TÉCNICO LISBOA

Assessing mannosylerythritol lipids sustainable production and use in oil spill response strategies

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Thesis to obtain the Master of Science Degree in

Biotecnology

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December 2021

Acknowledgments

First of all, my deep gratitude goes to my advisors Dr. Nuno Faria and Prof. Dr. Frederico Ferreira. For introducing me to such an interesting and stimulating field, for all the support during my research, their guidance, knowledge and important questions, thank you. Without their help this work would not be possible.

Next, I am very thankfull to Miguel Nascimento for all his advice, encouragement, continuous availability, and being an overall awesome friend. I am also thankfull to Petar Keković for his support, his suggestions and ideas. They were both great lab mates and always made me feel welcome.

To all iBB-BERG members and staff, but in particular to Ana Rita Valente, Flavio Ferreira, Leonor Resina, Maryna Bondar, Dona Rosa, Ricardo Pereira, Drª Carla Carvalho, and Drª Teresa Esteves, thank you for all the assistance.

I would like to thank all the partners that helped and supported this work. To the Portuguese Yeast Culture Collection (PYCC) for the supply of yeast strains, thank you. To IPMA, in the person of Miguel Caetano, I am grateful for all the help in obtaining the sea water and his advice. To LA-IST, in particular Engineer Georgina Sarmento and her team, thank you for the help with the FTIR analysis. Finally, to Lisbet Sørensen and Roman Netzer from SINTEF, I am grateful for the partnership, the work in the development of the formulation for oil spill remediation, and for the meetings without which the bioremediation assays would not have been done.

I would also like to thank for the funding received from the FCT granted to the project Mero (MIT-EXPL/ISF/0115/2017), to the Research Unit iBB - Institute for Bioengineering and Biosciences, UIDB/04565/2020 and UIDP/04565/2020, and to i4HB - the Associate Laboratory Institute for Health and Bioeconomy (LA/P/0140/2020), allowing me to get all the necessary resources to develop this work.

I wish to thank my fellow dorm mates, friends, and colleagues for making me feel at home away from home.

Finally, to my parents and my brother, I am grateful for their unconditional support, understanding and patience. I am not the best daughter or sister, but you are the best family anyone could wish for.

Abstract

The aim of this work consisted in accessing the sustainable production of mannosylerythritol lipids (MELs) and their use in more eco-friendly oil spill response strategies.

Towards MEL's sustainable production, it was demonstrated that higher aeration rates and the feeding strategy can be used to optimize MEL production. The highest productivity, 0.27 g/l/h was achieved with a Moesziomyces bullatus bioreactor fermentation started with 40 g/l of D-glucose, 20 g/l of waste frying oil (WFO), with 2 feeds of 20 g/l of WFO added at the 3th and 6th day of fermentation, with agitation of 150-800 rpm, and air flow of 2 vvm.

A MEL based oil spill response agent (MEL-OSRA) was developed. The marine toxicity of this and other oil spill response agents (OSRAs) was determined. MELs have low toxicity, and when added to the developed MEL-OSRA, this formulation has a lower toxicity than the solvent matrix-OSRA, without MEL. The developed formulation is an eco-friendlier alternative to conventional surfactants.

One factor found to affect bioremediation is the effectiveness of dispersion for each OSRA tested. Unlike Corexit 9500 and the developed formulation, MELs have a poor dispersion ability, thus, in the formulation the other more toxic components are the ones helping in the dispersion of oil even if, as shown in literature, MELs might then have an important role in the bioremediation process.

Keywords

Biosurfactants; Mannosylerythritol Lipids; Sustainable production; Oil spills; Bioremediation;

Resumo

O objetivo deste trabalho consistiu em avaliar a produção sustentável de manosileritritolípidos (MEL) e seu uso em estratégias mais ecológicas de resposta a derrames de crude.

Com o objectivo de alcançar uma produção sustentável de MEL, foi demonstrado que taxas de arejamento mais altas e a estratégia de alimentação podem ser usadas para otimizar a produção de MEL. A mais elevada produtividade, 0.27 g/l/h, foi alcançada com uma fermentação em bioreactor de Moesziomyces bullatus iniciada com 40 g/l de D-glucose, 20 g/l de óleo de fritura usado (OFU), com suplemento de 20 g/l de OFU adicionado ao 3º e 6º dia de fermentação, agitação de 150-800 rpm, e 2 vvm.

Um agente de resposta a derrames de crude baseado em MELs (ARDC-MEL) foi desenvolvido. A toxicidade em ambiente marinho deste e outros agentes de resposta a derrames de crude (ARDCs) foi determinada. Os MELs apresentam menor toxicidade e quando adicionados ao (ARDC-MEL) este apresenta uma toxicidade menor do que o ARDC com apenas a matriz solvente, sem os MELs. A formulação desenvolvida é uma alternativa mais ecológica a surfactantes convencionais.

A eficácia da dispersão de cada ARDC testado afeta a biorremediação. Ao contrário do que acontece com Corexit 9500 e a formulação desenvolvida, os MELs têm baixa capacidade de dispersão. Assim, são os outros componentes mais tóxicos da formulação que auxiliam na dispersão do óleo. Mesmo que, conforme a literatura indica, os MELs possam desempenhar um importante papel no processo de biorremediação.

Palavras Chave

Biosurfactantes; Manosileritritolípidos; Produção sustentável; Derrames de crude; Bioremediação;

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Acronyms

ANOVA analysis of variance

API American Petroleum Institute

CAGR compound annual growth rate

CDW cell dry weight

CFU colony forming units

CMC critical micelle concentration

DCM dichloromethane

EU European Union

FTIR fourier transform infrared spectroscopy

GC gas chromatography

GC-FID gas chromatography equipped with flame ionization detection

GI germination index

HLB hydrophilic-lipopholic balance

HPLC high performance liquid chromatography

IOPC Fund International Oil Pollution Compensation Fund

IPMA Instituto Português do Mar e da Atmosfera

ITOPF International Tanker Owners Pollution Federation Limited

kLa oxygen mass-transfer coefficient

LC50 median lethal concentration

MAH monocyclic aromatic hydrocarbon

MEL mannosylerythritol lipid

MEL-OSRA MEL based oil spill response agent

MMT million metric tonnes

OSRA oil spill response agent

PAH polycyclic aromatic hydrocarbon

PCB polychlorinated biphenyl

rpm rotations per minute

RRE relative root elongation

RRF relative response factor

RSG relative seed germination

solvent matrix-OSRA solvent matrix based oil spill response agent

THC total hydrocarbon content

TLC thin layer chromatography

vvm volume of gas per volume of liquid per minute

WFO waste frying oil

1

Introduction

Contents

1.1 Overview

Liquid petroleum hydrocarbon (also known as crude oil) is extensively extracted, refined and used. During such operations, and upon its transformation, often occur leakages to the environment. Crude oil is toxic, persistent and has negative influence on living organisms. Therefore, oil spills have the potential to cause serious impacts to natural resources and the livelihoods that depend on them. [1]

The frequency of oil spills has dramatically declined in the last decade as a result from numerous international agreements and standards which sought to prevent, prepare for and reduce the environmental and economic impacts of these incidents. [2] Still, there is a call for solutions that can be efficiently applied when an oil spill takes place. [3]

Bioremediation, defined as the act of adding materials to a contaminated environment to encourage the natural biodegradation process, is one of the more cost-effective and environmentally friendly clean-up methods. [4] [5]

Chemical dispersants, whose formulations have a mixture of solvents and surface-active agents present in different proportions, are one of such materials. [6] [7] [8] By applying them to the oil, they emulsify it and large clumps are converted into droplets, ranging in size from microns to millimetres. This reduces the interfacial tension and increases the surface area. [6] Nonetheless, they have several limitations [7], and their accumulation in the ecosystem could also lead to drastic environmental problems. [9]

An alternative strategy that follows the use of chemical dispersants methods, but aims to be a more ecofriendly solution, is the use of natural surfactants. Those have low toxicity, high biodegradability, environmental compatibility and an effectiveness at extreme conditions. [10] These properties lead companies to replace chemical surfactants with these sustainably produced biosurfactants. [11]

One of these biosurfactants are mannosylerythritol lipids (MELs). MELs are surface active glycolipids that are biosynthesized by a variety of microorganisms. [12]

However, there are some challenges associated with the industrial production of MEL. The development of new integrated processes, with improved fermentation processes and the use of alternative substrates are key for a decrease in manufacturing costs.

1.2 Objectives and challenges

There is a call for more eco-friendly formulations in the response to marine oil spills. In this work, the use of MELs in the formulation of OSRAs is suggested.

To achieve this goal, the sustainable production of MELs needs to be optimized and their environmental impact, more specifically their toxicity, evaluated. Furthermore, the performance in bioremediation studies requires evaluation.

1.3 Research questions and strategies

To address this thesis objective, 3 main research questions were made:

1. What are some of the relevant parameters for MEL production in a bench scale reactor fermentation? How can they be used to optimize MEL production?
2. How is the toxicity, in a marine environment, of different glycolipid biosurfactants and other OSRAs? And the phytotoxicity of the MEL OSRA formulation?
3. How is the performance of different OSRA formulations in sea water bioremediation? And their performance in suspended sediments bioremediation?

To answer the previous questions, and achieve this thesis objective, the following research strategies were pursued:

- In preliminary studies, and due to MEL's amphiphilic nature, *Moesziomyces bullatus* fermentations in shake flask were performed using two carbon sources (D-glucose and waste frying oil (WFO)), and following the methods previously optimized. A 1st set of experiments aimed to study the effect of aeration and inoculum to medium volume.
- Subsequent the oxygen mass-transfer coefficient (kLa) studies conducted within the research group, and aiming to optimize MEL titres, *Moesziomyces bullatus* fermentations in a bench scale reactor were conducted utilizing an optimized feeding strategy (starting with 40 g/l of D-glucose and 20 g/l of WFO and with 2 feeds of 20 g/l of WFO at the 4th and 7th day of fermentation). The previously used condition with 500 rotations per minute (rpm) and 1 volume of gas per volume of liquid per minute (vvm) was compared to the condition with the highest kLa in a fermentation with 800 rpm and 2 vvm.
- To assess how the feeding strategy can be used to optimize MEL production in reactor, further fermentation experiments were conducted. The previous tested conditions were compared to a condition of 800 rpm and 2 vvm fermentation started with 40 g/l of D-glucose and 20 g/l of WFO and with feeds of 6 g/l of WFO per day.
- To find the toxicity of different glycolipid biosurfactants and other OSRAs a 24h LC_{50} bioassay was carried out with *Artemia franciscana* and according to the standard operational procedures of the marine toxicity screening test Artoxkit M.
- Afterwards, to find the phytotoxicity of the MEL OSRA formulation, screening tests were performed using lettuce (*Lactuca sativa*) and tomato (*Solanum lycopersicum*) seeds.

- To investigate the performance of different OSRA formulations, several bioremediation experiments were conducted in a contaminated seawater medium and in contaminated sand sediments.

2

Literature review and State-of-the-art

Contents

2.1 Oil spills

2.1.1 Historical context and environmental role

The extensive extraction, refining, transport and use of liquid petroleum hydrocarbon (also known as crude oil) is often associated with its release into the environment. Crude oil is toxic, persistent, and has a negative influence on living organisms. Therefore, after crude oil spills, it contaminates marine/-coastal waters, shorelines, and land, requiring an effective clean-up of the polluted sites to mitigate environmental impacts. [3] [13]

Crude oil can enter water bodies by a number of anthropogenic and natural sources. Acute anthropogenic sources of crude oil are accidental oil spills or intentional operational discharges. The primary source of accidental oil spills is from tanker vessels carrying crude oil or petroleum products. [14] This contamination from ships can differ substantially from oil spills originating from oil and gas extractions, refining, and installations. The potential spills from this installations may represent larger quantity and prolonged release of oil, and thus imply environmental impacts and safety hazards more severe than for accidental oil spills from tanker transporter vessels. [15]

Other major sources of oil contamination to the marine environment are the chronic anthropogenic ones, such as runoff and atmospheric deposition from terrestrial environments. The main contributors are the chronic natural sources of contamination such as oil seeps and other naturally occurring hydrocarbons. [14]

In Europe there are over 1,000 offshore oil and gas installations. However, the European oil and gas industry is changing and the number of oil and gas installations is increasing, which may increase the probability of incidents that could lead to oil spills. As a consequence, existing public and private pollution response capabilities and contingency plans are being developed and continually updated and reviewed to be ready to respond to the challenges posed by the nature of spills from offshore operations. [15]

International Tanker Owners Pollution Federation Limited (ITOPF) has collected data on oil spills worldwide since the 1970's and now holds a database including more than 10 000 incidents. The 20 largest spills recorded occurred all over the world and 19 of them have taken place before the year 2000 (Figure 2.1). The data collected clearly indicates that the frequency of spills greater than 7 tonnes from tankers have shown a marked downward trends and the number of major ($>$700 tons) and medium (7-700 tons) oil spills have declined (Figure 2.2). [16]

The yearly average recorded of large spills ($>$700 tonnes) in the 2010s was 1.8 spills, which is less than a tenth of the average recorded in the 1970s. Similarly, there has been a a 95% reduction since the 1970s in the quantity of oil spilled. [16] This dramatic decline in the frequency of oil spills resulted from numerous international agreements and standards which sought to prevent, prepare for and reduce the environmental and economic impacts of maritime incidents. This agreements are a reflex of a wider and

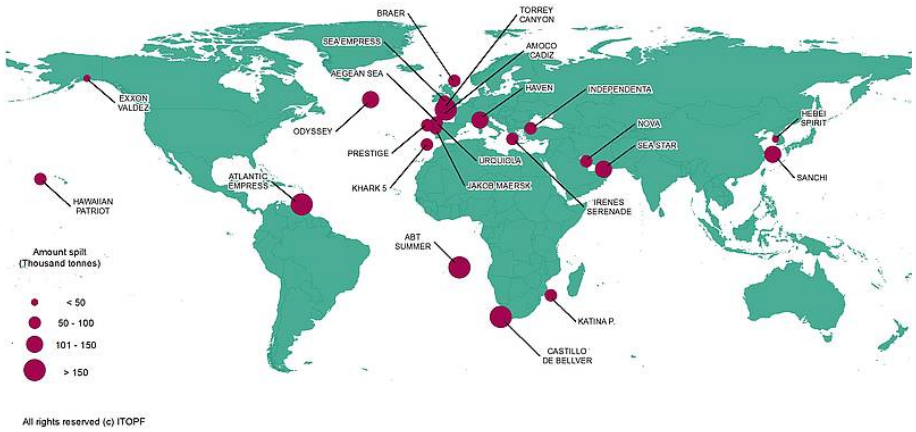

Figure 2.1: Location of the major oil spills in history In this figure we can see the location of the 20 largest tanker oil spills that have occurred since the TORREY CANYON in 1967. Retrieved from the ITOPF Oil tankers spill statistics 2020 and accessed in *https://www.itopf.org/knowledge-resources/data-statistics/statistics/* at March 6th 2020.

growing international realisation of the negative impacts oil spills can have on the environment and the importance of solutions to mitigate their occurrence and impact. [2]

Industry has an important role to play and undertakes initiatives to improve the safety and environmental standards of oil activities and to limit the extent of incidents that can affect human life and the environment. [15] Nowadays, the precautionary approach and the polluter pays principle are well-established and widely applied. Moreover, the additional costs incurred by clean-up activities and serious economic losses experienced by industries and individuals dependent on coastal resources have tremendous economic and social impacts. [2]

2.1.2 Formation, composition, and properties of crude oil

Marine oil spills have the potential to cause serious impacts to natural resources and the livelihoods that depend on them. The extent of impact however is influenced by a number of factors among them the type and characteristics of the oil spilled. [1]

Petroleum is a naturally occurring mixture of hydrocarbons, whether in a liquid, gaseous, or solid state. [17] One of the most chemically complex materials on Earth, petroleum is derived from ancient living organisms deposited as geological sediments. Its origins begin in certain depositional conditions that favor the accumulation and preservation of organic matter from once living organisms. Petroleum is

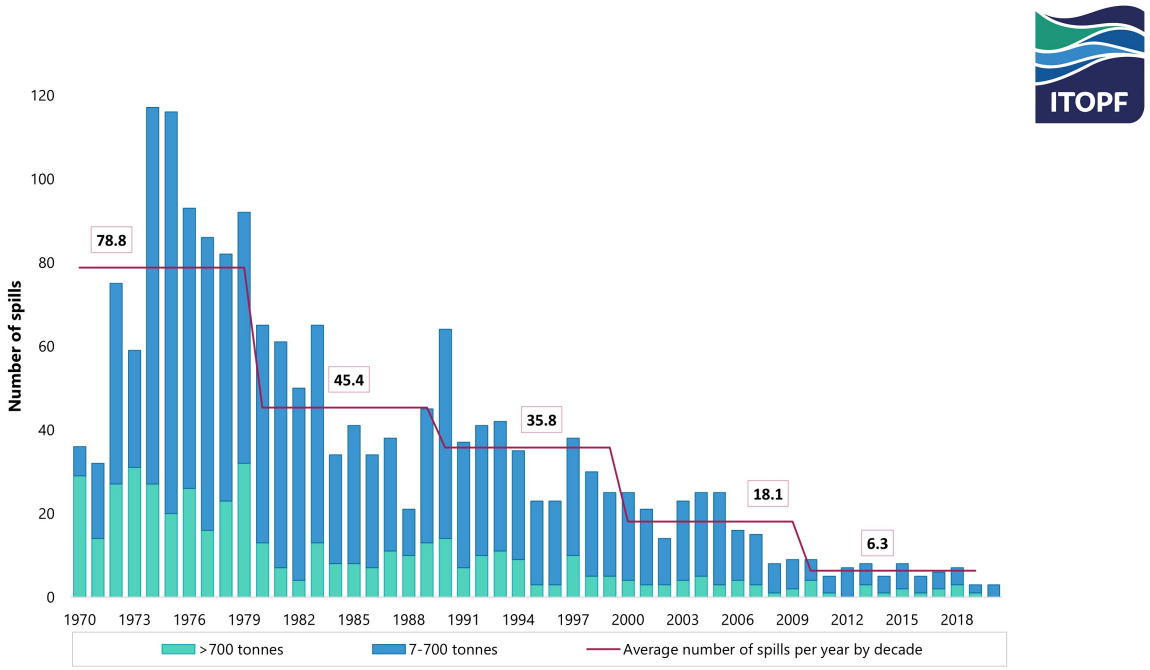

Figure 2.2: Global oil spill trend. This graph shows the number of spills (>7 tonnes) per year from 1970 to 2020 and the average number of spills by decade. Retrieved from the ITOPF Oil tankers pill statistics 2020 and accessed in *https://www.itopf.org/knowledge-resources/data-statistics/statistics/* at March 6th 2020.

formed when these organic-rich rocks are heated and the expelled fluids move into reservoirs. [18]

This complex mixture contains thousands of compounds with different chemical functionality including alkanes, cycloalkanes, aromatics, and compounds containing sulfur, oxygen, and nitrogen. [19] This compounds can be grouped into four main broad categories: saturates (branched, unbranched and cyclic alkanes), aromatics (ringed hydrocarbon molecules such as monocyclic aromatic hydrocarbons (MAHs) and polycyclic aromatic hydrocarbons (PAHs)), resins (polar oil-surface structures dissolved in saturates and aromatics), and asphalthenes (dark-brown amorphous solids colloidally dispersed in saturates and aromatics). [3]

Crude oil, a sub-category of petroleum, is a liquid mixture that includes a huge variety of different compounds and has a variation in composition due to the corresponding variation in natural processes and conditions that govern its formation. Such variation can be in both molecular weight and the types of molecules present. [14] [17] Crude oil ranges from a brownish green to black liquid, its boiling temperature range varies from 20°C to above 350°C, and it has a specific gravity (at 15.6° C) that varies from 0.75 to 1.00 (57° to 10° API gravity). [17] The formal classification of crude oil as heavy or light is based on this American Petroleum Institute (API) gravity, witch is a measure for the density of the crude oil in relation to water. Crude oil classified as heavy has an API gravity lower than 22.3° while medium and light crude oils have API gravities between 22.3° and 31.1° and equal to or >31.1°, respectively. However, some crude oils with the same API gravity exhibit different physical properties. [20]

Historically, the concept of persistent and non-persistent oils was related to the likelihood of the material to naturally dissipate in sea water and whether or not cleanup would be required. However, in the definition adopted by the International Oil Pollution Compensation Fund (IOPC Fund), a non-persistent oil is one that, at the time of shipment, includes a 50% and 95% volume of hydrocarbon fractions that distil, respectively, at temperatures of 340°C and 370°C, when tested by the ASTM Method D86/78 or any subsequent revision thereof. [1]

Crude oils can contain dramatically different amounts of material with different boiling temperature point ranges, according with crude oil origin and maturity. [21] Non-persistant oils, composed of lighter hydrocarbon fractions and generally volatile, when released to the environment, will dissipate rapidly through evaporation and rarely require a response. However, at high concentrations can result in acute toxicity to marine organisms. Persistent oils, containing a considerable proportion of heavy fractions or high-boiling temperature point material, do not dissipate quickly and will therefore pose potential threats to natural resources when released to the environment. [1]

2.1.3 Biodegradation and Bioremediation

Oil spills pose a serious environmental challenge, threatening both terrestrial and marine ecosystems. Hence, attention has been drawn towards identifying reliable and efficient oil spill clean-up methods. [3] Biodegradation, the natural process whereby microorganisms metabolize compounds, breaking down organic molecules into other substances, is one of the most important long-term natural processes for removal of oil from the marine environment. [22] [4]

As shown in section **??**, petroleum, and therefore crude oils, have a large variety of different components. [19] [17] This components have differences in their liability to microbial degradation, which follows the order: alkanes $>$ light aromatics (MAHs) $>$ cycloalkanes $>$ heavy aromatics (PAHs) $>$ asphalthenes. [3]

Biodegradation is known to be the main natural process for the removal of the nonvolatile fraction of oil from the environment. [23] Regardless, this process is usually too slow to mitigate serious ecosystem negative impacts resulting from an oil spill, and as a result, several oil spill remediation techniques have been established. [3]

Bioremediation, defined as the act of adding materials to a contaminated environment to promote the natural biodegradation process, is one of the more cost-effective and environmentally friendly clean-up methods . [3] [22] [4] [23] [5] Depending on the degree of intervention, this process is generally considered to include, among others, natural attenuation, when little or no human action occurs; bio-stimulation, when nutrients and electron donors/acceptors are added to promote the growth or metabolism of certain microorganisms; or bio-augmentation, the deliberate addition of natural or engineered microorganisms with the desired catalytic capabilities. [4]

In bioremediation, numerous contaminated components are converted into intermediate and final products, such as carbon dioxide, water, and biomass. Therefore, though the toxicity of the oil spill is reduced, bioremediation is not a rapid cleanup process, and is often ineffective in cleaning up the heavy components of crude oil, due to nutrient and oxygen limitations, as well as suboptimal abiotic conditions (pH, temperature). [3] [23]

