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BIOREMEDIATION ENHANCEMENT OF MARINE SEDIMENTS CONTAMINATED BY CRUDE OIL WITH BIOGENIC POLLUTANTS MOBILIZING AGENTS AND BIOSURFACTANTS

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Esame finale anno 2017

To my mother…

Abstract

Although the number of accidental oil spills has significantly decreased in the last decades, they still represent a serious global concern due to their environmental and economic impact on marine ecosystems, in particular on the sediments where the heavy hydrocarbon oil sink. One of the main limitation to the bioremediation of oil-contaminated marine sediments are the low bioavailability of hydrocarbons. The use of biosurfactants and pollutant mobilizing agents could enhance the hydrocarbons availability to indigenous microbial communities and their biodegradation. Surfactant releasing formulations for the deployment of these agents to the sediment should be developed in order to prevent the random dispersion of the surfactants in the marine water. The aim of my PhD research project is to assess the effectiveness of biogenic non-toxic and biodegradable pollutant mobilizing agents of plant and animal origin and of microbial surfactants in enhancing the hydrocarbons availability and biodegradability of oil-contaminated marine sediments. For this purpose, two types of cyclodextrins having different hydrophilicity, namely hydroxypropyl-β-cyclodextrins (HPB-CD) and randomly methylated β-cyclodextrins (RAMEB-CD), two commercial soy lecithin products (Textrol F and Solec C) having different hydrophilic/lipophilic balances (HLB 4 and 7, respectively), two microbial surfactants (rhamnolipids and sophorolipids), and bile acids, were tested.

The effectiveness of the surfactants in increasing the hydrocarbons biodegradation was studied in slurry microcosms of actual oil-contaminated marine sediment from Gela (Sicily), revealing that sophorolipids, cyclodextrins and to less extent, soy lecithins stimulated the *n*-alkanes anaerobic degradation. In addition, molecular analysis of 16S rRNA gene suggested that the same *Acidobacteria* phylotype was enriched in the microcosms where the higher *n*-alkanes removal occurred, and was probably responsible for the anaerobic biodegradation. Under aerobic condition, the same biosurfactants/pollutant mobilizing agents had a complete inhibitor effect on *n*-alkanes degradation in Gela sediment, and only HPB-CD and de-oiled soy lecithin were able to significantly increase the degradation in Ravenna sediment (contaminated in the laboratory with a crude oil).

The evaluation of hydrocarbons (bio)availability were analyzed via the measurement of hydrocarbons concentration in the porewater through passive sampling with polydimethylsiloxane (PDMS) fibers. An increase of *n*-alkane bioavailability in oil-contaminated Gela sediment occurred in the presence of the two cyclodextrins and Textrol F, both immediately after oil contamination as well as after the complete adsorption of hydrocarbons to sediment.

Investigations of surfactant releasing formulations for the deployment of these agents to the sediments showed that HPB-CD (bioavailability and biodegradation enhancers) could be efficiently encapsulated in agar hydrogels, while sophorolipids (biodegradation enhancers) in polybutylene succinate (PBS) microspheres (95% encapsulation efficiency) via double emulsion methods. The release rate of encapsulated HPB-CD (50-70% release in the first hours) was higher than

encapsulated sophorolipids (76% release in 40 days) when formulations were incubated in marine water, while similar release rates (81-87% release in 65 days) were observed when incubation was performed in oil contaminated sediment slurry.
Both encapsulated surfactants were able to reduce remarkably adsorption of freshly spiked hydrocarbons to sand; conversely, only agar-encapsulated HPB-CD were able to desorb *n*-alkanes in weathered contaminated sands and increase their bioavailability. While the encapsulation of sophorolipids in PBS microspheres reduced the *n*-alkanes anaerobic biodegradation in Gela slurries, the effects of HPB-CD encapsulated in agar on *n*-alkanes removal were comparable than that of free cyclodextrins.
Therefore, HPB-CD encapsulated in agar capsules might be the most promising solution for the enhancement of bioremediation in marine sediment.

Keywords: oil hydrocarbons; *n*-alkanes; marine sediments; biodegradation; bioavailability; biosurfactants; pollutants mobilizing agents; encapsulation.

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1 Introduction

1.1 Oil spills: an overall view

Hydrocarbons contamination of the marine environment due to accidental oil spills is still of high concern, resulting in an extensive and long-term deterioration of coastal and ocean ecosystems. Throughout history, many events of oil spills occurred worldwide, and there will surely be more spills in the future as long as fossil fuels are still in use as a main source of power (Murphy et al., 2016). Globally, approximately 10 million tons of oil are used every day for several purposes ranging from the energy field to the chemical industry for the production of vital products as plastics, fertilizers and raw materials. The movement of petroleum from oil fields to consumers involves 10-15 transfers (tankers, pipelines, railcars and tank trucks) during which accidents could easily occur. The situation would remain almost unchanged in the future as well as also the risk of oil pollution (Fingas, 2014).

The total volume of oil released into the ocean reaches from 1.7 to 8.8 million tons annually, evaluating also the natural seepage. Approximately 35% of this amount comes from human activities such as urban runoff or improper disposal of petroleum products; 12% is from transportation (tankers and pipelines) and about 3% is from petroleum extraction activities (Cohen et al., 2013). In particular, the amount of oil spilled into the ocean during accidents has ranged from a few hundred tons to several hundred thousand tons (Seah et al., 2016). Examples of notorious large oil spills since 1960 are the *Torrey Canyon* off the south-west coast of the United Kingdom in 1967 (Hawkins and Southward, A, 1992), *Amoco Cadiz* on Portsall Rocks, few kilometers from the coast of Brittany, France on March 1978 (Boucher, 1980), *Atlantic Empress* (1979) in the Caribbea, *Prestige Oil Spill* in Galicia on November, 2002 (Laffon et al., 2006) and *British Petroleum Deepwater Horizon* in the Gulf of Mexico on April 2010 (Shultz et al., 2015). Furthermore, smaller spills have already proven to have a great impact on marine ecosystems, such as the Exxon Valdez oil spill of Prince William Sound, Alaska on March 1989 (Atlas. et al., 2009).

The most common and dangerous effects of oil pollution involves influx of oil on shore, its adsorption in sediments and consequent deterioration of coastal area (Kingston, 2002). However, damages and costs can vary by different factors depending not only on the size of the spill but also on the type of oil, organisms exposed, weather conditions, and proximity to human activities. Among the oil spill results are injuries to ocean ecosystems, risks on humans health directly or indirectly through the food chain (Laffon et al, 2016), business impact (fishing, tourism, etc.), and marine natural resources deterioration (beaches, marshes, etc.).

A combination of laws, such as requirements for training, equipment, and emergency plans and double-hulled tankers, as well as liability laws should be required to avoid oil spills from occurring (Cohen et al., 2013).

1.2 Petroleum

Petroleum is a thick, flammable, yellow-to-black mixture of gaseous, liquid, and solid hydrocarbons that occurs naturally beneath the earth's surface. It can be separated into fractions including natural gas, gasoline, naphtha, kerosene, fuel and lubricating oils, paraffin wax, and asphalt and it is used as raw material for a wide variety of derivative products (Hu, 2017; AHD, 2017).

When humans have realized that naturally occurring petroleum had numerous uses, they organized means and methods to accumulate and utilize it to its fullest potential. The history of petroleum exploration around the world is very interesting; each country has its own different path of discovery that influences it to this day (AAPG, 2016).

The word petroleum comes from the Latin *petra*, meaning "rock," and *oleum*, meaning "oil". It consists of hydrocarbons of different molecular weights and other organic compounds, containing nitrogen, oxygen and sulfur, and trace amounts of metals such as iron, nickel, copper and vanadium (Sachanen, 1945; Arene and Kitwood, 1979; Bankole and Ogunkoya, 1978). The petroleum includes both naturally occurring unprocessed crude oil, existing as a liquid in natural underground reservoirs (Figure 1), and petroleum products derived from refined crude oil, including from gasoline and kerosene to asphalt and chemical reagents used to make plastics, paints, cosmetics and pharmaceutical compounds (Sweeney, 1950). By 1965, more than 90% of organic chemicals were synthesized from petroleum (Eneh, 2011).

Figure 1 One of the largest natural petroleum seeps, located in the upper Ojai Valley, California (AAPG, 2016).

Petroleum is generated by sediments (source rocks) containing organic matter originating from biological materials, i.e. large quantities of dead marine organisms, usually zooplankton and algae (Nelson, 1954). The following increase of temperature results in several geochemical reactions that leads from biopolymers to geopolymers (called kerogen), which are the precursors of petroleum. The amount, type and composition of petroleum is dependent on the nature and geological history of the source rock (Brooks et al., 1987).

The different properties of petroleum affect the behavior of oil into the oceans, determining the fate and effects on the environment, and consequently the efficiency

of remediation. It is, therefore, essential to know the composition of oil to understand the chemical and physical processes that trigger after the oil spill and to determine which measures must be taken in order to minimize the environmental impact. (Fingas, 2014).

The oil industry classifies "crude" by the location of its origin and by its relative weight or viscosity. The crude oil ranges in density from 0.78 to 1.00 gcm^{-3} and it consists of more than 90% by alkane, compounds of hydrogen and carbon only (Eneh, 2011). In particular, the oil hydrocarbons can be divided into “light”, “intermediate” and “heavy” fraction according to their molecular weight. The “light” fraction, that represents the 95% of oil’s soluble portion, consists of alkanes and cycloalkanes having up to 10 atoms of carbon, characterized by a low solubility in water (few mg/L), and of mono-aromatics hydrocarbons, such as benzene, toluene and xylene, more soluble than aliphatic compounds. The “intermediate” fraction consists of aliphatic hydrocarbons having from 11 to 22 atoms of carbon and aromatics having two or more rings (naphthalene, phenanthrene, anthracene, etc.). Finally, the “heavy” fraction consists of aliphatic hydrocarbons having a number of carbon atoms equal to or greater than 23, but also from resins and asphaltenes (ISPRA, 2011). The relative content of sulfur in natural oil deposits also results in referring to oil as "*swee*t," which contains relatively little sulfur, or as "sour," which instead contains substantial amounts of sulfur (AAPG, 2016).

However, the use of fossil fuels, such as petroleum had a negative impact on ecosystems through events such as oil spills and releasing a several pollutants into the air, such as ozone and sulfur dioxide. The burning of fossil fuels also plays a negative role due to the emission of carbon dioxide (CO_2), a greenhouse gas that is linked with the actual global warming (USDE, 1999).

1.2.1 Crude oil composition

The analysis of crude oils composition provides essential information that affects petroleum exploitation at every step, from exploration and production to transportation and refining. Crude oil components can be separated into four different fractions, saturate hydrocarbon (saturates), aromatic hydrocarbon (aromatics), resins and asphaltenes (SARA), each containing a great number of structures (Karlsen, Larter, 1991; Bissada et al., 2016).

Saturates are hydrocarbons containing no double bonds, which are further classified into alkanes (paraffins) and cycloalkanes (naphthenes) according to their chemical structures. Alkanes have either a branched or unbranched carbon chain(s), while cycloalkanes have one or more rings of carbon atoms, mainly cyclopentanes and cyclohexanes. The majority of cycloalkanes in crude oil have an alkyl substituent(s) (Harayama et al., 1999). Figure 2 shows some representative hydrocarbons present in the saturated fraction of crude oil.

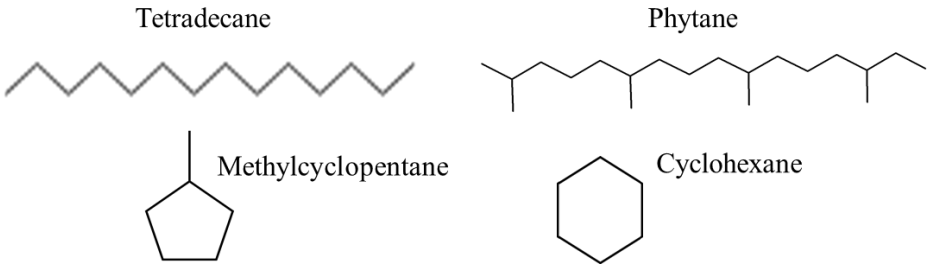

Figure 2 Chemical structure of representative hydrocarbons present in the saturated fraction of crude oil: tetradecane (an *n*-alkanes), phytane (a branched alkane), methylcyclopentane (a cycloalkane), a cyclohexane (a methyl-substituted cycloalkanes).

Aromatics are hydrocarbons having one (such as benzene and toluene) or more (such as naphthalene and pyrene) aromatic rings with or without an alkyl substituent(s) (Figure 3). Alkyl-substituted aromatics are usually more abundant than non-substituted compounds in crude oil (Mater and Hatch, 1994).

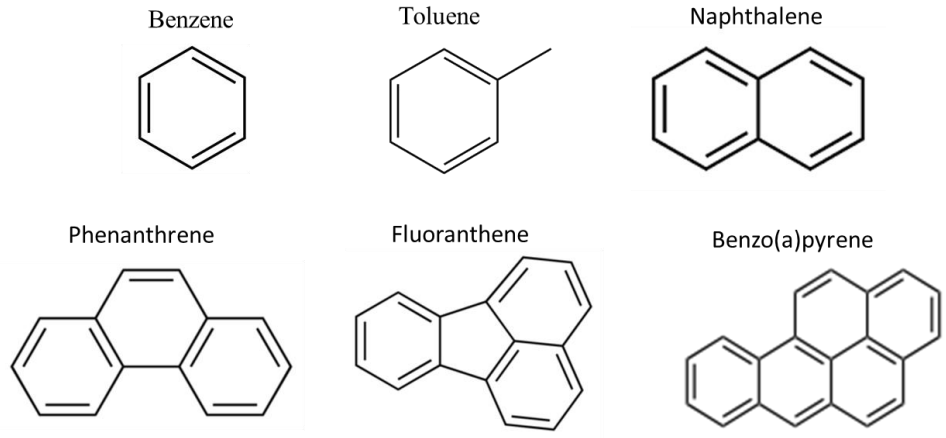

Figure 3 Skeletal formula of representative hydrocarbons present in the aromatic fraction of crude oil: benzene, toluene, naphthalene, phenanthrene, fluoranthene, benzo(α)pyrene.

In contrast to the saturated and aromatic fractions, both the resin and asphaltene fractions consist of non-hydrocarbon polar compounds, containing trace amounts of nitrogen, sulfur and/or oxygen in addition to carbon and hydrogen. These compounds often form complexes with heavy metals (Harayama et al., 1999).

Resins are *n*-heptane-soluble polar molecules, including pyridines, quinolines, carbazoles, sulfoxides and amides (Figure 4). Conversely, asphaltenes consist of high-molecular weight compounds that are not soluble in a solvent such as *n*-heptane; among these there are phenols, fatty acids, ketones, esters, porphyrins (Figure 5) (Colwell et al., 1977).

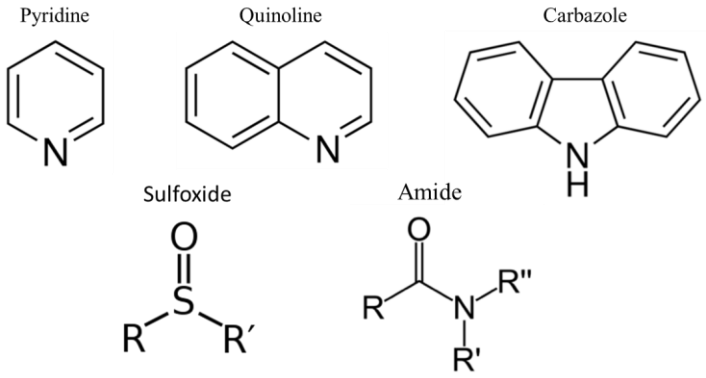

Figure 4 Chemical structure of representative compounds present in the resin fraction of crude oil: pyridine, quinoline, carbazole, sulfoxide and amide.

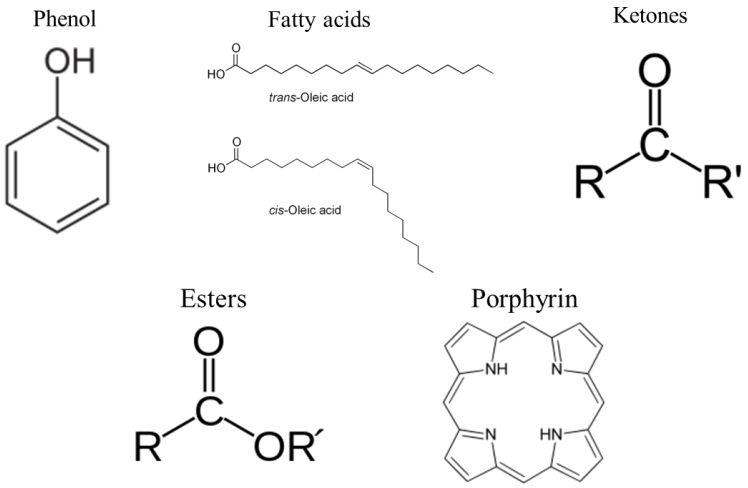

Figure 5 Chemical structure of representative compounds present in the asphaltene fraction of crude oil: phenol, fatty acids, ketones, esters, porphyrin.

Approximate composition of the crude oil was obtained from the study of more than 500 oils. As explained by Tissot and Welte, 1984), approximately 58% is made from saturated hydrocarbons, about 28% aromatics and the remaining 14% from non-polar compounds, i.e. resins and asphaltenes (Figure 6).

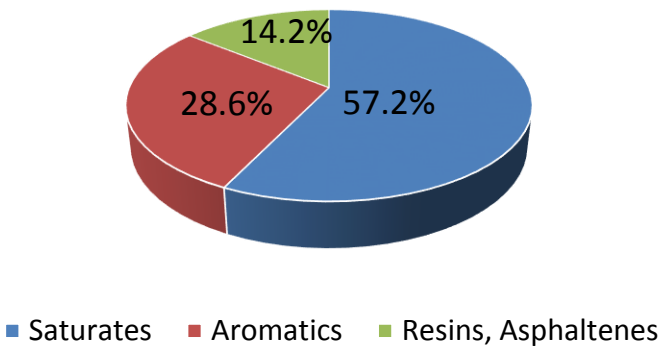

Figure 6 Gross composition of crude oils (wt. % of the fraction boiling above 210 °C; Tissot and Welte, (1984).

1.2.2 Physical-chemical characteristics of crude oil

As mentioned above, each petroleum type consists of a mixture of different molecules, which define its physical and chemical properties. The composition of the crude oil spilled in the oceans must be studied case by case in order to estimate its persistence in the environment and the most appropriate remediation alternative, but also to know the physical-chemical characteristics that affect the fate and behavior of the oil (ISPRA, 2011). *Viscosity*, *density*, *specific gravity* (density relative to water), *flash point*, *pour point*, *distillation* and *interfacial tension* are the most interesting properties of the crude oil (Fingas, 2014).

- *Viscosity* represents the resistance to flow in a liquid; it is affected by the temperature (lower temperatures giving higher viscosities) and the oil composition. The oil is a low viscosity when the amount of saturates and aromatics is high and the amount of resins and asphaltenes is low. The evaporation of the light components during the oil weathering leads to increased viscosity. An accurate determination of oil viscosity is essential because it is a fundamental factor in simulating reservoirs, forecasting production as well as in oil recovery methods (Hemmati-Sarapardeh et al., 2014). In terms of oil spill cleanup, viscous oil do not spread rapidly, do not penetrate soil readily, and affects the ability of pumps and skimmers to handle the oil. The oil viscosity can be measured with a viscometer at controlled temperatures.
- *Density* is the mass of a unit volume of oil, usually expressed as grams per millilitre (g/mL), or kilograms per cubic metre (kg/m^3). It is used to definite light or heavy oil and it is important because it indicates whether a particular oil will float or sink in water. Since the oil density ranges from 0.7 to 0.99 g/mL, the most oil usually float on water. However, since density increases as the light components of the oils evaporate, a heavily weathered oil, long after a spill event, may sink, while the fresh oil, immediately after the spill, may float rapidly.
- *Specific gravity* is a related measure, an oil's density relative to that of water. Since the densities of both water and oil change with the temperature, this propriety can be highly variable. The American Petroleum Institute (API) uses the specific gravity of petroleum at 50 °F (15.56 °C). Pure water has an API gravity of 10. Oils with lower specific gravities have higher API gravity. Heavy oils have less than 25 API, medium oil from 25 to 35 and light from 35 to 45. API gravities usually vary inversely with viscosity and asphaltene fraction.
- *Interfacial tensions* are the net stresses at the boundaries between different substances. They are expressed as the increased energy per unit area (relative to bulk materials) or as force per unit length. The Standard International (SI) unit are milliNewtons per meter (mN/m). Surface tension is related to the final

size of a slick. Decreasing the interfacial surface of oil with water, increase the extent of spreading and thinner terminal thickness of oil.

- *Flash point* of an oil is the temperature at which the vapor over the liquid can be ignited. A liquid is flammable if its flash point is less than 60 °C. This parameter is important during the spill clean-up operations. Since gasoline and other light fuels can ignite under ambient condition, they represent a serious risk when spilled. Several freshly crude oils have low flash point until the lighter components are evaporated.
- *Pour point* of an oil is the temperature at which no flux of the oil is visible over a period of 5 seconds from a standard measuring vessel. It ranges from -60 to 30 °C. Lighter oils with low viscosities have usually lower pour points. The pour point represents a consistent temperature at which an oil will pour very slowly and therefore has limited use as an indicator of the state of the oil.

The commodity classification API, accepted internationally, divides crude oils into four classes according to their °API density. Combining the classification API to the empirical concept of persistence of oils at sea, these are mainly divided into persistent (crude oils, fuel oils and asphalt) and not persistent (gasoline, kerosene and diesel). On the base of this classification, four main groups of crude oils and products are distinguished, as shown in the Table 1 (Biliardo and Mureddu, 2005).

GROUP	**SPECIFIC GRAVITY**	**°API DENSITY**	**PERSISTENCE**	**EXAMPLE**
I	< 0.8	> 45	*not persistent*	gasoline, kerosene, naphtha
II	0.8 – 0.85	35 – 45	*not very persistent*	diesel fuel, Abu Dhabi Crude
III	0.85 – 0.95	17.5 – 35	*average persistent*	Arabian Light Crude
IV	> 0.95	< 17.5	*very persistent*	Heavy fuel oil, Venezuelan crude oils

Table 1 Classification of crude oil in four main groups.

1.3 Marine oil spills

Although the number of accidental oil spills has significantly decreased since the 1970s, some important incidents still occurred in the last two decades, e.g. Aegean Sea 1992, Sea Empress1996, Ievoli Sun 2000, Prestige, 2002, MSC Napoli, 2007, etc. (Neuparth et al., 2012, Carpenter, 2016).

Figure 7 shows the number of large spills (>700 tonnes) from 1970 to 2015 as reported by the ITOPF (International Tanker Owners Pollution Federation), which maintains a database of oil spills from tankers, combined carriers and barges. This contains information on accidental spillages of persistent and non-persistent oil since 1970. The data include the type of oil spilt, the spill amount, the cause and location of the incident and the vessel involved. For historical reasons, spills are generally categorised by size, <7 tonnes, 7-700 tonnes and >700 tonnes. Information is now held on approximately 10,000 incidents, the vast majority of which (81%) fall into the smallest category i.e. <7 tonnes. Information is taken from both published sources, such as the shipping press and other specialist publications, as well as from vessel owners. Unsurprisingly, information from published sources generally relates to large spills, often resulting from collisions, groundings, structural damage, fires or explosions, whereas the majority of individual reports relate to small, operational spillages. Reliable reporting of small spills (<7 tonnes) is often difficult to achieve.

It should also be noted that the data for the amount of oil spilt in an incident include all oil lost to the environment, including that which burnt or remained in a sunken vessel. Since there is a considerable annual variation in both the incidence of oil spills and the amounts of oil lost, ITOPF can not guarantee that the information taken from the shipping press and other sources is complete or accurate. Consequently, the data and any averages derived should be viewed with an element of caution (www.itopf.com).

As shown in the figure below (Figure 7), in the last three decades the average number of incidents involving large oil spills from tankers has reduced progressively and since 2010 stands at an average of 1.8 large oil spills per year. Two large spills (>700 tonnes) were recorded for 2015 as a result of a collision. The first, in Singapore in January, resulted in a spill of approximately 4,500 tonnes of crude oil and the second in Turkey in June resulted in a spill of approximately 1,400 tonnes of naphtha. Six medium spills (i.e. between 7 and 700 tonnes) of various oils were also recorded for 2015 including cargoes of asphalt, naphtha and slurry oil, as well as bunker fuels. While this is slightly higher than the average of medium sized spills for this decade, it is still far below the averages for previous decades. Therefore, the total amount of oil lost to the environment in 2015 was approximately 7,000 tonnes, the vast majority of which can be attributed to the two large spills recorded in January and June.

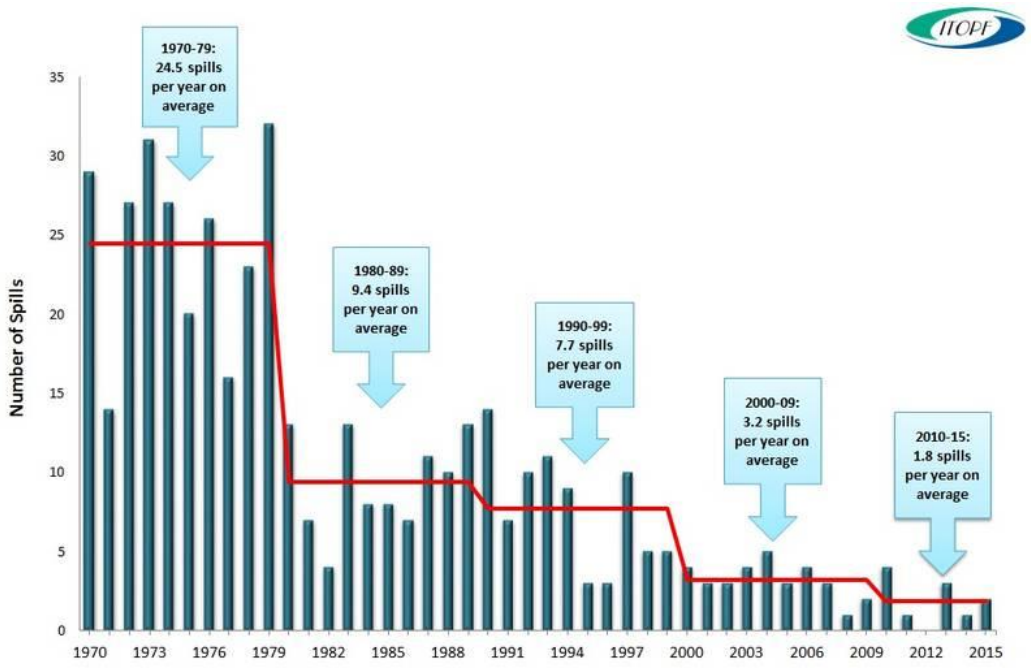

Figure 7 Number of large spills (>700 tonnes) from 1970 to 2015 (www.itopf.com).

In fact, when looking at the frequency and quantities of oil spilt, a few very large spills are responsible for a high percentage of oil spilt. For example, in 1990 there were 358 spills of 7 tonnes and over, resulting in 1,133,000 tonnes of oil lost: 73% of this amount was spilt in just 10 incidents. Similarly, in 2000 there were 181 spills of 7 tonnes and over, resulting in 196,000 tonnes of oil lost: 75% of this amount was spilt in just 10 incidents. Finally, in the period 2010-2015 there have been 42 spills of 7 tonnes and over, resulting in 33,000 tonnes of oil lost: 86% of this amount was spilt in just 10 incidents.

In the period 1970 to 2015, 50% of large spills occurred while the vessels were underway in open water; collisions and groundings accounted for 59% of the causes for these spills. While increased movement of petroleum could lead to an increased risk, the downward trends in oil spills continue, although an overall increase in the oil trade since the mid-1980s (Figure 8).

Figure 9 shows the 20 major oil spills that have occurred since the TORREY CANYON in 1967, whose names are listed in the Table 2; it is of note that 19 of the largest spills occurred before the year 2000. A number of these incidents, despite their large size, caused little or no environmental damage as the oil was spilt some distance offshore and did not impact coastlines. These oil spills must be added to those not caused by accidents of oil tankers. The most serious spill occurred during the Gulf War in 1991, and was caused by the Iraqi forces that deliberately destroyed oil tankers, wells, and terminals in Kuwait, resulting in fires and releases into air, water, and land. The second most important is the Deepwater Horizon spill in 2010, caused by an oil drilling platform explosion on deepwater well in the Gulf of Mexico (Cohen, 2013).

Figure 8 Seaborne oil trade and number of tanker spills 7 tonnes and over, 1970 to 2014 (Crude and Oil Product) (www.itopf.com).

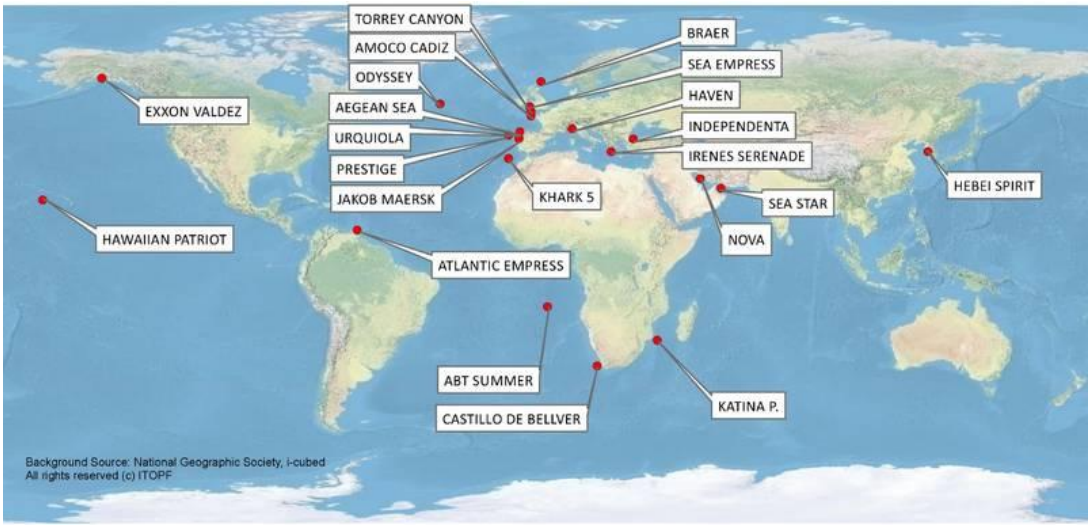

Figure 9 Map of the 20 major oil spills since 1967 (www.itopf.com).

Ship name	Year	Location	Spill Size (tonnes)
ATLANTIC EMPRESS	1979	Off Tobago, West Indies	287,000
ABT SUMMER	1991	700 nautical miles off Angola	260,000
CASTILLO DE BELLVER	1983	Off Saldanha Bay, South Africa	252,000
AMOCO CADIZ	1978	Off Brittany, France	223,000
HAVEN	1991	Genoa, Italy	144,000
ODYSSEY	1988	700 nautical miles off Nova Scotia, Canada	132,000
TORREY CANYON	1967	Scilly Isles, UK	119,000
SEA STAR	1972	Gulf of Oman	115,000
IRENES SERENADE	1980	Navarino Bay, Greece	100,000
URQUIOLA	1976	La Coruna, Spain	100,000
HAWAIIAN PATRIOT	1977	300 nautical miles off Honolulu	95,000
INDEPENDENTA	1979	Bosphorus, Turkey	94,000
JAKOB MAERSK	1975	Oporto, Portugal	88,000
BRAER	1993	Shetland Islands, UK	85,000
AEGEAN SEA	1992	La Coruna, Spain	74,000
SEA EMPRESS	1996	Milford Haven, UK	72000
KHARK 5	1989	120 nautical miles off Atlantic coast of Morocco	70,000
NOVA	1985	Off Kharg Island, Gulf of Iran	70,000
KATINA P	1992	Off Maputo, Mozambique	67,000
PRESTIGE	2002	Off Galicia, Spain	63,000
EXXON VALDEZ	1989	Prince William Sound, Alaska, USA	37,000
HEBEI SPIRIT	2007	South Korea	11,000

Table 2 Major oil spills (www.itopf.com).

Finally, Figure 10 shows the distribution of oil spills, both detected and suspected in European seas in 2013.

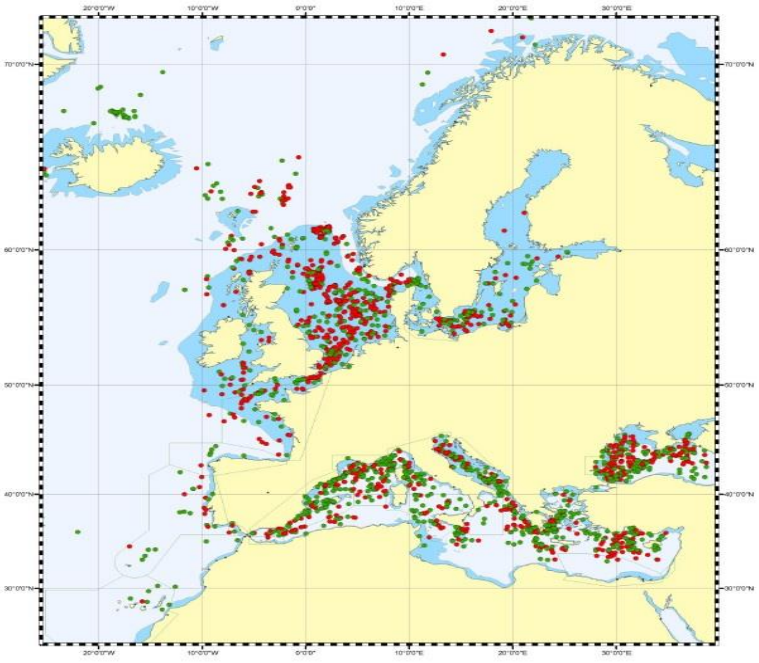

Figure 10 Distribution of oil spills in European seas in 2013 (www.safety4sea.com).

1.3.1 Fate of oil spills

The estimated amount of oil released into the sea annually is approximately 9 million barrels. However, compared to the vastness of the ocean it is literally "a drop in the bucket", representing only about 4/100 of 1% of the world's oceans. The problem after an oil spill is not the volume of the released oil, but its fate that became known in a qualitative and semi-quantitative manner by the early 1970s (Cohen, 2013; Burwood and Speers, 1974; Hoult, 1972). Research of the last 40 years has expanded the knowledge of these processes for different oils and ecosystems. These processes operate on multiple scales: from the scale of molecules to small-scale turbulence and large-scale interactions of tides, winds, and ocean currents. In particular, scientific research has expanded the understanding about of the processes that regulate the fate of chemicals in spilled oil by studying several accidental oil spills and small oil spills simulated by laboratory experiments and microcosm/mesocosm studies (Farrington, 2014).

The various physical, chemical, and biological processes after an oil spill occur with different kinetics: early stage processes, i.e. spreading, evaporation, dispersion, emulsification and dissolution, and late stage processes, i.e. oxidation, sedimentation and biodegradation that determine the ultimate fate of the spilled oil (Tarr et al., 2016). These processes are collectively termed "weathering" (Figure 11), and their rate and importance depend on different factors, such as the amount and duration of spilled oil, the wind velocity and direction, the oil's initial physical and chemical characteristics, the current and tides, the temperature (White and Blum, 1995), and the atmospheric and sea conditions (Al-Majed et al., 2012).

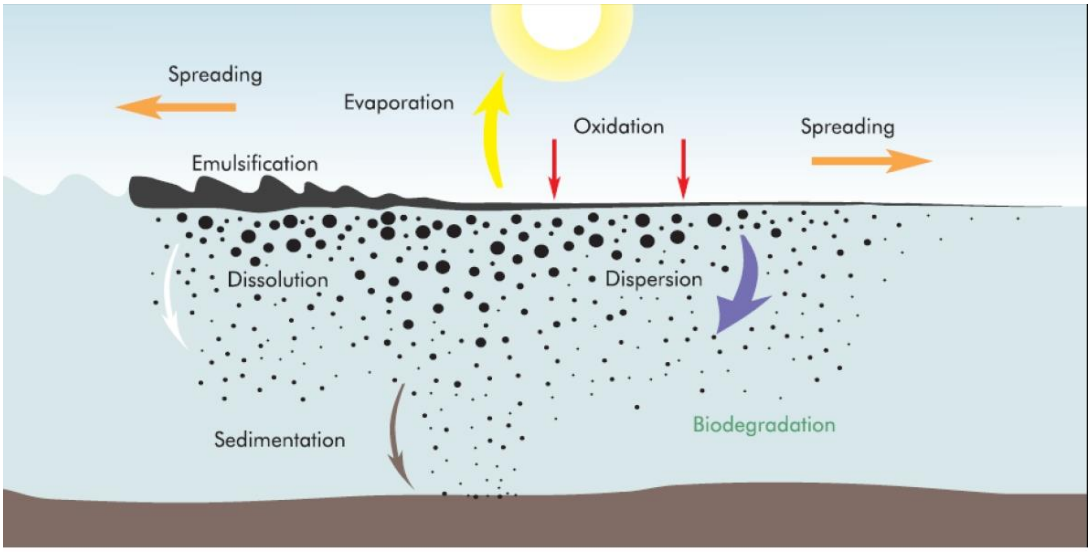

Figure 11 Weathering processes (ITOPF).

The Figure 12 shows the temporal evolution of physical-chemical weathering processes from hours to years.

Some of these processes, like the dispersion lead to the removal of the oil from the sea surface, facilitating its natural breakdown in the marine environment. Others, like the emulsification, cause the oil to become more persistent, which remain at sea or on the shoreline for a long time. A number of models are available in order to predict the fate of oil spills, understand the oil behavior and evaluate the scale of the impact triggered by this event (Wang et al., 2005a; Peishi et al., 2011; Afenyo et al., 2016).

The individual weathering processes are explained in more detail below (www.itopf.com[1]) and the Figure 13 illustrates some images related to these processes.

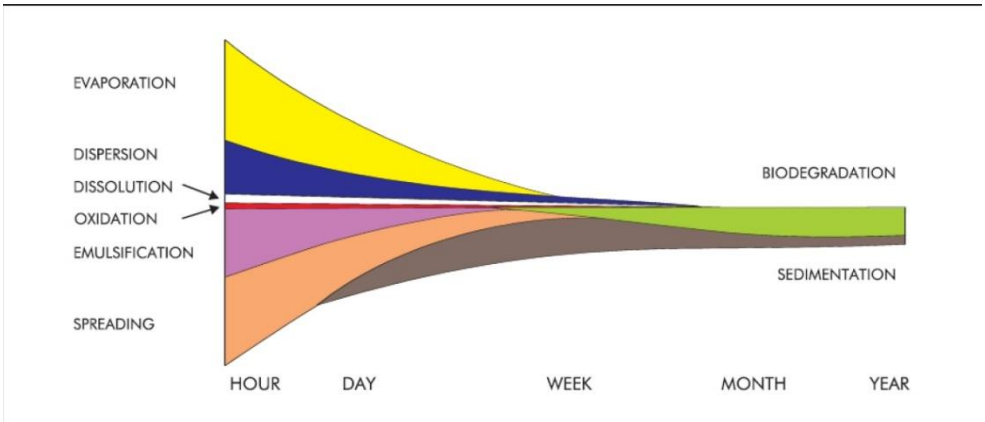

Figure 12 Temporal evolution of physical-chemical processes (ITOPF).

Spreading

Oil spilled into the sea initially spreads out and moves onto the water surface due to wind and current. The spreading depends upon the viscosity of the oil, which in turn depends on both oil composition and temperature. Low viscosity oils spread more quickly than those with a high viscosity; and the viscosity in turn is inversely proportional to temperature. Spreading is rarely uniform but large variations in the

thickness of floating oil are typical. After a few hours, the slick breaks up due to the action of winds, wave and water turbulence, and often forms narrow bands parallel to the wind direction. Therefore, the rate of spreading depends also upon environmental conditions such as temperature, wind, water currents. High temperatures, strong winds and sea surface currents usually result in a more rapid rate of spreading (www.itopf.com[1]).

Evaporation

Evaporation is the most important weathering process during the first 24-48 hours after an oil event, and it depends on the volatility of the oil components. An oil with a large percentage of light and volatile compounds evaporates more quickly than an oil containing a greater percentage of heavier compounds. Therefore, light products such as gasoline, kerosene and diesel could evaporate almost completely within a few days. Conversely, little evaporation occurs from a spilled heavy fuel oil. This process is controlled by other factors, such as the water and air temperatures and the vapor pressure of the chemicals present in the oil. It is more rapid in tropical regions than in temperate regions, and slowest in colder polar regions (Pan et al., 2017). The rate of evaporation generally increase with the spreading due to the increased surface area of the slick.

Dispersion

The slick could break up into fragments and droplets of varying sizes due to the waves and turbulence at the sea. Some of the smaller droplets remain suspended in the seawater, while the larger ones can rise back to the surface and reform a slick or spread out. Since the dispersed oil droplets have a greater surface area to volume ratio than floating oil, other natural processes such as dissolution, biodegradation and sedimentation are promoted. The dispersion depends upon the type of the oil and the sea conditions. It is faster if the oil is light and of low viscosity and if the sea is very rough. The addition of chemical dispersants can accelerate the process of natural dispersion (Pan et al., 2017).

Emulsification

The physical mixing due to the turbulence at the sea surface promotes the water-in-oil emulsion that is usually very viscous and more persistent than the original oil and can exist in the marine environment for over 100 days in a peculiar form called “chocolate mousse” because of its appearance. The emulsion reduces the other oil weathering processes and its stability increases with decreasing temperature. Emulsions may again separate into oil and water again if heated by the sun under calm conditions or when stranded on shorelines.

Dissolution

Water-soluble compounds in an oil may dissolve into the water column. Light aromatic hydrocarbons compounds, such as benzene and toluene, are the most soluble components in seawater. However, these compounds are also easily lost through evaporation, which is much faster than dissolution. Since most crude oils and all fuel

oils contain small proportions of these compounds, the dissolution is one of the less significant processes.

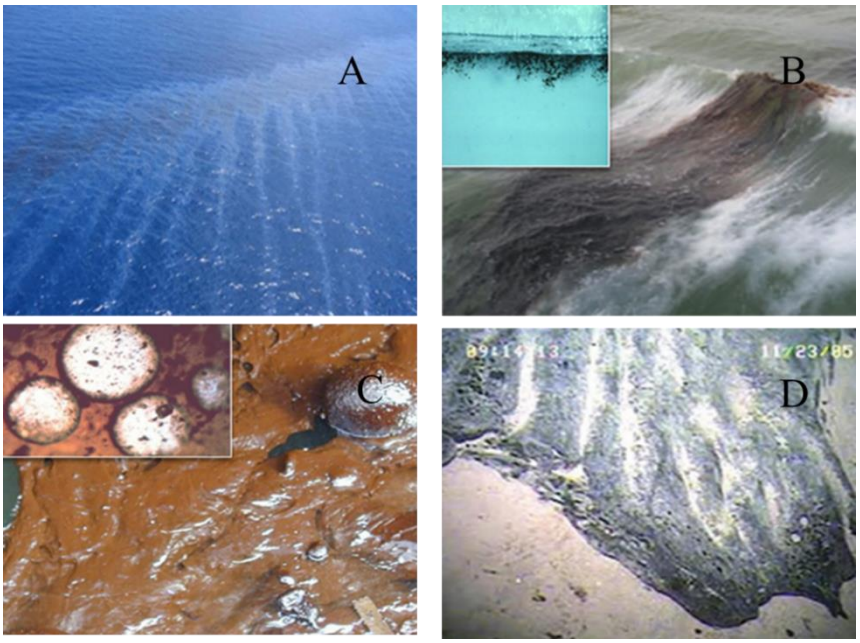

Figure 13 A: Spreading of the oil on the sea surface to a thin layer. B: Waves and turbulence at the sea surface cause the break up of the slick into droplets. C: Magnified image of water-in-oil emulsion and close view of emulsified heavy fuel oil. D: Heavy fuel oil on the seabed (www.itopf.com[1]).

