The Effects of Marine Oil Pollution and Remediation Strategies on Microbial Community Structure and Function

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SUMMARY

Due to the demand for oil consistently increasing over the past decade (currently approximately 105 million barrels per day), so does the risk of open water oil spills. Thus the need for strong remediation strategies has become much more apparent. The present thesis thus aims to bring to light the impact remediation responses and marine oil pollution on microbial community structures and functionality. As a considerable point of debate chemical dispersants were the main focus of these studies, investigating their effectiveness in enhancing biodegradation processes and their impact on ecological processes such as marine snow formation and microbial community composition. Due to a spill occurring during the writing of this thesis focus on other remediation strategies and the impact of an *in-situ* spill on microbial community structure and function was also implemented to highlight the effectiveness and usefulness of metagenomic analysis for post spill monitoring *in-situ*. As an important tool for mitigating the environmental impacts of marine oil pollution, the evaluation of chemical dispersant effectiveness in enhancing biodegradation under increasing oil concentrations was the main focus of Chapter 2. As oil concentrations increased the percentage of hydrocarbons degraded over time significantly declined, however, the rate of degradation only declined at oil concentrations of 1000 mg L-1. With the addition of dispersant use degradation rate across all concentrations was enhanced. Bacterial communities were also shown to be impacted, notably with declines in *Alteromonadaceae* and *Cellulophaga* as oil concentrations increased above 100 mg L-1 compared to lower concentration of oil (1 – 10 mg L-1) . Another point of debate surrounding dispersant use was marine oil snow (MOS) formation, thus chapters three and four focused on MOS formation and their associated microbial communities under surface spill conditions. Using the ecological index of hydrocarbon

exposure floc formations were shown to be sites of high hydrocarbon degradation potential, with upwards of 50% of the community being associated with the potential to utilise hydrocarbons as a viable carbon source. Observations of the bacterial succession of flocs further highlighted their hydrocarbon degrading potential with a clear succession over time from *Oleispira* dominated communities to a highly diverse community of obligate hydrocarbonoclastic bacteria and other generalistic potential degraders. This study also indicated microbial eukaryotes (algae, protists) as excellent biomarkers for hydrocarbon exposure and that they should also be considered alongside bacterial biomarkers for future spill monitoring models. Chapter four provided the opportunity to explore the impacts of the *in-situ* Poole Harbour 2023 spill on microbial communities and their functionality. Impacted communities within the Spill Site indicated potential biomarkers for hydrocarbon contamination such as *Thiobacillus* and *Dechloromonas*. Further analysis showcased the creeks capacity for hydrocarbon degradation through the presence of 24 hydrocarbon degrading genes targeting both alkane and aromatics and indicated an uplift in the presence of anaerobic hydrocarbon degradation genes at the spill-site compared to the rest of the creek and control site. This thesis highlights the effectiveness of remediation in mitigating the effects of marine oil pollution, whilst emphasising the environmental considerations that must be made when performing laboratory experiments. This thesis progresses our understanding of microbial and hydrocarbon interactions to improve biomonitoring techniques and decision-making via the Net Environment Benefit Analysis (NEBA).

Publications

The following publications occurred during the course of this PhD:

Chapter Two, Conference Paper (Appendix 1): Smallbone, J.A., McGenity, T.J., Holland, R.D., Thomas, G.E., Coulon, F., Cowley, T. and McKew, B.A. 2024. The effect of crude oil concentration on hydrocarbon degradation and associated bacterial communities under experimental conditions. In *International Oil Spill Conference Proceedings*. 2024: 1.

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Chapter One

Introduction

Marine oil pollution

Oil has increasingly become a major commodity within both developed and developing countries across the globe. Since 2010, oil production has increased at a steady rate from approximately 73 million barrels (11.6 billion litres) per day, to approximately 105 million barrels per day as of the start of 2025, seeing a brief decline of 97 million barrels post-COVID. Current production and consumption of crude oil are predicted to stay above 105 million barrels per day by the end of 2025 (U.S. Energy Information Administration, 2025).

The current global demand has substantially increased the need for transportation, production and exploration of oil, which in turn has increased the likelihood of oil/petroleum-based products entering the marine environment. To date, estimating the amount of oil entering the marine environment has proven difficult, with previous best estimates being 1.3 million tonnes per annum worldwide; sitting within a range of 470,000 and 8.3 million tonnes per annum, with approximately 46% of oil entering the marine environment coming from natural seeps and the remaining (best estimate: 600,000 tonnes) entering from anthropogenic sources (Table 1.1) (National Research Council, 2003). These estimates have lowered considerably since 1985 in which our best estimates were 3.2 million tonnes per annum. However other sources have quoted global estimations to be 4.63 million tonnes and 1-3 million tonnes respectively (Carpenter, 2019). Estimations made by the National Academies of Science have compared the best estimates previously made to newer estimations (National Academies of Science, 2022), however this only includes oil entering marine environments across North America (Table 1.2). According to these new estimates,

significantly more oil has entered our marine environments in the last decade when compared to previous decades. However, the majority of this increase has been created by the relative increase in extraction and consumption of oil as opposed to the transportation of oil. Although varied and potentially outdated these estimations showcase the difficulties faced in quantifying accurate estimates of oil entering marine environments. Even so, they represent a very real global issue associated with marine oil pollution.

Although recent estimates suggest transportation and exploration (e.g. Tanker spills and oil blowouts) make up a small proportion of anthropogenic sources of marine oil pollution (majority of anthropogenic sources being land-runoff) (Table 1.2), they are well documented and heavily researched. This is because of the significant impact such events have on the surrounding environment due to the sheer quantity of oil being introduced within such a small spatial and temporal scale when compared to that of natural seeps and other anthropogenic sources. Explorative oil pollution through deep-sea drilling was thrown into the spotlight with the occurrence of the infamous 2010 Macondo oil spill or Deep-water Horizon (DWH) spill, occurring in the Gulf of Mexico (Brakstad et al., 2018). During this event, according to the United States a net discharge of approximately 4.19 million barrels was released into the environment during the blowout, after adjustments were made for the collected oil (Barbier, 2015). Although, BP approximated this to be 2.45 million barrels, with various other bodies estimating amounts between 2.4 – 5.4 million barrels. Ultimately the United States court concluded that 4 million barrels were released, calculating 3.19 million barrels entered the Gulf of Mexico after collection . Before the DWH incident, oil well blowouts have caused a significant amount of marine oil pollution in the past (Table 1.3) (Jernelv, 2010).

Table 1.1: Worldwide estimates (minimum, best and maximum) of annual oil release (Thousands of tonnes) in the marine environment between 1990 – 1999 (National Research Council, 2003)

Source	Min estimate	Best Estimate	Max estimate
Natural seeps	200	600	2000
Extraction of Petroleum	20	38	62
Platform	0.29	0.86	1.4
Atmospheric deposition	0.38	1.3	2.6
Produced waters	19	36	58
Transport of Petroleum	120	150	260
Pipeline spills	6.1	12	37
Tank vessel spills	93	100	130
Operational discharge (cargo washings)	18	36	72
Coastal Facility Spills	2.4	4.9	15
Atmospheric deposition	0.2	0.4	1
Consumption of Petroleum	130	480	6000
Land-based (river/runoff)	6.8	140	5000
Recreational marine vessel	Nd	nd[d]	Nd
Spills (Non-tank vessels	6.5	7.1	8.8
Operational discharge (vessels ≥100 GT)	90	270	810
Operational discharge (vessels <100 GT)	Nd	nd[e]	Nd
Atmospheric deposition	23	52	200
Jettisoned aircraft fuel	5.0	7.5	22
Total	470	1300	8300

Notes:
Totals may not equal sum of components due to independent rounding
GT = Gross Tonnage (1 GT = 100 cubic feet/2.83 cubic metres)

Table 1.2: ‘Best estimate’ of annual oil release across North America (Thousands of Tonnes) into the marine environment, comparison between Oil in the Sea III and IV (National Academies of Sciences,

