco, R. Serpa, A. J. M. Stams, A. J. Cavaleiro, M. A. Pereira, M. M. Alves, "Carbon nanotubes accelerate methane production in pure cultures of methanogens and in a syntrophic coculture" vol. 19, pp. 1–40, 2017.

[148] A. F. Salvador, A. J. Cavaleiro, A. M. S. Paulo, S. A. Silva, A. P. Guedes, M. A. Pereira, A. J. M. Stams, D. Z. Sousa, and M. M. Alves "Inhibition Studies with 2-Bromoethanesulfonate Reveal a Novel Syntrophic Relationship in Anaerobic Oleate Degradation" *Appl. Environ. Microbiol.*, vol. 85, no. 2, pp. 1–12, 2019.

[149] S. Handl, S. E. Down, J. F. Garcia-Manzcorro, J. M. Steiner, and J. S. Suchodolski, Massive parallel 16S rRNA gene pyrosequencing reveals highly diverse fecal bacterial and fungal communities in healthy dogs and cats. *FEMS Microbial. Ecol.* 76,301-310, 2011.

[150] D. A. MacIntyre, M. Chandiramani, Y. S. Lee, L. Kindinger, A. Smith, N. Angelopoulos, [...], The vaginal microbiome during pregnancy and postpartum period in ahe vaginal microbiome during pregnancy and postpartum period in a European population, *sci. Rep.* 5:8988, 2015

[151] V. S. Cerqueira, M. do C. R. Peralba, F. A. O. Camargo, and F. M. Bento, "Comparison of bioremediation strategies for soil impacted with petrochemical oily sludge" *Int. Biodeterior. Biodegrad.*, vol. 95, no. PB, pp. 338–345, 2014.

[152] M. S. Safdari *et al.*, "Development of bioreactors for comparative study of natural attenuation, biostimulation, and bioaugmentation of petroleum-hydrocarbon contaminated soil" *J. Hazard. Mater.*, vol. 342, pp. 270–278, 2018.

[153] M. M. Nakazawa, S. Gavazza, M. T. Kato, and L. Florencio, "Evaluation of rhamnolipid addition on the natural attenuation of estuarine sediments contaminated with diesel oil" *Environ. Sci. Pollut. Res.*, vol. 24, no. 33, pp. 25522–25533, 2017.

[154] U.S. Environmental Protection Agency, "DSSTox (Distributed Structure-Searchable Toxicity) database" 2016. [Online]. Available: https://comptox.epa.gov/dashboard/ . [Accessed: 15-Jun-2020].

[155] D. Bhattacharya, P. M. Sarma, S. Krishnan, S. Mishra, and B. Lal, "Evaluation of genetic diversity among *Pseudomonas citronellolis* strains isolated from oily sludge-contaminated sites" *Microbiology*, vol. 69, no. 3, pp. 1435–1441, 2003.

[156] D. Zhao, C. Liu, L. Liu, Y. Zhang, Q. Liu, and W. M. Wu, "Selection of functional consortium for crude oil-contaminated soil remediation" *Int. Biodeterior. Biodegrad.*, vol. 65, no. 8, pp. 1244–1248, 2011.

[157] R. Thavasi, S. Jayalakshmi, and I. M. Banat, "Biosurfactants from marine bacterial isolates" *Appl. Microbiol. mircobial Biotechnol.*, pp. 1367–1373, 2010.

[158] A. Wedulo, D. K. Atuhaire, S. Ochwo, V. Muwanika, A. J. J. Rwendeire, and L. Scanakavuma, "Characterisation and evaluation of the efficiency of petroleum degrading bacteria isolated from soils around the oil exploration areas in western Uganda" *African J. Agric. Res.*, no. Vol. 13 No. 48 (2014), 2014.

[159] R. Lumactud, S. Y. Shen, M. Lau, and R. Fulthorpe, "Bacterial endophytes isolated from plants in natural oil seep soils with chronic hydrocarbon contamination" *Front. Microbiol.*, vol. 7, no. MAY, pp. 1–10, 2016.

[160] Y. Subhash and S. S. Lee, "Roseomonas deserti sp. nov., isolated from crude oil contaminated desert sand" *Int. J. Syst. Evol. Microbiol.*, vol. 68, no. 2, pp. 675–680, 2018.

[161] S. M. Seyedi, S. Rajaei, F. Raiesi, B. Shiran, and J. Raheb, "Characterization and potentials of indigenous oil-degrading bacteria inhabiting the rhizosphere of wild oat (*Avena Fatua* L.) in south west of iran" *Iran. J. Biotechnol.*, vol. 11, no. 1, pp. 32–40, 2013.