Oxidation

Oil reacts chemically with oxygen either breaking down into soluble products or forming persistent compounds called tars. This process, promoted by sunlight is very slow. The formation of tars forms an outer protective coating of heavy compounds that results in the increased persistence of the oil.

Sedimentation/Sinking

Once the lighter compounds have evaporated, some oils can be close to the density of seawater and the sedimentation occurs. Dispersed oil comes into contact with sediment, and bind to it especially in shallow waters. In addition, if the oil catches fire or is voluntarily burnt (in-situ burning) after it has been spilled, the residues can be sufficiently dense to sink.

Biodegradation

Several marine indigenous microorganisms are capable to use oil hydrocarbons as a source of carbon and energy and can partially or completely degrade them to water soluble compounds or eventually to carbon dioxide and water. Different species of microorganism are able to biodegrade a particular group of compounds present in a crude oil. However, some compounds in oil are very resistant to attack and may not readily biodegrade. The creation of oil droplets through dispersion, increases the surface area to volume ratio of the oil, and therefore increases the area available for microorganisms to attach to the surface of the oil and for biodegradation to take place. The main factors affecting the efficiency of biodegradation are the levels of nutrients

(nitrogen and phosphorus), the temperature and the presence of oxygen. As biodegradation of oil requires oxygen, this process could only take place at the oil-water interface. However, several anaerobic hydrocarbon degraders has been recently found in marine sediment, making possible the biodegradation process also in absence of oxygen (Acosta-González and Marqués, 2016).

1.3.2 Impact of oil spills

The effects of oil spills can have wide ranging impacts depending on different factors such as the quantity and type of oil spilled and its interaction with the marine environment. Other key factors include the weather conditions, the time of year in which the oil spill occurs, and the biological and ecological characteristics of the area, such as the presence of species able to biodegrade petroleum hydrocarbons. The selected clean-up techniques have also an effect on the environmental impact of an oil spill (www.itopf.com[2]). The different released oil contaminants can lead to serious levels of pollution.

Accidental spills can cause serious problems to human health. In particular, the polycyclic aromatic hydrocarbons (PAH) present in the oil are potent carcinogens with mutagenic properties. The oil also contains heavy metals that are among the most dangerous pollutants for the environment and can cause, in addition to cancer, acute poisoning and damage to the nervous system, muscles and bones. Furthermore, some metals, such as cobalt, lead and manganese may persist in the marine environment up to 1000 years, while others, such as cadmium, copper, nickel and zinc for a period between 1000 and 100000 years after an oil spill event. Other substances present in the oil can also accumulate in the fatty tissues of animals and move up the food chain reaching the human species (Baawain et al., 2011). Generally, the human effects following exposure to oil spills are mental (prevalence of depression, anxiety disorder and event-related psychological stress), physical/physiological effects (respiratory problems, irritations, headache, nausea/vomiting, back pain, injuries), and genotoxic, immunotoxic, and endocrine toxicity (Laffon et al., 2016).

Plankton, fish, seabirds, marine mammals and reptiles are the organisms commonly subjected to an oil spill in open sea. Several planktonic organisms, including bacteria, eggs and larvae, and a variety of animal and plant species are also subject to oil exposure after a spill event. Although the eggs and larvae of fish may be susceptible to the effects of oil, adult fish tend to be more resilient. Reductions in wild fish stocks have rarely been detected, since the fish can swim away to avoid unfavorable water conditions. Extended mortalities is present especially in shallow or confined water where very high and localized concentrations of dispersed oil has occurred. Seabirds are the most vulnerable animals after an oil spill. The rehabilitation practices of birds, often linked to the species of bird, are emerging and outcomes are improving. Since oils tend to float at the sea surface, marine mammals and reptiles that must go to the surface to breathe are susceptible to oil, which causes damage to nasal tissues and eyes. If the fur of some mammals becomes matted with the oil, they can die due to

hypothermia or overheating (www.itopf.com[3]). The impact of oil in nearshore waters depends on the high concentration of naturally or chemically dispersed oil at which the marine organisms are subjected, although sometimes the natural dilution capacity of tidal flushing is sufficient to keep the concentrations of dispersed oil below dangerous levels. The impacts of oil spills in shorelines are difficult to predict due to the large number of and variety of organisms present in this marine zone. Coral reefs, saltmarshes and mangroves, very important in order to provide coastal protection for many invertebrate and fish species, are particularly sensitive to oil pollution (www.itopf.com[4]).

The economic impacts of an oil spill are given by two contributions: "private" and "external" costs. The first consist of the damage to the parties involved in the accident (oil tanker, oil platform, pipelines, etc.) and the cost of oil lost. The others include strategies for limiting the spill and clean up the contaminated area, restructuring of public infrastructure, damage to natural resources, and legal disputes. This category also includes losses of species of commercial interest and reduction of tourism. Very small spills are of negligible cost, because oil tends to disperse in the water causing few dangers. When the spill reaches damaging coastal tracts, the higher costs are obviously those related to the impact on the environment and natural resources (Cohen, 2013).

1.3.3 Clean-up and recovery

Cleaning up of the petroleum hydrocarbons from the environment is a serious world problem. Once oil is spilled at open sea, it may naturally spread, dissolve, disperse and often drift to the shore due to the action of wind, waves and currents. In order to recover the oil floating on the sea and clean up the oil that reaches the shore, several strategies can be employed, which depend upon the weather and sea conditions, and the nature of the spilled oil. The immediate intervention techniques after an oil spill, that are most commonly used, can be classified into three categories: (i) containment and recovery, (ii) *in-situ* burning, and (iii) dispersant application (www.itopf.com[5]).

Containment and recovery is the primary solution to an oil spill adopted by several governments, as this strategy aims to physically remove oil from the marine environment. However, the weather and sea condition must be sufficiently good to permit the correct function of the equipment and the operator safety, and the oil must be in such a way that it can be easily recovered (up to 10-15%). For the containment and recovery strategy, several equipment are required. The two most important are the *boom*, which limits the spread of oil and concentrate it for recovery, and the *skimmer*, which recovers the oil from the sea surface and pump it into a proper storage container (www.itopf.com[6]).

In-situ burning is the process of burning floating oil, at or close to the site of a spill, which might remove a large amount of oil from the sea surface. The oil must be concentred and an ignition source applied. However, the resultant fire and potentially toxic smoke can have a negative impact on human health; therefore, it is better to use

it in the open sea away from populated areas. The condition of the oil is also important; weathering process like the evaporation of the lighter fraction of the oil and the oil emulsification may make the oil more difficult to ignite and burn. In order to contrast the cooling effect of the wind and sea and maintain a fuel source to the fire, the layer of oil on the sea surface needs to be at least 2-3 mm thick. Ignition can be obtained using several devices ranging from a diesel-soaked rag to more sophisticated equipment not easily available in many parts of the world. The slick needs to reach sufficiently high temperatures to keep the fire burning. As the slick becomes thinner due to the removal of the lighter fractions, the cooling effect of the wind and sea can extinguish the fire. Oil residues can sink and therefore contaminate the bottom of the sediments (Mullin and Champ, 2003; www.itopf.com[7]).

The other oil spill response strategy is the use of the dispersants that remove large amounts of oil from the sea surface by facilitating its transfer into the water column where it is degraded by natural processes. This strategy is very effective as it can be applied in conditions that make unusable the other techniques, although it also depends on the characteristics of the spilled oil and the sea and weather conditions. Dispersants have two main components: the surfactant molecules, made up of a hydrophobic part (with an affinity to oil) and a hydrophilic part (with an affinity to water), and the hydrocarbon-based solvent that transports the surfactants to the oil/water interface where they reduce the surface tension and allow small oil droplets to break away from the slick. Although larger droplets may rise back to the surface, most remain in suspension and are biodegraded by the indigenous bacteria. Dispersants can be applied by different methods, ranging from spraying dispersants from vessels or small aircraft for smaller spills and nearshore areas, to the use of large multi-engine planes for handling large offshore spills (Kujawinski et al., 2011; www.itopf.com[8]).

When the oil spill occurs or reaches the shoreline, the most appropriate techniques and a good organization are required to rightly clean up the contaminated areas. In order to select the best strategy, it is therefore important a rapid evaluation of the degree and type of contamination, together with the length, nature and accessibility of the affected coastline. Several techniques are available for the correct shoreline clean up. Liquid bulk oil can be removed from the shore by the use of vacuum trucks, pumps and skimmers; instead, for very viscous or emulsified bulk oil or oiled-soaked sediment, non-specialized engineering or agricultural machinery could be used to collected and remove stranded oil and contaminated material. The clean-up might also involve *flushing*, which uses high volumes of low-pressure water to wash stranded or buried oil from shorelines, and the *surf washing*, where the oil is released from the sediment by the natural cleaning action of the shoreline waves. Other techniques may be deployed to complete the clean up. High pressure washing using either hot or cold water can be used on most hard surfaces, particularly on man-made structures where natural cleaning is insufficient.

Furthermore, once the oil droplets sink to the water column and reach the bottom sediments, hydrocarbons rapidly and tightly adsorb to the organic matter of the sediment, which represents an extremely complicated environmental matrix with physical and chemical characteristics and types of contamination very different. The selection of the most suitable intervention depends on several characteristics: volume of sediment to be treated, physical characteristics of the sediments (such as particle size, presence of organic matter), chemical characteristics (such as pH, oxide-reducing conditions), and type of contamination. In-situ capping is an attractive, non-intrusive and cost-effective method of remediating contaminated sediments, which consists in covering the sediments by stable layers of clean materials (Mohan et al., 2000). The type of material can be simple, consisting of granular material, such as clean sediment, sand or gravel, or complex, consisting of multiple layers made of geotextiles and other materials, permeable or impermeable, possibly covered with other materials useful to the remediation (i.e. active carbons, organic material).
The functions of the capping consist in physical and chemical isolation and stabilization of contaminated sediment and its protection from the erosion by reducing resuspension and transport, in order to limit the exposure and thus the risk to polluted sediments. In certain conditions, the cap reduces contaminant mobility and subsequent its interaction with aquatic organisms. The main advantages are short time to reduce exposure to contaminants, request of less infrastructure in terms of material management, reduction of the risks associated with the transportation and the disposal of contaminated sediments, minor impact on local communities, and reduction of the cost if the capping materials are available in the site.
Conversely, the main limitation are the permanence of the contaminated sediments in marine environments, a possible re-suspension of contaminated sediments in the implementation process, the restrictions in the utilization of the site (reduction of depth, navigation restrictions), the habitat changes and conditions not favorable to the growth of the biological community. Therefore, it is necessary a slow and uniform application that allows the accumulation of the coating material in layers in order to avoid the dispersion or the mixing with the underlying contaminated sediments. To improve the effectiveness is possible to add additives such as carbons active, exploiting the adsorption properties, or alternative materials that decrease the permeability of the capping (Cornelissen et al., 2011).

Finally, both on off-shore and shoreline oil spill, *bioremediation* has become a very important method employed in the decontamination of petroleum polluted environments (Varjani, 2017). The *bioremediation* involves the use of natural microbial biodegradation activity and can be carried out basically by means of two different strategies: *biostimulation*, which consists in providing limiting nutrients to stimulate the growth of bacteria able to biodegrade the oil hydrocarbons, and *bioaugmentation*, which increases the rate of degradation through the addition of microorganisms. The problem with this strategy is the difficulty of provide effectively nutrients or microorganisms to the affected area after the spill due to the dispersion caused by the waves and currents.

1.4 Biodegradation of oil hydrocarbons

When oil spilled at sea, a number of chemical and physical processes, called collectively "weathering", changes the chemical composition and physical characteristics of the oil over time. Microbial degradation plays a major role in the weathering process determining the last fate of the oil spilled in marine environments. Petroleum biodegradation is carried out by microorganisms, which use hydrocarbons as energy and carbon source. They are ubiquitous in nature and are able to biodegrade various types of hydrocarbons. Some of them can degrade alkanes (normal, branched and cyclic paraffins), others aromatics, and others both paraffinic and aromatic hydrocarbons (Atlas, 1981). The rate and the extent of biodegradation depend on several factors, such as the recalcitrance and the bioavailability of the oil compounds, the presence of microorganisms with the capability to biodegrade the contaminants, the growth and other activity factors, i.e. temperature, pH, availability of nutrients, electron acceptors that influence the indigenous microbial metabolism (De Jonge et al., 1997). Bioremediation is a natural or 'green solution' that involves the use of microorganisms to remove pollutants from a contaminated site, causing minimal ecological effects and accelerating the natural fate of oil pollutants (Atlas, 1995). The aim of bioremediation is to transform organic contaminants into harmless compounds or to mineralize them to CO_2 and H_2O. It is necessary the presence of microorganisms that are able to quickly adapt to the substrates and to biodegrade them efficiently and in a reasonable time. Biodegradation by indigenous populations of microorganisms is one of the main mechanisms by which petroleum can be removed from the marine environment. Oil hydrocarbon-degrading microorganisms are ubiquitously present in soil/sediment and aquatic environments, but represent less than 1% of the total microbial population. However, these microorganisms tend to increase (usually to 10%) when oil hydrocarbons are present in the site (Atlas, 1995). *Achromobacter, Acinetobacter, Alcaligenes, Archrobacter, Bacillus, Flavobacterium, Coryneforms, Microbacterium, Micrococcus, Pseudomonas, Actinomycetes, Nocardia, Aureobasidium, Candida, Rhodotorula, and Sporobolomyces* are typical oil-degrading bacteria present in marine environment (Xue et al., 2015).

The microorganisms are capable of biodegrading the organic pollutants both in the presence and in the absence of oxygen.

1.4.1 Aerobic biodegradation

The most rapid and complete biodegradation of organic pollutants occurs under aerobic conditions. Several studies have reported that microbial species such as *Pseudomonas aeruginosa* (Zhang et al., 2011), *Pseudomonas putida* (Grant et al., 2011), *Rhodococcus* (Song et al., 2011), *Mycobacterium* (Nicolau et al., 2009) and *Acinetobacter* (Liu et al., 2014) are able to aerobically biodegrade the hydrocarbons. Although different studies reported that only strictly anaerobic microorganisms can be considered as actual indigenous of oil contaminated reservoirs (Grassia et al., 1996, Magot, 1996), also aerobic (Orphan et al., 2000, Borzenkov et al., 2006), facultative anaerobic (Bernard et al., 1992) and microaerophilic microorganisms

(Telang et al., 1997) were identified in petroleum field samples. For example, in oil samples collected from Brazilian offshore basin, aerobic biodegradation has been demonstrated for *Bacillus* spp., *Micrococcus* spp., *Achromobacter xylosoxidans*, *Dietzia* spp. and *Bacillus pumilus* (de Vasconcellos et al., 2009). Generally, the main bacteria detected in soil contaminated with aliphatic and aromatic hydrocarbons, polycyclic aromatic hydrocarbons and chlorinated compounds belong to *Pseudomonas* spp., *Acinetobacter* spp., *Alcaligenes* sp., *Flavobacterium/Cytophaga* group, *Xanthomonas* spp., *Nocardia* spp., *Mycobacterium* spp., *Corynebacterium* spp., *Arthrobacter* spp *and Bacillus* spp. (Fritsche and Hofrichter, 2008).
However, the presence of petroleum significantly affects the diversity of indigenous microbial communities, and the oxygen played an important role in the enrichment of the microbial consortium after an oil spill contamination. In particular, *Gammaproteobacteria*, including the genera of *Alcanivorax*, *Marinobacter*, *Pseudomonas* and *Acinetobacter* are dominant in oil-contaminated marine sediments (Kostka et al., 2011; Chen et al., 2017). Studies reported that *Alcanivorax* is able to use alkanes as sole carbon source (Harayama et al., 2004), and *Marinobacter* is responsible for the degradation of benzene, toluene, ethylbenzene, xylenes (Berlendis et al., 2010). Figure 14 shows the main pathway of aerobic hydrocarbons biodegradation; the oxygen is essential at the initial attack of the substrate and at the end of the respiratory chain.

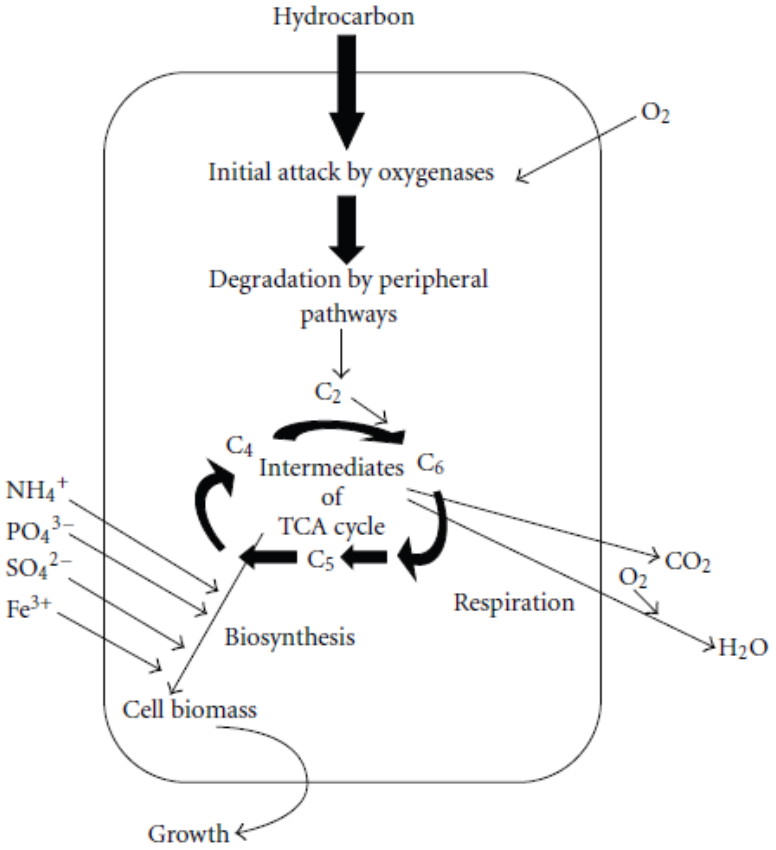

Figure 14 Aerobic hydrocarbons biodegradation by microorganisms: main pathway (Das and Chandran, 2010).

Enzymatic key reaction of aerobic biodegradation is the incorporation of oxygen into the substrate that consist of an oxidative process catalyzed by oxygenases and peroxidases. Organic contaminants are converted through peripheral pathways degradation into specific metabolites, such as acetyl-CoA, succinate, pyruvate that represent the intermediates of the central metabolism, for example the tricarboxylic acid (TCA) cycle (or Krebs cycle). Cell biomass can be biosynthesized from these intermediates. Sugars, required for biosynthesis and growth, are synthesized by gluconeogenesis pathway (Fritsche and Hofrichter, 2008).

The degradation of petroleum hydrocarbons can be mediated by specific enzyme systems that are required to introduce oxygen in the substrate in order to initiate biodegradation. In particular, the cytochrome P450 alkane hydroxylases are a family of ubiquitous heme-thiolate monooxygenases that play a central role in the microbial degradation of oil, chlorinated hydrocarbons and many other organic compounds (Das and Chandran, 2010).

The degradation of organic pollutants can occur by means of two different processes: growth or cometabolism. In the first case, the contaminants, that are directly used as the sole source of carbon and energy, are completely mineralized. In the second process, the pollutants are metabolised in the presence of another growth substrate that is used as the main carbon and energy source. Organic contaminants in the oxic site are predominantly biodegraded by chemo-organotrophic species, which are able to use several natural and xenobiotic compounds as carbon sources and electron donors for the energy generation. However, the presence of different microbial populations determines a greater efficiency of biodegradation, since genetic information of more microorganisms is necessary to biodegrade a complex mixture of organic pollutants (Fritsche and Hofrichter, 2008).

1.4.1.1 Growth-associated biodegradation

Attack on xenobiotics by oxygenases represents the initial step in the degradation process of aliphatic and cycloaliphatic hydrocarbons. Monooxygenases incorporate one atom of oxygen of O_2 into the substrate, while the second atom is reduced to H_2O (Figure 15); dioxygenases incorporate both atoms into the substrate.

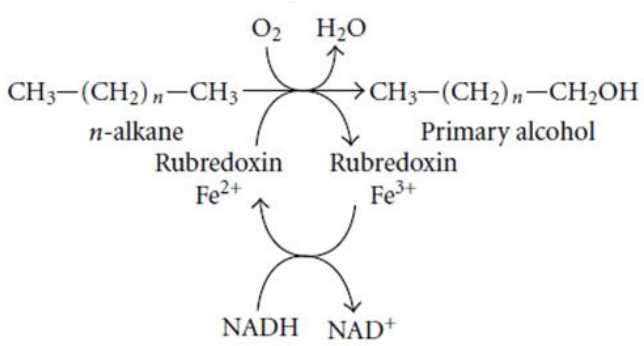

Figure 15 Enzymatic reaction of monooxygenases involved in the biodegradation of hydrocarbons (Fritsche and Hofrichter, 2008).

Oxidation of *n*-alkanes, the main constituents of crude oil, can be *terminal* or *diterminal*, depending on the addition of the oxygen to one or both the ends of the molecule. The oxygen can be also added to an internal carbon of the compound in the *subtermina*l process (Figure 16). The predominant pathway is the monoterminal oxidation: the organic compound is transformed into the corresponding alcohol, aldehyde and fatty acid through *n*-alkane monoxygenase, alcohol dehydrogenase and aldehyde dehydrogenase, respectively. Fatty acid is then converted in acetyl-CoA through β-oxidation. Lower (C_3-C_6) and longer alkanes follow the *subterminal* oxidation passing through the formation of a secondary alcohol, ketone and ester. Another pathway can proceed via an epoxide that is transformed to a fatty acid. The presence of methyl side groups do not significantly decrease the rate of biodegradation, whereas complex branching chains reduce the biodegradability (Fritsche and Hofrichter, 2008). In general, long-chain *n*-alkanes (C_{10}-C_{24}) are more easily biodegraded, whereas short-chain alkanes (less than C_9), which are more toxic to many microorganisms tend to evaporate rapidly from oil contaminated sites.

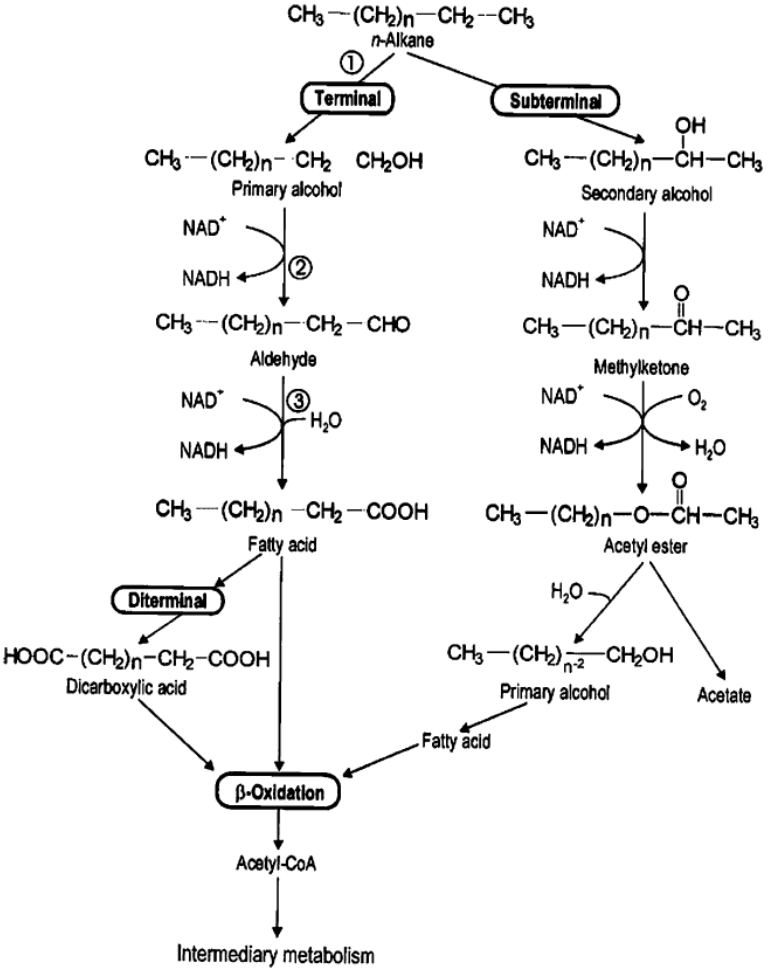

Figure 16 Degradation pathways of *n*-alkane (Fritsche and Hofrichter, 2008).

Cyclic alkanes represent a minor part of total oil components and are relatively resistant to microbial degradation, which is more complicated due to the absence of an exposed terminal methyl group. Whereas, the presence of alkyl side chains

increase the biodegradation. Few species are able to use cycloalkanes as sole carbon and energy source, although it is more frequently the cometabolic action by mixed cultures. The metabolic pathway of cyclohexane biodegradation is shown in Figure 17.

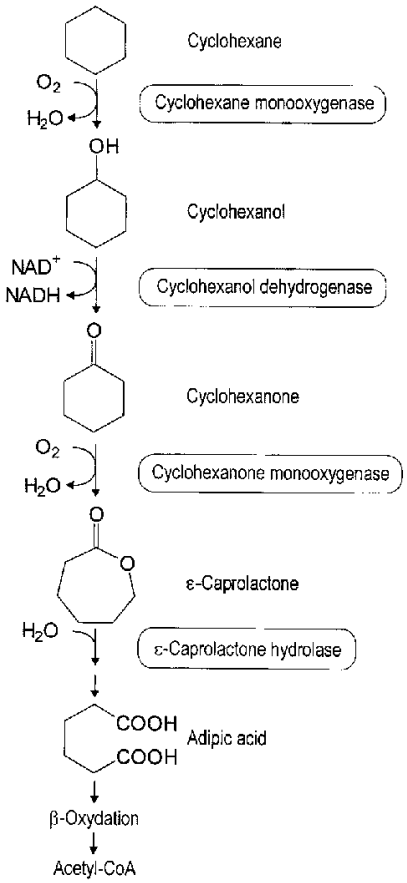

Figure 17 Degradation pathway of cyclic alkanes (cyclohexane) (Fritsche and Hofrichter, 2008).

In marine environments, the *n*-alkanes can be effectively biodegraded by *Acinetobacter calcoaceticus*, *Alcanivorax dieselolei* (Hassanshahian et al., 2012) and *Corynebacterium variabile* (Hassanshahian et al., 2014), and although with lower capability also by *Pseudomonas aeruginosa*, *Gordonia amicalis*, R*hodococcus wratislaviensis*, *Pseudomonas stutzeri*, *Halomonas halodurans*, H*alomonas organivorans*, *Marinobacter hydrocarbonoclasticus* and *Microbacterium aquimaris*. For all these species, the *n*-alkanes C_{12}-C_{18} are more rapidly and extensively biodegraded than *n*-alkanes with lower (C_9-C_{11}) and higher (C_{19}-C_{25}) molecular weight due to the microbial toxicity of lower *n*-alkanes and the less water solubility of the higher *n*-alkanes that reduce their bioavailability to microorganisms (Hassanshahian et al., 2012). In oil polluted soils, medium molecular weight *n*-alkanes were efficiently biodegraded by species belong to *Rhodococcus erythropolis*, *Acinetobacter baumanii*, *Burkholderia cepacia* e *Achromobacter xylosoxidan* (Tanase et al., 2013). In marine sediments, it has been demonstrated the capability of *Alcanivorax* spp. on the biodegradation of *n*-alkanes C_{13}-C_{30}, and branched *n*-akanes,

pristane and phytane (Liu et al., 2010). *Pseudomonas aeruginosa*, *Bacillus* and *Paracoccus* were able to grow in the coastal area using aliphatic hydrocarbons as carbon and energy source, such as tetradecane (C_{14}), hexadecane (C_{16}), octadecane (C_{18}) and eicosane (C_{20}), and heavy oils. Although more slowly, they can also grow on hexane (C_6), octane (C_8), octacosane (C_{28}), paraffins, pristane and cyclohexane, while generally they are not able to use aromatics as carbon source (Chaerun et al., 2004).

Among the constituents of crude oil, aromatic hydrocarbons, such as benzene, toluene, ethylbenzene and naphthalene are characterized by a higher thermodynamic stability than aliphatics. However, many bacteria isolated from contaminated soils possess self-transmissible catabolic plasmids encoding enzyme for the biodegradation of natural aromatics. Toluene, for example, is degraded by bacteria through five alternative pathways, each of which is encoded by a specific plasmid (Harayama et al., 1999).

The first step in the aerobic degradation of these compounds is the hydroxylation catalyzed by a dioxygenase that proceed with the conversion of the product (a diol) to a catechol or protocatechuate, common intermediates of several degradation processes, through a dehydrogenase (Figure 18).

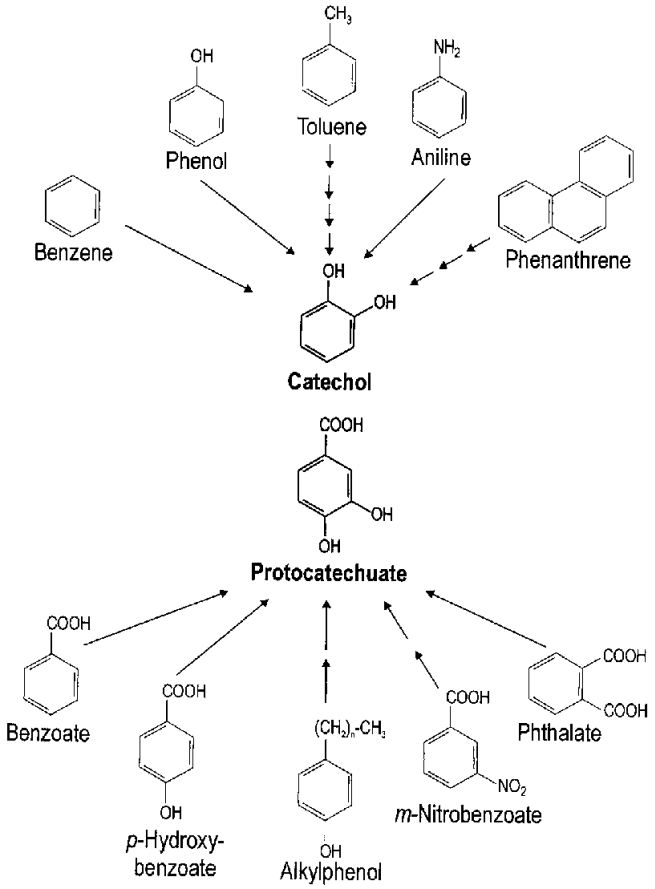

Figure 18 Degradation of different natural and xenobiotic aromatic compounds into two central metabolic intermediates: catechol and protocatechuate (Fritsche and Hofrichter, 2008).

Several microorganisms have evolved catabolic pathways to biodegrade aromatics, probably because these compounds are produced by all organisms (e.g., as aromatic amino acids or quinones). Therefore, synthetic molecules (xenobiotics) can be degraded by many microorganisms if the respective molecules are similar to natural compounds. The key step is the enzymatic conversion of xenobiotic into catechol or protecatechuate, the natural intermediates of the biodegradation.

In order to continue the biodegradation, it is necessary the opening of the aromatic ring of catechol or protocatechuate, via ortho- or meta-cleavage, so as to obtain the conversion into molecules used in the subsequent metabolic processes, such as the Krebs cycle (Figure 19). Both oxygenolytic ortho- and meta-cleavage reactions are catalyzed by specific dioxygenases. The molecule produced by the o-cleavage is subject to following enzymatic reactions that transform it into succinate and Acetyl-CoA, while the product of m-cleavage is metabolized by hydrolytic enzymes to acetaldehyde and pyruvate, which then are used in the central metabolism. Protocatechuate is metabolized by a homologous set of enzymes. The presence of a substituent group on the benzene ring triggers alternative mechanisms of degradation that can occur on the aromatic ring or on the same substituent groups, still leading to the formation of catechol or protocatechuate (Fritsche and Hofrichter, 2008).

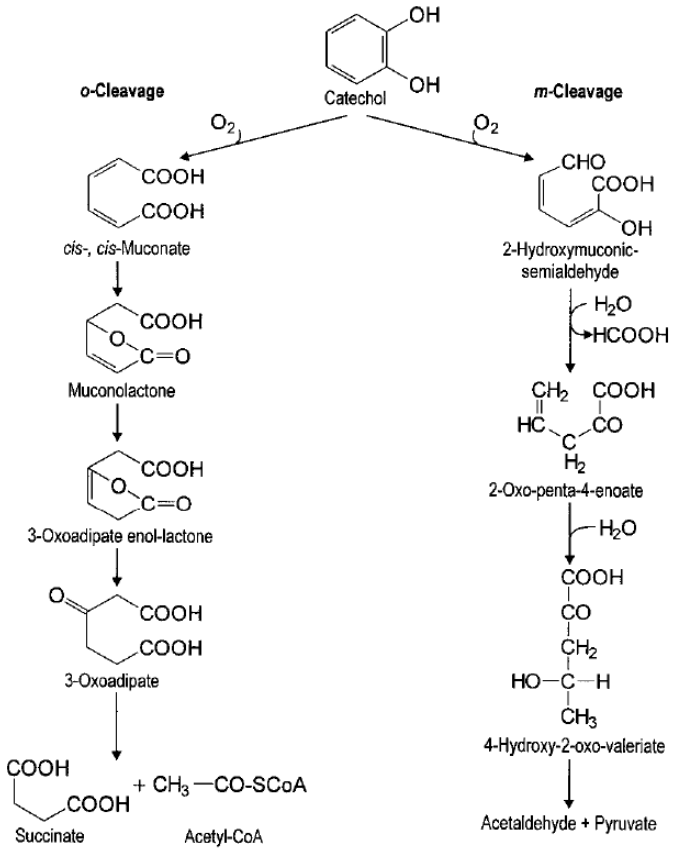

Figure 19 Pathways of aromatics aerobic biodegradation: oxygenolytic ortho- and meta-cleavage.

The biodegradation generally occurs thanks to a consortium of different microorganisms. Mazzeo et al., (2010) identified five main bacteria species in

effluents from petroleum refinery and, in particular, the bacteria *Pseudomonas putida* was probably responsible for the biodegradation of monoaromatic hydrocarbons, such as benzene, toluene, ethylbenzene and xylene (BTEX), which are environmental contaminants due to their toxicity to different organisms. Two species of *Pseudomonas* have also been isolated in petroleum contaminated wastewater for their ability to grow using many hydrocarbons as sole carbon source and to synthesize rhamnolipids as surfactants. One of these species, was also able to biodegrade high concentrations of benzene, toluene and xylene (Di Martino et al., 2012).

Considering the polycyclic aromatic hydrocarbons (PAHs), the simplest molecules, such as naphthalene, biphenyl and phenanthrene are rapidly biodegraded in aerobic conditions. The process usually begins with the dihydroxylation of one aromatic ring and continues with the opening of the rings from the hydroxylated one (Harayama et al., 1999) coming to the formation of catechol or protocatechuate; then the process can continue as previously explained. In marine environments, the PAHs can be biodegraded by *Achromobacter* sp., which can effectively degrade the *n*-alkanes (Deng et al., 2014). Sediment contaminated with high molecular weight PAHs, such as phenanthrene, pyrene and benzo(α)pyrene, however, appear to be biodegraded by the species of the genus *Rhodococcus*, which are also able to remove aliphatic hydrocarbons (Song et al., 2011). Furthermore, the phenanthrene were degraded in coastal sediments thanks to a microbial consortium of a various species of the genus *Bacillus*, *Pseudomonas*, *Staphylococcus*, *Geobacillus*, *Alcaligenes*, and *Stenotrophomonas maktphilia*, also in the presence of other contaminants (Patel et al., 2013). The aromatic hydrocarbons having four or more rings can be degraded by some bacteria, which were able to completely mineralize molecules such as pyrene, fluoranthene and benzo(α)pyrene (Harayama, 1997). Heterocyclic aromatic hydrocarbons and PAHs with substituent groups can be also biodegraded in aerobic conditions by microorganisms belonging to the genera *Acidovorax*, *Arthrobacter*, *Brevibacterium*, *Burk*holderia, *Comamonas*, *Mycobacterium*, *Pseudomonas*, and *Sphingomonas* (Seo et al., 2009).

1.4.1.2 Cometabolic biodegradation

Organic pollutants might be aerobically biodegraded through cometabolism, common phenomenon of microbial activities. During the cometabolic reaction, the pollutant (co-substrate) can be incidentally oxidized by enzymes and cofactors that are produced during the metabolism of another compound (growth substrate). The microorganisms, in fact, do not derive any nutritional benefit from the transformation of the pollutant, and therefore require another substrate as a source of carbon and energy. Although the co-substrate is not assimilate, the product may be an available substrate for other microorganisms present in the mixed indigenous microbial communities. The enzymes of the growing cells and the synthesis of cofactors necessary for enzymatic reactions, e.g., hydrogen donors (reducing equivalents, NADH) for oxygenases are requisites important in the cometabolic process (Fritsche and Hofrichter, 2008).

The low biodegradation efficiency of some petroleum compounds because of their complicated structures could be improved with the co-metabolism of other compounds. This type of process occurs, for example, in the biodegradation of some cycloalkanes without substituent groups, which are used as a substrate in the co-oxidation reactions, with the formation of a ketone or an alcohol (Atlas, 1981). Several microorganisms are also able to biodegraded PAHs through cometabolic process; for example, Xue at al., 2015 have demonstrated an improvement of pyrene biodegradation by adding Osmocote and Chitosan, as co-substrates.

1.4.2 Anaerobic biodegradation

Hydrocarbons are usually recalcitrant to the biodegradation due to the low reactivity of their C-H bonds. As shown in the previous paragraph, well known aerobic hydrocarbon-degrading microorganisms overcome this reactivity barrier thanks to oxygen-dependent mono- or dioxygenases for the initial attack at the hydrocarbons.
Therefore, since oxygen plays a crucial role in the first enzymatic reaction of hydrocarbon degradation, the mineralization of aliphatic and aromatic hydrocarbons was thought to be possible only under aerobic conditions. In the last two decades, however, biological hydrocarbon degradation was surprisingly shown to take place also in the absence of molecular oxygen (Heider and Schühle, 2013). Several studies have reported processes of hydrocarbons biodegradation in different anoxic environments under nitrate-, iron-, manganese-, and sulfate- reduction condition and methanogenesis (Spormann and Widdel, 2000; Chakraborty and Coates, 2004; Chakraborty et al., 2005; Foght, 2008). Anaerobic degradation of hydrocarbons was demonstrated to occur in marsh sediments (Boopathy et al., 2012), soil (Grishchenkov et al., 2000; Hasinger et al., 2012), groundwater (Yeung et al., 2013), aquifers (Kümmel et al., 2015), estuaries (Sherry et al., 2013), and both aquatic (Phelps and Young, 1999; Tsai et al., 2009; Cai et al., 2015, Ghattas et al., 2017) and marine (Massias et al., 2003; Acosta-González et al., 2013; Jaekel et al., 2015, Stagars et al., 2016) sediments.
In anaerobic environment, hydrocarbons act as substrate and electron donors in the respiratory chain leading to the reduction of nitrates, iron, manganese, sulphate or H_2O. However, since these electron acceptors have a redox potential lower than oxygen, the anaerobic process is much less exergonic and the anaerobic microbial growth is slower compared to the aerobic condition. Due to the absence of molecular oxygen, microorganisms must develop alternative systems to initiate the metabolic pathway. The hydrocarbon must be converted into a compound with an essential functional group for the subsequent reactions that lead the substrate to the main oxidative pathways, such as the β-oxidation or the Krebs cycle (Spormann and Widdel, 2000). Many hydrocarbons could be biodegraded under anaerobic condition by several facultative or strictly anaerobic bacteria by involving oxygen-independent hydrocarbon-activating reactions that are completely different from the “aerobic” initial reactions.

The biodegradation of hydrocarbons in the absence of molecular oxygen was demonstrated via methanogenesis of toluene and benzene (Vogel and Grbic-Galic 1986; Grbic-Galic and Vogel 1987). Subsequently, Lovley et al. (1989) showed that toluene could be completely oxidized to CO_2 by pure cultures of the Fe(III)- reducing *Geobacter metallireducens*. These studies conducted to the isolation and characterization of new microorganisms capable to oxidize hydrocarbons under anoxic conditions by involved sulfate, nitrate, oxidized metals, such as iron(III), manganese(IV) and CO_2 as electron acceptor (Heider et al. 1999; Chakraborty and Coates 2004).

Boopathy et al. (2012) compared the biodegradation of petroleum hydrocarbons in marsh contaminated sediments from the coastal Louisiana without any addition of electron acceptors and under different electron acceptor conditions. The largest oil degradation by the indigenous anaerobic bacteria was obtained in the presence of a mixture of different electron acceptors. Significant hydrocarbons degradation also occurred under sulfate-reducing and nitrate-reducing conditions. Further works on groundwater contaminated by oil hydrocarbons showed that bacteria belonging to different species, such as *Pseudomonas*, *Thiobacillus* and *Geobacter* are involved in anaerobic degradation of hydrocarbons. In particular, it was found that the microbial processes utilize nitrate, manganese(IV), iron(III) sulfate as electron acceptors, confirming their importance in the anaerobic degradation of hydrocarbons (Yeung et al., 2013). Under methanogenic conditions, the biodegradation of petroleum hydrocarbons has been also confirmed by studies in aquatic sediments conducted by Cai et al. (2015). Changes in the composition of oil were observed in 200 days, during which occurred a complete biodegradation of *n*-alkanes, a partial degradation of *n*-alkyl benzene and long chain *n*-alkyl toluene, a reduced degradation of n-alkyl cyclohexane and a very low removal of PAHs.

Microbial anaerobic oxidation of alkanes has been described for gaseous alkanes (Kniemeyer et al., 2007; Jaekel et al., 2012), mid-chain alkanes (Davidova et al., 2006) and long-chain alkanes (Zengler et al., 1999). In the anaerobic biodegradation of *n*-alkanes, the initial activation of the molecule is a critical and long studied step. Different general mechanisms have been proposed for the initial activation of alkanes involving biochemical reactions completely different from those used in the aerobic environment (Grassi et al., 2008; Mbadinga et al., 2011):

- addition of fumarate;
- carboxylation;
- alternative pathways.

Addition to fumarate and carboxylation were also observed during the anaerobic biodegradation of some aromatic hydrocarbons (Spormann and Widdel, 2000).