Source	Oil in the Sea III (1990 – 1999)	Oil in the Sea IV (2010 – 2019)
Natural Sources (Oil Seeps)	160	100
Extraction of Petroleum (inc DWH, 2010)	3.0	66.5
Excluding DWH	NA	9.5
Platforms	0.16	1.1
Atmospheric Deposition	0.12	Not reported
Produced Waters	2.7	6.8
Transportation of Petroleum	9.1	0.82
Pipeline Spills	1.9	0.38
Tank Vessel Spills	5.3	0.2
Commercial Vessel Spills	0.099	0.008
Coastal Terminal Spills	1.9	0.22
Coastal Refinery Spills	Inc in terminal spills	0.01
Atmospheric Deposition	0.01	Not reported
Consumption of Petroleum	84	1200.4
Land-based Runoff	54	1200
Recreational Marine Vessels	5.6	Not reported
Spills (non-tank vessels)	1.2	0.39
Op. Discharge (vessels > 100 GT)	0.10	0.009
Op. Discharge (Vessels < 100 GT)	0.12	0
Atmospheric Deposition	21	Not Reported
Aircraft Jettison	1.5	Not Reported
Total	260	1400

2022

Notes:
Totals may not equal sum of components due to independent rounding
GT = Gross Tonnage (1 GT = 100 cubic feet/2.83 cubic metres)

Table 1.3: The largest marine oil blowouts recorded before 2010’s DWH incident (Jernelv, 2010)

Well	Country	Year	Tons Spilled
Deepwater Horizon	United States	2010	650,000
Ixtoc I	Mexico	1979	475,000
Nowruz	Iran	1983-85	100,000
Nowruz	Iran	1983	40,000
Ecofisk	Norway	1977	27,000
Funiwa 5	Nigeria	1980	26,000
Montara	Australia	2009	20,000

As the production of oil increases so does the amount of oil transported across the world, with much of this transport (63%) being by ship (U.S. Energy Information Administration, 2019). This is expected to increase by 35%, from 2.5 billion barrels of

oil per year to 3.3 billion by 2030 in need of transportation (Rystad Energy, 2022). As a consequence of increased transportation, the likelihood of an oil spill occurring can be high. Since the 1960s, major tanker spills (>50,000) have occurred across the globe (Table 1.4), with the largest case introducing 287,000 tonnes (Atlantic Empress) of oil into the marine environment (Figure 1.1) (ITOPF, 2020). However, over the past 50 years with the increased importance of oil spill response, we have seen a continuous advancement in oil transportation technology (Double-hull designs) and regulations (Paris Memorandum of Understanding on Port State Control; aimed to reduce and eliminate substandard ships) (Chen et al., 2019), leading to a substantial decrease in the severity of oil-related incidences and total spillage of oil. Notably, during 2019 and 2020 zero large spills (>700 tonnes) and only three medium spills (7-700 tonnes) were recorded (ITOPF, 2020). Even so, although rarer, major accidents can still occur, notably the Sanchi oil spill in 2018; being the 9th largest spill since 1967. Therefore, even with the advancements being made, the transportation of oil remains a major factor in marine oil pollution.

Table 1.4: Top 20 major (>50,000) Tanker spills between 1967 – 2020 (ITOPF, 2020)

Ship name	Year	Location	Amount of Oil Spilt (Tonnes)
Atlantic empress	1979	Off Tobago, West Indies	287,000
ABT Summer	1991	700 nautical miles off Angola	260,000
Castillo De Bellver	1983	Off Saldanha Bay, South Africa	252,000
Amoco Cadiz	1978	Off Brittany, France	223,000
Haven	1991	Genoa, Italy	144,000
Odyssey	1988	700 nautical miles off Nova Scotia, Canada	132,000
Torrey Canyon	1967	Scilly Isles, UK	119,000
Sea Star	1972	Gulf of Oman	115,000
Sanchi	2018	Off Shanghai, China	113,000
Irenes Serenade	1980	Navarino Bay, Greece	100,000
Urquiola	1976	La Coruna, Spain	100,000
Hawaiian Patriot	1977	300 nautical miles of Honolulu	95,000
Independenta	1979	Bosphorus, Turkey	94,000
Jakob Maersk	1975	Oporto, Portugal	88,000
Braer	1993	Shetland Islands, UK	85,000
Aegean Sea	1992	La Coruna, Spain	74,000
Sea Empress	1996	Milford Haven, UK	72,000
Khark 5	1989	120 nautical miles off Atlantic coast of Morocco	70,000
Nova	1985	Off Kharg Island, Gulf of Iran	70,000
Katina P	1992	Off Maputo, Mozambique	67,000

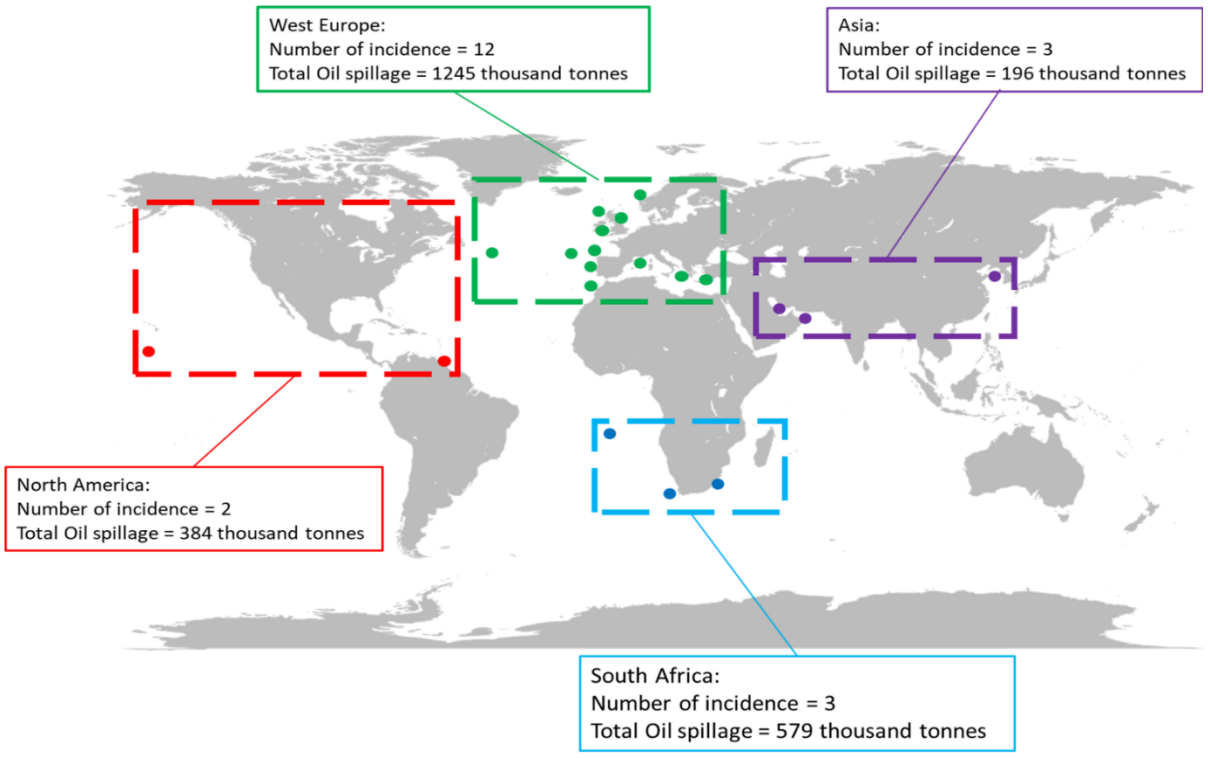


Figure 1.1: Distribution of Major Tanker oil spills between 1967 – 2020, number of incidences within each region and total Oil Spillage "Thousands of Tonnes". (Adapted from Chen et al., 2019), Data from ITOPF, 2020.