[162] X. W. Xu *et al.*, "*Pelagibacterium halotolerans* gen. nov., sp. nov. and *Pelagibacterium luteolum*

sp. nov., novel members of the family hyphomicrobiaceae" *Int. J. Syst. Evol. Microbiol.*, vol. 61, no. 8, pp. 1817–1822, 2011.

[163] S. Cappello, R. Denaro, M. Genovese, L. Giuliano, and M. M. Yakimov, "Predominant growth of *Alcanivorax* during experiments on 'oil spill bioremediation' in mesocosms" *Microbiol. Res.*, vol. 162, no. 2, pp. 185–190, 2007.

[164] A. Angelov, P. Bergen, F. Nadler, P. Hornburg, A. Lichev, [...], Novel Flp pilus biogenesis-dependent natural transformation. *Front. Microbial*. 6:84, 2015.

[165] L. Craig, K. T. Forest, and B. Maier, Type IV pili: dynamics, biophysics and functional consequences. *Nat. Rev. Microbial.* 17, 429-440, 2019.

[166] E. Z. Ron and E. Rosenberg, Natural roles of biosurfactants. *Environ. Microbial.* 3, 229-236, 2001.

[167] N. L. Oliveira, M. L. Nieves, M. Lozada, G. Del Prado, H. M. Dionisi, F. Siñeris, Isolation and characterization of biosurfactant-production *Alcanivorax* strains: hydrocarbon accession strategies and alkanes hydroxilasegene analysis, *Res, Microbial*. 160, 19-26, 2009.

[168] S. Siebenhaller, J. Gruninger, C. Syldatk, "Enzymatic synthesis of glycolipid surfactants", in *Lipid Modification by Enzymes and Engineered Microbes*, ed. U. T. Bornscheuer (Urbana, IL: AOCS Press), 2018

[169] S. De, S. Malik, A. Ghosh, R. Sahab, B. Saha, A review on natural surfactants. *RSC Adv.* 5:65757, 2015

[170] I. Del Castillo, P. Hernández, A. Lafuente, I. D. Rodríguez-Llorente, M. A. Caviedes, and E. Pajuelo, "Self-bioremediation of cork-processing wastewaters by (chloro)phenol-degrading bacteria immobilised onto residual cork particles" *Water Res.*, vol. 46, no. 6, pp. 1723–1734, 2012.

[171] M. Dias-Machado, L. M. Madeira, B. Nogales, O. C. Nunes, and C. M. Manaia, "Treatment of cork boiling wastewater using chemical oxidation and biodegradation" *Chemosphere*, vol. 64, no. 3, pp. 455–461, 2006.

[172] A. Franzetti, P. Caredda, C. Ruggeri, P. L. Colla E. Tamburini, M. Papacchini, G. Bestettia, "Chemosphere Potential applications of surface active compounds by Gordonia sp . strain BS29 in soil remediation technologies" *Chemosphere*, vol. 75, no. 6, pp. 801–807, 2009.

[173] P. Arulazhagan and N. Vasudevan, "Biodegradation of polycyclic aromatic hydrocarbons by a halotolerant bacterial strain *Ochrobactrum* sp . VA1" *Mar. Pollut. Bull.*, vol. 62, no. 2, pp. 388–394, 2011.

[174] J. Brzeszcz and P. Kaszycki, "Aerobic bacteria degrading both *n*-alkanes and aromatic hydrocarbons: an undervalued strategy for metabolic diversity and flexibility" *Biodegradation*, vol. 29, no. 4, pp. 359–407, 2018.

[175] J. Trögl, C. O. Esuola, S. Křížanecká, P. Kuráň, L. Seidlová, P. Veronesi-Dáňová, J. Popelka, O. O. Babalola, P. Hrabák, M. Czinnerová, E. Kakosová, A. Ševců, D Tischler, "Biodegradation of high concentrations of aliphatic hydrocarbons in soil from a petroleum refinery: Implications for applicability of new actinobacterial strains" *Appl. Sci.*, vol. 8, no. 10, pp. 1–12, 2018.

[176] R. M. Silva, A. R.Castro, R. Machado, M. A. Pereira.. Dissolved oxygen concentration as a strategy to select type and composition of bacterial storage lipids produced during oilfield produced water treatment. *Environ. Technol. Innov*. 23:101693, (2021a).

[177] R. M. Silva, A. M.Fernandes, F.Fiume, A. R.Castro, R.Machado, M. A. Pereira Sequencing batch airlift reactors (SBAR): a suitable technology for treatment and valorization of mineral oil wastewaters towards lipids production. *J. Hazard. Mater*. 409:124492, (2021b).