The first suggestion for the addition of exogenous carbon at the subterminal position (C-2) of alkanes resulted after a study conducted by So and Young (1999a, 1999b). During the growth of a sulfate reducing bacterium, they observed formation of fatty acids (FAs) with chain lengths correlating with those of the alkanes. Mass

spectrometric analysis revealed that the original carbon chain of the alkane was preserved in the FAs and that the methyl group was systematically the original terminal carbon of the alkane substrate.

Later, Rabus et al. (2001) demonstrated a denitrifying bacterium able to activate *n*-hexane through homolytic C–H bond cleavage at C-2 and addition to fumarate, giving (1-methylpentyl)succinate as the first stable product. Studies in the following years led to the determination of the *n*-alkanes degradation pathway for anaerobic bacterial by addition of fumarate, shown in Figure 20. A glycyl radical enzyme catalyses the addition of *n*-alkane to fumarate. Subsequently, a carbon skeleton rearrangement of the resulting methylalkyl succinate and a decarboxylation yields a 4-Me-branched fatty acid, which can be further degraded by β-oxidation via a 2-Me-branched and a linear fatty acid having two carbon atoms less than the original *n*-alkane. A similar degradation pathway was described later for the marine sulfate reducing bacteria *Desulfatibacillum aliphaticivorans* (Cravo-Laureau et al., 2004) and strain AK-01 (Callaghan et al., 2006) grown on longer chain *n*-alkanes. This process generates fatty acids (FAs) with even number of carbon atoms from *n*-alkanes having also an even number of carbon atoms, and vice versa.

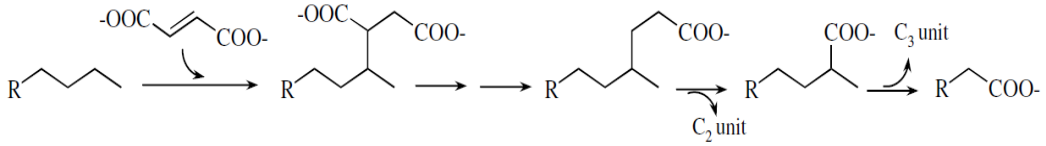

Figure 20 Mechanisms of *n*-alkane activation via addition to fumarate in anaerobic bacteria.

Studies of So et al. (2003) on a sulfate-reducing bacterium (Aeckersberg et al., 1991) have allowed to determine the second pathway for anaerobic *n*-alkane oxidation by carboxylation with inorganic carbon (bicarbonate), predominantly on C-3, and removal of two subterminal carbon atoms from the alkane chain (Figure 21). This mechanism usually leads to even and odd numbered fatty acids (FAs) from odd and even numbered *n*-alkanes, respectively. However, this relationship between the carbon chain length of *n*-alkanes and fatty acid needs other investigations (Grossi et al., 2008).

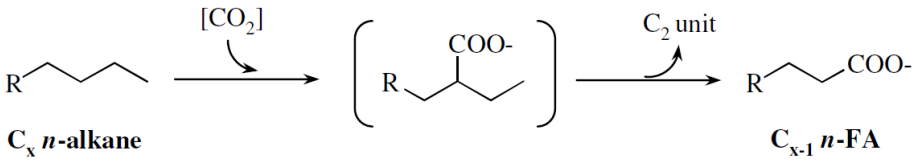

Figure 21 Mechanisms of n-alkane activation via carboxylation in anaerobic.

Alternative mechanisms not involving the addition of fumarate and the carboxylation, have been proposed for the initial attack of the alkanes in anoxic environments, which depend on the type of microorganism and the electron acceptors that are available. Guyoneaud R. isolated a denitrifying bacterium, *Pseudomonas balearica*, able to grow on *n*-alkanes C_{15}-C_{18} and produce fatty acids predominantly with an even number of carbon atoms from the *n*-alkanes with an even number of carbon atoms

and vice versa, as in the case of the addition of fumarate. However, metabolites characteristic of activation of the alkane through addition to fumarate (i.e. alkyl succinates) have never been observed with this strain, suggesting that the activation of *n*-alkanes occurs through a mechanism different from the activation with fumarate (Grossi et al., 2008).

Mehboob et al. (2009) observed that the bacterium *Pseudomonas chloritidismutans* can obtain molecular oxygen via chlorate respiration; the *n*-alkane thus can be subject to an "unusual oxygenation" for the incorporation of the molecular oxygen generated from the dismutation of chlorite in complete absence of air, as illustrated in Figure 22a. A similar mechanism of "intra-cellular oxygen production" is also possible with nitrates and nitrites as electron acceptors (Ettwig et al., 2010); it was also hypothesized that nitrite or nitric oxide produced by the reduction of nitrate can be directly involved in the activation of alkanes, as seen in Figure 22b (Zedelius et al. 2011). Finally Head et al. (2010) have assumed an anaerobic hydroxylation mechanism of oil alkanes, as illustrated in Figure 22c (Mbadinga et al., 2011).

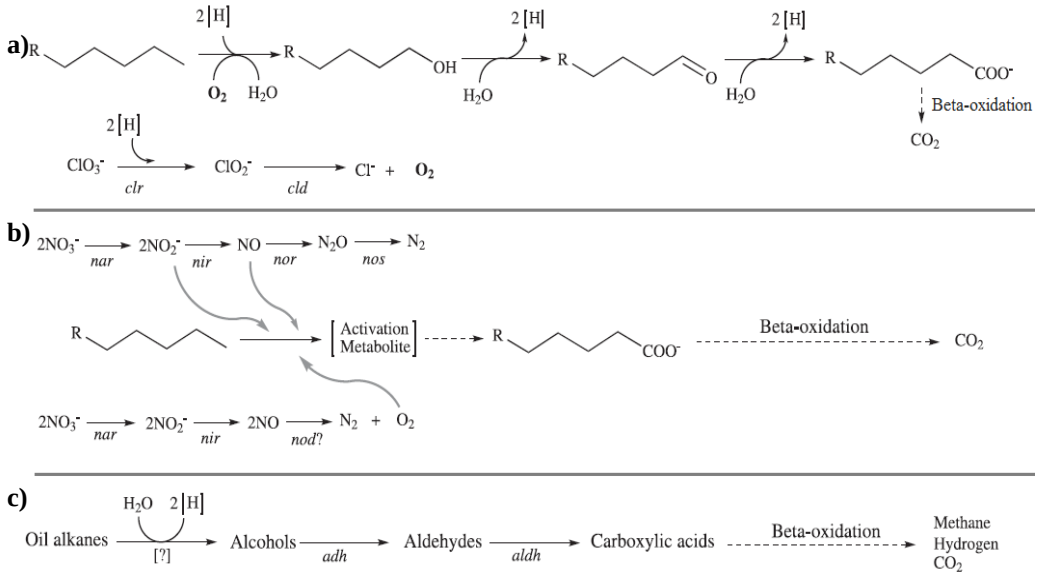

Figure 22 Mechanisms of *n*-alkane activation through chlorate respiration (a), nitrates and nitrites utilization (b) or hydroxylation (c) (Mbadinga et al., 2011).

The mechanisms of anaerobic biodegradation of alkanes in oil reservoirs is not well known due to low concentrations of signature metabolites (Bian et al., 2015). Aitken et al. (2013) identified significant differences between the metabolites produced by the biodegradation of *n*-alkanes under sulfate-reducing and methanogenic conditions, suggesting that the alkane activation mechanisms are different in the two situations. Under sulfate-reducing conditions, fumarate addition and the formation of alkyl succinate metabolites was the main mechanism for the anaerobic biodegradation of *n*-alkanes and branched chain alkanes. Conversely, in sediment microcosms where methane generation was quantitatively linked to *n*-alkane degradation, the low alkyl succinate metabolites production would seem to be not correlated to the biodegradation process. This indicates that the degradation of the n-alkanes under

methanogenic conditions, unless a very rapid turnover of succinate, does not proceed through the addition of fumarate, but alternative mechanisms of alkane activation may operate.

More recent study on the microbial community and functional gene composition of alkane-degrading methanogenic cultures, has reported that bacterial sequences affiliated to *Thermodesulfovibrio* spp. and *Anaerolineaceae* and archaeal sequences falling within the genus *Methanoculleus* are the principal members involved in the anaerobic degradation of *n*-alkanes. The presence of *assA* functional genes encoding the alkyl succinate synthase α subunit suggested that a possible initial activation of *n*-alkanes could be the fumarate addition mechanism (Liang et al., 2016).

Work on a soil contaminated by oil hydrocarbons showed that species of *Pseudomonas* and *Brevibacillus* are able to anaerobically degrade some *n*-alkanes and PAHs (Grishchenkov et al., 2000). Anaerobic biodegradation of hydrocarbons in petroleum contaminated soils can depend on the length of the chain and the reducing conditions. Hasinger et al. (2012) reported that under nitrate-reducing conditions alkanes C_{11}-C_{13} are the most recalcitrant, while medium molecular weight alkanes showed excellent level of biodegradation, higher not only than those reported in the sulfate-reduction condition, but also than those obtained under aerobic conditions. Conversely, under sulfate-reducing conditions the biodegradation was limited for *n*-alkanes with low molecular weight and larger for the high molecular weight molecules, suggesting a correlation between the level of degradation and the length of the carbon chain. Anaerobic biodegradation in oil-contaminated marine sediments may be depend on both the length of the chain and the presence of branching. (Massias et al., 2003).

Isolated or enriched anaerobic alkane degraders generally belong to two phyla: *Proteobacteria* and *Firmicutes*. In marine environments, alkane degradation is predominantly performed by sulfate-reducing bacteria within the class *Deltaproteobacteria* and in particular with members of the family *Desulfobacteraceae*. Members of the *Desulfosarcina/Desulfococcus clade* have been shown to be important in seep sediments (Acosta-González et al., 2013; Kleindienst et al., 2014). Another study on anaerobic hydrocarbons biodegradation in oil-contaminated sediment collected from an estuary under sulfate-reducing conditions, showed a high biodegradation of *n*-alkanes C_7-C_{34}. The bacterial communities involved in degradation process was prevalently composed by *Gammaproteobacteria*, closely related to *Marinobacterium* sp. and *Firmicutes*, connected to *Clostridiales*. When the most of the biodegradation was occurred and the sulfates was exhausted, a change in the species has been detected. In fact, *Chloroflexi* (*Anaerolineaceae* family), *Firmicutes* and the most common *Deltaproteobacteria* sulfate-reducers were identified. The results suggest that in addition to the conventional sulfate-reducing bacteria, other groups of microorganisms are important in the degradation of *n*-alkanes in the sulfate-reducing conditions (Sherry et al., 2013).

Generally, cycloalkanes not substituted or monosubstituted are subject to rapid anaerobic biodegradation, while those with more substituents are much more recalcitrant. Furthermore, the biodegradation of cycloalkanes is often limited by sulfates concentration (Townsend et al., 2004). Jaekel et al. (2015) obtained an enrichment culture of cyclohexane-degrading sulfate-reducing bacteria from hydrocarbon-contaminated intertidal marine sediments; the dominant phylotype affiliated with the *Desulfosarcina-Desulfococcus* cluster of the *Deltaproteobacteria*, was proposed to be responsible for the biodegradation of cyclohexane in sulfate-reducing conditions. Further studies have suggested that these bacteria are also able to biodegrade other cycloalkanes and *n*-alkanes, including the gaseous alkane *n*-butane. The identification of the metabolite cyclohexyl succinate has also led to the conclusion that activation of the cyclohexane occurred by the addition of fumarate. The detection of other metabolites, such as 3-cyclohexylpropionate and cyclohexanecarboxylate suggested that the process of cyclohexane biodegradation in anoxic conditions is analogous to that of the linear *n*-alkanes.

The first demonstration of anaerobic benzene mineralization was reported by Grbic-Galic and Vogel (1987) in a methanogenic culture that was originally prepared from sewage sludge and pre-enriched with ferulic acid as substrate. Many other studies showed the anaerobic biodegradation of aromatic hydrocarbons, in particular BTEX (benzene, toluene, ethylbenzene and xylene) in nitrate-, iron-, sulfate-reducing and methanogenic conditions (Phelps and Young, 1999; Chakraborty et al., 2005; Kasai et al., 2006; Musat and Widdel, 2008; Cervantes et al., 2011; Masumoto et al., 2012; Meckenstock et al., 2016). Removal of BTEX in anaerobic sediments depends on the inoculum used and the terminal electron acceptor available. For example, in a work of Phelps and Young (1999), marine cultures have showed strong biodegradation activity in sulfate-reducing conditions, while freshwater cultures only in methanogenic conditions. Instead, both sediment cultures have biodegraded rapidly some aromatic compounds in nitrate-reducing conditions.

Although most of studies on microbial degradation of polycyclic aromatic hydrocarbons (PAHs) have been conducted under aerobic conditions, the anaerobic biodegradation of these compounds has been also reported in soil and marine sediment under various electron-accepting conditions (Coates et al., 1997; Rothermich et al., 2002; Meckenstock et al., 2004). Liang et al. (2014) isolated a species of *Pseudomonas* from sediments of Shantou (China) offshore able to degrade phenanthrene, benzo(α)pyrene, fluoranthene, and pyrene in aerobic or anaerobic conditions. Sodium sulfite, iron oxide, manganese dioxide, potassium chlorate; maltose; glycine, and a salinity of 20 % significantly stimulated anaerobic degradation of benzo(α)pyrene.

These studies facilitate the understanding of the molecular mechanism underlying hydrocarbons biodegradation under different condition, and could promote microbial remediation of oil-polluted environments in the future.

1.5 Bioavailability of oil hydrocarbons

After oil spill events, marine sediments may represent the final sink for weathered petroleum hydrocarbons because of their strong tendency to adsorb on the organic matter of the sediment through weak Van der Waals interactions or hydrogen- or covalent bonding (Dec and Bollag, 1997). Consequently, their biodegradation rate is reduced due to their low bioavailability to microorganisms. The soil/sediment plays thus an important role in the fate of hydrophobic organic compounds. The term "bioavailability" refers to the fraction of organic contaminant that can be uptake or transformed by living organisms, becoming therefore a crucial regulatory factor for the biodegradation of oil hydrocarbons or other organic compounds in contaminated soil/sediment. Some bioremediation strategies, like the addition of nutrients and/or electron acceptor might be not efficient when biodegradation rate is limited by the low bioavailability of the contaminant (De Jonge et al., 1997).

A concept closely related to the bioavailability is the "bioaccessibility", although some believe that the difference between them is purely semantics. Bioavailable compound is chemically active to degrader organisms, whereas bioaccessible compound is per se chemically inactive but potentially exploitable (Semple et al., 2004).

Bioavailability depends on different factors, such as the soil/sediment type (e.g. mineral and organic matter content, pH, ion exchange capacity, porosity), the physico-chemical properties of the contaminants (e.g. aqueous solubility, polarity and molecular structure), the time and environmental conditions and the microbial characteristics (Semple et al., 2003). In particular, contaminants are removed from the sediment with different rate and extent depending on their proprieties (Jones et al., 1996). Furthermore, increasing the contact time between the contaminant and the sediment, the chemical and biological availability of the contaminant decrease. This process, called "ageing", is describe by two mechanisms collectively termed sequestration: (i) diffusion through the porewater in the sediment and (ii) sorption on mineral and organic matter fractions of the sediment. The diffusion is retarded by the sorption within nano or micro-pores containing organic matter. In addition, some pores are too small to allow the presence of microorganisms, thus contaminants in such pore become not bioavailability (Pignatello J.J. and Xing B, 1996). The interaction sediment/contaminant depends on nature and amount of the sediment organic matter, microbial activity and contaminant concentration (Hatzinger P.B. and Alexander M. 1995). Organic contaminants aged in soil/sediment are not accessible to the microorganisms even though fresh biodegradable compounds are added. Bioavailability is more limiting when the concentration of available oil contaminants is low (De Jonge et al., 1997). The evaluation of contaminants bioavailability in sediment is an essential means for a successful bioremediation.

1.5.1 Methods for bioavailability measurement

Bulk solid-phase concentration is generally used to assess sediment quality, but it is not useful for appraising the bioavailability of organic compounds in soil or sediment

(Burton, 1991). However, there are number of chemical techniques used to predict the accessibility of organic contaminants to the microorganisms. Assessments of bioavailability are mainly based on mild organic solvent and non-exhaustive extraction conditions that remove only the labile and readily extractable compounds, which are more representative of bioavailability (Hatzinger and Alexander, 1995). An alternative to convention solvent-extraction for the evaluation of bioavailability is the chemical solid-phase soil extractions; a solid phase adsorbent material is placed into contact with a slurry and the organic compounds can therefore diffuse out of the sediment and on to the adsorbent material.

Other techniques have been developed for the evaluation of the availability that allow a strong correlation between the fraction microbially degradable and the fraction available to the chemical extractants. These methods include persulfate oxidation that was validated by Cuypers et al. in 2000 in order to predict polycyclic aromatic hydrocarbon (PAH) bioavailability in soils and sediments, and it was subsequently adapted also for petroleum hydrocarbons (Cuypers et al., 2001). The advantages are its simplicity and high reproducibility.

The *in situ* porewater concentration may be another and more effective tool for estimating the bioaccumulation of sediment-associated contaminants and therefore their bioavailability. However, measurement dissolved hydrocarbons concentrations in porewater is often difficult due to the long and rigorous analytical procedures required to obtain large volumes of porewater to detect a sufficient level of hydrocarbons (Mayer et al., 2000). An alternative monitoring approach is the passive sampling of the interstitial water (or porewater) in contaminated sediments (Thomas et al, 2014, Hong et al, 2015). Several passive sampling techniques might be used, including semi-permeable membrane devices (SPMDs), polyoxymethylsoloxane (POM) sheets, polyethylene (PE) sheets and polydimethylsiloxane (PDMS) fibers. These passive samplers are directly placed *in situ* until equilibrium is reached and the contaminants porewater concentration is then calculated through partition coefficients. A large amount of time could be required to reach the equilibrium, although for such fibers only few days are sufficient to attain the equilibrium of some reference compounds with the porewater (Lampert et al., 2015).

1.6 Bioremediation of oil-contaminated marine environments

Biodegradation of oil hydrocarbons by indigenous bacterial populations is one of the main mechanisms to remove petroleum from the marine environments. Bioremediation is an environmentally "green" method that stimulates microorganisms, either naturally-occurring or introduced, to biodegrade pollutants, representing thus the most practical and cost-effective promising mean of treating soil, water and sediments contaminated by oil hydrocarbons.

Several environmental factors affect the bioremediation process, such as temperature, pH, salinity, nutrients and soil/sediment type. For example, physical nature and chemical composition of oil and microbial metabolisms usually depend on the temperature. In general, with decreasing temperature the oil viscosity increases and the microbial metabolisms decreases. The ideal pH for hydrocarbons degradation ranges from 6.5 and 8. Since pH is usually between 2.5 and 12.0 in environmental sites, it can be modified by adding alkaline or acid buffer. Although the little information on the effect of the salinity on hydrocarbons degradation, it is presumed an inverse relationship between the salinity of the environment and the oil biodegradation, even if indigenous microorganisms in the marine environment may be less impacted by high salt concentration due to their acclimation (Margesin and Schinner 2001). Nutrients, in particular nitrogen, phosphorous, and micronutrients, such as calcium, magnesium, sulfur and zinc, must be present in amounts enough for the microbial growth, otherwise it would be necessary to add this substances to the polluted site. Soil/sediment type influences the permeability that is a crucial factor for the bioremediation. Since water is important to the transport of nutrients and electron acceptors, low permeability could have a negative effect on the hydrocarbons biodegradation. Proprieties such as organic matter that influence the adsorption of the petroleum hydrocarbons also have an impact on biodegradation: hydrophobic pollutants tend to adsorb on the organic matter of the soil/sediment and result less bioavailability to degrading bacteria. Therefore, changing the environmental condition could increase the biodegradation activities of the indigenous communities (Fingas, 2016).

Bioremediation can be performed *ex si*tu or *in situ*. *Ex situ* bioremediation technologies involve the removal of the contaminated material to be treated in a separate place, while *in situ* technologies involve the treatment of the pollutants at the contaminated site itself. The *in situ* bioremediation offers several advantages, including the affordability, the reduction of impacts on the site, the elimination of the transportation costs and the waste production, and the applicability to dilute and widely diffused contaminants (Iwamoto and Nasu, 2001, Azubuike et al., 2016).

Since microorganisms are capable to biodegrade a large variety of synthetic compounds, bioremediation can be applied to sites contaminated with different pollutants.

Bioremediation processes utilized in the field are classified into three main categories: bioattenuation, biostimulation and bioaugmentation. Biostimulation and bioaugmentation could be used for both *in situ* and *ex situ* bioremediation (Fingas, 2016).

- Bioattenuation is based on the monitoring of the natural degradation of the contaminants over time without human intervention. It is widely used for underground storage tank sites with petroleum-contaminated soil and groundwater (Dojika et al., 1998).
- Biostimulation involves the manipulation of the environment in order to stimulate and increase the biodegradation of contaminants by indigenous microorganisms. This method includes the addition of various limiting nutrients and electron acceptors, such as nitrogen, phosphorus, oxygen and substrates like methane, phenol and toluene (Iwamoto and Nasu, 2001).
- Bioaugmentation is the inoculation of bacteria with the desired biodegradation capabilities in order to enhance the removal of pollutants in contaminated sites. This approach is used in the case of very recalcitrant chemicals where bioattenuation or biostimulation does not have a sufficient effect. However, large attention should be taken for the application of bioaugmentation because large amounts of bacteria are added to contaminated sites and their impact on both human and environment must be known. Moreover, the inoculated bacteria have to perish after the remediation in order to not affect the indigenous microbial community for a long time. Nakamura at al. (2000) performed bioaugmentation experiment in Japan for the first time under strict control by the Ministry of International Trade and Industry. A phenol-utilizing bacterium, *Ralstonia eutropha* (KT- 1) isolated from the same contaminated site, was injected without adding any substrates. An emerging strategy for the bioaugmentation is the use of genetically engineered microorganisms. For example, Munakata-Marr (1996) conducted a study of bioaugmentation using a modified strain (*Burkholderia cepacia*), which can degrade trichloroethylene growing on lactate. This strategy avoids the utilization of other toxic compounds as substrate and could be used for other contaminants like oil hydrocarbons.

Bioremediation has emerged over the last decade as a promising technology for combating oil-contaminated environments. However, relatively few studies have been conducted on hydrocarbon contamination in marine sediments (Kim et al., 2008; Frenzel et al., 2009; Long et al., 2009; Da Silva et al., 2009; Beolchini et al., 2010). Experiments on field and laboratory showed that biodegradation processes of oil-contaminated sediments might be accelerated by enhancing the activity of hydrocarbon-degrading microorganisms through biostimulation as well as bioaugmentation strategies. Most of experimental tests have been conducted in aerobic conditions since microbial oxygenases and peroxidases, which catalyse the initial attack of hydrocarbons as well as the incorporation of oxygen, is the enzymatic

key reaction in the biodegradation process (Swannell and McDonagh, 1996; Kim et al., 2006). Increasing detection that the hydrocarbons biodegradation can take place also in anaerobic environment has opened new perspectives for the *in situ* bioremediation of contaminated sediments, where reducing conditions below the sediment surface limit the usefulness of O_2 as an electron acceptor. In anoxic marine sediments, sulfate, Mn(IV) and Fe(III) usually are the primary terminal electron-accepting processes; thus, the microbial metabolism of hydrocarbons may be effective if the hydrocarbon-oxidizers are sulfate, Fe(III), or Mn(IV) reducers. Dell'Anno et al. (2009) demonstrated that the inoculation of anaerobic microcosms of hydrocarbons-contaminated sediment from Ancona (North Adriatic Sea, Mediterranean Sea) with acetate and sulfate-reducing bacteria decreased the redox potential values and hydrocarbons concentration. A recent laboratory study of Mohajeri et al. (2017) showed a significant hydrocarbons biodegradation in coastal sediment samples artificially contaminated with weathered crude oil by the supplementation of acclimatized microorganism as well as nitrogen and phosphorus.

One of the main limitations in the bioremediation of oil-contaminated marine sediments is the low bioavailability of the oil pollutants, resulting in a limited biodegradation efficiency. Petroleum hydrocarbons are highly hydrophobic and tend to adsorb strongly to the sediment particles, reducing the availability of oil compounds to microorganisms. A strategy to enhance the bioavailability of the oil contaminants is to transport the pollutants from the organic matter of sediment to the aqueous bulk phase. One of the effective ways to increase the bioavailability (or solubility) of petroleum hydrocarbon pollutants is the use of surfactants or biodegradable pollutant mobilizing agents that enhance the desorption and solubilization of petroleum hydrocarbons, facilitating their assimilation by microorganisms. Several studies showed that surfactant agent has the ability to effectively solubilize and mobilize organic compounds adsorbed on soil constituents (Zang et al., 1992; Robinson at al., 1996; Volkering et al., 1997, Bezza and Chirwa, 2017). Therefore, non-toxic and biodegradable pollutant mobilizing agents of plant and animal origin and of microbial surfactants can be used to enhance hydrocarbons bioavailability and thus biodegradation in oil-contaminated marine sediments.

1.6.1 Biosurfactants/biogenic pollutants mobilizing agents

The term "surfactants", SURFace ACTive AgeNTS, indicates molecules that, among other properties, decrease the surface tension (or interfacial tension) between two liquids or between a liquid and a solid. Surface tension is the force acting on the surface of a liquid leading to minimization of the area of that surface. The surfactants peculiar characteristic is to be amphipathic molecules, as they have both a hydrophilic portion (polar) said head, and a hydrophobic portion (apolar) said tail: therefore, they contain both a water-insoluble (or oil-soluble) and a water-soluble component. Surfactants play a strategic role in the intervention process after an oil spill event increasing the concentration of hydrophobic compounds in the water phase. They could create, in fact, an emulsion of oil in water by forming, if present in

concentration above their Critical Micellar Concentration (CMC), specialized structures vital to their action, called micelles, which solubilize the hydrophobic molecules (Christofi and Ivshina, 2002). A typical micelle is an aggregation of surfactant molecules with the hydrophilic "head" regions in contact with the surrounding solvent and the hydrophobic “tail” regions in the micelle centre (Figure 23). The micelle incorporates the contaminant inside its central hydrophobic core thanks to the affinity with the apolar tails, increasing the dispersion of the contaminant in solution above its water solubility limit. This solubilization may also lead to mobilization of hydrophobic pollutants adsorbed to soil/sediment.

Important characteristic of surfactant is the Hydrophile–Lipophile Balance (HLB) that is relates to the relative abundance of hydrophilic and hydrophobic portions and influence the physico-chemical properties of the surfactants (Tiehm, 1994). In general, a surfactant with a low HLB is lipophilic, whereas a surfactant with high HLB has a higher solubility in water. According to the ionic charge of the polar part, the surfactants can be classified into anionic, cationic, non-ionic or zwitterionic (Christofi and Ivshina, 2002).

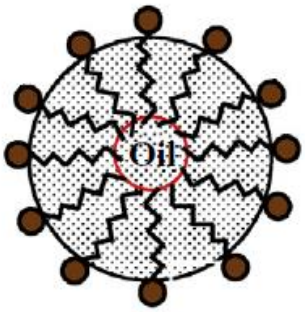

Figure 23 Representation of a micelle containing oil (Gong et al., 2014).

Furthermore, the surfactants can be distinguished in synthetic and natural depending on their origin. The first are chemically and generally used for the recovery after oil spills; their disadvantage is that they are not biodegradable and can be toxic for the environment (Banat, 1995). The surfactants of natural origin are mostly synthesized by living organisms, such as plants, animals and microorganisms; the latter are called biosurfactants (Silva et al., 2014). The biosurfactants have numerous advantages compared to synthetic ones, including higher biodegradability, lower toxicity, better surface activity and greater environmental compatibility. They are also more resistant to the extreme conditions of pH, temperature and salinity and more effective than chemical surfactants in enhancing the hydrocarbons bioavailability (Ron and Rosenberg, 2002). Therefore, biosurfactants seem to be the better candidates for the use in bioremediation of contaminated sediments. Among these, there are glycolipids, lipopeptides, phospholipids, fatty acids, neutral lipids, polymeric and particulate compounds. The biosurfactants are classified based on their chemical structure and microbial origin. The hydrophilic portion can be a polysaccharides, amino acid, cationic/anionic peptide, phosphate, carboxylic acid or alcohol, while the hydrophobic portion is made up of long-chains of saturated or unsaturated hydrocarbons or fatty acids (Banat et al., 2010). Some type of biosurfactants and

microorganisms that produced these compounds are shown in Table 3. The action of surfactants on the bioavailability enhancement might depend by the salinity of marine water; ions present in seawater affect the surface activity of some pollutant mobilizing agents (Marti et al., 2014).

Type of surfactant	Microorganisms
Trehalose lipids	*Arthrobacter paraffineus, Corynebacterium* spp., *Mycobacterium* spp., *Rhodococus erythropolis, Nocardia* sp.
Rhamnolipids	*Pseudomonas aeruginosa, Pseudomomas* sp., *Serratia rubidea*
Sophorose lipids	*Candida apicola, Candida bombicola, Candida lipolytica, Candida bogoriensis*
Glycolipids	*Alcanivorax borkumensis, Arthrobacter* sp., *Corynebacterium* sp., *R. erythropolis, Serratia marcescens, Tsukamurella* sp.
Cellobiose lipids	*Ustilago maydis*
Polyol lipids	*Rhodotorula glutinus, Rhodotorula graminus*
Diglycosyl diglycerides	*Lactobacillus fermentii*
Lipopolysaccharides	*Acinetobacter calcoaceticus* (RAG1), *Pseudomonas* sp., *Candida lipolytica*
Arthrofactin	*Arthrobacter* sp.
Lichenysin A, Lichenysin B	*Bacillus licheniformis*
Surfactin	*Bacillus subtilis, Bacillus pumilus*
Viscosin	*Pseudomonas fluorescens*
Ornithine, lysine peptides	*Thiobacillus thiooxidans, Streptomyces sioyaensis, Gluconobacter cerinus*
Phospholipids	*Acinetobacter* sp.
Sulfonylipids	*T. thiooxidans, Corynebacterium alkanolyticum*
Fatty acids (corynomycolic acids, spiculisporic acids, etc.)	*Capnocytophaga* sp., *Penicillium spiculisporum, Corynebacterium lepus, Arthrobacter paraffineus, Talaramyces trachyspermus, Nocardia erythropolis*
Alasan	*Acinetobacter radioresistens*
Streptofactin	*Streptomyces tendae*
Particulate surfactant (PM)	*Pseudomonas marginalis*
Biosur PM	*Pseudomonas maltophilla*

Table 3 Type and microbial origin of biosurfactants (Mulligan, 2005)

Some surfactants/ biogenic pollutants mobilizing agents used in this study to enhance the hydrocarbons bioavailability and biodegradations, are briefly described:

- glycolipids;
- cyclodextrins
- soya lecithin;
- bile acids.

Glycolipids, consisting of a carbohydrate portion linked to fatty acids, are low molecular weight microbial surfactants produced by several types of microorganisms. They are promising biosurfactants since they can find application in different fields thanks to their low toxicity, high biodegradability, biological activities and chemical stability (Paulino et al., 2016). Furthermore, glycolipids are characterized by high structural diversity including rhamnolipids, sophorolipids, trehalolipids and mannosylerythritol lipids, which contain mono- or disaccharides combined with long-chain aliphatic acids or hydroxyaliphatic acids (Banat et al., 2010).

Rhamnolipids, containing rhamnose and β-hydroxydecanoic acid (Figure 24), have been discovered for the first time by Bergstrom et al. (1946) growing *Pseudomonas pyocyanea* (now *Pseudomonas aeruginosa*) on glucose (Abdel-Mawgoud et al., 2010), and since then these microbial surfactants have been intensively studied.

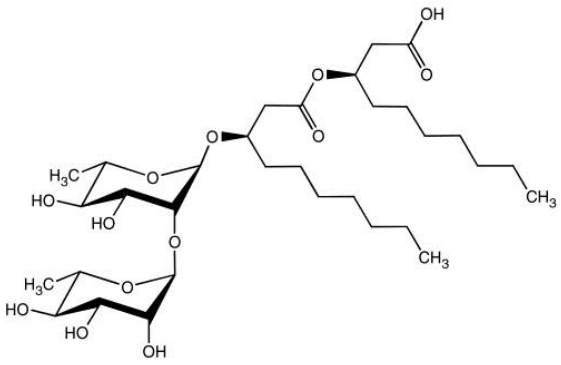

Figure 24 Chemical structure of the first identified rhamnolipids, named as *α*-*L*-rhamnopyranosyl-*α*-*L*-rhamnopyranosyl-*β*-hydroxydecanoyl-*β*-hydroxydecanoate (Abdel-Mawgoud et al., 2010).

Several studies mainly focused on rhamnolipids biosynthesis and isolation, and analytical determination of their chemical structures and physico-chemical properties, particularly in terms of surface activity and potential applications (Maier and Soberón-Chávez, 2000, Abdel-Mawgoud et al., 2010). Rhamnolipids are very important in the bioremediation since they have a potential application in the removal of petroleum from contaminated areas (Lang and Wullbrandt, 1999). In particular, they were used to enhance the bioavailability of crude oil (Nikolopoulou and Kalogerakis, 2008), diesel (Whang et al., 2008) and polycyclic aromatic hydrocarbons (PAHs) (Sponza et al., 2009) in different places such as wastewater, contaminated soil and marine environments. For example, rhamnolipids produced by *Pseudomonas aeruginosa J4*, were able to increase diesel solubility, indigenous

biomass growth and then diesel biodegradation in both water and soil systems (Whang et al., 2008).

In contrast to rhamnolipids, which are mainly produced by bacteria belonging to the species *Pseudomonas aeruginosa*, sophorolipids are synthesized by a selected number of non-pathogenic yeasts. They consist of the dimeric sugar sophorose (hydrophilic head) linked to a long chain hydroxy fatty acid containing 16 or 18 carbon atoms (hydrophobic tail) (Figure 25). Sophorolipids have good surfactant activities, excellent skin compatibility and they can be used in different sectors due to their emulsifying, antimicrobial and other beneficial properties (Van Bogaert et al., 2007). The application of sophorolipids in the bioremediation has been shown by several studies. For example, the addition of sophorolipids extracted from culture supernatants after growth of *C. bombicola ATCC 22214* on glucose and corn oil as substrate, enhanced the biodegradation of both saturated and aromatic hydrocarbons (Kang et al., 2010). The yeast *Candida tropicalis*, isolated from petroleum-contaminated soil in India, is also a strong producer of sophorolipids and an efficient degrader of diesel oil (Chandran and Das, 2012).

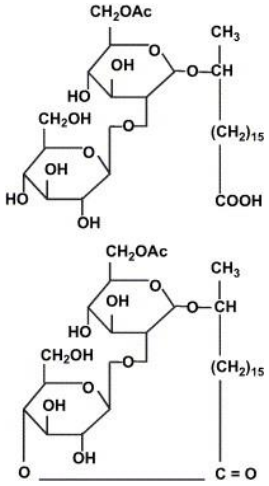

Figure 25 Structure of sophorolipid from *C. bombicola* (Mulligan, 2005).

Cyclodextrins are cyclic oligosaccharides composed of α-(1,4) linked glucopyranose subunits. They are of three different types: α-, β- and γ-cyclodextrins (Figure 26), composed of six, seven and eight α-(1,4)-linked glycosyl units, respectively (Del Valle, 2004).

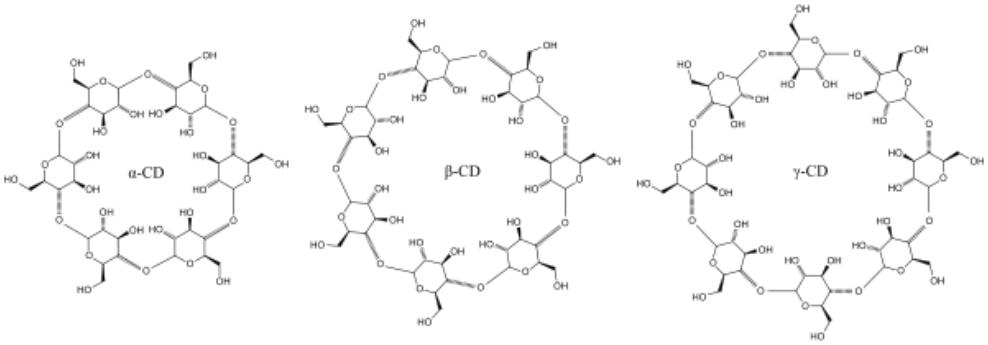

Figure 26 Chemical structure of α-, β- and γ-cyclodextrins.

Cyclodextrins assume a particular three-dimensional conformation consisting of toroidal hydrophobic cavities surrounded by a hydrophilic shell (Figure 27); they are thus water-soluble and can form inclusion complexes with hydrophobic molecules of a size compatible with their hydrophobic core (Bardi et al., 2000).

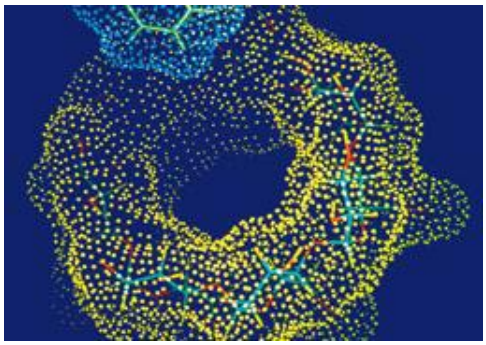

Figure 27 Three-dimensional conformation of cyclodextrin.

Cyclodextrins represent a valid option for bioremediation due to their low cost, biodegradability, biocompatibility and effective capability to enhance the pollutants bioavailability. For example, β-cyclodextrins have accelerated the biodegradation of different hydrocarbons, particularly naphthalene, in petroleum-polluted soil by stimulating the growth of indigenous microbial population (Bardi et al., 2000).
In order to reduce the costs due to the use of pure cyclodextrins, which are expensive, other applicable alternatives have been studied. For example, it has been analysed the possibility to use a randomly methylated-β-cyclodextrin (RAMEB-CD) to improve the aerobic biodegradation of polychlorinated biphenyls (PCBs) in soils, which is often slow and incomplete due to the low bioavailability of these pollutants. In different tested laboratory conditions, it has been shown that the RAMEB-CD were able to increase the bioavailability and biodegradation of PBCs with effects that dependent on various factors, such as the concentration of RAMEB-CD, the physico-chemical nature of the amended soil, and the employed soil treatment conditions (Fava and Ciccotosto, 2002). Hydroxypropyl-β-cyclodextrin (HPB-CD) is another important cyclodextrin used in bioremediation. Studies showed that it is able to increase the bioavailability and thus biodegradability of several contaminants, such as pyrene and other polycyclic aromatic hydrocarbons (Wang et al., 2005b).

The term “lecithin” indicates a mixture of phospholipids, natural constituents of animals and plants. The first animal source of lecithin is the egg yolk; since it is too expensive, lecithin is now mainly obtained from plant seeds, in particular soybean oilseeds due to their abundant availability and low cost. Crude soybean oil contains 1-3% of phospholipids that are extracted as a by-product during oil refinement. The composition of lecithin varies according to the method of extraction and purification. Commercial soy lecithin consists of phospholipids (65-75%), triglycerides (34%), and smaller amounts of carbohydrates, pigments, sterols, and sterol glycosides (Dickinson, 1993). Phospholipids are diglycerides with a phosphate group attached to the third carbon atom of the glycerol molecule and their fatty acid composition may differ in chain length and saturation due to their response to environmental

conditions (Figure 28). Since they are amphipathic molecules with hydrophilic polar heads and hydrophobic nonpolar fatty acid tails, they are able to decrease the surface or interfacial tension.

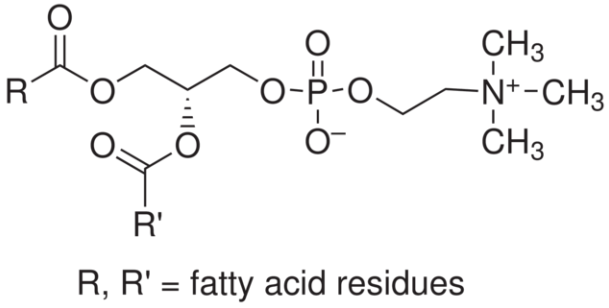

Figure 28 Soy lecithin: chemical structure

A process, called *de-oiling*, allows to obtain lecithins with a high content of phospholipids (Xu et al., 2011). A conventional method to remove triglycerides is the acetone de-oiling due to the fact that, in contrast to phospholipids, the triglycerides are soluble in acetone (Wu and Wang, 2003). Another method uses a silica column with hexane solution; the separation is obtained by the adsorption of neutral lipids while phospholipids pass through the column (Schneider, 1989). Soy lecithin is extensively used as an emulsifier, antioxidant, stabiliser, lubricant, wetting agent and nutritional supplement thanks to its structure and physical-chemical properties (Xu et al., 2011). They may also find application in bioremediation, both in untreated and in de-oiled form.

Bile acids are organic compounds biosynthesized in the liver of mammals and other vertebrates from cholesterol and stored in the gall bladder. They are lipid-carriers able to solubilize many lipids by forming mixed micelles with fatty acids, cholesterol and monoglycerides. Bile acids are secreted into the bile largely in a conjugated form with taurine or glycine, forming bile salts. In humans are mainly taurocholic acid and glycocholic acid (derivatives of cholic acid), and taurochenodeoxycholic acid and glycochenodeoxycholic acid (derivatives of chenodeoxycholic acid).
The chemical structure of cholic acid and taurocholic acid are showed in the Figure 29. Bile acids are amphipathic molecules, i.e. they contain both a hydrophilic (polar) part consisting of a rigid steroid core and a hydrophobic (lipid soluble) portion consisting of a short aliphatic side chain. There are two major classes of bile acids depending on the length of the side chain: C27 and C24 bile acids, constituted by 27 and 24 carbon atoms, respectively (Mukhopadhyay and Maitra, 2004).

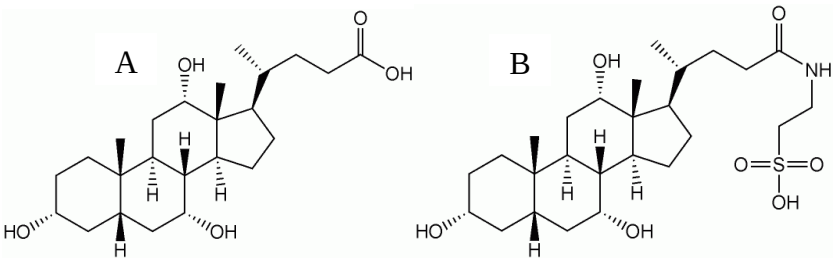

Figure 29 Chemical structure of cholic acid (A) and taurocholic acid (B)

In aqueous solution, bile salts aggregate to form micelles. However, bile acids tend to aggregate in a manner different from conventional surfactant molecules due to their rigid steroid backbone having polar hydroxyl groups on the concave α-face and methyl groups on the convex β-face. Aggregation of bile salts in aqueous solution is given by the hydrophobic association of apolar β-faces of steroid backbones, while further aggregation occurs through hydrogen bonding interactions (Mukhopadhyay and Maitra, 2004). Thanks to their function, bile acids may be also employed as surfactants in bioremediation of oil hydrocarbons contaminated marine environments. Furthermore, studies have shown that bile acids are products not only of eukaryotic cells, but also of prokaryotic cells. *Myroides* sp. strain SM1 isolated from seawater produced cholic acid, deoxycholic acid and their conjugates with glycine in their culture supernatants when grown on marine broth (Maneerat et al., 2005).