The use of bioremediation in oil spills faces several challenges, including resistance of asphalthenes to biodegradation, delay of heavy or high molar mass PAHs biodegradation, eutrophication caused by biostimulation, unsustainability of bio-augmentation, poor bioavailability of spilled petroleum, and inefficient biodegradation in anoxic environments. [3]

A successful bioremediation approach should be capable of breaking down all crude oil components within a reasonable time-frame at a minimum cost. Therefore, it may be necessary to employ integrated remediation technologies in order to create an aggressive synergistic approach which has the potential to be more effective. [3]

2.1.4 Dispersion of oil, dispersants and their use

The environmental and economic impacts of an oil spill along with its response strategy are affected by how long the oil will remain on the sea surface. The natural dispersion of oil spilled at sea is a key process in determining the expected lifetime of the contaminants on the sea surface. [24] Weathering, emulsification, and mousse formation, are processes that dissolve and disperse the spilled oil. Turbulent conditions at the sea surface, such as breaking waves, naturally disperse and break up the oil slicks into small droplets ($<$100 µm) that are injected to the depth of the water column where they will be further biodegradated. [25]

The application of chemical dispersants can be an effective means of enhancing the natural dispersion of oil from the sea surface into the water column and are applied as an emergency response to oil spills. [6] [7] In the dispersants formulations, a mixture of solvents and surface-active agents, meaning surfactants, are present in different proportions. [8] By applying dispersant formulations to the crude oil, they emulsify it and large clumps are converted into droplets, ranging in size from microns to millimetres. This reduces the interfacial tension and increases the surface area (Figure 2.3). [6]

Chemical dispersants can accelerate dilution and biodegradation of the oil, reducing its environmental and economic impact. [7] However, the dispersed oil can have an impact on sub-surface resources and it is essential that the limitations of dispersants to be recognised, including possible toxic effects the chemical dispersants may have on aquatic organisms. [7] [8]

In most European Union (EU) Member States, the use of dispersant is secondary to mechanical containment and recovery. In several other states the use of dispersants is either not allowed or is highly restricted, as is the case of countries with coastlines bordering the Baltic Sea due to the sensitive

ecological conditions and low water exchange. [7]

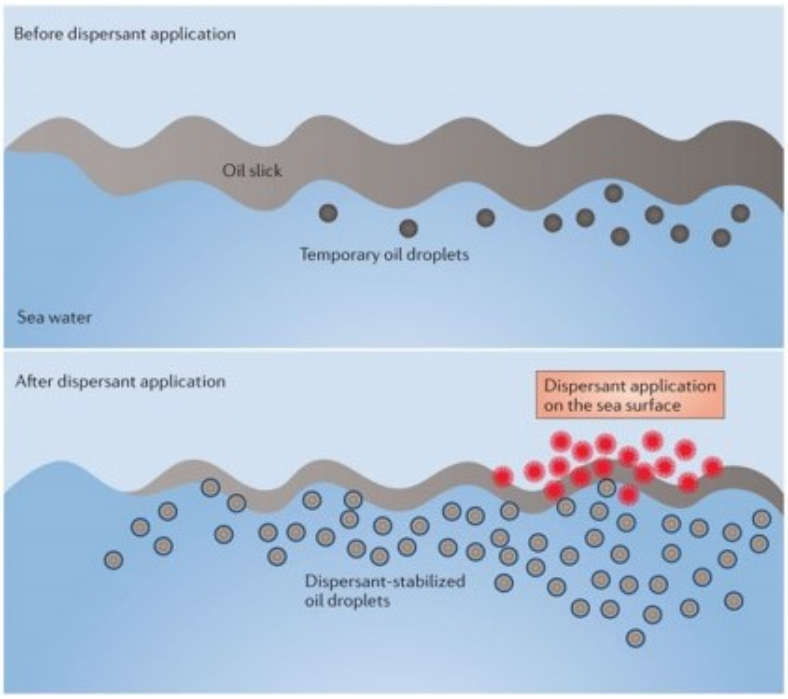

Figure 2.3: Dispersant application and its interaction with oil in sea water. The application of dispersant that interacts with the oil in sea water, the oil slicks are broken up and dispersant-stabilized oil droplets are dispersed in the water column. Retrieved from [6].

2.2 Surfactants

2.2.1 Surfactants characteristics, their role and applications

As seen in section 2.1.4 chemical dispersants are a mixture of surfactants and solvents. [26] The surfactants themselves are not new, and in fact soap, the oldest known surfactant, appeared well over 2000 years ago. Although only since the Second World War has the modern surfactant industry emerged. [27]

Surfactants, also designated as surface active agents due to their ability to reduce the surface and the interfacial tensions, are amphiphilic molecules with at least one 'solvent-loving' lyophilic group and one 'solvent-fearing' lyophobic group. When the solvent is water or an aqueous solution then we can use the term hydrophilic for the polar head and hydrophobic for the non-polar polar (or ionic) tail, that is usually hydrocarbon chain (Figure 2.4). [27] [28] [29]

The polar head group is usually attached at one end of one or more alkyl chain(s), a hydrophobic tail that usually has between 8 to 18 carbon atoms. The properties of any given surfactant will depend on the degree of chain branching, the position of the polar head group and the length of the chain. [28]

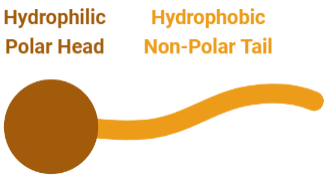

Figure 2.4: Schematic illustration of a surfactant molecule. This illustration shows the structure of a surfactant molecule with one hydrophilic, polar head group and one hydrophobic, non-polar tail. Created with *BioRender.com*

Adsorption and aggregation are two phenomena that result from the opposing forces within the same molecule. Adsorption occurs when, in an aqueous media, the surfactant molecules migrate to the interfaces and orientate in such a fashion as to minimise the contact between their hydrophobic groups and the water. Alternatively, the surfactant molecules can aggregate in the bulk solution with the hydrophilic 'head groups' orientated towards the aqueous phase as a way of limiting the contact between the hydrophobic groups and the water. This aggregation process, called micellisation, leads to the formation of aggregates, or micelles, that vary in shape depending on concentration (Figure 2.5). Micelles begin to form at a very low concentration, known as critical micelle concentration (CMC). [27]

Figure 2.5: Schematic illustration of a spherical micelle. This illustration shows the configuration of a spherical micelle aggregate with the hydrophilic 'head groups' orientated towards the aqueous phase on the outside, limiting the contact between the hydrophobic 'tail groups' and the water. Created with *BioRender.com*

Surfactants are generally classified by ionic types depending on their chemical structure. They are anionic when the hydrophilic portion of the surfactant carries a negative charge, cationic when it carries a positive charge, amphoteric when its both positive and negative charges, and non-ionic when it carries no charge at all. [27]

The most common non-ionic surfactants are ethoxylates, ethylene and propylene oxide co-polymers and sorbitan esters. Examples of commercially available ionic surfactants include fatty acids, ester sulphonates or sulphates (anionic) and quartenary ammonium salts (cationic). [29]

Surfactants have a tendency to accumulate at interfaces, such as the boundaries between two immiscible phases, increasing the solubility of hydrophobic compounds in an aqueous solution or the solubility

of water in a hydrophobic solution. The tendency for a surfactant to accumulate at a specific boundary depends on the surfactant structure and nature of each of the two phases that meet at the interface. Therefore, there is no universally good surfactant, suitable for all uses and the choice will always depend on the application. [28]

Surfactants use is widespread in different human applications, from detergents and personal hygiene or for the fabrication of many of the products we use. Selection of a surfactant for a specific use is not only governed by their intrinsic surface active properties, but its toxicological, environmental, regulatory and application-specific requirements should also be considered, as such properties may dictate their suitability for a given purpose. [27]

2.2.2 Surfactants and their market

Surfactants constitute an important class of industrial chemicals widely used in almost every sector of modern industry. [29] The global surfactants market size was valued at $41.3 billion in 2019 and at the time was projected to reach $58.5 billion by 2027, corresponding to to an expected compound annual growth rate (CAGR) of 5.3% from 2020 to 2027. [30] However, the market was negatively impacted by COVID-19 in 2020. The global surfactants market was over $36 billion in 2020 and a 4% CAGR is now estimated for the period from 2021 to 2026. [31]

In 2018, when the global surfactants market size was valued at $39 billion and the global demand was of 16.8 million metric tonnes (MMT), household detergents were the application with more demand and anionic surfactants were the surfactant type with more global demand (Figure 2.6). [32]

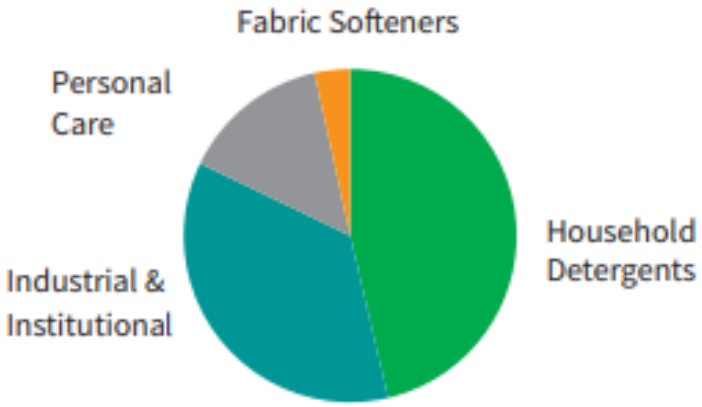

Figure 2.6: Graphical representation of the global surfactants market, by application, in 2018. This graph shows the global demand of surfactants in 2018, by application. Retrieved from [32]

In 2020 anionic surfactants still account for approximately half of the total share of the surfactants market and a growing focus on environmental regulations is expected to increase the demand for these surfactants (Figure 2.7). The largest market and also the fastest growing one is the Asia-Pacific. This

region is expected to dominate the market and is also likely to witness the highest CAGR from 2021 to 2026. [31]

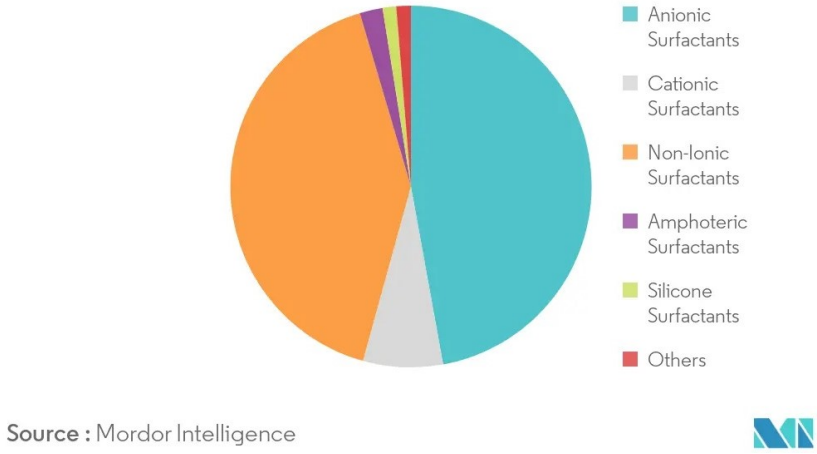

Figure 2.7: Graphical representation of the global surfactants market, by type, in 2020. This graph shows the global demand of surfactants in 2020, by type. Retrieved from [31]

2.2.3 Toxicity and other relevant environmental concerns

One of the critical challenges faced by the surfactants industry is the environmental impact of their use in various products. [31]

Surfactants were often regarded as harmless based on their biodegradability and speculated low concentrations in the environment. However, following statistical analysis of their worldwide amount, it was found that they are present in concentrations higher than the threshold at which they would not have an effect on the ecosystem. [9] The variability in test methods used to find surfactants' toxicity in the published studies make difficult to compare the toxicity among the different surfactants. The studies suggest that dispersants range from mildly to highly toxic to the environment and living organisms. [26]

The accumulation of these molecules in the ecosystem could lead to drastic environmental problems. From their synthesis, to their disposal, when released to the environment, surfactants can aggravate the problems related to global warming, climate change, ozone layer depletion, greenhouse gas emission and visible manifestations of surfactant toxicity are available in microbes, plants and animals. Surfactant production results in atmospheric emissions, waterborne wastes and solid wastes capable of causing eutrophication and acidification of rivers and lakes. [9]

Total banning of surfactants is impossible in the current modernized lifestyle, but the use of biosurfactants could lower the extent of synthetic surfactants prevalence in environment and its associated toxicity. [9]

2.3 Biosurfactants

2.3.1 Distinctive characteristics

Natural surfactants (biosurfactants), include surfactants produced from animal materials, from plant materials, and biosynthesized by microorganisms. [33] The later are microbial compounds which exhibit particularly high surface and emulsifying activity, [13] [29] [34] which were discovered as extracellular amphiphilic compounds when researching into hydrocarbon fermentation, and are usually produced using hydrocarbons as carbon source. [29] [33] Microbial surfactants attracted attention due to their low toxicity, high biodegradability, environmental compatibility, effectiveness at extreme temperatures, pH and salinity and their mild production conditions, when compared to chemically synthesized surfactants. [13] [10] [35] Biosurfactants can be produced from industrial wastes and by-products, allowing for a cheap production and simultaneously reducing their polluting effect. [13]

Microbial biosurfactants, like previously seen in chemical surfactants, have hydrophobic and hydrophilic regions allowing them to reduce surface and interfacial tension. [13] [29] [11] These compounds can increase the surface area of hydrophobic water-insoluble substances, increase the water bio-availability of such substances and change the properties of the bacterial cell surface. This surface activity makes them excellent emulsifiers, foaming and dispersing agents. [13] [11] In general, biosurfactants are more effective and efficient, and their CMC is about 10–40 times lower than that of chemical surfactants. This means that less surfactant is necessary to get a maximum decrease in surface tension. [9]

Most biosurfactants are considered to be secondary metabolites from the microorganisms that produce them. Regardless, by facilitating nutrient transport or microbe–host interactions or even by acting as biocide agents some play essential roles for the survival of the biosurfactant-producing microorganisms. [34] These microbial compounds also play an important part in the environment, as they increase the surface area and bio-availability of hydrophobic water-insoluble substrates and are involved in heavy metal binding, bacterial pathogenesis, quorum sensing and biofilm formation. [34] [10]

Microbial surfactants can be classified according to different criteria. They can be classified by their molecular weight, ionic charges, secretion type, mode of action, microbial origin or their chemical structure. [13] [33] Based on their molecular weight, they can be divided into low-molecular-mass biosurfactants, efficient in lowering surface and interfacial tensions, or high-molecular-mass biosurfactants, more effective at stabilizing oil-in-water emulsions. [13] However, the chemical structure based classification is the most accepted criteria to classify these microbial derived surface active compounds. [33] Based on their natural chemical structure and microbial origin, biosurfactants are classified into five classes: glycolipids, phospholipids and fatty acids, lipopeptides or lipoproteins, polymeric surfactants, particulate surfactants (Table 2.1). [36]

Table 2.1: Biosurfactants classification. Classification of biosurfactants by their natural chemical structure, resuming their 5 classes and examples of each class with the respective producing microorganism. Table adapted from [36]

Chemical structure	Biosurfactants	Origins
Glycolipids	Rhamnolipids	*Pseudomonas aeruginosa*
	Sophorolipids	*Candida bombicola*
	Cellobiolipids	*Ustilago sp.*
	Mannosylerythritol lipids	*Moesziomyces spp.*
	Trehalolipids	*Rhodococcus spp.*
Phospholipids (or fatty acids) Lipopeptides	Lipid phosphate	*Torulopsis maynoliae*
Lipopeptides (or lipoproteins)	Serrawettin	*Serratia spp.*
	Surfactin	*Bacillus spp.*
	Subtilisin	*Bacillus spp.*
	Polymyxins	*Bacillus polymyxa*
	Viscosin, amphisin and putisolvin	*Pseudomonas spp.*
Polymeric	Liposan	*Candida lipolytica*
	Emulsan, Biodispersan	*Acinetobacter calcoaceticus*
	Mannan-lipid-protein	*Candida tropicalis*
	Carbohydrate-protein-lipid	*Pseudomonas fluorescens*
Particulate	Vesicles and fimbriae	*Acinetobacter calcoaceticus*
	Whole cells	Variety of bacteria

2.3.2 Biosurfactants and their market

With the increasing consumer demand for products that are both “greener”, milder and more efficient, a new class surfactants has gained emphasis. [9] Companies using surfactants in their products are now looking to replace some or all of the chemical surfactants with sustainable biosurfactants, [11] and due to their scale-up capacity, rapid production, and multi-functional properties, microbial biosurfactants are being chosen over their plant-based counterparts. [37]

However, several issues need to be considered for a successful large-scale exploitation of biosurfactants. The low availability and the high price of feedstock, the low titres and productivity, [38] and availability of substitutes can hinder the markets’ growth. [39] In the specific case of rhamnolipids, safety and yield need to improve. The pathogenic status of the producer organism, *P. aeruginosa*, is a concern although some companies have overcome the problem with the identification of potential new non-pathogenic producer organisms. Nevertheless, failure to achieve high yields may eventually preclude rhamnolipids from use in many possible applications. On the other end, sophorolipids and mannosylerythritol lipids appear to have a much greater potential, having no obvious safety issues and being produced with higher yields and they have already been included in several commercial products. [11]

In the beginning of the 21st century one the largest possible markets for biosurfactant was the oil industry, including using biosurfactants in oil spill bioremediation and dispersion. [29] Due to their unique properties, biosurfactants can be applied in the remediation of both inorganic, meaning heavy metals, and organic micropollutants such as PAHs, petroleum hydrocarbons and polychlorinated biphenyls (PCBs). [40]

The detergents application accounts for a significant demand for biosurfactants, as home care is the largest industry using surfactants. [41] The agriculture industry is other sector with increasing demands for biosurfactants. Due to their properties, biosurfactants are replacing the synthetic chemical-based products that agriculture relies so heavily on. [38]

In 2016, the global biosurfactants market size was valued at $3.99 billion and was projected to reach $5.52 billion by 2022, at a CAGR of 5.6% from 2017 to 2022. At the time, Europe was the largest market for biosurfactants and was projected to lead the biosurfactants market during the forecast period, 2017 to 2022. In this region, the market was largely driven by the increasing awareness among consumers towards the protection of the environment from toxic chemicals and the stringent laws enforced by regulatory bodies. Notwithstanding, the Asia-Pacific market was projected to be the fastest-growing market for biosurfactants mainly due to the technologically advancing and emerging countries in the region demanding innovative, biodegradable, renewable, and less toxic biosurfactant products (Figure 2.8). [41]

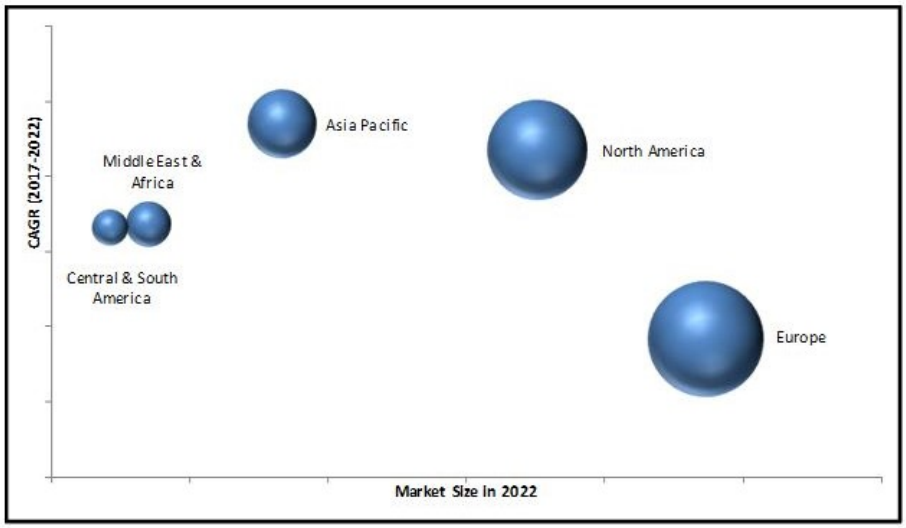

Figure 2.8: Biosurfactants market, by region, for 2022. Biosurfactants demand estimated for 2022, by region. The bubble size represents the market size for 2022. Retrieved from [41].