Biological and economic impacts of marine oil pollution

The Impacts of marine oil pollution are far-reaching and can vary dramatically dependent on oil type, the volume of oil entering the environment and the various environmental factors at play (Zhang et al., 2019a). These effects can be ecological, aesthetically damaging and can alter ecosystems by subjecting them to highly toxic substances but can also cause physiological harm at an individual species level. The toxicity of crude oil varies with the level of polycyclic aromatic hydrocarbons (PAHs) and volatile compounds (VOCs) which are present in all crude oils and in some cases are considered mutagenic and carcinogenic (Manzetti, 2013; National Toxicology Program, 2021). Once the environment has been subject to oil pollution there are a few exposure pathways present for hydrocarbons within marine biota, such as diffusion of soluble oil components across membranes and the ingestion of particulate matter and sediments contaminated with oil (Dupuis and Ucan-Marin, 2015). Exposure of PAHs through tissue has been observed within *Mytilus edulis* (Blue Mussels), where diffusion of PAHs was noted through gills, an extremely effective pathway given the role of gas exchange within gill structures (Axiak et al., 1988). Exposure through ingestion of particulate matter has been observed within invertebrates, notably from filter-feeding bivalves who take in suspended particulate matter (Payne and Driskell, 2005). Within marine mammals, birds and fish, exposure of these toxic components mainly occurs through accidental ingestion, entering their respiratory system whilst moving through the affected area (Zhang et al., 2019a).

A common occurrence during oil spill events is the contamination of high amenity coastal areas. The economic impacts can occur over two temporal scales, being immediate cost associated with response/clean-up and prolonged societal costs by communities dependent on proximity and/or reliance on the affected location.

These costs can vary drastically, for example, two spills that occurred in 1979 and 1991 (ABT Summer and Atlantic Empress) incurred no societal costs due to the remote nature of the spill being hundreds of miles offshore (Zhang et al., 2019a), whist other coastal spills may incur high societal costs from local communities. Societally tourism and fisheries sectors are some of the most impacted industries during an oil spill event.

Chemical composition of crude oils

Crude oil is a naturally occurring substance, constituting primarily of hydrocarbons (alkanes, cycloalkanes, and aromatics) and a low percentage of sulphur, nitrogen, and oxygen (Gad, 2014). The physical and chemical properties of crude oil can differ depending on the source of the crude oil; however, the main composition stays consistent.

The main components of crude oil consist of saturated hydrocarbons (aliphatic and alicyclic) and unsaturated hydrocarbons (aromatic) (Maier, 2019). Saturated hydrocarbons contain single-bonded carbon structures (C-C), with each carbon atom bonded to as many hydrogen atoms as possible forming straight, branched, and ringed chains. The general formula for linear saturated hydrocarbons is C_nH_{2n+2}, for example, the formula for ethane is C_2H_6 (Silberberg, 2015). Aliphatic hydrocarbons make up the largest fraction in crude oils consisting of straight-chain structures (n-alkanes) (for example n-Hexane "C_6H_{14}" and n-Hexadecane "$C_{16}H_{34}$") and branched-chain structures (isoalkanes), such as pristane "$C_{19}H_{40}$" and phytane "$C_{20}H_{42}$" (Maier, 2019; Wang et al., 2015) (Figure 1.2). Alicyclic hydrocarbons (Cycloalkanes) are another major component, making up between 20 – 67% of crude oils by volume (Maier, 2019) and consist of carbon chains in a ring formation. Cycloalkanes generally consist of cyclopentane (5 carbon rings) and cyclohexane (6 carbon rings) (Figure 1.2). As it is

difficult to isolate pure cultures of bacteria that degrade cycloalkanes, it is thought that the biodegradation of alicyclic hydrocarbons is a result of multiple microbes forming a microbial consortium.

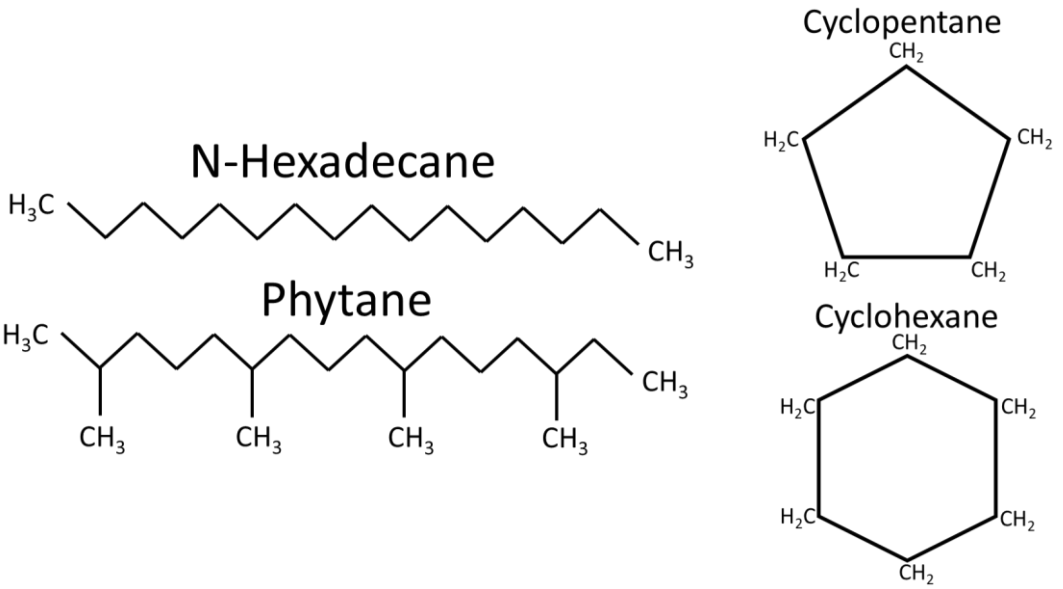


Figure 1.2: Chemical structure of straight chained (n-alkane: N-Hexadecane), branched chained (isoalkanes: Phytane) Aliphatic hydrocarbons and ring chained (Cycloalkane: Cyclopentane and Cyclohexane) alicyclic hydrocarbons.

Aromatic hydrocarbons are a separate group of unsaturated hydrocarbons, containing at least one unsaturated ring and are present in all crude oils, as well as some petroleum products. For example, the parent hydrocarbon of this class benzene (C_6H_6), being a component of gasoline (Maier, 2019). Often aromatics can be highly toxic, carcinogenic, and mutagenic and follow a 6-carbon benzene ring structure. These consist of both monoaromatic hydrocarbons and polyaromatic hydrocarbons (PAHs). Monoaromatics consist of benzene, toluene (Figure 1.3), ethylbenzene and xylene, often referred to as BTEX hydrocarbons (Camphuysen and Heubeck, 2001). PAHs consist of at least two fused benzene rings and contribute to a large proportion of crude oil compositions. Examples of PAHs within crude oil include naphthalene (2-ringed), phenanthrene (3-ringed) (Speight, 2017), pyrene (4-ringed) and Benzo[a]pyrene (5-ringed) (Itodo *et al.*, 2019) (Figure 1.3). Crude oils also contain methylated PAHs. Although hydrocarbon structures make up the majority of the

composition of crude oil, they also include polar molecules known as asphaltenes and resins. The structure of which consists of carbon, hydrogen, nitrogen, oxygen, and sulphur, with trace amounts of vanadium and nickel. In oil, asphaltenes are defined as n-heptane (C_7H_{16}) – insoluble and resins – soluble.