1.6.2 Encapsulation of biosurfactants

Surfactant releasing formulations for the delivery of the surfactants to the marine sediments is important in order to prevent their random dispersion in marine water.
Encapsulation of drugs and other chemical compounds is widely used in medical biotechnology, food and cosmetic industry (Brannon-Peppas, 1993; Barichello et al., 1999; Schwendener and Schott, 2017). Compounds encapsulation aims to control their delivery to the proper site as well as to protect them from environmental degradation. Biodegradable polymers, such as polysaccharides, poly α-esters, polyalkylcyanoacrylates and polyamidoamine dendrimers, can be used to encapsulate several active principles. Polymeric carriers have found large applications in cosmetics, personal care markets and pharmaceutical fields since they offer different advantages compared to other carrier materials. In fact, the polymers can increase the stability, efficacy and bioavailability of encapsulated compounds, and they are also removed from the body via normal metabolic pathways (Ammala, 2013).
Preparation of polymers microparticles were widely applied in drug delivery system since they have demonstrated high effectiveness, stability and lower toxicity. This system provides prolonged delivery of an active compound and improves its bioavailability in terms of pharmacological effect (Barakat and Ahmad, 2008; Naha et al., 2008). Polymer microparticles can be produced with various parameters through different methods such as oil/water single emulsion solvent evaporation, spray drying, ionotropic gelation, coacervation, air suspension and polymerization (Dong and Bodmeier, 2006; Giri et al., 2013). Efficient encapsulation of compounds characterized by hydrophilic profile might be performed using water/oil/water double emulsion solvent evaporation method (DEM). Aqueous solution of desired compound is firstly emulsified in a solution of polymer dissolved in an organic solvent. Organic solvent should be characterized by high volatility and low boiling point in order to enhance the removal of residual solvent and the recovery of microparticles. For this reason, some organic solvents as acetonitrile, ethyl acetate, chloroform, benzene and methylene chloride are preferred. This first oil in water emulsion is then dispersed in a water phase usually containing a stabilizing agent, e.g. polyvinyl alcohol. Formation

of double emulsion and subsequent removal of residual solvent yields the formation of solid polymeric microparticles containing the surfactants that are finally separated by filtration or centrifugation, washed and dried. Addition of stabilizing compounds such as polyvinyl alcohol, improves the viscosity of the microspheres during the procedure increasing the process of compounds loading. The double emulsion method has been widely applied mainly for the controlled delivery of drugs (Giri et al., 2013), but it can be adapted for the encapsulation of surfactants.
The use of encapsulated biosurfactants/pollutant mobilizing agents and their predicted delivery to marine sediment represents a challenge for scientists since marine environment is highly dynamic. Development of new solutions for the targeted deployment of surfactants is still under studies. Polymers that are rapidly biodegraded in the marine environment can be also used to encapsulate biosurfactants obtaining an environmental friendly formulation.

Depending on the type of polymer used for the encapsulation, the surfactants can be released from the microparticles by two main mechanisms: (i) diffusion and (ii) polymer biodegradation or hydrolysis. In particular, the release of the encapsulated molecules from polymers (e.g. hydrogels) via diffusion is expected to be faster than the release upon hydrolysis or biodegradation of polymers (e.g. polyesters).
A successful encapsulation of surfactants and pollutant mobilizing agents in biodegradable polymers must involve a high encapsulation efficiency and a welol known release rate of the surfactants from the formulation.

1.7 Gela refinery oil spill

Most of the experimental tests conducted in this thesis have involved the use of a crude oil actual-contaminated marine sediment from Gela (Sicily, Italy). The geographical position of Gela is shown in the Figure 30.

Figure 30 Geographical position of Gela refinery.

In 1960 in the locality of Piana del Signore, in the town of Gela started the construction of a refinery (Figure 31). Two years later came into operation the first production plants. Since 2003, Gela refinery has become an exclusive property company of Eni Group S.p.A. (Eni, 2007). The refinery currently works each year 5 million tons of crude oil, derived for more than 20% by local production, and produces fuels and chemical compounds with a conversion that reaches 85% (Eni, 2008). The transport of incoming and outgoing materials from petrochemical refinery occurs mainly by sea through tankers (Eni, 2007).

Figure 31 Gela refinery (www.italianinsider.it).

The crude oil is initially subjected to a desalting process to remove the sea water and then passes to the topping installations where takes place the fractional distillation

that separates the oil fractions on the base of the different density and boiling ranges in order to obtain gas, gasoline, diesel and fuel oil. The heaviest components of the oil, which can not be separated by distillation, are sent to a coking plant that allows to transform them in the same products derived from topping, but leading also to the production of a residue that is combusted in the thermoelectric plant of the refinery. It is subsequently reduced the sulfur content of gas oils, which are then subjected to catalytic cracking in order to increase the final production of gasolines and gas. Gasolines instead undergo a catalytic reforming process and desulphurization to produce finished gasoline (Eni, 2008). The wastewater is collected and sent to treatment plants. The same also happens to gases, which in fact are conveyed to treatment plants in order to remove sulfur and nitrogen compounds and recover the most interesting substances (www.eni.com).The refinery has a thermoelectric power plant that provides power plants and the Network Energy Services Manager (Eni, 2013). In 2013, in order to make the system financially sustainable the production of gasoline and polyethylene was stopped, maximizing the production of diesel, and the different processes have been made more eco-friendly (www.eni.com).
A serious accident occurred in the Topping 1 of Gela refinery on June, 4 2013 (Figure 32).; oil has leaked for about an hour pouring into the river Gela, adjacent to the implant, and then up to the sea (www.ilfattoquotidiano.it). Estimates indicate that about 5,000 liters of crude oil have leaked from the plant (www.rinnovabili.it)

Figure 32 Oil into the sea after the accident of Gela refinery (belicenews.it).

The cause was identified in a breakdown of a heat exchanger that resulted an incorrect functioning of a safety valve, without counting the gaps in the safety operations (www.ilmessaggero.it). The works to remove pollutants have lasted for weeks involving large amounts of personnel and equipment, both at sea and on land, in order to reduce the damage to the marine flora and fauna, which threatened the coast of the Sicily (http://livesicilia.it). In November 2014, the Eni oil refinery of Gela was converted to biofuel production after a job-saving agreement of the company with the government. The agreement consists of the 'green' conversion of the Sicilian refinery in Gela, which will become one of Italy's major producers of biofuel (www.italianinsider.it).

1.8 KILL SPILL PROJECT

The present study of thesis is part of the European Project "Integrated Biotechnological Solutions for Combating Marine Oil Spills", with the acronym KILL SPILL, founded by the European Union in cooperation with the Seventh Framework Programme (FP7) (www.killspill.eu).

The *Alma Mater Studiorum* - University of Bologna (UNIBO), as a partner, worked in different sections of this European project. My research activity is included in the Work Package 5 (WP5), entitled "Efficient cleanup of contaminated sediments due to oil spills. Emphasis on biotechnological solution".

The general aim of KILL SPILL is to improve existing technologies and to develop new highly efficient, economically and environmentally, viable biotechnological solutions for the clean-up of oil spills caused by maritime transport or offshore oil exploration and related processes,

In particular, the focus of WP5 is the enhancement of hydrocarbons biodegradation in contaminated sediments by providing extra electron acceptors, such as solid electrodes or oxygen, infauna, micro/macro nutrients, possibly in combination with contaminant mobilizing agents under controlled environmental conditions. This Work Package is addressed to different contamination scenarios, i.e., from "freshly" contaminated sediments to those subjected to chronic pollution, and contrasting climate regions, i.e., from Mediterranean to Norwegian Sea.

2 Aims

Nowadays hydrocarbons contamination of marine environments due to accidental oil spills and activities related to the petrochemical industry is of high concern, resulting in extensive and long-term deterioration of coastal and ocean ecosystems, in particular of sediments where heavy oil hydrocarbons sink.
The main limitations to the bioremediation of oil-contaminated marine sediments are the prevailing of anoxic conditions and the low bioavailability of hydrocarbons, which rapidly and tightly adsorb to the organic matter of the sediment. However, several anaerobic microbial communities are able to biodegrade both aliphatic and aromatic compounds, and various type of biosurfactants and biogenic mobilizing agents can be used to enhance the hydrocarbons bioavailability and biodegradation. Their effect has been less studied under anaerobic conditions, where the hydrocarbons may be slower biodegraded and in marine environments, where the high salinity might negatively affect their pollutant mobilizing activity.

The aim of this PhD research project was to identify an environmental friendly approach to increase oil hydrocarbons bioavailability and their biodegradation in oil-contaminated marine sediments.

In particular, the experimental activities focused on:

i. The selection and the evaluation of different biogenic non-toxic and biodegradable pollutant mobilizing agents and biosurfactants in enhancing the hydrocarbons biodegradability in slurry microcosms of oil-contaminated sediment suspended in marine water under both aerobic and anaerobic conditions.

ii. The characterization of stimulated indigenous microbial anaerobic communities with molecular fingerprinting method (PCR-DGGE) and clonal library of bacterial 16S rRNA genes.

iii. The assessment of the different pollutant mobilizing agents and biosurfactants in enhancing the oil hydrocarbons bioavailability via the measurement of hydrocarbons concentration in the porewater through passive sampling with polydimethylsiloxane fibers.

iv. The development and the optimization of encapsulation/entrapment procedures of the more effective pollutant mobilizing agents/biosurfactants in biodegradable polymers for their deployment into the sediment.

3 Materials and methods

3.1 Selection of microbial surfactants/pollutant mobilizing agents and sediments

For the selection of biosurfactants and commercial pollutant mobilizing agents, attention was paid to the choice of compounds with different characteristics, such as a more hydrophilic (hydroxypropyl-β-cyclodextrins, Solec C) and a more hydrophobic (randomly methylated-β-cyclodextrins, Textrol F) product within cyclodextrins and soy lecithins, a biosurfactant of animal origin (bile acids) and microbial surfactants produced by different microorganisms (rhamnolipids and sophorolipids) (Table 4). These surfactants were singly added in the different experimental tests.

SELECTED SURFACTANTS	CHARACTERISTICS
• Rhamnolipids (from *P. aeruginosa*) • Sophorolipids (from yeast strain)	Microbial surfactants, kindly provided by Prof. Ibrahim Banat, Ulster University UK
• Randomly methylated β-cyclodextrins (RAMEB-CD) • Hydroxy propyl-β-cyclodextrins (HPB-CD)	Cyclodextrins, provided by Amaizo-Cerestar, USA
• Solec C • Textrol F	Soy lecithins, $HLB_{TEXTROL\ F} = 4$; $HLB_{SOLEC\ C} = 7$, provided by Solae Italia srl, Italy
• Bile acids	Biosurfactants of animal origin (cholic acid, taurocholic acid, glycocholic acid, deoxycholic acid, etc.), provided by ICE srl, Italy

Table 4 Selected biosurfactants/commercial pollutant mobilizing agents, and their main characteristics.

The actual oil-contaminated sediment selected for this study, was collected by project Partner CNR-IAMC from an area located in the proximity of the refinery of Gela (Caltanissetta, Sicily, Italy), where an oil spill from pipeline of "Topping1" installation of ENI Refinery of Gela occurred on 4th June 2013 releasing approximately 5,000 liters of heavy crude oil (Gela sediment).

The effect of biosurfactants/commercial pollutant mobilizing agents on the biodegradation of oil hydrocarbons was also investigated in a sediment collected by UNIBO from the harbor of Ravenna (Italy) on June 2011 (Ravenna sediment). This sediment was contaminated in the laboratory immediately before experiments set up with a Dansk blend crude oil provided by Geodis Wilson Denmark A/S through Partner UCPH (viscosity at 50 °C: 4.04 cSt; density: 810 g/L).

3.2 Oil hydrocarbons biodegradation tests

3.2.1 Slurry microcosms set up

The tests were conducted in 120-mL slurry microcosms, containing 70 mL of sediment suspended in marine water (20% dry weight/volume) under aerobic and anaerobic conditions (Fava et al., 2003a; Fava et al., 2003b; Zanaroli et al., 2006).
As shown in Figure 33, the slurry microcosm consists of a small batch bioreactor (glass vial), containing a known amount of sediment and water (volume of slurry) and a headspace (volume of gas).

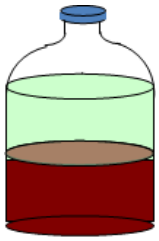

Figure 33 Representation of a slurry microcosm.

To maintain aerobic conditions, a CaO2-based slow oxygen-releasing compound (OXYGEL, Biorem Engineering BVBA, Belgium) was periodically added at the final concentration of 4 mL/$kg_{sediment}$; whereas, to maintain strict anaerobic conditions, microcosm's headspace was replaced with a mixture of N_2:CO_2, 70:30 v/v.
Three sets of microcosms were set up (Table 5). One was prepared with Gela sediment under anaerobic conditions (Gela anaerobic microcosms), a second with the same sediment under aerobic conditions (Gela aerobic microcosms), and a third with the Ravenna sediment contaminated in the laboratory with the Dansk Blend crude oil to the final concentration of 5 g/$kg_{sediment}$ under aerobic conditions (Ravenna aerobic microcosms).
All sets included microcosms supplemented with the biosurfactants and pollutant mobilizing agents previously selected (Table 4): two microbial surfactants (rhamnolipids and sophorolipids), two types of cyclodextrins (hydroxypropyl-β-cyclodextrins, HPB-CD and randomly methylated β-cyclodextrins, RAMEB-CD), two commercial soy lecithin products (Solec C and Textrol F) and bile acids, which were singly spiked.
All surfactants were added to microcosms at the final concentration of 1 g/L (5 g/$kg_{sediment}$), except for rhamnolipids and sophorolipids that were provided at 0.2 g/L (1 g/$kg_{sediment}$), given their very low critical micelle concentrations (CMC), which is 0.1-0.2 g/L. In addition, not amended biologically active and autoclave-sterilized controls were prepared for all sets, whereas autoclave-sterilized controls amended with each surfactant were also prepared for the two aerobic sets, to monitor the oxygen release in the presence of OXYGEL. The sterile controls were subjected to three cycles of autoclave (1 hour at 121 °C) in order to remove the indigenous microbial communities present in the sediment. The aerobic set microcosms were spiked with 1,2,3,4,5,6,7,8,9,10-decamethyl-anthracene (DMA, 30 mg/$kg_{sediment}$)

(Sigma-Aldrich), a polycyclic aromatic hydrocarbon (PAH) that is recalcitrant to biodegradation and so can be used as an internal standard (Sanni et al., 2015). OXYGEL was supplied to the aerobic sets after 0, 3, and 8 weeks of incubation (Gela sediment) or 0 and 8 weeks of incubation (Ravenna sediment).

Gela anaerobic microcosms *Actual oil-contaminated sediment* *Triplicate conditions* *N_2:CO_2 (70:30) headspace gas*	**Gela aerobic microcosms** *Actual oil-contaminated sediment* *Duplicate conditions* *OXYGEL, 4 mL/kg sed.*	**Ravenna aerobic microcosms** *Crude oil, 5 g/kg sed.* *Duplicate conditions* *OXYGEL, 4 mL/kg sed.*
Sterile un-amended control	Sterile un-amended control	Sterile un-amended control
Biotic un-amended control	Biotic un-amended control	Biotic un-amended control
+ Rhamnolipids (1 g/kg sed.)	+ Rhamnolipids (1 g/kg sed.)	+ Rhamnolipids (1 g/kg sed.)
+ Sophorolipids (1 g/kg sed.)	+ Sterile Rhamnolipids (1 g/kg sed.)	+ Sterile Rhamnolipids (1 g/kg sed.)
+ RAMEB-CD (5 g/kg sed.)	+ Sophorolipids (1 g/kg sed.)	+ Sophorolipids (1 g/kg sed.)
+ HPB-CD (5 g/kg sed.)	+ Sterile Sophorolipids (1 g/kg sed.)	+ Sterile Sophorolipids (1 g/kg sed.)
+ Solec C soy lecithin (5 g/kg sed.)	+ RAMEB-CD (5 g/kg sed.)	+ RAMEB-CD (5 g/kg sed.)
+ Textrol F soy lecithin (5 g/kg sed.)	+ Sterile RAMEB-CD (5 g/kg sed.)	+ Sterile RAMEB-CD (5 g/kg sed.)
+ Bile acids (5 g/kg sed.)	+ HPB-CD (5 g/kg sed.)	+ HPB-CD (5 g/kg sed.)
	+ Sterile HPB-CD (5 g/kg sed.)	+ Sterile HPB-CD (5 g/kg sed.)
	+ Solec C soy lecithin (5 g/kg sed.)	+ Solec C soy lecithin (5 g/kg sed.)
	+ Sterile Solec C soy lecithin (5 g/kg sed.)	+ Sterile Solec C soy lecithin (5 g/kg sed.)
	+ Textrol F soy lecithin (5 g/kg sed.)	+ Textrol F soy lecithin (5 g/kg sed.)
	+ Sterile Textrol F soy lecithin (5 g/kg sed.)	+ Sterile Textrol F soy lecithin (5 g/kg sed.)
	+ Bile acids (5 g/kg sed.)	+ Bile acids (5 g/kg sed.)
	+ Sterile Bile acids (5 g/kg sed.)	+ Sterile Bile acids (5 g/kg sed.)

Table 5 Sets of slurry microcosms: Gela anaerobic microcosms, Gela aerobic microcosms and Ravenna aerobic microcosms.

Triplicate microcosms were prepared for each condition of the anaerobic set, while duplicates were prepared for each condition of the two aerobic sets. All microcosms, hermetically closed with a septum and metal ring, were incubated statically at 20 °C in the dark. Incubation lasted 40 weeks for the anaerobic set and 28 weeks for the

aerobic ones. Anaerobic microcosms were sampled at weeks 0, 3, 8, 13, 17, 30 and 40; while aerobic microcosms of Gela sediment were sampled after 0, 3 and 28 weeks of incubation and those of Ravenna sediment after 0, 8 and 28 weeks.
In order to evaluate the stimulation of hydrocarbons biodegradation in the presence of the different selected surfactants, the following parameters were periodically monitored at each sampling:

- concentration of n-alkanes (C12-C32) in the sediment,
- pH,
- biogas composition, redox potential and sulfate concentration for the anaerobic set only,
- dissolved oxygen concentration for aerobic sets only.

Furthermore, the structure and composition of the bacterial communities was also analyzed in anaerobic microcosms of Gela sediment.

3.2.2 Sampling and hydrocarbons extraction

The microcosms were placed in agitation on a plate with magnetic stir bar and a volume of slurry (7 mL) was collected at each sampling: 2 mL were stored in sterile eppendorf and frozen at -20 °C for the following DNA extraction, 5 mL were transferred into glass vials (previously weighed) for the measure of pH, redox potential (for the anaerobic set) or dissolved oxygen (for the aerobic sets), sulfates and *n*-alkanes concentration. The Gela anaerobic microcosms were insufflated with N_2 for approximately 10 minutes in order to maintain the anaerobic conditions and a third of their headspace volume was replaced with CO_2 to restore the condition N_2:CO_2 70:30. Instead, the aerobic microcosms were supplemented with the OXYGEL.
After centrifugation (5000 rpm for 10 minutes) of the slurry, the water was removed and used for the analysis of the sulfates, while hydrocarbons were batch extracted (Santos et al., 2014) from the sediment (approximately 1 g) with 2.5 mL of a hexane:dichloromethane (DCM) mixture (1:1) in the presence of 1 g anhydrous sodium sulfate (Na_2SO_4) for 40 h at 30 °C and 150 rpm. Batch extraction was assisted with ultrasonication for 5 minutes before and after incubation under mixing. The solvent was recovered after centrifugation (5000 rpm for 10 minutes) for the gas chromatography analysis. The residual sediment, instead, was dried for 16 hours at 105 °C, in order to calculate the correct dry weight of the sediment from which hydrocarbons have been extracted, using the following formula:

$$W_{sed} = W_{fin} - (W_{vial} + W_{Na_2SO_4})$$

where:
W_{sed} is the dry weight of the sediment from which hydrocarbons have been extracted,
W_{fin} is the final weight of the vial after 16 hours at 105 °C,
W_{vial} is the initial weight of the vial before the sampling (tare),
W_{Na2SO4} is the correct weight of Na_2SO_4 used during the batch extraction.

3.2.3 Analytical methods

3.2.3.1 Gas-chromatograph analysis

Quali-quantitative analysis of *n*-alkanes in the organic extracts was performed with an Agilent Technologies gas-chromatograph (GC) 6890N (Figure 34A) equipped with a HP-5 capillary column 30m x 0,250 mm (Figure 34B) and a flame ionization detector (FID). The gas-chromatograph analysis is a common type of chromatography used in analytical chemistry to separate components of a mixture, which can be vaporized without decomposition, thanks to their different affinities with a stationary or mobile phase. The mobile phase is usually an inert (non-reactive) carrier gas, while the stationary phase is a stable substance inside the chromatography column that consists in a long capillary tube wrapped on itself and placed inside the thermostat oven of the GC.

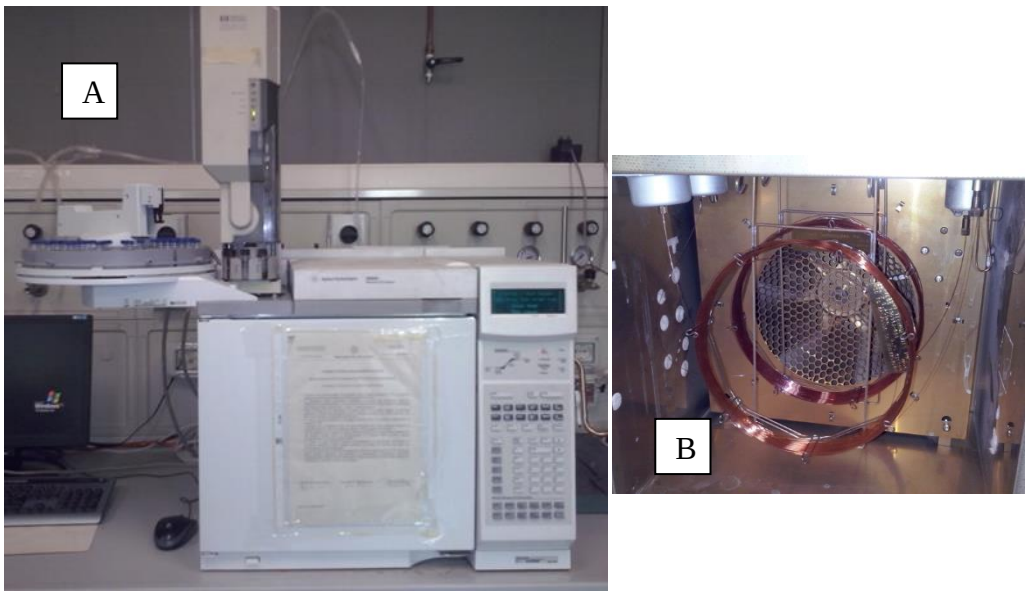

Figure 34: Agilent Technologies gas-chromatograph 6890N (A) equipped with a HP-5 capillary column (B).

The samples, consisting of the solvent extracts, are placed into 2 mL GC vials and loaded into the autosampler of the instrument. Autosampler carousel and robotic arm provide the sample transport to the inlet, which injects it into the chromatographic column through the continuous flux of gas carrier (nitrogen). Mobile phase and sample, mixed together, passing through the column come in contact with the stationary phase. During this step the components present in the mobile phase are separated according to their different affinity with the stationary phase and consequently leaving the column at different times (retention time): components with lower affinity come out first, while those with higher affinity have a greater retention time. The flame ionization detector (FID) burns the sample as it comes off the column; in fact the gas from the column is mixed with hydrogen and air and passing through a flame is subjected to a combustion reaction. This creates ions that produce an electrical current representing the signal provided by the FID. Several factors

affect separation efficiency in GC, including column temperature and carrier gas flow rate. The main parameters, used in the oil hydrocarbons analysis, are reported in the following table (Table 6).

Gas chromatograph parameters

	Injection volume	1 µl
Inlet	Temperature	270 °C
	Pressure	19 psi
	Gas flow rate	51.7 mL/min
Column	Initial pressure	19 psi
Detector	Temperature	320 °C
	H_2 flow rate	30.0 mL/min
	Air flow rate	300 mL/min
	Make up flow	N_2

Table 6 Gas chromatograph parameters for the analysis of *n*-alkanes.

The gas chromatograph provides a graph said chromatogram (Figure 35), which reports on the x-axis the retention time (in minutes), and on the y-axix the electrical signal (in mV) given by the detector. This graph is characterized by several peaks, which indicate the compounds present in the mixture; in this specific study the *n*-alkanes C_{10}-C_{40}.

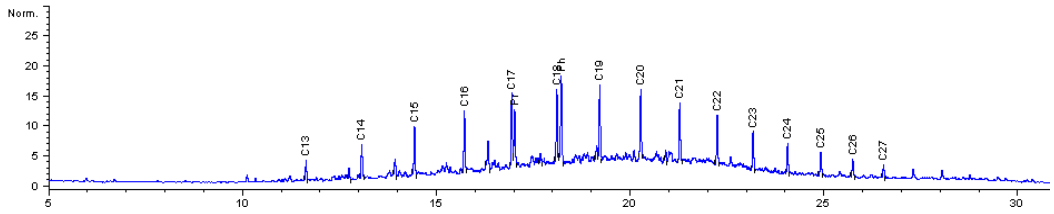

Figure 35 Example of a chromatogram: *n*-alkanes (C_{13}-C_{27}), pristane (Pr) and phytane (Ph)

The chromatogram allows an immediate qualitative analysis by identifying the components present in the mixture, given the correspondence of their retention times with those of a standard mixture C_{10}-C_{40}.

Furthermore, it is possible to obtain a quantitative analysis through a calibration curve by considering the area of the individual peak proportional to the concentration of each *n*-alkane. A set of standard solutions of known concentration is prepared and GC-analyzed producing a series of measurements. The calibration curve is a graph where concentrations are plotted along the x-axis and the detected areas of each GC peak are plotted along the y-axis. The plot of instrument response (area) versus concentration shows a linear relationship. The data (concentrations of the analyte and the area for each standard) can be fit to a straight line, using linear regression analysis. This yields a model described by the equation:

$$A = mC + q,$$

where A = area of the chromatographic peak;
C = concentration;
m = slope (also called the regression coefficient);
q = intercept.

The peak area of the unknown sample is measured and using the calibration curve, it is interpolated to find the concentration. The analyte concentration (C) of unknown samples may be calculated from this equation.
A standard mixture of *n*-alkanes from C_{10} to C_{40} and a mixture containing *n*-C17, *n*-C18, pristane and phytane were used to obtain 6-point calibration curves (0.125, 0.25, 0.5, 1, 2.5 e 5 mg/L). Calibration curves were verified constantly.
Figure 36 shows the calibration curve of C_{10}, as example.

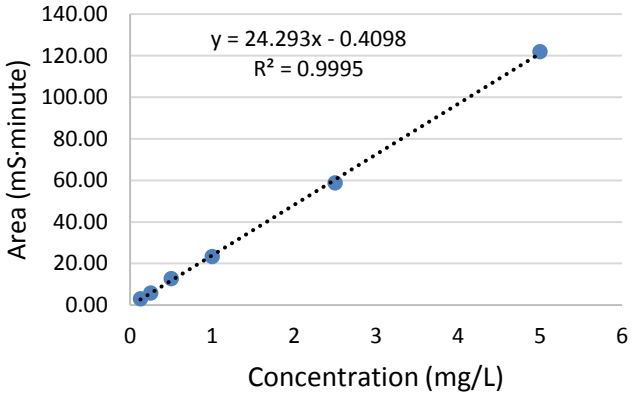

Figure 36 Calibration curve of C_{10}

Attention was placed on pristane (Pr) and phytane (Ph), two aliphatic hydrocarbons having 19 and 20 carbon atoms, respectively. Based on their retention times, the pristane comes off the chromatography column immediately after the C_{17}, while the phytane immediately after the C_{18}. These two molecules are recalcitrant to degradation under anaerobic conditions, so their concentration should remain almost constant over time. The concentration of pristane and phytane was used for the normalization of the results in anaerobic conditions (Nikolopoulou and Kalogerakis, 2009). Conversely, because under aerobic conditions these two compounds are more easily biodegraded, the normalization of the results for the aerobic sets was made using another more recalcitrant compound, the decamethyl anthracene (DMA), which was amended during the microcosms sets up. A standard sample of DMA was used to obtain 5-point calibration curve (1, 2.5, 5, 10, 20 mg/L).

For each sample, after the identification of the peaks of the *n*-alkanes (from C_{10} to C_{40}), Pr and Ph (for anaerobic set) and DMA (for aerobic sets), the corresponding areas were detected in order to calculate the concentrations (mg/L) through the calibration curve,

$$C = \frac{A-q}{m}.$$

The *n*-alkanes concentrations (g $_{n\text{-alkanes}}$/kg $_{\text{sediment}}$) were obtained using the following formula:

$$C\left(\frac{g}{kg}\right) = C\left(\frac{g}{L}\right) \cdot V_{solvent}(L) \cdot \frac{1}{DW\ (kg)}$$

where, $V_{solvent}$= volume of solvent hexane:DCM used during the extraction (L);
DW = dry weight of the sediment (kg),
C (g/L) = hydrocarbon concentration (g/L) obtained from the calibration curve.

The concentration g $_{n\text{-alkanes}}$/kg $_{\text{sediment}}$ was normalized using the concentration of Pr and Ph (for anaerobic test) and DMA (for aerobic sets) with the formula:

$$C_{norm} = \frac{C}{Pr+Ph} \cdot A(Pr, Ph) \text{ and } C_{norm} = \frac{C}{DMA} \cdot A(DMA)\ ,$$

where C = concentration of *n*-alkanes (g/kg$_{\text{sediment}}$);
Pr = concentration of pristane (g/kg$_{\text{sediment}}$);
Ph = concentration of phytane (g/kg$_{\text{sediment}}$);
A (Pr, Ph) = average concentration of Pr and Ph obtained from the triplicate sterile microcosms in the first sampling (g/kg$_{\text{sediment}}$);
DMA = concentration of DMA (g/kg$_{\text{sediment}}$);
A (DMA) = average concentration of DMA obtained from the triplicate sterile microcosms in the first sampling (g/kg$_{\text{sediment}}$).

For each sample, the normalized concentrations of each *n*-alkane (C_{10}-C_{40}) were added, obtaining the total concentration of *n*-alkanes, normalized to Pr and Ph (anaerobic set) or DMA (aerobic sets).
Moreover, for each microcosm were calculated the C/C_0 ratio, where C is the normalized concentrations at a determined sampling time and C_0 the concentration at the first sampling (time zero) or in the sterile un-amended control. For the different experimental conditions, it was so possible to evaluate the changes of the hydrocarbons concentration (C/C_0) over time. All the data presented are the mean of replicate microcosms (± standard deviation).

3.2.3.2 Biogas production and composition

The production and composition of biogas were monitored before sampling the slurry from the microcosms. Microorganisms, in fact, could produce biogas, mainly composed of methane and carbon dioxide, as results of anaerobic respiration. The production of biogas could indicate that methanogens indigenous bacteria are present in sediment and they may be responsible for the biodegradation processes of oil hydrocarbons.

The volume of produced biogas was measured with an airtight glass syringe. Since a possible production of biogas leads to an increase of pressure in the headspace of the microcosms, the gas tends to fill the syringe to bring the headspace to atmospheric pressure, and thus the volume of the biogas in the syringe could be measured.

Instead, the composition of biogas was analyzed using a micro gas chromatograph A3000 (Agilent Technologies, Figure 37) equipped with a thermal conductivity detector (Scoma et al. 2011).

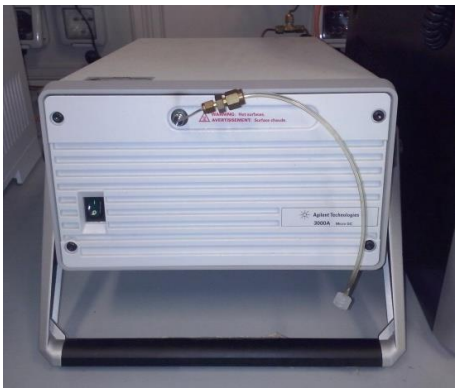

Figure 37 Micro gas chromatograph A3000 (Agilent Technologies).

3.2.3.3 pH and Redox potential

The pH and the redox potential were measured with a pH/mV/°C/F meter (model 700, Eutech Instruments). The electrodes were calibrated using appropriate solutions and inserted in microcosms the time required for the stabilization of the final value.

3.2.3.4 Concentration of dissolved oxygen

Dissolved oxygen was measured with an oxygen-meter, OXI 45 P (Crison Instruments, S.A.) during the sampling and before the addition of OXYGEL (Figure 38).

Figure 38 Oxygen-meter OXI 45 P (Crison Instruments, S.A.)

3.2.3.5 Analysis of sulfates

The concentration of sulfate ions, SO_4^{2-}, over time is evaluated to verify the possible presence in the slurry microcosms of sulfate reducing indigenous bacteria that might be responsible for the biodegradation. These microorganisms could use the hydrocarbon molecules present in the sediment as a carbon and energy source, reducing sulfates to sulfides as products of their metabolism. Consequently, a decrease over time of the concentration of SO_4^{2-} ions could suggest that sulfate reducing bacteria can be directly involved in the biodegradation processes.

Quali-quantitative analysis of sulfates in the microcosms water phase was performed with a Dionex DX-100 ion-exchange chromatograph equipped with a conductivity detector (Figure 39) (Zanaroli et al. 2006). This instrument allows to determine the

concentration of several anions in the solutions and works according to the principle of ion exchange chromatography.

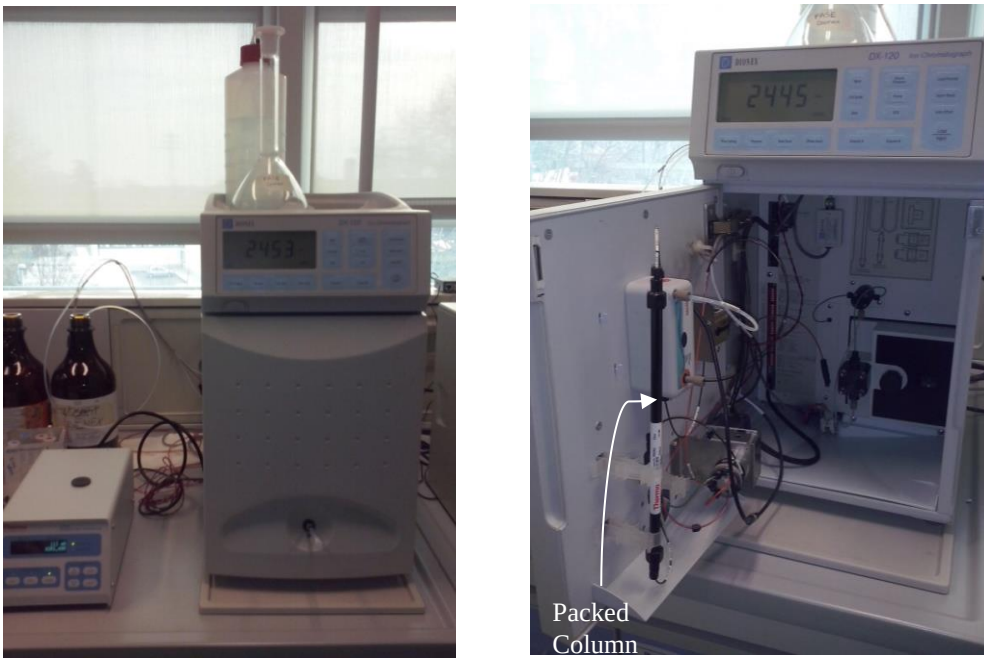

Figure 39 Dionex DX-100 Ion-exchange chromatograph

In ion chromatography, IC, the ions present in a solution are separated based on the different affinities with the stationary phase, which is contained in the packed column and is constituted by macromolecules with ionized active sites initially in equilibrium with the counterions present in the stationary phase. During the analysis, the sample is dissolved in a fluid called mobile phase, which carries it through the stationary phase of the column and activates a competitive mechanism in which the new counterions tend to replace those present in the stationary phase. A solution consisting of 8.0 mM Na_2CO_3 and 1.0 mM $NaHCO_3$ was prepared as mobile phase.

The separation of the anions present in the sample occurs according to their different affinities for the positively ionized sites of the stationary phase. The time in which the anions come from the chromatographic column is greater with increasing their affinity; therefore, the different anions come from the column separately and after characteristic times, said retention time, which is specific for each anion.

The response of the instrument is a graph, the chromatogram, which has on the x-axis the retention time of the different ions, in minutes, and y-axis the electric conductance detected by the instrument, in µS.

The chromatogram is constituted by a series of peaks that allow an immediate qualitative analysis of the anions present in the sample on the basis of their retention time, while for the quantitative analysis must be considered the areas of individual peaks, that are proportional to the concentrations of the anions in the sample. The quantitative analysis is carried out through a calibration curve, which is obtained by analyzing different solutions of known concentration. The values of concentration and area thus obtained are reported in a new graph which has in the x-axis the

concentration and in the y-axis the area. The determined points are interpolated, in order to obtain the equation of the calibration curve:

$$A = mC + q$$

where A = area of the chromatographic peak;
C = sulfates concentration;
m = slope (also called the regression coefficient);
q = intercept.

The sulfates concentration (C) of unknown samples may be calculated from this equation. For the calibration were prepared and analyzed seven standard solutions at the final concentration of 2, 5, 10, 20, 50, 70, 100 mg/L of sulfates; the results are shown in Table 7.

Sulfate Concentration mg/L	**Sulfates Area µS·minute**
2.00	0.07
5.00	0.33
10.00	0.75
20.00	1.82
50.00	3.6
70.00	5.62
100.00	7.10

Table 7 Concentration of sulfates standard solutions (mg/L) and the respective obtained area (µS·minute).

The pairs of values (concentration, area) were reported in the graph of Figure 40 and the equation of the calibration curve was calculated.

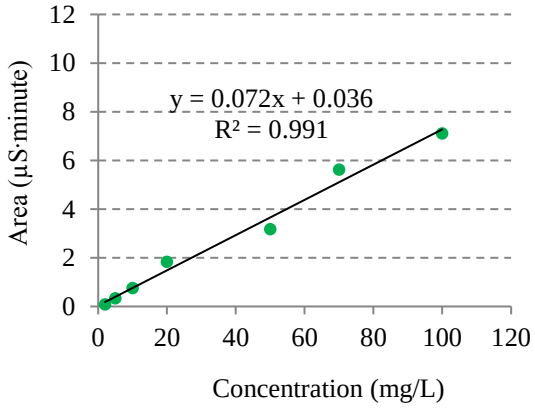

Figure 40 Calibration curve of the sulfates.

After centrifugation of the slurry at 5000 rpm for 10 minutes, the water was recovered, filtered and analyzed through ion-exchange chromatograph. For each sample, the analisys has provided the area of the peak relative to sulfates; thus, solving the

equation of the calibration curve, the concentration, in mg/L, was calculated. The sulfate reduction was conducted only for the Gela anaerobic microcosms.

3.2.4 Analysis of the microbial communities

The indigenous microbial communities of Gela anaerobic microcosms were characterized using the PCR-DGGE (Polymerase Chain Reaction-Denaturing Gradient Gel Electrophoresis) analysis of the genes coding for 16S ribosomal RNA. The molecular analysis was performed at the beginning and at the end of incubation, in order to investigate possible changes of the bacterial communities over time.

3.2.4.1 DNA extraction

Metagenomic DNA was extracted from each replicate with the UltraClean Soil DNA kit (MoBio Laboratories, Carlsbad, CA, USA) from approximately 250 mg of sludge pellet.
The extraction result of metagenomic DNA was analyzed by electrophoresis on a 1% agarose gel. The samples obtained were stored at -20 ° C for the PCR.

3.2.4.2 Polymerase Chain Reaction (PCR)

Total DNA is amplified using Polymerase Chain Reaction (PCR) technique of the 16S rRNA gene target, whose sequence is used in microbial ecology to characterized different microbes. The V3-V5 variable regions of the bacterial 16S rRNA gene were PCR amplified with primers GC-357f and 907r.
The PCR is a method very used in molecular biology to amplify specific fragments of nucleic acids of which are known the initial and final nucleotide sequences. Developed in 1983 by K. B. Mullis, thanks to which he was awarded the Nobel Prize for Chemistry, PCR is now used in many applications because of its flexibility, rapidity and reproducibility Mullis, K.B. (1994). The PCR generates thousands to millions of copies of a particular DNA sequence, which is very important in medical and biological research laboratories, for different applications, including DNA cloning for sequencing, functional analysis of genes and diagnosis of inherited and infectious diseases.
The sequence is selected and amplified by the replication of a cycle consisting of three steps: *denaturation*, *annealing* and *elongation*. *Denaturation* is the opening of the double helix of DNA, which separates the two strands, so that the complementary copy can be created. The following *annealing* process consists in the hybridization to the single-stranded DNA template of the two primers, which are short sequences of DNA that, for complementarity, identify the initial and final portion of the nucleotide sequences to be amplified, and bind to them. At this point, thanks to the action of the DNA polymerase, may start the phase of *elongation* that consists in the addition, starting from the primer, of the nucleotides complementary to the DNA template strand in 5' to 3' direction, in order to synthesize a new copy of the original DNA strand. Two identical DNA sequences are obtained from a single DNA template.

Completed the first duplication, the cycle is repeated several times, resulting in an exponential increase of the DNA fragments.

The PCR sample is the metagenomic DNA mixed in a solution, Master Mix, which contains all the components required for the amplification process.
In particular, the Master Mix is constituted by:

- deionized water;
- buffer, containing mainly TE (Tris-EDTA), important for the DNA stabilization;
- magnesium (1.5 mM), an essential cofactor for the DNA polymerase;
- two primers, GC-357 forward (0.4 µM) and 907 reverse (0.4 µM), which identify the initial and the final nucleotide sequence of the DNA fragment to be amplified, respectively. The GC-357 forward primer contains several nitrogenous bases guanine (G) and cytosine (C) allowing to create fragments with the GC-clamp that, containing a high amount of G and C, prevents the complete denaturation of the fragments during the DGGE analysis.
- nucleotide solution (200 µM), which provides the nucleotides for the creation of new strands;
- Taq polymerase (0.02 u/µL). The DNA polymerase is the enzyme that synthesizes in nature the new DNA strand from a template; in the PCR, it is necessary to use a polymerase of thermophilic microorganisms, such as Thermus aquaticus, since it is able to resist the high temperatures required for denaturation.

During PCR analysis, the sample is subjected to the specific temperatures required for the denaturation, annealing and elongation. The PCR thermocycler used in this study was Biometra® Tgradient (Figure 41) and the PCR program is described in Table 8:

PCR PROGRAM

1.	Initial Denaturation Step		95 °C	5 minutes
2.	30 cycles	Denaturation	95 °C	30 seconds
		Annealing	55 °C	30 seconds
		Elongation	72 °C	1 minute
3.	Finale Elongation Step		72 °C	10 minutes

Table 8 PCR program for the amplification of 16S rRNA gene target.

Figure 41 Biometra® Tgradient thermocycler.