Amid the COVID-19 crisis, the global market for biosurfactants estimated at $4.5 billion in the year 2020, and is projected to reach a revised size of $6.5 billion by 2027, growing at a CAGR of 5.3% over the analysis period 2020-2027. The glycolipids segment is projected to record a 5.7% CAGR and

reach $2.6 billion by the end of the analysis period. After an early analysis of the business implications of the pandemic and its induced economic crisis, growth in the lipopeptides and lipoproteins segment was readjusted to a revised 5.2% CAGR for the following 7-year period. [42] Europe still leads the biosurfactants market, closely followed by North America, and the Asia-Pacific market is still projected to be the fastest-growing market for biosurfactant. [39]

While one report in 2019 states that the global biosurfactants market is fragmented, competitive and with numerous players, [38] other report claims that it is consolidated and with only a few players operating in global and regional markets. [39] Regardless, AGAE Technologies LLC, Akzo Nobel N.V., BASF SE, BioFuture Ltd, Biotensidon GmbH, Ecover UK Ltd, Evonik Industries AG, Givaudan, Jeneil Biotech Inc., Logos Technologies, Saraya Co. Ltd. and Soliance SA were some of the identified players in this market, [38] [39] [42] with BASF SE, Ecover, and Evonik Industries AG as the major players in 2019. [38]

2.3.3 Renewable substrates and their use in biosurfactants production

The price of substrates is one of the main constrains to the wide spread exploitation of biosurfactants. [38] [43] This, coupled with the need for environmental preservation has led to the research of low-cost renewable substrates for biosurfactant production. [44] The use of some industrial wastes, or raw substrates with negligible value, has been proposed for the economical production of biosurfactants. [43] The waste materials selected should have a proper balance of nutrients that permits cell growth and product accumulation. [43] [44] Industrial wastes with a high content of carbohydrates or lipids are ideal low-cost substrates. [44]

Studies have been conducted with a variety inexpensive agro-industrial wastes and byproducts that can be used as feedstock for biosurfactant production. [43] Some examples of this renewable substrates from the industry with the potential to be used as a carbon source include crops and their residues, animal fat, dairy industry wastes, food processing industry wastes such as frying oil wastes or vegetable oils, oil distillery wastes, among others. [44]

Vegetable oils, one of these renewable substrates, are among the most used to produce biosurfactants. These oils are saturated compounds or unsaturated fatty acids with a 16-18 carbon atoms chain, and can lead to high production rates of biosurfactants. [43] Other substrates used as carbon sources to produce biosurfactants are molasses, a by product of the sugarcane industry with a high content in sugar compounds, and dairy industry products with high content in lactose and amino acids. [45]

2.4 Mannosylerythritol Lipids

2.4.1 Properties and advantageous characteristics

As seen in section 2.3.1, mannosylerythritol lipids (MELs) are surface active compounds belonging to the glycolipid class of biosurfactants and synthesized by a variety of microorganisms, including yeast strains of the *Pseudozyma* and *Ustilago* genera. [12] Within the *Pseudozyma* genus, MELs are secreted in abundance by *Pseudozyma antarctica*, *Pseudozyma aphidis*, and *Pseudozyma rugulosa* among others. [46] [10] *Ustilago* sp. also reportedly secretes MEL as a minor component along with cellobiose lipid. [47] However, it is important to mention that after a phylogenetic and taxonomic revision the *Pseudozyma* genus was renamed *Moesziomyces*. [48]

Pseudozyma antarctica, previously referred as *Candida antarctica*, is now *Moesziomyces antarcticus*. [49] *Moesziomyces aphidis* and *Pseudozyma rugulosa* are now the same species and named *Moesziomyces bullatus*. [50]

MELs are surfactants contain a 4-O-β-D-mannopyranosyl-meso-erythritol as the glycosidic/hydrophilic group and two short-chain fatty acids (usually C8–C12) as the hydrophobic groups. [10] According to their degree of acetylation at C4 and C6 position, and their order of appearance on the thin layer chromatography (TLC), [47] MELs can be identified as MEL-A when di-acetylated at the C-4 and C-6 positions of mannose, MEL-B when mono-acetylated at the C-6 position of mannose, MEL-C when mono-acetylated at the C-4 position of mannose or acMEL-D when deacetylated (Figure 2.9). [51]

The strains *Moesziomyces antarcticus* and *Moesziomyces bullatus* are able to produce large quantities of MELs. In these strains, and despite its low water solubility, MEL-A is a predominant product constituting about 70% of the total MELs. On the other hand, MEL-B and MEL-C have a higher hydrophilicity and lower critical aggregation concentrations and hence are suitable for use in emulsifiers, dispersants, and in other applications. [52]

2.4.2 MEL biosynthesis and its metabolic pathways

The biosynthesis of MEL, with the assembly of its 3 main moieties: mannose, erythritol and short-chain fatty acid, is regulated by a cluster of 4 genes. [53] This cluster consists of four enzymes and a transporter: mannosyltransferase (EMT1p) responsible for the formation of mannosylerythritol by mannosylation of erythritol, two acyltransferases encoded by *MAC1p* and *MAC2p* that lead to the acylation of mannosylerythritol, acetyltransferase encoded by *MAT1p* catalyzing the acetylation of mannosylerythritol at both the C4 and C6 hydroxyl groups of mannose, and the putative transporter encoded by *MMF1* (Figure 2.10). [51] [54]

MEL production from D-glucose requires de novo production of lipidic chains. However, when MEL is produced using vegetable oils as a carbon source, the triglycerides are cleaved by lipases forming

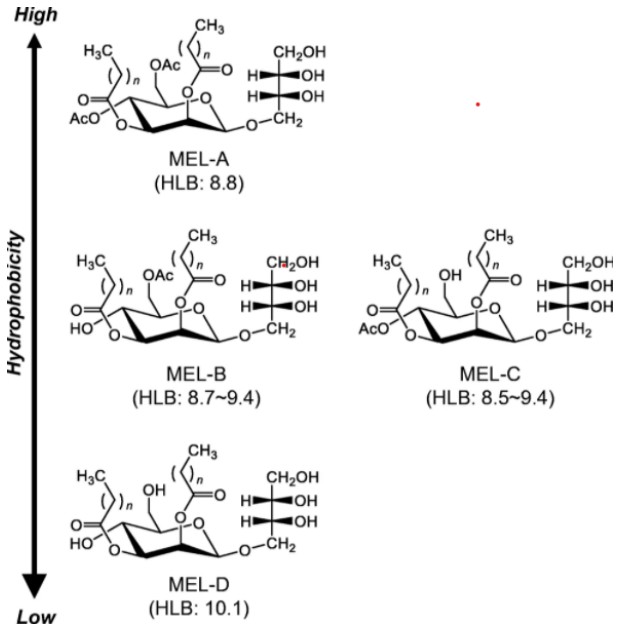

Figure 2.9: Chemical structures of the different types of MELs. Di-acetylated MEL-A, mono-acetylated in C6 MEL-B, mono-acetylated in C4 MEL-C, deacetylated MEL-D and their hydrophilic-lipopholic balance (HLB) calculated by Griffin's method from the weight percentage of the hydrophilic group to the hydrophobic groups in the molecules and with values ranging from 1 to 20. Retrieved from [51].

glycerol and fatty acids. For MEL production, regardless the substrate used, a partial β-oxidation of fatty acids, also known as chain shortening pathway, will occur. [55] This 'chain-shortening' pathway is distinct from the complete β-oxidation. [56] The substrates used in MEL production relate to the product structure obtained. When fatty alcohols or acids of chain length of C_n are used, the products are formed with chain length of C_{n-2}, C_{n-4}, C_{n-6}, etc. [47]

2.4.3 Fermentation processes to produce MEL

The carbon source is one of the main factors that affects MEL production. [12] A study from 1990, using *M. antarcticus* strain cultures, tested 6 different types at an initial concentration of 80 ml/l.In this study it was demonstrated that the best vegetable oil to be used as a carbon source is soybean oil. It had the highest yield producing 34 g/l of MEL, using *M. antarcticus*. [57] Still, the same study shows that almost all vegetable oils (except palm oil and coconut oil) are good carbon sources for the production of MEL. [57]

Water-soluble carbon sources like D-glucose and sucrose simplify the production and recovery steps when compared to the vegetable oils. In a 2006 study, *M. antarcticus* was able to produce MEL in the presence of D-glucose. After 10 days of cultivation, and using 40 g/l of D-glucose, a maximum of 3.5 g/l of MEL was obtained. When the researchers used a fed-batch mode the concentration of MEL increased with the cultivation time and reached approximately 12 g/l after 21 days of supplying to the medium 120

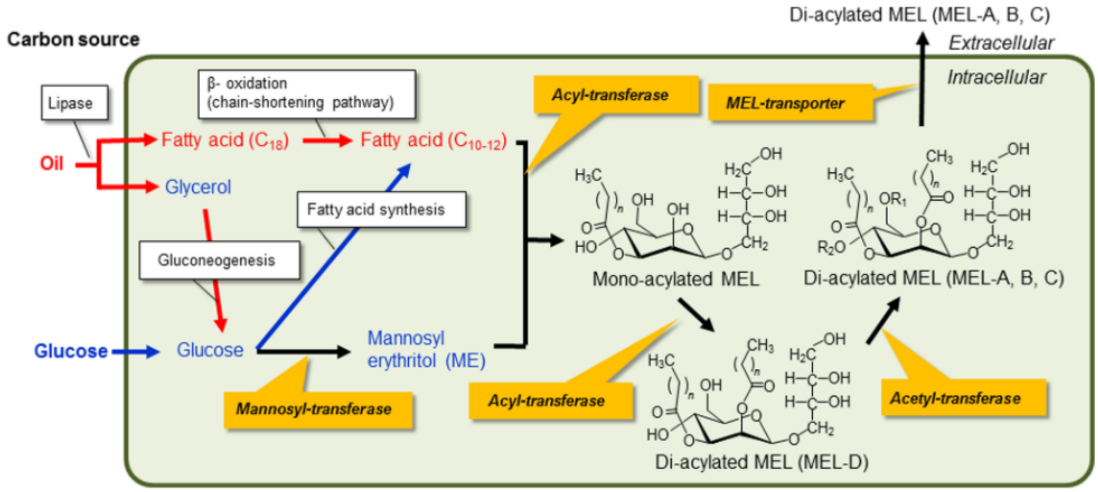

Figure 2.10: MEL biosynthesis. Schematic representation of possible metabolic pathways to produce MELs and the resulting chemical structures. Retrieved from [55].

g/l of D-glucose. [58]

Several renewable substrates were also tested as carbon sources for MEL production. One study performed at iBB-IST successfully demonstrated the conversion of cellulosic materials into MEL for the first time, obtaining 4.5 g/l of MEL from a model cellulosic substrate, and 2.5 g/l of MEL from a natural lignocellulosic substrate, after 14 days using a fed-batch strategy with *M. antarcticus*. [59] Other study has evaluated several waste products for the synthesis of biosurfactants, among witch MEL. The best results were obtained using *M. bullatus* cultivated in a medium with soapstock and whey permeate or molasses, producing 90 g/l of MEL. [60] Using residual honey as an hydrophilic carbon source, one study optimized MEL production and obtained 5 g/l of MEL. [61] Researchers demonstrated the production of MEL from coconut water, without adding other medium components, and obtained 3.85 g/l of MEL. [62] Another study used sugarcane juice as the carbon source, obtaining 12.7 g/l of MEL. [63] Crude glycerol can also be used as a carbon source in MEL production, as proven by a study that obtained 6.7 g/l of MEL. [64]

After the carbon source, the nitrogen source is the second most important supplement for the production of biosurfactants by microorganisms. In the fermentation process the C/N ratio affects the buildup of metabolites. A high C/N ratio with low nitrogen level limits bacterial growth and favours the cell metabolism towards the production of some secondary metabolites. On the other hand, excessive nitrogen leads to the synthesis of cellular material and limits the buildup of products. [44]

A study from 2005 proved that the type of nitrogen source affected the type MEL produced. The concentration of MEL-C was highest at the beginning, but decreased in favor of MEL-B at later stages as the cultivation time increased. This study also showed that the highest yield was attained with $NaNO_3$ and that acidic nitrogen sources, such as NH_4Cl and $(NH_4)_2SO_4$, resulted in a low final pH and low yields

of MEL. [65]

Using pentoses and pentose/hexose mixtures as the carbon source, a study performed at iBB-IST demonstrated that MEL production depends of a balanced sugar/nitrate ratio and can be improved using fed-batch strategies, where nitrate supplementation has crucial to promote sugar consumption and further MEL production. The higher MEL titres were obtained with sugar feeding at day 4, reaching 7.3 g/l and 5.8 g/l of MEL from D-glucose and D-xylose, respectively, in media with nitrate supplementation. [10]

MEL production can also be affected by the addition of the hydrophilic carbon source and in all the cases, the amount of MEL increased with an increase in the concentration of the carbohydrate added. [12]

Biosurfactants are more effective and efficient that their chemical contrerparts. [9] However, the industrial use of MELs will require overcoming several challenges, including the necessity of establishing highly productive processes, reducing the cost of production, and developing efficient recovery processes. [51] Thus, a successful commercialization of MELs will also depend on the optimization and enhancement of their production in bioreactor systems. [66]

While MEL production in shake flask is well reported, very few attempts have been made to produce it in a bioreactor.

One study tested the use of bioreactors on batch mode or fed-batch mode. In the batch mode, MEL production reached 37 g/l after 200 h and the highest production yield resulted from a pH controlled at 4.0. In the fed-batch fermentation, D-glucose and soybean oil were used as the initial carbon sources for cell growth, and soybean oil was used as the feeding carbon source during the MEL production phase. This secondary feeding of soybeen oil resulted in the disappearance of any foam and a sharp increase in the MEL production to 95 g/l at 200 h. [66]

2.4.4 Applications of MEL

MELs, due to their excellent interfacial properties and versatile biochemical actions are promising materials, with anticipated applications in various industries including cosmetics, pharmaceuticals, agriculture, food, and environmental fields. [51]

Studies have shown that MELs has numerous cosmetic applications. They can act as effective topical moisturizers and can repair damaged hair. Furthermore, these compounds have been shown to exhibit both protective and healing activities, to activate fibroblasts and papilla cells, and to act as natural antioxidants. [54] [67]

This biosurfactant also presents excellent properties in the field of medicine. MEL has antimicrobial activity against gram-positive bacteria, can induce cell differentiation and apoptosis and can be used as a vehicle for gene and drug delivery due to its ability to form thermodynamically stable vesicles with the ability to fuse with the membrane. [12]

Biosurfactants have very promising applications in environmental biotechnologies. [13] The effects of the biosurfactants produced by *M. antarcticus* strain T-34, MEL and BS-UC (a new type of biosurfactant produced by this strains that grows on n-undecane, $C_{11}H_{24}$), on the biodegradation of petroleum compounds was been investigated. The study found that, when in comparison with chemical surfactants, BS-UC and MEL could enhance the biodegradation of petroleum hydrocarbons such as single n-alkanes, mixtures of n-alkanes, kerosene, and crude oil. In a shake flask culture, the addition of 1% MEL and using crude oil as carbon source resulted in a degradation rate of crude oil of 74.1%, the second highest value after the one obtained by supplementing 1% BS-UC to *M. antarcticus* culture (76.6% crude oil degradation). Note that in this set-up *M. antarcticus* culture alone is responsible for 53% crude oil degradation. In a laboratory scale bioreactor immobilized with *M. antarcticus*, 1% MEL or 1% BS-UC addition improved not only the emulsification of kerosene in simulated wastewater, the initial concentration of kerosene increased from 800 mg/l to 4785 and 4975 mg/l, respectively, but also its biodegradation rate from 80% with no biosurfactant, to 87% and 90%, respectively. [68]

3

Materials and Methods

Contents

3.1 Materials

In this section, all the materials used will be detailed. Afterwards, the methodologies used will explored, starting with the ones related to MEL production, and followed by those used in toxicity and bioremediation experiments.

3.1.1 Reagents

For MEL production, Yeast extract (Oxoid), Malt extract (Oxoid), Peptone (Merck®), D-glucose (Fischer®), Agar (José M Vaz Pereira, S.A.), $NaNO_3$ (LabKem), $MgSO_4$ (Sigma-Aldrich®), KH_2PO_4 (Chemlab) and waste frying oil (WFO) were used.

In the toxicity experiments several glycolipid biosufactants and oil spill response agents (OSRAs) were used. Regarding the glycolipid biosufactants, mannosylerythritol lipids (MELs) used were obtained from *M. bullatus* and *M. antarcticus* fermentations, according to the methods to be described in section 3.2. Supernatant was obtained from the *M. bullatus* fermentation, containing 3.25 g/l of MEL. Sophorolipids and rhamnolipids were also tested. As for OSRAs, MEL-OSRA, solvent matrix-OSRA and Corexit 9500 (Nalco Holding Company), a commercially available chemical dispersant, were tested.

For the bioremediation studies, sea water medium, several OSRAs formulations, MEL, Statfjord C fresh crude oil and alkanes solutions were used.

The seawater medium used contains 50% v/v Bushnell-Haas medium, prepared according to the manufacturer instructions and 50% v/v of filtered seawater. The sea water was collected from coordinates 38°24.977N, 8°58.073W at a depth of 4 m with collaboration of Instituto Português do Mar e da Atmosfera (IPMA). This sampling was done at a distance of 3 to 5 km from the coast of Setubal, Portugal and the water column depth was of 74 m. The seawater collected was sent to iBB labs and kept at 4 °C. Before use seawater was placed at 25 °C and filtered using 25 μm paper filter in a sterilized filtering system vacuum filter. For the OSRAs formulations, Corexit 9500 (Nalco Holding Company), a commercially available chemical dispersant, was compared to formulated MEL-OSRA and solvent matrix-OSRA. The MEL used was obtained from a *M. bullatus* fermentation. Besides a commercially available n-alkane solution (C7-C40) from Sigma-Aldrich®, a solution of 5 n-alkane (C10, C12, C14, C16, C17) was also prepared.

3.1.2 Organic Solvents

The organic solvents used for MEL isolation and characterization, as well as for the toxicity tests and for the total hydrocarbon content (THC) extractions in bioremediation experiments, were: Ethyl Acetate, Acetone, n-Hexane, Methanol, Isopropanol, Acetyl Chloride, dichloromethane (DCM) from Fisher®.

3.2 MEL production

3.2.1 Yeast strains and their maintenance

Moesziomyces antarcticus PYCC 5048^T (CBS 5955) and *Moesziomyces bullatus* PYCC 5535^T (CBS 6821) (former *Moesziomyces aphidis*), were provided by the Portuguese Yeast Culture Collection (PYCC), CREM, FCT/UNL, Portugal. These yeasts were cultivated for 3 days at 25 ºC on Yeast Malt Agar (YM–agar) medium (yeast extract, 3 g/l; malt extract, 3 g/l; peptone, 5 g/l; D-glucose, 10 g/l; agar, 20 g/l). From the plates, stock cultures of each specie were also prepared. For that, each specie was grown in liquid medium and stored in 20% (v/v) glycerol aliquots at -80 ºC.

3.2.2 Media and cultivation conditions

The inoculum was prepared by transferring the yeast colonies of *M. antarcticus* and *M. bullatus* into a previously sterilized Erlenmeyer flask with 1/5 working volume of medium containing 3 g/l $NaNO_3$, 0.3 g/l $MgSO_4$, 0.3 g/l KH_2PO_4, 1 g/l yeast extract, 40 g/l D-glucose, and incubated in the orbital shaker at 27 ºC with 250 rotations per minute (rpm), for 48 h. Afterwards, at a volume of 10% of the total medium volume, the inoculum was used to start the fermentations in fresh cultivation media and maintained in an orbital shaker at 250 rpm and 27 ºC for the duration of the fermentation.

3.2.3 Shake flask cultivations

Aiming at increasing MEL titres and gain insights for optimization of MEL production, several shake flask experiments were conducted on the effects of aeration and inoculum to medium volume on *M. bullatus* culture fermentations.

To study the effect of aeration in MEL production, 4 conditions were assessed, varying the working volume in 250 ml shake flasks for increasing medium to headspace ratios in M. bullatus cultures incubated during 7 days at 27ºC and starting with 10% (v/v) of inoculum, 40 g/l of D-glucose (hydrophilic carbon source), 20 g/l of WFO (hydrophobic carbon source), and mineral medium (0.3 g/l $MgSO_4$, 0.3 g/l KH_2PO_4, 1 g/l yeast extract and 3 g/l $NaNO_3$).

- (ratio of 0.25) - For a ratio of 0.25, a total medium volume of 200 ml was used in 250 ml shake flasks, with resulting headspace of 50 ml;
- (ratio of 1.5) - For a ratio of 1.5, a total medium volume of 100 ml was used in 250 ml shake flasks, with resulting headspace of 150 ml;
- (ratio of 4) - For a ratio of 4, a total medium volume of 50 ml was used in 250 ml shake flasks, with resulting headspace of 200 ml;

- (ratio of 9) - For a ratio of 9, a total medium volume of 25 ml was used in 250 ml shake flasks, with resulting headspace of 225 ml;

To study the effect of the inoculum to total medium ratio in MEL production, 4 conditions were assessed, varying the amount of inoculum added to 250 ml shake flasks filled with 1/5 working volume. This M. bullatus cultures incubated during 7 days at 27ºC and starting with 40 g/l of D-glucose (hydrophilic carbon source), 20 g/l of WFO (hydrophobic carbon source), and mineral medium (0.3 g/l $MgSO_4$, 0.3 g/l KH_2PO_4, 1 g/l yeast extract and 3 g/l $NaNO_3$).

- (5% inoculum) - 5% of inoculum added to 250 ml shake flasks filled with 1/5 working volume for a total media volume of 50 ml;
- (10 % inoculum) - 10% of inoculum added to 250 ml shake flasks filled with 1/5 working volume for a total media volume of 50 ml;
- (20 % inoculum) - 20% of inoculum added to 250 ml shake flasks filled with 1/5 working volume for a total media volume of 50 ml;
- (30 % inoculum) - 30% of inoculum added to 250 ml shake flasks filled with 1/5 working volume for a total media volume of 50 ml;

3.2.4 Bioreactor cultivations

Aiming at optimizing a sustainable MEL production, several fermentation parameters in bench scale bioreactor were tested. The experiments were performed during 12 or 9 days in a 2.5L bioreactor filled with 1L of culture medium (1/5 working volume) constituted by mineral medium (3 g/l $NaNO_3$, 0.3 g/l $MgSO_4$, 0.3 g/l KH_2PO_4, and 1 g/l yeast extract), 10% of inoculum, and as carbon source added at day 0 of fermentation, 40 g/l of D-glucose and 20 g/l WFO. The temperature was controlled at 27ºC, while the pH was not controlled.

This bioreactor *M. bullatus* fermentations compared 2 conditions for agitation speed (rpm) and air flow (volume of gas per volume of liquid per minute (vvm)), plus 2 conditions regarding supplement of WFO during the fermentation.

- (500 rpm — 1 vvm — 2 feeds) - agitation speed set in a cascade mode between 150 rpm and 500 rpm, varying according to the dissolved oxygen, air flow set to 1 vvm, and a 2 feeds strategy with supplement of 20 g/l of WFO at the 4^{th} and 7^{th} day of fermentation;
- (800 rpm — 2 vvm — 2 feeds) - agitation speed set in a cascade mode between 150 rpm and 800 rpm, varying according to the dissolved oxygen, air flow set to 2 vvm, and a 2 feeds strategy with supplement of 20 g/l of WFO at the 3^{rd} and 6^{th} day of fermentation;

- (800 rpm — 2 vvm — several feeds) - agitation speed set in a cascade mode between 150 rpm and 800 rpm, varying according to the dissolved oxygen, air flow set to 2 vvm, and a semi-continuously feeding strategy with supplement of 6 g/l of WFO per day until the same amount of carbon from the 2 feeds strategy bioreactors is reached;

3.2.5 Yeast growth and viability

Cell growth was quantified by cell dry weight (CDW) and/or by counting colony forming units (CFU) of viable yeast cells. CDW was determined with 1 ml of culture broth. This culture broth was centrifuged at 10000 rpm for 6 min, the pellet was washed twice in deionized water, dried at 60 ℃ for at least 24 h and finally weighted. Viable yeast cells were determined by platting 10 µL of an appropriate dilution of the culture broth in YM-agar and after incubation at 25 ℃, for 48 h, CFU were counted.

3.2.6 Sugar and Nitrate profiles

Supernatants were collected, diluted with a sulphuric acid 0.05 M solution filtered through a 0.45 µm- pore-size filter and analysed for monosaccharides and nitrate quantification using high performance liquid chromatography (HPLC) system (VWH Hitachi, Darmstadt, Germany) equipped with a refractive index detector (L-7490, VWH Hitachi, Darmstadt, Germany) and a RezexTM RHM-Monosaccharide H+ (8%) column (300 mm x 7.8 mm, Phenomenex), at 50 ℃. Sulfuric acid (0.005 M) was used as mobile phase at 0.5 ml/min.

3.2.7 MEL and fatty acids profiles

1 ml of fermentation broth or 1 ml of isolated crude MEL were freeze-dried and methanolysis was performed, the resulting methyl esters were extracted and analysed by gas chromatography equipped with flame ionization detection (GC-FID) (Hewlett-Packard, HP5890). Methanolysis of freeze-dried samples was performed as follow: pure methanol was cooled down and acetyl chloride was added to it in a proportion of 20:1 (v/v), methanol to acetyl chloride, generating a water-free HCl/methanol solution. Freeze-dried samples were weighted and mixed with 2 ml of the HCl/methanol solution and 100 µL of internal standard. This internal standard has 4% (v/v) of heptanoic acid and 96% (v/v) of n-hexane. Then, the samples were incubated for 1 h at 80℃, allowing for the reaction into methyl esters. The resulting product was extracted with 1 ml of water and then 1 ml of n-hexane. The organic phase was retrieved, filtered with cotton and sodium sulphate, and analysed by gas chromatography (GC) to quantify the methyl ester concentrations. The GC system (Hewlett-Packard, HP5890), equipped with a FID detector and a HP-Ultra 2 column, was used. 1 µL of the retrieved organic phase was injected. The GC oven was programmed with an initial temperature of 140 ℃, raised to 170 ℃ at 15 ℃/min, then to 210

℃ at 40 ℃/min, and finally to 310 ℃ at 50 ℃/min. The final time at 310 ℃ was of 3 minutes. Nitrogen gas was used at a flow rate of 50 ml/h. MEL production was quantified through the amount of C8, C10 and C12 methyl esters.