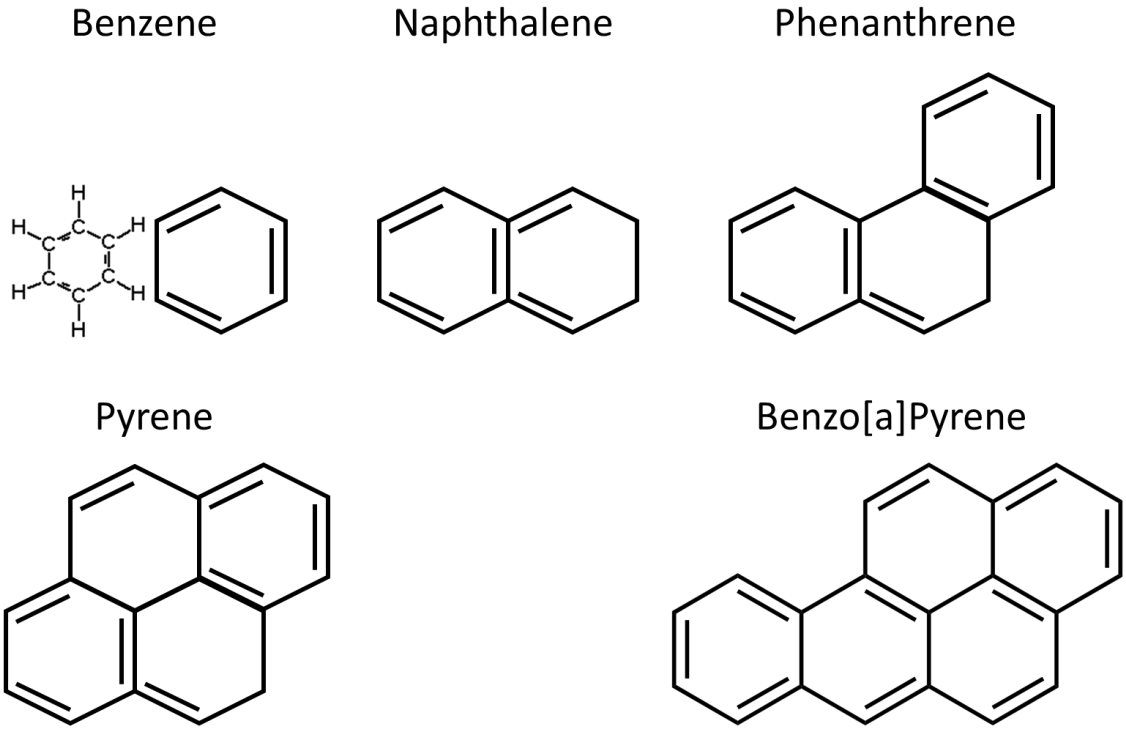


Figure 1.3: chemical structure representative of aromatic hydrocarbons: Monoaromatic – Benzene and polyaromatic (PAHs) – Naphthalene, Phenanthrene, Pyrene and Benzo[a]pyrene.

Physical and chemical attenuation processes

During an oil spill event, various physical and chemical processes occur within the marine environment collectively known as the "weathering process" and include, spreading, evaporation, dispersion, emulsification, dissolution, photo-oxidation and sedimentation/sinking (Figure 1.4). Although all are a part of the weathering process of oil, the relative importance and contribution each process has are time-dependent (Figure 1.5) (Al-Majed *et al.*, 2012). The weathering of oil is also dependent on a variety of factors including environmental conditions (Sea temperature, Wind speed, Turbulence, nutrients, etc) and type of oil. As soon as an oil spill occurs, weathering begins with the process of spreading, occurring within hours to days. During this, the

oil starts to spread across the sea surface forming a slick, the rate at which is determined by wind speed. Usually forming a narrow coherent slick, following the wind direction. The thickness then starts to reduce, and the slick may break down, directly influencing the other stages of weathering (ITOPF, 2011a).

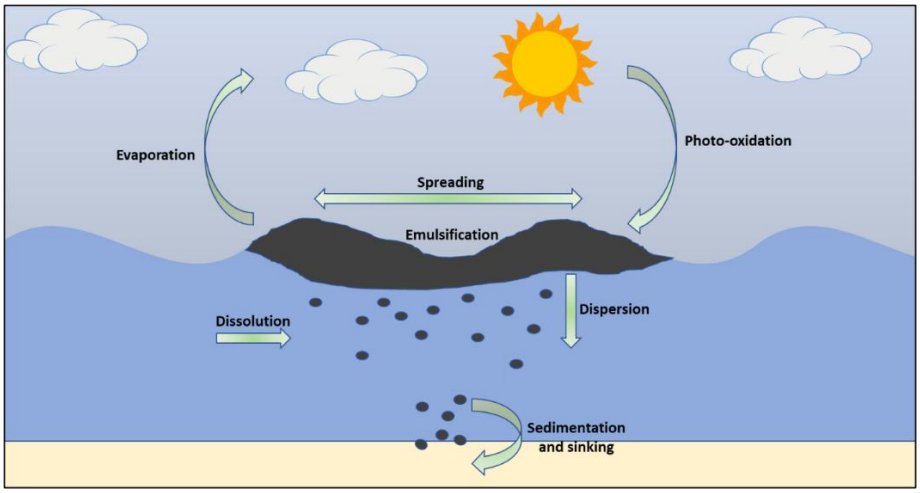


Figure 1.4: Schematic of the weathering processes that occur during an oil spill event in the natural attenuation of marine oil pollution.

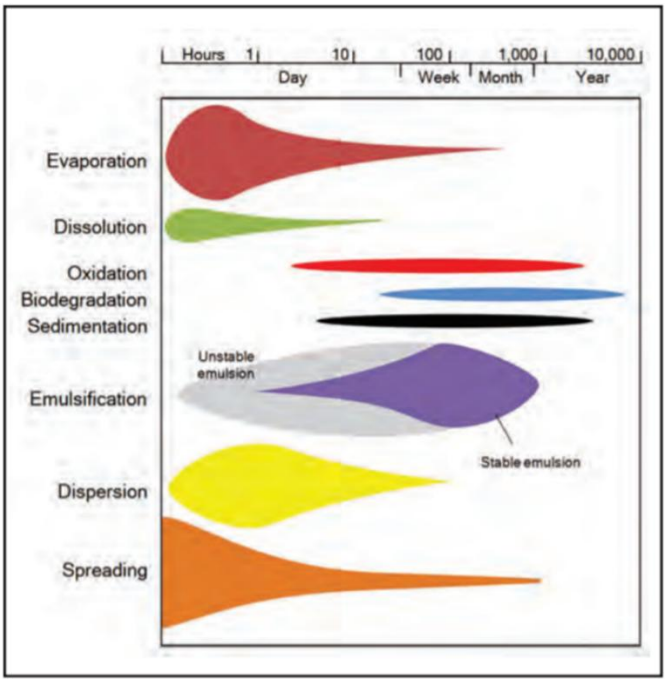


Figure 1.5: Schematic representing the importance of each weathering process of crude oil over time. The width indicates the importance of each process. Taken from ITOPF, 2011a

Within hours of an oil spill the evaporation of more volatile components can occur, the rate of which is dependent on the ambient water temperatures and wind speed. Components of oil with a boiling point of 200°C tend to evaporate within 24

hours in temperate conditions. Within this time period, light and medium crude oils can be reduced by 75% and 40% of their initial volume respectively; however heavier oils may only see reductions of 5% (Fingas, 1995).

Dispersion of the oil can also occur quite soon after an oil spill event, the rate of which is dependent on the nature of the oil (less viscous = easier dispersion) and the current sea state. Wave action at the sea surface is a major factor in the breakdown of parts or all of the oil slick into the water column, in the form of small droplets (Harayama *et al.*, 1999). Smaller droplets will then stay suspended in the water column; however, larger droplets risk resurfacing and joining the slick once again. Continuous turbulence within the water column tends to keep these droplets in suspension (oil-in-water emulsion) increasing surface area and thus increasing biological processes such as biodegradation. The process of dispersion can directly influence further weathering processes such as dissolution, and sedimentation (ITOPF, 2011a). The use of chemical dispersants during an oil spill can speed up this natural process “4.3.1 Impact on microbial biodegradation pg 34”. The rate of dissolution during the weathering process is directly dependent on the oils composition, environmental factors (turbulence, water temperature) and other weathering processes (Spreading, dispersion). Dissolution has been shown to decrease with a decrease in API°, as the oil composition consists of less light monoaromatic compounds decreasing its solubility within water (Hamam *et al.*, 1988). Early on during an oil spill water – in – oil emulsions can occur, in which the oil takes up water which can increase the volume of the pollutant by a factor of five times. These formations occur more readily in oils with a combined concentration (> 15ppm) of Nickel/Vanadium, or with an asphaltene content of >0.5%. The rate of emulsification is usually determined by these differences in composition and by wind speeds

(between 3-5ms^{-1}). Over time these emulsions can become more viscous and stable, as asphaltene compounds precipitate and coat the emulsion. The formation of water – in – oil emulsions is the main reason for the preservation of light and medium oils during a spill event, as they reduce the rate of other weathering processes (National Research Council, 2003).