The PCR products were analyzed by electrophoresis on 1.2% agarose gel to verify the amplification of the fragment, using as molecular weight marker the 100 bp DNA ladder (SibEnzyme®). The gel was stained by Atlas ClearSight DNA Strain (BIOATLAS) and its image, captured in a trans UV illuminator, Chemidoc (BIORAD), showed a band for each sample containing the amplified DNA fragment (Figure 42).

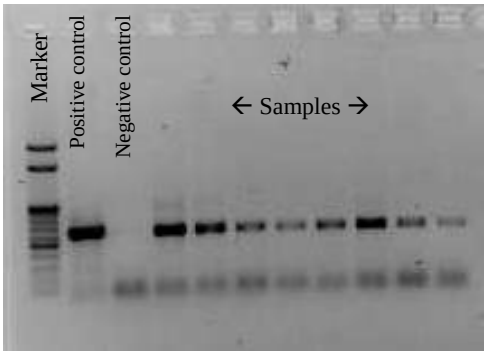

Figure 42 PCR products after gel electrophoresis. The gel also shows a positive and negative control, and a DNA ladder containing DNA fragments of defined length for sizing the bands.

3.2.4.3 Denaturing Gradient Gel Electrophoresis (DGGE)

Denaturing gradient gel electrophoresis (DGGE), developed by L. Lerman, is a molecular fingerprinting method used to identify and characterize the microbial communities present in the environmental samples.

DGGE consists of an electrophoretic run on a polyacrylamide gel that allows separation of PCR-generated DNA products of equal length depending on their sequence, thanks to a gradient of DNA denaturants and the temperature. This technique separates the amplified DNA products belonging to different microbial species, which are denatured to different temperatures (melting temperatures) and concentrations of denaturant. The 16S rRNA gene, in fact, is characteristic for each microbial species and the DGGE allows to separate sequences that differ by only one nucleotide.

Therefore, DGGE provides a profile of microbial communities, where each DNA fragment appears as a band and represents a bacterial species and the number of bands in a lane represents the microbial diversity in the sample (number of different species), while their relative intensity is correlated with their abundance.
Comparing different microbial communities:

- a band in the same horizontal position in different samples on a DGGE gel represents the same bacterial species shared amongst different microbial communities;
- a band presents only in a single lane is a species unique of that microbial community.

In this study, the amplicons from replicate microcosms were combined together and resolved with a D-Code Universal Mutation Detection System (Bio-Rad, Milan, Italy) (Figure 43) on a 7% (w/v) polyacrylamide gel (acrylamide-N,N'-methylenebisacrylamide, 37:1) containing a denaturing gradient from 40% (top) to 60% (bottom) denaturant (100% denaturant: 7M urea, 40% v/v formamide). The electrophoresis was run at 55 V for 16 hours at 60°C. After the run, the gel was immersed for 20 minutes, in the dark, in 250 mL of TAE (Tris-acetate-EDTA) buffer containing the dye Syber Green for the next acquisition phase with the Chemidoc system (BIORAD).

Figure 43 DCode™ Universal Mutation Detection System (Bio-Rad)

3.2.4.4 Clonal library

To characterize the composition of selected microbial communities, metagenomic libraries of the 16S rRNA genes were constructed. The production of metagenomic libraries by direct cloning the environmental DNA has a great potential for sequencing applications (Knietsch et al., 2003).
The 16S rRNA genes were PCR amplified from the metagenome with the primer pair 27f/1525r and the amplicons were cloned in pCR®4-TOPO® cloning vector and inserted in chemically competent One Shot® TOP10 *E. coli* cells (Invitrogen, Paisley, UK).
Each transformation sample were spread on pre-warmed LB agar plates containing 100 μg/mL ampicillin, in order to perform a rapid chemical transformation procedure. Plasmids, in fact, having the ampicillin resistance gene, confer resistance to this specific antibiotic to the bacteria carrying them. The presence of an antibiotic

resistance gene on plasmids allows to easily isolate in the presence of the antibiotic the bacteria containing the plasmid from the bacteria that do not contain it. Luria broth (LB) is a nutrient-rich media, consisting of tryptone (10 g/L), yeast extract (5 g/L) and NaCl (10 g/L), commonly used to grow culture bacteria in the laboratory. LB agar plates are frequently used to isolate individual colonies of bacteria carrying a specific plasmid. The plates were incubate at 37°C overnight. Single colonies from LB agar plate were selected using sterile pipette tips that were subsequentely dropped into LB liquid containing ampicillin (100 μg/mL). The bacterial cultures in LB liquid were incubate at 37°C for 16 hours in a shaking incubator (150 rpm) to allow their growth. PureLink® Quick Plasmid Miniprep Kit (Invitrogen, Paisley, UK) was used to isolate high quality plasmid DNA from *E. coli* cells. The purified DNA plasmids were stored in aliquots at −20°C for the following analysis (PCR and DNA sequencing). The 16s rRNA genes were PCR amplified from the purified plasmids with primers GC-357f and 907r and the library screening was performed by DGGE analysis as described above. Amplicons obtained from the metagenome were loaded on DGGE gels together with amplicons obtained from clones in order to assign each clone to a DGGE band of the community during the screening. Full inserts of clones giving a unique band in DGGE gels were PCR-amplified with primers 27f and 1525r and amplicons sequenced with primers T3 and T7 (BMR Genomics, Padova, Italy). Sequences were aligned to the Ribosomal Database Project (RDP, release 11) sequence database and closest relatives retrieved with the Seqmatch tool (Valentino et al. 2015).

3.3 Oil hydrocarbons bioavailability tests

3.3.1 Polydimethylsiloxane (PDMS) fibers

The evaluation of hydrocarbons bioavailability was monitored via the measurement of *n*-alkanes concentration in the porewater through passive sampling with polydimethylsiloxane (PDMS) fibers (Figure 44). The outer diameter of the PDMS is 558.8 µm and the inner diameter 486 µm (i.e., the thickness annulus of PDMS is 35.4 µm and the fiber volume is 0.597 µL/cm).

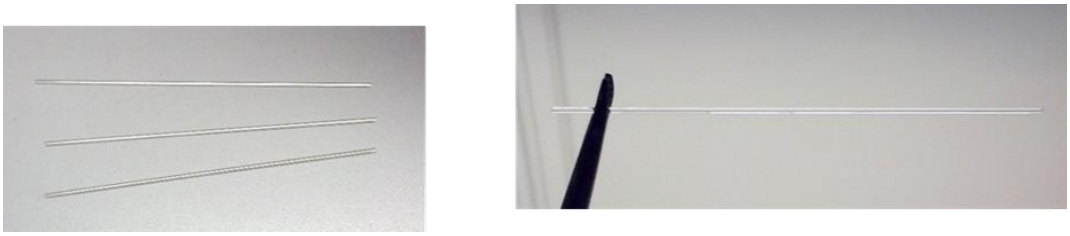

Figure 44 Polydimethylsiloxane (PDMS) fibers.

Before their use, the fibers were cut in 5 cm length and subsequently washed in hexane for 4 hours, then acetonitrile for other 4 hours and three times in water for 15 minutes, under mixing (150 rpm). The washing step of the fibers is necessary to eliminate anything that could interfere with the hydrocarbons adsorption and extraction and the reliability of the results.

3.3.2 Gela microcosms set up

The tests were conducted, in triplicate, in 120 mL slurry microcosms containing 80 mL of Gela sediment suspended in marine water (20% dry weight/volume) under sterile condition. The Gela sediment was contaminated in the laboratory immediately before the microcosms set up with a Dansk Blend crude oil to the final concentration of 5 g/kg sediment. The microcosms were amended with the same surfactants previous tested during the hydrocarbons biodegradation experiments: two types of cyclodextrins, namely randomly methylated β-cyclodextrins (RAMEB-CD) and hydroxylpropyl-β-cyclodextrins (HPB-CD), two microbial surfactants (rhamnolipids and sophorolipids), two commercial soy lecithin products (Solec C and Textrol F) and bile acids. All surfactants were added at the final concentration of 1 g/L, except the rhamnolipids and sophorolipids that were provided at 0.2 g/L given their low critical micelle concentrations (0.1-0.2 g/L). In addition, not amended controls were also set up (Table 9).

Polydimethylsiloxane (PDMS) fibers (5 cm of length) were incubated in the microcosms at 20 °C, 150 rpm, and replaced every 20 days to sample water-dissolved hydrocarbons. Bioavailability of hydrocarbons was measured after 20, 40 and 60 days of incubation.

Gela anaerobic microcosms *Dansk Blend crude oil, 5 g/kg sed.* *Triplicate conditions* *N_2:CO_2 (70:30) headspace gas*
Sterile un-amended control
Biotic un-amended control
+ Rhamnolipids (1 g/kg sed.)
+ Sophorolipids (1 g/kg sed.)
+ RAMEB-CD (5 g/kg sed.)
+ HPB-CD (5 g/kg sed.)
+ Solec C soy lecithin (5 g/kg sed.)
+ Textrol F soy lecithin (5 g/kg sed.)
+ Bile acids (5 g/kg sed.)

Table 9 Gela anaerobic microcosms: different tested conditions.

3.3.3 Analysis of *n*-alkanes porewater concentration

Hydrocarbons bioavailability was evaluated by measuring *n*-alkanes concentration in the porewater through passive sampling with polydimethylsiloxane fibers.
Every 20 days, PDMS fibers were removed from the microcosms, rinsed with water to remove any residual matter and cut into 1 cm segment. The cut fibers were placed into auto sampling GC vial containing an insert previously filled with 100 µl of hexane. After elution into the organic solvent for 16 h, PDMS fibers were removed from the insert and *n*-alkanes in the solvent were analyzed with an Agilent Technologies gas-chromatograph 6890N equipped with flame ionization detector (GC-FID) as describe above. The measured concentration of *n*-alkanes in the solvent was converted to total mass and divided by PDMS volume in the 5 cm fibers in order to determine the *n*-alkanes concentration on fibers, which was divided by the PDMS-water partition coefficient:

$$C_{pw} = \frac{C_a \cdot V_{solvent}}{V_{PDMS} \cdot K_{PDMS-W}},$$

where C_{pw} = *n*-alkanes porewater concentration (ng/L);
$V_{solvent}$ = volume of hexane used for *n*-alkanes extraction from 5 cm fiber (L);
C_a = *n*-alkanes concentration (ng/L);
V_{PDMS} = volume of 5 cm glass, cylindrical PDMS-fiber (L);
K_{PDMS-W} = fiber (PDMS)-water partition coefficient.

The fiber-water coefficient (K_{PDMS-W}) relates the concentration of *n*-alkanes in the fibers (C_{PDMS}) to the concentration in the porewater (C_{pw}):

$$K_{PDMS-W} = \frac{C_{PDMS}}{C_{pw}}$$

K_{PDMS-W} which is correlated with the octanol–water partition coefficient (K_{ow}) is given by the following equation (Thomas et al., 2014)

$$logK_{PDMS-W} = 0.725\ logK_{ow} + 0.479 \quad (R^2 = 0.99)$$

where K_{OW} is given by SPARC estimates (Table 10).

***n*-alkanes**	**logK_{OW}**	**logK_{PDMS-W}**
C10	5.25	4.285
C11	5.74	4.641
C12	6.23	4.996
C13	6.73	5.358
C14	7.22	5.714
C15	7.71	6.069
C16	8.20	6.424
C17	8.69	6.779
Pr	9.38	7.280
C18	9.18	7.135
Ph	9.87	7.635
C19	9.67	7.490
C20	10.16	7.845
C21	10.65	8.200
C22	11.15	8.563
C23	11.64	8.918
C24	12.13	9.273
C25	12.62	9.629
C26	13.11	9.984
C27	13.60	10.339
C28	14.09	10.694
C29	14.58	11.050
C30	15.07	11.405
C31	15.56	11.760
C32	16.05	12.115
C33	16.54	12.471
C34	17.03	12.826
C35	17.52	13.181
C36	18.01	13.536

Table 10 logK_{OW} and logk_{PDMS-W} of the different *n*-alkanes (C_{10}-C_{36}) (archem.us).

3.4 Encapsulation of surfactants

Sophorolipids and hydroxylpropyl-β-cyclodextrins (HPB-CD) were chosen for the surfactants encapsulation based on previous experimental results obtained in the hydrocarbons biodegradation and bioavailability tests.

Two different approaches were carried out:

i. encapsulation in biodegradable organic polymers using the double emulsion method;
ii. encapsulation in hydrogel made of agar.

3.4.1 Encapsulation in biodegradable organic polymers

Encapsulation of surfactants in biodegradable organic polymers was made in collaboration with Prof. P. Fabbri, Prof. L. Sisti, Prof. A. Celli via double emulsion method (DEM).
Different polymers (Figure 45) were selected and compared in terms of surfactant encapsulation efficiency (Brunner et al., 2011):

- polybutylene succinate (PBS);
- polybutylene succinate-co-adipate (PBC-coA);
- polycaprolactone (PCL);
- polylactic acid (PLA).

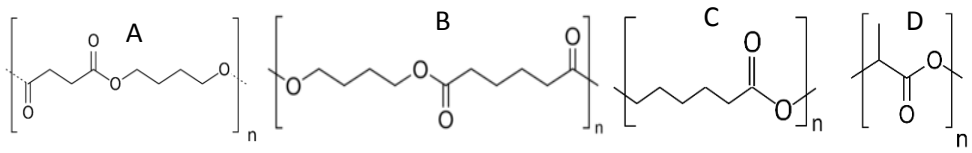

Figure 45 Chemical structures of the polymers used for surfactants encapsulation procedure via DEM. A: polybutylene succinate (PBS); B: polybutylene succinate-co-adipate (PBC-coA); C: polycaprolactone (PCL); D: polylactic acid (PLA).

In the double emulsion method (Figure 46Figure 46), the aqueous biosurfactant solution (S2) was emulsified with the polymer organic solution (S1), containing glycerol as water-soluble plasticizer at high mixing rates (13000 rpm) obtained through the Ultra-Turrax® T18 (IKA) (Figure 47).
The emulsion was then dropped in a water solution (S3) of a polyvinyl alcohol (0.1 weight/volume), an anticoagulant stabilizer under vigorous mixing (6000 rpm). The obtained microspheres were washed in 0.5 L of demineralized water, recovered by filtration and dried.

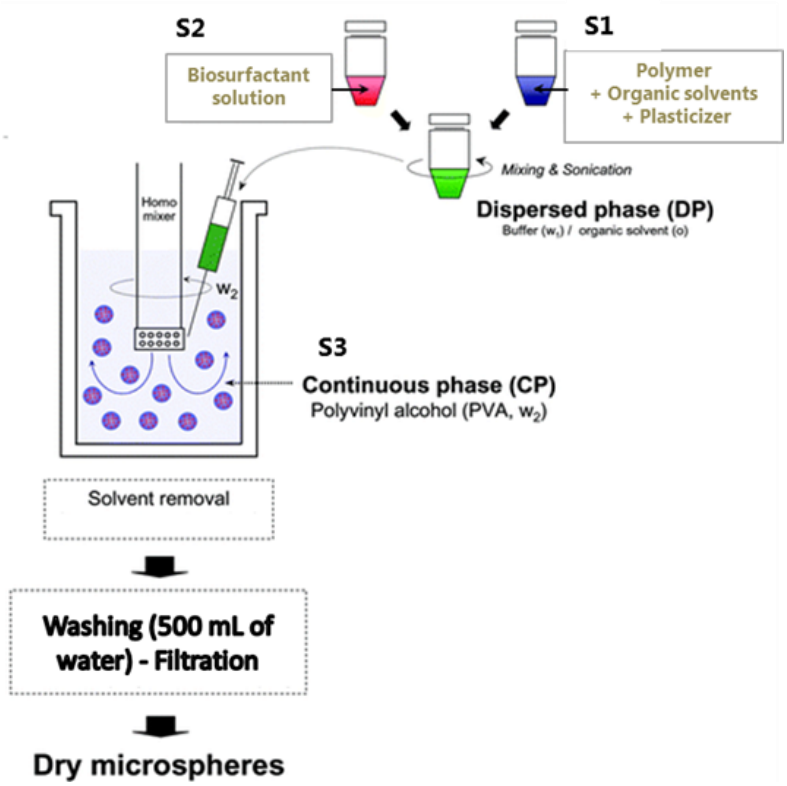

Figure 46 Double Emulsion Method (DEM): schematic representation.

Figure 47 Ultra-Turrax® T18 (IKA).

The solutions (S1, S2, S3) used for the preparation of microcapsules by DEM are shown in the diagram of Figure 48.

Solution 1 (S1)	Solution 2 (S2)	Solution 3 (S3)
•Chloroform ($CHCl_3$): 20 mL •Polymer: 2.0 g •Plasticizer (glycerol): 0.4 g	•Biosurfactant solution: 1 mL - HPB-CD (200 g/L) - Sophorolipids (30 g/L)	•polyvinyl alcohol (PVOH) 0,1 g •H_2O 100 ml

Figure 48 Solutions used for the preparation of microcapsules by DEM

The encapsulation of HPB-CD via DEM were tested in PBS, PLA, PBS-coA and PCL, while sophorolipids only in PBS. For HPB-CD encapsulated in PLA were used different concentrations of glycerol as plasticizer: 0.2, 0.4 and 0.8 g in order to increase the stability of the microspheres (Table 11).

SURFACTANTS	POLYMERS
Sophorolipids	PBS
HPB-CD	PBS PLA (0.2 g glycerol) PLA (0.4 g glycerol) PLA (0.8 g glycerol) PBS-coA PCL

Table 11 Conditions (surfactant, polymers and glycerol concentration) tested for the preparation of microcapsules via DEM.

3.4.1.1 Encapsulation efficiency in polymers

In order to assess the encapsulation efficiency, the dried polymer microspheres obtained via DEM were subjected to disruption and the surfactants concentrations were analyzed through high-performance liquid chromatography (HPLC).

The HPLC is a chromatographic technique that allows to separate, identify and quantify each component present in a mixture due to their different affinities between the "stationary phase", a solid adsorbent material placed inside the chromatographic column and the "mobile phase", a liquid solvent that flows through it. It relies on pumps to pass a pressurized liquid solvent containing the sample through the stationary phase. Pressures of hundreds of atmospheres are applied. Each component of the sample interacts differently with the adsorbent material of the stationary phase, causing different flow rates for the different components that flow out the column at different time, said retention time. Finally, the sample passes to the detector, which provides a signal directly proportional to the concentration of the compound present in the mixture, i.e. the chromatographic peak.

The encapsulation efficiency in the polymers was evaluated using two different approaches for the two surfactants, sophorolipids and HPB-CD.

A known amount of sophorolipids microspheres (0.1-0.2 g) were suspended in water and subjected to an acid hydrolysis of both polymers and sophorolipids (protocol kindly provided by Dr. Fabrizio Beltrametti and Dr. Adriana Bava, ACTYGEA S.r.l., Gerenzano, Italy). The concentrations of the microbial surfactants were then indirectly estimated via HPLC-IR analysis (Agilent Technologies 1260 Infinity) of the released glucose. The characteristics of the instrument, the set parameters and the composition of the mobile phase used for the HPLC analysis of glucose are reported in Table 12 (Rebecchi et al., 2016).

Agilent Technologies 1260 Infinity	
Column	Hi-Plex H column (8 pm, 300 x 7.7 mm, Agilent)
Mobile Phase	sulfuric acid, 5 mM
Injection volume	0.5 µl
Flow rate	0.6 mL/min
Column temperature	65 °C
IR detector temperature	40 °C

Table 12 Analytical conditions for the glucose analysis with HPLC-IR.

Figure 49 Agilent Technologies 1260 Infinity

The calibration was performed by means of standard solutions at known concentration of glucose (range 0.1-5 g/L) in order to associate a determined concentration to a peak area. In the Table 13 are indicated the concentrations of the standard solutions used for the glucose calibration and the relative areas revealed by the detector. The calibration curve of glucose, which shows the chromatographic areas as a function of the relative concentrations of the standard solutions are represented in the Figure 50.

Glucose concentration (g/L)	Peak Area
0.1	8886.8
0.25	17851.9
0.5	32251.7
1	71023.6
2.5	185829.5
5	359182.2

Table 13 Table: Concentrations of glucose standard solutions (g/L) and the corrisponding areas of the peaks.

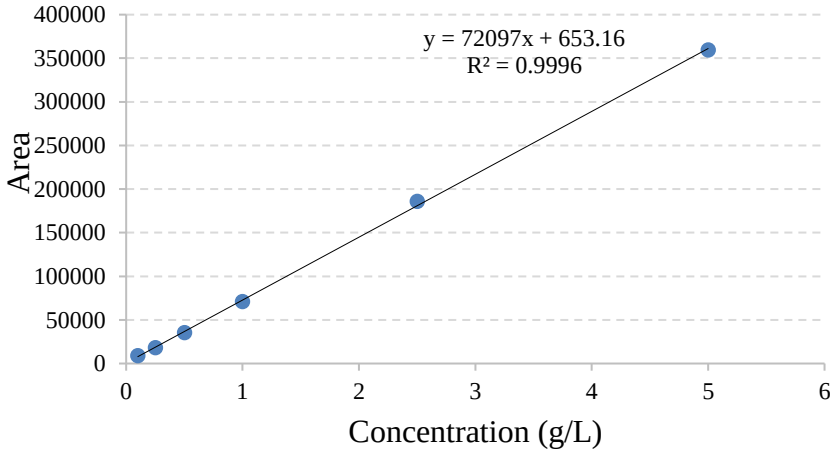

Figure 50 Calibration curve of glucose

The acid hydrolysis can not be used for the cyclodextrins because it would results in their chemical destruction. Therefore, the HPB-CD microspheres (1 g) were dissolved into chloroform, the same organic solvent used during their preparation via DME (2 mL), and after liquid-liquid extraction with water, the aqueous phase (containing the HPB-CD) was recovered and analyzed through HPLC. Table 14 shows the instrument characteristics and the main set parameters used for the HPLC analysis of cyclodextrins (Wei, et al., 2014). Several standard solutions of HPB-CD was used to obtain a 7-point calibration curve (range 0.1-10 g/L), that were verified constantly (Table 15).

Agilent Technologies 1260 Infinity	
Column	Alltech™ Alltima™ HP HILIC
Mobile Phase	Acetonitrile:Water 80:20
Injection volume	20 µl
Flow rate	2 mL/min
Column temperature	35°C
IR detector temperature	35°C

Table 14 Analytical conditions for the HPB-CD analysis with HPLC-IR.

HPB-CD concentration (g/L)	Peak Area
0.1	9524.7
0.5	26695.4
1	67575.5
2.5	186107.7
5	374002.0
7.5	555160.4
10	755825.5

Table 15 Concentrations of HPB-CD standard solutions (g/L) and the corresponding areas of the peaks.

The calibration curve of the HPB-CD is shown in Figure 51.

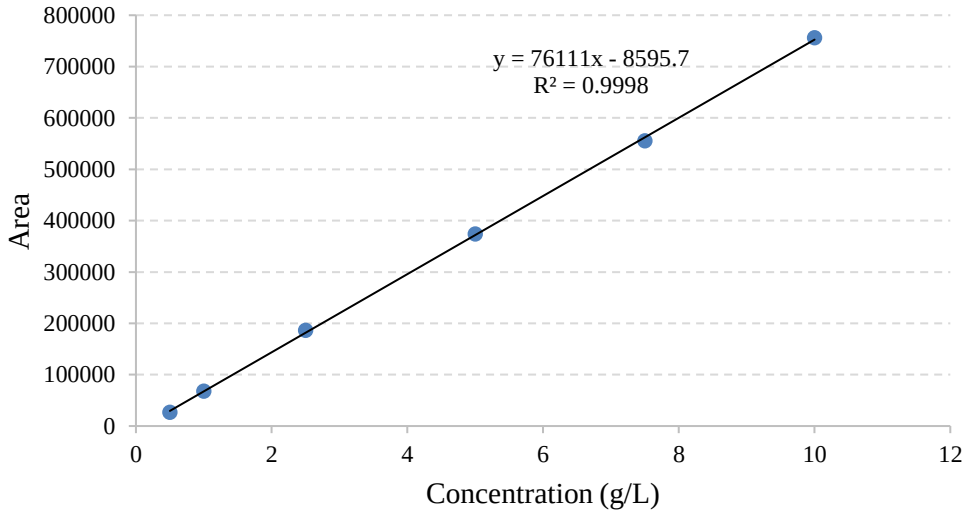

Figure 51 Calibration curve of HPB-CD

Figure 52 summarizes the different approaches used to evaluate the efficiency of encapsulation in polymers for the two surfactants (sophorolipids and HPB-CD).

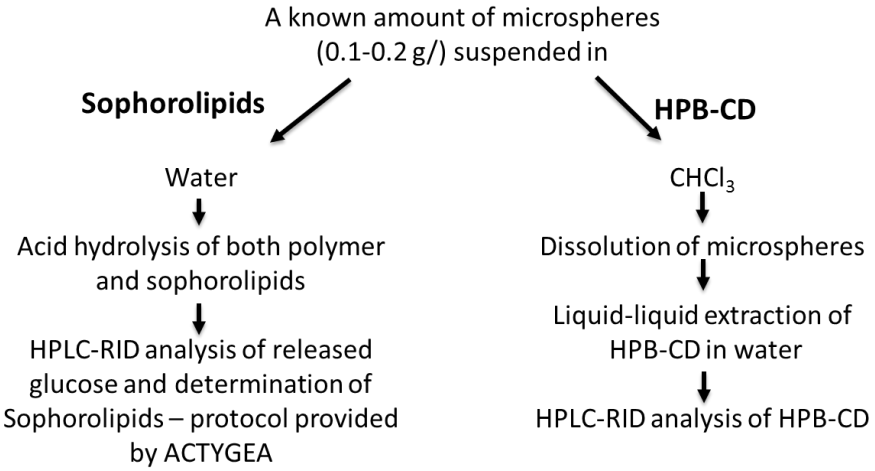

Figure 52 Different approaches used to evaluate the encapsulation efficiency in polymers for sophorolipids (on right) and HBP-CD (on left).

In order to assess the amount of surfactants that could be lost during the microspheres preparation via DEM, the water before and after the washing step was subjected to HPLC analysis as describe above.

3.4.2 Encapsulation in hydrogels

The encapsulation in hydrogels was made of polysaccharides agar extracted from marine algaes. Agar consists of a mixture of agarose and agaropectin (Figure 53). Agarose, the predominant component of agar, is a linear polymer, composed of the repeating monomeric unit of agarobiose, which is a disaccharide made up of D-galactose and 3,6-anhydro-L-galactopyranose. Agaropectin is a heterogeneous mixture of smaller molecules that occur in lesser amounts, and it is composed of alternating units of D-galactose and L-galactose highly modified with acidic side-groups, such as sulfate and pyruvate.

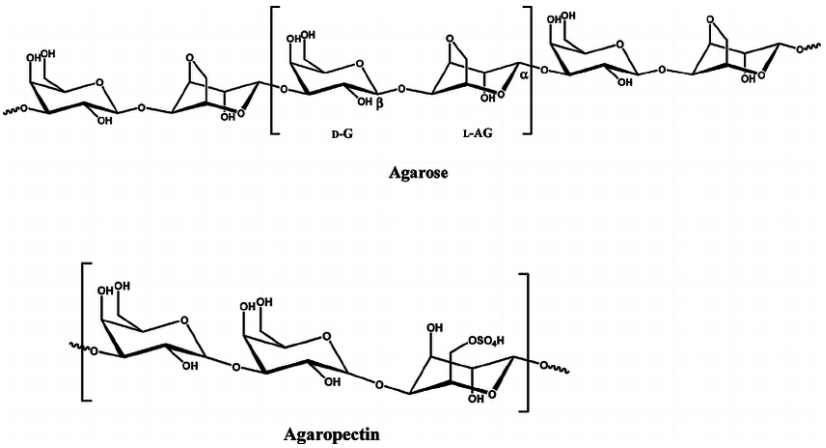

Figure 53 Chemical structure of agarose and agaropectin.

Agar capsules were prepared in multiwell plates (96 x 200 µL, Figure 54). A know concentration of surfactants (30 and 50 g/L for sophorolipids and HPB-CD, respectively) were dissolved in autoclave-sterilized agar solution at the temperature of about 50 °C; 0.2 mL of this agar-surfactants solution was introduced with a pipette into the wells of the plate. The capsules were then recovered from the multiwell plate after approximately 1 hour to ensure the polymerization of the agar capsules containing the surfactants. All operations were conducted in sterile condition. Different concentrations of agar hydrogel (5, 15 and 50 g/L) were tested.

Figure 54 Multiwell plates (96 x 200 µL) used for the encapsulation of surfactants in agar capsules.

3.4.3 Surfactants release investigation

3.4.3.1 Surfactants release rate in water

PBS-encapsulated sophorolipids and agar-encapsulated HPB-CD were compared in terms of surfactants release rate in sterile water. The tests were conducted in duplicate.

Microspheres (1 g) of PBS-encapsulated sophorolipids were incubated statically at 20 °C in 0.1 L of sterile water (Figure 55A); the maximum sophorolipids concentration in the water that correspond to the complete release of the biosurfactants is 0.27 g/L. The water was periodically sampled, subjected to acid hydrolysis and HPLC analyzed as previously described in order to evaluate the sophorolipids release over time.

Likewise, 96 capsules of agar-encapsulated HPB-CD were incubated statically at 20 °C in 0.2 L of sterile demineralized and marine water (Figure 55B). The capsules were prepared used a solution containing 200 g/L of HPB-CD and 5, 15 or 50 g/L of agar; thus the maximum concentration of cyclodextrins expected in water is 4.8 g/L. The HPB-CD concentration in the water, sampled at different time of incubation, was monitored via HPLC analysis.

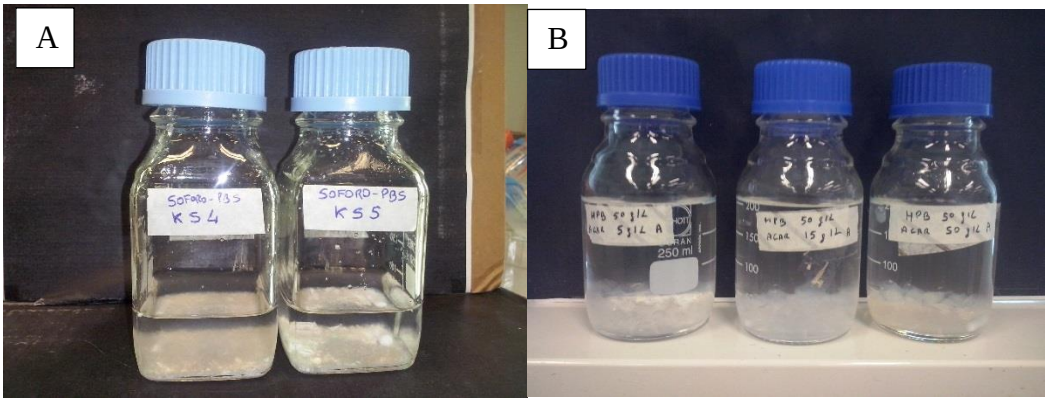

Figure 55 Sophorolipids (A) and HPB-CD (B) release tests from PBS microspheres and agar capsules, respectively.

3.4.3.2 Surfactants release rate in slurry microcosms

The release of sophorolipids from PBS microspheres and of HPB-CD from hydrogel capsules (made of agar 50 g/L) was also investigated in sterile slurry microcosms of sand contaminated with Dansk Blend crude oil (5 g/kg$_{sand}$) and suspended in synthetic marine water (20% w/v) under anaerobic condition.

PBS-encapsulated sophorolipids and agar-encapsulated HPB-CD were singly supplemented in the microcosms at the final concentration of 0.2 and 1 g/L, respectively. Synthetic marine water (2 mL) were sampled from slurry microcosms at days 28, 40 and 65 to evaluate the sophorolipids and cyclodextrins release over time. Water samples were analyzed via HPLC using the different approaches previously described for the two surfactants. The tests were conducted in triplicate.

3.5 Effect of encapsulated surfactants on *n*-alkanes bioavailability

3.5.1 Set up of "freshly spiked" and "weathered" sand slurry microcosms

Effectiveness of encapsulated surfactants in enhancing the bioavailability of *n*-alkanes was monitor in sand slurry microcosms under sterile condition. Autoclave-sterilized sand were contaminated in the laboratory with a mixture of Dansk Blend crude oil (5 g/kg_{sand}) and 1,2,3,4,5,6,7,8,9,10-decamethyl-anthracene (70 mg/kg_{sand}, as internal standard) dissolved in hexane. After the solvent evaporation the oil-contaminated sand was used to set up triplicate 80 mL slurry microcosms suspended in synthetic marine water (20% dry weight/volume) under anaerobic conditions (N_2:CO_2 70:30 headspace gas).

The effect of surfactant encapsulation on *n*-alkanes bioavailability was investigated in "freshly spiked" and "weathered" sand slurries in order to obtain data on both adsorption and desorption of contaminants under the influence of encapsulated surfactants. The evaluation of hydrocarbons bioavailability was monitored via the measurement of *n*-alkanes concentration in the porewater through passive sampling with polydimethylsiloxane (PDMS) fibers.

In freshly spiked sand slurries biosurfactants and PDMS fibers (5 cm length) were incubated immediately after contamination with crude oil, while in "weathered" spiked sand slurries surfactants and fibers (5 cm length) were incubated 40 days after contamination in order to allow the complete adsorption of the oil contaminants to the sand (Table 16).

Based on previous results, PBS-encapsulated sophorolipids and agar-encapsulated HPB-CD were chosen for the evaluation of *n*-alkanes bioavailability. After determining the amount of surfactants present in 1 gram of PBS microspheres and in each agar capsule, PBS-encapsulated sophorolipids and agar-encapsulated HPB-CD were added to the sand slurries at the final concentration of 1 g/kg_{sand} and 5 g/kg_{sand}, respectively.

To measure the amount of sophorolipids present in 1 g of PBS microspheres, a known weight of microsphere was analyzed through HPLC following the procedure previously described that involves an acid hydrolysis of the samples. Whereas, the amount of HPB-CD in each capsule was obtained by multiplying the concentration of surfactant used during the capsules preparation (50 g/L) by the volume of each capsule (0.2 mL).

Un-amended controls and microcosms spiked with not encapsulated surfactants at the same final concentration of the encapsulated ones (1 g/kg_{sand} for sophorolipids and 5 g/kg_{sand} for HPB-CD) were also set up. Both conditions (freshly spiked and weathered slurries) were incubated at 20 °C in the dark at 150 rpm.

“Freshly spiked” sand slurry microcosms *Dansk Blend crude oil, 5 g/kg sed.* ***Surfactants and PDMS fibers immediately after contamination***	**“Weathered” sand slurry microcosms** *Dansk Blend crude oil, 5 g/kg sed.* ***Surfactants and PDMS fibers 40 days after contamination***
Un-amended control	Un-amended control
+ Sophorolipids (1 g/kg_{sand}, 0.2 g/L)	+ Sophorolipids (1 g/kg_{sand}, 0.2 g/L)
+ PBS-encapsulated sophorolipids (1 g/kg_{sand}, 0.2 g/L)	+ PBS-encapsulated sophorolipids (1 g/kg_{sand}, 0.2 g/L)
+ HPB-CD (1 g/kg_{sand}, 5 g/L)	+ HPB-CD (1 g/kg_{sand}, 5 g/L)
+ Agar-encapsulated HPB-CD (1 g/kg_{sand}, 5 g/L)	+ Agar-encapsulated HPB-CD (1 g/kg_{sand}, 5 g/L)

Table 16 “Freshly spiked” and “weathered” sand slurry microcosms: conditions tested to evaluate the effect of not encapsulated (sophorolipids and HPB-CD) and encapsulated (PBS-encapsulated sophorolipids and agar-encapsulated HPB-CD) surfactants on hydrocarbons bioavailability.

Porewater concentration of *n*-alkanes in freshly spiked slurry microcosms was measured after 20, 40 and 60 days of sand crude oil contamination, while in weathered sand slurry microcosms after 60, 80 and 100 days. The PDMS fibers were replaced every 20 days to sample water-dissolved *n*-alkanes using the same approached previous described to investigate the effect of not encapsulated biosurfactants on hydrocarbons bioavailability.

The sterile sand slurry microcosms supplemented with the encapsulated surfactants (i.e. PBS-encapsulated sophorolipids and agar-encapsulated HPB-CD) were also used to investigate the release of encapsulated surfactants over time. The slurry of microcosms were periodically sampled and the water, recovered after centrifugation of the slurry sample (10 minute, 5000 rpm), were HPLC analysed to measure the concentration of sophorolipids and HPB-CD. In particular, the slurry of the sand microcosms were sampled after 28, 40 and 65 days of incubation.

3.6 Effect of encapsulated surfactants on *n*-alkanes biodegradation

The effectiveness of encapsulated surfactants in enhancing hydrocarbons biodegradation were investigated in weathered sand slurry microcosms inoculated (10% v/v) with the hydrocarbon-degrading marine microbial community previously enriched from Gela anaerobic microcosms.

3.6.1 Set up of weathered slurry microcosms

Effect of encapsulated biosurfactant on hydrocarbons biodegradation was monitored in weathered sand slurry microcosms under anaerobic conditions. Autoclave-sterilized sand were contaminated in the laboratory with Dansk Blend Crude oil (5g/kg_{sand}) through the same approach describe above (3.5.1 Set up of "freshly spiked" and "weathered" sand slurry microcosms) and used to set up triplicate 80 mL of slurry microcosms suspended in synthetic marine water (20% dry weight/volume). The microcosms were incubated statically at 20°C in the dark for 40 days to allow the adsorption of the oil contaminants to the sand. After this period, the sand slurry microcosms were inoculated (10% v/v) with a mixed culture enriched from Gela anaerobic microcosms. In particular, the Gela enrichment cultures amended with sophorolipids and HPB-CD and un-amended were chosen as inoculum. Slurry microcosms were singly spiked with sophorolipids, PBS-encapsulated sophorolipids, HPB-CD and agar-encapsulated HPB-CD. Un-amended sterile and biotic controls were also set up (Table 17). The sand slurries were also supplemented with nitrate (0.2 g/L) and phosphate (0.04 g/L) and incubated statically at 20 °C under anaerobic conditions (N_2:CO_2 70:30 headspace gas). Sand slurries were sampled at weeks 0, 6, 11 and 32. The following parameters were measured: pH, redox potential, biogas production and composition, hydrocarbons concentration and sulfate reduction.

Weathered sand slurry microcosms *Dansk Blend crude oil, 5 g/kg sed.* *Triplicate conditions* *N_2:CO_2 (70:30) headspace gas*
Sterile un-amended control
Biotic un-amended control
+ Sophorolipids (0.2 g/L, 1 g/kg_{sand})
+ PBS Sophorolipids (0.2 g/L, 1 g/kg_{sand})
+ HPB-CD (1 g/L, 5 g/kg_{sand})
+ Agar HPB-CD (1 g/L, 5 g/kg_{sand})

Table 17 Sand slurries: conditions tested to evaluate the effect of encapsulated surfactants on *n*-alkanes biodegradation.

4 Results and Discussion

4.1 Hydrocarbons biodegradation under anaerobic conditions

4.1.1 Gela anaerobic microcosms

The effectiveness of different surfactants/pollutants mobilizing agents in enhancing the hydrocarbons biodegradation were performed in slurry microcosms of actual oil-contaminated sediment from Gela harbor (Sicily) suspended in marine water (20% w/v) under anaerobic condition. The selected surfactants are shown in Table 4. The microcosms were periodically sampled and the following parameters were investigated: *n*-alkanes concentration, pH, redox potential, biogas production and composition. The indigenous microbial community were also analyzed at the begging and the end of the incubation, i.e. after 40 weeks.

4.1.1.1 Biodegradation of *n*-alkanes

The gas chromatographic analysis provided the concentrations of total *n*-alkanes (*n*-C_{12} to *n*-C_{32}) in the Gela slurry microcosms at different sampling times for all tested conditions. The results are presented as ratio C/C_0 (Figure 56), where C is the concentration of total *n*-alkanes at a determined sampling time (3, 8, 13, 17, 30 and 40 weeks), while C_0 is the concentration at the beginning of incubation (time zero). Data are the mean of replicate microcosms ± standard deviation.

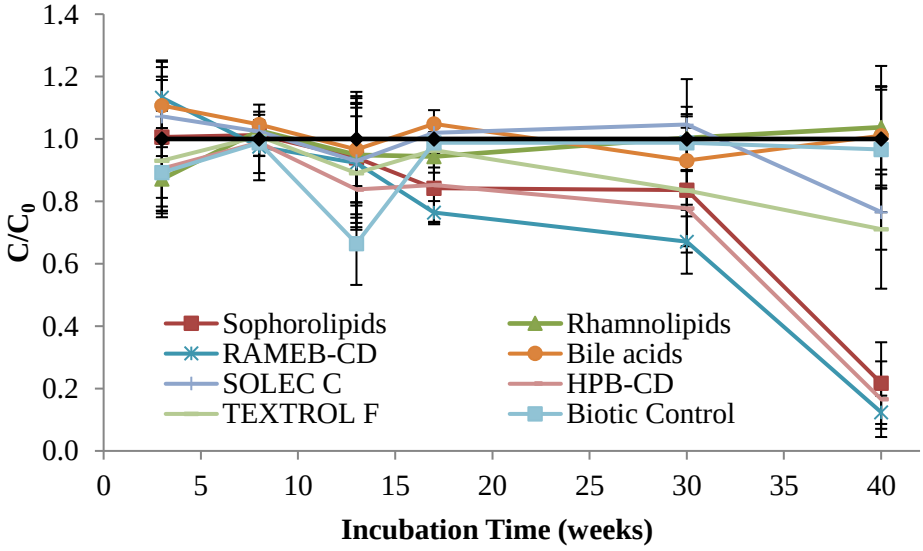

Figure 56 *n*-alkanes biodegradation in anaerobic Gela microcosms (C/C_0) at different sampling time.

During 40 weeks of incubation, biodegradation of weathered *n*-alkanes (*n*-C_{12} to *n*-C_{32}) was absent in the not-amended sterile and biologically active control microcosms, as well as in microcosms supplemented with rhamnolipids and bile acids

(Figure 56, Figure 58). A limited *n*-alkanes biodegradation (approximately 25-30%) was observed after 40 weeks of incubation in the microcosms spiked with the two soy lecithin products, Solec C and Textrol F (Figure 56, Figure 58). Conversely, 78 ± 13% of biodegradation was observed after 40 weeks of incubation in the microcosms amended with sophorolipids and 83-88% in the microcosms supplemented with the two cyclodextrins (HPB-CD and RAMEB-CD). In the latter, the biodegradation activity (22-33% of removal) was detected also after 30 weeks (Figure 56, Figure 57) and 17 weeks of incubation (approximately 15% removal, Figure 56).

