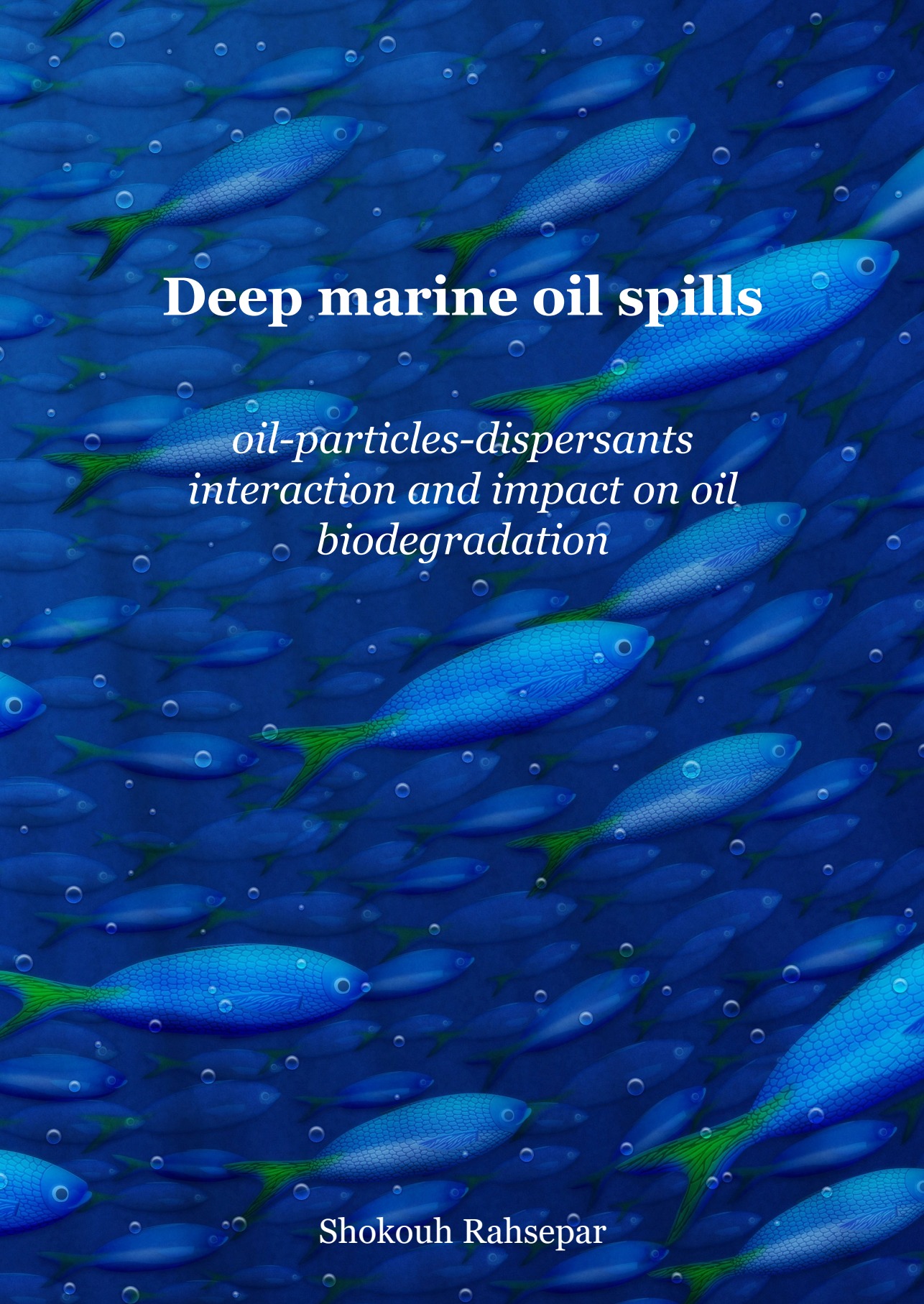

Deep marine oil spills

oil-particles-dispersants interaction and impact on oil biodegradation

Shokouh Rahsepar

Propositions

1. Transferring visible oil from the water surface to invisible oil sedimented on the seafloor and calling this clean-up suits magicians, not scientists.
   (this thesis)

2. Deep marine oil spill clean-up with current physico-chemical based technologies do more harm than good.
   (this thesis)

3. Biology sciences have shown that viruses like *Covid-19* are essential to the evolution of species.

4. Discovering new ways of thinking is more important than discovering new facts.

5. Yoga practices provide the opportunity to live in alignment with our integrity.

6. In an oil-based society, it is difficult to acknowledge the limits of current clean-up technologies for deep marine oil spills.

Propositions belonging to the thesis, entitled

Deep marine oil spills
oil-particles-dispersants interaction and impact on oil biodegradation

Shokouh Rahsepar
Wageningen, 01 December 2021

Deep marine oil spills

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Shokouh Rahsepar

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Dedicated to marine nature

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Summary

1.1 Summary

The world still runs on oil and such an oil-based society with a yearly increase in global demand urge to explore new oil sources. As a result, oil exploration and extraction have been moved to the deeper ocean marine environments. Oceans are a vital part of the ecosystem, contain phytoplankton and other crucial microorganisms responsible for CO_2 absorption, O_2 production, the global carbon cycle, and global climate control. While technologies for oil exploration and extractions in more remote and hard-to-reach locations such as deep marine environments have been developed, technologies for potential oil spill mitigation and clean-up remained under-developed and relied on the knowledge, experiences, and technologies adjusted for surface water oil spills. As a result, mitigation and cleaning up the deep marine oil spills often occur by unadjusted methods and technologies, which can cause unforeseen long-term negative impacts on the oceans and disturbing their performance in ecological and many other ecosystem functions.

One example of a deep marine oil spill was the Deepwater Horizon (DWH) oil spill, which occurred 1500 m below the water surface in the Gulf of Mexico in 2010. The clean-up technology applied for this first very deep marine oil spill was the injection of Corexit, an oil chemical dispersant, at the oil wellhead. Chemical dispersants have been used frequently for mitigating surface water oil spills. The application of dispersants to break down the oil into smaller droplets is based on the formation of smaller oil droplets that more readily dissolve into waters, and the dissolved oil compounds are then biodegraded rapidly by oil-degrading bacteria and other microorganisms. Injection of Corexit at the DWH wellhead as well as to the water surface kept the spilled oil in the water column and reduced the pollution of the shorelines. However, in the aftermath of the DWH and researching the consequences to the marine ecosystem, it was discovered that a significant portion of the oil ended up at the seafloor through unknown or not well understood processes and mechanisms. This lack of understanding was the main driver for setting up a research program called C-IMAGE (Center for Integrated Modeling and Analysis of Gulf Ecosystems) on the fate and effects of such deep-water oil spills, which this PhD research was part of it.

This PhD thesis offers a broad picture and aims at building a step-wise understanding of some critical processes determining the fate and biodegradation of oil in deep marine oil spills and the influence of chemical dispersants in each step. The DWH oil spill was taken as the practical example to refer to in our studies, which are described in six chapters.

Chapter 1 (the introduction) provides a brief overview of the state of the art knowledge of oil spills history, oil composition and compound's behavior in water, weathering processes, clean-up technologies, and compares surface and deep oil spill scenarios. Chapters two to five provide results and discussions of specially designed laboratory experiments to allow a step-wise elucidation of the fate and biodegradation of oil in deep marine spills in the absence and presence of chemical dispersants. In these experiments, the field conditions during and after the DWH spill were mimicked as far as possible, using several sets of batch and microcosms experimental systems. The batch experiments were at the scale of 10–100 milliliters, dedicated to oil compound's dissolution by Corexit, interactions with mineral and organic particles, and impacts on biodegradation of oil compounds. The microcosms experiments were performed in microcosm systems at 10–1000 liters scale dedicated to oil-organic particle-biota interactions mimicking conditions in the water column and in and at the seafloor.

In **Chapter 2**, biodegradation of oil compounds by two bacterial cultures (*n*-alkane and aromatic degraders) in the absence and presence of Corexit with various dispersants to oil ratios was studied. Our results show that the presence of Corexit does not in all cases enhances biodegradation of (all or some) oil compounds. When the Corexit was at the highest application concentration, the biodegradation was inhibited, especially when only the *n*-alkane degrading bacterial culture was present. However, with two bacterial cultures, the inhibition was not effective after ten days. This indicates that the initial inhibition of oil biodegradation can be overcome when different bacteria are present in the environment. We conclude that the observed inhibition is related to the enhanced dissolution of aromatic compounds into the water, inhibiting the *n*-alkane degrading bacteria.

In **Chapter 3**, the biodegradation of oil compounds was studied in batch experiments, while the oil interacted with marine snow, clay particles, and Corexit. Marine snow are organic particles produced by algae and plankton in the upper layers

of the ocean water column, a natural process essential for the whole functioning of the marine ecosystem. During the DWH oil spill and application of chemical dispersants, excessive production of marine snow was observed in the field. Results of our lab experiments show that the presence of marine snow particles enhances oil biodegradation. On the other hand, the presence of Corexit alone or in combination with organic or mineral particles (clay) hampers oil biodegradation. Clay and Corexit have a synergistic effect in increasing the dissolution of benzene, toluene, ethylbenzene, and xylenes (BTEX) compounds in the water and cause a delay in biodegradation of oil compounds through increasing the dissolution of toxic oil compounds. However, the delay in recovery of the biodegradation was reduced by the presence of marine snow particles that adsorbed toxic BTEX compounds from the water phase.

In **Chapter 4**, the effect of marine snow on the biodegradation of oil compounds in the sediment layer is described based on the results from microcosm experiments. These results show that the presence of marine snow reduces the depletion of oil *n*-alkanes by 40% on top sediments due to the preferred biodegradation of marine snow organics. Biodegradation of marine snow reduces the oxygen concentration in the sediment layers, resulting in a lower biodegradation of oil compounds in the sediment.

In **Chapter 5**, invertebrates were included in the microcosm experiments to study the effects of settled oil-associated marine snow. Benthic invertebrate survival and behavior were investigated and compared to the biodegradation of oil compounds in the presence and absence of invertebrates. Bioturbation activities by invertebrates strongly contribute to the oxygenating and nutrient cycling in the sediment layers and ease the aerobic biodegradation. Sedimentation of oil disturbs the benthic ecosystem by slowing down the bioturbation activity and inhibits the biodegradation of the oil compounds in the sediment. The benthic system itself is affected and has a reduced ability to restore conditions favourable for the biodegradation of oil compounds at the seafloor, presumably leading to long-term impacts on sediment ecosystem functioning through the oil at the seafloor.

In **Chapter 6**, the results from previous chapters are discussed and placed in a broader context. In short, deep marine injection of chemical dispersants in the DWH oil spill protected the shorelines and their habitats from oil pollution. However, the

side effect is that this clean-up response puts the deep parts of the marine ecosystem in danger. The most pronounced effects of such an approach are the intensive oil dissolution in the water column, oil components toxicity and stress impulses to the marine (micro) organisms, excessive formation of marine snow, and scavenging of oil particles from the water column to extensive oil sedimentation towards the seafloor. This new understanding generated in our studies suggests that it may be better to design clean-up strategies that are so-called "nature-based", which means using naturally occurring processes that support the self-healing capacities of the environment. This would also entail no or very limited use of chemical dispersants, and if these are used, it should be done with a full understanding of the effects of these dispersants on the complete deep marine system. In this way, seafloor pollution and long-term consequences on the marine ecosystem will be diminished.

We need to improve our understanding of deep marine systems and their response to oil spills much further. The various environmental conditions and processes are far from completely elucidated, and this is needed for a careful evaluation of scenarios for the specific environmental conditions that may dictate the oil spill behavior. This knowledge is required to further minimize damages from future deep oil spills. Though limiting petroleum oil consumption and transition to a non-fossil economy is a fundamental and final solution for oil pollution, however, inevitably, we will still live for decades in a society that will use fossil oil as an energy and chemical resource. If we transform to a renewable and biobased economy, biofuels and bio-lubricants may gradually replace fossil oils; hence, oily compounds are likely to stay in the world's economy. This pleads for budgets to continue this type of research also when the full attention of the global society is focused on climate mitigation and the energy transition. Such research is highly needed to offer the knowledge base to protect our seas and oceans during the coming transition decades and thereafter.

Chapter 1

General introduction

1.1 General background

In our current society, crude oil is still an important energy source and responsible for over one-third of the world's energy supply (Wu and Chen, 2019). Though non-fossil energy is proposed more and more in the light of climate change, the high demand for oil and related intensive exploration and transportation activities will continue still for several decades. These oil explorations, the offshore and onshore petroleum industry will have negative environmental consequences, of which accidental oil spills are important ones. These are expected to continue in the coming decades, despite safety and prevention measures taken by the oil industry. These spills have proven to severely affect our environment by causing adverse impacts due to pollution of soil, water, and sediment systems (Davies and Hope, 2015). In the last decades, much knowledge has been acquired on oil spill prevention and spilled oil attenuation, both in science and technology-related research and in best practices at the oil-related industries (Board and Council, 2014; Burns et al., 2002; Doshi et al., 2018; National Research Council, 2013; Vanem et al., 2008). However, major and new bottlenecks have appeared. Especially more extreme conditions need to be considered, such as those related to large-scale deep oil spill outbursts in the marine environment, such as the Deepwater Horizon (DWH) oil spill in 2010 in the Gulf of Mexico (Murawski, 2020).

With current developments in new routes for oil exploration and transportation, such as north arctic routes coming feasible due to the melting sea ice around the North Pole, new and again extreme condition-related challenges in dealing with oil pollution can be expected. Hence, oil spill prevention and mitigation will remain of the highest environmental importance for the coming decades. This PhD thesis addresses some of these new bottlenecks in oil spill pollution mitigation, especially those related to the DWH oil spill in the Gulf of Mexico, 2010. This introductory chapter presents a brief overview of the state of the art knowledge of oil composition, natural processes to which oil may get subjected to when entering the marine environment, and environmental technologies, including natural attenuation approaches, that can be applied to treat and mitigate environmental risks of spilled oil. After this, knowledge gaps, research objectives, and research questions addressed in the various research chapters of this thesis are described.

1.2 Marine oil spills

Marine oil spills are considered major environmental problems and refer to sudden releases or discharges of petroleum to surface or deep marine waters and result in immediate and long-term environmental damage. A wide range of marine habitats and wildlife targets may get affected in case of a surface spill, while in a deep marine spill, undersea habitats and related natural ecosystem supporting processes get disturbed, and fishery grounds can be severely damaged (Ainsworth et al., 2018). At many locations, petroleum also naturally seeps into the marine environment, often gradually, and it is known that the marine system can adjust itself naturally to mitigate this natural pollution (Head et al., 2006; Kvenvolden and Cooper, 2003). The key herein is that local microbial communities adapt to this and develop mechanisms for taking up and degrading the seeped oil (Kvenvolden and Cooper, 2003). In the case of an anthropogenic oil spill, a sudden and continued increase of oil concentration generally overwhelms the capacities of the marine environment to self-mitigate this pollution, and immediate response actions by man are needed to minimize damage and protect coastal and deep-water resources (Scoma et al., 2017).

1.2.1 History of large oil spills

Marine oil spills are regrettably common worldwide; previously, most spills were surface spills due to shallow water oil explorations, oil transportation, and tanker accidents. However, deep spills were reported in the last few decades due to the increasing number of deep-water offshore oil discoveries and extractions (Kvenvolden and Cooper, 2003; Michel and Fingas, 2015). As said, spills are expected to continue to occur for decades, as long as global societies depend on petroleum for energy and chemical resources. According to an overview of the largest oil spills in Table 1.1, the Gulf War spill (1991) is the largest yet, where 400 million gallons of crude oil were released into the Persian Gulf (Michel, 2011). One year after that spill, the assessments showed oil penetration up to 40 cm in the heavily burrowed sand at the shoreline (Michel, 2011), indicating long-lasting effects on the environment.

The second-largest and the deepest spill is the Deepwater Horizon (DWH) (2010), where 210 million gallons (4.1–4.9 million barrels, 490–584 million liters) of crude

oil were spilled into the Gulf of Mexico (GoM). The spill occurred due to a drilling rig explosion at a water depth of 1500 m below the surface (Crone and Tolstoy, 2010; McNutt et al., 2012b). In the aftermath of this oil spill, new unknowns and bottlenecks appeared about the oil's long-term fate, dispersion into the marine environment, and biodegradation in the water column and sediments. Understanding these bottlenecks improves environmental consequences assessments and response technologies in case of deep oil spills. Below, the various processes relevant for oil in water are described (Section 1.3), followed by explanations on state-of-the-art clean-up technologies (Section 1.4), after which these new bottlenecks will be elaborated further (Section 1.5).

Table 1.1 Overview of the largest oil spills in history (de la Huz et al., 2011; Huang et al., 2019; Jernelöv, 2010).

Oil spill name	Oil spill quantity (million gallons*)	Oil spill depth (meter)
Gulf War (1991), Persian Gulf	400	Surface
Deepwater Horizon (2010), Gulf of Mexico	210	1500
IXTOC I (1979), Gulf of Mexico	140	50
Fergana Valley (1992), Uzbekistan	88	Surface
Nowruz Fields Platform (1983), Persian Gulf	80	Surface
Amoco Cadiz oil tanker (1978), France	69	Surface
Odyssey oil tanker (1988), Canada	43	Surface
Torrey Canyon oil tanker (1967), United Kingdome	36	Surface
Exxon Valdez (1989), Alaska	11	Surface

* 1 gallon = 3.79 liters

1.3 Behavior of oil in water

The oil distribution and fate in marine systems are dominated by *oil composition* and *oil weathering processes* (Pierre, 1980; Tarr et al., 2016). Responses to a spill also impact the fate and behavior of the oil. Petroleum oil is a complex mixture of thousands of chemical compounds, and when released into the marine environment, each group of compounds behaves differently. Therefore, to understand the behavior and fate of a spilled mass of oil, it is needed to address the classification of oil compounds, the response of different oil compounds to the natural marine processes, and changes in oil composition and properties over time.

1.3.1 Oil composition

Crude oil (petroleum) is a carbon-based assembly of compounds that occurs naturally through the thermal decay of compressed organic materials over millions of years. The composition of oil varies depending on the geographical location and oil field where it originates. Each oil field produces an oil with a different composition and proportion of compounds, making each oil unique and easily subjective to fingerprinting (Desideri et al., 1985). Hydrocarbons are the most abundant compounds in crude oil (85-95%) with small amounts of oxygen (0.05-1.5%), nitrogen (0.1-2%), sulfur (0.05-6%) and metals (<0.1%) (Bagheri Garmarudi et al., 2019). Hydrocarbons in crude oil can be classified into three groups, namely *alkanes*, *cycloalkanes*, and *aromatic*s. Figure 1.1 illustrates the chemical structures of these three hydrocarbon groups.

Alkanes or paraffins are saturated hydrocarbons with linear (*n*-alkanes) or branched (*iso*-alkanes) chains. *n*-alkanes are hydrocarbons containing only hydrogen and carbon and are readily subjected to biodegradation when exposed to the most favourable condition for biodegradation, an oxygen-rich (aerobic) environment (Lofthus et al., 2018), while *iso*-alkanes are partially or fully resistant to biodegradation depending on the structure of the molecule (Hasinger et al., 2012; Siddique et al., 2015).

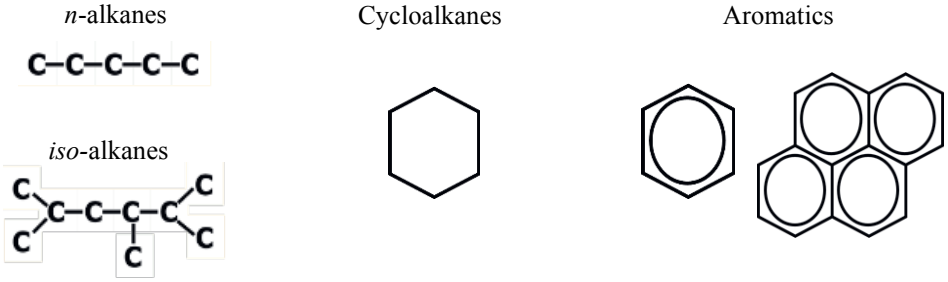

Figure 1.1 Chemical structure of three hydrocarbon groups in oil: *n*/*iso*-alkanes, cycloalkanes, and aromatics.

Cycloalkanes are saturated cyclic hydrocarbons with one or more carbon rings, and each ring may have one or more alkane side chains. Cycloalkanes are stable molecular structures and even more resistant to biodegradation than *iso*-alkanes (National Research Council, 1985).

Aromatics are unsaturated cyclic hydrocarbons (some with alkane branches) containing one benzene ring, such as Benzene, Toluene, Ethylbenzene, and Xylenes (BTEX), or more than one benzene ring such as naphthalene or other Polycyclic Aromatic Hydrocarbons (PAHs). The BTEX group is a highly volatile compound group with high solubility and direct toxicity when organisms are exposed to vapours or solutions with BTEX. Some compounds are even mutagenic (Benzene) to microorganisms, animals, and humans. PAHs are considered the most acute but also chronic toxic components of crude oil due to their hydrophobicity and tendency to accumulate in biota and animal/human tissues and organs, where they form mutagenic intermediary compounds during their mineralization and detoxification (Almeda et al., 2013). Aromatics in the aqueous environment have a diverse resistance to biodegradation, which depends on the stability of their molecular structure and/or their affinity to adsorb to suspended and sediment particles, making them unavailable to degradation by microorganisms (Cerniglia, 1992).

Based on the LC chromatography, the oil composition classifies into four fractions: Saturates, Aromatics, Resins, and Asphaltenes (SARA) (Bissada et al., 2016). Saturates and aromatics are of the greatest concern in terms of human and ecological toxicity. This is because chemicals in the saturated and aromatic fractions are more soluble and mobile in the environment to varying degrees and are more amenable to

degradation. On the other hand, resins and asphaltenes are relatively insoluble and recalcitrant.

1.3.2 Oil weathering processes

When oil is massively spilled into the marine system, it undergoes a continuous series of natural processes that partitions it over the various phases available, i.e., into the air, the water column, and the benthic sediment system. When these processes occur, a fraction of the oil components dissolve, evaporate or biodegrade to carbon dioxide and water (Mishra and Kumar, 2015). These natural processes impact the properties of the remaining oil mass and change "fresh crude oil" to "weathered oil" with the time of residence in the water. Figure 1.2 illustrates a schematic overview of oil weathering processes, and below these processes are described.

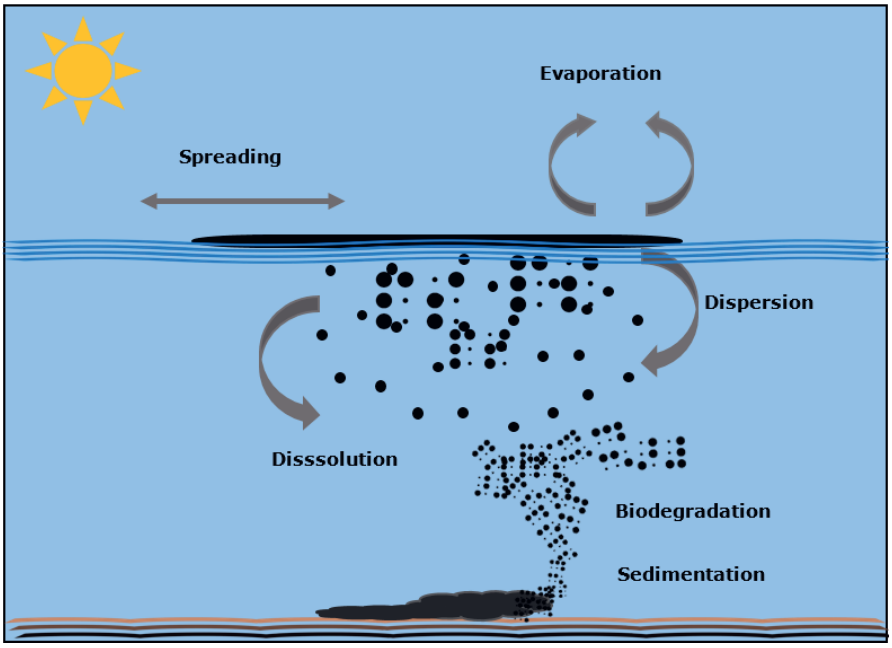

Figure 1.2 Overview of the natural processes oil gets through after a spill in the marine environment (weathering processes).

Evaporation of oil components with a low boiling point (the volatile compounds) to the atmosphere occurs as soon as the oil gets in contact with the atmosphere. The rate of evaporation has a linear relation with temperature and wind speed (Mishra and Kumar, 2015). A consequence of evaporation is that volatile compounds with low density leave the mass of oil, while the non-volatile compounds with higher density are retained; thus, the density of the remaining mass of oil increases, and the oil is transferred from floating at the surface to becoming submerged in the water column.

Spreading refers to the horizontal movement of spilled oil over the water surface and occurs immediately after the oil spill on the surface. The rate of spreading is dominated by the viscosity and surface tension of oil and the environmental temperature (Fay, 1971). Low viscous oil spread more quickly over the surface than those with a high viscosity, and as viscosity is inversely proportional to temperature, oil is spread slowly and less at low-temperature.

Dispersion refers to the horizontal and vertical distribution of spilled oil over the water column by water energy/turbulence. Like spreading, dispersion starts immediately after an oil spill and increases over the spill time, depending on the type of oil and the water energy. The more currents in the water, the more oil is dispersed to micrometer-sized droplets over the water column (Li et al., 2017). The formation of micrometer-sized oil droplets increases oil dissolution and improves oil bioavailability. When dispersed oil droplet sizes are less than 20 μm, they tend to remain in the water column.

Dissolution is the transfer of compounds from bulk oil mass to compounds dissolved in the water column. The soluble oil compounds (lower molecular weight compounds with less than 6 to 8 carbons) dissolve relatively quickly in water, and non-soluble ones (with more than 10-12 carbon atoms per molecule) remain in the pure phase. However, this pure phase fraction can be emulsified as pure phase micro-droplets and are then stabilized in the water column in this form. The dispersion mechanism increases the oil-water interface and can strongly enhance the dissolution process. Dissolution (directly or following dispersion) generally strongly spreads the toxicity to the aquatic environment because the water-soluble fractions of the oil generally have the highest direct toxicity (Liu and Kujawinski, 2015; National Research Council, 2003). The highest solubility is found in smaller aromatic

compounds such as BTEX and lowest/negligible in long-chain saturated and unsaturated aliphatic hydrocarbons and high molecular weight PAHs (National Research Council, 2003). Table 1.2 shows the solubility of some aliphatic and aromatic oil compounds in ambient environmental conditions.

Table 1.2 Solubility of some aliphatic and aromatic oil compounds in water at 25 ºC (McAuliffe, 1966; National Research Council, 2003).

Compound	Solubility (mg/L)
Benzene	1700
Toluene	530
Ethylbenzene	170
Xylene	150
Naphthalene	30
Phenanthrene	1
Dibenzothiophene	1.1
Chrysene	0.002
Propane	62
Hexane	9.5
Dodecane	0.003

Sedimentation results from the settling of weathered oil and can be enhanced by the adsorption or association of oil compounds to the more dense and available suspended particles in the water and eventually settling as mixed oil-mineral aggregates to the seafloor by gravity. Sedimentation is a natural mechanism in the marine system. During an oil spill, dissolved and dispersed oil in the water column get involved in this natural mechanism and may get transferred in this way to deep sea and sediment systems. In general, the interaction of oil with suspended particles takes dispersed oil particles away from the process of re-coalescence (regrouping into larger, more bulky oil particles). Depending on the buoyancy, this either prolongs the presence of oil particles in the water column (in case of low density/high buoyancy sediment particles, i.e., biological flocs) or enforces sedimentation (in case of high density/low buoyancy sediment particles, i.e., clay and silt) (National Research Council, 2003; Payne et al., 2003).

Oil droplets may interact with organic suspended particles such as marine snow or mineral ones such as clay. Marine snow formation and sedimentation is a natural

mechanism in the marine system, which forms in the upper layers of the water and slowly settles down to the sediments and transports the nutrients and carbon sources to the deep (Passow et al., 2012; Turner, 2002). During an oil spill, oil droplets associate with these marine snow and clay particles and get transferred to the deeper layers and eventually reach the sediment system of the seafloor.

Biodegradation is referred to the decomposition of oil into smaller compounds by naturally occurring microorganisms in the marine environment. Oil degrading microorganisms are usually capable of degrading a wide range of oil compounds, especially hydrocarbons, except for certain oil compounds which are not biodegradable (Das and Chandran, 2011; Xu et al., 2018). In general, 60% of crude oil constitutes of saturates hydrocarbon (Ławniczak et al., 2020), the most biodegradable compounds in the oil, which through biodegradation can be converted to non-toxic and less harmful compounds.

Duration of oil biodegradation depends on oil type and environmental conditions such as oxygen or other electron acceptor availability, redox potential, nutrients, salinity, temperature, pressure, and pH (Al-Hawash et al., 2018). Under aerobic conditions, at optimal temperature and high oil-water interface maximizing dissolution, as often occurs at the water surface in warmer climates, biodegradation can be optimal. However, in deep sea layers, low temperatures and reduced oxygen availability are expected to limit oil biodegradation (Vergeynst et al., 2018). Therefore, when comparing the conditions at the surface and in deep marine systems, biodegradation has higher rates in well-aerated dynamic surface water than in a deep sea compartment and the sediment layer. Oil biodegradation occurs mostly under aerobic conditions; however, several species are capable of degrading some oil compounds under anaerobic conditions, although at a much slower rate (Atlas and Hazen, 2011). Saturated hydrocarbons and monocycle aromatics are the most biodegradable compounds (Ławniczak et al., 2020), while Hopanoids are resistant to biodegradation and are often used as a conservative biomarker in oil spill studies (Prince et al., 1994).

1.4 Oil clean-up technologies

Response to an oil spill is a high-priority action during and after an oil spill. A proper clean-up response action generally has considerable impacts on minimizing the

environmental consequences of a spill. Oil spill responses can be categorized into three types of technology: *biological, physical/mechanical*, or *chemical*. In the case of a spill, one or a combination of these responses is generally applied, depending on the oil characteristics and environmental conditions (Chang et al., 2014; National Research Council, 2013). These response technologies are generally taken only for floating or beached oil, in other words, "visible oil". Even in the case of a deep-water spill, these response techniques, especially the chemical ones, are taken to prevent deep oil from surfacing. If oil of a deep spill does not rise to the surface, it generally does not subject to clean-up technology. The main reason is that it is extremely difficult to clean-up dissolved or seafloor settled oil, and current practice is to leave it to nature to heal itself, i.e., by natural attenuation in the water column or by sediment coverage and subsequent slow degradation processes.

1.4.1 Biological technologies

Oil degrading microorganisms are the ultimate cure for oil pollution, and in the case of a spill, biological technologies aim to optimize the environmental conditions to enhance natural oil biodegradation. During oil spills, oil-degrading microorganisms increase logarithmically, and nutrients such as nitrogen and phosphorus may become a limiting factor (Xu et al., 2018). Therefore, the addition of oil-degrading bacteria (bioaugmentation/seeding) and nutrients and oxygen (biostimulation) to stimulate bacterial growth and biodegradation of oil are technologies to facilitate the (enhanced) natural healing (Baniasadi and Mousavi, 2018; Nikolopoulou and Kalogerakis, 2010; Simpanen et al., 2016).

1.4.2 Physical/mechanical technologies

Physical/mechanical methods such as oil skimming from the water surface are typical response techniques used to physically collect the spilled oil (Broje and Keller, 2006; Etkin and Nedwed, 2020). However, the effectiveness of these methods depends on specific environmental conditions such as wind, currents, waves, oil viscosity, and they are less efficient in dealing with extensive oil spills (Etkin and Nedwed, 2020).

1.4.3 Chemical technologies

The aim of chemical techniques is to break down a continuous oil phase into smaller droplets and thereby facilitate oil dilution and dissolution in the water column. The chemical techniques are basically used to shift the "visible oil" from being at the water surface or polluted shorelines to the (invisible) water column. This can be achieved by applying chemical dispersants and chemical cleaning agents on floated or beached types of oil (National Academies of Sciences and Medicine, 2019) or when injected into the deep marine to minimize the risk of oil surfacing. Dispersants do not reduce the amount of oil entering the environment but push the effects of the spill underwater, keeping these away from water surfaces and shorelines.