3.2.8 MEL extraction

To extract MEL from the fermentation broth a liquid-liquid extraction was performed using ethyl acetate. Ethyl acetate was added in equal amounts to the fermentation broth and the organic phase was retrieved. Following 3 repetitions, the organic phases were then transferred to a round bottom flask and concentrated using a rotary evaporator, recovering ethyl acetate and obtaining crude MEL.

To obtain the supernatant fraction the fermentation broth was centrifuged for 6 minutes at 5000 rpm.

3.3 MEL based oil spill response agent formulation

The most widely used commercial OSRA is Corexit 9500 from Nalco Holding Company. It comprises of the anionic surfactant dioctyl sodium sulfosuccinate (DOSS), non-ionic surfactants, and a solvent base. However, it has been reported that the use of DOSS reduces the rate of oxidation of crude oil by microbial catalysis and has toxic effects on microalgae and other marine organisms. [69] Moreover, the currently used surfactants/dispersants are not effective over wide temperature and salinity ranges. Towards this, an environmentally friendly oil dispersant formulation based on biological compounds with high ecological performance and high dispersion effectiveness was developed in partnership between our group and SINTEF. This MEL based oil spill response agent (MEL-OSRA) was prepared according to the methods optimized by the partners at SINTEF and comprises a blend of MELs (42% w/w), TWEEN 80 (28% w/w), and a solvent base (30% w/w). The solvent base contains 66.7% (v/v) of a lighter fuel and 33.3% (v/v) of 2-ethylhexyl acetate. The term “lighter fuel” is defined as a gas, oil, or other fuel used in a lighter.

For comparison, a OSRA was prepared following the same methods and concentrations used for the MEL-OSRA but without addition of MEL. This formulation was given the name solvent matrix based oil spill response agent (solvent matrix-OSRA) and includes TWEEN 80 (48% w/w) and the solvent base (52% w/w) containing the same 66.7% (v/v) of a lighter fuel and 33.3% (v/v) of 2-ethylhexyl acetate.

3.4 Toxicity analysis

3.4.1 Marine toxicity screening tests using *Artemia franciscana*

A 24h LC_{50} bioassay was carried out with the glycolipid biosurfactants along with other OSRAs. This bioassay was conducted according to the standard operational procedures of the marine toxicity screening test Artoxkit M, obtained from Micorbiotests Inc. and developed by the research team of Prof. Dr. G. Persoone at the State University of Ghent in Belgium. [70] This bioassay uses instar II-III larvae of the brine shrimp *Artemia franciscana* hatched from cysts and standard seawater medium with a salinity of 35 ppt, provided by the commercial kit. The hatching of the cysts is carried out through exposure to a light source, with a minimum of 3000-4000 lux, during 30 h at 25 ℃. The hatching starts after about 18-20 hours, and after 30 hours most of the larvae will have moulted into the desired instar II-III stage. Using the provided multiwell test plate with 24 test wells, the larvae are transferred from the hatching medium to a rinsing well containing 1 ml of the test solution, thus exposing the larvae to the appropriate test solution before they enter the actual test well and minimizing dilution of the test solution during transfer. Then, ten larvae taken from the respective rinsing well are exposed in triplicate to 1 ml of each concentration of the test sample in the remaining wells. This bioassy design is based on one control and five increasing toxicant concentrations, each with 3 replicates of 10 animals. The incubation is carried out in the dark at 25 ℃ and after 24 hours the dead larvae in each test well are counted. In the end the % mortality and, for the definitive tests, the median lethal concentration (LC50) are calculated.

3.4.2 Phytotoxicity screening tests using *Lactuca sativa* and *Solanum lycopersicum*

The phytotoxicity of the MEL OSRA formulation was assessed on lettuce (*Lactuca sativa*) and tomato (*Solanum lycopersicum*), with 10 and 3 seeds respectively. The seeds were sterilized with sodium hypochlorite and for each condition distributed, in triplicate, into separate Petri dishes. The test solutions were prepared in distilled water with MEL concentrations of 0.02, 0.2, 1 and 2 g/l. Distilled water was used as the negative control and vitamin D as the positive control.

After five days (120 hours) of incubation in the dark at 25 ℃, the germinated seeds were counted, root and hypocotyl lengths were measured with a ruler. The relative seed germination (RSG), relative root elongation (RRE) and the germination index (GI) were calculated using the following formulas:

$$\text{RSG}(\%) = \frac{\text{n° seeds germinated in the test solution}}{\text{n° seeds germinated in the positive control}} \times 100 \tag{3.1}$$

$$\text{RRE}(\%) = \frac{\text{mean root length in the test solution}}{\text{mean root length in the positive control}} \times 100 \tag{3.2}$$

$$\text{GI}(\%) = \frac{\%RSG \times \%RRE}{100} \tag{3.3}$$

3.5 Bioremediation

3.5.1 Strategy for oil and OSRAs mixture

OSRAs formulations were premixed with Statfjord C fresh crude oil in a OSRA to oil ratio of 1 to 25 at 500 rpm for at least 3 h at room temperature. In the cases where the resulting mixture was not used straightforward it was stored at 4 ℃. Before using the stored mixtures, they were always mixed for at least half an hour at 500 rpm and at the same temperature as the biodegradation test.

3.5.2 OSRAs dispersion test

With the objective of testing 2 methods of dispersing OSRAs, OSRAs formulations premixed with Statfjord C fresh crude oil in a OSRA to oil ratio of 1 to 25 were added to baffled shake flasks with 100 ml of seawater medium. For a final crude oil concentration of 50 mg/l, 6.37 μl of the mixture were added. Alternatively, to baffled shake flasks containing 100 ml of seawater medium, 1st Statfjord C fresh crude oil was directly dispersed onto the surface of the seawater medium, and then the OSRAs formulations were dispersed onto the center of the oil slick.

Afterwards, the baffled shake flasks were placed on the orbital shaker and mixed for 10 minutes at a rotation speed of 200 rpm. At the end of the shaking period, the flasks were removed and allowed to remain stationary on the bench top for 10 minutes. At the conclusion of the settling time, the first 2 ml of sample were collected for possible later analysis, and then 30 ml of sample were collected for extraction with DCM. In a separation funnel, 5 ml of DCM was added to the samples and after vigorous mixture, the organic fraction was collected. This procedure was repeated 2 more times, and in the end all the organic phases were transferred to a round bottom flask where they were reduced to a final volume of 20 ml in a rotary evaporator. In the end the THC was quantified trough spectrophometry analysis.

3.5.3 Contamination of sea water and sediments samples

3.5.3.1 Sea water medium contamination

Crude oil / OSRA solutions were added to 100 ml of seawater medium for a final concentration of 50 mg/l of oil, in 250 ml baffled shaking flasks, which were incubated at 25 ℃ for 7 days at 200 rpm. Besides endogenous microbial consortium already present on the sea water, no additional microbial species were inoculated. In addition to those carried out from the sea water and crude oil / OSRA

solution, no carbon sources were fed to these experiments. Separate controls, prepared as previously stated but with or without the addition of azida (0.08% v/v), were also used.

At the end of each experiment 10 mL of HCl (15% v/v) was added to the samples in order to prevent further hydrocarbon degradation by microorganisms. In order to later quantify extraction losses 0.1 mL of pristane (2 g/l in n-hexane) was also added as an internal standard. Samples were then stored at 4 ℃ to avoid loss of hydrocarbons due to volatilization and degradation while waiting extraction.

3.5.3.2 Contamination of suspended sediments

Dispersant and oil solutions, mixed for at least half an hour at 500 rpm beforehand, were added to 1.4 l of filtered seawater with a final concentration of 67.2 mg/l of oil. The seawater was dispersed with oil by stirring at 500 rpm for 4 hours. Afterwards, 7.5 g of sediments were added to the glass flasks containing the seawater dispersed with oil and stirred for 55 additional minutes at approximately 250 rpm, followed by 5 minutes stirring at approximately 50 rpm. The speed was chosen by visual observation of the speed that allows for suspension of all sediment without adding a torque.

After the samples settled for 24 hours, residual oil on the water surface and glass walls was removed by using an absorbent pad. The sample was then filtrated on a Buchner funnel with a 25 μm paper filter. The sediments were flushed three times on the filter paper with 30 mL of clean seawater, left to dry at room temperature and then transferred to glass containers and covered by aluminium foil. The sediments and the remaining water phase, transferred to 2 l glass bottles, were stored at 4 ℃ while awaiting extraction.

3.5.4 Total hydrocarbon content extraction strategies

3.5.4.1 Total hydrocarbon content extraction from seawater

To assess the THC in contaminated seawater medium, it was extracted using n-hexane ($\geq$95%, HPLC grade, Fisher Chemical) as the extracting solvent. The content of each baffled shake flask of the bioremediation studies was transferred to a separation funnel, to witch a total 50 ml of hexane was added and vigorous mixed to help the transfer of the hydrocarbons to the organic phase. The organic fraction was transferred to a round bottom flask. Another 25 ml of hexane were used to rinse each baffled shake flask and then added to the separation funnel. After vigorous mixing, this last organic fraction was collected and transferred to the same round bottom flask.

In the combined organic phases, the solvent, n-hexane, was evaporated using a rotary evaporator until the volume was roughly 2 ml. The concentrated sample was then filtered with a Pasteur pipette containing cotton, silica gel and anhydrous sodium sulphate and transferred to a glass vial. Its volume was further reduced to 0.9 ml using liquid nitrogen and the samples were then completed with the

addition of 0.1 ml of 5-α-androstane (2 g/l in n-hexane) as an external standard.

A blank solution was prepared using n-hexane and both the internal and external standard solutions in a ratio of 8:1:1. For each new standard solution prepared, a respective new blank solution must be made with it in order to validate their and the GC-FID analysis integrity.

For the water phase collected in the experiments using sediments, the extraction of the THC was performed using DCM as the separation solvent and following the same procedures. After collecting the organic phases with the hydrocarbons, the DCM solvent was reduced with a rotary evaporator until the final volume was 20 ml.

All the samples were stored at -20 ℃ prior to GC-FID or spectrophotometry analysis.

3.5.4.2 Total hydrocarbon content extraction from sediments

In an attempt to extract the THC from sediments, the Soxhlet extraction technique for the extraction of chemicals from solid matrices was used.

The Soxhlet thimble was fitted and rinsed three times with DCM and the sediment samples were transferred to the thimble and covered with cotton wool. An internal standard (100 µL of pristane at 2 g/l) was added to the thimble and it was placed in the Soxhlet glassware. Then, 70 ml of DCM were added to the round bottomed flask connected to the Soxhlet glassware with a Liebig cooler placed on top. In a fume hood, using a warming plate at around 80-90 ℃, the round bottomed flask was placed in a bath at 60 ℃ and 250 rpm.

After an extraction time of 24 hours (plus the time to complete the current cycle), the flask with the extract was cooled and filtered through Bilson cotton and anhydrous sodium sulphate (Na2SO4, baked) onto a round bottomed flask and reduced to approximately 5 ml by evaporation of solvent, using a rotary evaporator at 40 ℃ and 900 pressure (as close to atm as the rotary evaporator allows). The samples were transferred to glass vials. The round bottom flask used was rinsed three times with DCM, that was then transferred to the vials, totaling a final volume of 20 ml.

The sample solvent could be then changed from DCM to n-hexane by further evaporating the DCM in the rotary evaporator till “dry” and then adding 3x2 ml of n-hexane, allowing a GC analysis after addition of a second internal standard (100 µL of androstane at 2 g/l) to 0,9 mL samples.

3.5.5 Total hydrocarbon content analysis and quantification

3.5.5.1 Gas chromatography with flame ionization detection analysis

The bioremediation samples THC were analysed by GC-FID (Hewlett-Packard, HP5890) using the following cycle parameters. Initial oven temperature was 40 ℃ and held for 2 minutes. Temperature was then increased at a rate of 6 ℃/min until reaching 310 ℃, where it was held for 10 minutes. Injector

and detector temperatures were 300 ℃ and 310 ℃ respectively. Purge was set to turn on after 0,66 minutes. Injected sample volume was 1 μL. Before each set of analysis, and between the analysis of 3 consecutive samples, a cleaning blank solution, with the internal and external standard solutions, was injected and processed through the complete analysis cycle.

Calibration curves were made for crude oil using a series of dilutions (1 mg/l, 10 mg/l, 50 mg/l in n-hexane) with 5-α-androstane as an internal standard. The relative response factor (RRF) was calculated using equation 3.4.

$$RRF = \frac{A_{std} \times C_{andr}}{A_{andr} \times C_{std}} \tag{3.4}$$

For the crude oil samples, the total area (A_{total}) was obtained by automatic integration of peaks from 6 min to 50 min of the GC-FID spectrum. The THC area (A_{THC}) was then calculated by subtracting the area of the internal standards (A_{andr}, A_{pris}) to the total area (equation 3.5).

$$A_{THC} = A_{total} - A_{andr} - A_{pris} \tag{3.5}$$

Pristane internal standard and the 5-α-androstane external standard peak areas in the sample and in the cleaning blank solutions were used to quantify the percentage of hydrocarbons recovered in the extraction using equation 3.6.

$$Recovery(\%) = \frac{\frac{A_{pris\ sample}}{A_{pris\ blank}}}{\frac{A_{ands\ sample}}{A_{ands\ blank}}} \times 100 \tag{3.6}$$

The concentration of hydrocarbons in each sample (C_{THC}) was calculated using the equation 3.7, optimized in appendix C and were the RRF is of 1.8.

$$C_{THC} = \frac{A_{THC} \times C_{andr}}{A_{andr} \times RRF \times Recovery} \tag{3.7}$$

3.5.5.2 Spectrophotometry analysis

The bioremediation samples THC could also be analysed through a spectrophotometry method. Aiming towards the creation of calibration curves for this method, premixed solutions of OSRAs formulations and Statfjord C fresh crude oil, in a OSRA to oil ratio of 1 to 25, were mixed with solvent, either DCM or n-Hexane, in increasing concentrations, at a 1 to 10 ratio of oil plus dispersant to solvent. Afterwards, this stock solutions were added to 30 ml of standard seawater medium with a salinity of 35 ppt. The THC of these samples was extracted using either DCM or n-Hexane as the separation solvent. In a separation funnel, 5 ml of solvent were added to the samples and vigorous mixed to help the transfer of the hydrocarbons to the organic phase. The organic fraction was then transferred to a round

bottom flask. This procedure was repeated 2 more times and after collecting all the organic phases with the hydrocarbons, the DCM solvent was reduced with a rotary evaporator until a final volume of 20 ml. A 1 ml sample was then transferred to a glass cuvette and the absorbance of the solution at 410 nm was measured using a UV–vis absorbance spectrophotometer (UH5300, Hitachi). For each solvent, after plotting the absorbance (at 410 nm) values against the concentration of THC in the seawater (mg/ml), a calibration curve was obtained. Respectively, $C_{\text{THC in seawater}} = \frac{Abs_{\text{at 410 nm}}}{1.8315}$ for n-Hexane and $C_{\text{THC in seawater}} = \frac{Abs_{\text{at 410 nm}}}{1.1832}$ for DCM.

4

Results and Discussion

Contents

4.1 MEL Production

MEL are glycolipids produced by *Moesziomyces* spp. from different substrates, preferably vegetable oils [65]. Within the iBB group, the use of alternative substrates is already well established.

With the objective of testing the MEL production method and studying the effect of aeration and inoculum % on *M. bullatus* culture, in preliminary studies, and due to MEL's amphiphilic nature, *Moesziomyces bullatus* fermentations in shake flask were performed using D-glucose as the hydrophilic carbon source and waste frying oil (WFO) as the hydrophobic carbon source. Subsequent oxygen mass-transfer coefficient (kLa) studies conducted within the research group, with the aim of optimizing and scaling up the fermentation process to produce MEL, several *M. bullatus* fermentations in a bench scale reactor were conducted.

4.1.1 Effect of aeration on *Moesziomyces bullatus* culture and MEL production

To study the effect of aeration in MEL production, 4 conditions were assessed, varying the working volume in 250 ml shake flasks for increasing medium to headspace ratios in M. bullatus cultures incubated during 7 days at 27ºC.

Observing figure 4.1, the results show a steady increase of biomass and a decrease of D-glucose consistent with its consumption throughout the fermentation time. However, it is interesting to observe that while D-glucose is not entirely consumed in conditions where the ratio of headspace to medium volume is below 1, the residual lipids are consumed in the conditions with higher ratios (1.5, 4 and 9). The lower the ratio, and consequently less the aeration, the higher is the concentration of D-glucose remaining at the end of the fermentation, suggesting a lower metabolic activity. This is further illustrated by the observation of higher production rates of MEL and less lipidic residues at the final day of fermentation for fermentations with higher ratios of medium to headspace, and thus higher aeration rates (figure 4.2).

In the end of the fermentations, after the extraction process, MEL's purity and the productivity of its production was calculated for each condition tested (table 4.1). The worst results were obtained for a 0.25 ratio of headspace to medium volume, with MEL at 13.97% purity and 0.05 g/l/h of productivity. By comparison, with a ratio of headspace to medium volume of 9 the best results are obtained. For a maximum MEL obtained of 25.63 g/l and 3.2 g/l of residual lipids, MEL had 87.35% of purity and a productivity of 0.19 g/l/h.

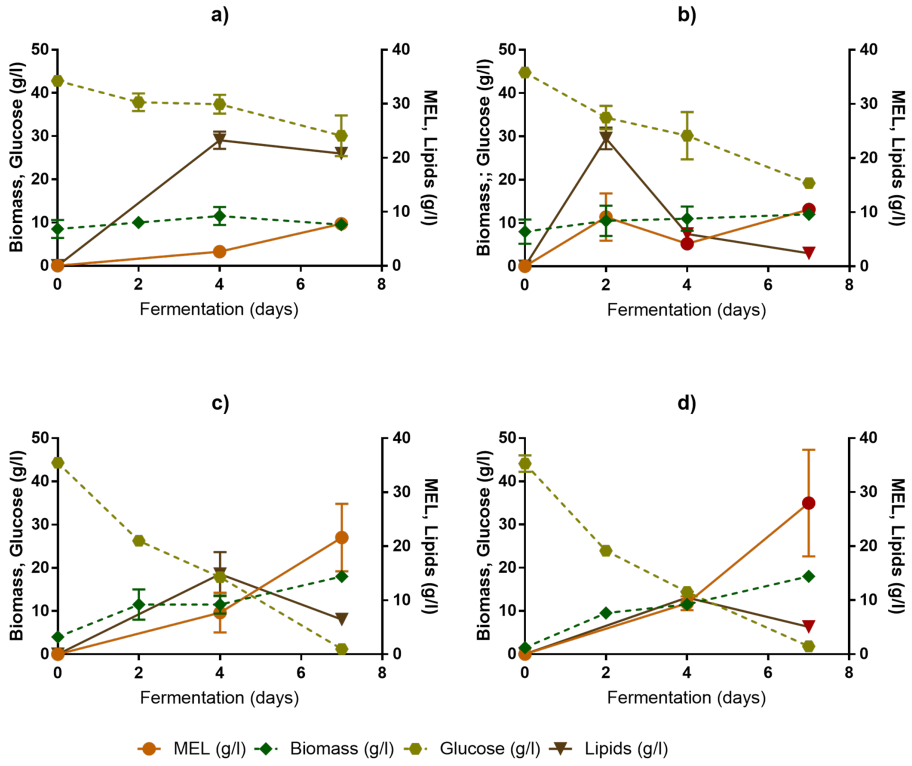

Figure 4.1: Effect of aeration on *M. bullatus* shake flask fermentations. MEL (orange filled line), biomass (green dotted line), D-glucose (yellow dotted line) and lipids (brown filled line) concentration, in g/l, throughout the days of *M. bullatus* fermentations incubated during 7 days at 27ºC using 40 g/l of D-glucose and 20 g/l of WFO as carbon source, varying the ratios of headspace to medium volume at 0.25, a); 1.5, b); 4, c); and 9, d).

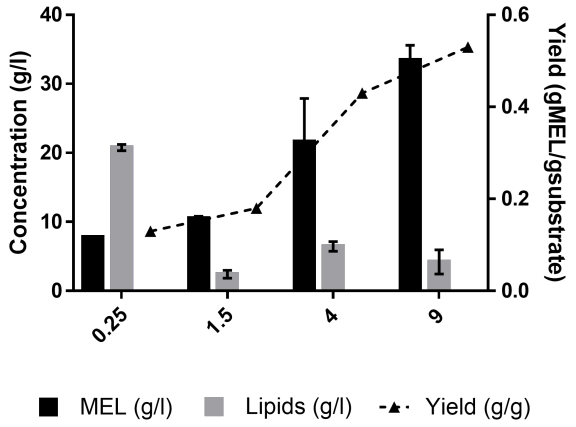

Figure 4.2: Aeration influence on *M. bullatus* shake flask fermentations. MEL (black bar), and lipids (grey bar) concentration, in g/l, plus the yield (black dashed line), in g/g, on the last day of *M. bullatus* fermentations incubated during 7 days at 27ºC using 10% inoculum, 40 g/l of D-glucose and 20 g/l of WFO as carbon source, varying the ratio of volume headspace and medium volume (0.25, 1.5, 4 and 9 for a total volume of 200, 100, 50 and 25 mL, respectively).

Table 4.1: Aeration influence on *M. bullatus* shake flask fermentation parameters. Maximum MEL obtained (g/L), maximum productivity (g/l/h), purity (%), and residual lipids remaining (g/l) at the end of *M. bullatus* fermentations incubated during 7 days at 27ºC using 40 g/l of D-glucose and 20 g/l of WFO as carbon source, varying the ratio of volume headspace and medium volume (0.25, 1.5, 4 and 9 for a total volume of 200, 100, 50 and 25 mL, respectively).

Parameters	**Ratio headspace/medium volume**			
	0.25	**1.5**	**4**	**9**
MEL_{max} (g/L)	6.5	10.52	20.4	25.63
$Productivity_{max}$ (g/L/h)	0.05	0.06	0.16	0.19
MEL purity (%)	13.97	32.71	78.46	87.35
Residual lipids (g/L)	18.4	2.42	6.12	3.2

Clearly oxygen plays a crucial role in MEL production and there are significantly differences comparing ratio of 0.25 and 1, with 4 and 9. Concluding, the conditions with lower medium volumes have an higher flask-to-medium ratio and consequently an higher aeration and % of oxygen available resulting in more productivity.