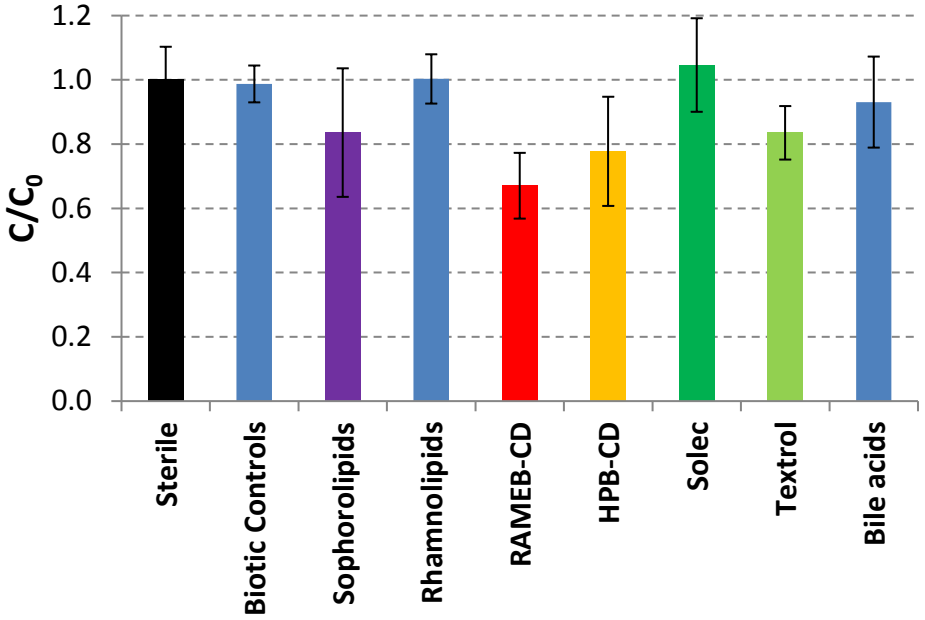

Figure 57 *n*-alkanes biodegradation in anaerobic Gela microcosms (C/C$_0$) after 30 weeks of incubation.

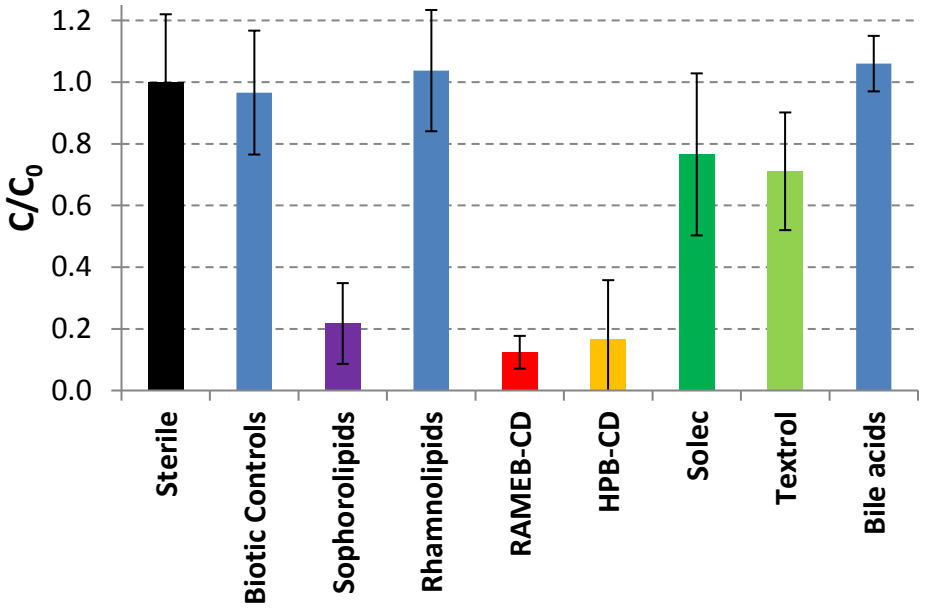

Figure 58 *n*-alkanes biodegradation in anaerobic Gela microcosms (C/C$_0$) after 40 weeks of incubation.

Figure 59 shows the chromatograms of samples in which a different biodegradation of hydrocarbons was observed after 40 weeks of incubation: (i) sterile control where no biodegradation occurred, (ii) microcosms amended with Solec C soy lecithin where was detected more than 20% of *n*-alkanes biodegradation, (iii) microcosms amended with HPB-CD where high biodegradation activity (approximately 80% of *n*-alkanes removal) occurred. Hence, the different height and area of the peaks indicates that different concentration of *n*-alkanes was observed after 40 weeks of incubation in the sterile un-amended control and in the microcosms supplemented with the soy lecithins and the cyclodextrins.

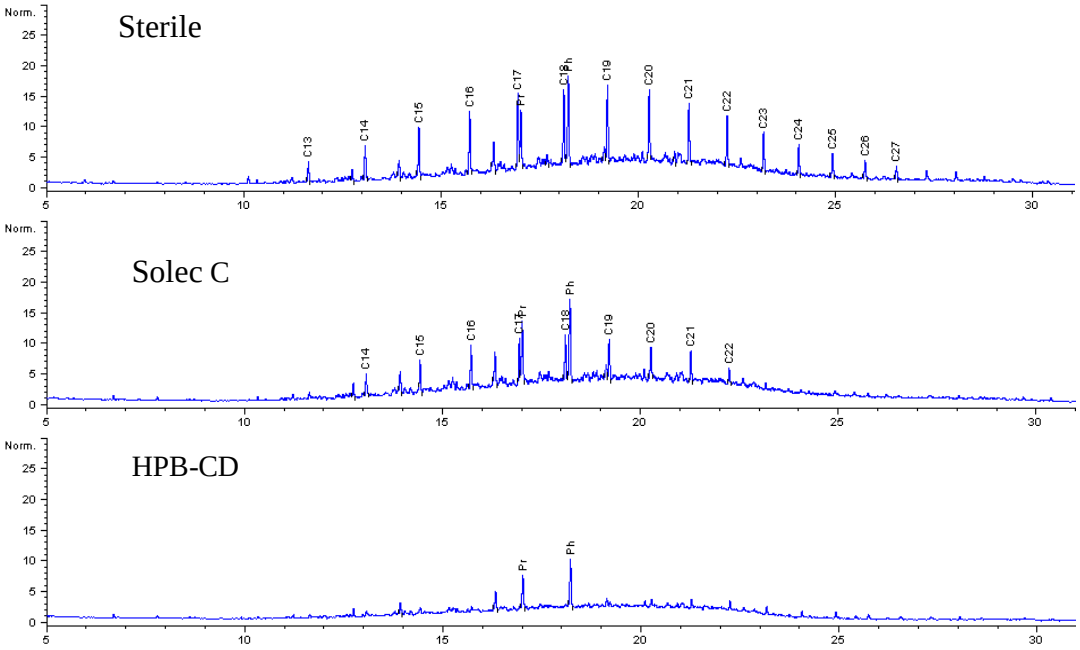

Figure 59 GC-FID chromatograms of sterile control (no *n*-alkanes biodegradation) and microcosms supplemented with Solec C (approximately 25% of *n*-alkanes removal) and HPB-CD (more than 80% of *n*-alkanes removal) after 40 weeks of incubation. In each chromatogram, the peaks of *n*-alkanes (*n*C12-*n*C32), Prystane (Pr) and Phytane (Ph) are indicated.

The stimulation of contaminants biodegradation with the use of surfactants has been largely demonstrated by several recent studies, although their effect under anaerobic condition has been poorly investigated, so far. In the last decades, several non-toxic and biodegradable microbial surfactants and pollutant mobilizing agents have been proposed for the enhancement of bioavailability and thus biodegradation of different hydrocarbons in actual-contaminated soils under aerobic conditions (Fava et al., 2001; Fava et al., 2003c; Silva et al., 2014), as well as their use in the bioremediation (Aparna et al., 2011). In particular, the cyclodextrins, mainly random methylated β-cyclodextrins (RAMEB-CD) and hydroxypropyl β-cyclodextrins (HPB-CD), have been very studied in the recent years for the increase of biodegradation in soil by improving the mobility and the bioavailability of oil pollutants (Molnár et al., 2005; Fenyvesi et al., 2005).

Although the effectiveness of surfactants in enhancing the hydrocarbons biodegradation has been less studied in marine environments, where the high salinity

might negatively affect their pollutant mobilizing activity, the potential application of glycolipids has been also verified for the bioremediation of marine oil pollution (Makkar et al., 2011).

The biodegradation activity was different for *n*-alkanes having different molecular weight (MW). Several studies have demonstrated that the biodegradation rate of oil hydrocarbons usually decreases with the increase of carbon number; low MW *n*-alkanes are the most easily biodegradable compounds, followed by branched alkanes, aromatic hydrocarbon, cyclic aromatic hydrocarbon, asphaltenes, and lastly heavy crude oil (Xue et al., 2015).
In Gela anaerobic sediment both low, medium and high MW *n*-alkanes were biodegraded after 30 and 40 weeks in the present of cyclodextrins, sophorolipids and soy lecithins. However, biodegradation of *n*-alkanes with different molecular weights (MW) was stimulated in a different way by different surfactants. Both cyclodextrins (HPB-CD and RAMEB-CD) enhanced the biodegradation of only low and medium MW *n*-alkanes during the first 30 weeks of incubation (

Figure 60), and then also the biodegradation of *n*-alkanes with high MW (Figure 61). Conversely, the other surfactants (soy lecithins and sophorolipids) stimulated predominantly the biodegradation of high MW n-alkanes after 40 weeks of incubation (Figure 61).

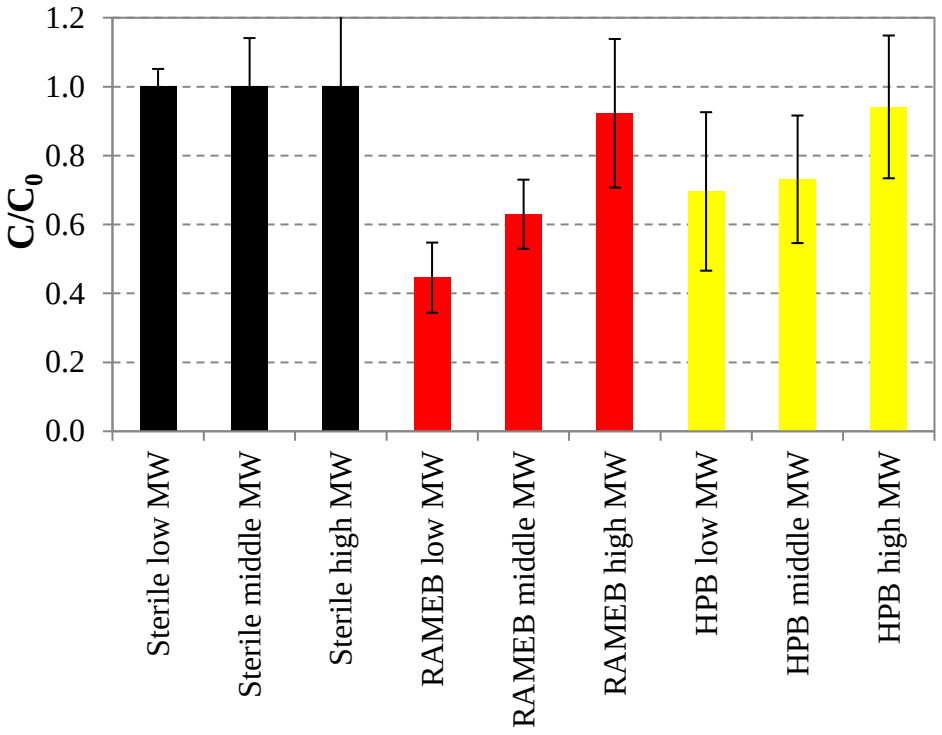

Figure 60 Effect of molecular weight (MW) on *n*-alkanes biodegradation after 30 weeks of incubation. Low MW:C_{11}-C_{17}; middle MW: C_{18}-C_{25}; high MW: C_{26}-C_{33}.

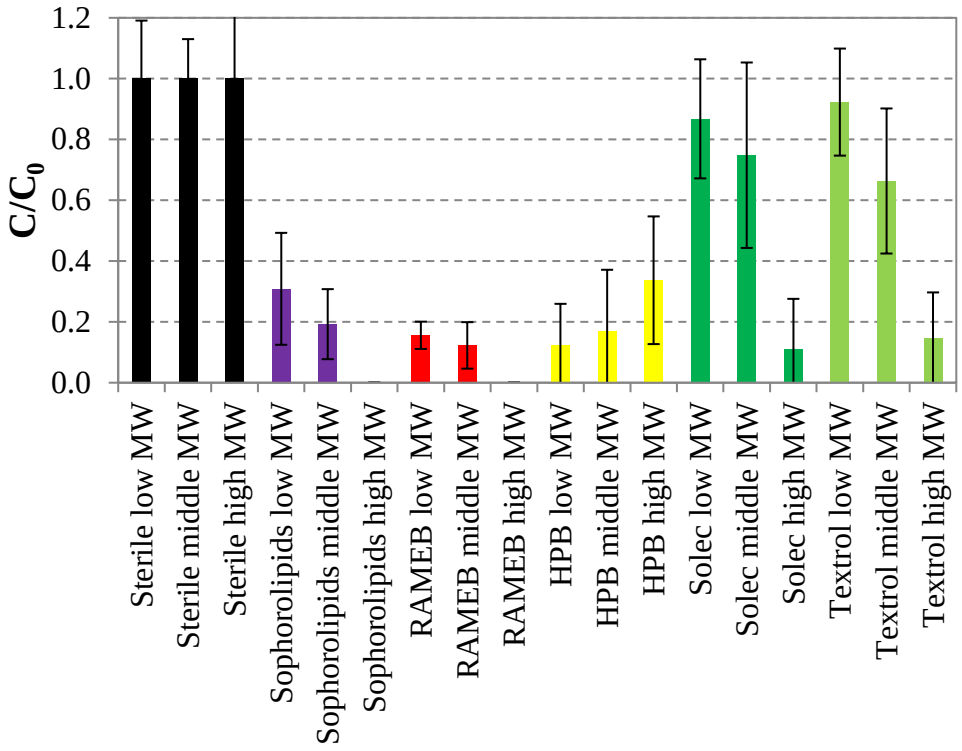

Figure 61 Effect of molecular weight (MW) on n-alkanes biodegradation after 40 weeks of incubation. Low MW:C_{11}-C_{17}; middle MW: C_{18}-C_{25}; high MW: C_{26}-C_{33}.

Such different biodegradation selectivity might be either due to

- a different mobilizing selectivity of surfactants with different chemical structures and/or
- the biostimulation of anaerobic indigenous communities with different biodegradation capabilities by the different surfactants (Megharaj et al, 2011).

4.1.1.2 Redox potential and pH

The redox potential was positive during 40 weeks of incubation in sterile controls and decreased to -90/-130 mV after 8 weeks in the biotic controls, i.e., not amended with biosurfactants/mobilizing agents. This indicates that the anaerobic metabolism could be limited by the low amount of indigenous anaerobic microorganisms and/or by a scarce bioavailability of carbon sources in Gela sediment.

Instead, in the surfactant-supplemented microcosms the redox potential decreased to -150/-200 mV after only 3 weeks of incubation (Figure 62), suggesting that the supplementation of biodegradable biogenic surfactants stimulated the activity of indigenous anaerobic microbes, increasing the bioavailability of hydrocarbons and/or because the biosurfactants were probably used as energy and carbon source (Volkering et al, 1997).

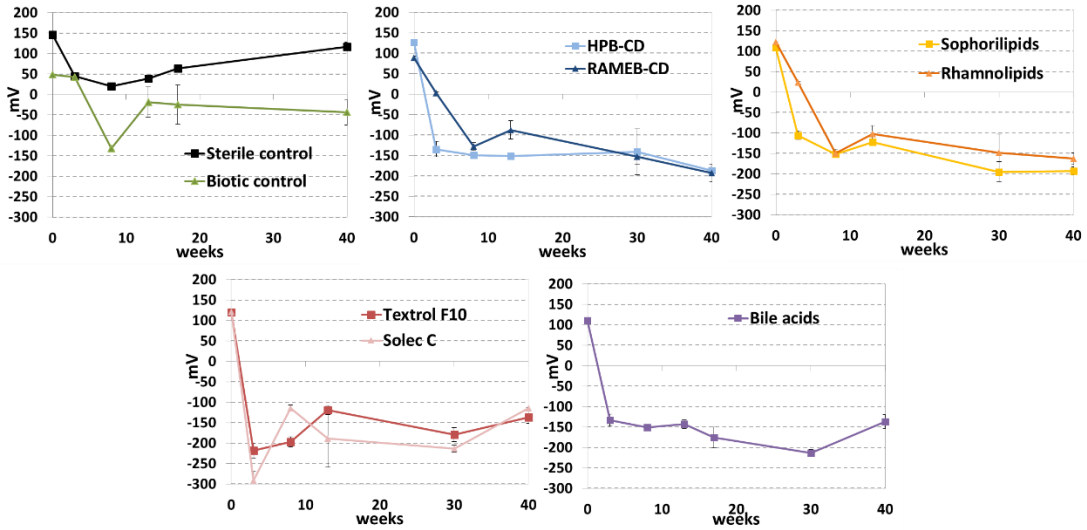

Figure 62 Redox potential (mV) during 40 weeks of incubation in the sterile un-amended control (Sterile control), biotic un-amended control (Biotic control) and surfactant-supplemented microcosms (HPB-CD, RAMEB-CD, Bile acids, Textrol F, Solec C, Sopholipids, Rhamnolipids).

Constant pH (in the range 7.0-8.0) were detected during 40 weeks of incubation both in the un-amended sterile and active controls and in the microcosms amended with the different biosurfactants (Figure 63).

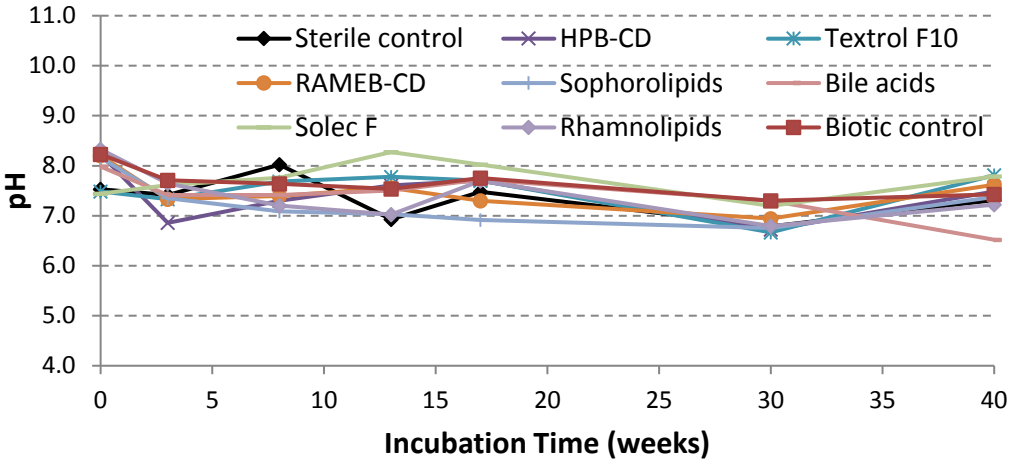

Figure 63 pH during 40 weeks of incubation in the sterile un-amended control (Sterile control), biotic un-amended control (Biotic control) and surfactant-supplemented microcosms (HPB-CD, RAMEB-CD, Bile acids, Textrol F10, Solec C, Sopholipids, Rhamnolipids).

4.1.1.3 Other microbial activities

No biogas and methane production were observed during 40 weeks of incubation both in the un-amended sterile and biotic controls, and in the surfactant-supplemented microcosms. Although, petroleum hydrocarbons could be biodegraded under methanogenic conditions (Berdugo-Clavijo and Gieg, 2014; Zhou, et al., 2012; Fowler et al., 2016), these results could exclude the present of methanogenic degradation in Gela sediment.

Sulfate reduction was absent in the un-amended controls over 40 weeks and was stimulated by the supplementation of some biosurfactants/mobilizing agents, in particular the two soy lecithin (Solec C and Textrol F), where approximately 70% of sulfate consumption occurred. A limited sulfate removal was observed in the presence of sophorolipids, HPB-CD and bile acids, (23%, 15% and 24%, respectively), while it was completely absent in the microcosms amended with the other surfactants, i.e. rhamnolipids and RAMEB-CD. The Figure 64 shows the sulfate reduction as concentration ratio between the end (after 40 weeks) and the beginning of the incubation (C/C_0).

The terminal electron-accepting processes in marine sediments under anaerobic conditions are usually Mn (IV), Fe (III) and sulfate reduction, so the bioremediation could be effective in presence of Mn (IV), Fe (III) and sulfate reducers among the indigenous population of the contaminated sediment. Due to the large amount of sulfate in marine environments, biodegradation of hydrocarbons would be most effective under sulfate-reducing conditions (Coates et al., 1997).

However, since the same sulfate reduction occurred in Gela microcosms exhibiting no biodegradation and in some having a high biodegradation activity the extent of sulfate reduction is apparently not correlated to the hydrocarbons biodegradation observed, suggesting that sulfate-reducing bacteria were probably not involved directly in degradation.

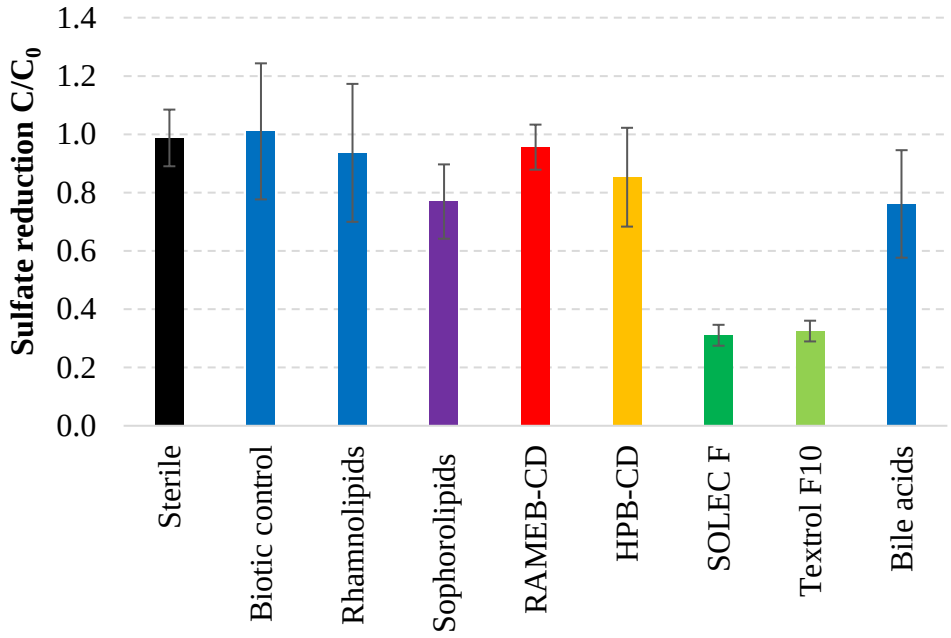

Figure 64 Sulfate reduction (C/C_0) after 40 weeks of incubation.

The extent of sulfate reduction and biodegradation of *n*-alkanes with different chain length were directly correlated. During 40 weeks of incubation low molecular weight *n*-alkanes (C_{10}-C_{17}) were preferentially biodegraded in the microcosms amended with the two cyclodextrins, where no sulfate reduction occurred. Conversely, an inverted behaviour was observed in the microcosms exhibiting sulphate reduction (soy lecithins and sophorolipids), where high molecular weight *n*-alkanes (C_{26}-C_{33}) were

predominantly removed. Under aerobic condition, the more extensive biodegradation of low molecular weight *n*-alkanes depends on biological factors, i.e. enzymatic activity, steric hindrance and chemical factors, i.e. water solubility, surface tension (Setti et al., 1993). Metabolic reaction involving high molecular weight *n*-alkanes have not been largely explored under anaerobic conditions. One possible hypothesis is given in terms of biochemical energy yield from alkane mineralization that is directly related to *n*-alkane chain length; the Gibbs free energy per mol of terminal electron acceptors that is reduced on the expense of hydrocarbon oxidation is increasing with increasing hydrocarbon chain length (Spormann and Widdel, 2000). Furthermore, hydrocarbon oxidation under sulphate reducing conditions presents a lower $\Delta G^{\circ\prime}$ compared to other conditions as denitrification. A small increase in energy yield led to the preferential degradation of high molecular weight alkanes under sulfate-reduction conditions (Hasinger et al., 2012).

4.1.1.4 PCR-DGGE analysis of 16S rRNA genes (Bacteria)

The PCR-DGGE analysis of the 16 rRNA genes was performed at the beginning and at the end of incubation in un-amended biotic control and microcosms that exhibited some biodegradation activity, i.e., microbial surfactants (rhamnolipids and sophorolipids), cyclodextrins (HPB-CD and RAMEB-CD) and soy lecithins (Solec C and Textrol F), to evaluate the presence of some differences in microbial populations, which could be related to the presence of the different surfactants. The DGGE profile (Figure 65) of the bacterial community showed the presence of the same band with a greater relative intensity (DGGE band D) after 40 weeks of incubation in the microcosms presenting the higher *n*-alkanes biodegradation, i.e., supplemented with sophorolipids, RAMEB-CD and HBP-CD. This suggests that the phylotype represented by band D might be the main indigenous microbe involved in *n*-alkane anaerobic biodegradation in the Gela sediment.

The microcosms amended with RAMEB-CD were selected to prepare a clonal library of bacterial 16S rRNA genes at the end of incubation to provide information on the main bacterial members of the community that were enriched and possibly to correlate them with the main DGGE bands.

To this purpose, the library was constructed using almost full length amplicons, in order to obtain higher phylogenetic information, and screened via DGGE analysis with the same set of primer used to obtain the community profile, in order to match OTUs with DGGE bands. Twelve OTUs have been identified and sequenced (Table 18), and six of which matched DGGE bands (Figure 65).

The molecular analysis has confirmed the presence of bacteria previously identified in marine environments; most of them belong to uncultured *Acidobacteria*, *Bacteroidetes*, *Alpha*- and *Deltaprotebacteria*, and *Spirochaeta* (Acosta-González and Marqués, 2016).

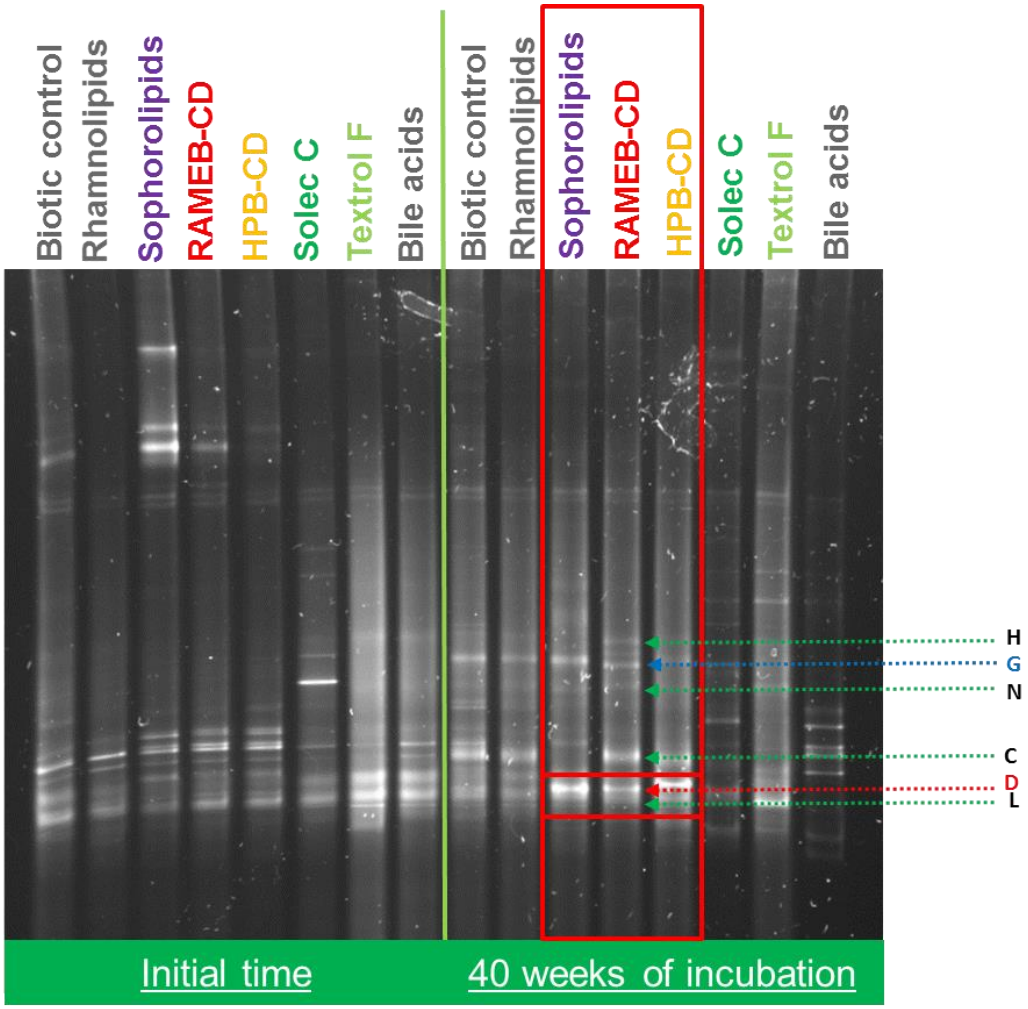

Figure 65 16S rDNA PCR-DGGE analysis of the indigenous microbial communities (Bacteria) in un-amended control and hydrocarbons degrading Gela anaerobic microcosms at initial time and after 40 weeks of incubation.

Band D, which represented a major member of the community according to both band intensity and clone abundance (20%) was 95% identical to an uncultured *Acidobacteria* bacterium previously identified in oil-polluted subtidal sediment after Prestige oil spill (Acosta-Gonzàlez et al. 2013). Therefore, this suggests the hypothesis that this most abundant phylotype could be directly involved in hydrocarbons biodegradation.

The other most abundant clone (OTU G, 14%) was 92% identical to an uncultured *Bacteroidetes* (*Draconibacterium orientale*; SS4; KF041475), a facultative anaerobic gram-negative rods isolated from enrichment cultures of a marine environment (Du Z.J., 2014).

OTU C (Sass et al., 2002.; Jiang et al., 2009), F (Fuseler et al., 1996), H (Suzuki et al., 2014) and O (Cravo-Laureau et al., 2004), represented by sulfate-reducing *Deltaproteobacteria* were less abundant (9%, 7%, 5% and 2% of relative abundance, respectively), concordant with the limited sulfate-reduction activity. This confirms

that sulfate-reducing bacteria, although commonly involved in hydrocarbons anaerobic biodegradation in marine environments, were not stimulated by the supplementation of cyclodextrins and were probably not responsible for the biodegradation activity.

OTU N (2% of relative abundance) was 99% identical to a *Bacteroidetes bacterium* (clone DT-1962; AM292393), which was found in a bacterial community enriched on *n*-hexane from the sediments composed mainly of *gammaproteobacteria* and *bacteroidetes*. This sediment was collected from a polyhaline (17 % salinity) Mediterranean lagoon located near the Etang de Berre (France) where deposits of petroleum residues were covered by saltwater (Alain et al., 2012).

Finally, OTU M (2% of relative abundance) was 88% identical to *Spirochaeta isovalerica* (T) type strain: DSM 2461; FR749931 (Harwood and Canale-Parola, 1983), firstly isolated from a marine intertidal mud. Although less common than sulfate reducing bacteria, various fermentative bacteria, such as *Thermotoga*, *Spirocheta* were isolated from crude oil reservoirs and were involved in their biodegradation (Magot et al., 2000; Xiong et al., 2015).

OTUs	Relative abundance	Phylogenetic group	Closet match [accession #]	Identity	Closest described bacterium [accession #]	Identity
B	7%	Unclassified_Bacteria	uncultured organism; MAT-CR-P5-F10; EU246239	594/621 (96%)	*Moorella thermoacetica* ATCC 39073; CP000232	547/658 (83%)
C	9%	*Deltaproteobacteria*	uncultured bacterium; MidBa65; EF999389	1421/1523 (93%)	*Desulfobulbus mediterraneus* (T); 86FS1; AF354663	1300/1439 (90%)
D	20%	*Acidobacteria*	uncultured *Acidobacteria* bacterium; RII-AN010; JQ580389	1426/1520 (95%)	*Thermotomaculum hydrothermale*; AC55; AB612241	1239/1414 (88%)
E	9%	*Alphaproteobacteria*	*Celeribacter indicus*; P73; CP004393	1413/1456 (97%)		
F	7%	*Deltaproteobacteria*	uncultured bacterium; A0-041; JN977177	1491/1554 (96%)	*Desulfobulbus propionicus* DSM 2032; CP002364	1416/1552 (91%)
G	14%	*Bacteroidetes*	*Draconibacterium orientale*; SS4; KF041475	1406/1526 (92%)		
H	5%	*Deltaproteobacteria*	uncultured *delta proteobacterium*; CB1129; DQ831550	1459/1517 (96%)	*Desulfobacterium anilini* (T); DSM 4660; AJ237601	1404/1533 (92%)
L	2%	*Alphaproteobacteria*	uncultured bacterium; LGH02-B-049; HQ916584	1359/1424 (95%)	*Thermotomaculum hydrothermale*; AC55; AB612241	1243/1414 (88%)
M	2%	*Spirochaetia*	*Spirochaeta isovalerica* (T); type strain: DSM 2461; FR749931	1360/1537 (88%)		
N	2%	*Bacteroidetes*	*Bacteroidetes bacterium* enrichment culture clone DT-1962; AM292393	599/603 (99%)	*Alkaliflexus imshenetskii* (T); type strain: Z-7010; AJ784993	639/764 (84%)
O	2%	*Deltaproteobacteria*	*Desulfatibacillum alkenivorans* (T); PF2803; AY493562	734/784 (94%)		
P	2%	unclassified_Bacteria	uncultured bacterium; LGH02-B-049; HQ916584	1340/1412 (95%)	*Holophaga foetida* (T); TMBS4T (DSM 6591T); X77215	1120/1324 (85%)

Table 18 Phylogenetic classification of bacteria corresponding to the DGGE bands of microcosms amended with RAMEB-CD.

4.2 Hydrocarbons biodegradation under aerobic conditions

4.2.1 Gela aerobic microcosms

The effect of the same surfactant/pollutant mobilizing agents (Table 4) on the hydrocarbons biodegradation were also investigated under aerobic condition provided through the supplementation of an oxygen-releasing compound (OXYGEL). The slurry microcosms were set up with the same actual oil-contaminated sediment from Gela harbor, which was tested under anaerobic condition.

4.2.1.1 Biodegradation of *n*-alkanes

The results of the hydrocarbons biodegradation were presented as ratio $C/C_{sterile}$, where C is the concentration of total *n*-alkanes (C_{12}-C_{34}) in biological active microcosms and $C_{sterile}$ is the concentration in sterile controls at the same sampling time.

Little biodegradation of *n*-alkanes (less than 15%) was apparently detected only in the presence of both cyclodextrins after 3 weeks of incubation. No hydrocarbons biodegradation occurred in un-amended sterile and biologically active controls and in microcosms supplemented with the other biosurfactants (Figure 66Figure 66 *n*-alkanes biodegradation in Gela aerobic microcosms ($C/C_{sterile}$) after 3 weeks of incubation.).

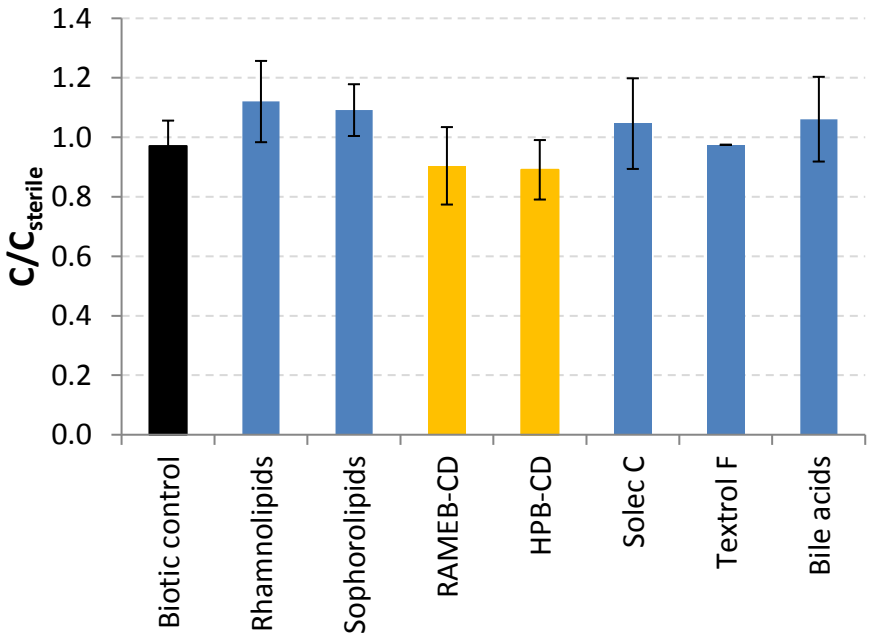

Figure 66 *n*-alkanes biodegradation in Gela aerobic microcosms ($C/C_{sterile}$) after 3 weeks of incubation.

After 28 weeks of incubation, approximately 70% of *n*-alkanes (C_{12}-C_{34}) biodegradation was detected in the un-amended biotic controls (Figure 67). Therefore, the aerobic biodegradation activities occurred after incubation time shorter compared to the anaerobic conditions where a significant removal of hydrocarbons in Gela sediment started only in the presence of some surfactants after 40 weeks of incubation. The supplementation of surfactants could produce positive or negative effects in bioremediation processes, depending on their types and concentrations (Tian et al, 2016). In Gela aerobic slurry microcosms, the addition of the soy lecithin product Textrol F and the two cyclodextrins (HPB-CD and RAMEB-CD) partially inhibited the biodegradation (approximately 20-30% removals of *n*-alkanes). Instead, the addition of the two microbial surfactants (rhamnolipids and sophorolipids) and the soy lecithin product Solec C completely inhibited the aerobic biodegradation of *n*-alkanes during the 28 weeks of incubation (Figure 67).

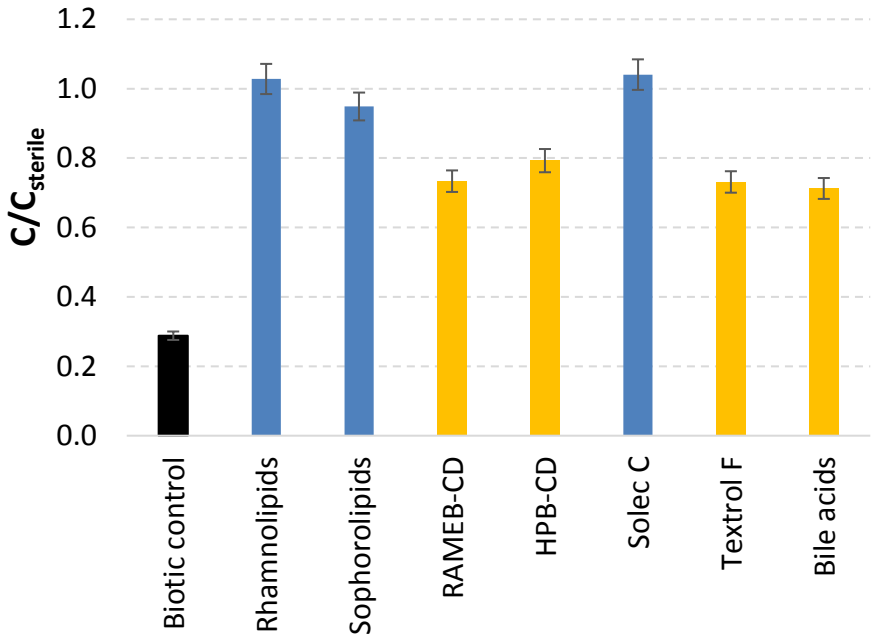

Figure 67 *n*-alkanes biodegradation in Gela aerobic microcosms ($C/C_{sterile}$) after 28 weeks of incubation.

As example, Figure 68 shows the chromatograms of the organic extracts obtained from the un-amended biotic controls (where approximately 70% of biodegradation was observed) and microcosms amended with rhamnolipids and Textrol F soy lecithin (where 0% and approximately 25% of biodegradation occurred, respectively) at the initial time and after 28 weeks of incubation. In each chromatogram the peaks of *n*-alkanes (C_{13}-C_{33}), Prystane (Pr), Phytane (Ph) and 1,2,3,4,5,6,7,8,9,10-decamethyl-anthracene (DMA) are indicated.

Although many surfactants are not toxic to the microorganisms at the concentrations near their critical micelle concentration (CMC) values, the microbial toxicity of the surfactants might be a possible cause of the *n*-alkanes biodegradation inhibition (Van Hamme et al., 1999; Silva, 2014).

Another possible motivation for the reduced contaminants removal in the presence of some surfactants might be due to an enhanced toxicity of petroleum hydrocarbons that become more bioavailability (Silva et al., 2014).
Furthermore, in the presence of several biodegradable surfactants, the pollutants could be slower removed by the indigenous microbial community due to the preferentially biodegradation of the mobilizing agents (Robles-González et al., 2008).

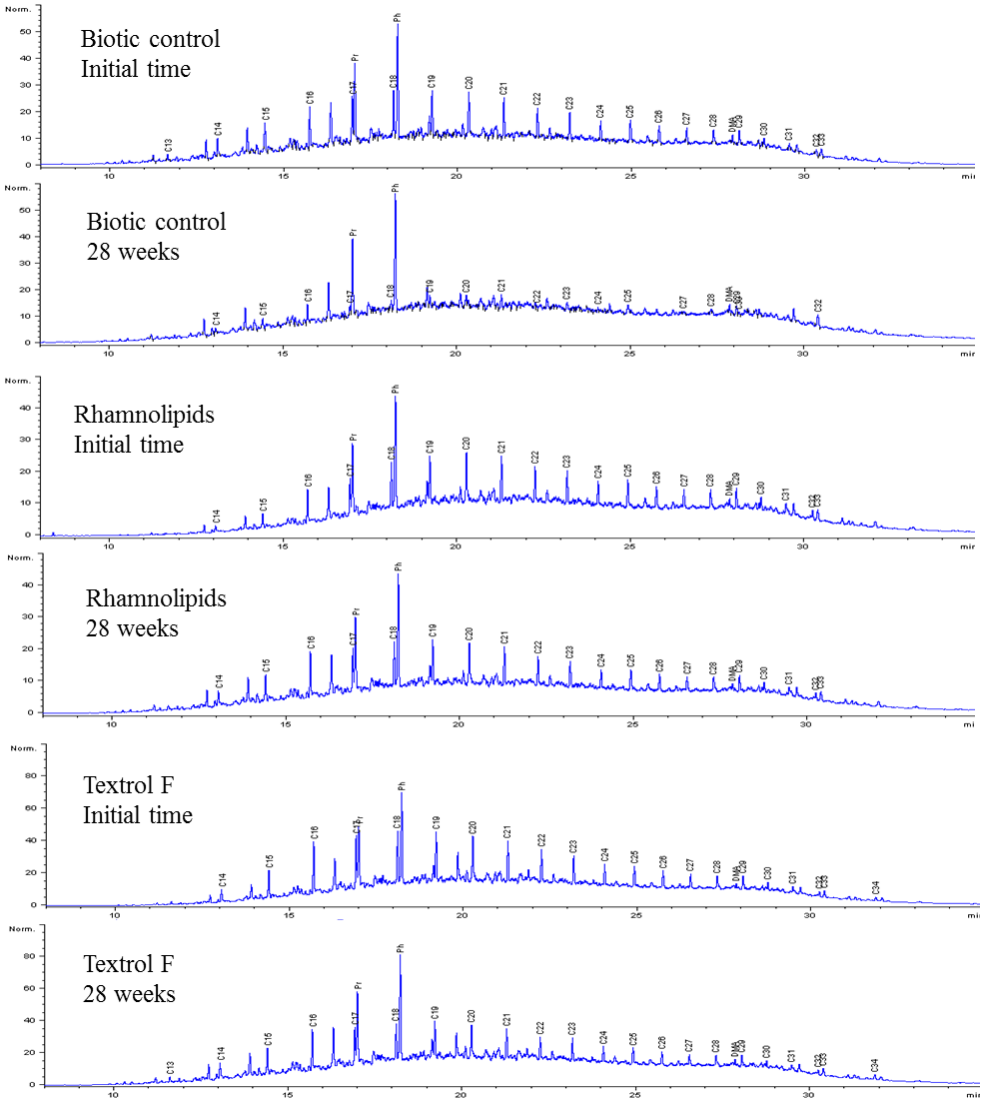

Figure 68 GC-FID chromatograms of biotic control and microcosms supplemented with rhamnolipids and Textrol F soy lecithin at the initial time and after 28 weeks of incubation. In each chromatogram, the peaks of *n*-alkanes (*n*C12-*n*C34), Prystane (Pr), Phytane (Ph) and 1,2,3,4,5,6,7,8,9,10-decamethyl-anthracene (DMA) are indicated.