The mechanism through which dispersants work is similar to detergent soap, based on molecules with a water-compatible head and an oil-compatible tail to reduce the interfacial tension between oil and water, enhancing dispersion. With the help of natural waves or manual energy, the oil mass is broken into smaller oil particles that become dispersed in the water column, and from which soluble compounds can more easily dissolve in the water phase. Theoretically, the application of chemical dispersants can increase oil biodegradability in the water phase. This is either by enhancing the flux of oil compounds through dissolution and diffusion from bulk oil towards the oil-degrading microorganisms or by enhancing direct uptake of compounds by microorganisms that adhere at the oil-water interface. Figure 1.3 illustrates the interaction of one type of chemical dispersant with an oil droplet in water.

The application of chemical dispersants has been a popular spill response option since 1960. One of the earliest records of using dispersants was at the Torrey Canyon oil spill (1967), where a mixture of solvents, detergents, and powdered chalk was used to sink 119,000 tonnes of spilled oil and prevent it from reaching shorelines (Etkin, 1998). Unfortunately, these detergents caused environmental damages because almost 60% of the dispersants contained toxic aromatic solvents (Law, 2011). Since then, the development of a "new generation" of chemical dispersants with a low amount or absence of aromatic compounds, and as a consequence, with lower toxic effects started. Corexit® is one of these "new generation" chemical dispersants with enhanced formulation and is the most applied chemical dispersants worldwide in the last decade (Lessard and DeMarco, 2000).

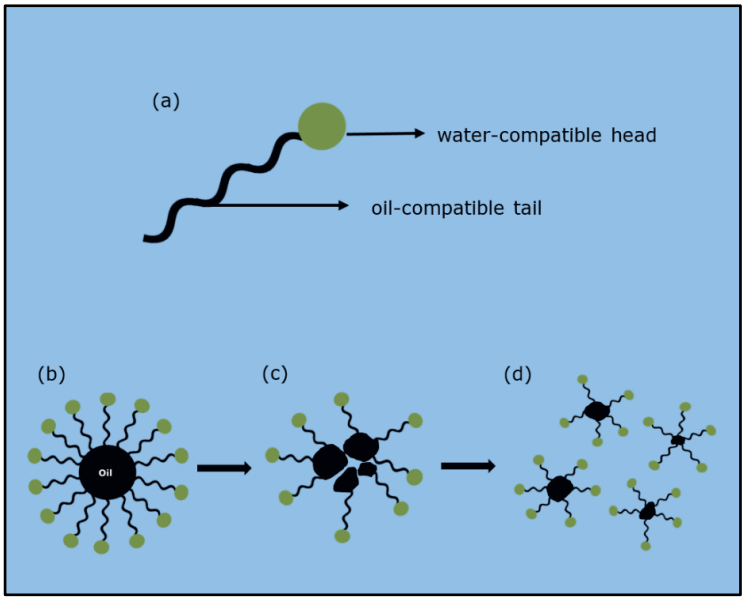

Figure 1.3 Interaction of a dispersant molecule with an oil droplet in water (a) dispersant molecule, (b) dispersant stabilized oil droplet in water, and (c) with small physical energy such as wave energy oil droplet breaks and (d) smaller oil particles surrounded with dispersants.

The application of dispersants to a surface spill results in oil removal from the water surface, avoiding shoreline impacts and rapid dispersion of oil into the top few meters of the water column. Furthermore, under optimal environmental conditions such as temperature and nutrients availability, it improves oil bioavailability and biodegradation (Tremblay et al., 2017). In deep spills, dispersants are applied directly to the spill source as it enters the water. Deep sea currents distribute dispersed droplets over a wide range, reduce the chance of oil surfacing and evaporation of volatile compounds into the atmosphere. These factors reduce human and surface wildlife exposures, increase exposure to crucial habitats such as phytoplankton and zooplankton to soluble hydrocarbons (National Research Council, 2005), increase localized toxicity, and reduce dissolved oxygen within the water column (Coelho et al., 2013; Kessler et al., 2011). However, dispersants are applied on deep spills only once, and our understanding is limited on the impacts on long-term effects and oil fate (Gregson et al., 2021). Therefore, the decisions regarding the use of dispersants involve balancing the potential advantages and disadvantages.

1.5 Oil spill in perspective: surface versus deep spills

As mentioned in section 1.2, oil spill accidents are shifting from surface spills only to surface and deep spills recently. The few deep spills that occurred last decennia (Table 1.1) were characterized by a much more massive oil release than experienced before, released from one point source into the environment, over a time frame of weeks to months, and under extreme conditions such as low temperature, high pressure, limited oxygen, and absence of light. In addition to heterotrophic also autotrophic microorganisms come in these environments into play (Brussaard et al., 2016). Thus, completely new challenges came forward by the DWH accident, and researchers were asked to assess the fate of oil, including its dissolution, suspended particle interaction, and biodegradation under these conditions (Murawski et al., 2020). Research groups such as the Center for Integrated Modeling and Analysis of Gulf Ecosystems (C-IMAGE) is a research consortium focused on these new challenges to provide a comprehensive understanding of the fate and biodegradation of oil in deep spills (C-IMAGE, 2010; Murawski et al., 2020). Comparing the scenarios of surface and deep oil spills and what process phases the spilled oil goes through in these two different situations may guide us to a first understanding of the differences in fate and biodegradation of the spilled oil under deep water conditions, and which abatement routine would be most feasible.

The oil released during surface spills is immediately or shortly after the occurrence of the spill in contact with the dynamic water surface and overlying atmosphere. A major difference between surface and deep spills is that the oil released from a deep spill travels hundreds to thousands of meters through the water column before reaching the surface. Traveling through the water column, the oil first interactions are with the surrounding water environment accompanied by changing pressure, temperature and oxygen concentration regimes, and exposure to different types and concentrations of suspended particles. This traveling can last for hours to days or even weeks, and in some cases, the oil never reaches the surface (National Research Council, 2003; Pesch et al., 2017). Hence dissolution, dispersion, sedimentary particle interactions, and interactions with the aquatic microbial life (in the form of toxic and biodegradative responses) dominate the long-term fate of oil released at great depth. The interactions of oil while traveling through the water column and before being subjected to surface dynamics (waves and wind) and evaporation is expected to alter the oil fate and biodegradation (Murray and Boehm, 2017).

However, the precise effects and consequences for spill abatement were, at the start of the studies reported in this thesis, not known.

During the first stage of a surface spill, "spreading" expands the floating oil layer and facilitates the evaporation of volatile compounds to the atmosphere (Brussaard et al., 2016; Neff et al., 2000). After evaporation, the oil density increases, the oil gets denser (meaning heavier or lower buoyancy) and starts traveling down through the water column, either in the form of bigger oil blobs or in the form of finer emulsified oil droplets, depending on wave dynamics and/or dispersing chemicals applied as part of a mitigation strategy (National Research Council, 2003). Once submerged and traveling downwards, the oil particles get further subjected to ongoing processes such as dilution and dispersion, dissolution, biodegradation, suspended particle interactions, and sedimentation. Thus, in a surface spill, evaporation is the initial weathering process for volatile and lighter compounds to leave the spilled oil mass; however, in a deep spill, these compounds leave the oil mass initially only by dissolution into the water phase and are further diluted by convective and diffusive processes in the water column (Brakstad et al., 2018b; Passow and Stout, 2020).

Depending on conditions at a deep spill, high pressure at the point of release may facilitate the formation of smaller oil droplets and further enhanced by pressure gradient induced dispersion (Malone et al., 2018), which greatly enhances the dissolution of soluble and volatile compounds of the oil into the water column. The larger oil droplets travel up to the water surface while smaller droplets remain suspended in the water column and form submerged oil plumes (Spier et al., 2013). Such suspended oil droplet plumes interact with natural marine sedimentary processes such as the formation and settlement of marine snow or mineral particles slowly settling to the sediment system at the seafloor. How these interactions occur and how these affect oil biodegradation and the long-term fate of the oil is still a major void in current oil spill knowledge (Brakstad et al., 2018b; Daly et al., 2016).

Despite this lack of knowledge, deep oil spill mitigation responses have been accompanied by deep marine injection of chemical dispersants, close or into the source of the spill, though effects on fate and biodegradation of the oil were unclear. In the DWH oil spill, unforeseen phenomena occurred, such as an enormous formation of marine snow in the water column and high interaction with clay

particles leading to a strongly enhanced deposition of oil towards the sediment system at the seafloor (Romero et al., 2017). In this case, it was difficult to assess whether short and long-term biodegradation of the oil was enhanced, decreased, or remained indifferent due to the deep marine injection of chemical dispersants (Davis, 2017; Kleindienst et al., 2015b; Romero et al., 2017).

The overall aim of the studies described in this thesis is to better understand how oil biodegradation and fate are influenced by conditions occurring naturally and under mitigation responses in situations of deep oil spills. More specifically, the focus has been put on how the spilled oil interacts with various system components such as organic and mineral particles, how these interactions influence oil biodegradation, and how chemical mitigation (application of dispersants) influences all individual processes and the overall fate of the oil. Various knowledge gaps already introduced above are addressed in a number of research objectives and research questions and further elaborated in the thesis outline below.

1.6 Thesis outline

Currently, the fate and biodegradation of oil in deep oil spills is not clearly understood, and this PhD thesis offers a step-wise understanding of some key processes impacting the oil biodegradation in deep marine spills. The **main research objective** of this thesis is to better understand oil biodegradation and associated processes that can occur under conditions apparent at deep oil spills.

The Deepwater Horizon (DWH) oil spill was taken as the practical example to refer to in our studies. Oil biodegradation is studied in the water column and sediment, with the presence and absence of chemical dispersants, mineral and organic suspended particles (clay and marine snow), and invertebrates. Figure 1.4 provides an overview of this PhD thesis and the research chapters.

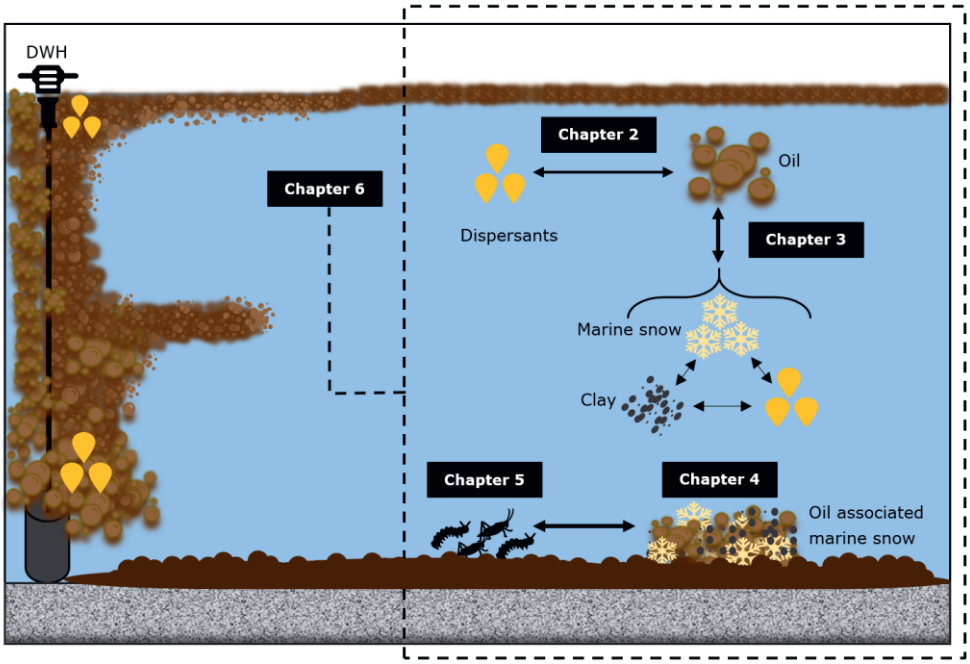

Figure 1.4 Overview of this thesis.

In our studies, the field conditions during and after the DWH spill were –as far as possible- mimicked using several sets of batch and microcosm experimental systems. A batch system is at the scale of 10 – 100 mL, dedicated to microscale oil component biodegradation, adsorption, and particle interaction processes. A microcosm system at 10 liters to 1000 liters scale dedicated to oil-sediment-water-biota interactions mimicking conditions in the water column and seafloor. Moreover, Corexit as the dispersant, Macondo oil, and synthetic seawater were taken as materials to mimic the conditions in the Gulf of Mexico during the DWH oil spill in 2010. Kaolin clay was used in studies focussing on clay-oil interactions, and an artificial (self-made) marine snow polymer matrix was used to research the effects of this material on oil-organic-clay aggregation processes and the subsequent effects on the sedimentation of these agglomerated particles, the biodegradation, and eco-toxicity of the oil in the formed sediment layer.

The following studies were performed to resolve missing knowledge on deep oil spill biodegradation and the fate and effects of the oil in the marine water column and sediment environments. Different dispersants to oil ratios (DOR) were tested in a

series of batch experiments to better understand the effect of chemical dispersants on crude and weathered oil biodegradation. These results are described and discussed in **Chapter 2**. Especially the effect of dissolution of more soluble oil fractions is described and how different microbial strains respond to this in terms of toxicity and biodegradation activity.

In **Chapter 3**, the previous study was extended to study the effects of the presence or absence of organic particles such as marine snow and mineral clay particles during oil spills and for different oil spill abatement strategies. A series of batch experiments were conducted, and the interactions of oil with kaolin clay (representing the clay particles present in the Mississippi discharge water that entered the Gulf of Mexico) were investigated under different dispersants to oil ratios (DORs). Oil-clay particle agglomeration and its effect on oil sorption/dissolution and subsequent biodegradation of different oil fractions are discussed in this chapter.

The effect of marine snow on oil biodegradation in the sediment layer was tested in a microcosm setup. In the first microcosm experiments, the oil biodegradation in the absence and presence of marine snow was tested, and the results are shown and discussed in **Chapter 4**. In the second set of microcosm studies, benthic invertebrates were included to study the effects of settled oil-associated marine snow on them. Benthic invertebrate survival and behavior were investigated and compared to the oil biodegradation in the presence and absence of invertebrates. These results are presented and discussed in **Chapter 5**.

The results of this thesis are generally discussed and placed in a broader context in **Chapter 6**. Especially the new knowledge generated in our studies on processes that occur in deep spills and during chemical responses to deep oil spills are discussed, both from scientific and practical perspectives. Finally, the chapter concludes with recommendations for future research on oil spill remediation and risk control and do's and don'ts in the practice of responses to future deep marine oil spills.

Chapter 2

Chemical dispersants: oil biodegradation friend or foe?

A modified version of this chapter has been published as

Rahsepar, S., Smit, M.P.J., Murk, A.J., Rijnaarts, H.H.M. & Langenhoff, A.A.M. (2016). Chemical dispersants: oil biodegradation friend or foe? Marine Pollution Bulletin, 108, 113-119.

Abstract

Chemical dispersants were used in response to the Deepwater Horizon oil spill in the Gulf of Mexico, both at the sea surface and the wellhead. Their effect on oil biodegradation is unclear, as studies showed both inhibition and enhancement. This study addresses the effect of Corexit on oil biodegradation by *n*-alkane and/or aromatic degrading bacterial culture in artificial seawater at different dispersant to oil ratios (DORs). Our results show that dispersants addition did not enhance oil biodegradation. At DOR 1:20, biodegradation was inhibited, especially when only the *n*-alkane degrading culture was present. With a combination of cultures, this inhibition was overcome after 10 days. This indicates that initial inhibition of oil biodegradation can be overcome when different bacteria are present in the environment. We conclude that the observed inhibition is related to the enhanced dissolution of aromatic compounds into the water, inhibiting the *n*-alkane degrading bacteria.

Keywords

Biodegradation; Dispersants; Oil spill; Enhanced dissolution

2.1 Introduction

Large oil spills in the marine environment have been occurring since the early 1900s when oil and gas industries started extracting oil offshore and using oil tankers for transportation (Burger, 1997). From 1970 to 2012, approximately 5.75 million tons of oil were released into the oceans as a result of tanker incidents (ITOPF, 2020). The release of oil into the marine environment is the main cause of marine pollution (Holliger et al., 1997). The largest accidental marine oil spill in the history of the petroleum industry is the Deepwater Horizon oil spill in April 2010 in the Gulf of Mexico (McNutt et al., 2012a).

Once the oil is discharged into the marine environment, the properties of the spilled oil change due to a variety of physical, chemical, and biological processes. These processes, collectively known as weathering (Boehm et al., 2008; Wardlaw et al., 2008), change the oil's composition, physical/chemical behavior, and toxicity. An important weathering process is evaporation which transfers light-weight and more volatile compounds to the atmosphere (Mansuy et al., 1997). Generally, this happens at the sea surface during the first few hours after a spill (Mansuy et al., 1997).

Another important weathering process is biodegradation, by which bacteria partially or completely transform oil to compounds that can be further degraded and become more soluble in water (Lepo et al., 2003; Pontes et al., 2013). The biodegradation rate depends on many parameters, such as temperature, electron acceptors and nutrients, the composition of the oil, and the active microbial population. Moreover, the presence of other compounds influences the biodegradation rate by either enhancing or inhibiting the microbial conversion or by changing the bioavailability of oil and its toxicity to bacteria. Therefore, weathering processes iteratively affect the ongoing degradation of the oil.

Traditionally, oil spill management often includes applying chemical dispersants on oil slicks to remove these from the water surface. Dispersants reduce the interfacial tension between the oil and seawater and stabilize the smaller oil droplets that are formed. As a result, the bioavailability of the oil increases, which can enhance oil biodegradation. At oil spills like the Deepwater Horizon, dispersants were injected into the oil wellhead (Kujawinski et al., 2011). In this case, the application of dispersants creates oil micro-emulsions, benzene, toluene, ethylbenzene, and xylene

(BTEX), and polycyclic aromatic hydrocarbons (PAHs) compounds dissolve faster. Since micro-emulsions cannot be separated easily from the water phase, this often leads to the higher apparent water solubility of these compounds (Zheng and Obbard, 2002).

Whether the addition of dispersants enhances or decreases oil degradation is not yet clear as in literature, contradicting results were published (Brakstad et al., 2015; Lindstrom and Braddock, 2002). Previous studies showed the positive effect of Corexit on the oil biodegradation by mixed bacterial communities (Hazen et al., 2010; Valentine et al., 2012). However, some other studies have reported a negative effect of Corexit on oil biodegradation (Hamdan and Fulmer, 2011). Clearly, the scientific and technical understanding of the physicochemical interactions taking place and how they affect subsequently biological activities is not (yet) complete.

We hypothesize that these contradicting results in the literature may at least partially relate to the chemical composition of different types of the oil (crude oil, weathered oil), the absolute and relative concentration of oil and dispersants, and the characteristics of the microbial population (presence or absence of active *n*-alkane and aromatic degraders) applied in the experimental work.

The aim of this study is a proof of principle of the effect of dispersants on oil degradation. We have systematically assessed the biodegradation of crude and weathered oil in the water phase with the different dispersant to oil ratios (DORs) and different bacterial cultures for either high or low energy hydrodynamic conditions by using dynamic or static experimental systems. This allows us to get insight into the competing effects of increased bioavailability on the biodegradation process under various conditions relevant to the marine environment. This will improve our understanding of the fate of chemically dispersed oil which is essential for assessing the added value of dispersants application.

2.2 Materials and methods

2.2.1 Oil and chemical dispersants

Macondo surrogate oil (MC252), kindly provided by BP (BP Gulf Science Data, 2013), was used in this study. MC252 is classified as a light sweet crude oil and contains a high number of light hydrocarbons, saturated *n*-alkanes, PAHs with a low sulfur content (Ryerson et al., 2011). To simulate the impact of the evaporative weathering process, the oil was artificially evaporated to 30% weight loss. The oil was continuously stirred with a magnetic stirrer at 70 °C for 3 h, while a light flow of nitrogen gas constantly flowed over the oil's surface. This resulted in a viscous oil with less light hydrocarbons and aromatic compounds, and without hydrocarbon compounds smaller than C14 (Zhanfei et al., 2012).

Corexit ®EC9500A (Nalco Holding Company, USA) was applied as a chemical dispersant. Dispersant solutions were prepared by diluting Corexit into demineralized water to make different ratios. Before addition to the batch bottles, the dispersant solutions were filtered sterilized (0.2 μm).

2.2.2 Bacterial cultures

Rhodococcus qingshengii TUHH-12 (DSMZ No. 46766), an *n*-alkane degrading culture, was used as inoculum in our experiments. The culture was isolated at the Technical University of Hamburg Harburg, Germany, from a seawater sample collected in Spitzbergen, Norway, with an optimal growth temperature of 28 °C. This culture was maintained in a mineral medium with *n*-hexadecane as the sole carbon source. The medium consisted of 2.6 g Na_2HPO_4, 1.33 g KH_2PO_4, 1 g $(NH_4)_2SO_4$, and 0.20 g $MgSO_4 \cdot 7\ H_2O$ dissolved in 1000 mL demineralized water. The medium was adjusted to pH 7. After sterilization, 5 mL of trace element solution and 1 mL of vitamin solution were added. The composition of both solutions is mentioned in the experimental setup section. The bacterial culture was incubated for three days, and four days prior to the experiments, the culture was transferred into artificial seawater amended with medium salts and *n*-hexadecane as a carbon source. This resulted in an active culture in its optimal growth phase, as controlled by measuring the Optical Density (OD) with a spectrophotometer (DR3900, Hach Lange) at a

wavelength of 600 nm. An OD of 0.98 was taken as a culture in its optimal growth phase.

Pseudomonas putida F1 is an aromatic degrading culture and was purchased as a freeze-dried culture from the German collection of microorganisms and cell cultures (DSMZ, No. 6899). After activation according to the DSMZ suggested procedure (DSMZ, 2014), *P. putida* F1 was transferred to the DSMZ medium No. 457 and supplemented with toluene as a sole carbon source. Four days prior to the experiments, the culture was transferred into seawater amended with medium salts and toluene. This resulted in an active culture in its optimal growth phase, as controlled by measuring the OD. An OD of 0.305 was taken as a culture in its optimal growth phase.

2.2.3 Experimental setup

The growth medium consisted of (per litre of water) 10.4 g Na_2HPO_4; 5.32g KH_2PO_4; 4 g $(NH_4)_2SO_4$; 0.8 g $MgSO_4.7H_2O$; 1 mL of trace element solution (2 g/L $FeCl_3.4H_2O$; 2 g $CoCl_2.6\ H_2O$; 1 g/L $CaCl_2.2H_2O$; 0.5 g/L $MnCl_2.4H_2O$; 30 mg/L $CuCl_2.2H_2O$; 50 mg/L $ZnCl_2$; 50 mg/L HBO_3; 90 mg/L $(NH_4)_6Mo_7O_{24}.4H_2O$; 100 mg/L $Na_2SeO_3.5H_2O$; 50 mg/L $NiCl_2.6H_2O$; 1 g/L EDTA; 1 mL/L 36% HCl); resazurin 0.5 g/L, and 1 mL of vitamin solution (0.106 mg/L biotin; 0.005 mg/L folic acid; 0.0025 mg/L pyridoxal-HCl; 0.015 mg/L lipoic acid; 0.0125 mg/L riboflavin; 0.266 mg/L thiamine-HCl; 0.413 mg/L calcium D pantothenate; 0.0125 mg/L cyanocobalamin; 0.0125 mg/L p-aminobenzoic acid; 0.0125 mg/L nicotinic acid). To avoid precipitation while mixing sea salt and growth medium, the phosphate and sulphate solutions were prepared separately and subsequently mixed while stirring.

Biodegradation of crude and weathered oil was tested in 125 mL bottles. The bottles contained 20 mL of medium, suitable for bacterial growth, in artificial seawater (32 g of artificial coral sea salt, AquaHolland, The Netherlands) in 1 L demineralized water. After autoclaving for 25 min at 121 ^oC, the bottles were opened in a laminar flow cabinet, and the filter-sterilized vitamin solution was added. Depending on the condition, 0.1 g crude or weathered oil, chemical dispersant (DOR 1:20 or 0:1), and 2 mL bacterial culture were added. The bottles were sealed with a Viton rubber stopper (Rubber BV, Hilversum, The Netherlands) and closed with aluminium caps. The bottles were incubated at 20 ^oC in the dark on a rotary shaker (120 rpm)

(dynamic conditions) or under static conditions. Sterilized abiotic controls were taken along as well.

2.2.4 Oxygen consumption of crude oil at different DORs

Oxygen consumption by *R. qingshengii* TUHH-12 was measured in batches with different DORs. The tested DORs (w/w) were 1:1, 1:10, 1:20, 1:50, 1:100, 1:1000, and 0:1 (no dispersant). These ratios were prepared by adding 5000, 500, 250, 100, 50, 10, 5, and 0 mg dispersant per L of the solution to which 0.1 g crude oil was added. Oxygen concentration was measured regularly, and pure oxygen was added when the oxygen concentration in the gas phase dropped below 10% (v:v). Based on the results, DORs 1:20 and 0:1 were chosen for our further experiments.

2.2.5 Effect of chemical dispersants on the biodegradation of BTEX and *n*-alkanes

A total of 6 sets of experiments were conducted, with either *R. qingshengii* TUHH-12 or *P. putida* F1, dynamic or static, and abiotic control (Table 2.1). Each set contained 6 conditions, representing different types of oil (crude, weathered, or no oil) and two DORs (1:20 and 0:1), and were tested in duplicate.

Table 2.1 Overview of the experimental sets.

	Crude oil	Weathered oil	No oil	Dynamic	Static
R. qingshengii TUHH-12	✓	✓	✓	✓	✓
P. putida F1	✓	✓	✓		✓
R. qingshengii TUHH-12 and *P. putida* F1	✓	✓	✓		✓
Abiotic control	✓	✓	✓		✓

During the incubations, the batches were monitored by regular analyses of oxygen and carbon dioxide in the headspace. In addition, the headspace of the batches was sampled for analyses of BTEX at selected time intervals, and the complete content of a batch was sacrificed for solvent extraction followed by analyses of *n*-alkanes (C11–C40).

2.2.6 Chemical analyses

Oxygen and carbon dioxide were analysed by gas chromatography (GC, Shimadzu (Shimadzu, Kyoto, Japan)). Headspace samples of 50 µL taken from batch bottles by glass syringe were injected directly into the GC. The GC was equipped with two packed columns in parallel (Porabond Q, 60-80 mesh, 2 m length, 3 mm internal diameter and Molsieve 5a, 60-80 mesh, 2 m length, 3 mm internal diameter; Varian, Middelburg, The Netherlands) and a thermal conductivity detector. Temperatures were constant with an injector of 120 °C, a column of 75 °C, and a detector of 150 °C. Helium was used as the carrier gas at a constant flow rate of 30 mL/min. Calibration was done with gas samples containing 2.98% oxygen and 24.80% carbon dioxide.

Concentrations of BTEX compounds were analysed on a GC (Fisions 8000) equipped with a CP-Sil 8CB column (25 m × 0.53 mm × 5.0 µm, Chrompack, Middelburg, The Netherlands). Samples were extracted from the headspace of the batches (10 mL) by solid-phase microextraction (SPME) with a 100 µm polydimethylsiloxane (PDMS) coated fibre. The fibre was placed in the headspace of the batch bottles for 2 min and desorbed in the injection port of the GC at 200 °C. The GC was operated in constant flow mode (2.5 mL/min). The column temperature increased from 40 °C to 130 °C at 5 °C/min, followed by a constant temperature for 5 min. BTEX compounds were detected with a Flame Ionisation Detector (FID) at 300 °C. The GC performance was tested by starting each GC sequence with an external standard. The identity of each individual BTEX compound was determined by using the retention time in a standard solution. Based on the peak area of each individual BTEX compound, the relative concentration of each compound was calculated and finally summed to calculate the total relative concentration of the BTEX compounds per sample.

Prior to *n*-alkanes quantification with gas chromatography, the samples were extracted with acetone and *n*-hexane, according to method NEN 5733. The *n*-hexane was dried with Na_2SO_4, and 1 µL was injected automatically by an autosampler into an HP 6890 GC with a CP-SIMDIST column (10 m × 0.32 mm × 0.1 µm) and Flame Ionization Detector at 320 °C with helium as the carrier gas. Following 5 min at an initial temperature of 40 °C, the temperature was increased at 10 °C/min to a final temperature of 300 °C. Each individual *n*-alkane (C11-C40) was determined using

the retention time in a standard solution. Based on the peak area of each individual *n*-alkane, the relative concentration of each *n*-alkane was calculated per data point and finally summed to calculate the total relative concentration of the *n*-alkanes. *R. qingshengii* TUHH-12 is an *n*-alkane degrading culture. Therefore we report the degradation of *n*-alkanes only and not of total petroleum hydrocarbons (TPH).

2.3 Results

2.3.1 Biodegradation of crude oil at different DORs

To determine the extent of crude oil biodegradation by *R. qingshengii* TUHH-12, the oxygen consumption was measured daily in batches with different DORs (Table 2.2). The oxygen consumption rate changed with the amount of Corexit. At DORs of 1:10-1:100, oxygen consumption rates were approximately 0.16 mmol/day. At a high DOR of 1:1, the lowest oxygen consumption rate was seen (0.13 mmol/day), while oxygen consumption rate was highest with DORs of 1:1000 and 0:1 (0.36 mmol/day).

Table 2.2 Oxygen consumption rates at different DORs.

DORs	1:1	1:10	1:20	1:50	1:100	1:500	1:1000	0:1
Corexit (mg/L)	5000	500	250	100	50	10	5	0
O_2 consumption (mmol/day)	0.13 ± 0.08	0.17 ± 0.05	0.17 ± 0.02	0.17 ± 0.01	0.15 ± 0.02	0.25 ± 0.02	0.34 ± 0.03	0.37 ± 0.08

Based on these results, DORs of 1:20 and 0:1 were chosen for further experiments. The targeted DOR was 1:20 as it was applied in the Gulf of Mexico in the response phase after the Deepwater Horizon oil spill in 2010.

2.3.2 Dispersants effect on oil biodegradation by *R. qingshengii* TUHH-12

The degradation of both crude and weathered oil at DORs of either 1:20 or 0:1 by *R. qingshengii* TUHH-12 was monitored by measuring the oxygen consumption and the concentration of *n*-alkanes under both dynamic and static conditions.

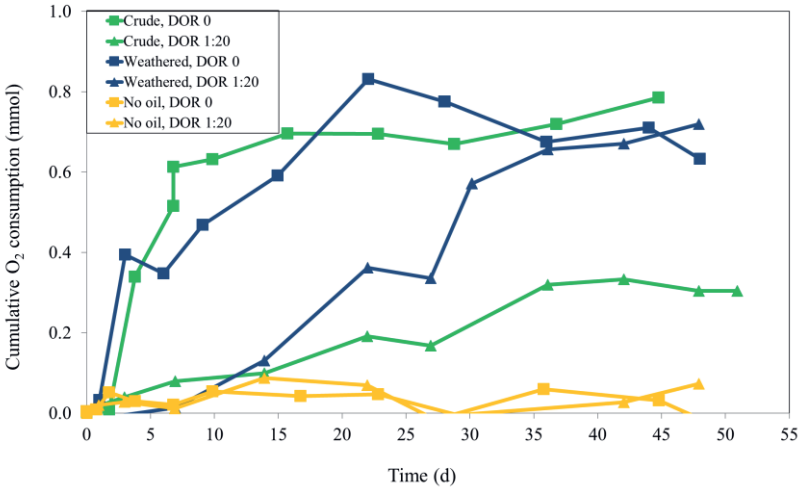

Figure 2.1 Cumulative oxygen consumption by *R. qingshengii* TUHH-12 during biodegradation of crude or weathered oil with and without Corexit.

***Oxygen consumption*:** In the absence of Corexit, high oxygen consumption rates were found already after a few days (Figure 2.1), both for crude and weathered oil degradation. They behaved similarly and reached their maximum oxygen consumption after 30 days. When Corexit was applied, the oxygen consumption was delayed for both crude and weathered oil. The low oxygen consumption for weathered oil lasted 10 days, after which it became comparable to the oxygen consumption in batches without Corexit. For crude oil, low oxygen consumption rates lasted at least 50 days (full incubation time) and never reached the high oxygen consumption as the other batches. In the control batches without oil, very limited oxygen consumption was observed, most likely due to the degradation of Corexit (results not shown). No differences were found between static and dynamic

incubations (results not shown), indicating that mass transfer was not a limiting factor under static conditions.

***Biodegradation of n-alkanes*:** The biodegradation of the *n*-alkanes from crude or weathered oil at DORs of either 1:20 or 0:1 was assessed by measuring the sum of individual *n*-alkanes (C11– C40) during our degradation experiments (Figure 2.2).