4.1.2 Effect of inoculum to total medium % on *Moesziomyces bullatus* culture and MEL production

To study the effect of the inoculum to total medium ratio in MEL production, 4 conditions were assessed varying the amount of inoculum (5, 10, 20, 30%) added to 250 ml shake flasks filled with 1/5 working volume.

Figure 4.3 shows an increase on biomass and consumption of D-glucose throughout the fermentation time similar for all the conditions. These results show that the % of inoculum used in the beginning of the fermentation is not affecting the cell activity and productivity of the cultures.

This is further proved by similar MEL titres and lipidic residues concentrations on the final fermentation day in all the conditions (figure 4.4).

In the end of the fermentations, after the extraction process, MEL's purity and the productivity of its production was calculated for each condition tested (table 4.2). In the 5% condition MEL at 73.26% purity was obtained with a productivity of 0.12 g/l/h, with the 10% condition MEL was produced with 73.09% purity and a productivity of 0.14 g/l/h. With 20% inoculum MEL had 69.79% of purity for a productivity of 0.16 g/l/h. Finally the 30% inoculum condition MEL had 65.63% purity and a productivity of 0.14 g/l/h.

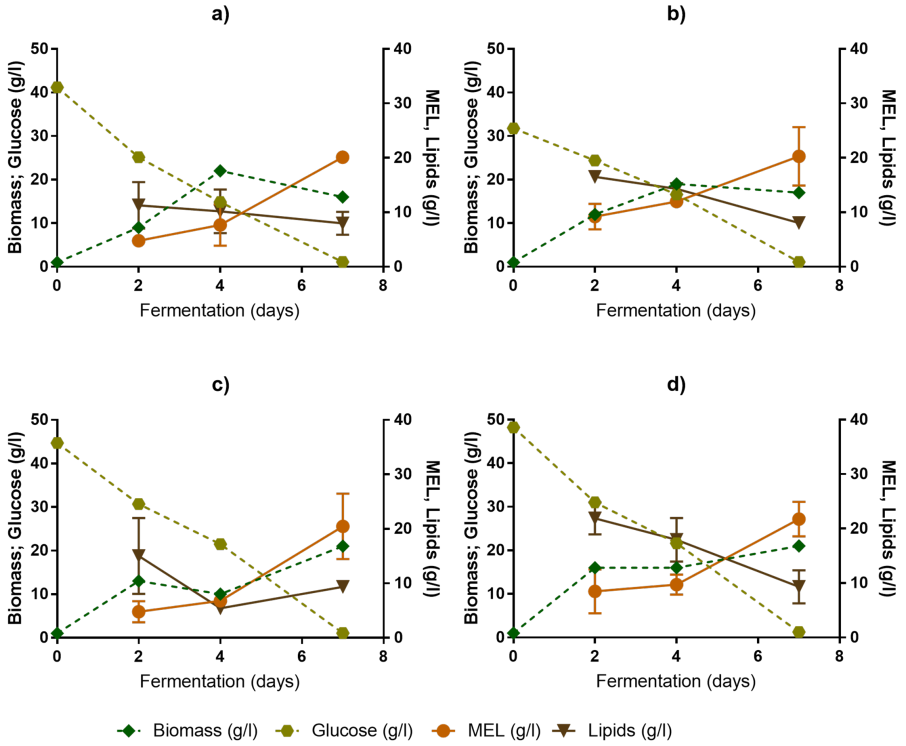

Figure 4.3: Influence of inoculum % on *M. bullatus* shake flask fermentations. MEL (orange filled line), biomass (green dotted line), D-glucose (yellow dotted line) and lipids (brown filled line) concentration, in g/l, throughout the days of *M. bullatus* fermentations incubated during 7 days at 27ºC using 40 g/l of D-glucose and 20 g/l of WFO as carbon source, and varying the the amount of inoculum added, either 5%, a); 10%, b); 20%, c); or 30%, d) to 250 ml shake flasks filled with 1/5 working volume (for a total of 50 ml of medium).

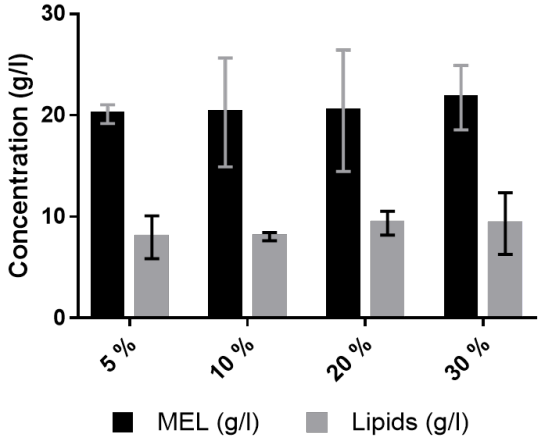

Figure 4.4: Inoculum to medium % influence on *M. bullatus* shake flask fermentation. MEL (black bar), and lipids (grey bar) concentration, in g/l, plus the yield (black dashed line), in g/g, on the last day of *M. bullatus* fermentations incubated during 7 days at 27ºC using 40 g/l of D-glucose and 20 g/l of WFO as carbon source, and varying the the amount of inoculum added, either 5, 10, 20, or 30%, to 250 ml shake flasks filled with 1/5 working volume (for a total of 50 ml of medium).

Table 4.2: Inoculum to medium % influence on *M. bullatus* shake flask fermentation parameters. Maximum MEL obtained (g/L), maximum productivity (g/l/h), purity (%), and residual lipids remaining (g/l) at the end of *M. bullatus* fermentations incubated during 7 days at 27ºC using 40 g/l of D-glucose and 20 g/l of WFO as carbon source, and varying the the amount of inoculum added, either 5, 10, 20, or 30%, to 250 ml shake flasks filled with 1/5 working volume (for a total of 50 ml of medium).

Parameters	**Inoculum to medium %**			
	5%	**10%**	**20%**	**30%**
MEL_{max} (g/L)	28.36	32.73	35.64	32.73
$Productivity_{max}$ (g/L/h)	0.12	0.14	0.16	0.14
MEL purity (%)	73.26	73.09	69.79	65.63
Residual lipids (g/L)	8.73	10.55	13.82	15.64

Concluding, no correlation could be found between the % of inoculum and MEL productivity. The % of inoculum used to start a fermentations does not have a significant effect on the productivity of the MEL production process.

4.1.3 Optimization of *Moesziomyces bullatus* fermentation in bench scale reactor

Subsequent the oxygen mass-transfer coefficient (kLa) studies conducted previously within the research group (in annex), and aiming to optimize MEL titres, *Moesziomyces bullatus* cultures were incubated in a 2.5L bioreactor filled with 1l of culture medium (1/5 working volume), during 12 or 9 days with controlled temperature at 27ºC, without controlled pH, using mineral medium as described in section 3.2.4, D-glucose and WFO as carbon source with feeds of WFO throughout the fermentation.

The previously state of the art condition of 150-500 rpm of agitation, varying according to the dissolved oxygen, and 1 vvm of air flow was compared with 150-800 rpm, also varying according to the dissolved oxygen, and 2 vvm, the condition that in the studies preformed within the research group had the highest kLa and consequently more head space with possibility for an improvement in the metabolic efficiency, as seen in 4.1.1. Both fermentations started with 40 g/l of D-glucose and 20 g/l of WFO as carbon source and had 2 feeds of 20 g/l of WFO added at either day 4 and 7 or at day 3 and 6 of fermentation.

Due to the quickly oil consumption and foam appearance a supplement of WFO is necessary during the fermentation. To assess how the feeding strategy of WFO can be used to optimize mannosylerythritol lipid (MEL) production in reactor, further fermentation experiments were conducted. The previous tested conditions were compared to a condition of 150-800 rpm, varying according to the dissolved oxygen, and 2 vvm fermentation started with 40 g/l of D-glucose and 20 g/l of WFO but with feeds of 6 g/l of WFO per day, only until day 6 of fermentation to ensure the amount of WFO is the same as in the previous

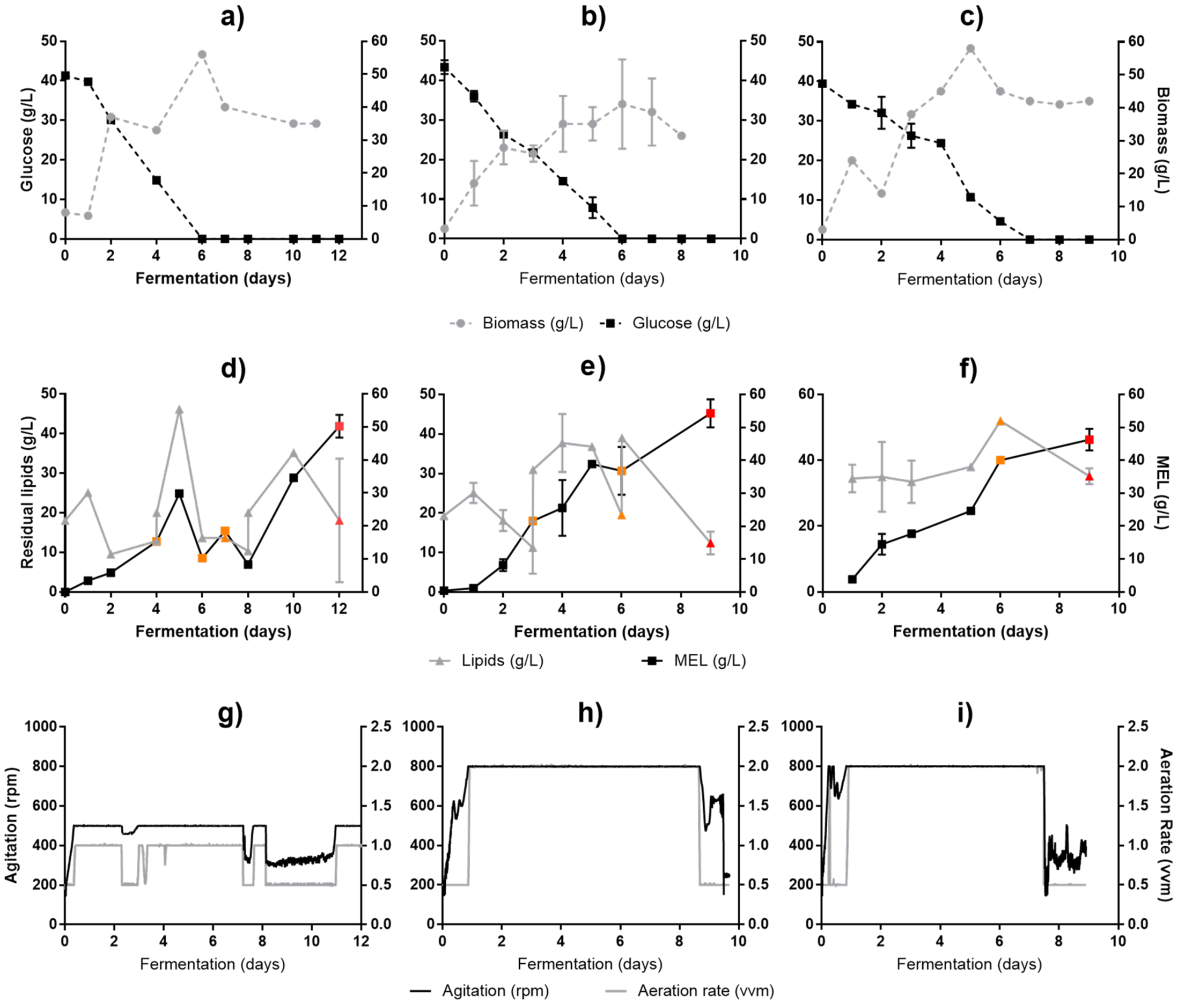

Figure 4.5: Optimization and scale up of *M. bullatus* fermentations to a bench scale reactor. Profile growth (grey dashed line with circles), D-glucose consumption (black dashed line with squares), residual lipids (grey filled line with triangles), MEL production (black filled line with squares), agitation (black filled line), and aeration rate (grey filled line) for *M. bullatus* cultures incubated in a 2.5L bioreactor filled with 1L of culture medium (1/5 working volume), during 12 or 9 days with controlled temperature at 27ºC, without controlled pH, using mineral medium, as described in section 3.2.4, 40 g/l of D-glucose and 20 g/l of WFO as carbon source plus two feeds of 20 g/l of WFO, at day 4 and 7 for the 150-500 rotations per minute (rpm), varying according to the dissolved oxygen, and 1 volume of gas per volume of liquid per minute (vvm) condition, a); d); g), or at day 3 and 6 for the 1st 150-800 rpm, varying according to the dissolved oxygen, and 2 vvm condition, b); e); h), or with semi-continously feeding of 6 g/l of WFO per day for the 2nd 150-800 rpm, varying according to the dissolved oxygen, and 2 vvm condition, c); f); i). The red point indicates the value of MEL and FFA, after extraction with ethyl acetate and the orange red point indicate the presence of beads in the medium.

bioreactor fermentations.

In the figure 4.5, observing the graphs a); b); and c) it is possible to verify a similar increase in biomass and a steady consumption of D-glucose in the 3 tested conditions. The graphs d) (500 rpm, 1 vvm and 2 feeds) and e) (800 rpm, 2 vvm and 2 feeds) have similar lipidic profiles, with marked increase after each feed. The graph f) shows consistent high lipidic values due to its semi-continously feeding of 6 g/l of WFO per day. Except the points that had beads present in the medium, compromising the measurements, all the conditions have similar MEL productions. The lower final value belongs to the 800 rpm, 2 vvm condition with several feeds, and the higher to the 800 rpm, 2 vvm condition with 2 feeds.

As seen in the previously in the subsection 4.1.1, a better aeration will result in a higher productivity. To explore these parameters and how the feeding strategy can be used to optimize MEL production in reactor, for the 3 conditions analysed, the purity, the productivity and the mass of CO_2 released were obtained (figure 4.6).

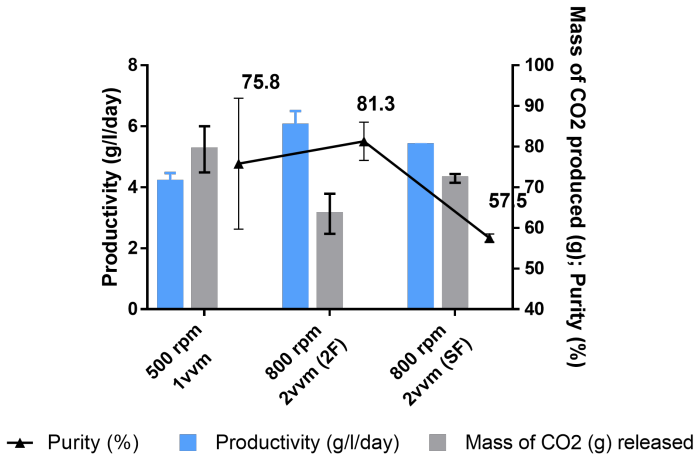

Figure 4.6: Efficiency of the *M. bullatus* fermentations in bench scale reactor. Purity % (black filled line), productivity in g/l/day (blue column) and mass of CO_2 (g) released (grey collumn) for *M. bullatus* cultures incubated during 12 or 9 days at 27ºC using 40 g/l of D-glucose and 20 g/l of WFO as carbon source plus two feeds of 20 g/l of WFO, at day 4 and 7 for the 500 rpm and 1 vvm condition, or at day 3 and 6, 2 feeds (2F), for the 800 rpm and 2 vvm (2F) condition, or with semi-continously feeding of 6 g/l of WFO per day, several feeds (SF), for the 800 rpm and 2 vvm (SF) condition.

The condition with less aeration, 1vvm, and agitation from 150-500 rpm performed worse than the conditions with a higher agitation and aeration rates. This condition, had the lowest MEL titres (47.8 and 52.66 g/l in each replica), the worst productivity (0.17 and 0.18 g/l/h in each replica), the higher mass of CO_2 released.

Regarding the feeding strategy, comparing between the conditions with higher aeration and agitation rates, the strategy with 2 feeds of 20 g/l of WFO, at day 3 and 6 performed better that the one with semi-continously feeding of 6 g/l of WFO per day. The 2 feeds strategy has the best productivity (0.27 and

0.24 g/l/h in each replica), with the lowest amount of CO_2 released, and high purity of the MEL obtained.

4.2 Toxicity analysis

4.2.1 Marine toxicity screening tests using *Artemia franciscana*

To access the toxicity, in a marine environment, of different glycolipid biosurfactants and other oil spill response agents (OSRAs), a 24h LC_{50} bioassay was carried out with *Artemia franciscana* and according to the standard operational procedures of the marine toxicity screening test Artoxkit M.

Before conducting definitive toxicity screening tests and thus obtaining the LC_{50}, meaning the lethal dose that leads to the mortality of 50% of individuals, of each tested OSRA or glycolipid biosurfactant, a range finding toxicity screening test was performed. An ideal interval to conduct each definitive toxicity screening tests was found as shown in appendix B.

Though an inhibitor dose-response fit with the equation $Y = 100/(1 + (X^{HillSlope})/(LC_{50}{}^{HillSlope}))$ the LC_{50} of the dispersant formulations, some of their components and alternative biosurfactants within the same class, was extrapolated (Figure 4.7).

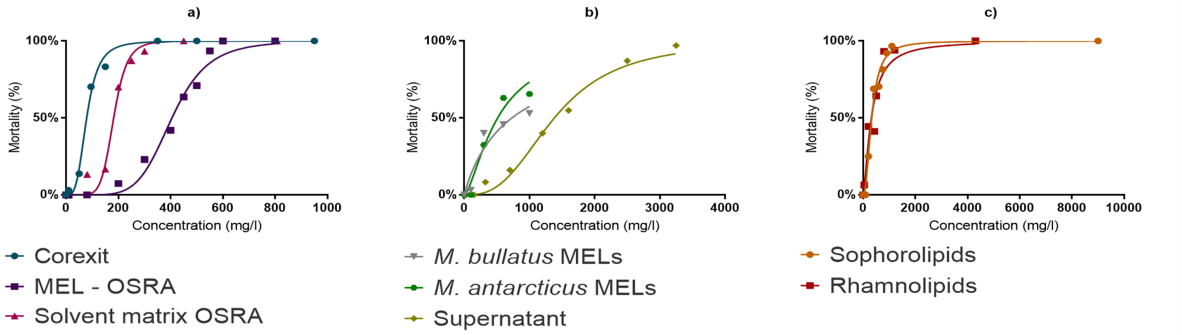

Figure 4.7: *Artemia franciscana* toxicity definitive tests of different OSRA formulations. Corexit commercial solution (blue line), developed MEL based oil spill response agent (MEL-OSRA) (purple line) and solvent matrix based oil spill response agent (solvent matrix-OSRA) (pink line), *M. bullatus* MELs (grey line), *M. antarcticus* MELs (green line), supernatant (yellow line), sophorolipids (orange line) and rhamnolipids (red line) 24h LC_{50} *A. franciscana* bioassay conducted according to the standard operational procedures of the marine toxicity screening test Artoxkit M.

Corexit has an LC_{50} of 78.92 +/- 2.77 mg/l with r square of 0.9955, the developed MEL-OSRA has an LC_{50} of 405.20 +/- 10.25 mg/l with r square of 0.9827 and the developed solvent matrix-OSRA has an LC_{50} of 182.20 +/- 4.44 mg/l with r square of 0.9894.

Regarding to the MELs and the supernatant collected in the extraction process, *M. bullatus* MELs have an LC_{50} of 731.60 +/- 174.7 mg/l with r square of 0.911, *M. antarcticus* MELs have an LC_{50} of 512.7 +/- 66.81 mg/l with r square of 0,958 and the supernatant has an LC_{50} of 1383 +/- 52.84 mg/l with

r square of 0.9897.

In other glycolipid class biosurfactants, sophorolipids have an LC_{50} of 327.1 +/- 20.05 mg/l with r square of 0,989 and rhamnolipids an LC_{50} of 316.3 +/- 47.98 mg/l with r square of 0.9571.

This results can be compared to the LC_{50} values found in the literature for these and other surfactants. Regarding glycolipid class biosurfactants, sophorolipids have an LC_{50} value of 600 mg/l in the literature, less toxic when compared to our LC_{50} of 327.1 mg/l [71]. Rhamnolipids, in the literature, have an LC_{50} of 525 mg/l, also less toxic [72]. However, their toxicity is still higher than the one obtained for MELs and the supernatant samples.

In other classes of surfactants, Triton X-100, a non ionic surfactant, has LC_{50} reported values of 80 and 58.35 mg/l in 2 separate studies [73], [74]. Quaternary ammonium compounds (QACs), cationic surfactants, have LC_{50} reported values between 5 to 30 mg/l [75]. Alkyl ethoxysulphates (AES), anionic surfactants, have LC_{50} reported values of 11.97 and 38.30 mg/l [76], [77]. This surfactants, not only are they much more toxic than MEL and the other biosurfactants, but the developed formulations as well. Only Corexit 9500 as comparable toxicity values. Further proving the added value of the developed MEL-OSRA formulation as a more eco-friendly alternative to conventional OSRA.

4.2.2 Phytotoxicity screening tests using *Lactuca sativa* and *Solanum lycopersicum*

To find the phytotoxicity of the MEL OSRA formulation, screening tests were performed using lettuce (*Lactuca sativa*) and tomato (*Solanum lycopersicum*) seeds incubated with increasing MEL concentrations, for 120 hours, in the dark, at 25 ℃. After the counting of germinated seeds and the measurement of root lengths, relative seed germination (RSG), relative root elongation (RRE) and the germination index (GI) % were calculated (table 4.3).

As the figure 4.8 shows, in both biological models until the 0.2 g/l of MEL we always have germination indexes higher that 60% and only in concentrations above 1 g/l of MEL do we find low germination indexes. However, more experiments should be conducted not only to further explore the concentrations between the 0.2 and 1 g/L interval but also to compare with other surfactants and biosurfactants, such as corexit, or other glycolipids.

Table 4.3: Phytotoxicity screening test of the MEL OSRA formulation using Lactuca sativa (lettuce) and Solanumlycopersicum (tomato) seeds. For each condition, the table shows the number of germinated seeds, the mean root elongation (mm), and using the positive control (0 g/l of MEL) the calculated RSG%, RRE% and GI%.