4.2.1.2 pH and dissolved oxygen concentration

Decrease of pH from approximately 7.80 to 6.50 was detected during the 28 weeks of incubation under all tested conditions (Table 19).

Initial dissolved oxygen concentrations varied from approximately 0.5 mg/L to approximately 2.0 mg/L (Figure 69). In general, increasing oxygen concentrations up to approximately 3.5 mg/L were detected over time in all sterile controls and the biologically active microcosms, indicating that oxygen was released from OXYGEL. However, despite the repeated additions of OXYGEL, the oxygen concentration did not increase above 3.5 mg/L in any case, and was comparable in biologically active and sterile microcosms after 28 weeks of incubation. This indicates that a limited oxygen consumption occurred due to aerobic growth, although it is well known that the incorporation of oxygen is the enzymatic key reaction catalyzed by oxygenases and peroxidases during the biodegradation of the main organic pollutants under aerobic conditions (Das and Chandran, 2010).

	WEEKS		
	0	3	28
Biotic control	7.9 ± 0.1	7.2 ± 0.1	6.6 ± 0.0
Sterile control	7.4 ± 0.1	7.2 ± 0.0	6.5 ± 0.1
Rhamnolipids	7.7 ± 0.1	7.1 ± 0.1	6.6 ± 0.1
Sterile Rhamnolipids	7.7 ± 0.3	7.3 ± 0.1	6.6 ± 0.1
Sophorolipids	7.2 ± 0.0	7.0 ± 0.0	6.6 ± 0.0
Sterile Sophorolipids	7.6 ± 0.0	7.3 ± 0.1	6.5 ± 0.1
RAMEB-CD	7.3 ± 0.0	7.2 ± 0.0	6.5 ± 0.0
Sterile RAMEB-CD	7.8 ± 0.2	7.4 ± 0.1	6.6 ± 0.0
HPB-CD	7.5 ± 0.0	7.0 ± 0.1	6.7 ± 0.0
Sterile HPB-CD	7.7 ± 0.1	7.1 ± 0.0	6.7 ± 0.0
Solec C	7.3 ± 0.0	6.9 ± 0.0	6.7 ± 0.1
Sterile Solec C	7.7 ± 0.1	7.2 ± 0.0	6.7 ± 0.1
Textrol F	7.3 ± 0.0	7.0 ± 0.2	6.7 ± 0.1
Sterile Textrol F	7.6 ± 0.3	6.8 ± 0.0	6.7 ± 0.0
Bile acids	7.4 ± 0.1	6.7 ± 0.1	6.6 ± 0.0
Sterile Bile acids	7.9 ± 0.1	7.1 ± 0.0	6.6 ± 0.0

Table 19 pH in Gela aerobic microcosms after 0, 3 and 28 weeks of incubation.

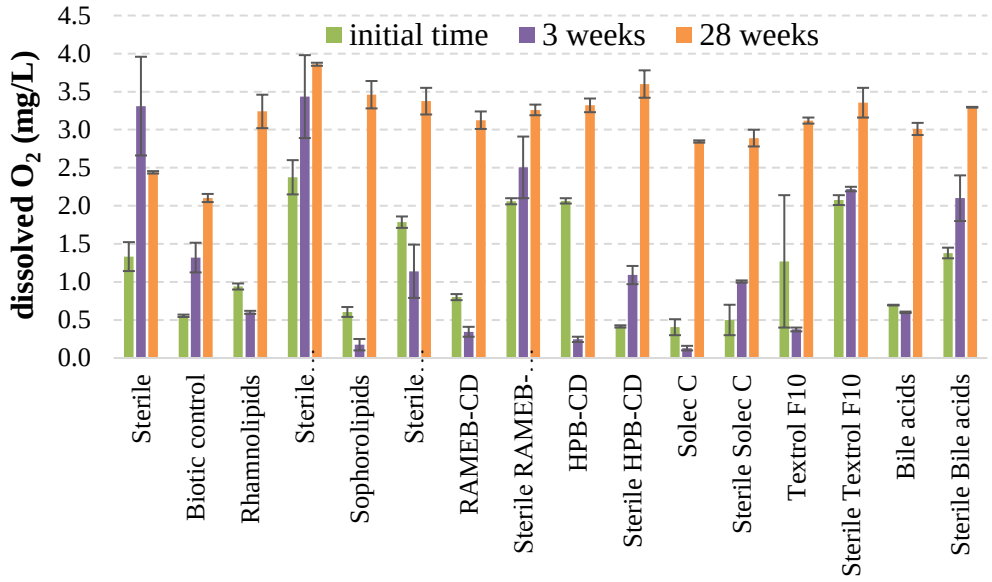

Figure 69 Dissolved oxygen concentration (mg/L) in Gela aerobic slurry microcosms at the initial time, and after 3 and 28 weeks of incubation.

4.2.2 Ravenna aerobic microcosms

The effect of biosurfactants/commercial pollutant mobilizing agents on the aerobic biodegradation was also investigated in slurry microcosms of sediment collected from the harbor of Ravenna (Italy), which was contaminated in the laboratory with a Dansk blend crude oil (5 g/kg$_{sediment}$).

4.2.2.1 Biodegradation of *n*-alkanes

Biodegradation of total *n*-alkanes (C_{12}-C_{32}) in Ravenna aerobic microcosms were analyzed as ratio between the concentration in biological active microcosms and the concentration in sterile controls at the same sampling time ($C/C_{sterile}$). A limited *n*-alkanes biodegradation (approximately 23%) was observed in the not-amended biologically active control microcosms after 8 weeks of incubation. The surfactants addition inhibited the biodegradation during the first 8 weeks of incubation in all the amended microcosms (Figure 70).

After 28 weeks of incubation, *n*-alkanes biodegradation of approximately 65% occurred in the not-amended biotic controls (Figure 71). The addition of the soy lecithin product Solec C and of the cyclodextrins HPB-CD enhanced the aerobic biodegradation of *n*-alkanes to approximately 80%. Conversely, a biodegradation comparable to that of occurring in not-amended biotic controls resulted in the microcosms amended with the soy lecithin Textrol F (61 ± 5% *n*-alkanes removal), RAMEB-CD (55 ± 1%) and rhamnolipids (55 ± 2%).

The microcosms treated with bile acids and sophorolipids (where only approximately 4 and 13% of *n*-alkanes removal occurred, respectively) showed a decrease of hydrocarbons biodegradation (Figure 71).

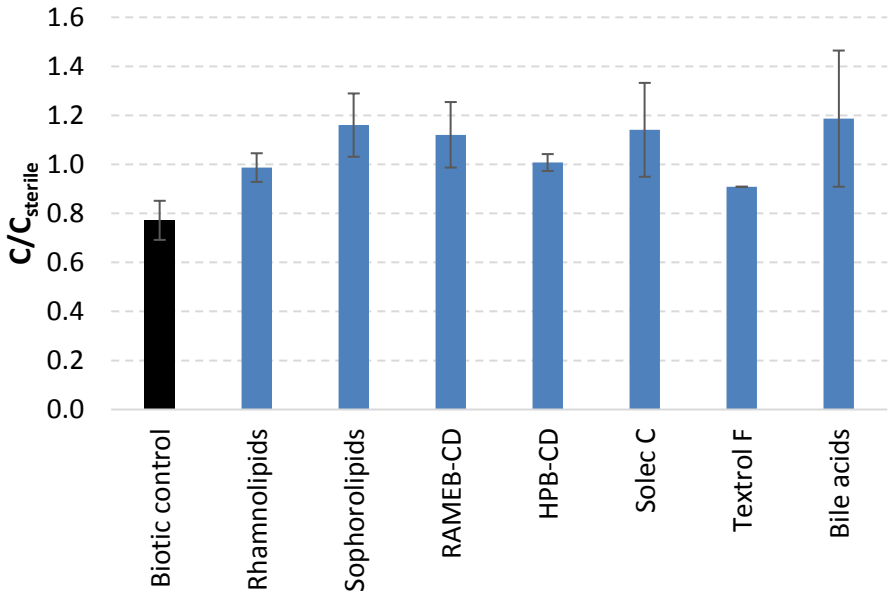

Figure 70 *n*-alkanes biodegradation in Ravenna aerobic microcosms ($C/C_{sterile}$) after 8 weeks of incubation.

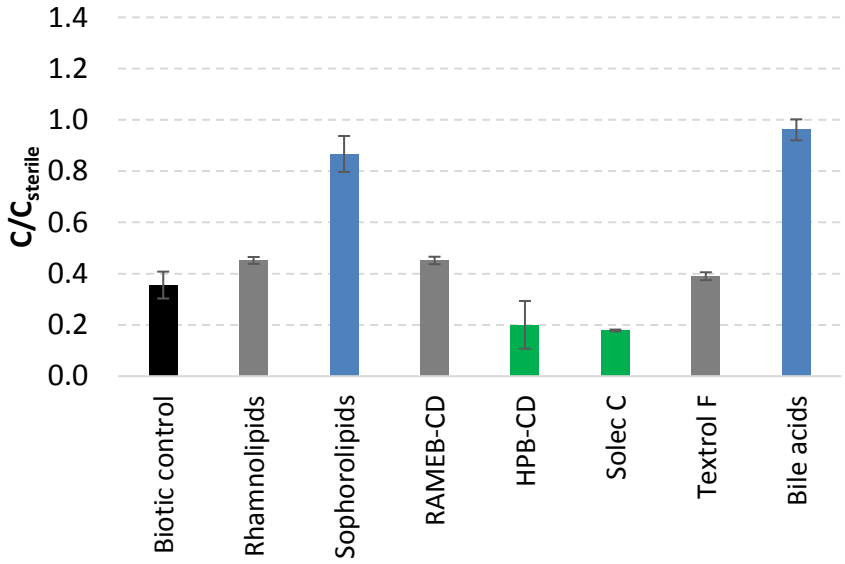

Figure 71 *n*-alkanes biodegradation in Ravenna aerobic microcosms ($C/C_{sterile}$) after 28 weeks of incubation.

The inhibitory effect caused by the presence of some surfactants (i.e. bile acids and sophorolipids) may depend on several factors, such as the microbial toxicity of biosurfactant or of their metabolites, the increased toxicity of the hydrophobic contaminants due to their increased solubility and the preferentially biodegradation of the pollutant mobilizing agent (Van Hamme et al., 1999, Silva et al., 2014, Robles-González et al., 2008).

However, while the supplementation of all biosurfactants inhibited hydrocarbons biodegradation in the Gela microcosms under aerobic condition, different effects on biodegradation activities (biostimulation, partial or complete inhibition) were exerted

by the different biosurfactants in the Ravenna sediment. Such differences might be due to: i) different sediment contamination (weathered vs. spiked, Gela Refinery oil vs. Dansk blend crude oil); ii) possible different solubilizing properties of the biosurfactants in the two sediments; iii) differences in the microbial communities and their response to hydrocarbons contamination.

4.2.2.2 pH and dissolved oxygen concentration

Low decrease of pH (from approximately 7.70 to 6.40) occurred during the 28 weeks of incubation in both sterile and biologically active microcosms under all tested conditions (Table 20**Errore. L'origine riferimento non è stata trovata.**).

Dissolved oxygen profiles over time (Figure 72) were very similar to those detected in the Gela aerobic microcosms (Figure 69). Its concentration ranged from approximately 0.5 mg/L to approximately 2.4 mg/L at the begging of the incubation and increased up to approximately 3.5 mg/L in all sterile and biologically active microcosms after 28 weeks, indicating that oxygen was released from OXYGEL. However, since the dissolved oxygen concentration is comparable in all biotic and sterile microcosms, a limited consumption of oxygen occurred.

	WEEKS		
	0	8	28
Biotic control	6.9 ± 0.0	6.8 ± 0.0	6.7 ± 0.1
Sterile control	7.5 ± 0.0	6.4 ± 0.1	6.4 ± 0.0
Rhamnolipids	7.0 ± 0.0	6.6 ± 0.0	6.8 ± 0.1
Sterile Rhamnolipids	7.7 ± 0.0	7.0 ± 0.0	6.7 ± 0.1
Sophorolipids	6.9 ± 0.1	6.7 ± 0.0	6.8 ± 0.0
Sterile Sophorolipids	6.9 ± 0.1	6.8 ± 0.1	6.6 ± 0.0
RAMEB-CD	7.0 ± 0.0	6.9 ± 0.0	6.8 ± 0.0
Sterile RAMEB-CD	7.5 ± 0.0	6.9 ± 0.0	6.7 ± 0.0
HPB-CD	6.9 ± 0.1	6.8 ± 0.0	6.9 ± 0.0
Sterile HPB-CD	7.2 ± 0.0	6.6 ± 0.1	6.5 ± 0.0
Solec C	7.0 ± 0.0	6.9 ± 0.0	6.9 ± 0.0
Sterile Solec C	6.8 ± 0.0	6.8 ± 0.0	6.6 ± 0.0
Textrol F	6.9 ± 0.0	7.1 ± 0.0	6.9 ± 0.0
Sterile Textrol F	7.4 ± 0.0	6.8 ± 0.0	6.6 ± 0.0
Bile acids	7.0 ± 0.1	6.8 ± 0.0	6.9 ± 0.0
Sterile Bile acids	7.7 ± 0.1	7.0 ± 0.1	6.6 ± 0.0

Table 20 pH in Ravenna aerobic microcosms after 0, 3 and 28 weeks of incubation.

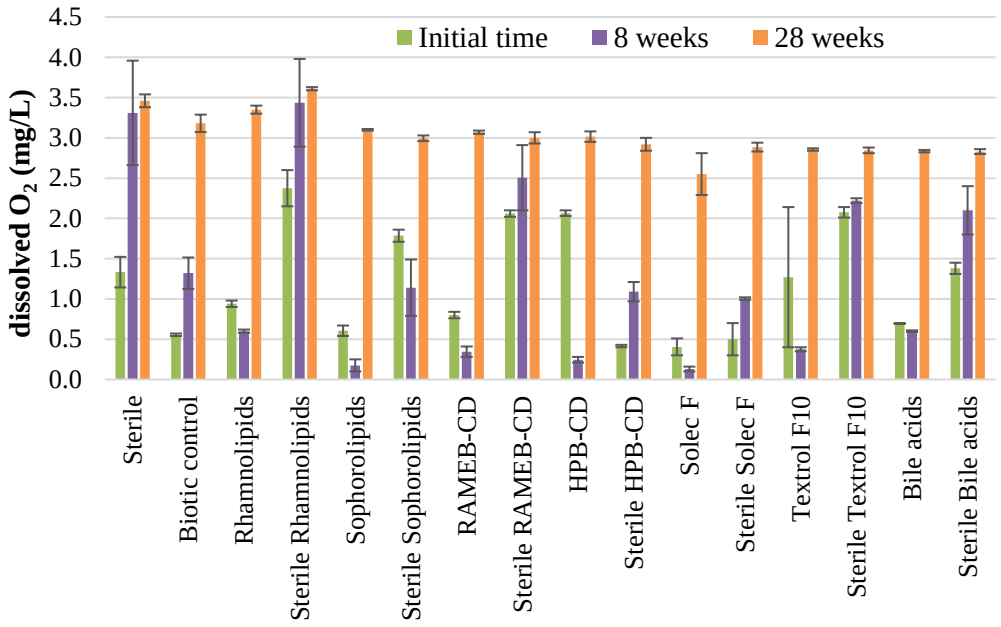

Figure 72 Dissolved oxygen concentration (mg/L) in Ravenna aerobic slurry microcosms at the initial time and after 8 weeks and 28 weeks of incubation.

4.3 Effect of free surfactants on bioavailability of *n*-alkanes

Sterile microcosms of Gela sediment were prepared to evaluate the effect of the different surfactants on hydrocarbons bioavailability by measuring the pollutants concentration in the porewater via passive sampling with polydimethylsiloxane (PDMS) fibers. The sediment was contaminated in the laboratory with a crude oil (5g/kg$_{sediment}$) and the fibers, incubated in the microcosms at 20 °C, 150 rpm, were replaced every 20 days to sample water-dissolved hydrocarbons.

4.3.1 Gela microcosms

High porewater concentration of *n*-alkanes, ranging from approximately 1200 ng/L to approximately 120 ng/L, was observed in all the Gela sediment slurries after 20 day of incubation (Figure 73). The *n*-alkanes porewater concentration decreased over the time, probably due to the progressive adsorption of spiked hydrocarbons to the Gela sediment. In fact, after 40 (Figure 74) and 60 (Figure 75) days of incubation a significant decrease of *n*-alkanes porewater concentration occurred in the un-amended controls, indicating that *n*-alkanes adsorption to the sediment was completed and equilibrium was reached after 40 days. Porewater concentration of *n*-alkanes comparable to the not-amended controls was also observed in the microcosm treated with both microbial surfactants (rhamnolipids and sophorolipids), Solec C soy lecithin and bile acids. Conversely, RAMEB-CD, HPB-CD and Textrol F soy lecithin reduced the adsorption rate of *n*-alkanes in the Gela sediment after 60 days of incubation. This indicates that both cyclodextrins and Textrol F are the most efficient surfactants in increasing the porewater concentration of freshly spiked *n*-alkanes.

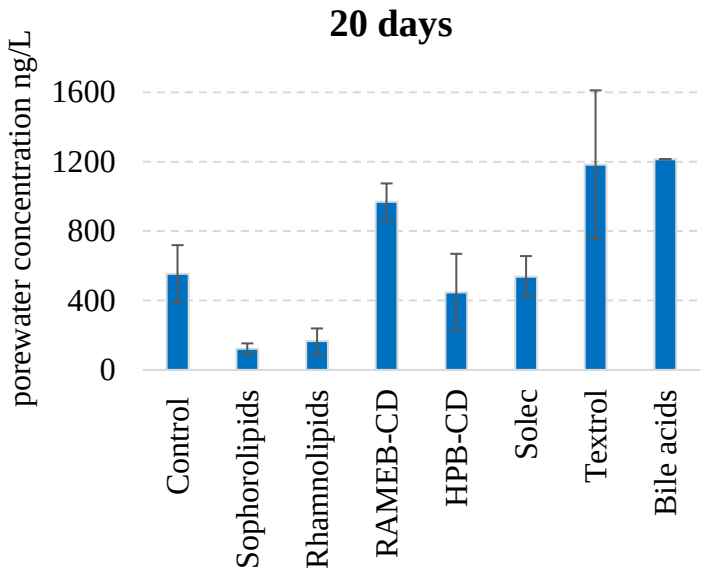

Figure 73 Porewater concentration of *n*-alkanes (ng/L) in sterile microcosms of Gela sediment spiked with Dansk Blend crude oil in un-amended control and in presence of different surfactants after 20 days of incubation.

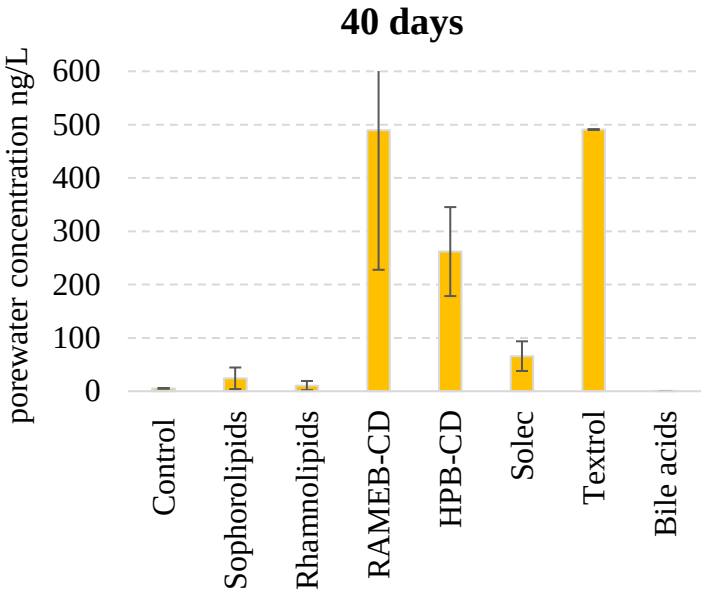

Figure 74 Porewater concentration of *n*-alkanes (ng/L) in sterile microcosms of Gela sediment spiked with Dansk Blend crude oil in un-amended control and in presence of different surfactants after 40 days of incubation.

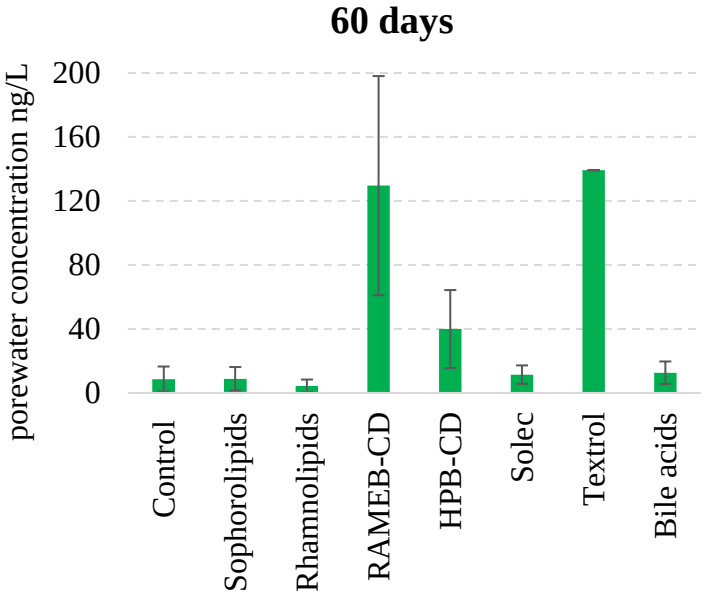

Figure 75 Porewater concentration of *n*-alkanes (ng/L) in sterile microcosms of Gela sediment spiked with Dansk Blend crude oil in un-amended control and in presence of different surfactants after 60 days of incubation.

To test the effect of both cyclodextrins and Textrol F soy lecithin on adsorbed *n*-alkanes, RAMEB-CD, HPB-CD and Textrol F were added to the un-amended controls, Sophorolipids and Rhamnolipids microcosms, respectively, after adsorption of spiked *n*-alkanes was completed (*n*-alkanes concentration in the aqueous phase was low and constant), i.e. after 60 days of incubation.

A significant enhancement of *n*-alkanes porewater concentration was observed 20 days after the addition of RAMEB-CD, and to less extent, 40 days after the addition

of HPB-CD. Instead, the supplementation of soy lecithin product Textrol F produced an increase of *n*-alkanes porewater concentration after 60 days (Figure 76). The enhancing of *n*-alkanes concentration in the porewater could be correlated to their capability to increase the anaerobic biodegradation of oil hydrocarbons detected in the previous studies.

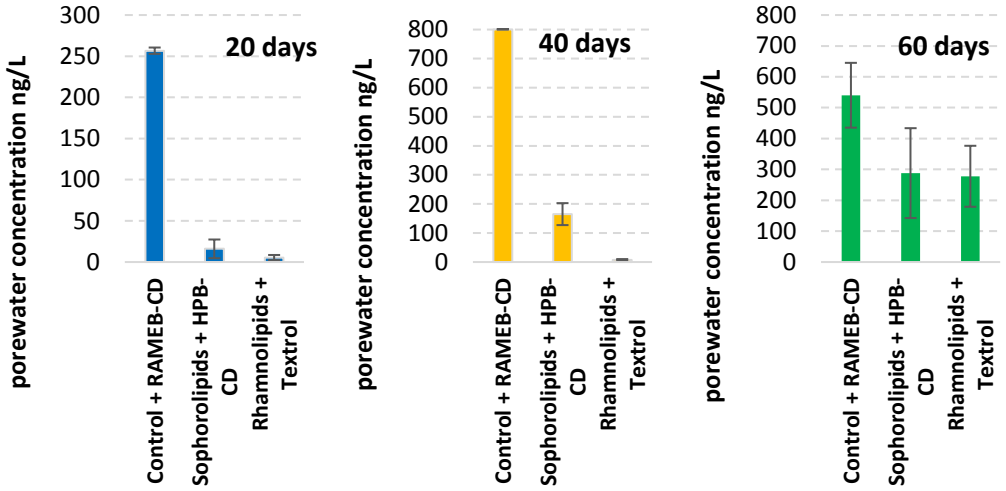

Figure 76 Porewater concentration (ng/L) in un-amended controls, Sophorolipids and Rhamnolipids microcosms 20, 40 and 60 days after the addition of RAMEB-CD, HPB-CD and Textrol F, respectively.

Sophorolipids, which stimulated the anaerobic hydrocarbons biodegradation (approximately 80 % of *n*-alkanes removal occurred), were not able to increase the *n*-alkanes bioavailability and were probably used as carbon and energy source by oil-degrading microorganisms (Matvyeyeva et al., 2014).

4.4 Surfactants encapsulation

Sophorolipids, which stimulated the anaerobic biodegradation and HPB-CD, which were able to enhance both biodegradation and bioavailability of *n*-alkanes were selected for the encapsulation within biodegradable organic polymers and agar hydrogels.

4.4.1 Surfactants encapsulation in biodegradable organic polymers

The encapsulation of the two selected biosurfactants in biodegradable organic polymers were prepared using the double emulsion method, as previously described in the paragraph 3.4.1 (Encapsulation in biodegradable organic polymers). Different polymers have been selected and compared in terms of encapsulation efficiency.

4.4.1.1 Encapsulation efficiency in polymers

The total amount of microspheres obtained using the double emulsion method (DEM) was approximately 1-1.4 g with all tested polymers.

Table 21 shows the loss of surfactants (in the water phase) during the preparation via DEM of the polymer microspheres (before and after the washing step) and the surfactant encapsulation efficiency obtained using different polymers:

- polybutylene succinate (PBS),
- polybutylene succinate-co-adipate (PBS-coA),
- polycaprolactone (PCL)
- polylactic acid (PLA).

Sample (surfactant, polymer)	Surfactant loss (%) before washing step	Surfactant loss (%) after washing step	Encapsulation efficiency of surfactant (%)
Sophorolipids, PBS	0	0	95
HPB-CD, PBS	30	90	7
HPB-CD, PLA_A	36	92	2
HPB-CD, PLA_B	27	93	0
HPB-CD, PLA_C	60	100	0
HPB-CD, PBS-coA	12	64	34
HPB-CD, PCL	14	71	27

Table 21 Loss of surfactants (%) during the encapsulation of sophorolipids and HPB-CD via DEM in different polymers and surfactant encapsulation efficiency (%); HPB-PLA_A, HPB-PLA_B and HPB-PLA_C were obtained using 0.2, 0.4, 0.8 g of glycerol, respectively.

The encapsulation of HPB-CD revealed a high loss of cyclodextrins in the water phase after washing procedure (64% to 100%) and a low efficiency of encapsulation (ranging from 0 to 34%) in each tested condition, indicating that HPB-CD was not effectively encapsulated within the selected polymers. Although different amount of glycerol as plasticizer (0.2, 0.4 and 0.8 g) during the preparation of PLA microspheres was used to increase the breaking strength, the encapsulation of cyclodextrins in this polymer via DEM was not effective (Table 21). Conversely, sophorolipids were efficiently encapsulated in PBS (95% of efficiency, Figure 77) and no loss of microbial surfactants was apparently detected in the water phase both before and after the washing step.

Therefore, the surfactants release from polymer microspheres and the evaluation of *n*-alkanes bioavailability and biodegradation were investigated only for PBS-encapsulated sophorolipids.

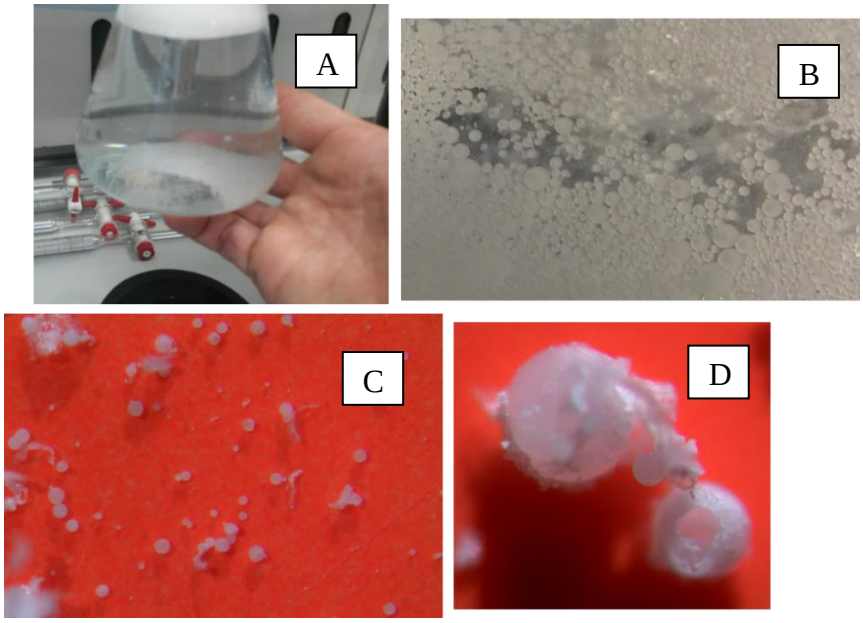

Figure 77 Microspheres of PBS with Sophorolipids: photos during preparation (A-B) and stereo microscope image of dried microspheres (C-D).

Researches on encapsulation of surfactants in biopolymers is very innovative and few studies are still present. However, cyclodextrins are commonly used as encapsulating agents because they can easily form inclusion complexes with different compounds (Marques, 2010). Sophorolipids were also used for other purposes e.g. the formation of nanoparticles (Privadarshini et al., 2016; Kasture, 2008).

4.4.1.2 Release of sophorolipids from polybutylene succinate microspheres in sterile water

The release of sophorolipids from polybutylene succinate (PBS) microspheres in the sterile water, analysed through HPLC, was slow; approximately 86% of total microbial surfactant (0.27 g/L) was released after 38 days of incubation (Figure 78). The black dashed line in the graph of Figure 78 represents the maximum estimated

concentration of sophorolipids in the water corresponding to the complete surfactants release.

Biodegradable organic polymers are totally eco-friendly since they can completely decompose in natural environments (Upreti and Srivastava 2003, Shah et al., 2014). Degradation of aliphatic polyesters includes chemical hydrolysis and microbial, enzymatic, thermal and oxidative degradation. Biopolymers can be degraded into water and CO_2 by several microorganisms in bioactive environments (Song. et al., 2009). In sterile condition, abiotic hydrolysis of these polymers is influenced not only by the chemical structure, but also by the ordered structure such as crystallinity, orientation, molecular weight, presence of impurities and residual monomers and other morphological properties (Chen et al., 2008). In particular, degradability of polybutylene succinate (PBS) has been widely investigated in the last years. Several studies showed that the PBS is subject to hydrolysis in the presence of humidity/water through the cleavage of ester linkages, leading to weight loss and decrease in mechanical properties in less than 30 days (Ahn et al., 2001; Muthuraj et al., 2015; Phua, et al., 2015). These research activities are in accordance with the results obtained in the tests of surfactants release for the PSB-encapsulated sophorolipids.

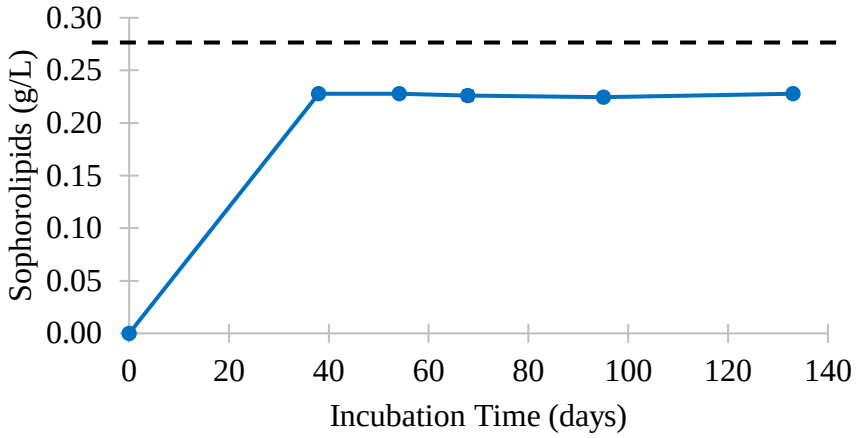

Figure 78 Sophorolipids (g/L) release from PBS microspheres in sterile water over time (days). The black dashed line indicates the maximum estimated concentration of sophorolipids in sterile water (their complete release).

4.4.2 Surfactants encapsulation in hydrogels (agar)

Capsules of hydrogel were prepared in multiwell plates using a known concentration of surfactants (30 and 50 g/L for sophorolipids and HPB-CD, respectively). Different concentrations of agar (5, 15 and 50 g/L) were tested.

Sophorolipids prevented the agar polymerization in the multiwell plates using all the different concentrations of agar. Thus, only the agar-encapsulated HPB-CD were used to evaluate the surfactants release from hydrogel capsules in sterile water, and to assess the effectiveness of the surfactants encapsulated in hydrogel in enhancing the hydrocarbons bioavailability and biodegradation. Figure 79 shows the hydrogel

capsules of HPB-CD made with a solution of 50 g/L of agar. The final concentration of HPB-CD in each capsule is 50 g/L.

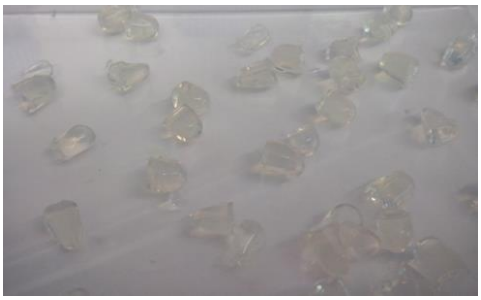

Figure 79 Agar capsules of HPB-CD (50 g/L).

4.4.2.1 Release of hydroxypropyl-β-cyclodextrins from agar capsules in sterile water

Concentration of agar in the capsules influenced the release of the cyclodextrins in marine water; increasing agar concentration reduced the release of HPB-CD. Depending on agar concentration, approximately 50-70% of HPB-CD was released within the first hours of incubation at 20 °C. In demineralized water, the release of cyclodextrins from the hydrogel capsules was more rapid and less influenced by the agar concentration. Figure 80 shows the release of HPB-CD (g/L) over time from capsules made using different concentration of agar (5, 15 and 50 g/L) in both sterile marine (in red) and demineralized (in blue) water. The black dashed line in the graphics represents the maximum estimated concentration of HPB-CD (4.8 g/L), i.e. the concentration that would detected if the surfactants were completely released in the water.

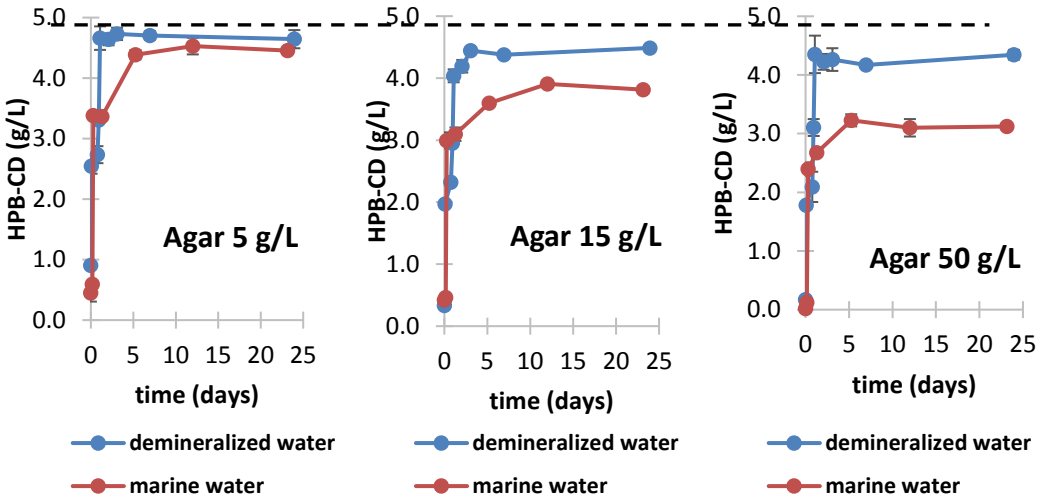

Figure 80 HPB-CD (g/L) release from capsules made using different concentration of agar (5, 15 and 50 g/L) in sterile marine (in red) and demineralized (in blue) water over time (days). The black dashed line indicates the maximum expected concentration of HPB-CD in the water.

4.4.3 Release of encapsulated surfactants in crude oil-contaminated sand slurry microcosms

The release of PBS-encapsulated sophorolipids and agar-encapsulated HPB-CD was also investigated in sterile slurry microcosms of sand contaminated by crude oil (5 g/kg_{sand}). A gradual release over time of sophorolipids and HPB-CD was observed from PBS microspheres and agar capsules, respectively. In particular, after 28, 40 and 65 days of incubation approximately 48%, 78% and 87% of total HPB-CD, and approximately 55%, 73% and 81% of total sophorolipids were released in sand slurry microcosms, respectively (Figure 81). The release rate of both surfactants agents in sand slurries is lower than that in marine water.

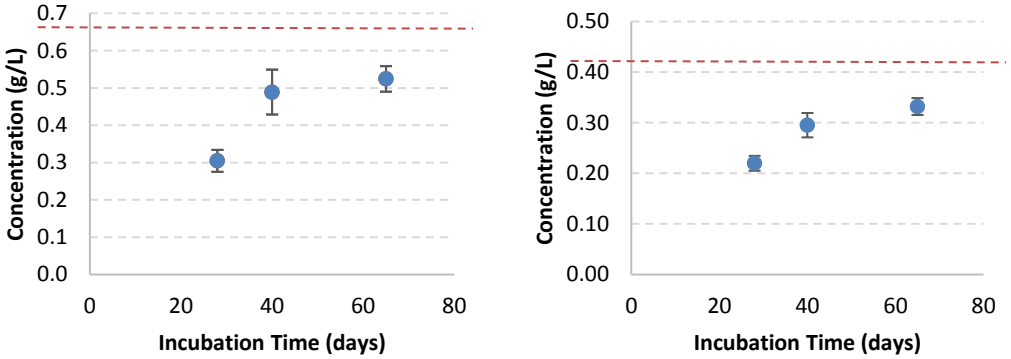

Figure 81 Release of HPB-CD from agar capsules (on left) and of sophorolipids from PBS microspheres (on right) over time. The red dotted line represents the maximum expected concentration (complete surfactants release).

4.5 Effect of encapsulated surfactants on bioavailability of n-alkanes

The effect of encapsulated surfactants on hydrocarbons bioavailability was analyzed in both "freshly spiked" and "weathered" sand slurry microcosms by measuring the pollutants concentration in the porewater via passive sampling with PDMS fibers.

4.5.1 "Freshly spiked" sand slurry microcosms

In "freshly spiked" sand slurries, surfactants and PDMS fibers were incubated immediately after contamination with crude oil. Thus, the results of *n*-alkanes porewater concentration obtained in these microcosms provide indication on the absorption of hydrocarbons in the sand, which can be compared with those obtained using free surfactants.

After 20 days of incubation, the porewater concentration of *n*-alkanes in freshly spiked sand slurries was comparable in all samples (range from approximately 320 to approximately 470 ng/L). Therefore, no substantial differences were found during the first 20 days between un-amended controls and the microcosms supplemented with the both surfactants encapsulated and not (Figure 82).

After 40 (Figure 83) and 60 (Figure 84) days of incubation, oil hydrocarbons adsorption occurred only in the un-amended control, where was observed a decrease of *n*-alkanes porewater concentration until approximately 80 ng/L. This indicates that complete adsorption of spiked hydrocarbons to the sand was reached after 40 days of incubation.

The supplementation of both free and encapsulated HPB-CD increased the porewater concentration of *n*-alkanes (about 1600 ng/L). Conversely, the enhancement of hydrocarbons bioavailability in the presence of PBS-encapsulated sophorolipids (where less than 500 ng/L of *n*-alkanes porewater concentration occurred) was about 50% of that observed with free sophorolipids (approximately 1000 ng/L). Both encapsulated surfactants were thus able to reduce remarkably hydrocarbons adsorption to sand thus increasing their bioavailability.

Compared to Gela sediment, *n*-alkanes adsorption in the sand microcosms was slower in the presence of sophorolipids and HPB-CD because the spiked oil hydrocarbons in the Gela sediment could be adsorbed more rapidly due to the presence of organic matrix.

In the microcosms supplemented with PBS-encapsulated sophorolipids, the porewater concentration of *n*-alkanes was lower than the microcosms amended with not-encapsulated biosurfactants probably due to the greater time required for the complete release of the biosurfactant from PBS microspheres. However, since previous studies showed that the release of both sophorolipids and HPB-CD from PBS microspheres and agar capsules in sterile slurry microcosms was more than 80% after 65 days of incubation, the presence of biopolymer may affect negatively the desorption process.

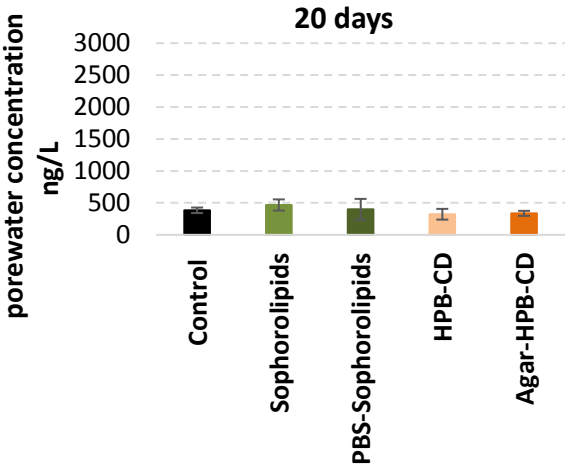

Figure 82 Porewater concentration of *n*-alkanes (ng/L) in "freshly spiked" microcosms after 20 days of incubation under different conditions.