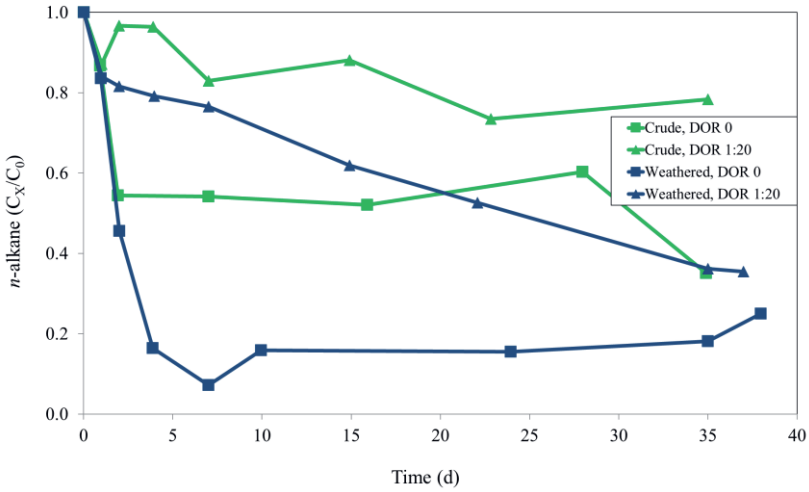

Figure 2.2 Relative *n*-alkane concentration in the presence of *R. qingshengii* TUHH-12 during biodegradation of crude or weathered oil with and without Corexit.

At a DOR of 0:1, biodegradation of *n*-alkanes in both crude and weathered oil started immediately. After 5 days, however, *n*-alkanes continued to be consumed from weathered oil while consumption of *n*-alkanes from crude oil stopped. At a DOR of 1:20, biodegradation of crude and weathered oil was delayed, and less degradation was found. However, the extent of *n*-alkane removal after 38 days was similar to incubations without dispersant for weathered oil.

2.3.3 Dispersants effect on oil biodegradation by *R. qingshengii* TUHH-12 and/or *P. putida* F1

Similar degradation experiments described for *R. qingshengii* TUHH-12 were also performed with the aromatic compounds degrading culture *P. putida* F1 and with both pure cultures combined in the presence and absence of Corexit.

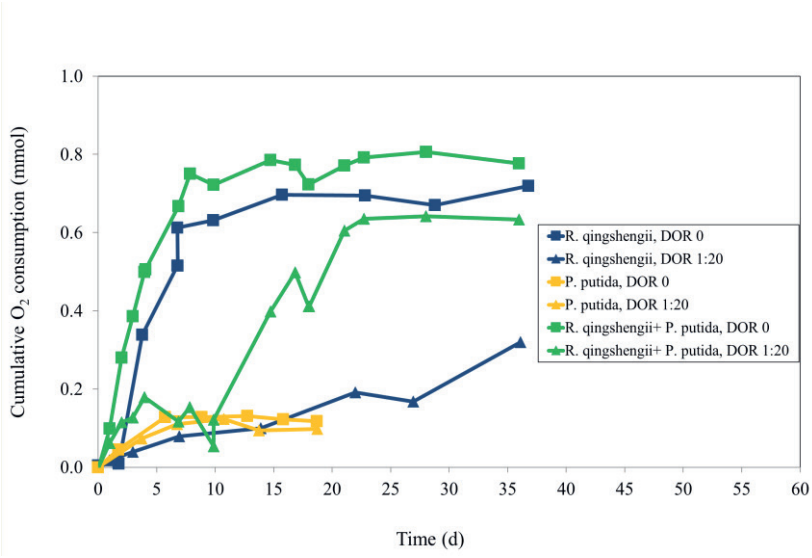

Figure 2.3 Cumulative oxygen consumption by *R. qingshengii* TUHH-12 and/or *P. putida* F1 during biodegradation of crude oil with and without Corexit.

***Oxygen consumption*:** profiles of oxygen consumption with *R. qingshengii* TUHH-12 or *P. putida* F1 or a combination of both pure cultures are depicted in Figure 2.3. High oxygen consumption was observed for incubations of crude oil with *R. qingshengii* TUHH-12 at DOR 0:1 and for the combination of both pure cultures at both DORs, although oxygen consumption at DOR 1:20 only increased after a lag phase of 10 days. On the other hand, oxygen consumption with the aromatic compounds degrading culture *P. putida* F1 was much lower compared to the other incubations with *R. qingshengii* TUHH-12 and/or a combination of both cultures as Macondo crude oil is light sweet oil and contains mostly aliphatic hydrocarbons and less aromatic compounds.

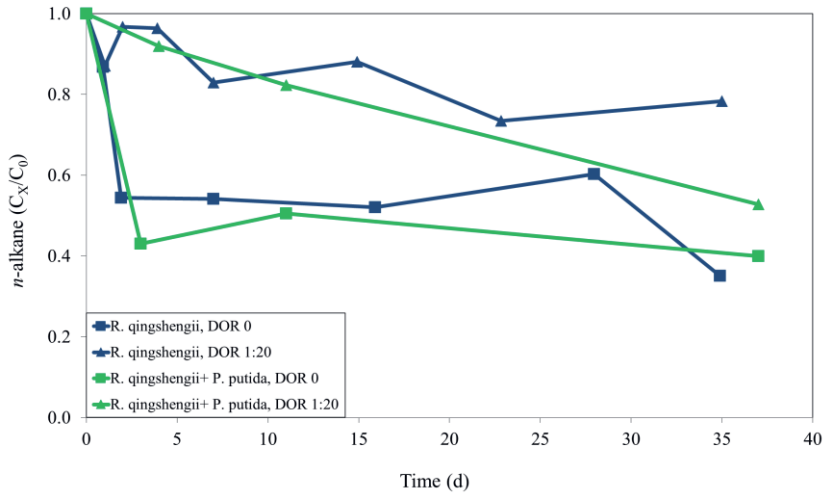

Figure 2.4 Relative *n*-alkane concentration in the presence of *R. qingshengii* TUHH-12 and/or *P. putida* F1 during biodegradation of crude oil with and without Corexit.

***Biodegradation of n-alkanes*:** The relative concentration of the summed individual *n*-alkanes for the tested conditions is given in Figure 2.4. Without dispersant, a faster *n*-alkane removal was observed compared to the DOR 1:20. Furthermore, a slower initial *n*-alkane degradation was observed for *R. qingshengii* TUHH-12 and the combined culture experiments during the first 10 days of incubation for DOR 1:20 than 0:1.

***Biodegradation of BTEX*:** The relative concentration of BTEX compounds in tested conditions is given in Figure 2.5. Limited BTEX compounds degradation was observed in the presence of *n*-alkane degrading *R. qingshengii* TUHH-12 (see Figure 2.5) since *R. qingshengii* TUHH-12 is an *n*-alkane degrading culture.

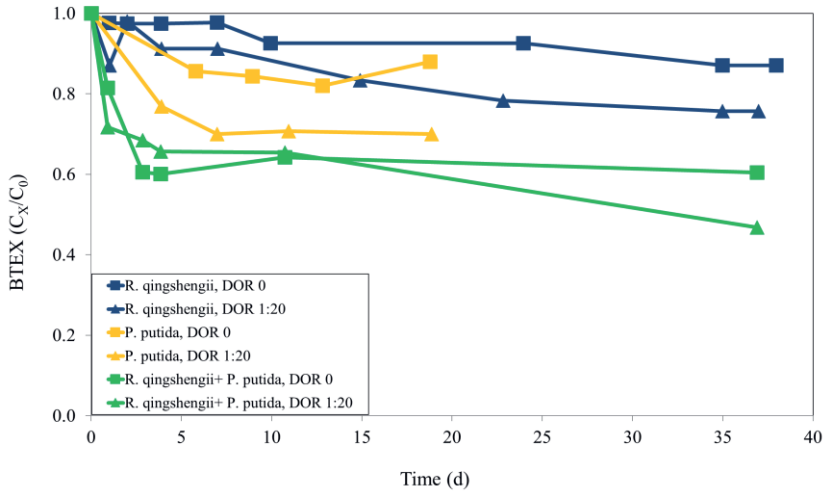

Figure 2.5 Relative BTEX concentration in the presence of *R. qingshengii* TUHH-12 and/or *P. putida* F1 during biodegradation of crude oil with and without Corexit.

A fast initial BTEX degradation was observed in the incubations with *P. putida* F1, especially when treated with Corexit. BTEX compounds degradation was highest when both cultures were present. The concentration of the BTEX compounds were increased when Corexit was applied (Table 2.3).

Table 2.3 Individual BTEX concentration (µg/L) analysed in the headspace of the batches with and without Corexit.

	Benzene	**Toluene**	**Ethylbenzene**	***o*-, *m*-, and *p*-Xylene**
DOR 0	133 ± 3.67	67 ± 1.24	9 ± 0.5	43 ± 1.06
DOR 1:20	155 ± 6.18	75 ± 1.64	10 ± 0.11	47 ± 0.22

2.4 Discussion

We studied the effect of adding chemical dispersants to crude and weathered oil and the consequential effect on oil biodegradation in the presence of *n*-alkane or aromatic compounds degrading culture or a combination of both pure cultures. To mimic the effect of the applied Corexit on the oil biodegradation at the surface or underwater, we used either crude or weathered oil in our experiments. Crude oil is representative of the freshly spilled oil at the sea depth/oil wellhead. Weathered oil represents the oil slicks at the surface, where a large part of the volatile compounds was evaporated into the atmosphere. Using pure cultures, we tried to limit uncertainties caused by population dynamics that may obscure the results when working with more complex microbial systems.

Chemical dispersants are often applied to manage and control oil spills in DORs that generally range from 1:1 to 1:50, depending on the type of dispersants, the application method, the oil type, and the environmental conditions (ITOPF, 2014). For example, during the Deepwater Horizon oil spill response phase, Corexit ®EC9500A was applied in a targeted overall ratio of 1:20 (Kujawinski et al., 2011).

We tested different DORs and found that the applied Corexit concentrations directly affected the oxygen consumption rate by *R. qingshengii* TUHH-12. A higher Corexit concentration (lower DOR) resulted in a lower oxygen consumption rate, indicating that the oil biodegradation was inhibited. In our detailed experiments with crude and weathered oil and the tested pure bacterial cultures, we were able to show the reason for the observed inhibition.

According to our results, when *R. qingshengii* TUHH-12 was applied, the inhibition of Corexit on crude oil biodegradation lasted at least 50 days (our incubation time), whereas weathered oil biodegradation was inhibited only 10 days. This is because weathered oil contains less light aliphatic and aromatic compounds compared with crude oil. Our chemical analyses showed that crude oil had high concentrations of BTEX compounds (around 2000 µg/g oil), whereas our weathered oil hardly contained BTEX compounds (< 0.25 µg/g oil). According to our results, the use of Corexit with crude oil resulted in higher concentrations of dissolved lighter aromatic compounds (BTEX) in the dispersed small oil droplets and the water phase (Prince, 2015). Those higher concentrations of BTEX compounds in the water column could

decrease the activity of the bacteria, which could explain the retardation effect we observed for *R. qingshengii* TUHH-12.

The experiments performed with the BTEX degrading culture, *P. putida* F1, in combination with the *n*-alkane degrading culture, *R. qingshengii* TUHH-12, resulted in an immediate degradation of BTEX compounds, followed by degradation of *n*-alkanes. With the combination of both cultures, the inhibitory effect of chemically dispersed crude oil on the degradation lasted only 10 days. We believe that the initial inhibition of the activity of *R. qingshengii* TUHH-12 by the dissolved light aromatic compounds is decreased through biodegradation of these compounds by *P. putida* F1. Since weathered oil is devoid of aromatic compounds, this inhibitory effect of a high concentration of dissolved BTEX for *R. qingshengii* TUHH-12 did not occur in chemically dispersed weathered oil. Finally, the biodegradation of crude and weathered oil was comparable under both dynamic and static conditions, indicating that mass transfer was not a limiting factor in the static condition, and the addition of Corexit does not tackle the rate-limiting step.

The oil-water interface increases, and consequently, the bioavailability of the oil increases with the addition of Corexit, thus enhancing its biodegradation (Southam et al., 2001). Some studies showed that Corexit improves oil biodegradation (Hazen et al., 2010). However, we did not find any case of improvement on oil biodegradation by using Corexit. Our study shows that chemically dispersed crude oil inhibits biodegradation and not Corexit itself.

We believe that the inhibitory effect of Corexit on biodegradation of the oil reported in the literature depends on the choice of weathered or crude oil, the experimental design, and the applied bacteria. There are various oil-degrading bacterial communities present in marine environments capable of degrading different oil compounds (Lloyd et al., 2010; Orcutt et al., 2010). These communities can consume oil from natural seeps, and their population will increase during a spill to cope with the high concentration of the spilled oil (Hazen et al., 2010; Joye et al., 2014; Mason et al., 2012). However, with deep marine dispersants injection, the maximum concentration of lighter aromatic compounds can be reached (Kleindienst et al., 2015b). We believe that the inhibition of crude oil biodegradation due to Corexit addition that we found in our lab experiments will not be limited in the natural marine

environment, as naturally existing bacterial communities can degrade the increased dissolved aromatic compounds.

2.5 Conclusion

The application of Corexit on crude oil resulted in increased solubility of the aromatic compounds of the oil in seawater. This resulted in higher concentrations of these aromatic compounds which inhibited oil biodegradation, especially when no aromatic compounds degrading culture were present. When a combined culture was used, this retardation was overcome after 10 days of incubation, as the aromatic degrading culture decreased the BTEX compounds concentration, which decreased the activity of the *n*-alkane degrading culture. However, this mechanism does not play a role for weathered oil as hardly any aromatic compounds are present.

Acknowledgements

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Chapter 3

Oil biodegradation: interactions of artificial marine snow, clay particles, oil and Corexit

A modified version of this chapter has been published as

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Abstract

During the Deepwater Horizon oil spill, interactions between oil, clay particles, and marine snow lead to the formation of aggregates. Interactions between these components play an important, but not well understood, role in oil biodegradation in seawater. This study aims to explore the effect of these interactions on the biodegradation of oil in the water. Laboratory experiments were performed, analyzing respiration and *n*-alkane and BTEX biodegradation in multiple conditions containing Corexit, alginate particles as marine snow, and kaolin clay. Two oil-degrading bacterial pure cultures were added, *Pseudomonas putida* F1 and *Rhodococcus qingshengii* TUHH-12. Results show that the presence of alginate particles enhances oil biodegradation. The presence of Corexit alone or in combination with alginate particles and/or kaolin clay hamper oil biodegradation. Kaolin clay and Corexit have a synergistic effect in increasing BTEX concentrations in the water and cause a delay in oil biodegradation.

Keywords

Biodegradation; Oil spill; Marine snow; Kaolin clay; Chemical dispersants; Corexit

3.1 Introduction

Suspended minerals and organic particles present in the seawater can interact with spilled oil and form oil-loaded aggregates (Lee et al., 2002b). During the Deepwater Horizon (DWH) oil spill in the Gulf of Mexico (GoM), 4.9 million barrels of oil were released into the deep sea from a depth of 1500 m. More than 65% of that oil rose to the water surface (Ryerson et al., 2012). As a response, 2 million gallons of chemical dispersants (Corexit ®EC9500A and EC9527A) were applied to prevent contamination of shorelines and marshlands (Joye et al., 2016; Kujawinski et al., 2011). Corexit was applied at the water surface and, for the first time, injected into the oil wellhead (Kujawinski et al., 2011). Moreover, during the oil spill, the Mississippi River was strongly flushed (Bianchi et al., 2011) to keep the oil away from the river system and sensitive marshlands, introducing great amounts of suspended particles, such as clay, into the GoM (Kourafalou and Androulidakis, 2013).

Chemical dispersants enhance the formation of smaller oil droplets and the dissolution of the more hydrophilic and toxic oil compounds (Zhao et al., 2015). Furthermore, chemical dispersants can trigger Extracellular Polymeric Substances (EPS) production by phytoplankton-associated microorganisms (van Eenennaam et al., 2016). This can at least partially explain the excessive production of marine snow that was observed during and shortly after the DWH oil spill (Passow et al., 2012). EPS forms glue between organic particles, suspended minerals, and dispersed oil. When the complex marine snow particles become larger (> 0.5 mm) and negatively buoyant, they sink to the seafloor by gravitational settling (Passow et al., 2012). As much as 14% of the oil interacted with suspended particles and marine snow and subsequently formed oil-mineral aggregates and oiled marine snow (Daly et al., 2016; Passow et al., 2012; Ziervogel et al., 2012), which eventually settled to the seafloor (Romero et al., 2015; Schwing et al., 2015).

During the DWH oil spill, the activity of oil-degrading bacteria increased (Kessler et al., 2011), probably because the bacterial biomass increased due to the availability of larger amounts of oil as a substrate for microbial growth. Whether this is related to the addition of chemical dispersants is not yet clear, as there is a debate in the literature about the impact of Corexit on oil biodegradation. Several studies report that Corexit hardly inhibits the biodegradation of Macondo crude oil (Brakstad et al.,

2015; Wang et al., 2016), and some studies show the opposite (Hamdan and Fulmer, 2011; Rahsepar et al., 2016). This difference in reported impacts of Corexit on oil biodegradation can be related to the different bacterial species used, and each has its specific mechanism for accessing and assimilating hydrocarbons from oily substrates (Bouchez Naïtali et al., 1999; Hua and Wang, 2014; Kapellos, 2017).

The role of marine snow on the fate of crude oil in the deep sea has attracted significant attention in the case of the Deepwater Horizon oil spill. The aggregation of oil droplets with marine snow results in oil sedimentation to the seafloor and enhanced biodegradation (Cai et al., 2017; Størdal et al., 2015); however, the mechanisms are unknown. Interactions in the seawater between marine snow, suspended particles, and dispersed oil droplets can change oil biodegradation. Within the oiled marine snow complexes, the accessibility of the oil for biodegrading bacteria may have changed to become less or more favorable. Chemical dispersion may influence the fate of spilled oil in marine environments in at least two ways: via changed transport and via changed biodegradation kinetics. In this study, multi-parameter laboratory experiments were conducted to explore how oil biodegradation is affected by interactions between oil droplets, alginate particles representing marine snow, Corexit as a chemical dispersant, and kaolin clay as suspended particles.

3.2 Materials and methods

3.2.1 Experimental setup

The biodegradation of oil was tested for six conditions in duplicate. Three conditions without dispersant: Oil + Snow, Oil + Clay and Oil + Snow + Clay, and three similar conditions with addition of dispersant (Corexit): Oil + Snow + Corexit, Oil + Clay + Corexit, and Oil + Snow + Clay + Corexit. In addition, three "no oil" control conditions of Snow only, Snow + Clay, and Clay only were used. Abiotic controls (sterile condition) were prepared similarly to the main conditions without bacterial culture and the addition of 10 mM sodium azide.

The experimental setup was similar to Rahsepar et al. (2016) but with different combinations of conditions. In short, batch experiments were performed in 125 mL glass bottles containing 20 mL bacterial growth medium in artificial seawater (32

g/L artificial sea salt in demi-water). Depending on the condition, 0.1 g of surrogate Macondo oil (BP Gulf Science Data, 2017b) and Corexit ®EC9500A (Nalco Holding Company, USA) with Corexit:oil ratio 1:20 was added to the batches. This ratio is a standard application ratio of dispersants to achieve an effective dispersion (Zeinstra-Helfrich et al., 2015b).

Kaolin clay (hydrated aluminium silicate, CAS 1332-58-7, Sigma Aldrich) with kaolin clay:oil ratio (KOR) 1:2.6 was added in the conditions containing kaolin clay. This KOR was chosen in order to obtain optimal aggregate formation (Stoffyn-Egli and Lee, 2002). During the DWH oil spill, a large amount of suspended solids entered the GoM due to the flushing of the Mississippi River (Bianchi et al., 2011). The Mississippi River contains high concentrations of kaolin clay (Sionneau et al., 2008); therefore, we used this type of clay as representative for suspended particles. In the batches with marine snow, 6 mL of alginate particles (Section 3.2.2) were added to the batches.

To start the oil biodegradation, 1 mL of *Pseudomonas putida* F1 (DSMZ, No. 6899) with an optical density of 0.305 and 1 mL of *Rhodococcus qingshengii* TUHH-12 (DSMZ No. 46766) with an optical density of 0.98 was added to all batches. After preparing the batches, the bottles were sealed with Viton stoppers and aluminium caps and incubated for 45 days at 20 ^{o}C in the dark under static conditions.

3.2.2 Production of artificial marine snow

Alginate particles representing artificial marine snow were used as an approximation of real marine snow as sufficient and reproducible quantities of realistic marine snow were not available. EPS, the glue of marine snow, contains substantial amounts of alginate-like exopolysaccharides (van Eenennaam et al., 2016). Therefore, artificial marine snow was prepared by adding 22.5 g of commercially available alginate (alginic acid sodium salt, CAS 9005-38-3 Sigma Aldrich), a gelling and nontoxic anionic polysaccharide, to 1.1 L of filtered natural seawater while stirring. Then, 9.45 g of kaolin clay (hydrated aluminium silicate, CAS 1332-58-7, Sigma Aldrich) and 19 g of fresh weight of phytoplankton biomass (Chlorella pasta, Ingepro BV, Borculo, The Netherlands) were added to the solution. Separately, 188 g of $CaCl_2$ (CAS 10043-52-4, Fluka Analytical) was dissolved in 13 L of demi-water, and this solution was added to the alginate solution while stirring. The calcium causes

coagulation of the alginate and precipitation of 600 mL marine-snow-like flocks. Finally, the overlying liquid was poured off, and the artificial marine snow was divided into three equal parts for the three microcosms replicates.

3.2.3 Particle size distribution test

Particle size distribution was tested in triplicate for all nine conditions described in 2.1 and two extra conditions: Oil only, Oil + Corexit. Particle size distribution was not tested for the abiotic controls.

Particle size distribution was determined with a Mastersizer 2000 laser light scattering instrument (Malvern Instruments Ltd, UK). Triplicates of samples of conditions as described in Section 3.2.1 were prepared separately but similarly to the batches of the oil biodegradation test. The samples were shaken manually just before injection into the Mastersizer in order to introduce a homogenous sample to the Mastersizer. The Mastersizer measures the volume percentage of particle size distribution ranging from 0.1 μm to 10,000 μm. The average of triplicates was obtained by the Mastersizer software and showed no significant standard deviation between the triplicates.

3.2.4 Chemical analyses

Bacterial respiration was monitored by analyzing oxygen consumption and carbon dioxide production by taking headspace samples and analysis by gas chromatography (GC-FID, Shimadzu, Kyoto, Japan) (Rahsepar et al., 2016). Oxygen was supplied to the batches by injection through the Viton stoppers when the oxygen concentration in the headspace of the bottles fell below 10%. Therefore, oxygen was never a limiting factor for bacterial respiration in our experiments. We believe that the dissolved oxygen was steadily distributed in the water phase of the batches since the water depth was around 2 cm, and oxygen could diffuse from the gas phase into the water phase.

Total Petroleum Hydrocarbons (TPH) were extracted from the liquid phase according to liquid-liquid extraction protocol NEN 5733 and analyzed using GC-FID (Fisions 8000) (Rahsepar et al., 2016). The sum of individual *n*-alkanes (C11-C40) over time was used to quantify the *n*-alkane biodegradation.

BTEX compounds (benzene, toluene, ethylbenzene, and *o*-, *m*-, and *p*- xylene) were extracted from the headspace of the batches by solid-phase microextraction (SPME) and were analyzed on a GC-FID (Rahsepar et al., 2016). First, the identity of each BTEX compound was determined by using the retention time in a standard solution. Then, based on the peak area of each BTEX compound, the concentration (µg/L) of each compound was calculated to determine the concentration of the ΣBTEX compounds per sample.

3.3 Results

3.3.1 Particle size distribution

Particle size distribution is expected to significantly affect the biodegradation of dispersed oil (Brakstad et al., 2015; Wang et al., 2016). Particle size distribution for six conditions and 3 no oil controls (Section 3.2.1) was tested. The smallest particle sizes belong to the "Oil + Corexit" condition, with particle sizes ranging from 0.1 µm – 1 µm (Figure 3.1). The largest particle size belongs to all conditions containing alginate particles, ranging from 100 µm – 10,000 µm. In this group, the combination "Oil + Snow + Clay + Corexit" had the largest particle size (100 µm – 10,000 µm), while the other conditions had similar particle sizes ranging between 100 µm – 1,000 µm. In this group, larger particles were observed in "Oil + Clay + Corexit" (10 µm–100 µm). This shows that alginate particles mediate the formation of larger oil-alginate particles.

Furthermore, it is known that Corexit contains dioctyl sodium sulfosuccinate (DOSS), an anionic surfactant that partitions at the oil-water interface, reduces the interfacial tension, and facilitates the formation and stabilization of oil droplets. The distribution for "Oil + Corexit" in Figure 3.1 shows that Corexit was added above the critical micelle concentration, and complete pseudo-solubilization was achieved with the formation of submicrometer-sized droplets. In addition, the added kaolin clay and/or alginate particles adsorbed some Corexit and possibly competed with each other for space at the oil-water interface. This resulted in a reduced solubilization effect of Corexit and a shift to larger particle sizes in the distributions of "Oil + Clay + Corexit" and "Oil + Snow + Clay + Corexit".

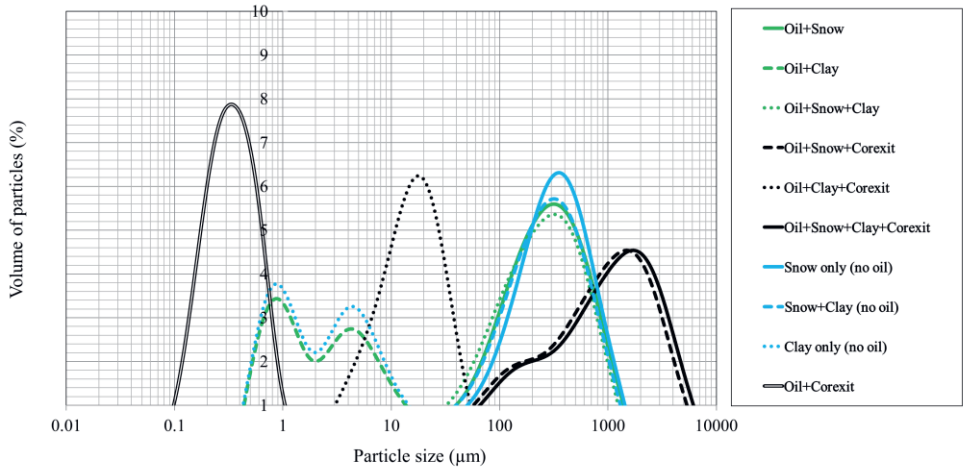

Figure 3.1 Particle size distribution in the tested conditions (average of triplicates): conditions without Corexit (green), conditions containing Corexit (black), and no oil controls (blue). Particle size distribution in the "Oil only" was hampered by the phase separation process and lead to no detectable oil particles.

Similar particle size was seen in "Clay only" and "Oil + Clay", ranging from 1 µm to 10 µm. These results were as expected because kaolin clay has the ability to disperse the oil to small particles. Overall, in conditions that included clay and alginate particles, particle sizes shifted considerably to larger values when Corexit was added. Particle size distribution in the "Oil only" condition is not presented in Figure 3.1 since oil droplet formation was hampered by phase separation processes, leading to no detectable particles in the Mastersizer equipment.

3.3.2 Oxygen consumption

The oxygen consumption (mmol) was tested in the headspace of the batches for 6 tested conditions and 3 no oil controls during the incubation time (Figure 3.2). The oxygen consumption was negligible in control "Clay only" and in sterile controls. Results of the conditions "Oil only" and "Oil + Corexit" are from Rahsepar et al. (2016).

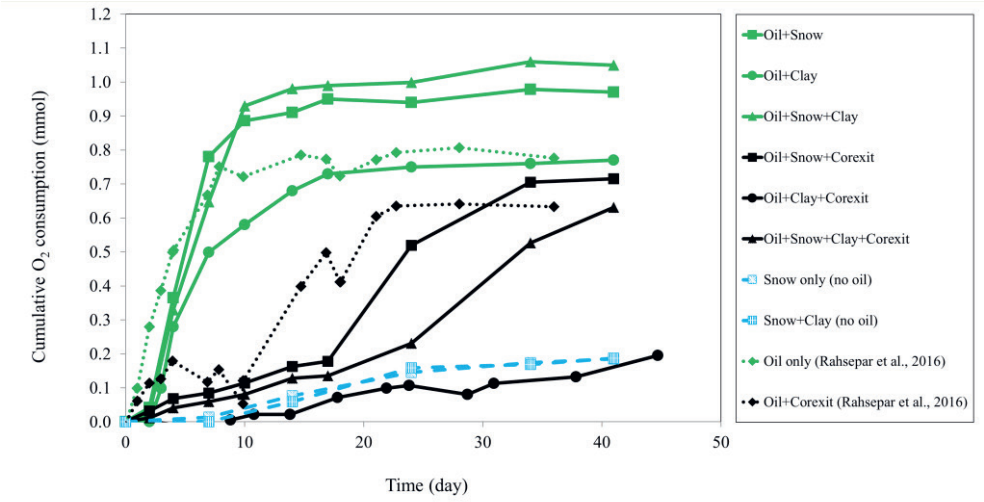

Figure 3.2 Cumulative oxygen consumption in batches during the incubation time (average of duplicates): conditions without Corexit (green), conditions containing Corexit (black), and no oil controls (blue). Oxygen consumption in the control condition "Clay only" and sterile controls was negligible and close to zero, these results are therefore not shown.

The oxygen consumption in conditions "Oil + Snow" and "Oil + Snow + Clay" was similar and was the highest of all conditions. Comparing the oxygen consumption in these two conditions with "Oil only" illustrates that alginate particles increase the oxygen consumption in the batches. Alginate particles also consume oxygen, as can be seen in "Snow only" and "Snow + Clay". The presence of Corexit reduced the oxygen consumption in all conditions, but the least in conditions containing alginate particles.

3.3.3 Biodegradation of *n*-alkanes

Results for *n*-alkane (C11-C40) biodegradation, presented as relative *n*-alkane peak area over time, are shown in Figure 3.3. No *n*-alkane biodegradation was observed in the sterile controls (results not shown).

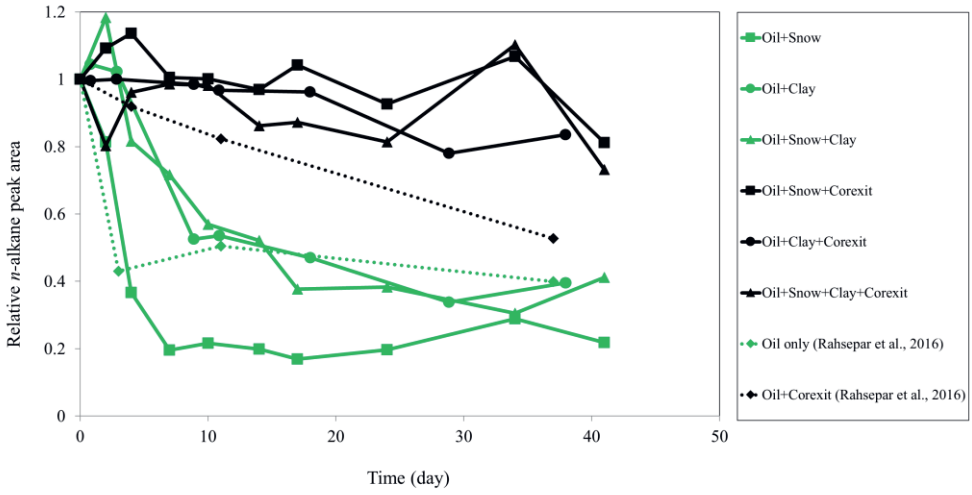

Figure 3.3 Peak area of *n*-alkanes (C11 – C40) extracted from the liquid phase of the batches during the incubation time, relative to day 0 (average of duplicates). Conditions without Corexit (green) and exposures containing Corexit (black).

The highest *n*-alkane biodegradation was observed in conditions containing alginate particles without dispersants. The addition of Corexit resulted in an inhibition in the biodegradation of *n*-alkanes. Comparing the *n*-alkane biodegradation in the "Oil only" condition from the previous study to our "Oil + Snow" condition illustrates that alginate particles enhance the biodegradation of *n*-alkanes.

3.3.4 Biodegradation of BTEX

Figure 3.4 shows the BTEX biodegradation profiles, presented as relative BTEX peak area. No BTEX biodegradation was observed in the sterile controls (results not shown). Similar to the results of the oxygen consumption and *n*-alkane biodegradation, BTEX biodegradation was lowest in the presence of Corexit. Comparing the BTEX biodegradation in the "Oil only" condition from the previous study (Rahsepar et al., 2016) to our "Oil + Snow" condition shows that alginate particles also enhance the BTEX biodegradation.