Over a longer period, a process known as photo-oxidation occurs in which hydrocarbons react with oxygen through light-catalysed reactions promoted by sunlight, degrading crude oil compounds into more soluble products (Shankar *et al*., 2015). Usually, crude oils are quite unreactive to oxidation, however, in the presence of water, these reactions are heightened following the oxidation formula of $C_nH_{2n}+2 + 0_2 \leftrightarrow CO_2 + H_20$ ($C_nH_{2n}+2$ representative of a Cycloalkane hydrocarbon compound). The photooxidation process includes two principal mechanisms, direct photolysis (Reactant absorbs light energy directly) and indirect photolysis, a process in which chromophores such as PAHs and asphaltene photochemically produce reactive oxygen species (ROS). These ROS can then indirectly react with not only light-absorbing components but with various crude oil constituents (Ward and Overton, 2020). These processes break down hydrocarbons by producing intermediates which then attack the molecules. Products formed through photooxidation tend to be more polar, naturally dispersible, and toxic in comparison to the parent material. The extent to which photooxidation occurs depends on (1) the spectrum and irradiance of the incident light, (2) the optical properties of both the surface water and the hydrocarbons themselves and (3) the presence of photosensitisers and photo degraders (Shankar *et al.*, 2015).

The last non-biological process to occur during a spill event within the marine environment is the sinking and sedimentation of crude oil. As previously stated, the sinking of crude oil will occur when said oil is denser than water (<10° API). This can occur in naturally heavy oils, with the loss of lighter hydrocarbons through weathering processes and via oil droplet interactions with organic matter and suspended particulate matter forming aggregates known as marine oil snow (MOS) dense enough for the oil to sink and settle on the benthos. As MOS is formed via dispersed oil interactions with organic matter (Microbes, faecal matter etc) and particulate matter, there is currently debate as to whether the use of chemical dispersants to speed up the dispersion process of oil enhances (Fu *et al*., 2014; Suja *et al*., 2017) MOS formation or inhibits it (Passow, 2016; Passow *et al*., 2017).

Biological attenuation processes

The ocean contains a vast array of microorganisms (microbes) that are capable of metabolising oil compounds in the form of hydrocarbons, including some 175 genera of bacteria, archaea and fungi which use hydrocarbons as either their sole or major carbon source (Hazen *et al.*, 2016). This process is known as biodegradation and has occurred naturally within the marine environment for millions of years via microbes taking advantage of natural seeps introducing approximately 600,000 tonnes of oil into the marine ecosystem annually (National Research Council, 2003) and can occur both aerobically and anaerobically. The metabolic process in which microbes utilise hydrocarbons as a carbon source to produce energy is known as oxidative phosphorylation respiration. A secondary process in biodegradation known as detoxification also occurs primarily as a mechanism in response to direct exposure of the microbes to oil, whereby the hydrocarbons are degraded into more water-soluble components that can be discharged from the body (National Research Council, 2003).

During biodegradation, various enzymes are involved dependent on the microbes and the degradation pathway taken, for example, the degradation of n-alkanes consists of four distinct pathways utilised by various microbial communities (Ji *et al.*, 2013).

During biodegradation, individual microbes tend to target specific types of hydrocarbons, generally acting together as a microbial consortium or in succession of each other during the degradation process (Brakstad *et al.*, 2017). For example, hydrocarbonoclasticus bacteria (*Alcanivorax*) associated with the metabolism of straight-chained and branched alkanes, may be succeeded by a consortium of PAH degraders (*Cycloclasticus*) (Harayama *et al.,* 2004; McGenity *et al.,* 2012). The formation of these diverse microbial communities is therefore capable of degrading complex hydrocarbon structures, using an array of pathways and enzymes when subjected to crude oil within the marine environment. Generally, it is observed that less complex saturated n-alkanes are most readily degradable, with $nC_{10} – C_{26}$ alkanes being the most readily utilised by hydrocarbon degraders (Head *et al*., 2006). As the hydrocarbon structure increases in complexity (Branched Alkanes), they become less readily available as a smaller number of microbes can utilise these structures. Large multi-ring aromatic hydrocarbons tend to be the most resistant to biodegradation, however, some low molecular weight aromatics such as naphthalene may be oxidised before some n-alkane structures. Even so, the majority of aromatics will degrade at a much slower rate, requiring specific degraders for the task (Venosa and Zhu, 2003). Polyaromatic hydrocarbons (PAH's) with a more complex structure may only degrade through co-metabolism, in which particular microorganisms will transform/modify compounds they cannot utilise themselves, whilst metabolising simpler hydrocarbons. The modified PAH compounds will then be utilised by a secondary microorganism that can use the product as a viable source (Dalton and Stirling, 1982). This process may

be done through multiple microorganisms until complete degradation has occurred and has been observed in the biodegradation of pyrene (Zhang *et al.*, 2019b).

The distribution of hydrocarbon degraders within a marine environment is likely closely related to historical exposure. Regions that are frequently exposed to crude oil through natural seeps and/or frequent oil pollution will likely have a higher percentage of naturally occurring hydrocarbon-degrading microbial communities, which can make up to 100% of the heterotrophic bacterial community during oil spillage. Within environments that are subject to zero crude oil input, hydrocarbon degraders may make up less than 0.1% of the overall community (Venosa and Zhu, 2003), namely taking advantage of other smaller potential sources of hydrocarbons outside of crude oil input (Gutierrez *et al*., 2019). This potential previous exposure to oil pollution may impact the rate at which biodegradation occurs. For example, in a heavily polluted region or area with regular natural seeps, the microbial community capable of degradation may already be present in abundance, whereas in a pristine environment there may be a “lag” between oil exposure and biodegradation as the microbial community will need to form and produce the required enzymes involved in degradation.

Biological limiting factors

In addition to crude oil composition, there are a variety of environmental factors that impact the rate of biodegradation of hydrocarbons. These include oxygen, nutrients, temperature, and pressure.

Oxygen is one of the most important factors for microbial degradation of hydrocarbons through aerobic pathways. This is because microbial communities will employ oxygen incorporating enzymes known as dioxygenase and monooxygenase as the first step in biodegradation (Rojo, 2010) These enzymes are used to transfer

oxygen molecules to the hydrocarbon molecule, therefore dissolved oxygen is required for this process to occur. Fortunately, oxygen is usually not a limiting factor and is plentiful during marine oil spill events. However, in certain environments microbial communities may experience limitations in oxygen supply, for example, anoxic zones in the water column, sediments and low energy beaches, mudflats, and saltmarshes where a depletion in oxygen can occur, decreasing the rate of biodegradation dramatically (Venosa and Zhu, 2003). Another example specifically in regard to spills, would be the oxygen anomaly that occurred during the Macondo 2010 deepwater horizon spill, in which dissolved oxygen concentrations significantly as a direct response to microbial activity associated with hydrocarbon degradation (Kessler *et al*., 2011). Although much of the site was still well oxygenated, some small pockets may have experienced oxygen limiting conditions. Anaerobic degradation of hydrocarbon has been observed to occur at much slower rates. For example, during the Amoco Cadiz spill in which biodegradation through anaerobic pathways within the sediment was found to be slower by several magnitudes to that of aerobic degradation (Hassanshahian and Cappello, 2013).