MEL Concentration (g/l)	0 Positive control	0.02	0.2	1	2	Vitamin D Negative control
			Lactuca sativa (lettuce)			
Number germinated seeds	21	21	19	9	0	0

Table 4.3 continued from previous page

MEL Concentration (g/l)	0 Positive control	0.02	0.2	1	2	Vitamin D Negative control
Mean root elongation (mm)	9.7	6.8	7.8	3.0	0.0	0.0
RSG (%)		100.0	90.5	42.9	0.0	0.0
RRE (%)		69.9	79.7	30.8	0.0%	0.0
GI (%)		69.9	72.1	13.2	0.0	0.0
Solanum lycopersicum (tomato)						
number germinated seeds	9	9	7	3	0	0
Mean root elongation (mm)	2.3	2.4	1.9	1.3	0.0	0.0
RSG (%)		100.0	77.8	33.3	0.0	0.0
RRE (%)		104.8	79.6	57.1	0.0	0.0
GI (%)		104.8	61.9	19.0	0.0	0.0

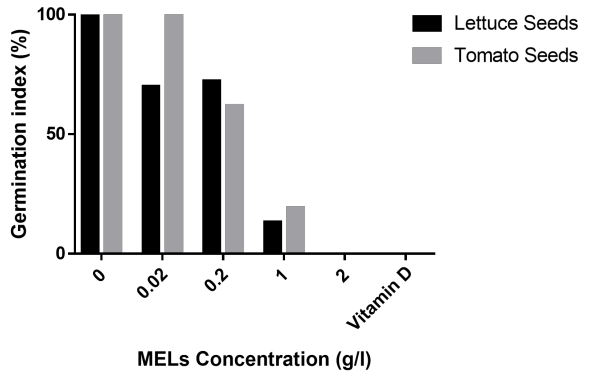

Figure 4.8: Phytotoxicity of the MEL OSRA formulation using *Lactuca sativa* (lettuce) and *Solanum lycopersicum* (tomato) seeds. Germination index (%) in lettuce (black columns) and tomato (grey column) seeds germination index (%) for MEL OSRA formulation concentrations of 0 (positive control), 0.02, 0.2, 1, and 2 g/l and using vitamin D as the negative control.

4.3 Bioremediation

4.3.1 Seawater bioremediation

Crude oil dispersion and further natural degradation can be promoted with OSRAs. To test the efficacy of different OSRAs, a bioremediation assay was carried out in 100 ml of seawater medium containing 50% v/v Bushnell-Haas medium and 50% v/v of filtered seawater. Solutions with a OSRA to oil ratio of 1 to 25 were added to the seawater medium in baffled shake flasks for a final concentration of 50 mg/l of oil. The OSRA were assessed: (i) commercially available chemical dispersant Corexit 9500; (ii) MEL-OSRA; (iii) solvent matrix-OSRA, where B is similar to A but does not include MEL; (iv) MEL obtained from a *M. bullatus* fermentation; and a negative control without an OSRA. After 7 days of

incubation at 25 ℃ and 200 rpm, the total hydrocarbon content (THC) of the baffled shake flasks was extracted and analysed by gas chromatography equipped with flame ionization detection (GC-FID).

After quantification of the THC, the concentrations data was analysed by ordinary one-way analysis of variance (ANOVA) followed by Tukey's multiple comparisons test using GraphPad Prism version 7.0.0 for Windows, GraphPad Software. The level of significance was set at $p<0.05$, 95% confidence interval (figure 4.9).

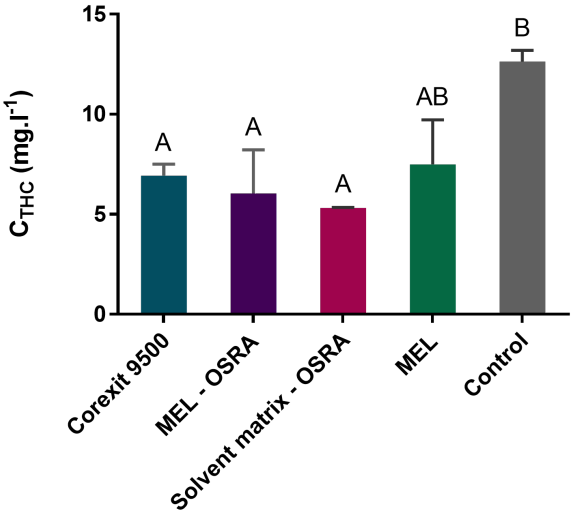

Figure 4.9: Concentration of total hydrocarbon content in seawater bioremediation assay done in March 2020. Concentration mean ± SD of total hydrocarbon content (THC), in mg/l, of the duplicate samples of OSRAs. Corexit 9500 (blue bar), MEL-OSRA (purple bar), solvent matrix-OSRA (pink bar), MELs (green bar), and negative control (grey bar). One-way ANOVA with Tukey's multiple comparisons test: columns topped with a different letter are significantly different, ($p < 0.05$). Columns topped with A have statistically significant similarities between them and columns topped with B have statistically significant similarities between them.

All the OSRA used present lower concentration of THCs, corresponding to higher degradation of crude oil during the incubation period than the control. Indeed, concentrations of THC obtained for assays using Corexit 9500, MEL-OSRA and solvent matrix-OSRA are statistically significantly lower than the one obtained for control. From these three conditions, solvent matrix-OSRA, without MEL, presents the lowest concentration of THC. However, these three conditions do not have statistically significant differences between themselves and with MELs. The condition using MEL alone has OSRA presents to be the less efficient condition, with a concentration of THC lower, but not statistically significant different than the one of the control.

Corexit 9500 has been previously applied in the field, such as in the 2010 Gulf of Mexico oil spill crisis, but as reviewed by Wise and Wise, this dispersant is in fact toxic to several species. [26] As seen previously, this dispersant is more toxic than the other OSRA tested. This 1st experiment results were

promising in establishing the developed formulations as viable alternatives to Corexit 9500.

Nonetheless, due to the optimization of the formulations, where 2-ethylhexylacetate replaced the previously used ethyl acetate, further experiments were conducted. As with the previous experiment, 2 bioremediation assays were carried out in baffled shake flasks and solutions with a OSRA to oil ratio of 1 to 25 were added to 100 ml of seawater medium for a final concentration of 50 mg/l of oil. The OSRA assessed were the same with the addition of a negative control that besides the lack of an OSRA had azida in a concentration of 0.08% added. After 7 days of incubation at 25 ℃ and 200 rpm, the THC of the baffled shake flasks was extracted and analysed by GC-FID exclusively in the case of the assay carried out in June 2020 or by GC-FID and fourier transform infrared spectroscopy (FTIR) in the assay carried out in September. This last analysis was done in LA-IST, by Engineer Georgina Sarmento and her team.

After quantification of the THC, the concentration data was analysed by ordinary one-way analysis of variance (ANOVA) followed by Tukey's multiple comparisons test using GraphPad Prism version 7.0.0 for Windows, GraphPad Software. The level of significance was set at $p<0.05$, 95% confidence interval (figure 4.10). However, as seen in the graphs a), b), and c) from figure 4.10, the conditions tested do not have statistically significant differences among themselves and the controls, where no additional OSRA was added, have lower THC concentration values. This is consistent with the main difficulties found in the laboratory, the chromatograms obtained had very different base lines, with many undefined peaks, making them difficult to analyse. As we can see in annex C, any slight contamination from previous runs or even from other components in the crude oil has a huge impact in the analysis.

In an effort to overcome the difficulties and better understand this results, a peak to peak analysis was conducted. Well defined peaks, with retention times consistent between GC-FID runs, were selected and the concentration of the hydrocarbon compound (mg/l) calculated using the established method. The results obtained are shown in graphs c) and d) from figure 4.10.

However, this results from the peak to peak analysis do not show consistency among the hydrocarbon compounds. The OSRA samples have distinct effects in the degradation of the different hydrocarbon compounds selected. In the case of the assay conducted in June, for corexit 9500, the C30 value seems clearly an outlier and might indicate the presence of other compound with very similar retention time. Nevertheless, excluding this value, the controls generally had higher hydrocarbon concentration values. The OSRA formulations tested have lower concentration of the different hydrocarbons, corresponding to an higher degradation of crude oil during the incubation period in this conditions and supporting the results obtained in the previous assay. The assay conducted in September, also has generally higher hydrocarbon concentration values in the control. However, the sterile control, where azida was added, and unlike what was expected, has consistently low values. This might indicate possible losses in the extraction process. The values for the C22 are also unexpected, with both controls with much lower

concentration values.

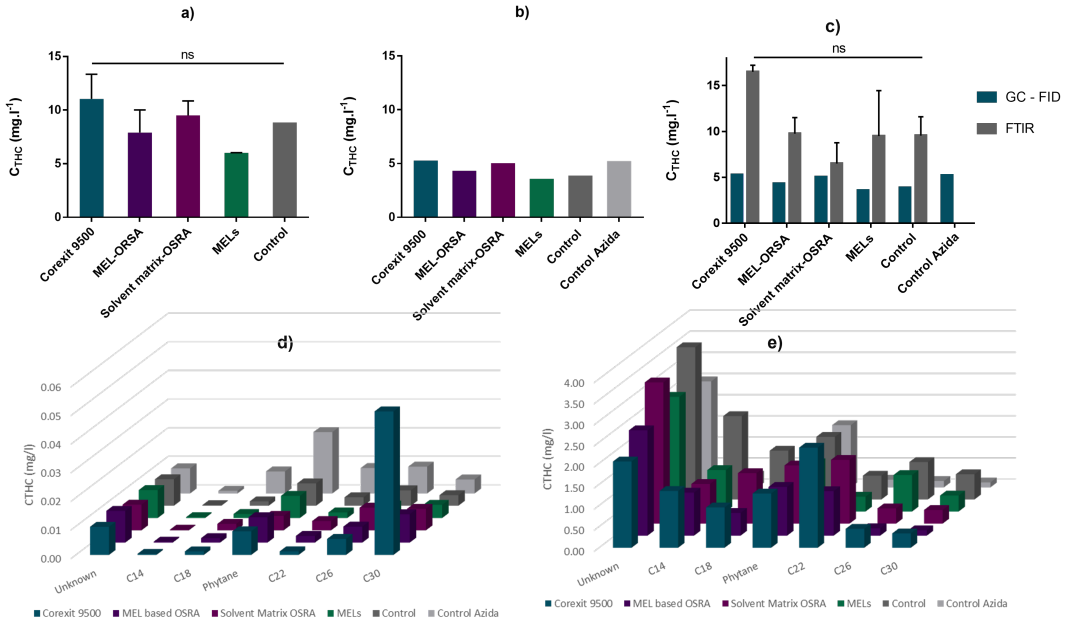

Figure 4.10: Concentration of total hydrocarbon content in seawater bioremediation assays done in June and September 2020. Corexit 9500 (blue bar), MEL-OSRA (purple bar), solvent matrix-OSRA (pink bar), MELs (green bar), negative control (dark grey bar), and negative control with azida (light grey bar). Concentration mean $\pm$ SD of total hydrocarbon content (THC), in mg/l, of OSRAs samples, trough GC-FID quantification a), b), and the blue column in c) or through FTIR quantification, grey column in c). Concentration mean (mg/l) of the hydrocarbon compounds selected, (C14, C18, Phytane, C22, C26, C30, and an unknown compound) and analysed peak to peak in each tested OSRAs sample, d) and e). Bioremediation assays conducted in June, a) and d), and in September, b), c) and e) of 2020. One-way ANOVA with Tukey's multiple comparisons test: ns, no statistically significant differences found, ($p < 0.05$).

One other factor that could be affecting our seawater bioremediation assays is the effectiveness of dispersion for each OSRA tested. To study this effect, OSRAs (corexit 9500, the developed MEL-OSRA, and MELs) premixed with Statfjord C fresh crude oil in a OSRA to oil ratio of 1 to 25 were added to baffled shake flasks with 100 ml of seawater medium. After a mixing and a resting period, the THC was extracted, quantified trough spectrophometry analysis, and compared to a negative control. Using corexit 9500, an alternative method of dispersing OSRAs was also analysed. 1st Statfjord C fresh crude oil was directly dispersed onto the surface of the seawater medium, and then the OSRAs formulations were dispersed onto the center of the oil slick.

After quantification of the THC, the concentrations data was analysed by ordinary one-way analysis of variance (ANOVA) followed by Tukey's multiple comparisons test using GraphPad Prism version 7.0.0 for Windows, GraphPad Software. The level of significance was set at $p<0.05$, 95% confidence interval (figure 4.11).

This results clearly show that with a direct application of corexit in the oil slick we recover more

hydrocarbon content and have the best dispersion in the water column. With corexit and MEL-OSRA pre mixed in the oil, we obtain statistically similar results. Though not being as well dispersed as the directly applied corexit, both perform better than the MELs and the control without an OSRA. This can also be observed in the photographs taken to each OSRA baffled shake flask prior to extraction in comparison to the negative control (figure 4.12). In the control, without the addition of an OSRA, the crude oil remained on the seawater surface and wasn't dispersed in the water column. In contrast, with both corexit conditions and with the MEL-OSRA, the crude oil is well dispersed in the seawater.

4.3.2 Suspended sediments bioremediation

To study the performance of different OSRA formulations in suspended sediments bioremediation, premixed solutions of OSRA and crude oil were added to 1.4 l of filtered seawater in a final concentration of 67.2 mg/l of oil. After the oil dispersion, 7.5 g of sediments were added to the glass flasks and stirred for 55 minutes at approximately 250 rpm, followed by 5 minutes stirring at approximately 50 rpm. Following 24 hours of settling time, the sediments were filtered and left to dry. Afterwards, the THC in both the seawater phase, in the sediments, and in the possible residues left in the glass material, was extracted and quantified trough spectrophometry analysis.

In the graph from the figure 4.13, the corexit 9500 condition has has most of the THC in the water phase. However, we saw that with corexit 9500, the crude oil had a very good dispersion in the water column. This is not consistent with the results now obtained. If well dispersed the crude oil could more easily reach the sediments in suspension. The condition with the MEL-OSRA, among all others, has the higher concentration of THC in the sediment phase. As seen before, with the MEL-OSRA, crude oil is well dispersed through the water column and consequently more crude oil reaches the sediments. Only a small fraction remains in the water phase and almost none is stuck to the glassware. The control condition has the higher concentration of THC left as residues in the glass material. Without OSRA, the crude oil does not disperse well through the water column and tends to remain on the seawater surface. Consequently, only small fractions reach the sediments. Most crude oil stays stuck to the glassware or in the water phase. The MEL condition has the higher concentration of THC in the water phase. This is probably the result of the poor dispersion ability of MELs and indicates that in the MEL-OSRA the other more toxic components are the ones helping in the dispersion of oil even if, as shown in literature, MELs might then have an important role in the bioremediation process.

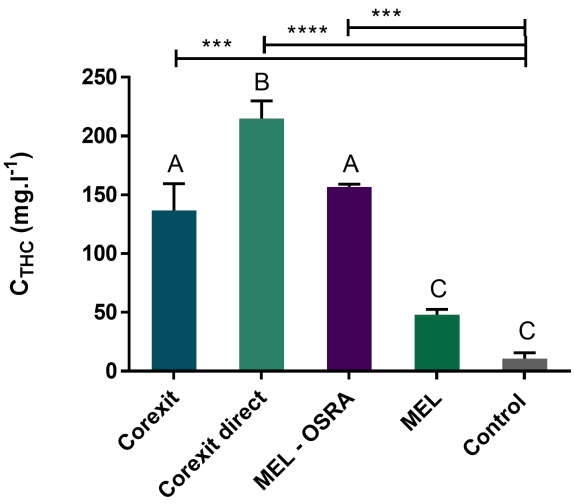

Figure 4.11: Dispersion effectiveness of the oil spill response agents (OSRAs) in study. Concentration mean ± SD of total hydrocarbon content (THC), in mg/l, in each OSRA and the control dispersion test. Corexit 9500 pre-mixed with crude oil (blue bar), corexit 9500 directly added to oil slick (light green bar), pre-mixed MEL-OSRA (purple bar) and MELs (green bar), negative control (dark grey bar). One-way ANOVA with Tukey's multiple comparisons test: columns topped with a different letter are significantly different, ($p<0.05$). Columns topped with A have statistically significant similarities between them and columns topped with C also have statistically significant similarities between them. Asterisks, *** significant at $p<0.001$, and **** significant at $p<0.0001$.

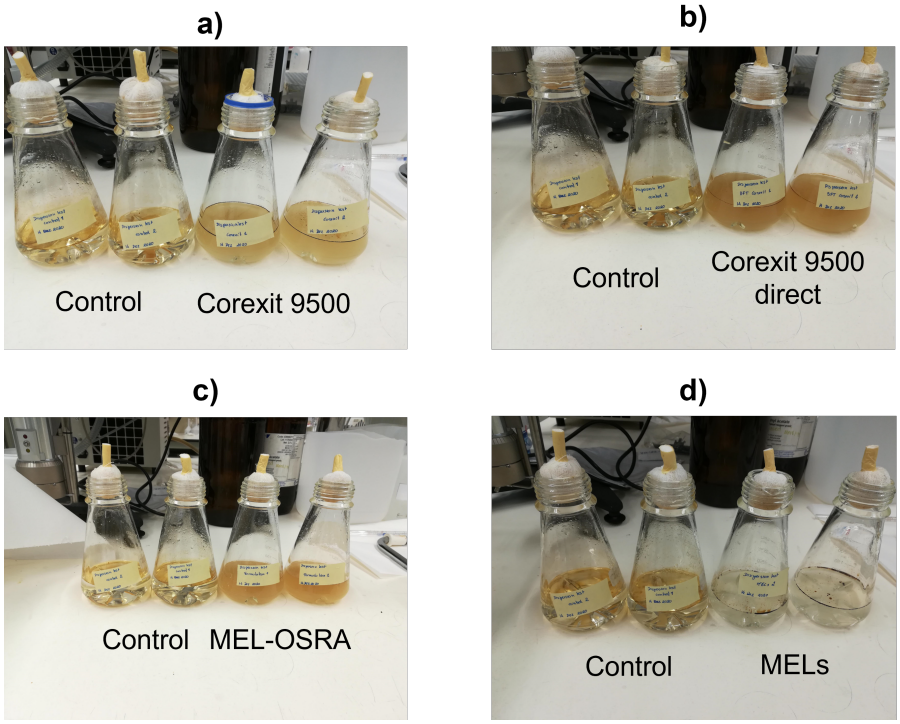

Figure 4.12: Dispersion effectiveness of the oil spill response agents (OSRAs) in study. Photographs of each OSRA baffled shake flask prior to extraction in comparison to the negative control. Corexit 9500 pre-mixed with crude oil, corexit 9500 directly added to oil slick , pre-mixed , and MELs (green bar).

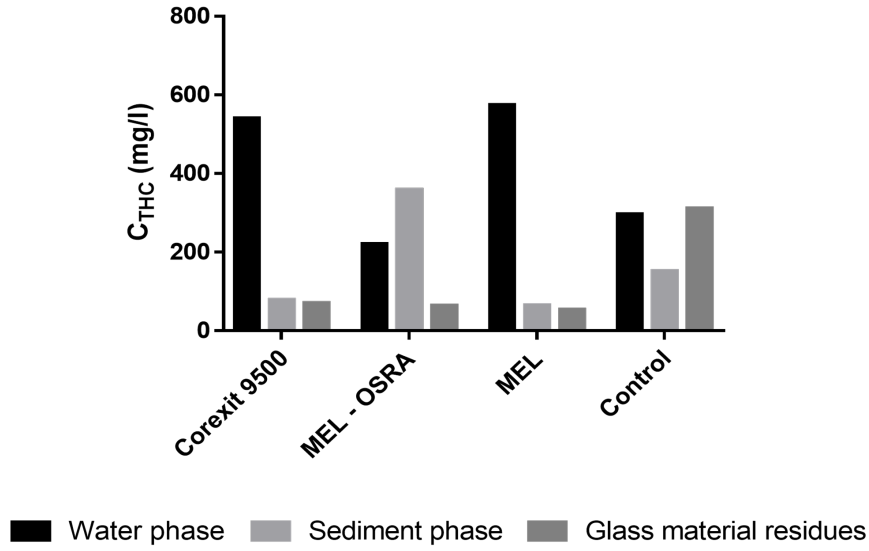

Figure 4.13: Concentration of total hydrocarbon content in suspended sediments bioremediation assay. Concentration mean $\pm$ SD of total hydrocarbon content (THC), in mg/l, in the water phase (black bar), the sediment phase (light grey bar), and glass material residues (dark grey bar), for each OSRA and the control. Corexit 9500, MEL-OSRA, MELs, and the negative control.

5

Conclusion and Future Work

Contents

5.1 Conclusions

The main objective of this thesis was to access the use of mannosylerythritol lipids (MELs) in the formulation of more eco-friendly oil spill response agents (OSRAs). Towards this, the sustainable production of MELs needed to be optimized and their environmental impact, more specifically their toxicity, estimated. Furthermore, the performance in bioremediation studies required evaluation.

This study showed that aeration is a defining parameter for MEL production. At the shake flask scale, the use of higher flask-to-medium ratios, and consequently more aeration in Moesziomyces spp. culture fermentations, results in cell cultures with more metabolic activity and higher MEL productivity. The best MEL productivity, at 0.19 g/l/h, was obtained with a ratio of medium to headspace of 9. At the bench reactor scale, the *Moesziomyces bullatus* fermentations conducted with an agitation of 150-800 rotations per minute (rpm) and 2 volume of gas per volume of liquid per minute (vvm), with the highest oxygen mass-transfer coefficient (kLa) tested, and consequently the best aeration, had higher MEL titres and more productivity.

The feeding strategy was also used to optimize MEL production. In bench scale reactor, *Moesziomyces bullatus* fermentations at 150-800 rpm and 2 vvm, with feeds of 20 g/l of waste frying oil (WFO) at the 3rd and 6th day, when compared to *M. bullatus* fermentations at 150-800 rpm and 2 vvm, with semi-continuous fedding of 6 g/l of WFO every day, had higher MEL titres, were more productivity and the MEL had more purity. In bioreactor fermentations, the best productivity observed in this study was achieved with this condition, at 0.27 g/l/h.

The environmental impact, more specifically the toxicity, in a marine environment, of different glycolipid biosurfactants and other OSRAs was found with a 24h LC50 bioassay was carried out with *Artemia franciscana*. Among the OSRAs tested, The commercial solution of corexit 9500 had the lowest LC_{50} at 78.92 mg/l, and corresponding to an higher toxicity. In contrast, the supernatant and MELs have the highest LC_{50}, respectively, 1383, 731.6, and 512.7 mg/l, for supernatant, *M. bullatus*, and *M. antarcticus*. More promisingly, not only do MELs have lower toxicity, but when added to the developed MEL based oil spill response agent (MEL-OSRA), it has a lower toxicity than the solvent matrix based oil spill response agent (solvent matrix-OSRA) with an LC_{50} of 405.20 mg/l when compared to an LC_{50} of 182.20 mg/l. Comparing to toxicity values found in the literature for other surfactants, they are they much more toxic than MEL and the other glycolipid biosurfactants, as well as the developed formulations. Further demonstrating the added value of the MEL-OSRA as a more eco-friendly alternative to conventional OSRAs.

In regards to the phytotoxicity of the MELs, in both biological models (*Lactuca sativa* and *Solanum lycopersicum*) until the 0.2 g/l of MEL we always have germination indexes higher that 60% and only in concentrations above 1 g/l of MEL do we find low germination indexes.

To study the performance of different OSRA formulations in sea water bioremediation several assays

were carried out. In a 1st experiment promising results were obtained to establish the developed formulation based on MEL as a viable alternative to Corexit 9500. Nonetheless, due to the optimization of the formulations, with one compound replaced, further experiments were conducted. However, the OSRA tested did not have statistically significant differences from the controls, where no additional OSRA was added. In an effort to better understand this results, a peak to peak analysis was then conducted. In the analysis, the OSRA formulations tested have, generally, lower concentration of the different hydrocarbons selected, corresponding to an higher degradation of crude oil during the incubation period and supporting the results obtained in the previous assay.