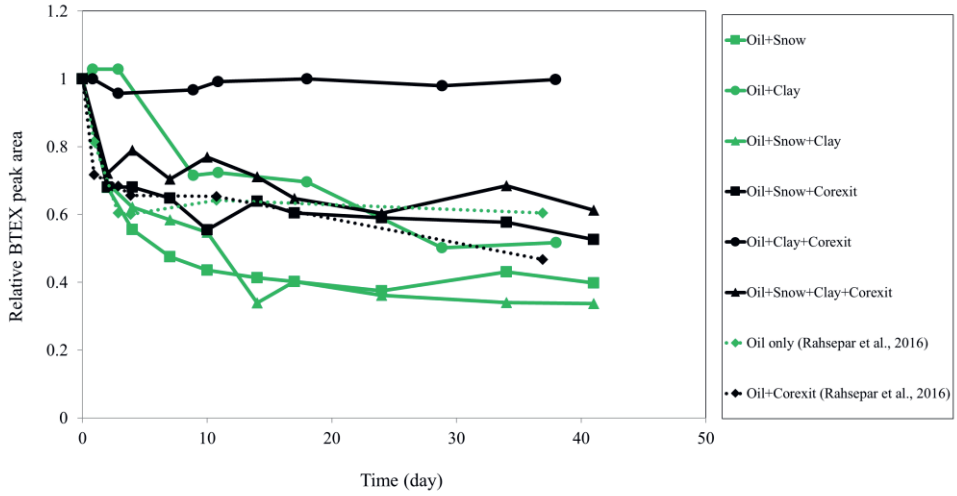

Figure 3.4 Peak area of BTEX in headspace samples during the incubation time, relative to the first day of incubation (average of duplicates). Conditions without Corexit (green) and exposures containing Corexit (black).

The concentration of ΣBTEX compounds in the headspace of six conditions is shown in Table 3.1. The ΣBTEX concentration was measured in the headspace of the batches on the first day of incubation to analyze the effect of Corexit on BTEX concentration before the oil biodegradation starts.

Table 3.1 ΣBTEX concentration (µg/L) analyzed in the headspace of the batches in the presence and absence of Corexit (average and standard deviation of duplicates).

Exposures	**ΣBTEX (µg/L) in absence of Corexit**	**ΣBTEX (µg/L) in presence of Corexit**
Oil + Snow	228 ± 2.2	266 ± 1.8
Oil + Clay	284 ± 1.3	317 ± 3.2
Oil + Snow + Clay	203 ± 1.3	251 ± 2.1
Oil only (Rahsepar et al., 2016)	252 ± 1.6	287 ± 2.1

The concentration of BTEX compounds in the headspace was highest when dispersant was applied, and the condition "Oil + Clay + Corexit" had the highest BTEX concentration. In the absence of Corexit, condition "Oil + Clay" had the

highest BTEX concentrations, illustrating that kaolin clay has the ability to disperse the oil and increase the dissolution of BTEX compounds.

3.4 Discussion

A previous study by Rahsepar et al. (2016) tested the effect of Corexit on oil biodegradation in a batch experiment study. The current study extended this by also including the interactions with alginate particles and kaolin clay. Thus, we can compare the biodegradation of oil in the absence and presence of Corexit and when interacting with alginate particles and kaolin clay. In addition, the previous study showed that the presence of Corexit reduces the oxygen consumption (bacterial respiration) during the first 10 days of incubation, coinciding with reduced *n*-alkane biodegradation (Rahsepar et al., 2016). We observed similar results in experiments described in this research, where additional components such as alginate particles and kaolin clay are added.

Results of Table 3.1 show that the application of Corexit increases the dissolution of toxic oil compounds such as BTEX. Kaolin clay particles have a similar effect as Corexit. Moreover, the presence of Corexit and kaolin clay has a synergistic effect on the dissolution of BTEX compounds. This confirms the findings of Rahsepar et al. (2016), showing increased toxicity of oil compounds in the presence of Corexit. Contrary, alginate particles reduce the BTEX concentration. The lowest concentration of BTEX was observed in the presence of alginate particles, illustrating that the alginate particles decrease the dissolution of BTEX compounds or introduce an alternative medium for BTEX sorption resulting in decreasing concentration of BTEX. When alginate particles and kaolin clay are both present (condition Oil + Snow + Clay), the concentration of BTEX compounds is low. This indicates that the sorption of BTEX on alginate particles outcompete the increased desorption in the presence of kaolin clay.

Our results show that the combination of oil with alginate particles increases oxygen consumption (bacterial respiration) (Figure 3.2) and increases the *n*-alkane and BTEX biodegradation. Alginate particles provide a substrate for bacterial attachment and are also prone to aggregation with oil droplets and other alginate particles. In such complex aggregates, bacteria are in close proximity with the oil droplets, and transport limitations are reduced. In turn, the rate of biodegradation is increased. On

the other hand, alginate particles formed larger oil-alginate particles (100 µm to 10,000 µm, Figure 3.1) that might indicate a reduced mass transfer of oil to water (Table 3.1). This may indicate that biodegradation activity is not only happening in the water phase but also at the inside of the alginate particles aggregate. In the presence of alginate aggregates, part of the BTEX and *n*-alkanes could be biodegraded while diffusing out of the oil phase in the aggregates towards the bulk solution. In addition, alginate particles were found to form a barrier around the oil droplets, prevent coalescing with other droplets (Khelifa et al., 2005) and enhance droplet stability. This can explain the lower availability of BTEX compounds in the presence of alginate particles (Table 3.1). Larger particles such as alginate and aggregates are known to potentially reduce the dissolution rate of oil, possibly resulting in aqueous oil concentrations below toxicity limits (Lee et al., 2003), thus leading to elevated biodegradation of the oil (Owens and Lee, 2003).

By adding Corexit to the combination of oil and alginate particles, bacterial respiration and removal of *n*-alkanes and BTEX are inhibited to similar levels as without alginate particles (Figures 3.3 and 3.4). Considering the particle size distribution in this condition (Figure 3.1), Corexit-induced inhibition of biodegradation is not due to oil mass transfer limitation but seems related to aqueous concentrations and activity of the Corexit itself. Corexit appears to counteract the stimulatory effect of alginate on the oil-degrading bacteria regarding BTEX and *n*-alkane biodegradation, i.e., by reestablishing the biodegradation inhibitory effects observed in oil-Corexit condition. In addition, the anionic surfactant DOSS of Corexit can also cover the surface of the oil droplets, thus preventing the adherence of bacteria to this surface (Neu, 1996). This will also result in reduced oil biodegradation.

Similar removal of both *n*-alkanes and BTEX were observed in the two conditions Oil + Clay and Oil + Snow + Clay, while condition Oil + Snow had the highest oil biodegradation. Possibly this is a result of sorption of oil compounds onto fine kaolin clay particles (< 2 µm), which can disperse the oil onto the solid matrix and thereby decrease the availability of the oil for biodegradation. Corexit addition to the Oil + Snow + Clay condition results in an inhibition in oil biodegradation. According to Figure 3.1, the addition of Corexit causes an increase in the particle size, but this did not enhance oil biodegradation. This confirms that inhibition of oil biodegradation in the presence of Corexit is not due to the mass transfer limitation but is due to the

high concentration of toxic compounds and toxicity on the oil-degrading bacteria. It has been shown that dispersants can reduce or delay the performance of oil-degrading bacteria (Joye et al., 2016). This toxicity can be either due to the toxicity of the dispersant itself (Techtmann et al., 2017) or by the toxicity of solubilized compounds released from the oil (Epstein et al., 2000), such as BTEX and PAHs (Kleindienst et al., 2015b; Rahsepar et al., 2016).

There is a synergistic effect between kaolin clay and Corexit leading to oil biodegradation inhibition. According to Table 3.1, kaolin clay can increase the dissolution of BTEX compounds in the batches. When kaolin clay and Corexit are both present, the concentration of BTEX compounds are at the highest level, which inhibited the oxygen consumption (Figure 3.2) and *n*-alkane and BTEX biodegradation (Figures 3.3 and 3.4). This shows that the role of kaolin clay is complex. Its addition results in oil emulsification (Figure 3.1) but cannot outperform the extent of biodegradation that is achieved without any addition (Figures 3.3 and 3.4).

Oxygen consumption in the condition "Oil + Snow" is higher than condition "Oil only". Oxygen consumption for the Corexit is shown to be negligible (Rahsepar et al., 2016). Considering the oxygen consumption in the "Snow only", it shows that alginate itself biodegrades and therefore consumes dissolved oxygen. Especially the carbohydrate compounds of alginate have oxidized groups in their molecular structures, which are readily available for further enzymatic conversions and mineralization (Alldredge, 1998; Bochdansky et al., 2010). The artificial marine snow (alginate particles) used in this study contained alginate, a gelling and nontoxic anionic polysaccharide, which provides easily degradable carbon compounds. Therefore, on the one hand, alginate particles are able to increase the oil biodegradation either by increasing the oil mass transfer by inner aggregate dispersion or by bringing oil-degrading bacteria closer to the substrate. On the other hand, alginate particles increase the oxygen consumption rate. Since oxygen was injected into the batches when it was below 10% in the headspace, oxygen did not become a limiting factor in our experiments. However, in conditions with limited oxygen availability, such as sediments layer, marine snow could limit the oil biodegradation by consuming the dissolved oxygen and creating anaerobic conditions (Kessler et al., 2011).

3.5 Conclusion

This study shows that alginate particles representing marine snow enhance oil biodegradation in the water phase in the abundance of dissolved oxygen. In the presence of Corexit, oil biodegradation is inhibited. Corexit and kaolin clay have the synergistic effect in increasing BTEX concentrations to high toxic levels and inhibiting oil biodegradation. These inhibitions on biodegradation are lower in the presence of alginate particles. Moreover, the presence of alginate particles prevents a build-up of toxic BTEX concentrations in the water phase. Alginate particles appear to enhance the surface area of the oil phase. An explanatory model for all these observations is that an inner-aggregate environment is created, causing oil phase redistribution with enlarged surface area and inner-aggregate settling places for oil-degrading bacteria. Thus, mass transfer is enhanced, leading to the release of degradable oil compounds, and the diffusion distances between the oil phase and the oil-degrading bacteria are shortened. The enhanced oil biodegradation effect is partially mitigated by adding Corexit because of its toxic effect on the oil-degrading bacteria. This hypothetic model needs to be further studied in defined oil-snow-bacteria aggregates to further elucidate the underlying processes as a basis to optimize oil biodegradation in the field.

Acknowledgements

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Chapter 4

Marine snow-oil interaction affects *n*-alkanes biodegradation in sediment

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Abstract

During the Deepwater Horizon oil spill, excessive production of marine snow was observed, and it was estimated that as much as 14% of the oil was transferred to the seafloor by MOSSFA (Marine Oil Snow Sedimentation and Flocculent Accumulation). MOSSFA is an important pathway for transferring oil to the seafloor. We performed experiments at laboratory scale in 15 microcosms, representing 5 exposures of marine snow with or without oil, oil plus clay, and controls with only clay or sediment. Results showed that the presence of marine snow reduces the depletion of oil alkanes by 40% due to the preferred biodegradation of marine snow organics. Biodegradation of marine snow reduces the oxygen concentration, which might result in an anaerobic layer in the sediment at the seafloor, reducing the oil biodegradation rate. Our results show that marine snow hampers benthic oil biodegradation.

Keywords

Biodegradation; Marine snow; Oil spill; Sediment; MOSSFA; Deepwater Horizon

4.1 Introduction

The Deepwater Horizon (DWH) oil drilling rig in the Gulf of Mexico (GoM) exploded in April 2010, causing the release of roughly 500,000 m^3 of Macondo well oil over the course of 87 days (Beyer et al., 2016). To prevent oil surfacing and to maintain the spilled oil in the water column, a response operation was applied. About 8000 m^3 chemical dispersants (Corexit EC9500A and EC9527A) were applied, of which 60% to the water surface and 40% to the DWH wellhead at a depth of 1500 m close to the seafloor (Beyer et al., 2016; Kujawinski et al., 2011). The released oil from the wellhead formed droplets in different sizes due to pressure differences between the oil reservoir and the water column, and droplet formation was further enhanced by the deep marine injection of dispersants (Malone et al., 2018; Peterson et al., 2012).

Dispersants reduce the oil-water interfacial tension (Soloviev et al., 2016), thereby breaking oil into smaller and more stable suspended droplets than occurs by natural dispersion (Zeinstra-Helfrich et al., 2015a). The resulting larger oil-water surface area increases oil dissolution and the bioavailability for oil-degrading bacteria (Doyle et al., 2018). Oil droplets migrate through the water column depending on droplet sizes and buoyancy. As a result, a portion of the spilled oil with smaller droplet sizes and lower density remained suspended while forming oil plumes, either in the water column or (partly) at the water surface. Although deep marine injection of chemical dispersants aimed to prevent oil from reaching the water surface and thereby threatening shorelines, however, some portion of the oil surfaced (MacDonald et al., 2015). Dispersants applied at the surface enhance further droplet dispersion, dissolution of lighter oil components and subsequently trigger settling into the water column (Kleindienst et al., 2015a; MacDonald et al., 2015; Zuijdgeest and Huettel, 2012).

The excessive formation of marine snow reported during the DWH oil spill was a consequence of the formation of extracellular polymeric substances (EPS) (Passow et al., 2012). The EPS is produced either as natural oil dispersants by free-living oil-degrading bacteria or as a stress response by bacteria living in symbiosis with phytoplankton (Wotton, 2004), which may trigger by the presence of chemical dispersants and oil compounds. Phytoplankton is generally considered as the main contributor to the natural formation of marine snow (Daly et al., 2016; Hastings et

al., 2016; Passow et al., 2012; Sohm et al., 2011; van Eenennaam et al., 2019; van Eenennaam et al., 2016). Marine snow occurs naturally in the marine environment and is responsible for the carbon cycle and nutrient transport from upper layers to the deeper layers of water by gravitational setting (Wimpenny and Poole, 2009). During oil spills, oil droplets interact and aggregate with marine snow and get transported to the seafloor. This mechanism was the main pathway of transferring the oil to the sediment layer in the case of the DWH oil spill (Hastings et al., 2016; Romero et al., 2015). The process of settling of marine snow-associated oil is called Marine Oil Snow Sedimentation and Flocculent Accumulation (MOSSFA).

Interaction of oil droplets with marine snow increases the oil surface area (Gregson et al., 2021; Lee et al., 2002a), oil mass transfer, and oil bioavailability. In the presence of marine snow, oil-degrading bacteria tend to attach to the marine snow, and this increases oil biodegradation (Rahsepar et al., 2017). Furthermore, mineral particles such as suspended clays can interact with marine snow and increase the marine snow gravitational settling speed. Elevated concentrations of suspended solids from the Mississippi river discharge and mud drilling close to the oil wellhead increased the oil droplets aggregations and eventually the settling to the sediment layer (Brooks et al., 2015; Yan et al., 2016). By transferring the oil droplets from the water column to the floor, the bioavailability of oil may decrease due to changes in the environmental conditions, such as changed redox conditions or mass transfer limitations (Bagby et al., 2017).

Marine snow accumulated on the seafloor up to 120-180 km from the wellhead (Stout et al., 2017), and deposition of marine snow-associated oil was observed as 1–6 cm thick oily deposits along the Northeast GoM slope (Brooks et al., 2015). It is estimated that after the DWH oil spill, up to 14% of the total spilled oil was transferred to the seafloor by the sedimentation of marine snow-associated oil (Daly et al., 2016; Dissanayake et al., 2018). Mass accumulation rates increased from pre-spill to after spill during 2011 and 2012 from 0.05- 0.16 g/cm^2/year to 0.48–2.40 g/cm^2/year (Daly et al., 2016). The oxygen concentrations at a depth of 1000 – 1200 m, e.g., in the GoM, are generally in the range of 2.57 ± 0.5 mg/L (Campbell et al., 2019). Aerobic processes can thus take place in the sediment layer, which will deplete the dissolved oxygen. Continued oxygen consumption in the sediment depends on the diffusion of oxygen from the water column into the sediment (Camilli et al., 2010; Schwing et al., 2020a). There are various papers that describe a

MOSSFA event and the transfer of oil to the sediment layers at the seafloor (Chanton et al., 2015; Quigg et al., 2020; Romero et al., 2017; Schwing et al., 2018; Stout et al., 2016); however, the fate and biodegradation of hydrocarbons in the deposited marine snow-associated oil is not well studied.

We hypothesize that increases in organic matter content at the seafloor will increase oxygen consumption, limiting the availability of dissolved oxygen for the degradation of oil compounds. The aim of this study is to elucidate the degradation of oil and, specifically, the degradation of *n*-alkanes when oil is associated with marine snow. The degradation of *n*-alkanes was studied in 15 microcosms with natural marine sediment in combination with artificial marine snow with or without oil, oil plus clay, and controls with only clay or only sediment. The degradation of *n*-alkanes in these microcosms was studied during an incubation period of 42 days.

4.2 Materials and methods

4.2.1 Experimental setup

Oil biodegradation experiments were performed in 15 microcosms of 25 × 25 × 25 cm, representing five different exposure conditions in triplicate (Table 4.1). Full glass microcosms were used to avoid the oil absorption by silicone rubber. Climate was controlled at 14 °C, with a day-night light regime of 16 h light and 8 h dark. The microcosms contained a 5 cm layer (≈3,2 L) of sieved (1 mm) natural uncontaminated sediment and a 15 cm layer (≈9.4 L) of filtered (0.45 µm) natural seawater originating from the Netherlands. The sediment and seawater were added to the microcosms one day prior to the exposure day (day 1) to settle. At the exposure day, depending on the exposure conditions, a 1cm layer of marine snow with or without oil or 3.15 g of clay with or without oil was applied on top of the sediment layer, as described in detail in 2.2. The concentration of oil in the oil-containing microcosms was 0.63 g oil per 625 cm^2, which is equivalent to 10 g/m^2 per microcosm. This is the highest concentration of oil found in the sediment layer of GoM, which makes it a worst-case scenario (Romero, personal communication, based on (Romero et al., 2017)).

Table 4.1 Overview of the exposure conditions for the microcosm experiment. '+' indicates present in the microcosm and '–' indicates absent from the microcosm.

Exposures	**Marine snow**	**Oil**	**Kaolin clay**
Sediment control	-	-	-
Clay control	-	-	+
Snow	+	-	+
Oil	-	+	+
Snow + oil	+	+	+

The microcosms were covered with acrylic plastic covers to minimize water evaporation, and the air was bubbled in the top 5 cm of the water column through two glass Pasteur pipettes per microcosm via an air pump. Seawater level was maintained by adding approximately 300 mL demi-water per week to the microcosms to correct for evaporation. The incubation period of the microcosms was 42 days.

Sediment core samples for chemical analyses were taken from each microcosm on days 1, 16, 30, and 42. In order to avoid disturbance of the surrounding sediment, a 2 cm diameter glass tube was pushed vertically into the sediment before sampling, and then a 1.5 cm diameter glass tube was placed inside the bigger tube to extract the sediment under vacuum. The outer tubes were left in the microcosms until the end of the experiment to avoid disturbance of the remaining sediment and water column. The water level inside the tubes was adjusted with filtered seawater after the sample was taken to prevent drawing surrounding water into the outer sampling tubes. At each sampling time, 4 samples were taken from different locations in one microcosm, which pooled to get a representative sample per microcosm. The samples were kept in a 300 mL glass jar at 4 ºC in the dark for 3 days to allow the sediment to settle. After settling, the overlaying water was carefully removed with a glass pipette, and the sediment samples were freeze-dried using a Christ Alpha 2-4 LDplus freeze dryer (Martin Christ Gefriertrocknungsanlagen GmbH, Osterode am Harz, Germany), followed by extraction for chemical analyses.

4.2.2 Exposure conditions of the microcosms

We used surrogate Macondo oil in our experiment, kindly provided by BP, a light oil chemically similar to the Macondo oil of the DWH oil spill (BP Gulf Science Data, 2017b). The oil was weathered for 24 h in the dark at room temperature while stirring with a magnetic stirrer to allow the evaporation of lighter hydrocarbons. Using weathered oil for our experiment gives a more realistic scenario than fresh oil since the marine snow-associated oil aggregates during the MOSSFA event also weathered due to evaporation and dissolution. The oil added to our experiments showed the typical unimodal distribution peaking at *n*-C17, which is characteristic of light oils, such as Macondo well oil.

Uncontaminated sediments were collected from the top 10 cm of an intertidal mudflat in the Dutch Wadden Sea (N 52° 56.112 E 004° 59.976) at low tide. Collected sediments were transported to the laboratory and sieved over a 1 mm sieve to remove larger organisms and particles. The sediment did contain background levels of *n*-alkanes and isoprenoid alkanes; however, they showed distribution characteristic of natural biogenic input (i.e., odd over even preference), such as algae (Gelpi et al., 1970; Li et al., 2016b). Uncontaminated natural seawater was collected from Eastern Scheldt, The Netherlands, filtered over a 0.45 µm filter, and stored in a tank in the laboratory.

In order to kick-start the biodegradation of oil, two oil-degrading microorganisms were added to the water column on day 1. *Rhodococcus qingshengii* TUHH-12 (DSMZ No. 46766) was used as an alkane-degrading culture, isolated by the Technical University of Hamburg, Germany, from a seawater sample (Schedler et al., 2014). *Pseudomonas putida* F1, an aromatic degrading culture, was purchased from the German collection of microorganisms and cell cultures (DSMZ, No. 6899). 1 mL of *Rhodococcus qingshengii* TUHH-12 (Optical Density of 0.98) and 1 mL of *Pseudomonas putida* F1 (Optical Density of 0.30) (Rahsepar et al., 2016) was added to each microcosm on day 1. All the exposures contained bacteria.

EPS, including proteins and polysaccharides, was produced in our laboratory by exposing phytoplankton communities to chemical dispersants (van Eenennaam et al., 2016). However, it was not feasible to produce sufficient amounts of EPS for the amount of marine snow needed for this study. Therefore, a method was developed

to produce artificial marine snow which resembles natural marine snow. Based on the natural marine snow composition reported for the MOSSFA event during the DWH oil spill, the following essential ingredients for the production of the artificial marine snow were defined; alginate-like exopolysaccharides, phytoplankton biomass, and mineral particles (Alldredge and Silver, 1988; Daly et al., 2016; Thornton, 2002; van Eenennaam et al., 2016). This artificial marine snow was used to study the effect of the association of oil with marine snow on *n*-alkanes (C13-C30) biodegradation in this study.

Artificial marine snow was prepared by adding 22.5 g of commercially available alginate (alginic acid sodium salt, CAS 9005-38-3 Sigma Aldrich), a gelling and nontoxic anionic polysaccharide to 1.1 L of filtered natural seawater while stirring. Then, 9.45 g of kaolin clay (hydrated aluminum silicate, CAS 1332-58-7, Sigma Aldrich) and 19 g of fresh weight of phytoplankton biomass (*Chlorella* pasta, Ingepro BV, Borculo, The Netherlands) were added to the solution. Separately, 188 g of $CaCl_2$ (CAS 10043-52-4, Fluka Analytical) was dissolved in 13 L of demi-water, and this solution was added to the alginate solution while stirring. The calcium causes coagulation of the alginate and precipitation of 600 mL marine-snow-like particles. Finally, the overlying liquid was poured off, and the artificial marine snow was divided into three equal parts for the three microcosm replicates.

To prepare each replicate of Snow-only exposure, 200 mL of this artificial marine snow was added to the microcosms resulting in an approximately 1 cm thick layer on top of the sediment. For the Snow + Oil exposure, the procedure was similar as described above, except that the kaolin clay was first mixed with 1.9 g of oil and 100 mL demi water before adding it to the alginate solution. For each replicate of the Clay control, the kaolin clay was mixed with approximately 500 mL seawater from the microcosm to form a suspension and then added to the microcosms. The clay settled on top of the sediment in a homogenous layer of approximately 0.5 mm thick. The Oil-only exposure was prepared per microcosm by mixing 3.15 g of kaolin clay with 0.63 g of oil and 100 mL of demi water to form a homogenous slurry that was added to the microcosm. To the Sediment control microcosms, nothing extra was added.

4.2.3 Monitored water parameters

Dissolved oxygen (mg/L), pH (-), temperature (°C), and salinity (‰) were monitored weekly during the experiment in the water phase at 10 cm above the sediment. The pH was measured with a Mettler Toledo pH probe, and the dissolved oxygen, temperature, and salinity with a Hach HQ40d multimeter using dissolved oxygen, salinity, and temperature probes.

4.2.4 Chemical analyses

For chemical analyses, 5 g of freeze-dried sediment was extracted with 8 mL dichloromethane. An aliquot of the whole extract was first analyzed using GC-MS to check if the oil fingerprint is present. Then, the whole extract was separated into saturate and aromatic fractions by loading onto a silica gel (0.6 g, 70-230 mesh) column, pre-washed with pentane, and eluting with 2 mL of pentane to obtain the saturate, followed by elution with 2 mL of dichloromethane and isopropyl alcohol, respectively, to obtain the aromatic fraction. The temperature program for the GC-MS analyses included a hold of 5 min at 40 °C, followed by a 4 °C/min ramp up to 325 °C, and finishing with an isothermal hold at 325 °C for 15 minutes. Separation was performed on an HP-5MS capillary column (30 m × 0.25 mm × 0.25 µm), and helium was used as the carrier gas at a flow of 1.0 mL/min. The mass spectrometer was acquiring data in a combined full scan/selected ion monitoring (SIM-SCAN) mode. SIM trace at m/z 85 was used to integrate the areas of *n*-alkanes (C13-C30) and isoprenoid alkanes (pristane and phytane). Integrated areas were then normalized to the area of C30-hopane (17α(H),21ß(H)-hopane) at SIM trace m/z 191. C30-hopane is considered a conservative biomarker due to its recalcitrance to weathering (Prince et al., 1994). This normalization approach is typically used in oil spill studies to detect and compare compositional changes due to evaporation, biodegradation, and other weathering processes (Aeppli et al., 2014; Radović et al., 2014).

To demonstrate the stability and persistence of C30-hopane during our experiments and to confirm the validity of C30-hopane normalization, additional batch experiments were performed. The concentration of C30-hopane was followed during 42 days in 4 batches (1 L), similar to the microcosm exposure Snow + Oil. The whole content of two bottles was extracted at day 0 and the other 2 extracted at day 42. The

samples were analysed using GC-MS as described above, and the C30-hopane was quantified against an internal standard of cholestane-d_4, assuming a relative response factor of 1 (Bennett and Larter, 2000).

4.2.5 Statistical analyses

GraphPad Prism 5 was used to perform statistical analyses using two-way ANOVA with Bonferroni multiple comparisons post-test.

4.3 Results

4.3.1 Physical parameters in the water phase

In the absence of marine snow, the oxygen concentration in the water column 10 cm above the sediment layer was stable during the incubation time of 42 days (8 mg/L ± 0.3 mg/L). In the Marine snow-only exposure, the oxygen concentration in the water phase dropped by 37% to 5.3 ± 0.3 mg/L at day 6, followed by a rebound to the initial value at day 10 (Figure 4.1). The highest oxygen consumption was observed in the Snow + Oil exposure. The oxygen concentration in the water dropped significantly with 52% from 9 ± 0.5 mg/L to values varying around 4.4 ± 0.5 mg/L in the first 6 days, whereafter it returned to the initial oxygen concentration within 4 days.

The pH, salinity, and temperature in the water phase remained constant during the incubation time in all the exposures at an average value of 8.5 ± 0.3, 14°C ± 1°C, and 34% ± 2%, respectively (Figure S4.1).

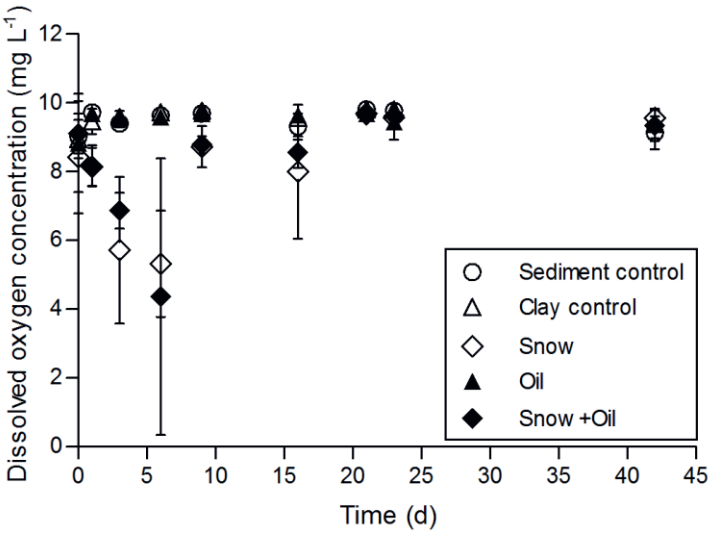

Figure 4.1 Dissolved oxygen concentration at 10 cm above the sediment layer in the water phase of the microcosms. Average and standard deviation of three microcosm replicates.

4.3.2 C30-hopane normalized *n*-alkanes (C13-C30) and isoprenoid alkanes

The batch experiments confirmed the persistence of C30-hopane during our experiments, with average duplicate concentrations of 180 and 210 µg of C30-hopane per g of sediment extracted at t=0 and t=42, respectively. Furthermore, the observed variability (approx. 10% relative standard deviation) is within the acceptable limit of analytical error accumulated during experimental setup, sampling, sample preparation, GC-MS analyses, and peak integration. Therefore hopane normalized peak areas were further used to determine the relative abundance changes of *n*-alkanes and isoprenoid alkanes.

The C30-hopane normalized peak areas of the *n*-alkanes (C13-C30) and isoprenoid alkanes determined in samples of the exposures Oil and Snow + Oil are presented in Figure 4.2. The results show that 2.5 times more *n*-alkanes and isoprenoid alkanes were degraded in the absence of marine snow (65%) than in the presence of marine

snow (25%). Limited degradation of *n*-alkanes and isoprenoid alkanes was observed in the presence of marine snow during the first 30 days (Figure 4.2).

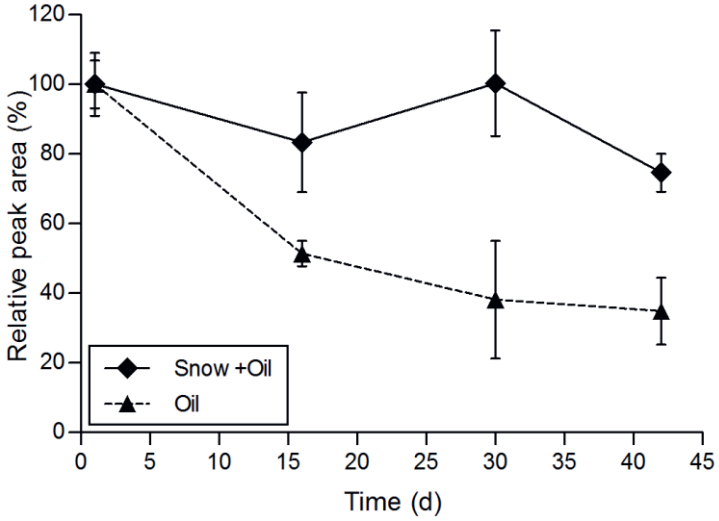

Figure 4.2 Relative peak area of C30-hopane normalized *n*-alkanes (C13-C30) and isoprenoid alkanes (pristane and phytane). Average and standard deviation of three microcosm replicates.

After 42 days of incubation of the oil, extended degradation of all individual *n*-alkanes and isoprenoids alkanes occurred (Figure 4.3) in the absence but not in the presence of marine snow. In addition, when looking at the individual removal percentages of the different *n*-alkanes and isoprenoid alkanes, the degradation of the smaller compounds (< C18) is far more pronounced in the absence of marine snow (Figure 4.4).