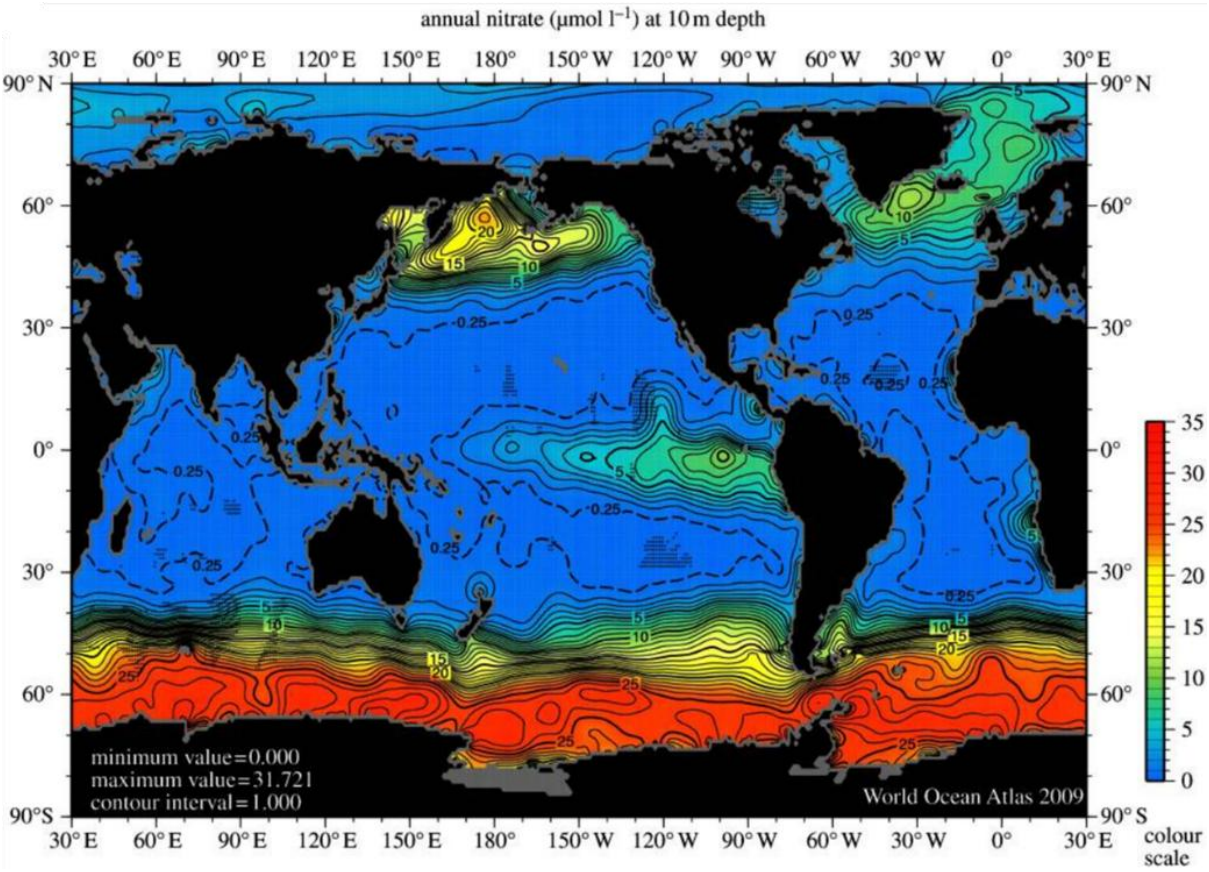


Figure 1.6: Nitrite concentration within the surface water of the ocean (Voss et al., 2013)

Nutrients such as nitrogen and phosphorus play a crucial role in the growth of microbial communities. Nitrogen in the form of ammonium (NH_4+), Nitrate (NO_3-) and Nitrite (NO_2-) is required for protein and nitrogenous base synthesis, whilst phosphorus, as phosphate (PO_4^{3-}) is required for the synthesis of nucleic acids and phospholipids (Madigan *et al*, 2018). Marine environments are usually naturally deficient in these nutrients (Figure 1.6), therefore due to this reliance, they can potentially limit biodegradation rates of hydrocarbons within this environment (Hassanshahian and Cappello, 2013). Approximately, 150mg of nitrogen and 30mg of phosphorus is utilised in the degradation of 1g of hydrocarbons (Venosa and Zhu, 2003). Consequently, during an oil spill, a large influx of carbon is introduced on a short timescale, making these vital nutrients quickly become limited due to an increased need for microbial growth. These limitations become more apparent as hydrocarbon-degrading microbes compete with non-hydrocarbon degraders that also

require these nutrients for growth, causing further deficit (Thingstad and Rassoulzadegan, 1995). Iron is another nutrient required for microbial growth that has also been found to limit biodegradation, being used for cellular respiration and protein synthesis (Madigan *et al*, 2018). Although generally abundant, the potential precipitation of iron as ferric hydroxide during alkaline reactions can cause this nutrient to limit biological processes such as microbial growth in surface water to a similar level of nitrogen and phosphorous, thus limiting hydrocarbon degradation. This is especially prevalent in regions of clear offshore water (Dibble and Bartha, 1976). To combat these limitations biostimulation is sometimes considered as a remediation tool, in which nutrients that may be limited during an oil spill event are supplied to the environment stimulating microbial growth, thus increasing biodegradation rates (Coulon *et al.*, 2007). However, the use of biostimulation as a remediation tool must be regulated accordingly due to the risks of potential eutrophication (Altieri and Diaz, 2019) and the financial/time costs that may ensue.

Marine oil pollution is a persistent and continual global issue, thus occurring across multiple habitats and environments. During oil spill events, crude oil may be exposed to a temperature range of 0^{o}C to 35^{o}C dependent on location. This can impact biodegradation significantly as the ambient temperature of the environment can affect both the properties of spilt oil and the activity of microbial communities associated with hydrocarbon degradation. Observations of hydrocarbon degradation associated with temperature have noted multiple aspects including the reduced volatilisation of crude oils in low-temperature environments (Atlas, 1981), during which, observations were made highlighting light oils consisting of volatile components evaporating much slower at 10^{o}C than at 20^{o}C delaying the onset of microbial degradation. During the same study, lighter oils were observed to be more susceptible to biodegradation than more

viscous heavy oils at 20°C (Atlas, 1975). Crude oil also tends to become more viscous in low temperatures reducing spreading, thus reducing surface area availability for biodegradation to occur (Yaghi and Al-Bemani, 2002). It is generally observed that degradation rates decrease with a decrease in temperature, believed to primarily be a result of a decrease in enzymatic activity within microbial communities associated with hydrocarbon degradation (Leahy and Colwell, 1990). Gibbs *et al.* (1974) observed a decrease in the rate of biodegradation of approximately 60% between 14°C – 4°C, reminiscent of seasonal changes that can be seen across seawater surrounding the British Isles. A change in the rate of biodegradation has also been observed within specific n-alkane hydrocarbons. Notably in nC12 – C20 a significant decrease in biodegradation via *Rhodococcus* was observed over a gradient of temperatures (30°C, 20°C, 10°C and 1°C) (Margesin *et al.*, 2013). Furthermore, during temperature experiments performed by Coulon *et al*. (2007), a significant reduction of total petroleum hydrocarbons (TPH) was observed over 90 days at a temperature of 20°C (8% remaining) in comparison to TPH at 4°C (15% remaining), thus presenting a significant increase in biodegradation with an increase in temperature. Dependent on the ambient temperature of the environment certain microorganisms may thrive or suffer during the biodegradation process, with species outcompeting one another depending on their physiological constraints. For example, at 30°C – 20°C both *Pseudomonas oleovorans* and *Rhodococcus* (Strain Q15) are capable of utilising dodecane. As the temperature decreases to 10°C the rate of degradation by *pseudomonas oleovorans* become negligible, whereas Q15 is capable of utilising dodecane and hexadecane at temperatures down to 0°C without any significant rate change. This strain was also capable of utilising both octacosane and dotriacontane at 5°C (Whyte *et al.*, 1998). Psychrotrophic hydrocarbon-degrading microbe

Pseudoalteromonas. Spp (P29) was shown to be very capable during petroleum degradation at temperatures of 5^{o}C showing a biodegradation efficiency of nearly 90 – 80% respectively for two types of oil (Lin *et al.*, 2009). During the same study, it was found they were capable of significant microbial growth between 5^{o}C – 35^{o}C with an optimum temperature of 25^{o}C, show casing the broad potential of this species in biodegradation across a range of temperatures. There is an increasing need to understand how hydrocarbon-degrading communities may respond to a range of temperatures and each other, as the exploration for oil is now expanding to regions and depths of extreme temperatures (Zhang *et al.*, 2019a)