[178] D. M. Bayer, A. C. O. Chagas-Spinelli, S. Gavazza, L. Florencio, and M. T. Kato, "Natural attenuation and biosurfactant-stimulated bioremediation of estuarine sediments contaminated with diesel oil" *Appl. Biochem. Biotechnol.*, vol. 171, no. 1, pp. 173–188, 2013.

[179] F. Widdel and R. Rabus, "Anaerobic degradation of saturated and aromatic hydrocarbons," *Curr. Opin. Biotechnol.*, vol. 12, pp. 259–276, 2001.
[180] L. M. Gieg, S. J. Fowler, and C. Berdugo-Clavijo, "Syntrophic biodegradation of hydrocarbon contaminants" *Curr. Opin. Biotechnol.*, vol. 27, pp. 21–29, 2014.
[181] B. Schink, "Degradation of unsaturated hydrocarbons by methanogenic enrichment cultures" *FEMS Microbiol. Lett.*, vol. 31, no. 2, pp. 69–77, 1985.
[182] F. Bianco, M. Race, S. Papirio, and G. Esposito, "Removal of polycyclic aromatic hydrocarbons during anaerobic biostimulation of marine sediments" *Sci. Total Environ.*, vol. 709, p. 136141, 2020.
[183] B. Song, J. Tang, M. Zhen, and X. Liu, "Effect of rhamnolipids on enhanced anaerobic degradation of petroleum hydrocarbons in nitrate and sulfate sediments" *Sci. Total Environ.*, vol. 678, pp. 438–447, 2019.
[184] S. Kato, K. Hashimoto, and K. Watanabe, "Methanogenesis facilitated by electric syntrophy via (semi)conductive iron-oxide minerals" *Environ. Microbiol.*, vol. 14, no. 7, pp. 1646–1654, 2012.
[185] H. M. Azam and K. T. Finneran, "Ferric iron amendment increases Fe(III)-reducing microbial diversity and carbon oxidation in on-site wastewater systems" *Chemosphere*, vol. 90, no. 4, pp. 1435–1443, 2013.
[186] C. Cruz Viggi, S. Rossetti, S. Fazi, P. Paiano, M. Majone, and F. Aulenta, "Magnetite particles triggering a faster and more robust syntrophic pathway of methanogenic propionate degradation" *Environ. Sci. Technol.*, vol. 48, no. 13, pp. 7536–7543, 2014.
[187] K. Yamada-Onodera, H. Mukumoto, Y. Katsuyama, and Y. Tani, "Degradation of long-chain alkanes by a polyethylene-degrading fungus, *Penicillium simplicissimum* YK" *Enzyme Microb. Technol.*, vol. 30, no. 6, pp. 828–831, 2002.
[188] L. Pereira, P. Dias, O. S. G. P. Soares, P. S. F. Ramalho, M. F. R. Pereira, and M. M. Alves, "Synthesis, characterization and application of magnetic carbon materials as electron shuttles for the biological and chemical reduction of the azo dye Acid Orange 10" *Appl. Catal. B Environ.*, vol. 212, pp. 175–184, 2017.
[189] G. Martins, A. F. Salvador, L. Pereira, and M. M. Alves, "Methane Production and Conductive Materials: A Critical Review" *Environ. Sci. Technol.*, vol. 52, no. 18, pp. 10241–10253, 2018.
[190] Z. Yang, X. Shi, C. Wang, L. Wang, and R. Guo, "Magnetite nanoparticles facilitate methane production from ethanol via acting as electron acceptors" *Sci. Rep.*, vol. 5, pp. 1–8, 2015.
[191] S. A. B. Weelink, M. H. A. van Eekert, and A. J. M. Stams, "Degradation of BTEX by anaerobic bacteria: Physiology and application" *Rev. Environ. Sci. Biotechnol.*, vol. 9, no. 4, pp. 359–385, 2010.
[192] N. D. Gray, A. Sherry,R. J. Grant,A. K. Rowan,C. R. J. Hubert,C. M. Callbeck,C. M. Aitken,D. M. Jones,J. J. Adams,S. R. Larter,I. M. Head, "The quantitative significance of Syntrophaceae and syntrophic partnerships in methanogenic degradation of crude oil alkanes," *Environ. Microbiol.*, vol. 13, no. 11, pp. 2957–2975, 2011.
[194] T. Siddique, T. Penner, K. Semple, and J. M. Foght, "Anaerobic Biodegradation of Longer-Chain *n*-Alkanes Coupled to Methane Production in Oil Sands Tailings" pp. 5892–5899, 2011.
[195] A. J. M. Stams, J. B. Van Dijk, C. Dijkema, and C. M. Plugge, "Growth of syntrophic propionate-oxidizing bacteria with fumarate in the absence of methanogenic bacteria" *Appl. Environ. Microbiol.*, vol. 59, no. 4, pp. 1114–1119, 1993.
[196] C. N. Sawyer and P. L. McCarty, "Chemistry for Environmental Engineering" 3th ed., McGraw-Hill Book Company, pp. 456–463, 1978.
[197] Y. S.-P. Gonzalez, "Bioremedation of Oil Spills", Escuela Técnica Superior de Ingenieros de Minas. Available online at: http://oa.upm.es/10795/1/YOLANDASANCHEZPALENCIA.pdf