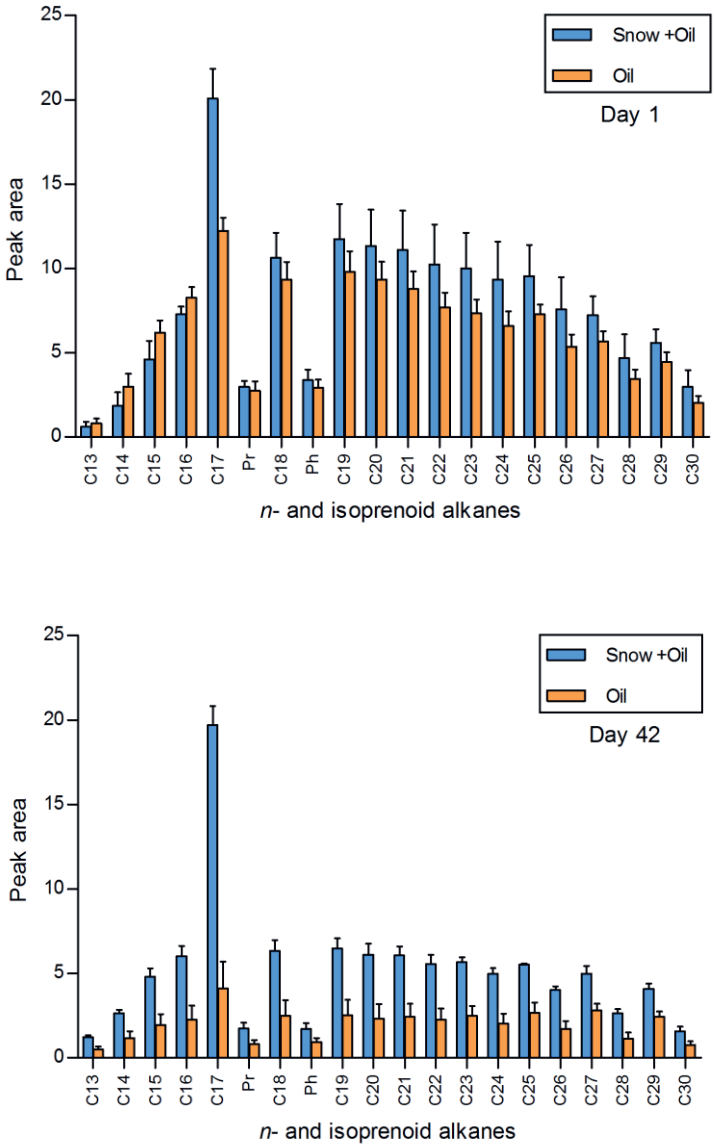

Figure 4.3 C30-hopane normalized peak areas of *n*-alkanes (C13-C30) and isoprenoid alkanes (pristane and phytane) in sediment samples on day 1 (top) and day 42 (bottom). Average and standard deviation of three microcosm replicates.

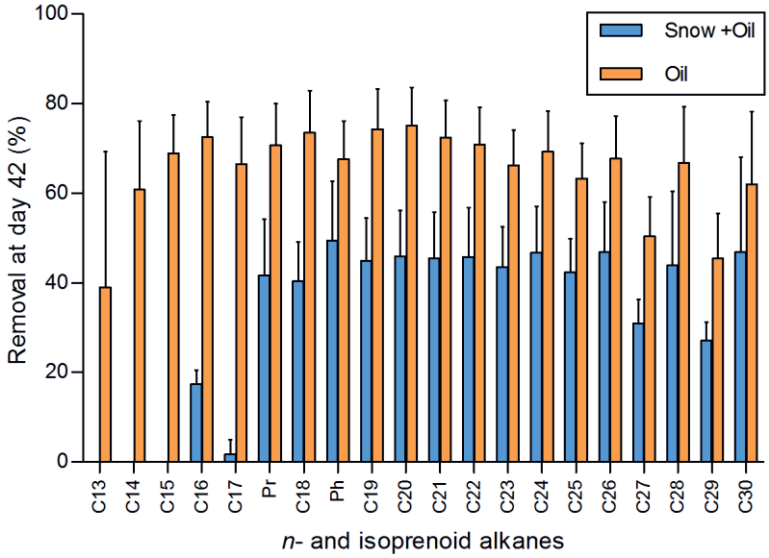

Figure 4.4 Profile of removal percentage of *n*-alkanes (C13-C30) and isoprenoid alkanes (pristane and phytane) in sediment samples at day 42 compared to day 0. Average and standard deviation of three microcosm replicates.

4.4 Discussion

In this research, we studied *n*-alkane biodegradation, and the effect of oil association with organic and clay particles, during and after settling at a sediment layer in microcosms. We observed 40% less *n*-alkane biodegradation when oil was in association with artificial marine snow. Our results align with findings from a field study with sediment samples collected in the Gulf of Mexico after the DWH oil spill. This report describes the depletion of oxygen in the sediment layers after the DWH event (Hastings et al., 2016), which results in less biodegradation for many alkanes after deposition to the seafloor (Bagby et al., 2017).

In the presence of marine snow, the oxygen concentration in the water 10 cm above the sediment was up to 52% after 6 days of incubation (Figure 4.1), despite continuous gentle aeration at the water surface, showing enhanced oxygen consumption due to marine snow degradation.

Hydrocarbon degrading bacteria are not necessarily limited to the consumption of hydrocarbons as a carbon source. In general, these bacteria utilize hydrocarbons and other oil compounds as carbon and energy sources when easily degradable carbon is not available. The physiological activity of bacteria and their metabolic enzymes determine the function and carbon source consumption by bacteria (Mukherjee et al., 2017; Xu et al., 2018). Oil compounds such as *n*-alkanes are saturated and considered more recalcitrant to bioconversion than the organic fraction of marine snow. Therefore, marine snow will be the preferred carbon source that depletes the present oxygen and thus will delay the biodegradation of the *n*-alkanes.

Both our artificial marine snow and natural marine snow contain carbon compounds that are easily degradable with concomitant oxygen use. Especially the carbohydrate of marine snow have oxidized groups in their molecular structures, which are readily available for further enzymatic conversions and mineralization (Alldredge and Silver, 1988; Bochdansky et al., 2010; Gutierrez et al., 2018; Shanks and Trent, 1980).

Relative to marine snow, oil is recalcitrant to biodegradation because of its stable reduced aliphatic or aromatic molecular structures that need to be activated for further mineralization, i.e., through oxygen inclusion by oxygenases (Chikere et al., 2011; Nzila, 2018; Shrivastava and Phale, 2012). As a result, biodegradation of oil components with half-lives up to 60 days, depending on the oxygen availability, will be slower compared to, e.g., marine snow (Bagby et al., 2017; Prince et al., 2017; Reddy et al., 2012). Therefore, when both marine snow and oil are present, marine snow will be a preferred substrate over oil for the aerobic microbial community. Oil degradation is then further hampered by oxygen depletion, as we have seen, especially during the first 6 days of incubation.

In our experiment, the air was continuously bubbled into the water at 5 cm below the water surface, but still, reduced oxygen concentrations were detected in microcosms with marine snow, especially within the first 6 days of incubation. Follow-up microcosm experiments, including eco-toxicological effects on invertebrate organisms, also demonstrated oxygen depletion in the sediment layer in the presence of marine snow (van Eenennaam et al., 2018). At the deep sea, oxygen availability in the water phase nearby the sediment layer could be limited, depending on the depth and hydrological conditions. Depleted oxygen in the deep sea top sediment

layer with more than 1 cm of marine snow-associated oil most likely contributed to the persistence of oil-sediment layers are reported for the DWH oil spill (Bagby et al., 2017) and the Ixtoc-I spill in 1979 (Lincoln et al., 2020), where MOSSFA had occurred as well (Lincoln et al., 2020; Vonk et al., 2015). Also, anaerobic conditions were detected in the sediments layer after the DWH oil spill (Bacosa et al., 2018), suggesting that marine snow may indeed have limited the oil biodegradation in these sediments. A study showed that the intensity of anaerobic conditions in the sediment layer of GoM lasted for up to three years after the DWH spill, likely as a result of excess organic matter and hydrocarbon burial and decomposition in the sediments (Daly et al., 2016; Foekema et al., 2020; Hastings et al., 2016).

Our results elucidate that the *n*-alkanes biodegradation rate in the sediment layer is influenced by two related factors: *carbon source competition* and *oxygen availability*. When high concentrations of marine snow or other easily degradable carbon sources are present, aerobic biodegradation of marine snow is preferred, followed by the biodegradation of *n*-alkanes.

The association of oil with marine snow by itself does not mean that the *n*-alkanes biodegradation will decrease. In conditions with no oxygen limitation, the inclusion of oil in marine snow could even increase the *n*-alkanes biodegradation, as shown for the water phase (Rahsepar et al., 2017). The increased degradation of organic carbon sources caused depletion of oxygen at the sediment layer up to 3 years following the DWH spill (Hastings et al., 2016).

This study is a first step in elucidating the effects of marine snow in the deep sea ecosystem. The laboratory setup of this experiment allowed us to specifically study the biodegradation of marine snow-associated oil on the marine sediment. This fundamental knowledge is highly relevant to understand more complex systems. The limited availability of oxygen in the marine sediment layer can be enhanced by the bioturbation activities of benthic organisms (Foekema et al., 2020; Pelegrí and Blackburn, 1994). This might further affect the biodegradation rate of oil, which we further investigated in a microcosm experiment with representative benthic invertebrates (Chapter 5).

4.5 Conclusion

This study shows that biodegradation of *n*-alkanes in top sediments was slowed down up to 40% when oil was associated with marine snow. We believe two correlated factors play a role in this slow biodegradation: carbon source competition and oxygen availability. Marine snow can enhance oil biodegradation in the abundance of oxygen. However, when the dissolved oxygen is a limiting factor, preferential oxygen consumption for the degradation of marine snow competes with oil degradation, thus slowing down the *n*-alkanes biodegradation. This needs to be taken into account when dealing with future oil spills.

Acknowledgements

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Supplementary Materials

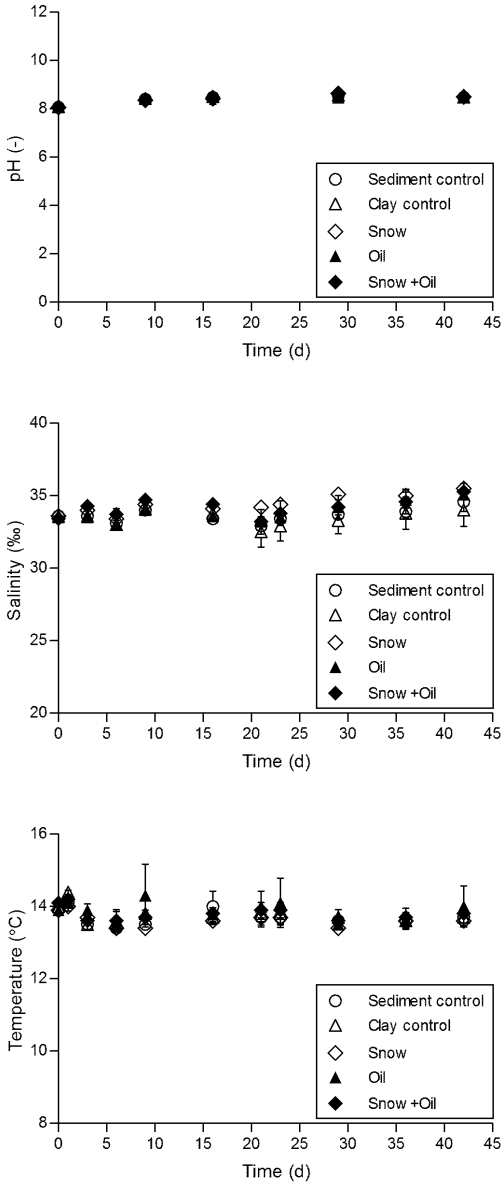

Figure S4.1 Water column measurements of pH (top), Salinity (middle), and temperature (bottom). Average and standard deviation of three microcosm replicates.

Chapter 5

Marine snow increases the adverse effects of oil on benthic invertebrates

A modified version of this chapter has been published as

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Abstract

After the Deepwater Horizon oil spill, a MOSSFA (Marine Oil Snow Sedimentation and Flocculent Accumulation) event took place, transporting an estimated 14% of total released oil to the sediment and smothering parts of the benthic ecosystem. This microcosm study describes the effects of oiled artificial marine snow on benthic macroinvertebrates. *Corophium volutator* survival was reduced by 80% in oil-contaminated snow. *Hydrobia ulvae* survival was reduced by 40% in oil-contaminated snow, possibly due to the consumption of oiled snow. *Macoma balthica* was sensitive to marine snow, and the addition of oil slightly decreased survival. This study reveals trait-dependent sensitivity to oil with or without marine snow. The main drivers for organismal response to marine snow and oil are sensitivity to hypoxia and oil toxicity, motility, and feeding habits. Adverse effects of MOSSFA events on benthos will have consequences for the benthic-pelagic habitat and food chain and should receive more attention in oil spill management.

Keywords

Benthic invertebrates; Marine snow; MOSSFA; Oil toxicity; Oil spill

5.1 Introduction

The Deepwater Horizon (DWH) oil spill in the Gulf of Mexico in 2010 was one of the largest marine oil spills in US history. Over three months, 4.9 million barrels of oil leaked into the sea (McNutt et al., 2012b). The main spill response was the use of 6.8 million liters of dispersants (Kujawinski et al., 2011), both applied at the sea surface as well as injected in the deep marine at the wellhead.

One of the unexpected events seen during the DWH spill was the unprecedented formation of marine snow. This was probably due to the increased production of extracellular polymeric substances (EPS) by the microbial and phytoplankton community as a biological stress response to dispersants and oil (Passow et al., 2012; van Eenennaam et al., 2016; Ziervogel et al., 2012). Laboratory-induced EPS made by phytoplankton-associated bacteria was found to contain substantial amounts of alginate-like exopolysaccharides (van Eenennaam et al., 2016), and a peak of polysaccharides in sediment from the Gulf of Mexico coincided with the marine snow formation during the spill (Hollander et al., 2016). In addition, during the oil spill, there was a large amount of suspended solids due to the flushing of the Mississippi river as a spill response (Bianchi et al., 2011) as well as a phytoplankton bloom (Hu et al., 2011; O'Connor, 2013).

Marine snow aggregates, made of dispersed oil, organic debris, phytoplankton, and suspended particles, glued together by the sticky EPS, settled on the seafloor in a process called MOSSFA: Marine Oil Snow Sedimentation and Flocculent Accumulation (Daly et al., 2016). MOSSFA increased sedimentation rates and caused a downward flux of oil to the sediment (Brooks et al., 2015; Hastings et al., 2016; MOSSFA Steering Committee, 2013). Estimates vary, but as much as 14% of the total oil released during the DWH oil spill may have ended up on the sediment due to MOSSFA (Daly et al., 2016). Estimates of the total area of sedimentary oil deposition range from 3,200 km^2 (Valentine et al., 2014) to 24,000 km^2 (Chanton et al., 2015). A review of large historical oil spills has indicated that the MOSSFA process may have occurred during other spills as well, such as the IXTOC-I blowout (Vonk et al., 2015).

The MOSSFA related oil contamination sparked interest in the potential long-term effects of sedimented oil for benthic habitats (Kinner et al., 2014). Benthic

organisms, especially those that are sedentary, are particularly at risk for the MOSSFA related oil contamination (Fisher et al., 2016) since they cannot easily escape the contamination. Moderate to severe reduction of macro- and meiofaunal abundance and diversity were found over an area of 172 km^2 around the wellhead (Montagna et al., 2013). Persistent reducing conditions in the sediment and the 2-3 fold increase in PAHs reduced benthic foraminiferal diversity and density (Schwing et al., 2016; Schwing et al., 2015).

MOSSFA could affect benthic ecosystems via two mechanisms: 1) direct toxicity of the oil and 2) reduced oxygen availability caused by the microbial degradation of the marine snow. Direct oil toxicity to benthic organisms is widely reported, both in experimental studies (e.g., (Foekema et al., 1996) and (Bhattacharyya et al., 2003)) and in oil spill observations (e.g., (Teal and Howarth, 1984), (Lee and Lin, 2013), and (Jewett et al., 1999)). The availability of oxygen in deeper layers of the sediment can be increased by the bioturbation activity of many benthic organisms (Pelegrí and Blackburn, 1994). Bioturbation enhances the sediment oxygenation, solute transport, and remineralization of organic matter and mixes horizontal sediment layers (Levin, 2003). Oxygen consumption by biodegradation of marine snow, the second mechanism, can impact benthic organisms after a MOSSFA event. The accumulation of organic material on the seafloor increased microbial respiration, resulting in decreased oxygen in sediment pore waters (Hastings et al., 2016). In laboratory studies, artificially produced marine snow was found to consume oxygen at a rapid rate (Rahsepar et al., 2017). This lower oxygen concentration in the sediment is detrimental to benthic invertebrates living in the top layers of the sediment.

Benthic organisms, in general, are an important part of the marine food web, and many pelagic species are dependent on the benthic ecosystem for feeding or reproduction, while some have benthic life stages. Most oil effect studies are performed with pelagic species and without marine snow or sediment. This article describes a microcosm experiment revealing the additional effect of marine snow on the toxicity of oil on marine benthic invertebrates. The effect of the presence of invertebrates on bioturbation and oil biodegradation is also presented. The experiment simulated the effect of marine snow and oil in small-scale microcosms with sediment, natural seawater, and four representative species of benthic invertebrates. These invertebrates reflect four different benthic lifestyles and feeding strategies (traits): an infaunal deposit and suspension feeding amphipod, an epifaunal

deposit feeding and grazing gastropod, an infaunal deposit and suspension feeding bivalve, and epi- and infaunal deposit and suspension feeding foraminifera.

5.2 Materials and methods

5.2.1 Experimental setup

The basic experimental design was similar to Chapter 4's, with the inclusion of benthic macroinvertebrates. In short, 21 microcosms of 25 × 25 × 25 cm were placed in a climate-controlled room at 14°C, with a day-night light regime of 16 h light and 8 h dark. Full glass microcosms were used to avoid the presence of silicone rubber.

Sediment was collected during low tide from the top 10 cm of an intertidal mudflat in the Dutch Wadden Sea, at approximate location N 52° 56.112 E 004° 59.976, and transported directly to the laboratory of Wageningen Marine Research, Den Helder, The Netherlands. Sediment was subsequently sieved over a 1 mm sieve to remove large organisms and particles and thoroughly mixed by hand to create homogeneous sediment. The microcosms were filled with a 5 cm thick layer of sediment and a 15 cm layer of natural seawater (0.45 µm filtered Eastern Scheldt water) on top. The sediment was left to settle for one day prior to the start of the experiment.

The next day, day 0, the treatments as described in section 5.2.3 were added to the microcosms. One day later (day 1), 40 *Corophium volutator* (amphipod), 20 *Macoma balthica* (bivalve), and 20 *Hydrobia ulvae* (gastropod) were randomly added to each microcosm. These test organisms were collected at the same time and location as the sediment and were kept in the lab at 14 ºC with natural seawater and aeration. The natural sediment already contained foraminifera.

Air was bubbled via two tubes with glass Pasteur pipette tips per microcosm in the top 5 cm of the water column, using an aquarium air pump. Microcosms were covered with acrylic plastic covers to minimize evaporation. Approximately once per week, the water level was adjusted with demineralized water to compensate for evaporation.

In order to stimulate biodegradation of the oil, 1 mL of *Rhodococcus qingshengii* TUHH-12 (DSMZ No. 46766), an alkane-degrading bacterial culture, with an optical density of 0.98, and 1 mL of *Pseudomonas putida* F1 (DSMZ, No. 6899), an

aromatic-degrading bacterial culture, with an optical density of 0.305, were added to the water column of each microcosm at day 0. See (Rahsepar et al., 2016) for the culturing procedure.

5.2.2 Treatments

Five treatments were tested in triplicate (Table 5.1). The Control treatment consisted of only a sediment layer. The Oil treatment consisted of oil mixed with clay particles to let the oil settle on top of the sediment. As a control for this treatment, a Clay only treatment was included with the same amount of suspended clay. The Snow + Oil treatment included artificial marine snow plus oil mixed with clay. As a control, the Snow treatment was the same but without oil. The clay was needed to make the marine snow and oil negatively buoyant. With the clay, no floating oil and/or marine snow were visible neither on the surface nor in the water column.

Table 5.1 Test treatments for the microcosm experiment. Artificial marine snow was composed of alginate, phytoplankton biomass, and kaolin clay.

	Treatment description	**Treatment code**	**Oil**	**Kaolin clay**	**Alginate and phytoplankton biomass**
Set 1: In vivo observations	Control	Control			
	Kaolin clay	Clay		✓	
	Marine snow	Snow		✓	✓
	Oil and kaolin	Oil	✓	✓	
	Marine snow and oil	Snow + Oil	✓	✓	✓
Set 2: Biodegradation sampling	Oil and kaolin	Oil/B	✓	✓	
	Marine snow and oil	Snow + Oil/B	✓	✓	✓

In addition to the five treatments in triplicate as mentioned above, in which invertebrate survival was tested for 16 days, a second set of 6 microcosms was run in parallel with only the Oil and Snow + Oil treatments used for oil biodegradation sampling. These microcosms in Set 2 had to be separate from the microcosms in Set 1, since taking sediment samples for oil biodegradation analysis from the same microcosms would interfere with the final observations of invertebrates survival. The microcosms in Set 2 ran for 42 days, and samples were taken on days 1, 8, 16, 30, and 42, using the method described in Chapter 4.

5.2.3 Test treatment preparation

Artificial marine snow was used as an approximation of natural marine snow since it was not feasible to create natural marine snow in the amounts needed for this study. To mimic the lab-induced EPS containing alginate-like exopolysaccharides (van Eenennaam et al., 2016), we used commercially available alginate, a gelling and nontoxic anionic polysaccharide, to create artificial marine snow with the addition of phytoplankton and kaolin clay particles, with or without oil.

Artificial marine snow was prepared for three replicates as one batch by slowly adding 22.5 g alginate (alginic acid sodium salt, CAS 9005-38-3, Arcos Organics; 7.5 g for each microcosm replicate) to 1.1 L filtered natural seawater while stirring. Then, 19 g fresh weight of phytoplankton biomass (*Chlorella* paste, Ingepro BV, Borculo, The Netherlands; 6.3 g for each microcosm replicate) and 9.45 g kaolin clay (hydrated aluminum silicate, CAS 1332-58-7, Sigma Aldrich; 3.15 g for each microcosm replicate) were added. Next, 13 liters of a 14.5 g L^{-1} $CaCl_2$ (CAS 10043-52-4, Fluka Analytical) in demi-water solution were quickly added to the alginate-phytoplankton-clay mix and shaken by hand a few times. The calcium caused the alginate to coagulate, incorporating the phytoplankton and clay particles in precipitated marine-snow-like flocks. The overlying liquid was poured off before dividing the marine snow into three equal parts for the microcosm triplicates. For the Snow + Oil treatments, 9.45 g kaolin clay was first mixed with 1.9 g oil (0.63 g for each microcosm replicate) and some demi water into a homogeneous slurry before adding it to the alginate-phytoplankton mix. The marine snow was gently added to the microcosms in order to cover the sediment with a 1 cm thick layer evenly.

The replicates of the Clay treatment were individually prepared. For this, 3.15 g kaolin clay was mixed with approximately 500 mL water from the microcosm and, this suspension was then poured into the microcosms. The clay settled homogeneously on top of the sediment in a layer that was <0.5 mm thick. The replicates of the Oil treatment were also individually prepared by mixing 3.15 g kaolin clay with 0.63 g oil and some demi water. Then, the suspensions were added to the microcosms.

In all oil exposures, there was an oil load per microcosm of 0.63 g per 625 cm^2, which is equivalent to 10 g m^{-2}. The 'Surrogate Macondo oil' that was used was

kindly provided by BP and is chemically similar to the oil from the Macondo oil well involved in the DWH oil (BP Gulf Science Data, 2017a). The oil was weathered for 24 h in the dark at room temperature while stirring on a magnetic stirrer to allow evaporation of lighter hydrocarbons. These lighter hydrocarbons, including aromatics, are the main cause of toxicity; therefore, weathering reduces the toxicity of the oil (Brils et al., 2002). Since the oil incorporated in marine snow aggregates during the MOSSFA event has also been weathered substantially due to evaporation and dissolution, using weathered oil in the microcosm experiment gives a more realistic toxicity scenario than fresh oil.

5.2.4 Water quality parameters

During the experiment, the following water quality parameters were regularly monitored in the middle of the water column: pH (-), temperature (°C), salinity (‰), dissolved oxygen saturation level (%). A Mettler Toledo pH meter was used to measure pH, and the other parameters were measured with a Hach HQ40d multimeter using salinity and dissolved oxygen probes. Both meters were calibrated prior to each measuring day. In addition, Oxygen Sensor Spots (PSt3, PreSens Precision Sensing GmbH, Regensburg, Germany) and a handheld Fibox 4 Optical Oxygen Meter (PreSens Precision Sensing GmbH, Regensburg, Germany) were used to monitor local concentrations of dissolved oxygen above and below the sediment-water interphase without disturbing the sediment integrity. Sensor spots were glued to the glass wall at four locations in Snow and Snow + Oil treatments, and because of practical reasons at only three locations in Oil, Clay, and Control.

5.2.5 Sampling for biodegradation analyses

The method to sample sediment over time for oil biodegradation analyses is the same as described in Chapter 4. In short, a glass tube with a diameter of 2 cm was inserted into the sediment to the bottom of the microcosm, from which the sediment was collected. The tubes were left in the microcosms until the end of the experiment to minimize disturbance of the surrounding sediment and suspension of sediment particles in the water column. Per sampling, four subsamples were combined to get a representative sample per microcosm. After removing the overlying water, the pooled sediment samples were freeze-dried using a Christ Alpha 2-4 LDplus freeze

dryer (Martin Christ Gefriertrocknungsanlagen GmbH, Osterode am Harz, Germany).

5.2.6 Chemical analyses

For chemical analysis, 5 g of freeze-dried sediment was extracted with 8 mL dichloromethane. An aliquot of the whole extract was first analyzed using GC-MS to check for the presence of the oil fingerprint. Then, the extract was separated to saturate and aromatic fractions by loading onto a silica gel column (0.6 g, 70-230 mesh), pre-washed with pentane and eluting with 2 mL of pentane to obtain the saturate fraction, followed by elution with 2 mL of dichloromethane and isopropyl alcohol, respectively, to obtain the aromatic fraction. The temperature program for the GC-MS analysis included a hold of 5 min at 40 °C, followed by a 4 °C/min ramp up to 325 °C, and finishing with an isothermal hold at 325 °C for 15 minutes. Separation was performed on an HP-5MS capillary column (30 m × 0.25 mm × 0.25 µm), and helium was used as the carrier gas at a flow of 1.0 mL min^{-1}.

The mass spectrometer acquired data in a combined full scan/selected ion monitoring (SIM-SCAN) mode. SIM trace at *m/z* 85 was used to integrate the areas of *n*-alkanes (C13-C30) and isoprenoid alkanes (pristane and phytane). Integrated areas were then normalized to the area of C30-hopane (17α(H),21ß(H)-hopane). C30-hopane is considered a conservative biomarker due to its recalcitrance to weathering (Prince et al., 1994). This normalization approach is typically used in oil spill studies to detect and compare compositional changes due to evaporation, biodegradation, and other weathering processes (Aeppli et al., 2014; Radović et al., 2014). The stability of C30-hopane was confirmed in our previous study described in Chapter 4.

5.2.7 Biological observations and sampling procedure

During the experiment, behavioral observations of the test organisms were made, and photos of the microcosms were taken. On day 16, a sample of approximately one tablespoon was taken from the top cm sediment for foraminifera analysis. Then, the entire content of the microcosm was gently sieved over a 1 mm sieve to collect the test organisms. The organisms were counted, and survival was assessed. *H. ulvae* were put on a glass petri-dish and inspected for movement. *M. balthica* were put on a glass petri-dish and observed for visible siphons to assess survival. After counting,

the organisms were preserved in 4% formaldehyde buffered with borax (sodium tetraborate decahydrate, Sigma-Aldrich, CAS Number: 1303-96-4). The foraminifera samples were stained with 2.5 mL of 3.3 mg L^{-1} rose Bengal (purity 95%, Sigma-Aldrich, CAS Number: 632-69-9) and preserved in 4% formaldehyde. Living (stained) and dead (unstained) foraminifera were counted using a binocular microscope.

5.2.8 Statistical analyses

GraphPad Prism 5 was used to draw graphs and perform statistical analysis using one-way ANOVA with post-hoc Tukey's test or two-way ANOVA with Bonferroni multiple comparisons post-test.

5.3 Results

5.3.1 Water quality

The water quality parameters temperature, pH, and salinity were measured in the water column (Figure S5.1). The temperature remained stable at 14.4 ± 0.2 °C, without significant differences between any of the treatments. In addition, there was no difference in salinity between any of the treatments over the course of the experiment; salinity was stable at 33.5 ± 0.7 ‰ throughout the experiment. Overall, the pH was stable at 8.26 ± 0.19. However, in the Snow and Snow + Oil treatments, pH was slightly, but statistically significant, lower than in the other treatments on days 3 and 10.

The dissolved oxygen saturation, measured halfway in the water column, was 97.7 ± 3.8% in all microcosms during the entire experiment, indicating no oxygen depletion in the water column. However, subtle decreases in the treatments with marine snow were observed on days 3-10 (Figure 5.1): oxygen saturation, expressed relative to the Control treatment at each day, was significantly lower in both marine snow treatments. On day 15, oxygen saturation was similar again in all microcosms.

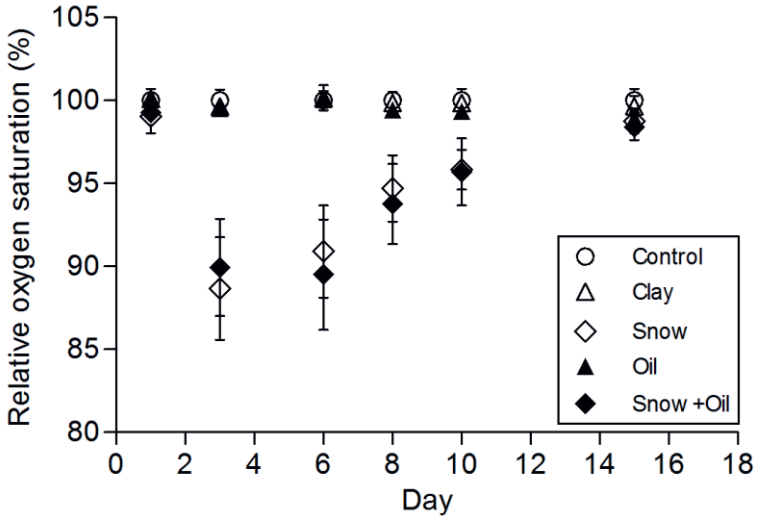

Figure 5.1 Dissolved oxygen saturation (%) in the water column during the 16 day experiment, relative to Control of each day (average and standard deviation of triplicate microcosms, except Control day 8: duplicate).

In addition to dissolved oxygen in the water column, the vertical oxygen profile in the water just above the sediment layer and in the sediment layer itself was measured with Oxygen Sensor Spots on days 1, 8, and 15 (Figure 5.2, Figure S5.2-S5.3). In the Snow and Snow + Oil treatments on day 1, the oxygen saturation in the marine snow layer just above the sediment interface was lower than in the Oil treatment: ~ 40% vs. ~ 90%. However, on days 8 and 15, the oxygen saturation just above the sediment interface in Snow and Snow + Oil increased again to levels comparable to day 1.

5.3.2 Chemical analyses

Clean sediment from the Control treatment contained background levels of *n*-alkanes; however, they showed distribution characteristics of natural biogenic input (i.e., odd over even preference), such as algae (Gelpi et al., 1970; Weete, 1976). On the contrary, in the oil-containing treatments, *n*-alkanes showed the typical unimodal distribution peaking at *n*-C17, characteristic of light oils, such as Macondo oil (Figure 5.3, Figure S5.4).