During the Deepwater Horizon oil spill event, it was estimated a large proportion of oil reached the seafloor of the Gulf of Mexico (GOM). During subsequent investigations, it became apparent that estimating the fate of this oil was difficult as a lack of data on factors such as temperature and pressure throughout the water column during the event meant these investigations were lacking vital information. During a recent study in 2018, 13 sediment samples were taken from a range of depths (62 – 1520m) to assess the potential inhibitory effect pressure may have on hydrocarbon degradation (Nguyen *et al.*, 2018). During which, the depletion of total n-alkanes (%) decreased as pressure increased using a range between 0.1 – 15MPa (simulate *in-situ* conditions), therefore representing a decrease in biodegradation. However, the degradation of PAHs stayed moderately the same between shallow and deep-water conditions with no significant difference. Multiple past studies also highlight significant reductions in biodegradation, for example, Schwarz *et al*. (1975) observed a significant decrease in hexadecane utilisation via microbes isolated at 4940 metres subjected to 50MPa in comparison to ambient pressure (0.1MPa). Again, a significant reduction in biodegradation of 33% from microbial communities subject to 15MPa in comparison

to 0.1MPa was recorded during such investigations (Prince *et al*., 2016c). However, specific microorganisms have been observed to potentially be physiologically capable of dealing with these high pressures. No inhibitory effects to pressure were present in *Marinobacter hydrocarbonoclasticus* (Strain #5) at 35MPa, whilst only slight inhibitory effects were noticed in *Rhodococcus qingshengii* (Strain TUHH-12) (Grossi *et al*., 2010; Schedler *et al*., 2014).

Oil degrading microbial communities

Approximately 320 genera of bacteria, as well as 12 genera of archaea have been suggested to have the capacity of utilising hydrocarbon sources (Prince *et al.*, 2019). Common hydrocarbon-degrading bacteria such as *Alcanivorax* and *marinobacter* have been shown to be ubiquitous, globally (Chernikova *et al*., 2020) found in both sea surface environments and deep sea regions, whilst rarer taxa tend to be unevenly distributed dependent on habitat and environmental condition (Jiao *et al*., 2016). An example of this can be seen in taxa *Desulfobacterota*, *Psedomanadota* and *Chloroflexota* dominating cold seep habitats across the mediterranean and Caspian Seas (Warkhade *et al*., 2025). This highlights both the generalistic and specialist geography of hydrocarbon degraders taxa being both ubiquitous globally and selecting for specific conditions dependent on environmental requirements. Approximately 214 fungal genera and 23 algal genera have also been reported to be capable of utilising various hydrocarbons (Prince, 2018). Generally, these microbes exist in low abundance. However, during an oil spill event that introduces large volumes of hydrocarbons over a short temporal scale to the surrounding environment, this can stimulate the growth of these organisms, causing structural changes to the microbial community present. The identification of such species and the characterisation of community structures, succession and metabolic pathways of

these microorganisms are important to developing and improving upon *in-situ* bioremediation strategies during an oil spill event (Hassanshahian and Cappello, 2013). No single hydrocarbon-degrading microbe is capable of degrading every component found in crude oil, therefore many different species are required to form large communities for significant degradation that are found worldwide. Although the majority of hydrocarbon degraders are generalistic, meaning they can utilise a variety of different carbons sources, there are group of microbes known as obligate hydrocarbonoclastic bacteria (OHCB). These specialised taxa almost exclusively utilise hydrocarbons as a carbon source, utilising a variety of hydrocarbons during biodegradation (Gutierrez, 2019), consisting of both n-alkane (*Alcanivorax, Thalassolitus*) and PAH (*Cycloclasticus, Neptunomonas*) (Yakimov *et al*., 2022). Although initially characterised as very specialised degraders, they are currently thought to possess a much higher metabolic versatility due to their presence in low abundance outside of spill events (Cafaro *et al.*, 2013). Even so, very little evidence suggests they compete for non-hydrocarbon recourses with other non-hydrocarbon degraders. Our current understanding of OHCBs makes them easily distinguishable from more metabolically diverse microbes that have the capacity to metabolise hydrocarbons but also utilise a wider range of metabolic pathways to obtain nutrients and energy by other means. Therefore, they will be treated as separate for the duration of this thesis.

Microbial communities have been shown to respond significantly when oil enters the marine environment, leading to significant changes within community structure/composition (Chakraborty *et al.*, 2012). However, this response is highly dependent on various factors such as oil composition, degree of weathering and a multitude of environmental factors (Temperature, nutrient concentration) (McGenity *et*

al., 2012). Even so, a general trend in microbial response has been observed. Notably a large influx in the abundance of alkane degraders (for example *Alcanivorax*) occurs during the initial oil spill event, focusing on the degradation of simpler straight/branched alkane hydrocarbons. These are then succeeded by an increase in PAH degrading microbes e.g., *Cycloclasticus* (Figure 1.7). During such an event OHCB can eventually constitute around 90% of the overall community, whereas they would only consist of approximately <1% beforehand (Coscolín *et al.*, 2019).

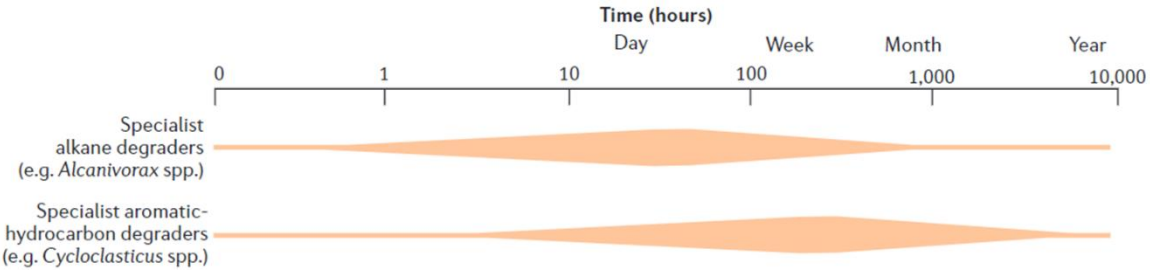


Figure 1.7: Change in abundance of specialised microorganisms involved in hydrocarbon degradation. Highlights *Alcanivorax Spp* as first responders, closely followed by *Cycloclasticus Spp.* Schematic represents the general changes in microbial community abundance and structure observed during several studies (Adapted from Head *et al.* (2006)).

Obligate hydrocarbonoclastic bacteria

As previously mentioned OHCB's are capable of utilising both n-alkanes and PAH's, being capable of exclusively using hydrocarbons as a carbon and energy source. To date, there are eleven recognised genera of OHCB's, with the newest addition being *Planomicrobium*, consisting of one known species *Planococcus alkanoclasticus* (Yakimov *et al*., 2022). This particular OHCB is the only known species of class *Bacilli* (Engelhardt *et al.*, 2001). The rest of the well-established OHCB consist of multiple genera within class *Gammaproteobacteria*. These include *Porticoccus*, *Algiphilus*, *Polycycolvorans*, *Alcanivorax*, *Neptunomonas*, *Oleibacter*, *Oleiphilus*, *Deispira*, *Thalassolituus* and *Cycloclasticus* (Dyksterhouse *et* al., 1995; Yakimov *et al., 1998;* Gutierrez *et al*., 2012; Gutierrez *et al.,* 2013; Yakimov *et al*., 2022).