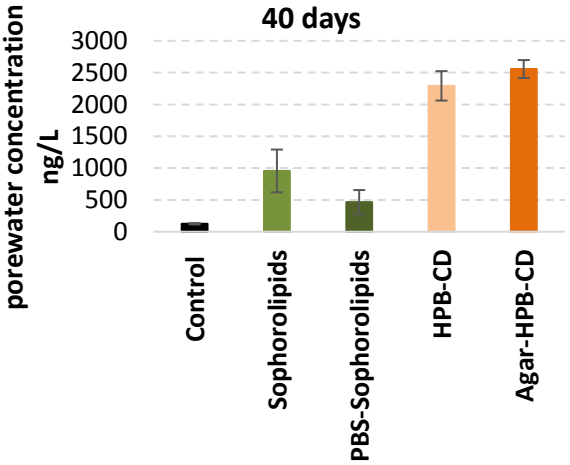

Figure 83 Porewater concentration of *n*-alkanes (ng/L) in "freshly spiked" microcosms after 40 days of incubation under different conditions.

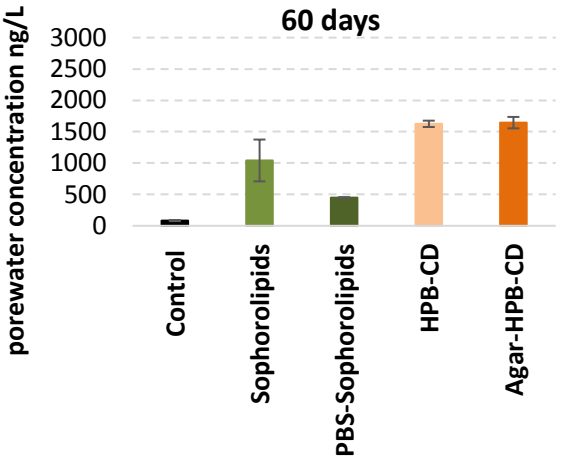

Figure 84 Porewater concentration of *n*-alkanes (ng/L) in "freshly spiked" microcosms after 60 days of incubation under different conditions.

4.5.2 “Weathered” sand slurry microcosms

Results on “weathered” sand allow to evaluate the capabilities of the selected surfactants (encapsulated and not) to desorb the *n*-alkanes from the sand. In these microcosms, in fact, surfactants and PDMS fibers were added 40 days after the crude oil contamination of the sand to allow the complete adsorption of the hydrocarbons.
A constant concentration of approximately 600 ng/L was observed at each sampling in un-amended control, confirming that hydrocarbons adsorption to sand was completed during the 40-day weathering (Figure 85, Figure 86 and Figure 87). Porewater concentration higher than un-amended control was observed in the microcosms amended with free sophorolipids (more than 1000 ng/L) and of both free and encapsulated cyclodextrins (approximately 1500 ng/L). Conversely, no significant effect was observed in the presence of PBS-encapsulated sophorolipids where *n*-alkanes porewater concentration was about 480-630 ng/L.

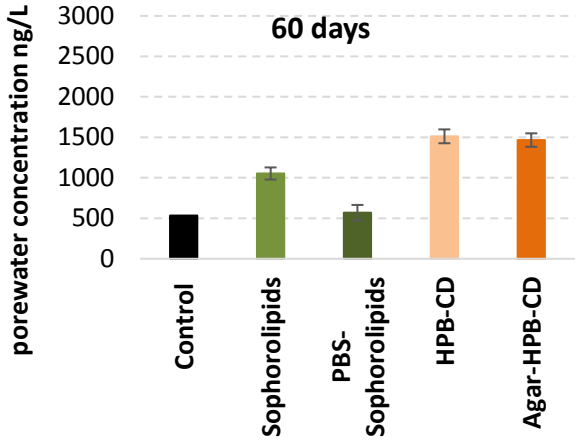

Figure 85 Porewater concentration of *n*-alkanes (ng/L) in “weathered” sand slurry microcosms after 60 days of oil contamination under different conditions.

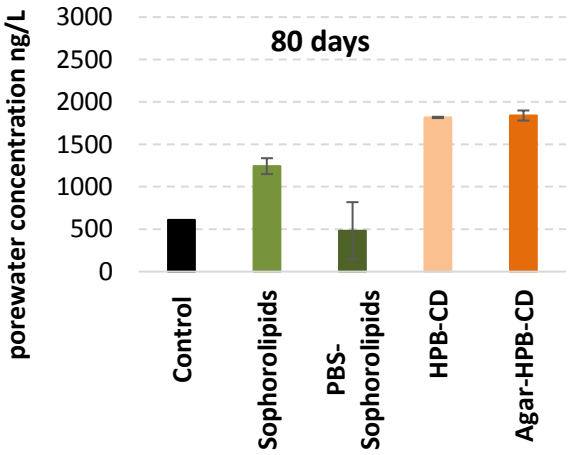

Figure 86 Porewater concentration of *n*-alkanes (ng/L) in “weathered” sand slurry microcosms after 80 days of oil contamination under different conditions.

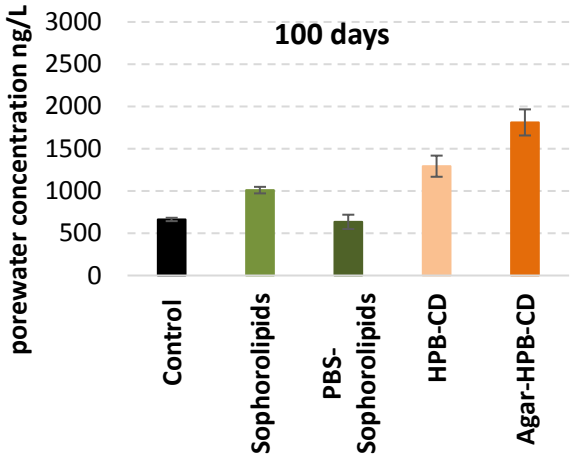

Figure 87 Porewater concentration of *n*-alkanes (ng/L) in "weathered" sand slurry microcosms after 100 days of oil contamination under different conditions.

Therefore, agar-encapsulated HPB-CD were able to desorb *n*-alkanes and increase their bioavailability in measure comparable to free cyclodextrins.

These results are in line with the experiments previously performed for the evaluation of *n*-alkanes porewater concentrations in the presence of not encapsulated surfactants in Gela microcosms.

4.6 Effect of encapsulated surfactants on biodegradation of *n*-alkanes

The effect of encapsulated surfactants on hydrocarbons biodegradation were investigated in weathered sand slurry microcosms inoculated with hydrocarbon-degrading anaerobic microbial communities, previously enriched from Gela anaerobic microcosms.

4.6.1 Weathered sand slurry microcosms

Concentration of *n*-alkanes, pH, redox potential and other microbial activities, i.e. sulfate reduction and biogas production and composition were measured after 6, 11 and 32 weeks of incubation.

4.6.1.1 Concentration of *n*-alkanes

Concentrations of total *n*-alkanes (*n*-C12 to *n*-C32) were evaluated via GC-FID analysis. The results are reported as ratio C/C_0, where C is the concentration of the total *n*-alkanes at determined sampling time (6, 11 and 32 weeks), while C_0 is the concentration at the beginning of incubation (Figure 88**Errore. L'origine riferimento non è stata trovata.**).

During 32 weeks of incubation *n*-alkanes biodegradation was very limited in un-amended sterile (5±1%) and biotic (10±3%) controls, and approximately 63% and 40% in the presence of not encapsulated sophorolipids and not encapsulated HPB-CD, respectively. The encapsulation of sophorolipids in PBS reduced the *n*-alkanes anaerobic biodegradation to approximately 14%, which is consistent with its low/negligible effect on hydrocarbons bioavailability that was observed on weathered contaminated sand. Conversely, *n*-alkanes removal in the presence of agar-encapsulated HPB-CD was comparable than that of the free cyclodextrins, in line with their ability to increase the hydrocarbons porewater concentration.

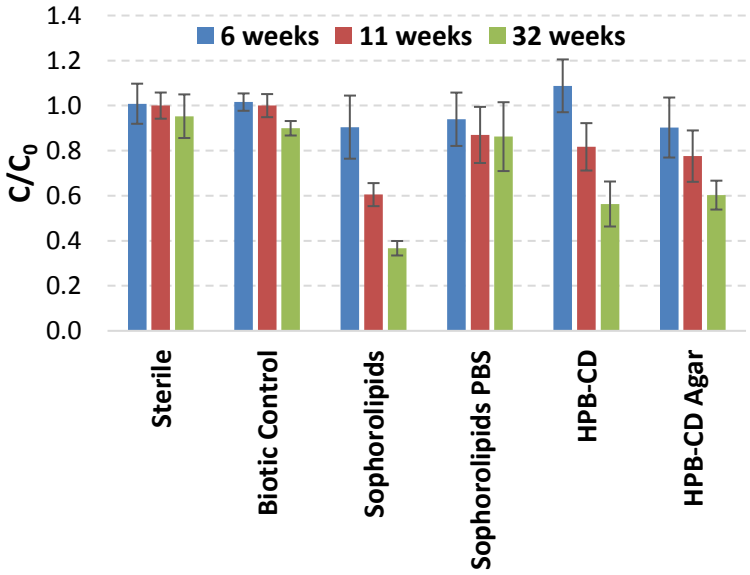

Figure 88 Biodegradation of *n*-alkanes in "weathered" sand slurry microcosms (C/C_0) after 6, 11 and 32 weeks of incubation.

4.6.1.2 pH and redox potential

No substantial change of pH (ranging from 7.8 to 6.3) occurred during 32 weeks of incubation in all tested conditions (Figure 89).

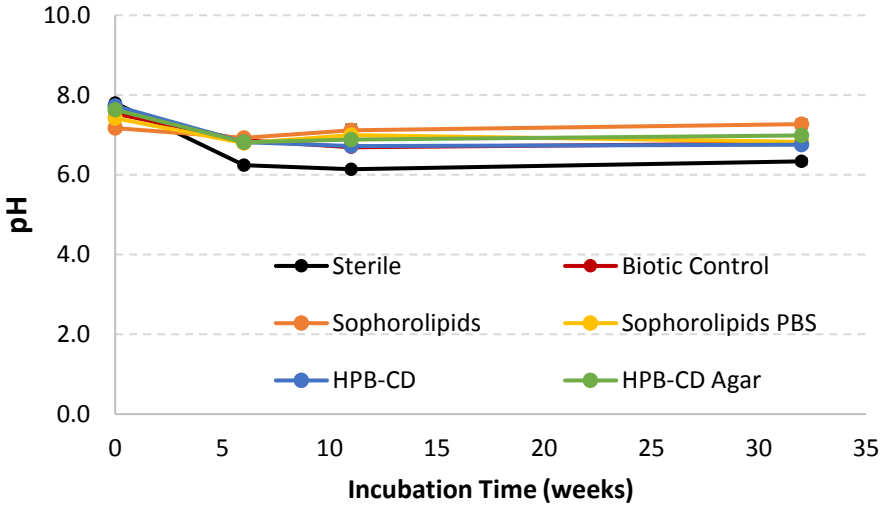

Figure 89 pH during 32 weeks of incubation in un-amended sterile and biotic controls and surfactant-supplemented sand slurry microcosms.

Redox potential was positive during 32 weeks of incubation in both un-amended sterile and biotic controls, supporting the hypothesis that the scarce bioavailability of carbon sources in the sand could limit the anaerobic metabolism.

Instead, redox potential decreased to -100/-250 mV after 6 weeks of incubation in the cultures supplemented with all tested surfactants, both encapsulated and not (Figure 90). This suggests that the supplementation of biodegradable biogenic surfactants stimulated the activity of anaerobic microorganisms, increasing the bioavailability of hydrocarbons and by consuming the biosurfactants or the polymers as energy and carbon source.

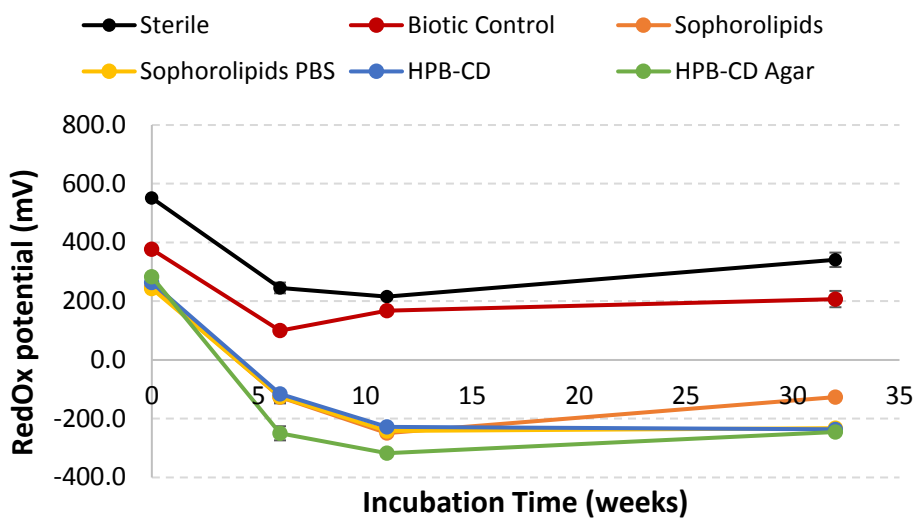

Figure 90 Redox potentials (mV) during 32 weeks of incubation in un-amended sterile and biotic controls and surfactant-supplemented sand microcosms.

4.6.1.3 Other microbial activities

No biogas and methane production were observed after 32 weeks of incubation in all tested conditions, excluding the methanogenic degradation in these cultures.

Figure 91 shows the sulfate reduction as ratio (C/C_0), where C is the sulfate concentration at determined sampling time, while C_0 at the beginning of the incubation.

During 32 weeks of incubation, sulfate removal was absent in the un-amended sterile and biotic controls, where no biodegradation occurred, and negligible in the presence of HPB-CD (approximately 3%).

Conversely, it was stimulated by the supplementation of agar-encapsulated cyclodextrins, where approximately 40% and 50% of sulfate removal was detected after 6 and 32 weeks of incubation, respectively. Since the extent of sulfate reduction was different in the cultures amended with free HPB-CD and agar-encapsulated HPB-CD where approximately the same amount of *n*-alkanes removal occurred, this mechanisms is probably not correlated to the hydrocarbons biodegradation and sulfate-reducing bacteria were not involved directly in the degradation of *n*-alkanes, but they could use agar as a carbon and energy source.

A significant sulfate consumption also occurred after 6 weeks of incubation in the presence of sophorolipids both encapsulated in PBS (approximately 30%) and not encapsulated (approximately 20%). Since approximately the same amount of sulfate reduction occurred in the cultures exhibiting limited biodegradation (i.e. sophorolipids encapsulated in PBS) and in those presenting higher biodegradation activity (i.e. not encapsulated sophorolipids), the extent of sulfate reduction is again not correlated to the hydrocarbons removal, and sulfate-reducing bacteria were not participated in *n*-alkanes removal.

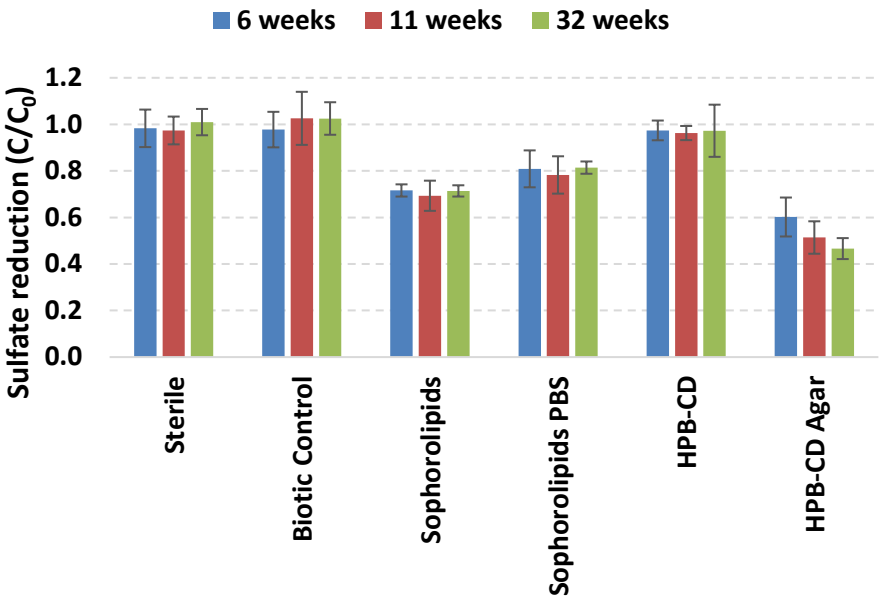

Figure 91 Sulfate reduction after 6, 11 and 32 weeks of incubation in un-amended sterile and biotic controls and in surfactant-supplemented sand slurry microcosms.

5 Conclusions

This PhD research project was aimed to identify an environmental friendly approach to increase oil hydrocarbons bioavailability and biodegradation in contaminated marine sediments. Several surfactants/pollutant mobilizing agents have been selected and applied to slurry microcosms: two microbial surfactants (sophorolipids and rhamnolipids), two types of cyclodextrins (hydroxypropyl- and randomly methylated-β-cyclodextrins; HPB-CD and RAMEB-CD), two commercial soy lecithin products (de-oiled and raw) and bile acids.

Under anaerobic condition, all biogenic agents stimulated the indigenous anaerobic metabolisms in an actual oil-contaminated sediment from Gela harbor. However, after 40 weeks only cyclodextrins (83-88% of *n*-alkanes removal), sophorolipids (78%) and, to less extent, soy lecithins (23-29%) stimulated the hydrocarbons biodegradation, with different selectivity towards *n*-alkanes having different molecular weights. In particular, higher molecular weight hydrocarbons were preferentially degraded in the presence of sophorolipids and soy lecithins, while cyclodextrins were more active on short chain alkanes. Sulfate reduction was absent in un-amended controls, and it was stimulated by the supplementation of some biosurfactants/pollutant mobilizing agents. However, the extent of sulfate reduction was apparently not correlated to the hydrocarbons biodegradation observed. Molecular analysis of the bacterial 16S rRNA gene revealed that the same *Acidobacteria* phylotype was enriched in the anaerobic microcosms where the higher hydrocarbons biodegradation occurred, and was probably responsible for the *n*-alkanes biodegradation.

Under aerobic condition, a partial or complete inhibition of the biodegradation was observed by all biosurfactants in Gela sediment after 28 weeks; whereas only Solec C soy lecithin and HPB-CD were able to increase the *n*-alkanes removal in a crude oil-contaminated sediment from Ravenna.

The two cyclodextrins (RAMEB-CD and HPB-CD) and the soy lecithin Textrol F were the most effective agents able to increase *n*-alkane porewater concentrations in Gela sediment, both immediately after contamination with a crude oil as well as after complete adsorption of hydrocarbons to sediment has occurred.

HPB-CD (bioavailability and biodegradation enhancers) was efficiently encapsulated in agar hydrogel capsules, whereas sophorolipids (biodegradation enhancers) in polybutylene succinate (PBS) microspheres via double emulsion methods (95% encapsulation efficiency).

The release rate of encapsulated HPB-CD in marine water (50-70% release in few hours) was higher than encapsulated sophorolipids (76% release in 40 days), indicating that partial loss of the active agent may occur during sedimentation when hydrogels are used. However, similar release rates (81-87% in 65 days) were observed

when the encapsulated surfactants were incubated in oil contaminated slurry microcosms.

In “freshly spiked” sand microcosms both surfactant formulations were able to reduce remarkably hydrocarbons adsorption to sand thus increasing their porewater concentration, while in “weathered” contaminated sand microcosms only agar-encapsulated HPB-CD were able to desorb *n*-alkanes and increase their bioavailability.

The encapsulation of sophorolipids in PBS microspheres reduced the *n*-alkanes anaerobic biodegradation in sand slurries. On the contrary, the effects of agar-encapsulated HPB-CD on *n*-alkanes removal were comparable than those of the free cyclodextrins, in line with their ability to increase the hydrocarbons porewater concentration.

In conclusion, agar-encapsulated HPB-CD are a promising solution for the enhancement of hydrocarbons (bio)availability and anaerobic biodegradation in contaminated marine sediments.

6 Research period abroad

6.1 Abstract

The large use of methyl tert-butyl ether (MTBE), an octane enhancer and a fuel oxygenate in reformulated gasoline, has led to extensive environmental contamination. Since MTBE is resistant to most physical methods of decontamination, biodegradation may be an effective and low-cost alternative. MTBE removal has been observed in soil and sediment under different redox conditions. However, information on anaerobic MTBE degradation is insufficient and a very few responsible microorganisms have been identified yet.

Aim of my research project at the Rutgers University (New Brunswick, USA) was the study of the anaerobic microbiology and biodegradation of MTBE in slurry microcosms. No anaerobic degradation of this contaminant was obtained during 15 weeks of incubation. However, the addition of methoxylated aromatic compounds (syringic acid or ferulic acid) as co-substrate produced an increase of MTBE removal, resulting in an effective method for the stimulation of MTBE biodegradation *in situ*.

6.2 Introduction

Methyl tert-butyl ether (MTBE) is a volatile colorless liquid, synthesized from methanol and isobutylene. It was introduced in the 1970s as an octane enhancer and was widely used during the 1990s as a fuel oxygenate in reformulated gasoline (Youngster et al. 2010). The largest production of MTBE in the USA has been reached with 9200 million kg/year in 1999 (Häggblom et al. 2007). Although the MTBE is now banned or substituted, it represents at present a wide world groundwater contaminant, mostly in aquifers near urban areas (Pankow et al. 1997, Heald et al. 2005).

MTBE is considered a possible human carcinogen (US EPA 1993), a skin and respiratory irritant, and causes reproductive mutations in zebrafish at concentrations present in polluted environments (Moreels et al. 2006).

Therefore, widespread groundwater contamination with MTBE has become a serious problem that requires efficient decontamination treatments.

However, the physical and chemical properties, including high water solubility and low Henry's Law, make the MTBE a more persistent pollutant than other components of gasoline, such as benzene, toluene, ethylbenzene, and xylene.

Furthermore, this contaminant is relatively resistant to biodegradation due to its tertiary carbon structure and stable ether bond. Initially considered to be completely recalcitrant to microbial degradation, several studies have demonstrated that MTBE can be removed in both aerobic (Salanitro et al., 1994) and anaerobic conditions (Mormile et al., 1994; Wei and Finneran, 2009).

Although different aerobic MTBE-utilizing microorganisms have been isolated, much less is known about anaerobic degradation of MTBE. Previous studies have reported that O-demethylation was the first step in MTBE biodegradation under

sulfate-reducing conditions, as a stoichiometric amount of tert-butyl alcohol (TBA) was found. Several aryl O-methyl ethers that are O-demethylated by acetogenic bacteria, may be also O-demethylated by the MTBE-utilizing enrichment cultures. The addition of the methoxylated aromatic compounds as co-substrates can stimulate the biodegradation rate of MTBE (Youngster et al. 2008).

6.3 Aim

My PhD research period at the "Department of Biochemistry and Microbiology", Rutgers University (New Brunswick, NJ, USA) was aimed to study the anaerobic microbiology and biodegradation of methyl tert-butyl ether (MTBE).

The main experimental activities focused on:

i. the selective enrichment of anaerobic cultures in the presence of MTBE;

ii. the evaluation of MTBE biodegradation in presence of syringic acid or ferulic acid, as co-substrate.

6.4 Materials and methods

6.4.1 Enrichment MTBE cultures

Anaerobic cultures were enriched from sediment microcosms (10% v/v) of two different locations:

(i) the Arthur Kill Inlet (AK), an intertidal strait between New Jersey and Staten Island, NY;

(ii) the New York Harbor (NYH).

The enrichment cultures (Figure 92) were maintained at 28 °C using strict anaerobic technique and sulfidogenic conditions. The sulfate-reducing medium consisted of (g/L): KCl, 1.3; KH_2PO_4, 0.2; NaCl, 1.17; NH_4Cl, 0.5; $CaCl_2*2H_2O$, 0.1; $MgCl_2*6H_2O$, 0.18; $NaHCO_3$, 2.5, and Na_2SO_4, 2.84 (20 mM).

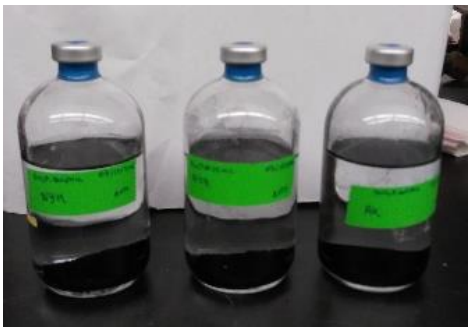

Figure 92 Enrichment cultures prepared under strict anaerobic and sulfidogenic (+ 20 mM sulfate) conditions.

Three sets of microcosms were prepared for each type of sediment (Table 22). One was amended with MTBE (Figure 93A) as sole carbon source (AK MTBE and NYH MTBE), a second with MTBE and syringic acid Figure 93B) as co-substrate (AK

MTBE + SYRINGIC ACID and NYH MTBE + SYRINGIC ACID), and a third with MTBE and ferulic acid (Figure 93C) as co-substrate (AK MTBE + FERULIC ACID and NYH MTBE + FERULIC ACID). In addition, autoclave-sterilized control were prepared from AK sediment microcosms only in presence of MTBE (Sterile Control MTBE). For all sets, the contaminant (MTBE) were added at the final concentration of 100 µM and the methoxylated aromatic compounds (syringic acid or ferulic acid) at 200 µM. The tests were conducted in triplicate.

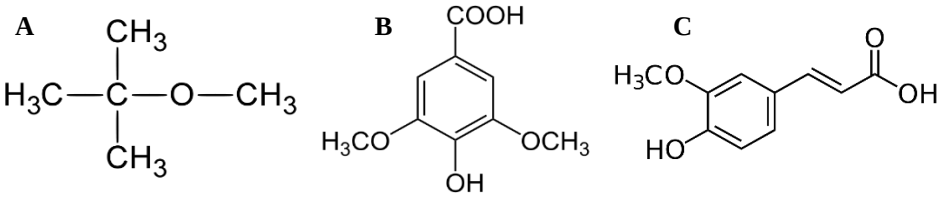

Figure 93 Chemical structures of MTBE (A), syringic acid (B) and ferulic acid (B).

The cultures supplemented with MTBE and syringic acid or ferulic acid were transferred into fresh sulfidogenic medium (5% v/v) after 21 weeks of incubation. The original and the second enrichment cultures were spiked again with MTBE and methoxylated aromatic compounds at the same final concentrations (MTBE: 100 µM, syringic or ferulic acids: 200 µM).

1. MTBE (100 µM)
- AK MTBE
- NYH MTBE
2. MTBE (100 µM) and SYRINGIC ACID (200 µM)
- AK MTBE + SYRINGIC ACID
- NYH MTBE + SYRINGIC ACID
3. MTBE (100 µM) and FERULIC ACID (200 µM)
- AK MTBE + FERULIC ACID
- NYH MTBE + FERULIC ACID
4. MTBE (100 µM) Sterile condition
- STERILE CONTROL MTBE (100 µM)

Table 22 Experimental conditions set up for each type of sediment, Arthur Kill Inlet (AK) and New York Harbor (NYH).

6.4.2 Analytical methods

Concentration of MTBE was determined, as described in Somsamak et al. (2001), through direct aqueous injection of a sample volume of 1 µL using a Hewlett-Packard 5890 series II gas chromatograph (Figure 94) equipped with a flame ionization detector (GC-FID). Compounds were separated on a DB1 capillary column (0.53 mm×30 m, J&W Scientific, Folsom, CA, USA).

Concentrations of methoxylated aromatic compounds (syringic acid and ferulic acid) were measured through an Agilent 1100 series high-performance liquid chromatograph equipped with a reversed-phase Sphereclone column (5μ ODS(2), 250 mm×4.60 mm; Phenomenex, Torrance, CA, USA) (Figure 95). Compounds were eluted with a linear MeOH–H_2O gradient (0.1% acetic acid) in which the MeOH concentration was increased from 35% to 65% at a column temperature of 30°C and a flow rate of 1 mL/min (Youngster et al. 2008).

Figure 94 Hewlett-Packard 5890 series II gas chromatograph

Figure 95 Agilent 1100 series high-performance liquid chromatograph

6.5 Results and discussion

The gas chromatographic analysis has provided the concentrations of methyl tert-butyl ether (MTBE) present in the different enrichment cultures. The results are shown as ratio C/C_0 (Figure 96), where C is the MTBE concentration after 15 or 21 weeks of incubation and C_0 is the concentration at the initial time.

During 15 weeks of incubation, the biodegradation of MTBE was completely absent in sterile control, as well as, in both AK and NYH enrichment cultures amended with

MTBE as sole carbon source (Figure 96A). Conversely, complete MTBE depletion occurred in the presence of the two co-substrates after 21 weeks of incubation (Figure 96B). Therefore, both syringic and ferulic acid promoted the anaerobic degradation of MTBE, suggesting that the same acetogenic bacteria involved in the O-demethylation of methoxylated aromatic compounds are able to mediate the initial ether cleavage and the utilization of the methyl group of MTBE. Sulfidogens may also increase MTBE degradation by utilizing the products of the acetogenic metabolism, so the overall carbon flow in these cultures can be coupled to sulfate reduction (Youngster et al. 2010).

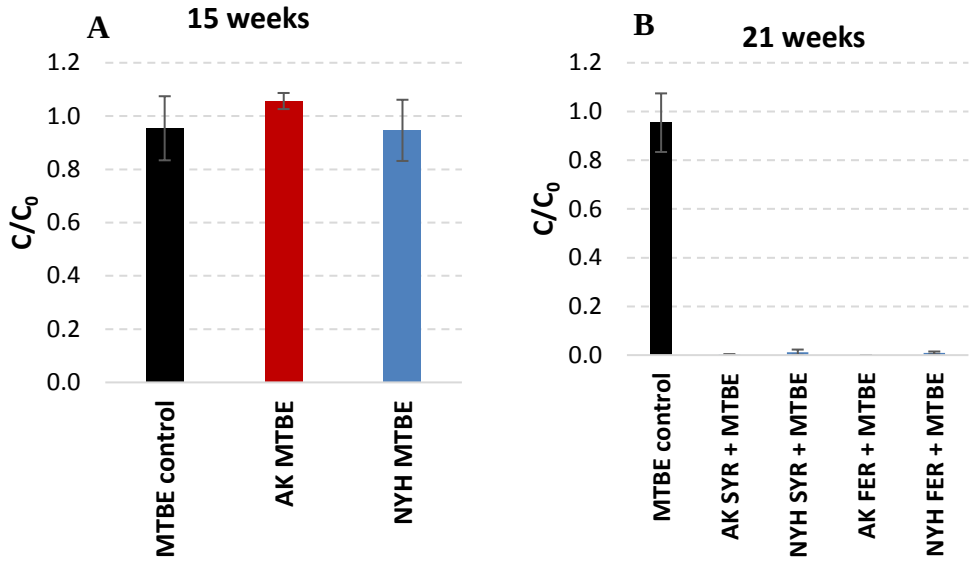

Figure 96: Biodegradation of MTBE in enrichment cultures amended with MTBE as sole carbon source (AK MTBE, AK MTBE and NYH MTBE) after 15 weeks of incubation (A), and in the presence of syringic acid (AK SYR + MYBE, NYH SYR + MTBE) and ferulic acid (AK FER + MYBE, NYH FER + MTBE) as co-substrate after 21 weeks of incubation (B).

The cultures supplemented with syringic or ferulic acid, which showed a complete removal of co-substrates and MTBE, were transferred into fresh sulfidogenic medium (5% v/v). Concentrations of co-substrates (syringic and ferulic acid) and MTBE over time in the original and second enrichment cultures (after the re-spiked with MTBE and methoxylated aromatic compounds) are shown in the Figure 97 and Figure 98Figure 98, respectively.

After 21 days of incubation the original enrichment cultures showed almost complete removal of co-substrate and less than 20% of MTBE degradation in the present of syringic acid as co-substrate, and approximately 40 and 60% of co-substrate removal (in AK and NYH enrichment cultures, respectively) and more than 20% of MTBE degradation in the presence of ferulic acid (Figure 99).

A comparable trend was observed in the second enrichment cultures (5% v/v), where complete co-substrate removal and approximately 20% of MTBE degradation occurred in the presence of syringic acid, while complete (NYH cultures) or approximately 50% (AK cultures) of co-substrate removal and less than 10% of

MTBE degradation was detected in the presence of ferulic acid (Figure 100) after 21 days of incubation.

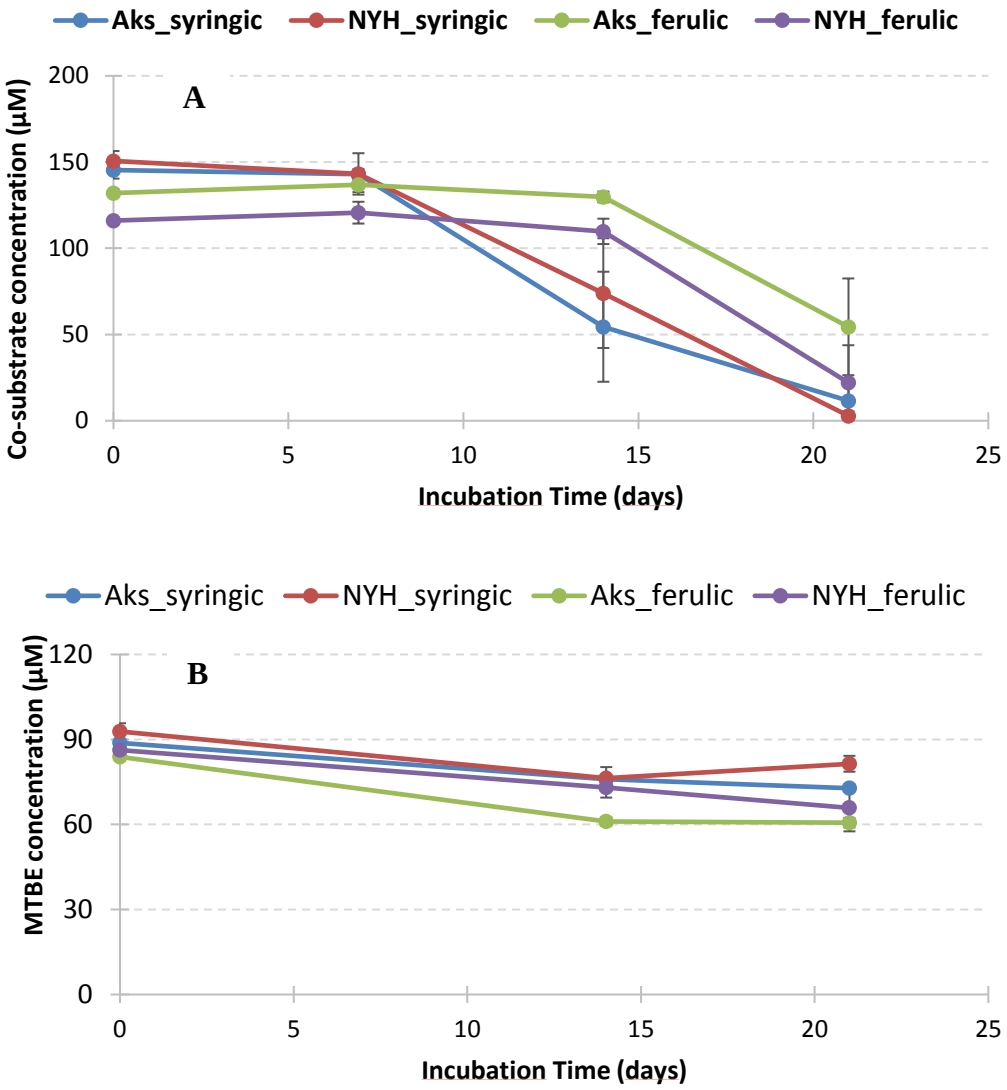

Figure 97 Co-substrates (A) and MTBE (B) concentration over time (days) in original enrichment cultures amended with MTBE and syringic (AKs_syringic and NYH_syringic) or ferulic acid (AKs_ferulic and NYH_ferulic) as co-substrate.

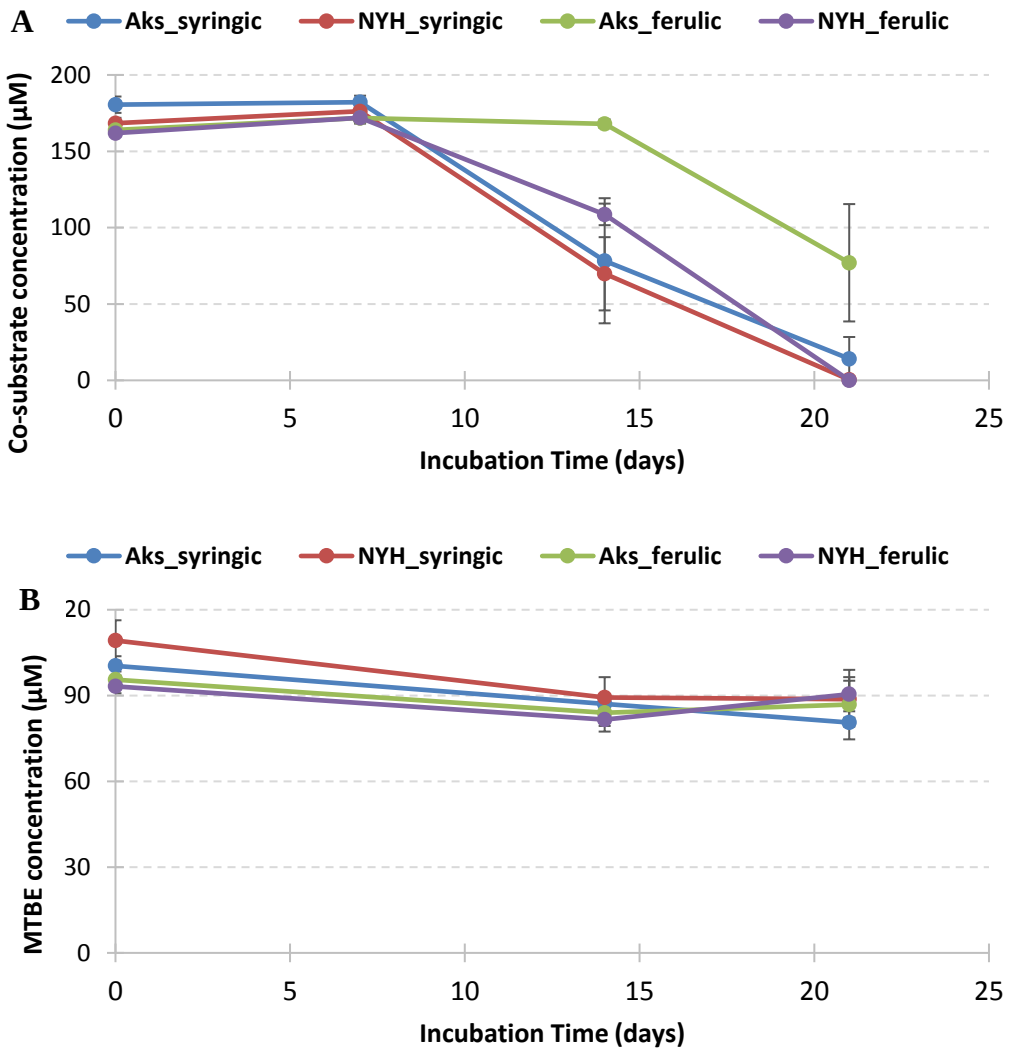

Figure 98 Co-substrates (A) and MTBE (B) concentration over time (days) in second enrichment cultures amended with MTBE and syringic (AKs_syringic and NYH_syringic) or ferulic acid (AKs_ferulic and NYH_ferulic), as co-substrate.

Although short incubation times were analyzed in these experimental tests, a little biodegradation of MTBE has been detected after less than 3 weeks in the presence of both aromatic methoxylated compounds, which were almost completely removed in all cultures. This suggests the hypothesis previously formulated regarding the initial O-demethylation step by acetogenic bacteria. The accumulation of tert-butyl alcohol (TBA) in the enrichment cultures where was observed a little or extensive biodegradation of MTBE, supports this theory (data not shown). Previous studies also showed that the microbial communities of these enrichment cultures can O-demethylate MTBE leading to accumulation of TBA, which was not subsequently biodegraded (Liu et al. 2016).

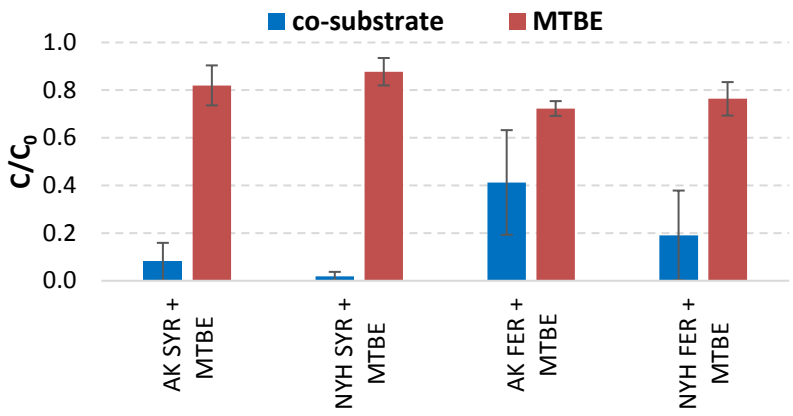

Figure 99 Co-substrate (syringic or ferulic acid) and MTBE biodegradation in original enrichment cultures amended with MTBE and syringic acid (AK SYR + MTBE and NYH SYR + MTBE) or ferulic acid (AK FER + MTBE and NYH FER + MTBE) after 21 days of incubation.

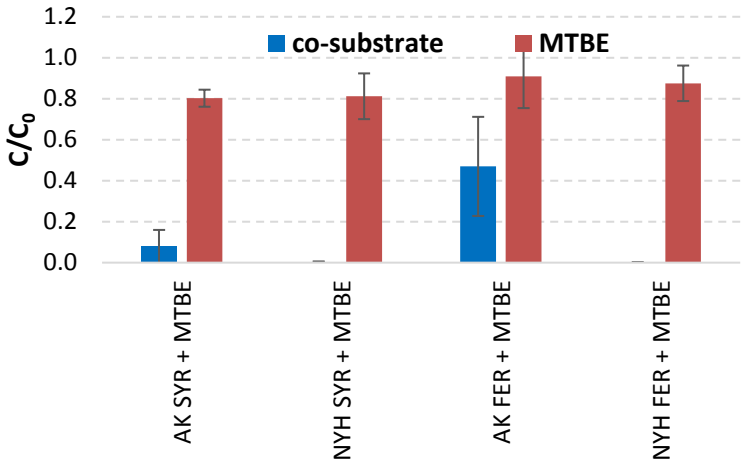

Figure 100 Co-substrate (syringic or ferulic acid) and MTBE biodegradation in second enrichment cultures amended with MTBE and syringic acid (AK SYR + MTBE and NYH SYR + MTBE) or ferulic acid (AK FER + MTBE and NYH FER + MTBE) after 21 days of incubation.

6.6 Conclusions

Anaerobic degradation of methyl tert-butyl ether (MTBE), a synthetic fuel oxygenate, was absent when it is present as sole carbon source in the enrichment cultures. The addition of methoxylated aromatic compounds (syringic acid or ferulic acid) as co-substrate showed a positive effect on MTBE depletion, and it may be an effective method to stimulate the MTBE biodegradation rate *in situ*. However longer incubation time and further investigations are necessary. Characterization of the indigenous microbial communities will be crucial to identify the main bacteria enriched/stimulated in these microcosms, and therefore responsible for MTBE biodegradation.

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AAPG: American Association of Petroleum Geologists.

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