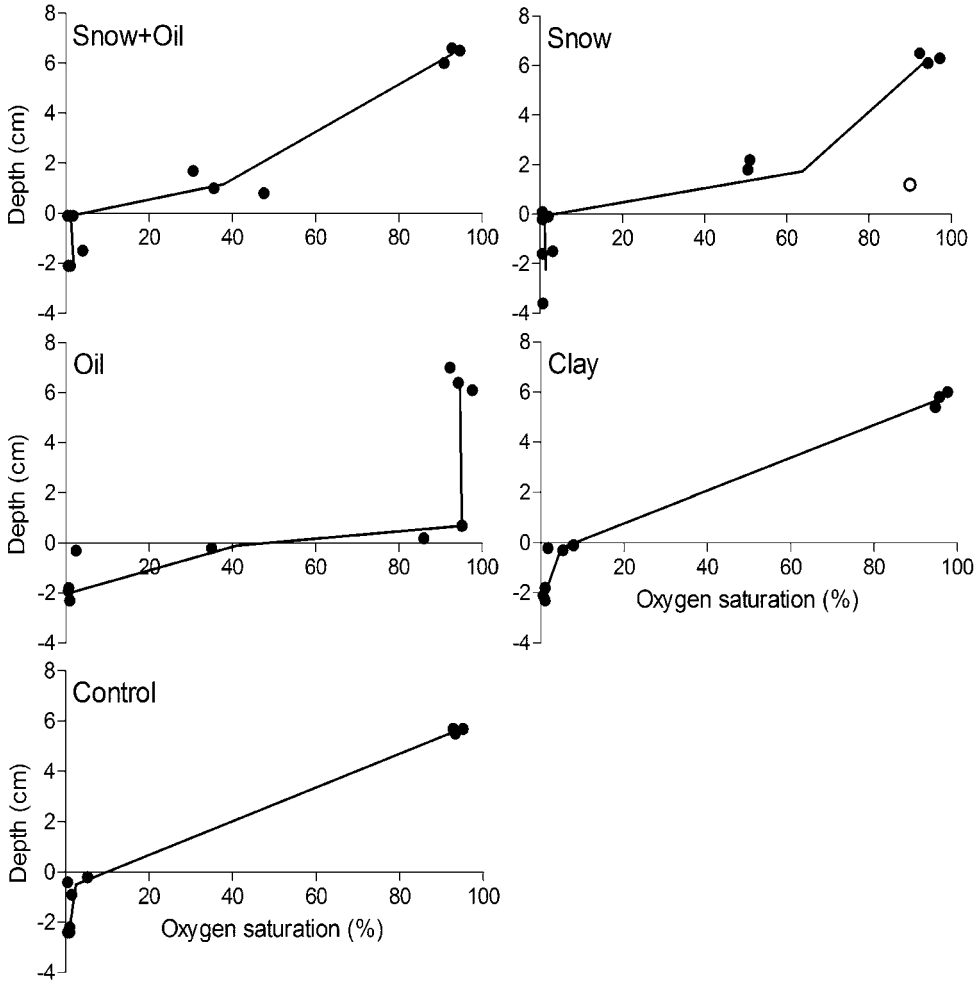

Figure 5.2 Oxygen saturation profiles of the microcosms at day 1. Depth refers to the relative position of each Oxygen Sensor Spot to the sediment-water interface at 0 cm. The data points are individual measurements of three replicate microcosms. The black line is the average of the three replicates. The open circle in the Snow graph represents an Oxygen Sensor Spot that was located above the marine snow layer, while the other two Oxygen Sensor Spots at similar depth were inside the marine snow layer. For this reason, the oxygen saturation at this location was much higher than the other two locations.

There was no difference in *n*-alkane and isoprenoid alkane hopane normalized peak areas on day 1 between Snow + Oil and Oil. However, on day 42, the normalized peak areas are much lower for both treatments compared to day 1, and a clear difference between the Snow + Oil and Oil treatments is observed. There is 61% biodegradation after 42 days in Snow + Oil, compared to 83% degradation in Oil (Table 5.2), indicating inhibition of oil degradation related to the presence of marine snow.

Table 5.2 Relative oil biodegradation over four-time ranges. Data from this study was calculated based on data from Chapter 4: a similar microcosm experiment with artificial marine snow, without benthic macroinvertebrates.

	Relative oil biodegradation (as decrease of C13-C30 relative peak areas compared to day 1)			
Day range	**Oil**	**Snow + Oil (this study)**	**Oil (Chapter 4)**	**Snow + Oil (Chapter 4)**
1 – 8	18%	1%		
8 – 16	51%	27%		
1 – 16	69%	28%	49%	17%
16 – 42	14%	33%	17%	9%
Total oil biodegradation over 42 days	83%	61%	66%	26%

In addition, a preferred degradation of the short-chain *n*-alkanes compared to the long-chain *n*-alkanes is obvious. In both treatments, the majority of degradation took place between day 8 and day 16 (Table 5.2), with 18% degradation between day 1 and day 8 in the absence of marine snow and hardly any degradation in the presence of marine snow.

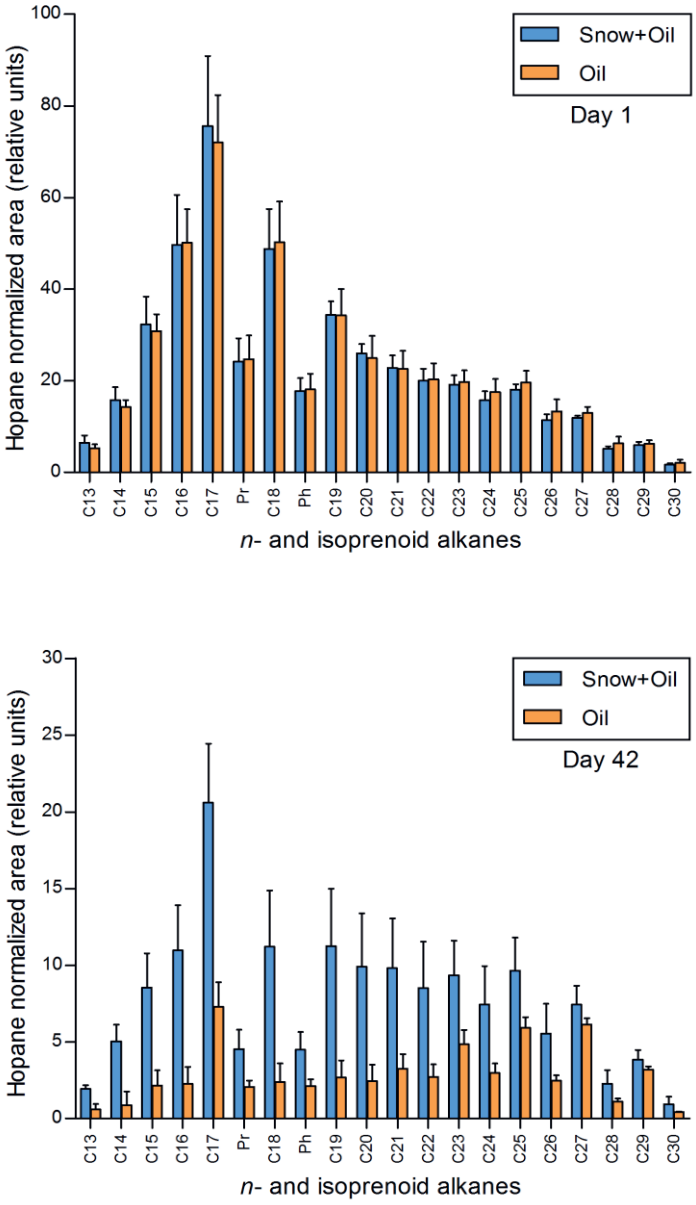

Figure 5.3 Hopane-normalized peak areas for *n*-alkanes and isoprenoid alkanes (pristane and phytane) on day 1 (top) and day 42 (bottom) (average with standard deviations of triplicates).

5.3.3 General in vivo observations

Throughout the 16 days of the in vivo experiment, the water in the microcosms with Oil stayed clear, while the water in Control and Clay turned turbid after three days. The microcosms with marine snow were more turbid than the others and had a dense fouling layer on the glass. After 6 days, the marine snow layer became thinner, and a white and black layer, possibly fungi, covered parts of the surface. After 16 days, the thickness of the oxygenated top layer was relatively thick (~5 mm or more, indicated by the yellow box in Figure 5.4A) in both Clay and Control treatments, with visible bioturbation activity. In contrast, in the Oil treatment, the oxygenated layer was thinner (~2 mm) without visible burrowing behavior and *H. ulvae* tracks on the sediment and the glass microcosm wall (Figure 5.4B). No oxygenated top layer was observed in the microcosms of Snow and Snow + Oil treatments, except where the marine snow layer did not completely cover the sediment due to a non-homogeneous marine snow layer (Figure 5.4C, Figure S5.5-S5.7).

5.3.4 Invertebrate survival

In the Control treatment, 25 out of 40 *C. volutator*, 13 out of 28 *H. ulvae*, and 17 out of 19 *M. balthica* were recorded alive after 16 days. Thus, for all three species, the survival in the Clay treatment was not significantly different from the Control treatment.

The survival percentage of *C. volutator* was significantly ($p < 0.05$) lower in Oil and Snow + Oil treatments, relative to Control (9% and 20% respectively, Figure 5.5, top panel). Snow without oil also reduced the relative percentage of surviving *C. volutator* to 69%, but this effect was not statistically significant.

The survival percentage of *H. ulvae* did not differ significantly between treatments (Figure 5.5, middle panel); however, there is a trend of lower *H. ulvae* survival in the Snow + Oil treatment, but not in Oil or Snow separately. More *H. ulvae* were attached to the glass wall or floating at the water surface in the Oil treatment than in the other treatments.

M. balthica survival was reduced in the Snow and Snow + Oil treatments (Figure 5.5, bottom panel). Survival in Snow + Oil was lower than either Snow or Oil

separately (21% compared to 32% and 102%, respectively) and was significantly different from the Control treatment ($p = 0.041$) but not from Snow ($p = 0.984$).

The ratio between living and dead foraminifera after 16 days was 0.28 ± 0.09 in the Control treatment. This ratio was significantly reduced by 68%, 78%, and 90% in Oil, Snow, and Snow + Oil treatments, respectively, compared to Control (Figure 5.6).

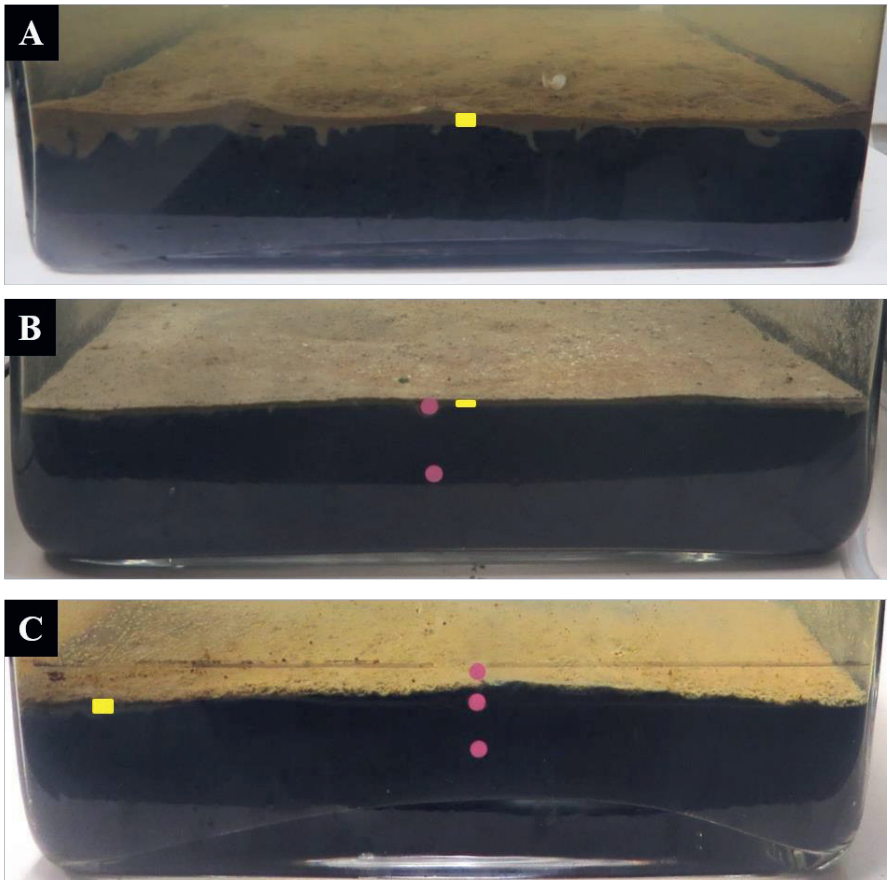

Figure 5.4 Examples (at day 16) of a thick (~5mm or more) oxygenated layer of a Control microcosm with abundant visible burrowing activity (A) and a thin (~2 mm) oxygenated layer of an Oil microcosm without visible burrowing activity (B). No oxygenated layer was present in microcosms with Snow + Oil (C), except for a patch in the left corner where no marine snow was accumulated. The height of the yellow boxes indicates the thickness of the oxygenated layer. The pink dots in B and C are the Oxygen Sensor Spots.

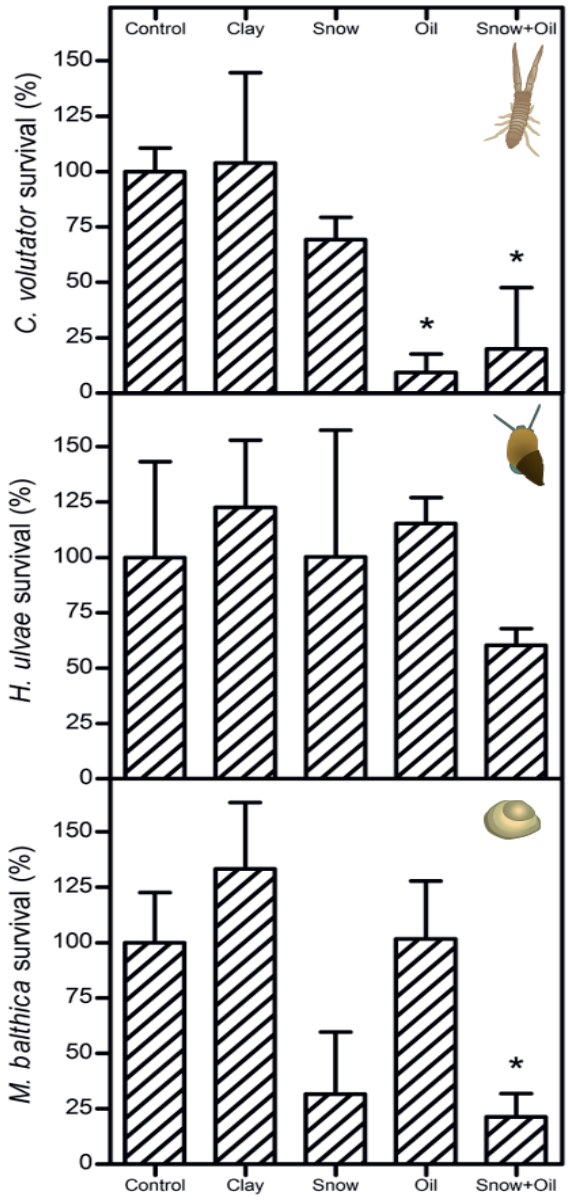

Figure 5.5 Survival of *C. volutator* (top panel), *H. ulvae* (middle panel), and *M. balthica* (bottom panel) after 16 days exposure, compared to Control (100%) (average and standard deviation of triplicate microcosms. * indicates statistically significant difference from Control at $p < 0.05$).

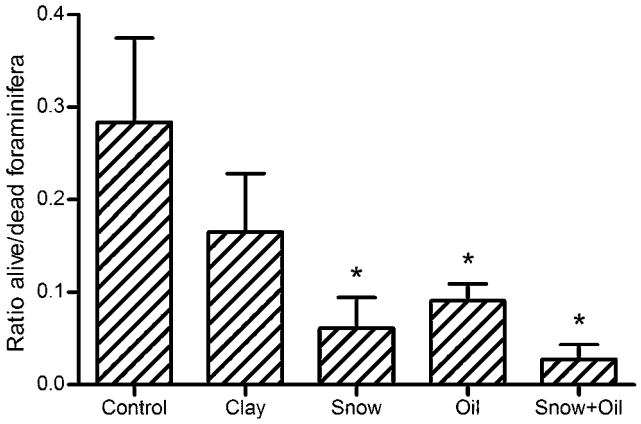

Figure 5.6 Ratio of alive/dead foraminifera after 16 days (average and standard deviation of triplicate microcosms. * indicates statistically significant difference from Control at $p < 0.01$).

5.4 Discussion

This study presents the impact of marine snow on the toxic effects of slightly weathered crude oil on marine benthic invertebrates and the biodegradation of the oil. Three single species and one naturally occurring community of species were studied: an infaunal deposit and suspension feeding amphipod (*Corophium volutator*), an epifaunal deposit feeding and grazing gastropod (*Hydrobia ulvae*), an infaunal deposit and suspension feeding bivalve (*Macoma balthica*), and epi- and infaunal deposit and suspension feeding foraminifera. This combination of organisms is representative of three main benthic niches to represent the benthic toxic effects of oil and marine snow. Our results reveal that the main driving factors for the responses to oil and marine snow are sensitivity to hypoxia, motility, and feeding habits. The ultimate net effect of oil-contaminated marine snow will be a balance between these different driving factors, determined by differences in traits of the species. The three main driving factors are discussed below.

Hypoxia, defined as dissolved oxygen concentrations of generally lower than 2 mg L^{-1} (Dale et al., 2010), can be caused by the degradation of marine snow, which consumes oxygen in the sediment and the water column just above the sediment

(Figure 5.2), ultimately resulting in a complete disappearance of an oxygenated top layer (Figure 5.4). Hypoxia did not occur higher in the water column (Figure 5.1) due to air bubbling in the top 5 cm of the water column. However, the lower pH and concomitant lower oxygen saturation in the water column in the first 10 days of the exposures with marine snow indicate that there was a slight acidification due to increased respiration.

Motility determines whether the organisms are able to escape unfavourable conditions, like hypoxia and toxicity, which can drive infaunal species, such as *C. volutator*, out of their infaunal habitat into the water column. Sedentary species, such as *M. balthica*, experience more severe effects of oil spills than motile species (Fisher et al., 2016; Lee and Lin, 2013) because of their inability to escape adverse conditions.

Organisms that are likely to use marine snow as a food source could actively expose themselves to the oil by consuming oil-contaminated marine snow. This was the case in our experiments with *H. ulvae*, who were able to escape the oil by climbing onto the glass wall, but remained on the sediment surface to eat the oiled marine snow. The artificial marine snow used in our study consisted of alginate, which is a nontoxic anionic polysaccharide, and phytoplankton biomass plus clay to make it negatively buoyant. Therefore marine snow is a suitable food source for especially grazers and deposit/suspension feeders.

5.4.1 Differential organismal responses to oil and marine snow

The four invertebrate species responded in different ways to the benthic exposure to oil and marine snow, related to their biological traits.

***Corophium volutator*:** In the present experiment, the amphipod *C. volutator* escaped from low oxygen saturation at the sediment-water interface due to the presence of marine snow. However, even though they escaped the sediment, the survival was still reduced by 31% to 69% compared to Control in the treatment with marine snow (Figure 5.5). *C. volutator* is sensitive to hypoxia, as was observed in both infield and laboratory experiments (Gamenick et al., 1996; van den Heuvel-Greve et al., 2007). The amphipods are typically burrowed in the sediment, but they can escape by swimming in the water column in adverse conditions like low sediment oxygen. In

contrast, the organisms did not escape to the water column when exposed to only oil because there was no hypoxia caused by marine snow. As a result, survival was severely reduced to 9% of Control due to oil toxicity. *C. volutator* are highly sensitive to oil (Foekema et al., 1996; Wake, 2005). In general, crustaceans are more sensitive to oil than other aquatic organisms (Anderson et al., 1974; Gesteira and Dauvin, 2000; Lee et al., 1977; Wake, 2005). The combination of oil and marine snow led to a low survival of 20%, which is higher than the survival in the oil-only exposure. This can be due to the avoidance behavior of the organisms, as well as the nutritional value of the marine snow itself. Most likely, the organisms escaped the low oxygen situation in the marine snow layer and thus reduced direct exposure to oil. The deposit and suspension feeding *C. volutator* might have been feeding on the oil-contaminated marine snow, given the additional 49% decrease in survival in this treatment compared to the treatment with clean marine snow, although direct contact with the oil cannot be excluded.

***Hydrobia ulvae*:** The response of H. *ulvae* in this study is driven by three factors: motility, sensitivity to hypoxia, and feeding habits. The epifaunal deposit feeding and grazing gastropod *H. ulvae* is motile, highly tolerant to hypoxia (Gamenick et al., 1996), and less sensitive to oil than *C. volutator* and *M. balthica* (Wake, 2005). Firstly, *H. ulvae* avoided oil on the sediment by attaching to the glass wall of the microcosm and floating at the water surface, as observed in the treatments with oil. A similar response was observed in a microcosm study (Chronopoulou et al., 2013), where *H. ulvae* were seen escaping experimentally oiled cores in microcosms. Secondly, their high tolerance to hypoxia allowed *H. ulvae* to escape the low oxygen saturation in the marine snow layer, as showed by the high survival in the treatment with clean marine snow (Figure 5.5). Thirdly, *H. ulvae* are grazers and deposit feeders whose diet includes macro- and microalgae and detritus (Aberle et al., 2009) and thus are likely to feed on marine snow. Therefore, they will be orally exposed to oil when feeding on oil-contaminated marine snow, as is suggested by the low survival in the treatment with oil-contaminated marine snow: 60% compared to Control (Figure 5.5). Longer exposure time and internal oil measurements can verify the possible pathway of oil from marine snow to organisms.

***Macoma balthica*:** The bivalve M. *balthica* is a deposit feeder with low motility, high vulnerability to hypoxia (Brafield, 1963), and lower sensitivity to oil than *C. volutator* (Wake, 2005). In this experiment, *M. balthica* was more affected by

oxygen depletion than by the presence of oil. In our experiment, the main driving factor for mortality was the low oxygen saturation in the marine snow layer because of the inability of *M. balthica* to escape adverse conditions due to its low motility. Therefore, *M. balthica* suffered more from the low oxygen saturation in the marine snow layer than the other two, more motile, species (Figure 5.5). The survival in treatments with marine snow (32%) and oil (102%) separately was higher than in the combination of both: survival in the treatment with oil-contaminated marine snow was only 21% compared to Control. This indicates a synergistic effect, likely because of internal oil exposure due to the deposit feeding strategy of *M. balthica*. The oil, at 10 g m^{-2}, did not reduce survival in the treatment with only oil (Figure 5.5). This is in line with the findings of Shaw et al. (1976), who performed a field mesocosm experiment with a simulated oil slick stranding at two oil loadings: 1.2 µL cm^{-2} and 5 µL cm^{-2}. At 1.2 µL cm^{-2}, which corresponds to 10 g m^{-2} of oil at relative density 0.838 (BP Gulf Science Data, 2017a), there was no significant mortality to *M. balthica*, while high mortality was found at 5 µL cm^{-2} (42 g m^{-2}).

***Benthic foraminifera*:** Benthic foraminifera is used as an indicator for sediment hypoxia because of their high sensitivity to changes in oxygen concentration (Dale et al., 2010). Our finding that oil and marine snow negatively affected the survival of the natural benthic foraminiferal community is in line with field studies in the Gulf of Mexico after the DWH spill. These field studies showed that the decrease in benthic foraminiferal diversity and density was related to the spill and the MOSSFA event (Schwing et al., 2016; Schwing et al., 2015). Internal oil measurements in the foraminifera tests could shed more light on the uptake of oil compounds by these organisms.

5.4.2 Effects of marine snow and benthic invertebrates on oil biodegradation

The current study with benthic invertebrates is a follow-up of the microcosm study of Chapter 4, in which the effect of marine snow on oil biodegradation in the sediment layer was studied. With the results of this study and the previous study without invertebrates, we can elucidate the effects of invertebrates on biodegradation.

Oil biodegradation in our experiment was reduced in the presence of marine snow (Figure 5.3, Table 5.2), which is in accordance with the results of Chapter 4, a similar microcosm experiment without invertebrates. Most likely, preferential oxygen consumption for the degradation of marine snow competes with oxygen consumption for oil degradation, thus inhibiting oil degradation. In the present study, the majority of the oil biodegradation takes place between day 8 and day 16 (Table 5.2), and a striking difference is visible during days 1-8: hardly any oil degradation takes place in microcosms with marine snow. This is in line with the hypothesis that marine snow consumes oxygen during degradation and thus inhibits oil degradation. Compared to results from Chapter 4, there is more oil biodegradation in the presence of benthic invertebrates (Figure 5.7, Table 5.2): after 42 days, 83% of the oil (without marine snow) is degraded in the presence of invertebrates, versus 66% without invertebrates. In the treatment with oil-contaminated marine snow, 61% of oil is degraded in the presence of the invertebrates versus 26% without invertebrates.

The reduction in oil biodegradation due to marine snow was 22% in the present study, compared to 40%, according to Chapter 4. The benthic invertebrates stimulate the degradation of oil with and without marine snow. Because of the bioturbation by the invertebrates, oxygen penetration into the marine snow layer and sediment layer can increase. This can partly counterbalance the negative effect of marine snow on oil biodegradation. Especially the amphipod *C. volutator* is an important bioturbator, which thoroughly mixes the top layer of the sediment. Bioturbation enhances oxygen penetration into the sediment and transport of compounds from anoxic layers to the oxic top layer (Pelegrí and Blackburn, 1994). *C. volutator* is able to increase the oxic sediment volume by as much as 150% at high densities (Hylleberg and Henriksen, 1980).

In our experiment, the oxygenated top layer in microcosms with only oil was thinner than in the control microcosms (2 mm vs. 5 mm), while the layer was completely absent in the microcosms with marine snow, either with or without oil (Figure 5.4). Lower oxygen concentrations in the sediment without benthic invertebrates hamper the oil biodegradation compared to sediment with invertebrates.

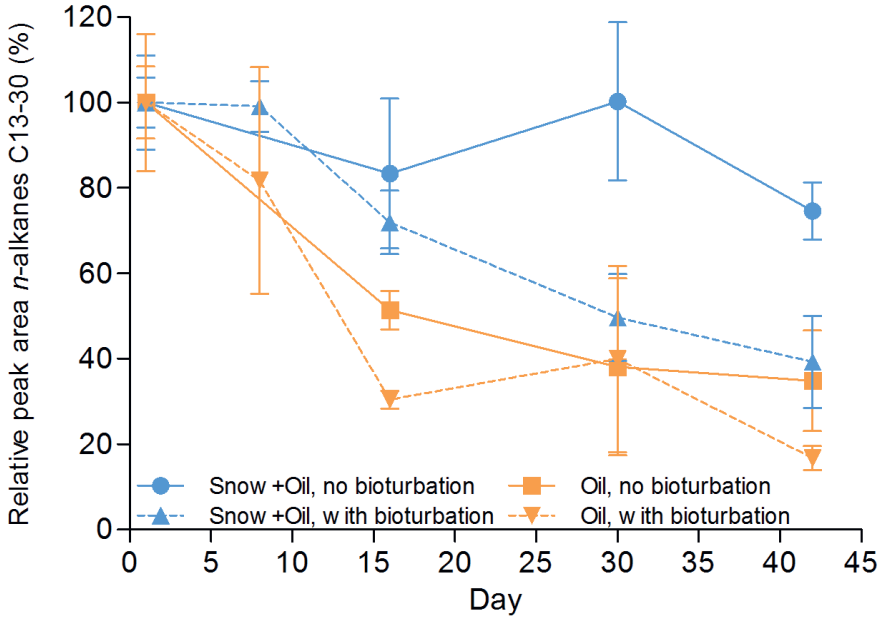

Figure 5.7 Relative peak area of *n*- and isoprenoid alkanes C13-C30. Solid lines: data from Chapter 4, microcosm experiment with marine snow and oil, no benthic macroinvertebrates. Dotted lines: data from this study, microcosm experiment with marine snow and oil, with benthic macroinvertebrates (average and standard deviation of triplicate microcosms).

5.4.3 The field relevance of the artificial marine snow and oil exposure concentration used

Marine snow is a complex material, including EPS (polysaccharides), organic debris, phytoplankton, and suspended particles. To be able to produce large quantities of reproducible marine snow, we developed a method to create flocks of artificial marine snow that resembled the marine snow that was found in the Gulf of Mexico, using commercially available alginate, phytoplankton paste, and kaolin clay. The artificial marine snow we produced contained all ingredients considered essential in the MOSSFA process in the field situation during the DWH oil spill (Daly et al., 2016): alginate-like exopolysaccharides (van Eenennaam et al., 2016), phytoplankton biomass (Hu et al., 2011; O'Connor, 2013), and mineral particles (Bianchi et al., 2011).

Interestingly, the homogeneity of the marine snow layer is an important factor that drives organism behavior. Small deviations of homogeneous coverage of sediment with marine snow led to significant spatial differences in the survival of *C. volutator*. Two of the replicate microcosms in the treatment with oil-contaminated marine snow had just one living *C. volutator* left, while the other replicate had 13 living individuals. The microcosm with the high survival rate had a clearly unequal distribution of the marine snow layer, leaving a patch of approximately 5 x 5 cm in one corner uncovered (Figure S5.5-S5.7). This served as a refuge for *C. volutator* to escape the oxygen-limiting effects of marine snow and oil. We observed that indeed this patch had an oxygenated top layer and visible burrowing activity of *C. volutator*, while the rest of the microcosm did not (Figure S5.7). In the field situation, oil deposition by MOSSFA on the seafloor is highly patchy (Daly et al., 2016). Uncovered patches can help organisms avoid the adverse conditions of covered patches, thus aiding the survival and later recovery. Less motile species will recover slower than species that can migrate to clean locations. The gastropods in our experiment were able to use the microcosm walls as a refuge, but our results also suggest that they were attracted by the nutritional value of the marine snow, leading to elevated oil exposure.

Approximately 99% of the oil-contaminated area impacted by the Deepwater Horizon oil spill in the Gulf of Mexico contained < 1 g m^{-2} of oil, and 0.7% of the area contained 11-43 g m^{-2} (Romero, personal communication, based on (Romero et al., 2017)). The oil loading we used in the microcosms, 10 g m^{-2}, is at the upper limit of what is found in the field, so it is a plausible worst-case scenario. However, exposure of organisms will be more chronic in the field than the 16 days exposure in our experiments. It is to be expected that invertebrates that can survive fasting for 2 weeks will start eating oiled marine snow upon fasting for a longer period.

5.4.4 Ecosystem implications of MOSSFA

The ecological consequences of the MOSSFA event on an ecosystem scale are not yet fully understood. It is, however, anticipated that the effects could be long-lasting (Bik et al., 2012; Daly et al., 2016). For example, changes in the benthic microbial communities can reduce the carbon flow to higher trophic levels, affecting zooplankton and fish species (Ortmann et al., 2012) and, in turn, may lead to long-term effects in higher food webs (Bik et al., 2012).

This study, with representative species of the benthic system, demonstrates that the effects of marine snow and oil on benthic invertebrate survival can be synergistic, and the impact on benthic organisms depends on their biological traits. The main driving factors for organismal response to marine snow and oil are motility, vulnerability to hypoxia and oil toxicity, and feeding habits. Situations with large amounts of suspended solids and high primary production, such as high river discharge periods and during algae bloom in spring time, are especially vulnerable to triggering a MOSSFA event (Daly et al., 2016). In addition, the use of dispersants may increase the chance for such an event to occur (van Eenennaam et al., 2016). The unanticipated benthic ecosystem effects after MOSSFA should thus be considered when deciding on oil spill response methods. Baseline data on benthic communities and monitoring programs are needed to assess the environmental effects of sedimented oil and track the recovery of ecosystems over time.

Acknowledgements

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Supplementary materials

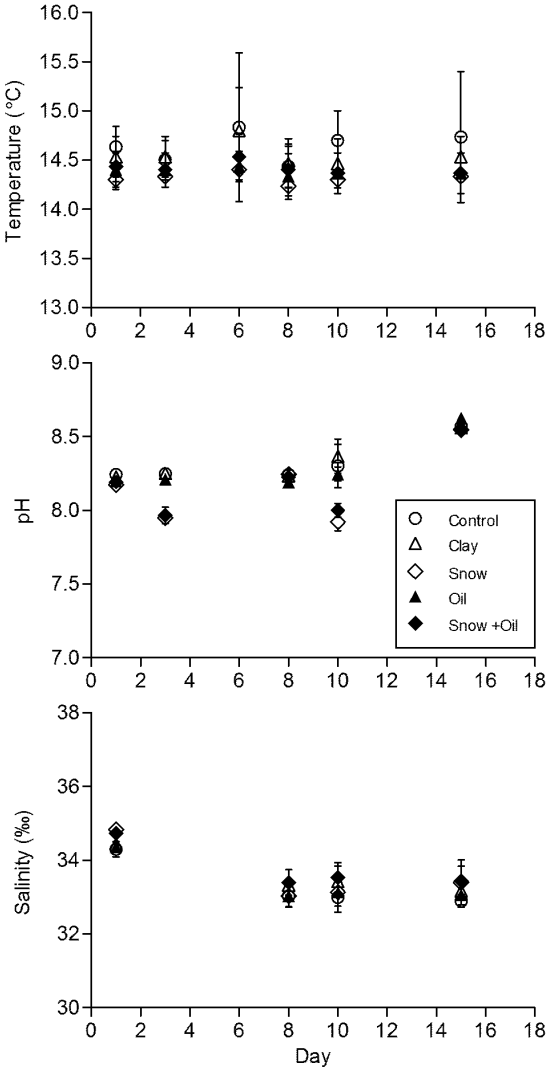

Figure S5.1 Water column measurements of temperature (°C, top panel), pH (middle panel), and salinity (‰, bottom panel). Averages and standard deviation of triplicates. Salinity at days 3 and 6 was measured with an incorrectly calibrated salinity probe, hence the higher values.