One factor found to affect bioremediation is the effectiveness of dispersion for each OSRA tested. The best dispersion, with more hydrocarbon content recovered, occurs with a direct application of corexit in the oil slick followed by corexit and MEL-OSRA pre mixed in the oil added to seawater. With MEL and in the control, a very low hydrocarbon content was recovered. The crude oil remained on the seawater surface and wasn't dispersed in the water column.

The performance of different OSRA formulations in suspended sediments bioremediation was also studied. In the developed formulation, crude oil is well dispersed through the water column and consequently more crude oil reaches the sediments. This condition, among all others, had the higher concentration of THC in the sediment phase. In contrast the MEL condition had the higher concentration of THC in the water phase. This is probably the result of the poor dispersion ability of MELs and indicates that in the MEL-OSRA the other more toxic components are the ones helping in the dispersion of oil even if, as shown in literature, MELs might then have an important role in the bioremediation process.

5.2 Future Work

This thesis, while exploring possible answers to a more sustainable production of MELs, still leaves much room to improvement and an in depth economical evaluation of the MEL production bioprocess should be conducted.

In regards to the environmental impact of MEL and MEL containing formulations, their toxicity in marine environment was determined using an *Artemia franciscana* 24h LC_{50} bioassay. Even so, and despite the reliability of this method, more biological models should be explored. And as for their phytotoxicity, only MEL was tested. The phytotoxicity of MEL containing formulations, other surfactants and biosurfactants should be explored.

In the bioremediation studies, due to the challenges presented by the analytical methods used, new and improved experimental designs should be explored.

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A

MEL Production

A.1 Bioreactor optimization: kLa study

The kLa often serves to compare the efficiency of bioreactors and their mixing devices as well as being an important scale-up factor. To improve MEL titres, the research group colleague, Miguel Nascimento, calculated the kLa for different agitations speeds. In the data collected, it is possible to confirm that in the reactor used the kLa increases with the increase of agitation speed and the vvm used. This increase improves the head space, allowing for more metabolic efficiency.

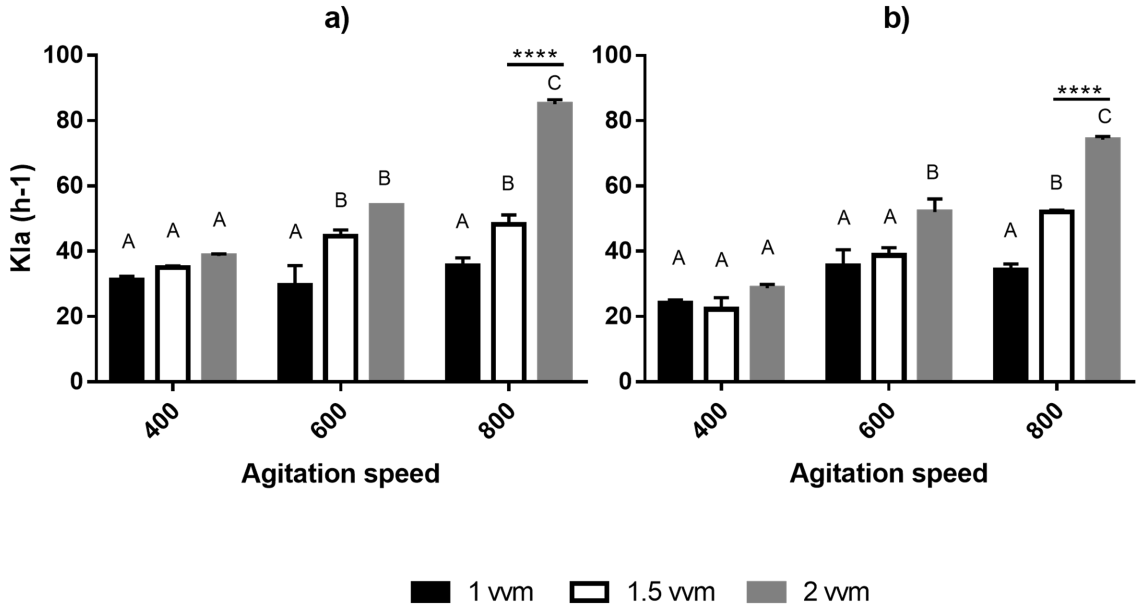

Figure A.1: KLa study. The graph shows the kLa in h-1 of a) mineral medium without oil and b) mineral medium with oil for agitations speeds of 400, 600 and 800 rpm and 1, 1.5 and 2 vvm.

A.2 Bioreactor optimization: comparison between *Moesziomyces bullatus* fermentations in a bench scale reactor

The state of the art condition for *M.bullatus* fermentations in a bench scale reactor was of 500 rpm and 1 vvm (starting with 40 g/l of D-glucose and 20 g/l of WFO and with 2 feeds of 20 g/l of WFO at the 4^{th} and 7^{th} day of fermentation). This condition was compared to the 800 rpm and 2 vvm condition with the same 2 feeds strategy (only at the at the 3^{rd} and 6^{th} day of fermentation) and with the 800 rpm and 2 vvm condition with semi-continously feeding of 6 g/l of WFO per day. The tables A.1, A.2, A.3 show, for each replica, all the calculated values.

Table A.1: Parameters of *M. bullatus* bioreactor fermentations with 150-500 rpm, 1 vvm, and 2 feeds. Parameters obtained for *M. bullatus* cultures incubated during 12 days at a controlled temperature of 27ºC, without pH control, in a 2.5l bench scale bioreactor with 1/5 working volume (total medium volume of 1l) using mineral medium as described in section 3.2.4, 10% inoculum, 40 g/l of D-glucose and 20 g/l of WFO as carbon source plus two feeds of 20 g/l of WFO, at day 4 and 7 of fermentation.

500 rpm - 1vvm - 2 feeds	1st replica	2nd replica
Final mass extracted	33.427	36.078
Final volume (mL)	720	650
Final titre of MEL (g/L)	47.8	52.66
Final titre of FFA (g/L)	7.0	29.08
Mass of MEL (g)	34.390	34.229

Table A.1 continued from previous page

500 rpm - 1vvm - 2 feeds	1st replica	2nd replica
Mass of FFA (g)	5.053	18.902
Mass of CO2 produced (g)	96.747	94.758
Mass of carbon in CO2 (g)	26.386	25.84318
Total Biomass (g/l)	30.0	35
Biomass (g)	21.6	22.75
Yield g of MEL/g of S	0.478	0.34229
Yield mol MEL/mol de S	0.166	0.183
Yield (mol of carbon in MEL/mol of C in susbtrate)	0.46	0.511
Productivity (g/l/h)	0.17	0.18
MEL purity (%)	87.19%	64.42%
Fermentation (days)	12	

Table A.2: Parameters of *M. bullatus* bioreactor fermentations with 150-800 rpm, 2 vvm, and 2 feeds. Parameters obtained for *M. bullatus* cultures incubated during 9 days at a controlled temperature of 27ºC, without pH control, in a 2.5l bench scale bioreactor with 1/5 working volume (total medium volume of 1l) using mineral medium as described in section 3.2.4, 10% inoculum, 40 g/l of D-glucose and 20 g/l of WFO as carbon source plus two feeds of 20 g/l of WFO, at day 3 and 6 of fermentation.

800 rpm - 2vvm -2 feeds	1st replica	2nd replica
Final mass extracted	26.465	26.275
Final volume (mL)	420	520
Final titre of MEL (g/L)	57.28	51.23
Final titre of FFA (g/L)	10.39	14.46
Mass of MEL (g)	24.057	26.639951
Mass of FFA (g)	4.364	7.5206235
Mass of CO2 produced (g)	66.959	60.000
Mass of carbon in CO2 (g)	18.261	18
Total Biomass (g/l)	26.0	26
Biomass (g)	10.9	13.52
Yield g of MEL/g of S	0.573	0.5123067
Yield mol MEL/mol de S	0.199	0.178
Yield (mol of carbon in MEL/mol of C in susbtrate)	0.56	0.497
Productivity (g/l/h)	0.27	0.24
MEL purity (%)	84.64%	77.98%
Fermentation (days)	9	

Table A.3: Parameters of *M. bullatus* bioreactor fermentations with 150-800 rpm, 2 vvm, and several feeds. Parameters obtained for *M. bullatus* cultures incubated during 9 days at a controlled temperature of 27ºC, without pH control, in a 2.5l bench scale bioreactor with 1/5 working volume (total medium volume of 1l) using mineral medium as described in section 3.2.4, 10% inoculum, 40 g/l of D-glucose and 20 g/l of WFO as carbon source plus several feeds of 6 g/l of WFO per day, until the same amount of carbon as the previous bioreactor fermentations is reached.

800 rpm - 2vvm - several feeds	1st replica	2nd replica
Final mass extracted	39.065	37.825
Final volume (mL)	550	610
Final titre of MEL (g/L)	48.30	48.60
Final titre of FFA (g/L)	34.64	36.88

Table A.3 continued from previous page

800 rpm - 2vvm (several feeds)	1st replica	2nd replica
Mass of MEL (g)	20.287	25.273219
Mass of FFA (g)	14.548	19.178816
Mass of CO2 produced (g)	71.460	72.97
Mass of carbon in CO2 (g)	19.489	19.90
Total Biomass (g/l)	34.0	30
Biomass (g)	18.7	18.3
Yield g of MEL/g of S	0.483	0.4860234
Yield mol MEL/mol de S	0.047	0.047
Yield (mol of carbon in MEL/mol of C in susbtrate)	0.47	0.472
Productivity (g/l/h)	0.22	0.23
MEL purity (%)	58.24%	56.86%
Fermentation (days)	9	

B

Toxicity

B.1 Marine toxicity: range finding tests using *Artemia franciscana*

To find the ideal interval to conduct each 24h LC_{50} definitive toxicity screening tests a range finding bioassay was carried out (figure B.1) and then the interval extrapolated (table B.1).

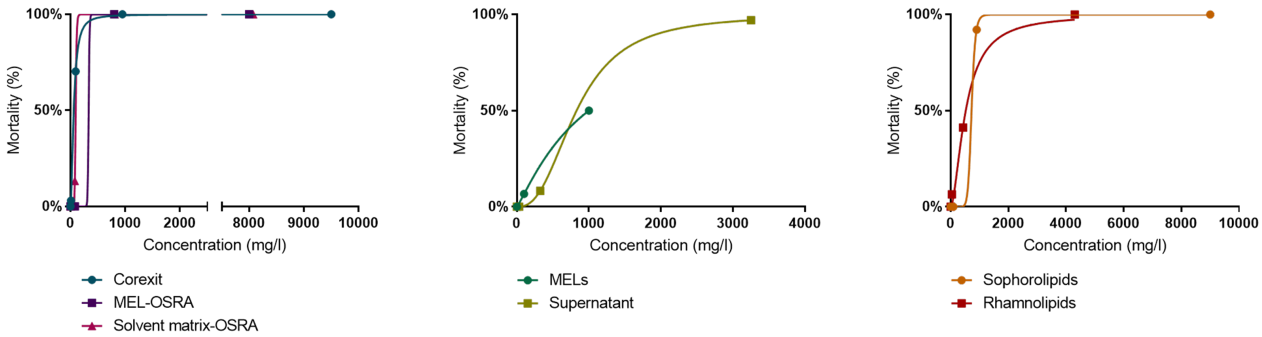

Figure B.1: *Artemia franciscana* toxicity range finding tests of different OSRA formulations. Corexit commercial solution (blue line), developed MEL-OSRA (purple line) and solvent matrix-OSRA (pink line), MELs (green line), supernatant (yellow line), sophorolipids (orange line) and rhamnolipids (red line) 24h LC_{50} *A. franciscana* bioassay conducted according to the standard operational procedures of the marine toxicity screening test Artoxkit M.

Table B.1: *A. franciscana* toxicity definitive test interval (mg/l) used for each different toxicant.

Toxicant	***A. franciscana* toxicity definitive test interval (mg/l)**
Corexit	0.95-950.00
MEL-OSRA	8.00-800
solvent matrix-OSRA	8.08-807.50
MELs	10-2700.00
Supernatant	32.50-3250.00
Sophorolipids	9.00-9000.00
Rhamnolipids	4.30-4300.00

C

Bioremediation

C.0.1 Establishing total hydrocarbon content: relative response factor for hydrocarbons and crude oil

Direct calibration standards for both a commercially available n-alkane solution (C7-C40) and Statfjord C fresh crude oil were prepared and analyzed by gas chromatography equipped with flame ionization detection (GC-FID) in order to determine the relative response factor (RRF) of each mixture and thus allow further quantification of total hydrocarbons.

A series of dilutions were made with pristane and 5-α-androstane, respectively as internal and external standard. Duplicates of 10, 100, 500 mg/l solutions of the mixture of n-alkane (C7-C40) in n-hexane were analysed. Duplicates of 50, 500, 2500 mg/l crude oil solutions in n-hexane were analysed (tables C.1 and C.2). This concentrations were selected through trial and error and smaller concentrations, though more similar to environmental conditions, are outside the sensitivity of our methods.

Table C.1: Alkanes direct quantification. This table shows the alkanes direct quantifications, obtaining, for each alkane, an average RRF using the equation $RRF = \frac{A_{std} \times C_{andr}}{A_{andr} \times C_{std}}$.

Alkanes						
Alkane	A_{std}	A_{andr}	$C_{andr}(mg/l)$	$C_{std}(mg/l)$	RRF	$AverageRRF$
C7	51744.9	531702.2	200	10	1.946	1.599
	54405.1	626145.6	200	10	1.738	
	511658.3	613110.1	200	100	1.669	
	527778.3	616506.2	200	100	1.712	
	2461381.5	806874.5	200	500	1.220	
	2433342.3	744862.8	200	500	1.307	
C8	48701.6	531702.2	200	10	1.832	1.646
	51445.3	626145.6	200	10	1.643	
	538067.5	613110.1	200	100	1.755	
	572504.0	616506.2	200	100	1.857	
	2724952.3	806874.5	200	500	1.351	
	2672984.5	744862.8	200	500	1.435	
C9	51563.1	531702.2	200	10	1.940	1.724
	54656.1	626145.6	200	10	1.746	
	561841.9	613110.1	200	100	1.833	
	597683.5	616506.2	200	100	1.939	
	2826484.1	806874.5	200	500	1.401	
	2763485.2	744862.8	200	500	1.484	
C10	52783.2	531702.2	200	10	1.985	1.782
	56850.2	626145.6	200	10	1.816	
	584745.3	613110.1	200	100	1.907	
	620399.1	616506.2	200	100	2.013	
	2912855.7	806874.5	200	500	1.444	
	2840730.9	744862.8	200	500	1.526	
	52614.6	531702.2	200	10	1.979	

Continued on next page

C11						1.801

Table C.1 – continued from previous page

Alkane	A_{std}	A_{andr}	$C_{andr}(mg/l)$	$C_{std}(mg/l)$	RRF	$AverageRRF$
	56859.8	626145.6	200	10	1.816	
	600643.1	613110.1	200	100	1.959	
	630162.4	616506.2	200	100	2.044	
	2967282.4	806874.5	200	500	1.471	
	2860760.0	744862.8	200	500	1.536	
C12	55902.2	531702.2	200	10	2.103	1.856
	58731.0	626145.6	200	10	1.876	
	614611.8	613110.1	200	100	2.005	
	644250.6	616506.2	200	100	2.090	
	3024626.9	806874.5	200	500	1.499	
	2907916.4	744862.8	200	500	1.562	
C13	56884.8	531702.2	200	10	2.140	1.878
	61332.4	626145.6	200	10	1.959	
	619539.2	613110.1	200	100	2.021	
	643487.2	616506.2	200	100	2.088	
	3020432.3	806874.5	200	500	1.497	
	2907783.7	744862.8	200	500	1.562	
C14	55909.8	531702.2	200	10	2.103	1.863
	59263.7	626145.6	200	10	1.893	
	619840.0	613110.1	200	100	2.022	
	644830.1	616506.2	200	100	2.092	
	3054044.4	806874.5	200	500	1.514	
	2896680.8	744862.8	200	500	1.556	
C15	57339.2	531702.2	200	10	2.157	1.883
	63667.0	626145.6	200	10	2.034	
	612073.7	613110.1	200	100	1.997	
	642578.8	616506.2	200	100	2.085	

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Table C.1 – continued from previous page

Alkane	A_{std}	A_{andr}	$C_{andr}(mg/l)$	$C_{std}(mg/l)$	RRF	$AverageRRF$
	3003603.0	806874.5	200	500	1.489	
	2868387.4	744862.8	200	500	1.540	
C16	57061.9	531702.2	200	10	2.146	1.858
	60228.3	626145.6	200	10	1.924	
	613845.1	613110.1	200	100	2.002	
	637944.5	616506.2	200	100	2.070	
	2979325.4	806874.5	200	500	1.477	
	2851477.2	744862.8	200	500	1.531	
C17	55634.9	531702.2	200	10	2.093	1.788
	58728.9	626145.6	200	10	1.876	
	586455.9	613110.1	200	100	1.913	
	607802.4	616506.2	200	100	1.972	
	2855088.0	806874.5	200	500	1.415	
	2721081.5	744862.8	200	500	1.461	
C18	53336.5	531702.2	200	10	2.006	1.740
	56918.9	626145.6	200	10	1.818	
	572741.6	613110.1	200	100	1.868	
	599885.3	616506.2	200	100	1.946	
	2782842.7	806874.5	200	500	1.380	
	2651401.9	744862.8	200	500	1.424	
C19	48380.1	531702.2	200	10	1.820	1.618
	53625.8	626145.6	200	10	1.713	
	534872.9	613110.1	200	100	1.745	
	557750.8	616506.2	200	100	1.809	
	2627867.3	806874.5	200	500	1.303	
	2452002.7	744862.8	200	500	1.317	
	45482.8	531702.2	200	10	1.711	

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C20						1.476

Table C.1 – continued from previous page

Alkane	A_{std}	A_{andr}	$C_{andr}(mg/l)$	$C_{std}(mg/l)$	RRF	$AverageRRF$
	49314.3	626145.6	200	10	1.575	
	486559.1	613110.1	200	100	1.587	
	503385.9	616506.2	200	100	1.633	
	2366039.3	806874.5	200	500	1.173	
	2191377.2	744862.8	200	500	1.177	
C21	120654.4	531702.2	200	10	4.538	1.817
	49411.8	626145.6	200	10	1.578	
	427260.8	613110.1	200	100	1.394	
	434337.6	616506.2	200	100	1.409	
	2003759.8	806874.5	200	500	0.993	
	1847287.6	744862.8	200	500	0.992	
C22	28238.3	531702.2	200	10	1.062	1.011
	35085.6	626145.6	200	10	1.121	
	356814.0	613110.1	200	100	1.164	
	357841.3	616506.2	200	100	1.161	
	1569305.7	806874.5	200	500	0.778	
	1458053.6	744862.8	200	500	0.783	
C23	23984.2	531702.2	200	10	0.902	0.816
	26903.6	626145.6	200	10	0.859	
	296488.8	613110.1	200	100	0.967	
	289313.7	616506.2	200	100	0.939	
	1277807.6	806874.5	200	500	0.633	
	1104505.6	744862.8	200	500	0.593	
C24	17840.5	531702.2	200	10	0.671	0.634
	21597.0	626145.6	200	10	0.690	
	245575.8	613110.1	200	100	0.801	
	230066.0	616506.2	200	100	0.746	

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Table C.1 – continued from previous page

Alkane	A_{std}	A_{andr}	$C_{andr}(mg/l)$	$C_{std}(mg/l)$	RRF	$AverageRRF$
	932712.9	806874.5	200	500	0.462	
	810627.9	744862.8	200	500	0.435	
C25	13244.9	531702.2	200	10	0.498	0.503
	17250.6	626145.6	200	10	0.551	
	207340.1	613110.1	200	100	0.676	
	186505.0	616506.2	200	100	0.605	
	736677.9	806874.5	200	500	0.365	
	596206.6	744862.8	200	500	0.320	
C26	9790.4	531702.2	200	10	0.368	0.406
	13436.2	626145.6	200	10	0.429	
	179851.1	613110.1	200	100	0.587	
	155224.5	616506.2	200	100	0.504	
	620456.5	806874.5	200	500	0.308	
	453813.5	744862.8	200	500	0.244	
C27	7471.1	531702.2	200	10	0.281	0.337
	10723.7	626145.6	200	10	0.343	
	157284.8	613110.1	200	100	0.513	
	131985.0	616506.2	200	100	0.428	
	532114.0	806874.5	200	500	0.264	
	359049.4	744862.8	200	500	0.193	
C28	5658.8	531702.2	200	10	0.213	0.306
	8671.3	626145.6	200	10	0.277	
	145641.1	613110.1	200	100	0.475	
	120093.5	616506.2	200	100	0.390	
	509752.5	806874.5	200	500	0.253	
	431554.5	744862.8	200	500	0.232	
	5227.1	531702.2	200	10	0.197	

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C29						0.271

Table C.1 – continued from previous page

Alkane	A_{std}	A_{andr}	$C_{andr}(mg/l)$	$C_{std}(mg/l)$	RRF	$AverageRRF$
	8044.7	626145.6	200	10	0.257	
	131307.0	613110.1	200	100	0.428	
	110953.6	616506.2	200	100	0.360	
	471322.3	806874.5	200	500	0.234	
	282974.3	744862.8	200	500	0.152	
C30	4898.8	531702.2	200	10	0.184	0.251
	7641.5	626145.6	200	10	0.244	
	117847.6	613110.1	200	100	0.384	
	101773.7	616506.2	200	100	0.330	
	446524.6	806874.5	200	500	0.221	
	260505.3	744862.8	200	500	0.140	
C31	4523.6	531702.2	200	10	0.170	0.233
	7383.9	626145.6	200	10	0.236	
	106316.1	613110.1	200	100	0.347	
	95047.6	616506.2	200	100	0.308	
	410420.8	806874.5	200	500	0.203	
	247312.3	744862.8	200	500	0.133	
C32	38279.4	531702.2	200	10	1.440	0.450
	6725.6	626145.6	200	10	0.215	
	96510.1	613110.1	200	100	0.315	
	115827.5	616506.2	200	100	0.376	
	392009.3	806874.5	200	500	0.194	
	303565.8	744862.8	200	500	0.163	
C33	3971.7	531702.2	200	10	0.149	0.203
	6695.4	626145.6	200	10	0.214	
	86870.6	613110.1	200	100	0.283	
	82913.3	616506.2	200	100	0.269	

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Table C.1 – continued from previous page

Alkane	A_{std}	A_{andr}	$C_{andr}(mg/l)$	$C_{std}(mg/l)$	RRF	$AverageRRF$
	361880.3	806874.5	200	500	0.179	
	228536.8	744862.8	200	500	0.123	
C34	3651.7	531702.2	200	10	0.137	0.186
	6553.5	626145.6	200	10	0.209	
	77203.9	613110.1	200	100	0.252	
	73126.5	616506.2	200	100	0.237	
	334359.0	806874.5	200	500	0.166	
	215442.5	744862.8	200	500	0.116	
C35	35210.3	531702.2	200	10	1.324	0.370
	5771.5	626145.6	200	10	0.184	
	66748.2	613110.1	200	100	0.218	
	59126.1	616506.2	200	100	0.192	
	293054.3	806874.5	200	500	0.145	
	290138.4	744862.8	200	500	0.156	
C36	3201.2	531702.2	200	10	0.120	0.159
	6210.9	626145.6	200	10	0.198	
	62163.0	613110.1	200	100	0.203	
	61111.1	616506.2	200	100	0.198	
	272005.0	806874.5	200	500	0.135	
	190459.1	744862.8	200	500	0.102	
C37	2735.7	531702.2	200	10	0.103	0.129
	5146.8	626145.6	200	10	0.164	
	49532.4	613110.1	200	100	0.162	
	47877.6	616506.2	200	100	0.155	
	209901.9	806874.5	200	500	0.104	
	159314.4	744862.8	200	500	0.086	

Table C.2: Crude oil direct quantification. This table shows the fresh crude oil direct quantifications, obtaining an average RRF using the equation $RRF = \frac{A_{std} \times C_{andr}}{A_{andr} \times C_{std}}$.