[accessed: 15-october-2020], p. 93, 2011.
[198] F. M. Kaser and J. D. Coates, "Nitrate, Perchlorate and Metal Respirers" *Handbook of hydrocarbon and lipid microbiology*, 2010.
[199] T. Siddique, P. M. Rutherford, J. M. Arocena, and R. W. Thring, "SHORT COMMUNICATION A proposed method for rapid and economical extraction of petroleum hydrocarbons from contaminated soils" pp. 2–5, 2006.
[200] U.S. Environmental Protection Agency, "Method 3510C: Separatory funnel liquid-liquid extraction" 2000. [Online]. Available: https://www.epa.gov/sites/production/file%0As/2015-12/documents/3510c.pdf. %0A. [Accessed: 16-May-2020].
[201] W. Quality, Determination of hydrocarbon oil index, Part 2: Method using solvent extraction and gas chromatography, reference number ISO 9377-2:2000. Geneva, Switzerland.
[202] J. E. Anderson, B. R. Kim, S. A. Mueller, and T. V. Lofton, "Composition and analysis of mineral oils and other organic compounds in metalworking and hydraulic fluids" *Crit. Rev. Environ. Sci. Technol.*, vol. 33, no. 1, pp. 73–109, 2003.
[203] J. Zhang and Y. Lu, "Conductive Fe3O4 nanoparticles accelerate syntrophic methane production from butyrate oxidation in two different lake sediments" *Front. Microbiol.*, vol. 7, no. AUG, pp. 1–9, 2016.
[204] G. Hu, J. Li, and G. Zeng, "Recent development in the treatment of oily sludge from petroleum industry: A review" *J. Hazard. Mater.*, vol. 261, pp. 470–490, 2013.
[205] J. Zheng, B. Chen, W. Thanyamanta, K. Hawboldt, B. Zhang, and B. Liu, "Offshore produced water management: A review of current practice and challenges in harsh/Arctic environments" *Mar. Pollut. Bull.*, vol. 104, no. 1–2, pp. 7–19, 2016.
[206] N. Jiménez, H. Richnow, and C. Vogt, "Methanogenic Hydrocarbon Degradation : Evidence from Field and Laboratory Studies" pp. 227–242, 2016.
[207] P. Xu, G. M. Zeng, [...], Z. F. Liu, "Use of iron oxide nanomaterials in wastewater treatment: A review" *Science of the Total Environment*, vol. 424. pp. 1–10, May-2012.
[208] R. D. Ambashta and M. Sillanp, "Water purification using magnetic assistance: A review" *Journal of Hazardous Materials*, vol. 180, no. 1–3. pp. 38–49, Aug-2010.
[209] H. Hu, Z. Wang, and L. Pan, "Synthesis of monodisperse Fe_3O_4@silica core-shell microspheres and their application for removal of heavy metal ions from water" *J. Alloys Compd.*, vol. 492, no. 1–2, pp. 656–661, 2010.
[210] S. Zhang, H. Niu, Z. Hu, Y. Cai, and Y. Shi, "Preparation of carbon coated Fe3O4 nanoparticles and their application for solid-phase extraction of polycyclic aromatic hydrocarbons from environmental water samples" *J. Chromatogr. A*, vol. 1217, no. 29, pp. 4757–4764, 2010.
[211] A. R. Castro, I. Rocha, M. M. Alves, and M. A. Pereira, "*Rhodococcus opacus* B4: a promising bacterium for production of biofuels and biobased chemicals" *AMB Express*, vol. 6, no. 1, p. 35, 2016.
[212] A. E. Rotaru, P. M. Shrestha, F. Liu, T. Ueki, K. Nevin, Z. M. Summers, and D. R. Lovley "Interspecies electron transfer via hydrogen and formate rather than direct electrical connections in cocultures of Pelobacter carbinolicus and Geobacter sulfurreducens" *Appl. Environ. Microbiol.*, vol. 78, no. 21, pp. 7645–7651, 2012.