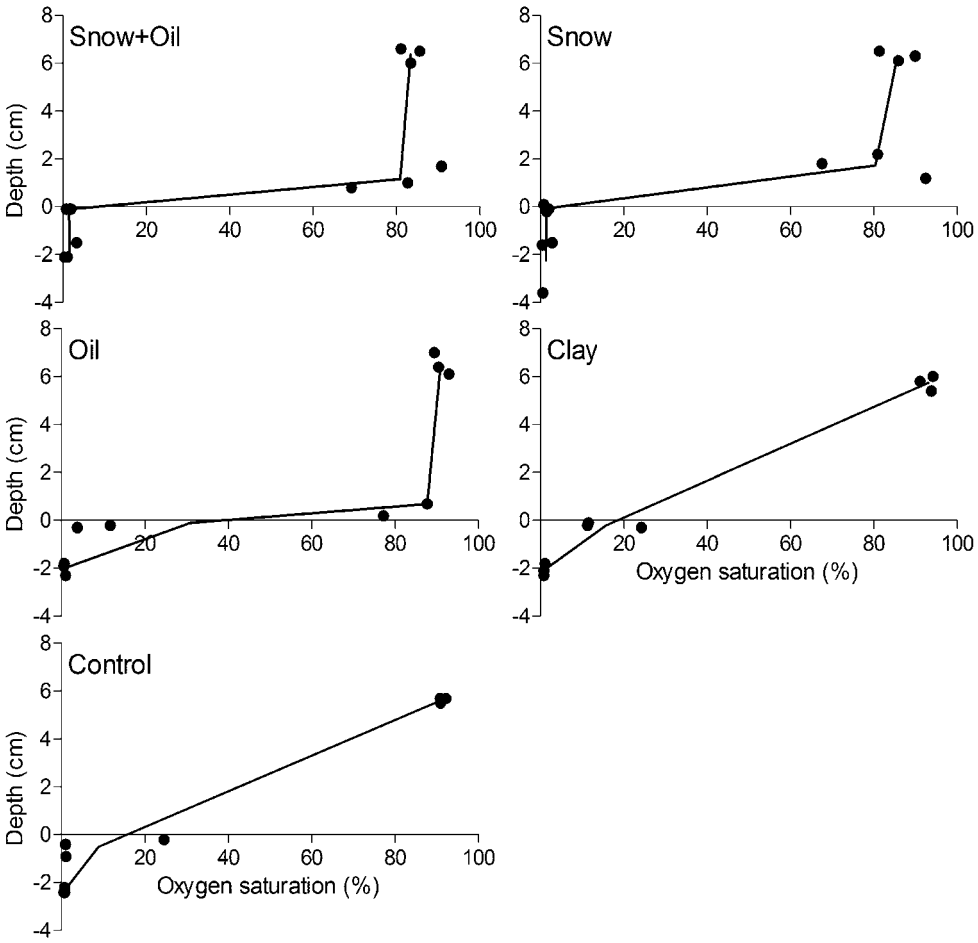

Figure S5.2 Oxygen saturation profiles of the microcosms at day 8. Depth refers to relative position of each Oxygen Sensor Spot to the sediment-water interface at 0 cm. The data points are individual measurements of three replicate microcosms. The black line is the average of the three replicates.

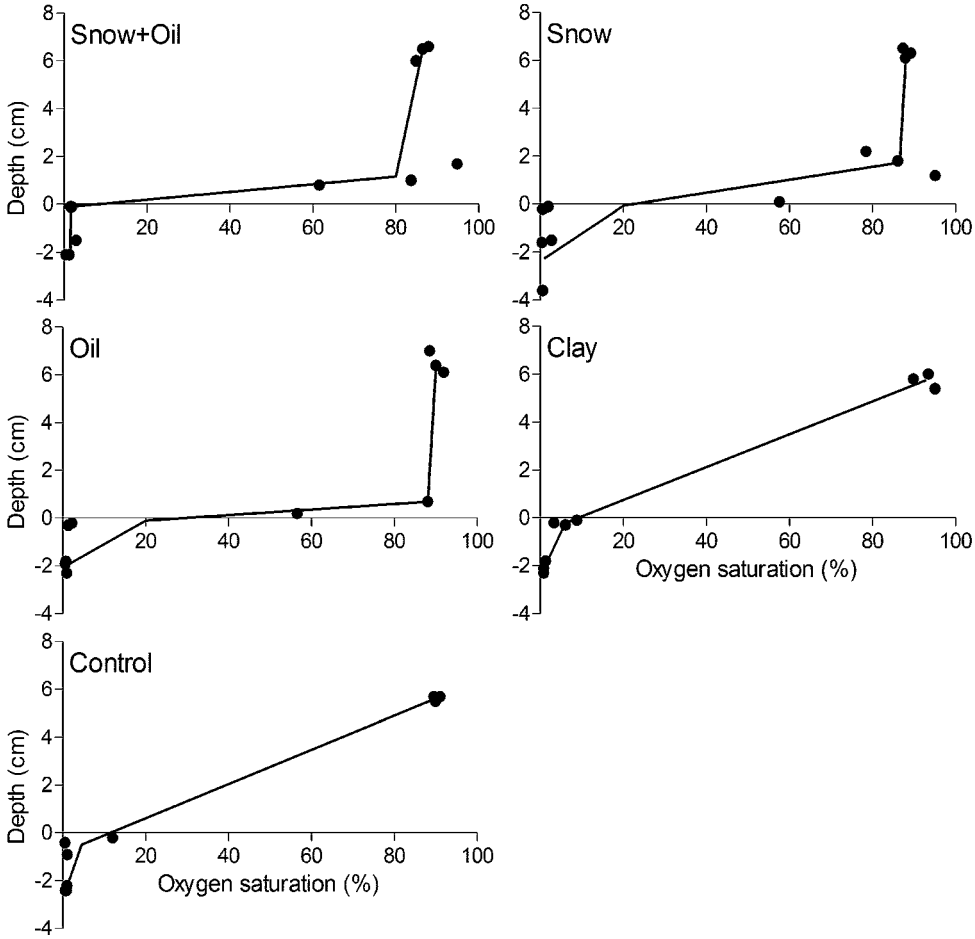

Figure S5.3 Oxygen saturation profiles of the microcosms at day 15. Depth refers to relative position of each Oxygen Sensor Spot to the sediment-water interface at 0 cm. The data points are individual measurements of three replicate microcosms. The black line is the average of the three replicates.

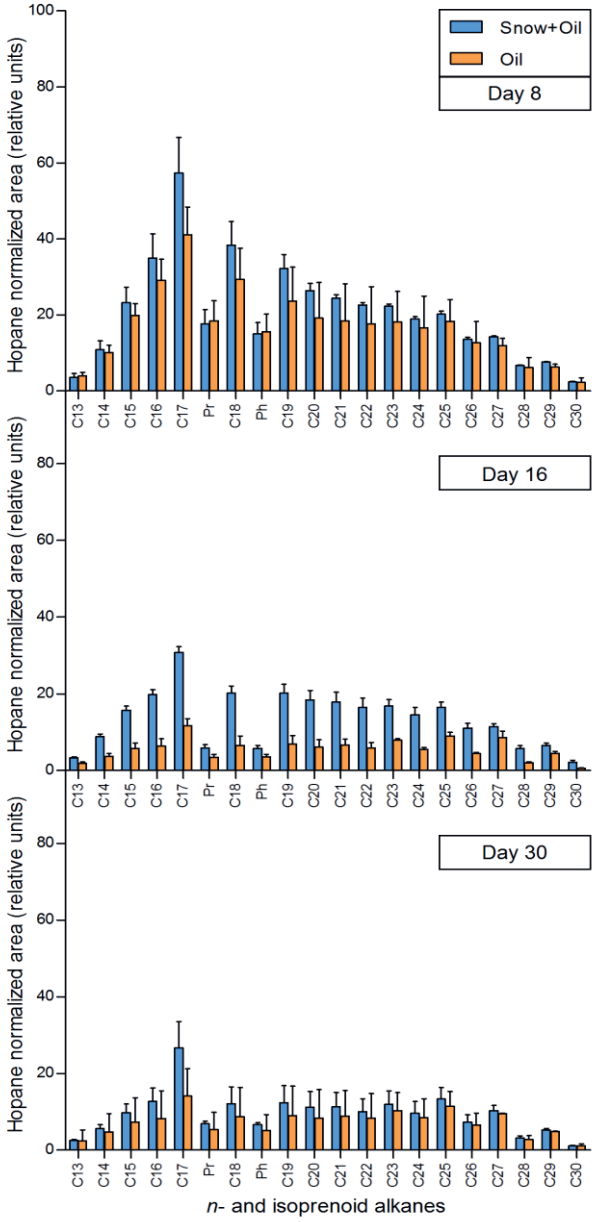

Figure S5.4 Hopane normalized peak areas for normal and isoprenoid alkanes on day 8 (top panel), day 16 (middle panel), and day 30 (bottom panel). Averages with standard deviations of triplicates.

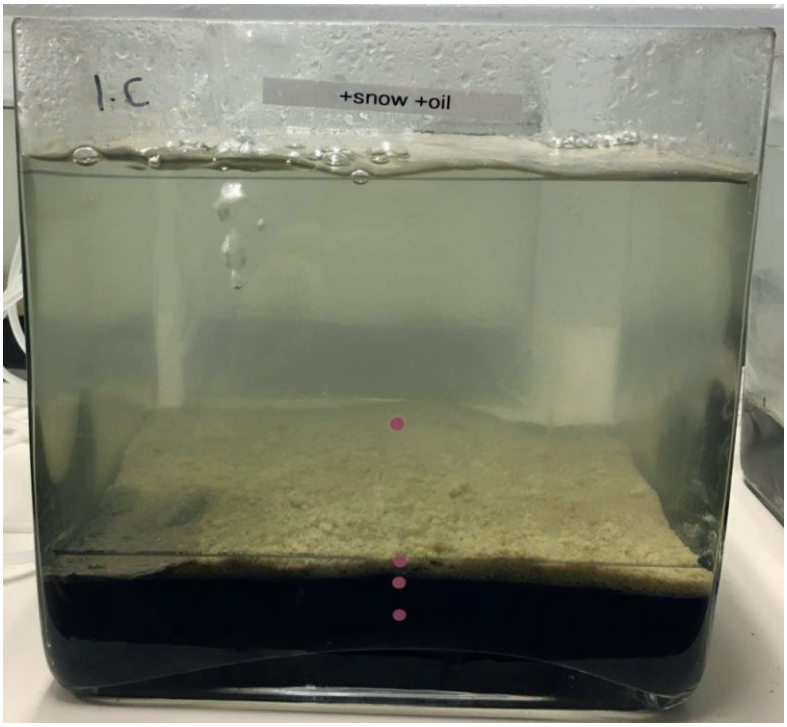

Figure S5.5 Unequal distribution of artificial marine snow in the lower left corner in microcosm replicate '1C', with treatment Snow + Oil. A patch of approximately 5×5 cm is uncovered. This led to a higher survival in this microcosm replicate compared to the other two replicates (13 vs. 1 surviving individual *Corophium volutator*).

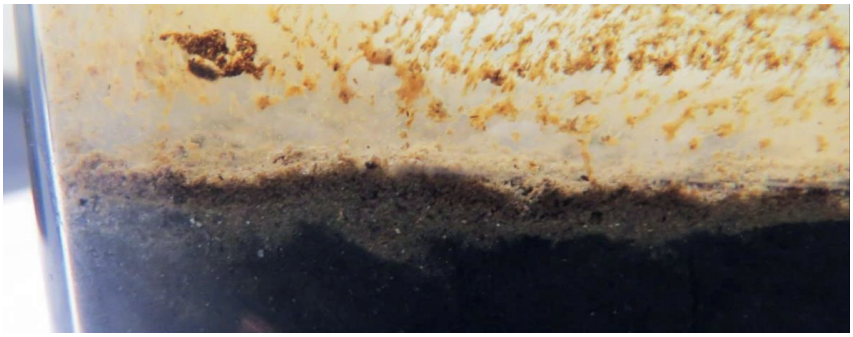

Figure S5.6 Close up of oxidized layer in microcosm replicate '1C' at day 16. A full view of the entire top layer can be found in Figure 5.4.

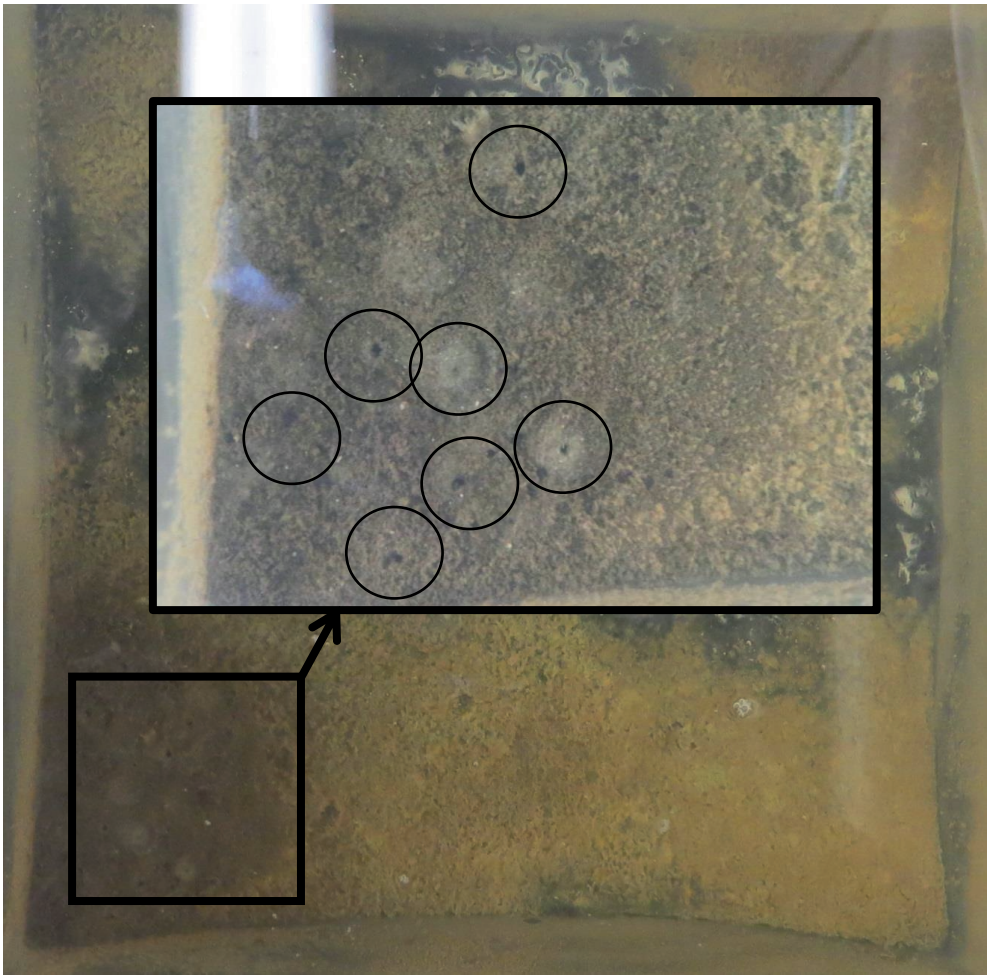

Figure S5.7 Close up of top of sediment of replicate '1C' at day 16. The lower left corner had visible burrowing activity. *Corophium volutator* burrows were present in this patch (black circles).

Chapter 6

General discussion on processes impacting oil biodegradation in deep marine oil spills

6.1 Introduction

Our current society is still dependent on oil. Though not beneficial for humankind's "biggest" challenge on planet earth facing global warming, climate change mitigation span into the second half of the 21st century (UNFCCC, 2016). Hence, the oil will still be used globally for at least a few decades (Chepeliev and van der Mensbrugghe, 2020; Watson, 2020). Off-shore oil production sites faced last decades a reduction in volume originating from easily accessible underground reservoirs. , This shifted oil production from shallow (≈ 50 m deep) to deep (≈ 1500 m deep) waters and hard-to-reach locations (Pinder, 2001). Over half of the oil resources explored in the 21st century are and will be located in deep marine areas (Zhang et al., 2019). Deep-water oil exploitation is expensive and challenging in keeping control, and so are related deep-water oil spills and their mitigation and clean-up. The ultimate cost estimated for mitigating the Deepwater Horizon oil spill (DWH), the largest deep marine oil spill on record, is US$144.89 billion (Gyo Lee et al., 2018). Harsh environmental situations such as low temperature and extreme pressure in the water adjacent to the well and the oil reservoirs make deep spill mitigation and clean-up a challenge with many unknowns (Lehr and Socolofsky, 2020; Li et al., 2016a). Deep oil spills such as the DWH spill in 2010 revealed part of these unknowns and challenges, with unforeseen impacts on the fate of the spilled oil, effects of mitigation measures, and harm to ecosystems (Beyer et al., 2016; Chang et al., 2014; Cope et al., 2020; Passow and Overton, 2020; Reuscher et al., 2020).

6.2 Consequences and impacts of deep marine oil spills

The environmental and ecological impacts of deep oil spills are incredibly vast, yet eleven years after the DWH spill, the full consequences are yet not fully understood (Bracco et al., 2020). Various investigations, including ecosystem research projects, started after the DWH spill, such as the Gulf of Mexico Research Initiative (GOMRI), supported by public and private funding, to discover and understand the spill impacts. The overview framework of the consequences of deep oil spills can be categorized as short-term and long-term harms on three intensively linked sectors: *humans and society*, the *economy*, and the *ecosystem* (Figure 6.1).

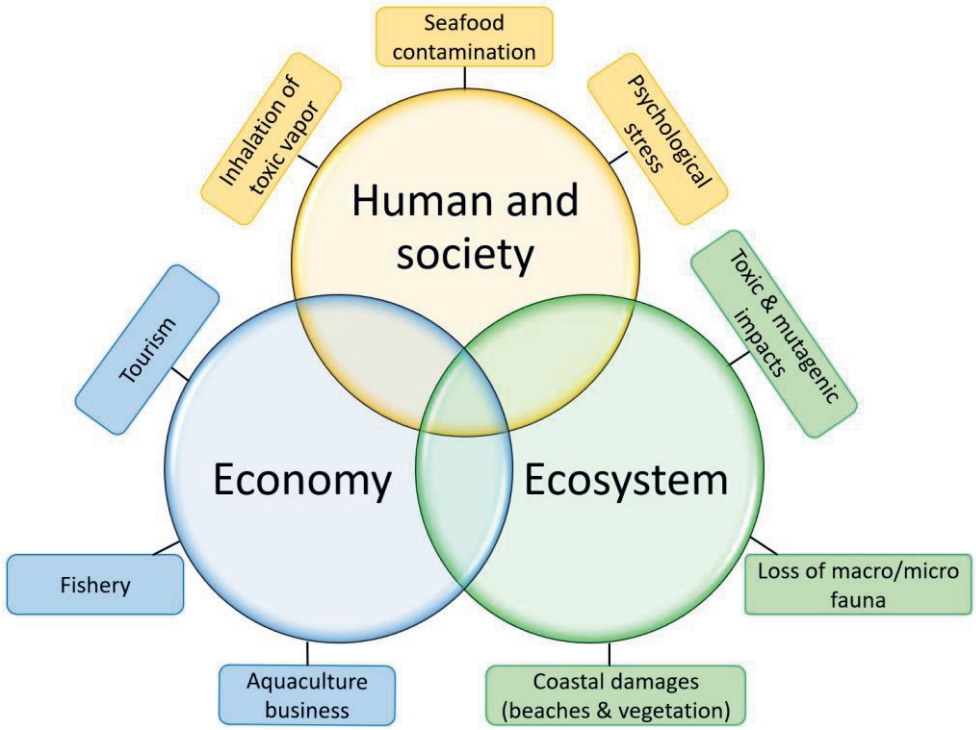

Figure 6.1 Overview framework of the consequences of deep marine oil spills.

The *humans and society* harms include direct and indirect health issues. The direct effects are related to the inhalation of volatile organic compounds, which can cause severe changes in DNA and cancer upon continued exposure. Indirect issues are related to consumption of contaminated seafood and bioaccumulation of oil toxins and psychological stress caused by the awareness of a damaged and toxified living environment. Especially the long-term effects of oil hydrocarbons toxicity to humans are less understood (Aguilera et al., 2010).

The *economic* harm includes coastal economy, tourism, recreational activities, commercial fisheries, aquaculture businesses, and sea-based transportation. The *ecosystem* damage includes coastal ecosystems, marine fauna and vegetation in ecosystems, toxic and mutagenic effects to marine life, and loss of macro and microfauna. The marine ecosystem contains various interacting species, where oil spills may impact differently on each of them, and the species also interact with oil pollution and influence the fate of the oil. For example, phytoplankton and

microorganisms, including some oil-degrading bacterial species, react to oil pollution by excreting EPS (Extracellular Polymeric Substance), which impacts the fate of oil, as was demonstrated in this thesis (Chapters 4 and 5).

Oil sedimentation also puts the seafloor ecosystem and the species that live in it or depend on it at risk. Oil biodegradation at the sediment layer will lead to oxygen depletion (Mason et al., 2014; Schwing et al., 2020b) and cause massive ecological damage to the deep marine species. This will be long-term harm because oxygen cannot be replenished in-situ by photosynthesis, i.e., by plants and their roots in the sediments like in shallow systems, since there is no light at this depth. Oxygenation of deeper sediment layers depends on intermittent mixing with water originating from the top surface sediment by macrofauna bioturbation, but those are severely inhibited by oil pollution. Therefore, hypoxia and anoxia conditions result in secondary damaging effects such as microbial community shifts, exclusion of oxygen-dependent components of the food web, and altered nutrient cycles. Some species swim away from the pollution, while sedentary species are exposed the most (Murawski et al., 2021). The reaction of some marine invertebrates to oil pollution is shown in Chapter 5.

As explained in Chapter 1, each oil spill is unique depending on the oil chemical composition and the geographical and environmental conditions. Consequently, the impacts and damage of oil spills are partly case-specific and unique due to the local conditions influencing processes and effects. Therefore a generic understanding of the influence of these conditions on the oil spill behavior is needed to assess the harm it may create and which disaster management policies are relevant for the local situation. Section 6.4 describes how a clean-up method, specifically the deep marine injection of chemical dispersants, can impact the fate of the oil and shift the damage and consequences.

The ultimate cure for a deep marine oil spill is biodegradation of the spilled oil. Naturally existing oil-degrading bacteria perform this self-healing power of the marine environment, and those generally increase in number during the oil pollution and degrade -at least a portion of- the oil. In shallow waters and coastal areas, the environmental conditions impact the efficiency of the oil remediation process. The impacts of these environmental conditions, such as temperature and nutrient and oxygen availabilities, have been studied previously (Al-Hawash et al., 2018; Neethu

et al., 2019). In contrast, other natural processes in the deep marine systems impact the oil-degrading bacteria and affect the natural oil biodegradation negatively. Hence, our understanding of oil biodegradation in deep marine spills was incomplete at the start of the research described in this thesis. The work described in this thesis improved our understanding of Deep Marine Oil Spills and especially the effects of oil-particle-dispersant interactions on oil biodegradation.

In this discussion chapter, a deep marine oil spill comprehensive overview is pictured, emphasizing the oil compound's movement and interactions with natural marine particles (organic and inorganic particles) in the water column and seafloor sediment and impacts on natural biodegradation. Then, it is evaluated how chemical dispersants, when applied as a spill clean-up method, impact these interactions and the natural oil biodegradation. Finally, we suggest using the comprehensive overview for different marine environmental conditions as a base for more wise mitigation of deep marine water oil spills.

6.3 Processes occurring during a deep marine oil spill

The ultimate fate of oil pollution in the deep marine environment is different from that in shallow marine systems and at the marine surface. Volatile compounds of the oil in floating layers, surface slicks, and dissolved in the upper water layers will tend to evaporate into the atmosphere, and sunlight degrades some portion of oil. In contrast, oil is moved directly upward or downward depending on the buoyancy of the droplets or indirectly by going through complex and interacting processes such as dissolution, partition into the water column, and deposition of heavy oil components to the sediment system. In deep marine waters, the fate of the lighter, more soluble, and volatile compounds is determined by their aqueous solubility, whereas the fate of insoluble oil components is determined by the interactions with particles and sedimentation. Figure 6.2 shows the processes and journey of oil droplets during a deep marine oil spill, followed by explanations of how these processes affect the ultimate fate of the deep oil spill.

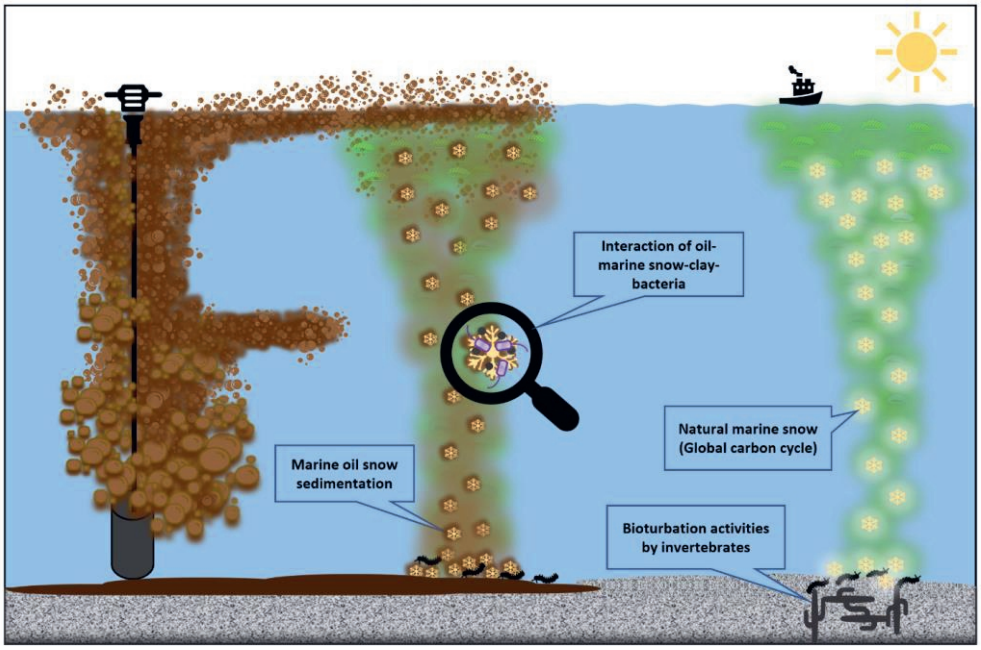

Figure 6.2 Processes occurring during a deep marine oil spill: interacting factors and mechanisms.

6.3.1 Oil dissolution at depth and compounds solubility

As soon as oil spills occur at greater depths (> 1000 m) in the marine environment, oil droplets are formed due to extremely high pressures and pressure differences between the reservoir, the wellhead, and the water column (Reddy et al., 2012). At the oil release point, the oil enters the water column in sprayed droplets creating a large oil-water interface per volume of oil, facilitating a rapid dissolution of the volatile soluble hydrocarbons into the water. Oil droplet size plays a vital role in the dissolution of oil compounds. The finer the oil droplets are, the higher the contact area with water is, and a faster dissolution of lighter oil compounds into the water phase occurs (Ryerson et al., 2012). Oil droplets may remain isolated droplets or coalesce into stretched pure phase and start spreading vertically and horizontally depending on their buoyancy and water currents. The lighter oil compounds dissolve further at a rate depending on their aqueous solubility (Arey et al., 2007). This rate of light compound dissolution and the volume/mass ratio of the droplets steers their dispersion behavior. From practice, it was learned that oil droplets larger than 100

microns in diameter tend to rise to the surface, while smaller droplets are trapped in the deep marine waters due to the increase in density over time in combination with droplet-droplet aggregation or association with suspended particles (Reddy et al., 2012; Ryerson et al., 2012) and precipitating to the seafloor (Yan et al., 2016).

Due to extensive oil dissolution, a cloud of oil droplets forms oil plumes at various depths of the water column that spread horizontally submerged for kilometers and then reach the water surface or the seafloor, often in the coastal area. This was the case in the DWH oil spill (Hu et al., 2017). Different concentrations of oil compounds are found in the horizontal spreading plumes, i.e., a portion of volatile organic compounds (VOCs) not yet dissolved into the water column and elevated concentrations of long-chain aliphatic compounds and polycyclic aromatic hydrocarbons (PAHs). The lighter compounds are easily dissolved in the water column, with short-term effects on the environment, and may eventually evaporate at the surface. The heavier compounds influence the natural ecosystem much more over a longer time frame. For a deep-water marine oil spill, compounds with high water solubility dissolve rapidly into the water column (Gros et al., 2014; Reddy et al., 2012), impacting microbial communities' performance and the population living in that water column. See Table 1.1 for the water solubility of some aromatic compounds.

6.3.2 Effects of deep oil dissolution on microorganisms and oil biodegradation

High dissolution of toxic oil compounds such as VOCs and lighter PAHs impacts marine organisms, including oil-degrading bacteria. A direct toxicity effect occurs when these compounds enter the bacteria by dissolving into and destabilizing their cell membranes (Xu et al., 2018). However, dilution (diffusion and current-induced convection) to the adjacent water volumes decreases the concentrations to non-toxic levels. Then the oil-degrading bacteria are able to biodegrade these compounds when optimal environmental conditions exist, often leading to the removal of biodegradable compounds from the water column (Kimes et al., 2014). The biodegradation rate is controlled by environmental conditions such as the temperature, the ratios of oxygen to carbon (oil), carbon to nutrient, natural concentrations of nitrogen, phosphorus, and microelements which are highly dependent on the specific local environment (Hazen et al., 2016; Liu et al., 2017).

Studies on spills such as the DWH, Ixtoc, and the Exxon Valdez have shown that small aromatics such as benzene, toluene, ethylbenzene, and xylenes (BTEX) and small saturates (< 7 C per molecule) dissolve quickly and reach solubility saturation concentrations early after release (Reddy et al., 2012). A fast dilution and decrease of these water concentrations are also observed down-current.

Our laboratory experiments on the impact of the dissolution of toxic soluble/volatile compounds on *n*-alkanes biodegradation were performed in closed systems (125 mL bottles), with limited dissolution and dilution possibility. Therefore, we could study the effect of the dissolution of *n*-alkanes on their biodegradation in a controlled way (Chapter 2). For this, we used R. *qingshengii TUHH-12*, an *n*-alkane degrading culture. We showed that *n*-alkanes were degraded at a higher rate when the volatile compounds were removed preliminary by evaporation (Figure 2.2). After introducing a culture of P. *putida F1*, aerobic aromatic compound degrading bacterial strain, degradation of *n*-alkanes improved in samples containing crude oil, where the toxic highly soluble/volatile compounds are still present. These findings indicate that the dissolution of volatile compounds in a closed system has an inhibitory impact on the *n*-alkane biodegradation, and the presence of oil-degrading bacteria helps overcome this inhibitory effect. Compared to oil spill situations, shallow and coastal oil spills are open systems with a high energy input in the water (currents, waves), increasing dissolution and evaporation. In deep waters, such high-energy inputs do not occur, and oil (droplet) plumes may be considered closed systems, as we mimicked in our batch experiments. In such natural systems, we predict that increased dissolution of volatiles positively influences the performance of bacteria and hydrocarbon biodegradation in the plume (Reddy et al., 2012), and this process can be an essential part of a natural process-based oil spill mitigation strategy.

6.3.3 Interaction of oil with mineral and organic particles and impacts on oil biodegradation

Oil droplets interact with their surrounding environment, associate/aggregate with either inorganic or organic dissolved compounds or suspended particles in the water column. These interactions can greatly influence the fate and biodegradation of the spilled oil by determining the oil's up or downwards movements, mass transfer, and bioavailability. Oil droplet-particle aggregates are diverse in density or buoyancy

and, therefore, show variations in settling velocity towards the seafloor. Some remain afloat at a certain depth with a higher chance for further biodegradation, others slowly settle, some settle quickly within hours (Li et al., 2017), and once reaching the seafloor, they generally form a layer and get covered by fresh sediment, which slows down oil biodegradation (Bagby et al., 2017; Chanton et al., 2015). This is further elaborated in Section 6.3.4. The fate and biodegradation of oil droplet-particle aggregates also depend on the type of particles involved in the interaction (Khelifa et al., 2005; Loh and Yim, 2016). Two frequently present types of particles in marine water will be discussed: *clay*, a predominant and ubiquitous mineral particle in the marine environment, and *marine snow*, an organic/semi-organic particle formed in the upper water column from biological (macrofauna, algae, and plankton related) processes, and that settle slowly to the deeper water layers ultimately reaching the seafloor.