Crude Oil						
	Sample ID	A_{std}	A_{andr}	$C_{andr}(mg/l)$	$C_{std}(mg/l)$	RRF
1)	50 mg/l_A	4106502.6	799181.7	200	50	20.554
2)	50 mg/l_B	2881674.2	816121.8	200	50	14.124
3)	500 mg/l_A	3889335.1	821415.3	200	500	1.894
4)	500 mg/l_B	8765780.5	605921.1	200	500	5.787*
5)	2250 mg/l_A	9844489.3	480414.6	200	2 250	1.821
6)	2250 mg/l_B	8921194.3	530633.9	200	2 250	1.494

*Outlier. The base line in the GC-FID graph is not clean and has contaminations from previous runs.

The RRF was plotted for each of the alkanes observed in the GC-FID chromatogram both with the mean RRF of all the solutions (figure C.1) and with mean of the duplicates for each dilution (figure C.2). For fresh crude oil, the RRF data was plotted for each dilution (figure C.3). The RRF data was also analysed by ordinary one-way analysis of variance (ANOVA) followed by Tukey's multiple comparisons test using GraphPad Prism version 7.0.0 for Windows, GraphPad Software. The level of significance was set at $p < 0.05$ (95% confidence interval).

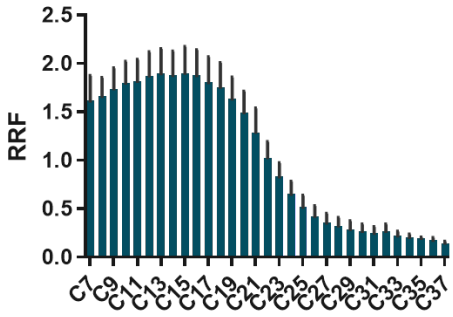

Figure C.1: Relative response factor of alkanes mixture Relative response factor (RRF) mean ± SD of the n-alkane mixture solutions for every alkane in the GC-FID chromatogram obtained by direct quantification of the alkanes.

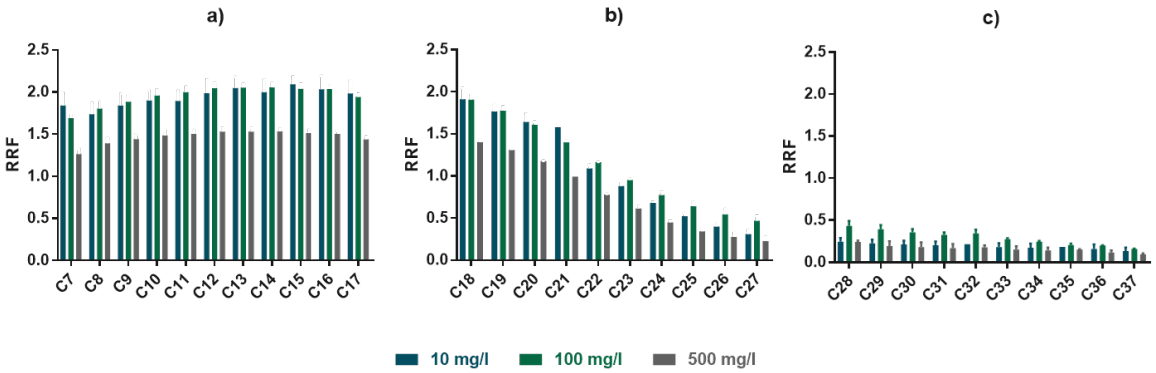

Figure C.2: Relative response factor of alkanes mixture Relative response factor (RRF) mean ± SD of each dilution (10 mg/l, 100 mg/l and 500 mg/l) of the n-alkane mixture solutions for every alkane in the GC-FID chromatogram obtained by direct quantification of the alkanes. a) RRF of the 3 dilutions for the carbons between C7 and C17; b) RRF of the 3 dilutions for the carbons between C18 and C27; c) RRF of the 3 dilutions for the carbons between C28 and C37.

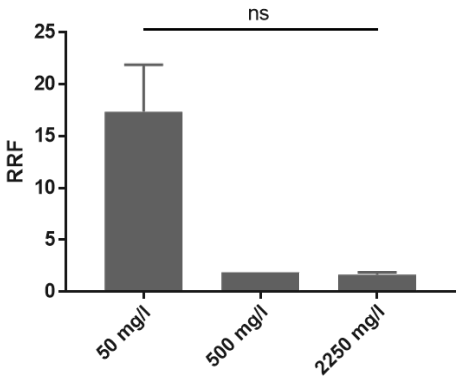

Figure C.3: Relative response factor of crude oil The relative response factor (RRF) mean ± SD for the total hydrocarbon area of each crude oil dilution (50 mg/l, 500 mg/l and 2250 mg/l) obtained from the GC-FID chromatogram obtained by direct quantification of the crude oil solutions. One-way ANOVA with Tukey's multiple comparisons test: n.s. (not significant).

In the quantification of alkanes using this direct method the RRF follows a clear tendency as the n-alkane scale increases. In the smaller carbons we observe a similar RRF, however for alkanes with high number of carbons, high RRF value was obtained.

This analysis is supported by the Tukey's multiple comparisons test. The alkanes with 7 through 20 carbons do not have statistically significant differences among them. Similarly, the carbons 25 through 37 also do not have among them statistically significant differences. In the carbons 20 to 25 we see a marked decrease of the RRF value. The RRF for the same alkane also changes depending on the concentration being tested.

The dilutions, according to the Tukey's multiple comparisons test performed, do not have statistically

significant differences among them. For the 50 mg/l crude oil concentration the RRF value does not follow the tendency of the higher crude oil concentrations. The direct quantification of crude oil in the lowest concentrations is very difficult to accomplish. For the higher concentrations, the RRF has values between 1,5 and 1,9 (one outlier where in the CG-FID chromatogram the base line was not clean and had a clear contamination from previous runs). Thus, the RRF selected when calculating the concentration of hydrocarbons in each sample (C_{THC}) is of 1.8.

C.0.2 Establishing total hydrocarbon content: THC extraction and analysis

With the objective of optimizing the total hydrocarbon content extraction and analysis, calibration standards for hydrocarbon extractions were made with Statfjord C fresh crude oil, and with a solution made from the alkanes C10, C12, C14, C16 and C17, using a series of dilutions. For both the alkanes mixture and for the crude oil we use duplicates of 1, 10 and 50 mg/l solutions in seawater medium.

Following the previously described hydrocarbons content extraction protocol, and using n-hexane as the separation solvent, pristane as the internal standard and 5-α-androstane (2 g/l in n-hexane) as external standard, the samples total hydrocarbon content (THC) was analysed by GC-FID.

For the alkanes mixture, the five hydrocarbon areas were individually integrated and the total area is the sum of those areas (table C.3). For the crude oil samples, the total area was obtained by automatic integration of peaks from the 7 minutes to the 50 minutes of the GC-FID spectrum. The THC area was then calculated by subtracting the area of the internal standards to the total area (table C.4).

Table C.3: Alkanes extraction and quantification. This table shows some selected alkanes extraction and subsequent THCquantification. The hydrocarbon area of each alkane was individually integrated and A_{THC} is calculated as the sum of those areas

Alkanes								
Sample ID		A_{andr}	$A_{andrref.}$	A_{pris}	$A_{prisref.}$	A_{THC}	C_{andr} (mg/l)	C_{pris} (mg/l)
1 mg/l A	C10	646236.3	633474.9	831762.0	1378488.0	14843.1	200	200
	C12	646236.3	633474.9	831762.0	1378488.0	36744.1	200	200
	C14	646236.3	633474.9	831762.0	1378488.0	48338.3	200	200
	C16	646236.3	633474.9	831762.0	1378488.0	51232.8	200	200
	C17	646236.3	633474.9	831762.0	1378488.0	43941.5	200	200
1 mg/l B	C10	484644.6	633474.9	1027442	1378488.0	13707.7	200	200

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Table C.3 continued from previous page

Sample ID		A_{andr}	$A_{andrref.}$	A_{pris}	$A_{prisref.}$	A_{THC}	C_{andr} (mg/l)	C_{pris} (mg/l)
	C12	484644.6	633474.9	1027442	1378488.0	31925.3	200	200
	C14	484644.6	633474.9	1027442	1378488.0	59499.9	200	200
	C16	484644.6	633474.9	1027442	1378488.0	60340.6	200	200
	C17	484644.6	633474.9	1027442	1378488.0	59313.8	200	200
10 mg/l A	C10	472090.7	633474.9	1014019	1378488.0	470150.2	200	200
	C12	472090.7	633474.9	1014019	1378488.0	727080.2	200	200
	C14	472090.7	633474.9	1014019	1378488.0	673817.4	200	200
	C16	472090.7	633474.9	1014019	1378488.0	632946.3	200	200
	C17	472090.7	633474.9	1014019	1378488.0	584945.3	200	200
10 mg/l B	C10	626306.5	640393.2	735861.6	979870.4	420169.7	200	200
	C12	626306.5	640393.2	735861.6	979870.4	690969.9	200	200
	C14	626306.5	640393.2	735861.6	979870.4	648322.7	200	200
	C16	626306.5	640393.2	735861.6	979870.4	596256.5	200	200
	C17	626306.5	640393.2	735861.6	979870.4	553499.1	200	200
50 mg/l A	C10	616081.0	640393.2	648840.4	979870.4	2391201	200	200
	C12	616081.0	640393.2	648840.4	979870.4	3630794	200	200
	C14	616081.0	640393.2	648840.4	979870.4	3296770	200	200
	C16	616081.0	640393.2	648840.4	979870.4	3081993	200	200
	C17	616081.0	640393.2	648840.4	979870.4	2873109	200	200
50 mg/l B	C10	623802.2	640393.2	728083.3	979870.4	2382241	200	200
	C12	623802.2	640393.2	728083.3	979870.4	3822449	200	200
	C14	623802.2	640393.2	728083.3	979870.4	3646844	200	200
	C16	623802.2	640393.2	728083.3	979870.4	3345810	200	200
	C17	623802.2	640393.2	728083.3	979870.4	3197879	200	200

Table C.4: Crude oil extraction and quantification. This table shows for the different crude oil concentrations, their extraction and subsequent THC quantification, using the equation $A_{THC} = A_{total} - A_{andr} - A_{pris}$.

Crude oil						
Sample ID	A_{total}	A_{hexane}	A_{andr}	A_{pris}	A_{THC}	$C_{andr}(mg/l)$
1 mg/l_A	157658464.6	152652921.0	978806.0	961551.9	3065185.7	200
1 mg/l_B	90833461.0	87857424.9	542620.3	993448.2	1439967.6	200
10 mg/l_A	97554233.3	92713612.4	581470.0	1199585.0	3059565.9	200
10 mg/l_B	95890077.3	92070547.9	463082.3	809663.1	2546784.0	200
50 mg/l_A	101941146.8	90688579.0	440788.1	1311258.1	9500521.6	200
50 mg/l_B	103984289.4	92764712.3	433445.5	1209679.1	9576452.5	200

Internal and external standards of, respectively, pristane and 5-α-androstane in the sample and in the wash solution were used to quantify the percentage of hydrocarbons recovered in the extraction process. The concentration of hydrocarbons in each sample was then calculated and analysed (tables C.5 and C.6).

Table C.5: Alkanes extraction and quantification. This table shows some selected alkanes extraction and quantification, with RRF I calculated previously by the direct method, RRF II calculated based on the extraction data and the expected concentration for 100% recovery. Recovery I was obtained by $Recovery(\%) = \frac{A_{pris}}{A_{andr}} \times 100$, recovery II by $Recovery(\%) = \frac{\frac{A_{prissample}}{A_{priswash}}}{\frac{A_{andrsample}}{A_{andrwash}}} \times 100$. $C_{THCI} = \frac{A_{THC} \times C_{andr}}{A_{andr} \times RRFI \times RecoveryII}$ and $C_{THCII} = \frac{A_{THC} \times C_{andr}}{A_{andr} \times RRFIImean}$.

Alkanes							
Sample ID		RRF_{II}	RRF_I	$Recovery(\%)_I$	$Recovery(\%)_{II}$	C_{THC} $(mg/l)_I$	C_{THC} $(mg/l)_{II}$
1 mg/l_A	C10	0.046	1.782	128.709	60.339	152.47	23.71
	C12	0.114	1.856	128.709	60.339	362.40	58.70
	C14	0.150	1.863	128.709	60.339	474.95	77.22
	C16	0.159	1.858	128.709	60.339	504.75	81.84
	C17	0.136	1.788	128.709	60.339	449.86	70.19
1 mg/l_B	C10	0.057	1.782	211.999	74.534	309.26	29.20
	C12	0.132	1.856	211.999	74.534	691.55	68.00

Continued on next page

Table C.5 continued from previous page

Sample ID		RRF_{II}	RRF_I	$Recovery(\%)_I$	$Recovery(\%)_{II}$	C_{THC} $(mg/l)_I$	C_{THC} $(mg/l)_{II}$
	C14	0.246	1.863	211.999	74.534	1284.02	126.74
	C16	0.249	1.858	211.999	74.534	1305.66	128.53
	C17	0.245	1.788	211.999	74.534	1333.69	126.34
10 mg/l_A	C10	0.199	1.782	214.793	73.560	11032.67	1028.09
	C12	0.308	1.856	214.793	73.560	16381.59	1589.93
	C14	0.285	1.863	214.793	73.560	15124.50	1473.46
	C16	0.268	1.858	214.793	73.560	14245.34	1384.08
	C17	0.248	1.788	214.793	73.560	13680.42	1279.12
10 mg/l_B	C10	0.134	1.782	117.492	75.098	5781.59	692.56
	C12	0.221	1.856	117.492	75.098	9128.75	1138.92
	C14	0.207	1.863	117.492	75.098	8533.14	1068.63
	C16	0.190	1.858	117.492	75.098	7868.97	982.81
	C17	0.177	1.788	117.492	75.098	7590.66	912.33
50 mg/l_A	C10	0.155	1.782	105.317	66.217	29983.25	4006.82
	C12	0.236	1.856	105.317	66.217	43711.32	6083.94
	C14	0.214	1.863	105.317	66.217	39540.86	5524.23
	C16	0.200	1.858	105.317	66.217	37064.34	5164.34
	C17	0.187	1.788	105.317	66.217	35905.00	4814.33
50 mg/l_B	C10	0.153	1.782	116.717	74.304	32694.39	3942.39
	C12	0.245	1.856	116.717	74.304	50368.51	6325.81
	C14	0.234	1.863	116.717	74.304	47873.99	6035.20
	C16	0.215	1.858	116.717	74.304	44040.36	5537.01
	C17	0.205	1.788	116.717	74.304	43741.11	5292.20

In the quantification of the hydrocarbons present in the extractions of n-alkane mixture solutions, the pristane peaks had always an area higher than the 5-α-androstane ones, resulting in recoveries above 100%.

Table C.6: Crude oil extraction and quantification. This table shows the extraction and subsequent THC quantification for the different crude oil concentrations, with RRF I calculated previously by the direct method, RRF II calculated based on the extraction data and the expected concentration for 100% recovery. Recovery estimated based on previous extraction, $C_(THCI) = \frac{A_{THC} \times C_{andr}}{A_{andr} \times RRFI \times RecoveryII}$ and $C_{THCII}) = \frac{A_{THC} \times C_{andr}}{A_{andr} \times RRFII}$.

Crude oil					
Sample ID	RRF_{II}	RRF_I	$Recovery(\%)$	$CTHC(mg/l)_I$	$CTHC(mg/l)_{II}$
1 mg/l_A	0.975	1.8	0.75	463.93	642.67
1 mg/l_B	0.975	1.8	0.75	340.21	544.61
10 mg/l_A	0.975	1.8	0.75	710.43	1079.85
10 mg/l_B	0.975	1.8	0.75	944.50	1128.66
50 mg/l_A	0.975	1.8	0.75	3276.16	4423.30
50 mg/l_B	0.975	1.8	0.75	3431.86	4534.18

However, as seen previously with the RRF studies, the peak area in the chromatogram is affected not only by the concentration of the solution but each peak, corresponding to an individual hydrocarbon, has a differential response. The pristane peak occurs usually around the 27 minute mark next to the C17, the 5-α-androstane peak occurs at around the 33 minutes mark after the C20. This difference in exit time is enough to explain why the pristane, added in the beginning of the extraction process, can have larger peaks.

Afterwards, the recovery calculations were adjusted to take this into account, $Recovery(\%)_{II}$, using not only the 2 different solutions added in the beginning and the end of the extraction process but by passing in the GC-FID a blank wash solution with both standards solutions, 2 g/l in n-hexane.

For a more comprehensive analyse, the THC concentration for the duplicate dilution samples was presented (figure C.4). The data obtained was also analysed by ordinary one-way analysis of variance (ANOVA) followed by Tukey's multiple comparisons test in the case of the crude oil samples and by two-way ANOVA followed by Tukey's multiple comparisons test of the concentrations means using GraphPad Prism version 7.0.0 for Windows, GraphPad Software. The level of significancewas set at $p<0.05$ (95% confidence interval).

In the quantification of THC in the crude oil solutions, and according to Tukey's multiple comparisons test, we don't see significant differences between the solution with 1 and 10 mg/l. Indicating that the method I is not precise enough for lower concentrations, unlike method II.

For the quantification of THC in the alkanes solutions, Tukey's multiple comparisons test of the con-

centrations means show significant differences between each concentration in both methods.

This is consistent with the main difficulties found in the laboratory. For the alkanes solutions, the cleaner runs in the GC-FID chromatogram with more well defined peaks contribute to an easier and more precise analysis in all dilutions. On the other end, the crude oil solutions chromatograms have much more undefined peaks and are very difficult to analyse. Any slight contamination from previous runs or even from other components in the crude oil can have a huge impact in the lower concentrations.

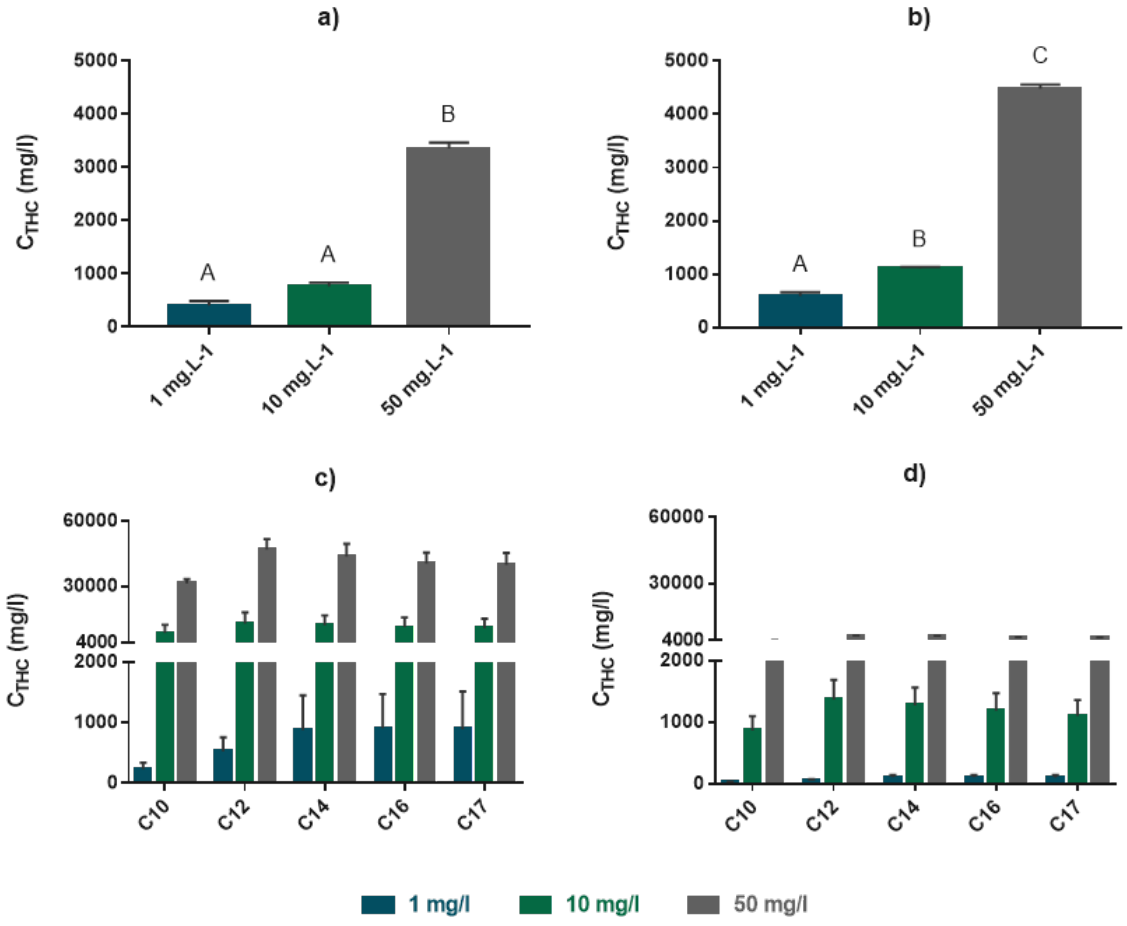

Figure C.4: Total hydrocarbon content concentration. The graph shows the mean ± SD of the total hydrocarbon content (THC) of the duplicate samples. a) THC concentration, in mg/l, of the crude oil samples using C_{THCI} formula; b) THC concentration, in mg/l, of the crude oil samples using C_{THCII} method; c) THC concentration, in mg/l, of the alkanes mixture samples using C_{THCI} formula; d) THC concentration, in mg/l, of the alkanes mixture samples using C_{THCII} method; One-way ANOVA with Tukey's multiple comparisons test: columns topped by the same letter are not significantly different, p $>$ 0.05).