***Interaction of oil with mineral clay*:** In general, minerals with higher hydrophobicity have more tendency to aggregate with oil (Wang et al., 2011). Clay has such a surface hydrophobicity and a higher tendency to aggregate with oil (Stoffyn-Egli and Lee, 2002). These clay particles have a high negative charge as expressed by cation exchange capacity (Stoffyn-Egli and Lee, 2002) and, when present in freshwater rivers, remain longer suspended in the water column due to electrostatic repulsion between the clay particles. When entering higher saline marine waters, often occurring in the vicinity of coasts and estuaries, this charge is compensated by adsorption of the cations from the seawater to the clay particles. The high ionic strength also suppresses the electrostatic repulsion, and consequently, clay particles tend to aggregate and settle to the seafloor. Hence, when present in marine waters contaminated with oil, suspended clay particles easily interact with oil droplets either by *adsorption onto the surface of the oil droplets* or even by *penetrating into the oil droplets*, creating cake layers in and around the oil droplets. These layers slow down the mass transfer of soluble oil compounds to the solution phase and bacteria by intra-aggregate sorption-retarded diffusion and hence slow down biodegradation. Such phenomena are reported in the literature for many aerobically degradable compounds (Kupryianchyk et al., 2012; Rijnaarts et al., 1990). When meeting clay particles, oil droplets get coated by clay particles and become resistant to dissolution and biodegradation and, therefore, more stable in their persistence in the environment. In the batch experiments described in this thesis, clay was shown to

increase the dissolution of BTEX compounds (Table 3.1) and decrease *n*-alkanes biodegradation (Figure 3.3).

Clay particles not only adsorb onto the surface of oil droplets but could also penetrate into oil droplets due to hydrodynamic forces, resulting in the increased dissolution of oil compounds. Other studies showed that interactions of oil and mineral particles also enhance the physical dispersion of spilled oil (Zhang et al., 2010). Another process occurring is the adsorption of dissolved oil compounds onto fine kaolin clay particles (< 2 μm), which can disperse the oil from the solution onto the solid matrix and, in this way, decrease the oil availability for biodegradation. On the other hand, because of the enlarged oil-water interface, bacteria-oil contacts may be enhanced, which should be beneficial for oil biodegradation (Wise and Wise, 2011). Hence, it depends on conditions whether clay can enhance or retard oil biodegradation.

In the case of the DWH spill, the natural mineral particles were present in the water column and could not fully explain the significant amounts of oil sedimented at the seafloor (Chanton et al., 2015; Schwing et al., 2015; Valentine et al., 2014). Hence another factor may play a role in oil droplet sedimentation, which leads to the hypothesis of the involvement of marine snow in oil droplet sedimentation (Passow and Hetland, 2016).

***Interaction of oil with marine snow*:** Organic rich suspended particles such as marine snow are important components impacting the fate and biodegradation of oil, and oil spills can trigger the formation of extra amounts of marine snow. These marine snow particles are formed in the upper water column from mainly algae and plankton bio-interactions and provide downward fluxes of carbon compounds to the deeper waters and the seafloor (Giering et al., 2020), and represent an essential pathway for carbon and nutrient cycling in the marine environment (Archer, 2003; Giering et al., 2020). Marine snow particles are a carbon source for bacteria living in the deeper water column and sediments that are ideal locations for the enrichment of microbial communities. These bacteria are at the base of the food chain and, via protozoa and planktonic organisms, feed higher trophic organisms (Kvale et al., 2020). Oil flocculent accumulation was observed to occur in response to oil pollution, most likely by algae and bacteria forming EPS, as a toxicity response or by converting oil compounds into EPS (Lauritano et al., 2020; Poli et al., 2010; Quigg et al., 2016). In the case of an oil spill, oil droplets interact with marine snow,

form aggregates by the inclusion of clay or other mineral particles, and thus gain in density, and merge into the natural downward nutrient and carbon flux to the deep sea environment (Brakstad et al., 2018b) (Chapters 4 and 5).

Association of marine snow and oil may improve or decrease the oil biodegradation. Improvement could be caused by bacteria attached to marine snow that are brought into close contact with oil, and these bacteria tend to be more metabolically active than those suspended in water (Gustitus and Clement, 2017; Xu et al., 2018). The attached bacteria may have a faster uptake of oil compounds, either by direct pure oil phase uptake (Chen et al., 2013) or by shorter dissolution-diffusion mass transfer distances between the oil droplets and the attached bacteria. Hence, oil-bacteria aggregation mediated by marine snow may enhance the bioavailability and mass transfer of oil compounds to the degrading bacteria and consequently improve the oil biodegradation. Furthermore, aggregation of oil droplets with organic marine snow particles appeared to decrease the concentration of dissolved BTEX compounds in the batch experiments and improved *n*-alkanes biodegradation (Chapter 3). Thus, marine snow particles may provide an alternative sorbing matrix for BTEX, decreasing the aqueous BTEX concentration in that particular experiment, and higher bacterial concentrations may have contributed to higher biodegradation.

In contrast, the association of marine snow and oil may decrease oil biodegradation. The organic matter may be preferentially biodegraded when the oil is initially captured in a concentrated form in the aggregate matrix, including marine snow organic matter. The oil then biodegrades after the organic matter is depleted and under the condition that sufficient oxygen influx is provided (Bælum et al., 2012). Aggregation of oil with marine snow particles indeed increased oxygen consumption in batch oil biodegradation experiments (Chapter 3). These results indicate that when oil is associated with marine snow, oil biodegradation improves. However, this only happens in the abundance of dissolved oxygen. Marine snow appears to be a preferable food source over oil compounds, even for oil-degrading bacteria (Passow et al., 2012) (Chapter 4). Our research demonstrated in Chapter 4 that organic compounds of marine snow are more oxidized than saturated oil compounds and are more easily degradable under low oxygen or even anaerobic conditions. In contrast, most oil compounds, especially saturated oil chemicals, are less degradable under low oxygen concentrations or anaerobic conditions (Bochdansky et al., 2010;

Gutierrez et al., 2018). Therefore, dissolved oxygen can quickly become a limiting factor and then controls the rate of aerobic biodegradation of oil-associated marine snow in the marine environment and the water column, as in the sediment layer.

Overall, the availability of particles and their interactions and aggregation with spilled oil can be a helper in resolving the deep-marine oil spills, especially in the waters with high energy and turbulence due to strong deep sea currents where the convective supply of water with dissolved oxygen is not limited. Therefore, the formation of marine snow and interactions with oil is a significant influencer in oil fate and biodegradation. On the one hand, marine snow can improve oil bioavailability and, consequently, oil biodegradation in oxygen-rich environments. However, on the other hand, it slows down biodegradation under anoxic conditions and transports the oil pollution to the seafloor and its habitats (see Section 6.3.4 for details), and has significant negative impacts on biodegradation and functioning of the ecosystem.

6.3.4 Oil sedimentation and impacts on biodegradation and benthic life

Oil droplets and oil aggregates gain in density due to dissolution and biodegradation of lighter compounds and increasing relative concentration of heavier oil constituents in oil droplets and aggregates. Hence, these are subject to increased settling rates over time and thus get transferred to the benthic zone by sedimentation processes. The process of Marine Oil Snow Sedimentation and Flocculent Accumulation (MOSSFA) was a significant pathway for the distribution of oil to the seafloor in the DWH spill (Vonk et al., 2015), where 1.8 to 21% of the oil released reached the benthic zone (Brooks et al., 2015; Daly et al., 2016; Passow and Hetland, 2016; Valentine et al., 2014). Oil-associated marine snow sedimentation also occurred in two other oil spills, Tsesis (1977) and Ixtoc I (1979), in which a significant amount of oil reached the seafloor (Passow et al., 2012).

Oil aggregate formation is a common process in many oil spills, and in the case of deep spills, the formation of droplets and long times of presence of these droplets in the water column makes the probability of aggregate sedimentation much higher compared to the shallow or surface spills. Therefore, it is essential to understand oil interactions after sedimentation and their impacts on fate and biodegradation.

***Limiting factors for oil biodegradation at depth*:** Oil biodegradation rates in the sediment layer are different from that in the water column because of the change in the oil composition (contains heavier and poorly degradable compounds) and various environmental conditions that are different in the sediment layers than in the water column. Changes in the environmental conditions can be due to reduced redox conditions and intense competition for energetically favorable electron acceptors, such as oxygen. Changes in the environmental conditions can also be due to a reduced oil/water interface in oil particle layers in the sediment, reducing the oil mass transfer to oil-degrading bacteria and bioavailability for biodegradation. Oil on top of sediment also can be mixed downwards in sediment by bioturbation activities and exposing the oil to limited oxygen concentrations and microbial communities (Grossi et al., 2002) for an unknown period.

As described previously, the association of oil with marine snow initially improves the oil mass transfer and bioavailability in the water column because the oxygen influx needed for biodegradation is well maintained in the oxygen-rich water column. However, upon reaching the sediment, this influx of oxygen decreases. At the same time, the degradation of marine snow continues and outcompetes the remaining degradable oil compounds for the low amounts of oxygen in the sediment. Hence, the marine snow organics create an anoxic sediment layer which becomes highly unfavorable for oil biodegradation. This was demonstrated by our research described in Chapter 4, where artificial marine snow was applied in microcosm studies mimicking oil-contaminated seafloor conditions. Here it was observed that the originally well-oxygenated top layer of the sediment completely disappeared and resulted in limited *n*-alkane biodegradation in the sediment. In practice, during and after the DWH oil spill, reduced redox conditions in the sediment were also observed (Hastings et al., 2016), which indicated anaerobic conditions, and a slow-down of biodegradation for oil accumulated at and in the seafloor.

***Effects of oil on benthic life and bioturbation activities*:** Sedimentation of oil to the seafloor can disturb the benthic ecosystem in two ways: 1) exposure of oil to benthic organisms creating direct toxicity, genetic modification or bio-accumulative effects, and change the redox condition to hypoxia or anaerobic conditions (Schwing et al., 2015), and 2) a slowed down bioturbation activity of the benthic ecosystem; under unpolluted healthy conditions, this activity strongly contributes to the oxygenating

and nutrient cycling in the sediment layers (Ferrando et al., 2015; Stauffert et al., 2013).

Despite the positive effect of the presence and activity of invertebrates and bioturbation on oil biodegradation, sedimentation of oil has adverse effects on invertebrate's survival, mainly when oil is associated with marine snow. Sedimented oil and its association with marine snow have a synergistic and negative impact on benthic invertebrate survival. The impact varies on their biological traits. The main driving factors for organismal response to marine snow and oil are motility, vulnerability to hypoxia and oil toxicity, and feeding habits. According to our results, sedentary invertebrates such as *Macoma balthica* experienced severe effects of deposited oil. This species is a natural organic matter deposit feeder with low motility and high vulnerability to hypoxia and suffered more from hypoxia and internal oil exposure due to feeding from marine snow contaminated with oil. *Corophium volutator*, a motile invertebrate, was able to escape the deposited oil layer, but even though they escaped the sediment, the survival was reduced compared to our control due to the sensitivity of this species to hypoxia. *Ulvae* is a motile invertebrate and highly tolerant to hypoxia, but its survival was reduced due to exposure to oil by oral intake when feeding on oil-polluted marine snow. Benthic *foraminifera* was used as indicators for sediment hypoxia because of their high sensitivity to changes in oxygen concentration, and also for this organism, we demonstrated that feeding from oiled sediment caused low survival in them.

Deposited oil causes long-term damage to the ecosystem. Changes in the benthic microbial communities can reduce the carbon flow to higher trophic levels, affecting zooplankton and fish species (Ortmann et al., 2012) and, in turn, lead to long-term effects in higher food webs (Bik et al., 2012). Due to the slowed-down bioturbation activity, oxygen influxes and biodegradation rates are reduced, increasing the residence time of oil and their exposure to benthic organisms, subsequently leading to bioaccumulation of oil compounds in the marine food web (Alsaadi et al., 2018). The persistence of oil in the sediment layer changes the density of benthic organisms for years after a spill and affect the genetic material of marine species, which can gradually result in changes in functions or abilities of those species or even result in the extinction of specific species (Suchanek, 1993). The results of the microcosm studies described in Chapter 5 reconfirm that the oil biodegradation rate in the sediment layer is influenced by carbon source competition and oxygen availability.

Although the association of settled oil compounds and particles and sediments improves the surface area of the oil-water interface, which is, in any case, beneficial for oil biodegradation in the seafloor sediment, the influx and concentration of oxygen remain the most critical controlling factor in oil biodegradation (Chapters 4 and 5).

Though the benthic system is generally heavily impacted by the pollution of sediments with oil, it also acts as a great contributor to oil biodegradation in the sediment (Grossi et al., 2002). Invertebrates, one of the groups of species that habitat the benthic zone, naturally improve the oxygen diffusion into the sediment pores by bioturbation. The studies in Chapters 4 and 5 showed that there is more *n*-alkane biodegradation in the presence of benthic invertebrates. Bioturbation enhances oxygen penetration into the sediment layer (Pelegrí and Blackburn, 1994) and partly counterbalances the oxygen lost for marine snow biodegradation.

Sedimentation of spilled oil transfers the pollution to the deep marine environment and creates severe and long-term harm to the marine ecosystem and consequently to nature, including humans, through effects on fisheries and ecosystem quality. Therefore, it is essential to understand the oil movements and interactions in the marine environment and find solutions and technologies to decrease the chance of oil sedimentation and technologies for improving oil biodegradation at the seafloor and in the water column before the pollution reaches the seafloor.

6.4 Deep marine injection of dispersants and impacts on deep marine oil spills

Technologies to attenuate marine spill pollution are presented in Chapter 1. Chemical technologies have been used frequently for mitigating surface water oil spills and, for the first time, applied into the Deepwater Horizon oil spill (Kujawinski et al., 2011) without knowing the consequences and interference in the essential marine processes. Since no specific technologies are designed/devoted to battle deep marine oil spills, knowledge and experiences with shallow water spill situations have been used to mitigate the DWH oil spill. As a result, the complex and harsh conditions in deep marine environments, the unknown consequences of dispersants on natural marine processes, and the effects on the marine habitats were unknown

when the DWH oil spill occurred. Moreover, impacts on the fate and biodegradation of oil in the short and long term were unknown when mitigating the DWH oil spill.

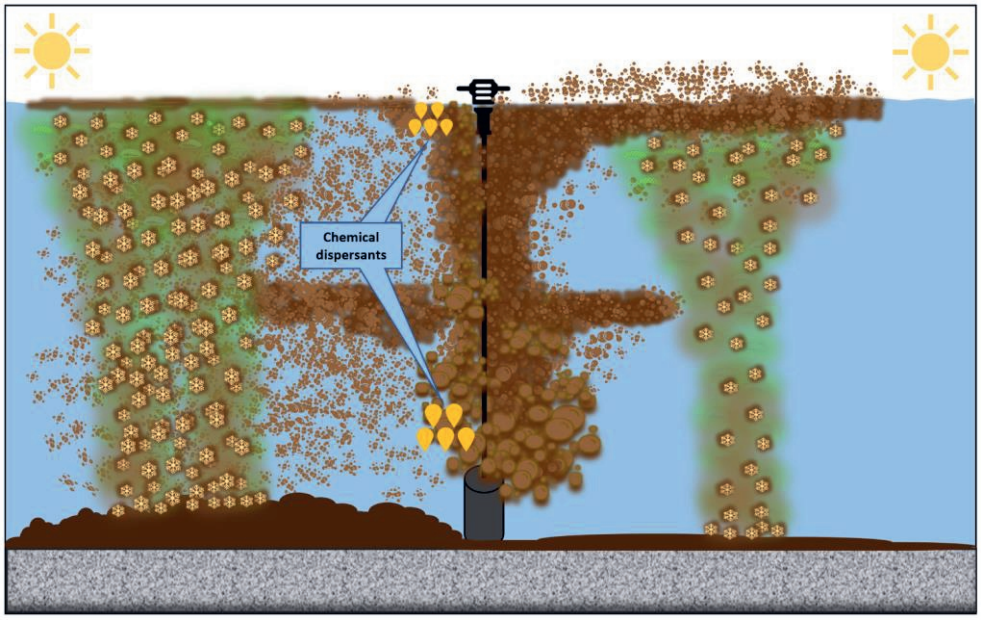

Figure 6.3 Impacts of deep marine injection of dispersants on oil spill processes: with (left) and without (right) dispersants.

The application of chemical dispersants has fundamental impacts on the oil composition, oil movements in the marine environment, oil-degrading bacteria, and natural marine processes (e.g., EPS and marine snow production and sedimentation). These impacts start as soon as the dispersants are applied to the deep spill and have a domino effect on the later processes and journey of oil compounds in the marine environment. The combination of dispersants and oil causes substantial dissolution of oil compounds and impacts marine microorganisms and oil-degrading bacteria. The reaction of bacteria to the substantial oil dissolution is the production of EPS and excessive marine snow formation and, therefore, increased oil sedimentation and prolonged oil presence on the seafloor. Figure 6.3 visualizes the impacts of deep marine injection of dispersants on oil spill processes.

6.4.1 Impact of dispersants on the oil compounds behavior and movement

The application of dispersants to disperse oil into smaller droplets is based on the assumption that smaller droplets are more easily dissolved into surrounding waters and then biodegraded more rapidly by bacteria. Chemical dispersion of oil combined with the physical dispersion at high pressure at deep-water results in even smaller microdroplets and promotes the oil dissolution, thus increasing the amount of oil partitioned into the water column (Camilli et al., 2010; Reddy et al., 2012). Therefore it is expected that less spilled oil reaches the surface, and the amount of surface oiling and coastal damage reduces. This is the most direct benefit of the application of chemical dispersants (Prince, 2015). However, the downside is the high risks put on the deep marine ecosystem due to increased oil sedimentation.

In laboratory experiments, we found elevated concentrations of BTEX in the liquid phase when Corexit was added to batches containing crude oil (Table 2.3). The formation of hydrocarbon-rich plumes of dispersed oil at the 1000–1200 m water depths during the DWH spill was associated with deep injection of dispersants (Kujawinski et al., 2011). These oil plumes contained a complex mixture and toxic concentrations of soluble and insoluble hydrocarbons, including alkanes, BTEX, and PAHs, which further impact the marine ecosystem and especially the oil-degrading bacteria. There are conflicting reports on the effects of deep injection of dispersants on oil biodegradation (Ferguson et al., 2017; Joye et al., 2016; Kleindienst et al., 2015a). Biodegradation rates could not be determined directly at in-situ conditions during the DWH oil spill. Instead, the role of dispersants on oil biodegradation is studied in laboratory experiments, and the results in the literature vary probably due to different experimental approaches and the lack of experiments under realistic environmental conditions.

6.4.2 Impact of dispersants on function of microbial communities and oil biodegradation

Dispersants application impulse three significant impacts on the functioning of microbial communities and oil biodegradation: 1) Enhance dissolution and toxicity,

2) Serve as a substrate for oil-degrading bacteria, and 3) Their residence time in the marine environment.

First, the chemical dispersants enhance the dissolution of oil compounds into the marine system, inducing various impacts on marine organisms, including oil-degrading bacteria. The response of oil-degrading bacteria is related to the toxicity of dispersants towards the bacteria (Liu et al., 2016; Xu et al., 2018) or increased toxicity due to the enhanced bioavailability of the toxic oil compounds (Bejarano, 2018). The presence of dispersants significantly altered microbial community composition (Kleindienst et al., 2015a), and this changed population drives changes in hydrocarbon-degradation rates and alters the oil-degradation efficiency. Change in the microbial community inside the oil plumes was assumed to be due to the high concentrations of aromatics and other hydrocarbons during the DWH spill (Dubinsky et al., 2013; Thomas et al., 2021). The dispersants also influence the microbial activity in the marine environment and have been known to increase (Prince et al., 2016) or suppress the activity of specific oil-degrading microorganisms (Kleindienst et al., 2015a). Some oil-degrading bacteria excrete extra EPS as a stress response to cope with a high dissolution and concentration of food sources (dispersants or enhanced dissolved oil compounds).

In laboratory experiments, the number of bacteria degrading aromatic compounds increased after the application of dispersants (Sun et al., 2019). It is hypothesized that this is a response mechanism of the bacteria to cope with the increased (toxic but degradable) concentrations of aromatic hydrocarbons in the water column. Other studies showed the growth of *Marinobacter*, a hydrocarbon-degrading bacterium, in the absence of dispersants and a decrease in abundance of *Marinobacter* when dispersants were added (Kleindienst et al., 2015a). This may indicate an inhibitory and toxic effect of the dispersant to these hydrocarbon-degrading bacteria. Similar results were observed in other studies where dispersants altered the microbial community composition to benefit aromatic compound degradation (Brakstad et al., 2018a; Kleindienst et al., 2015a; Sun et al., 2019). In our experiments described in Chapter 2, the lag phase during *n*-alkane biodegradation was shortened when an aromatic degrading bacteria was introduced to the batches. This indicates that a high concentration of BTEX inhibited the function of R. *qingshengii TUHH-12* (an *n*-alkane degrading culture) until the high concentrations of BTEX decreased to non-toxic levels by the added aromatic degrading bacteria.

Second, dispersants contain biodegradable components that serve as growth substrates for bacteria, including oil-degrading bacteria, that can be more preferred by the microorganisms than the degradable oil compounds (Chakraborty et al., 2012; Techtmann et al., 2017; Van Hamme et al., 2003). Corexit petroleum distillate fraction consists of hydrocarbons (C9 – C16) (McFarlin et al., 2018). Gas chromatography results shown in Chapter 2 indicate the presence of hexadecane in samples containing only dispersants, a C16 hydrocarbon. In addition, oxygen consumption measurements confirmed microbial activity (respiration) linked to the biodegradation of dispersants (Chapter 2 and 3). Hence oil-degrading bacteria can also biodegrade the dispersants (McFarlin et al., 2018). Since dispersants are water-soluble and have more oxidized moieties in their molecular structure, they generally form a more accessible substrate for biodegradation than oil, leading to competition between dispersants and oil compounds as substrates biodegradation (Xu et al., 2018). Kleindienst et al. enriched *Colwellia*, an aromatic compound degrading bacterial strain, in dispersant-only treatments and showed that dispersant components were preferred substrates for growth relative to degradable oil compounds (Kleindienst et al., 2015b). Similarly, the hydrocarbon components of Corexit were mineralized and served as a carbon source for microbial growth in other studies (Bælum et al., 2012). This could limit the biodegradation of oil compounds in the presence of dispersants when the dissolved oxygen concentrations are limited in the marine environment.

Third, the residence time of dispersants in the marine environment is important, i.e., the dispersants can remain in the marine environment for a considerable time. For example, Dioctyl Sodium Sulfosuccinate (DOSS), a key component of Corexit, was found in the DWH oil plumes, indicating that some dispersant components stayed in the water column without any degradation even 64 days after the Corexit deep injection (Kujawinski et al., 2011).

Overall, the effects of dispersants are specific to different bacterial species (Thomas et al., 2021). Deep marine application of dispersants increases the dissolution of oil compounds in the water column, as was shown in the deep oil plumes observed during the DWH spill and our results in Chapters 2 and 3. Long-term negative impacts of dissolution are not expected due to the dilution, spreading, and water currents in the marine environment. However, oil plumes can be considered mobile

but closed systems, with slow dilution and spreading of oil compounds to the fringes and outside the plume.

6.4.3 Impact of dispersants on the interaction of oil with mineral and organic particles and oil sedimentation

The application of chemical dispersants can trigger the oil-degrading bacteria and phytoplankton to produce extra EPS. Under the stressful conditions of high concentrations of dispersants and oil compounds, bacteria and phytoplankton can produce EPS, which was observed in large quantities during the DWH spill (Passow et al., 2012). Extra EPS production results in excessive marine snow formation, especially in the presence of mineral particles. As a result, marine oil snow accumulates on the seafloor, where biodegradation is inhibited due to oxygen depletion and causes extended impacts on the benthic ecosystem and long-term oil persistence on the seafloor (Daly et al., 2016; Passow and Hetland, 2016; Quigg et al., 2016). The EPS production was observed in the experimental studies when marine phytoplankton was exposed to Corexit (van Eenennaam et al., 2016). This supports the assumption that the application of dispersants facilitates the formation of EPS. Production of high concentrations of EPS facilitates the coagulation and aggregation of organic matter, oil droplets, dispersants, clay particles, and subsequent sedimentation of marine oil snow particles (Daly et al., 2016; Passow and Ziervogel, 2016; Quigg et al., 2016). Studies showed that some oil-degrading cultures, such as *Colwellia*, play an essential role in marine snow formation in the presence of chemical dispersants (Gregson et al., 2021). *Colwellia* is an aromatic compound degrading bacterium and was found in high abundance in the water column and oil plumes during the DWH spill.

Deep injection of dispersants impacts the oil droplet size distribution, dissolution, and the oil aggregation and flocculation processes. The application of dispersants results in smaller oil droplets favoring the formation of the oil aggregates that can be trapped by mineral clay or marine snow particles (Passow et al., 2017). However, Corexit can also disperse organic matter, with the consequence that natural marine snow concentrations are reduced in the presence of Corexit, and the net effect of Corexit on the oil transport via marine snow depends on the relative strength of these opposing processes (Passow et al., 2017).

The formation of marine oil snow is of central importance for the fate of oil. When marine snow associated with oil settles, it transports oil to depth and the seafloor. Therefore, deep injection of dispersants in waters with high suspended solids and phytoplankton increases the chance of fast transportation of the oil compounds to the seafloor and long-term persistence of the oil in the sediments. Hence, strategies for applying dispersants during a deep-water oil spill to either protect the water column or protect the seafloor sediments may be different (and even opposing) and depend on the situation and location of the spill.

6.4.4 Impact of dispersants on the benthic life

Deep injection of dispersants increases the oil sedimentation and prolongs the oil persistence. The addition of dispersants has been found to increase the absorption of PAHs in some aquatic species (Duran and Cravo-Laureau, 2016; Honda and Suzuki, 2020; Zuijdgeest and Huettel, 2012). Toxicity experiments performed on a closely related species indicated that oil had little effect on coral's health but that oil and dispersant mixtures were particularly toxic to corals (DeLeo et al., 2016; Goodbody-Gringley et al., 2013; Negri et al., 2018). The long-term persistence of elevated DOSS concentrations on beaches and in the deep sea demonstrates that at least some of the components of dispersants are persistent in the environment (Gofstein et al., 2020; Gray et al., 2014; Mascarelli, 2011).

6.5 Future deep oil spills

As long as our society is dependent on oil, the probability of oil spill occurrence exists. The chance for a spill significantly increases when oil production shifts from shallow to deep-waters and hard-to-reach locations. With the lack of validated technologies and knowledge for deep marine clean-up, the damages to humans, society, the economy, and the ecosystem will remain a significant concern. The understanding and knowledge gained on the fate and processes of the oil in the marine environment after the DWH spill accident though tragic, has been a big step forward in lessons learned in managing the damages in future deep spills. However, major knowledge gaps remain (Bracco et al., 2020).

The consequences and processes of an oil spill (Figures 6.2 and 6.3) teach us that oil fate and damages are dependent on the composition and properties of the oil and the

marine environmental characteristics and conditions. This provides a base of understanding and decision-making to address future deep marine oil spills. Variables such as depth and water temperature, availability of minerals and particles in the water column, sunlight penetration in the water column, and phytoplankton content are essential variables for determining the fate and biodegradation of oil and consequently the damages that may or may not occur. Moreover, the response strategies and clean-up technologies, such as the application of dispersants, are externally imposed factors (by humans) that will impact the oil fate and consequent damages. Moreover, effects of dispersants can have opposite effects for different parts of the marine environment: beneficial for the surface and coastal areas downstream but detrimental for the seafloor and related ecosystem below submerged deep-water oil plumes.

The consequences and processes of an oil spill described earlier (Figures 6.2 and 6.3) are based on oil composition and properties, and environmental conditions dominant in the DWH oil spill in the Gulf of Mexico. As mentioned in Chapter 2, the DWH oil is classified as a light sweet crude oil and contains a high number of light hydrocarbons, saturated *n*-alkanes, and PAHs. These light crude oils have a higher tendency to dissolve and spread into the water column and a higher tendency to interact with riverain and mineral particles suspended in the water column. In contrast, heavy oil with a limited number of light hydrocarbons will dissolve into the water column to a lesser degree, therefore triggering less pronounced EPS production, but might more directly interact with particles. So again, it is related to the conditions in-situ and how this crude oil will behave in the marine environment.

Water temperature impacts the behavior of oil by impacting viscosity. Oil compound dissolution is enhanced in warm waters, while cold waters in which oil and water attain a higher viscosity slow down the dissolution and spreading of oil compounds in the water column. This situation potentially intensifies/worsens by injecting dispersants (French-McCay et al., 2018), resulting in increased oil compound dissolution without spreading and dilution, which extends toxic effects of oil on the marine species and communities (Fisher et al., 2016). Changes in oil and water viscosity also impact the interaction of oil with particles. In cold viscose waters, improved adhesion properties of oil enhance the interactions and aggregation with suspended particles. This can improve the bioavailability of oil for oil biodegradation but also increase the chance of oil sedimentation. In laboratory experiments, pilot,

and field-scale tests, the formation of oil mineral aggregates is demonstrated in a clean-up response to the oil spills at cold temperatures (−1 to −4 °C) (Jézéquel et al., 2018; Wang et al., 2013; Wilkinson et al., 2017).

However, removing viscose oil from cold water can be obtained with proper oil-mineral particles- chemical dispersants ratios. This ratio impacts aggregates' mean sizes and size distribution and prevent oil surfacing or fast sedimentation (Wang et al., 2013). The proper ratio depends on the oil composition, type of particles, and dispersants, tested by laboratory experiments. Deep injection of chemical dispersants to the spills in cold temperature environments like the Arctic could also result in extensive oil sedimentation (MOSSFA). The main drivers for MOSSFA are a combination of high concentrations of EPS, mineral particles, dispersants, and oil droplets (Daly et al., 2016). Global warming is expected to facilitate the chance of MOSSFA in the Arctic by increasing the phytoplankton density and releasing minerals from melting ice fields. Studies showed that oil biodegradation rates in the microcosms at 4°C were only half compared to 20°C (Coulon et al., 2007). Therefore, extensive oil sedimentation and MOSSFA event in the Arctic could lead to long oil persistence and increased exposure of the benthic ecosystem to the oil.

6.6 Conclusion

This thesis aimed to improve the understanding of physical-chemical and biological processes affecting the fate and biodegradation of oil in deep marine oil spills and the influence of chemical dispersants used for pollution mitigation on these processes. The lesson learned from this and other studies is to learn from nature and base mitigation strategies on the self-healing capacities of nature, i.e., oil biodegradation. As soon as the oil is spilled and pollution of the environment has occurred, the human response could be optimal when it is in line with such a "Nature-based" response, taking advantage of natural biodegradation as much as possible. While humans successfully improved the formula of chemical dispersants to a less toxic level to enhance this natural biodegradation, other side effects and problems were born. Side effects such as intensive oil dissolution in water, oil components toxicity, stress impulses to the marine organisms inducing increased production of EPS, excessive formation of marine snow, and scavenging of oil particles from the water column to extensive oil sedimentation towards cold deep sea environments and eventually the seafloor.

The visible problem, oil on surface waters, is converted into an invisible problem of long-lasting oil-particle layers on the seafloor. These natural conditions are less favorable for biodegradation, and it will cost more effort and time to resolve the pollution problem by nature, and in case clean-up is demanded, also by humans. Even a complete clean-up in one human generation may have become unfeasible. After disasters such as the Deepwater Horizon oil spill, we learned the consequences of deep injections of dispersants into the deepsea. We need to improve our understanding of deep marine oil spills on various environmental conditions and carefully evaluate the scenarios for every specific environmental condition to minimize damages of future oil spills. Further studies on oil pollution and mitigation in deep and cold marine water environments are needed to further improve our understanding of deep marine oil spills. Though limiting petroleum oil consumption is a fundamental and final solution for this problem, further research into mitigation of marine oil pollution is highly needed to protect our marine ecosystems in the coming decades, the time that our societies need for a transition to such a non-fossil economy.

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Shokouh Rahsepar,

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About the Author

Shokouh Rahsepar was born in the mountain foothills of Alvand, Iran, on April 21, 1984. The beauty of nature has fascinated her since childhood. With a great love for nature, she pursued her bachelor's degree in Environmental science in Iran, followed by a Master's of Environmental Engineering at Universiti Teknologi Malaysia and a Ph.D. in Environmental Technologies at Wageningen University, The Netherlands. For this thesis, she collaborated with the Center for the Integrated Modeling and Analysis of the Gulf Ecosystem (C-IMAGE), which aimed to develop information needed for a decision support tool for applying chemicals to reduce the impact of deep oil spills. Shokouh will continue loving nature, learning more about its pollutants, and working towards zero pollution for nature.

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