Alkane degrading OHCB

Genus *Alcanivorax* is a prominent OHCB within the family Alcanivoracaceae, and part of the Order *Oceanospirillales*. To date, there have been a total of 29 species reported by 2024, with the most recent species named being *Alcanivorax quisquiliarum* in 2023 (An *et al*., 2023; Chem *et al*., 2024). The majority of these species were isolated from surficial seawater and tidal sediments, either accidentally or from chronically contaminated samples (McGenity, 2019). *Alcanivorax xenomutans* however, is an example that was isolated from deeper waters within the Indian ocean after carbon enrichment occurred via petroleum pollution (Fu *et al*., 2018). In pristine environmental conditions, they tend to be found in very low abundance, however, they rapidly increase to being the most abundant genus during the early stages of an oil spill event and biodegradation (Pal *et al.*, 2019). *Alcanivorax borkumensis (strain SK2)* was the first species to be identified and successfully isolated. Described as a Gram-negative, non–motile aerobic rod capable of utilising alkanes up to C32, some branched alkanes such as phytane and even simple aromatic hydrocarbons, providing a highly competitive range of utilisation over other hydrocarbon degraders of a smaller range (Yakimov *et at.,* 1998; Timmis *et al*., 2010). With the high prominence of the genus *Alcanivorax*, strain SK2 was the first hydrocarbon degrader to have its entire genome publicly reported. This provided an in-depth insight into its capacity for n-alkane degradation, metabolic capabilities, biosurfactant production, biofilm formation and ability to survive at oil-water interfaces, allowing *Alcanivorax* access to oil within a range of environmental conditions (Schneiker *et al.*, 2006). More recently, strain SK2 was observed to possess a type of extracellular iron-chelating molecule, thought to support a high-performance iron–uptake system even in reduced iron environments; vital to hydrocarbon metabolism (Denaro et al*.,* 2014). *Alcanivorax. Spp* produce

various enzymes integral to the metabolism of hydrocarbons, including, Alkane hydroxylase AlkB1 and AlkB2, three cytochrome P450s and the monooxygenase AlmA (Miri *et al.*, 2010; Wang and Shao, 2014). This variety of enzyme is what makes *Alcanivorax. spp* a prominent degrader as they target multiple ranges of hydrocarbons. AlkB1 preferably oxidise hydrocarbons *n*C_5 – C_{12} and AlkB2 oxidise hydrocarbons *n*C_8 – C_{16}, whilst Alm oxidise long-chained hydrocarbons from C_{18} up to C_{36} (Miri *et al*., 2010; Wang and Shao, 2012). *Alcanivorax borkumensis* (Strain MM1) has been shown to produce a biosurfactant of glycolipids, which has been shown to reduce the surface tension of water and interfacial tension of n-hexadecane, allowing increased dispersion and easy access to hydrocarbons for degradation (Passeri *et al.*, 1992). Another biosurfactant that has been identified was produced by *Alcanivorax dieselolei* (Strain B5). This biosurfactant was a novel lipopeptide, which was found to increase oil uptake within the water, thus increasing the emulsification of oil (Qiao and Shao, 2010).

Genus *Oleibacter* is another OHCB within class *Gammaproteobacteria*, order *Oceanospirillales*, currently consisting of one species *Oleibacter marinus* (Parte *et al*., 2020). However, this was reclassified as *Thalassolituus* in 2022 as *Thalassolituus Maritimus* (Dong *et al*., 2022). Prior to this, *Oleibacter marinus* was shown to be dominant in microcosms simulating oil spills using Indonesian seawater and has been identified in low abundance (0.6% and 0.1%) around Singapore and Japan. However, observations of *Oleibacter marinus* have also been made at mesophotic depths (397m) and even depths up to 10,400m within the Mariana trench, challenger deep, where a large abundance of hydrocarbons degraders including*, Alcanivorax* and *Thalassolituus* were observed (Whitman *et al*., 2016; Liu *et al*., 2019).

Genus *Thalassolituus* consist of six identified species *Thalassolituus oleivorans, Thalassolituus Marinus*, *Thalassolituus Maritimus*, *Thalassolituus alkanivorans*, *Thalassolituus pacificus* and *Thalassolituus Hydrocarbonoclasticus* (Parte *et al*., 2020; Dong *et al*., 2022; Wei and Quan, 2022) and are part of class *Gammaproteobacteria*, order *Oceanospirillales*. Described as a motile strictly aerobic, heterotrophic, gram-negative, curved bacteria, is capable of utilising a narrow spectrum of aliphatic hydrocarbons ($nC_7 – C_{20}$). Strain MIL-1 was isolated by extinction dilution from n – tetradecane enriched cultures using seawater sampled from the harbour of Milazzo, Italy. Optimal conditions for the growth of this strain consisted of 2 – 7% salinity and temperatures of 20 – 25^oC (temperature range of 0 – 30^oC). As they are strictly aerobic they are unable to grow via fermentation, nitrate reduction or phototrophic means and have been described as halophilic (Yakimov *et al.*, 2004). *Thalassolituus* are distributed worldwide, with *Thalassolituus* like microbial communities being observed across the Baltic, Barents, Mediterranean, Atlantic, and pacific and have also been observed in large abundance in North Sea microcosms when enriched with crude oil at 4 – 20^oC; being the most dominant species within the community at 20^oC consisting of 15% of the overall community (Yakimov *et al*., 2007; Coulon *et al*., 2007). Sequencing of strain MIL-1 has identified various genes associated with the utilisation of hydrocarbon compounds for example an alkane monooxygenase which was shown to be heavily expressed on C_{14}. However, it was also observed that this strain was capable of utilising long-chain alkane hydrocarbons up to nC_{32}, due to genes that coded for subterminal Baeyer-villiger monooxygenase (Gregson *et al*., 2018).

Genus Oleiphilus currently consists of two identified species, *Oleispira Antarctica* and *Oleispira lenta* and sit within the class *Gammaproteobacteria*, order

Oceanospirillales (Parte *et al*., 2020). Two strains of *Oleispira Antarctica* (RB – 8 and RB – 9) were isolated in 2003 within Rod Bay, Ross Sea, Antarctica and were described as gram-negative, psychrophilic, aerobic bacteria with a curved rod morphology and polar flagella. These strains were found to optimally grow at a salinity of 3 – 5% (w/v), at a broad temperature range of 1-15^{o}C. However, they were able to grow at a significant rate between the temperatures of -6.8 and 25^{o}C (Yakimov *et al*., 2003). Prior reports have stated *Oleiphilus* can be very dominating within a microbial community within colder environments, even outcompeting other OHCB's such as *Alcanivorax* and *Thalassolituus* (Coulon *et al*., 2007). Sequencing has revealed an array of genes associated with adaptation to these colder climates known as chaperonins (CPn60, CPn10), which have been suggested to protect the functioning of proteins at lower temperatures and a variety of genes for hydrocarbon degradations, including alkane monooxygenase such as C23040 (similar to AlkB2). This allows *Oleiphilus* to utilise a variety of aliphatic hydrocarbons (C_{10} – C_{24}). However, they have been observed to be unable to utilise longer/branched chains potentially due to a lack of AlmA genes (Gregson *et al*., 2020). In 2012 *Oleiphilus lenta* was identified using a Strain (DFH11T) from coastal water off Qingdao, China, which was characterised as a Gram-negative, motile, aerobic spirilli (Wang *et al*., 2012). This particular strain grew optimally at a salinity of 23% (w/v) at temperatures between 0 – 30^{o}C.

Aromatic degrading OCHB

Both genus *Cycloclasticus* and *Neptunomonas* have been observed to be highly efficient at utilising aromatic hydrocarbons as their main source of carbon. Genus *Cycloclasticus* resides within class *Gammaproteobacteria*, order *Thiotrichales* and currently consists of three named species (one validly published under ICNP) (Parte *et al*., 2020). Described as a gram-negative, rod-shaped bacteria *Cycloclasticus*

are obligatorily aerobic and motile via a single flagellum (Dyksterhouse *et al.*, 1995). *Cycloclasticus pugetii* (Strain PS-1), was isolated from Puget sound, Washington surface sediments using biphenyl (aromatic compound) and was found to grow on a limited selection of aromatics including toluene (Monoaromatic), anthracene, naphthalene and phenanthrene (PAH's). The key enzymes associated with PAH degradation are ring hydroxylating dioxygenase (RHDs), nine of which have been identified in *Cycloclasticus* (RHD - 1 to 9) (Wang *et al.*, 2018). The extent of aromatic degradation within *Cycloclasticus* has been observed to be extremely high in comparison to other aromatic degraders such as *Marinobacter* and *Sphingomonas (Kasai et al., 2002)*. Genus *Neptunomonas*, class *Gammaproteobacteria,* order *Oceanospirillales* was first identified in 1999 and currently consists of eight known species (Hedlund *et al*., Cyclo1999; Parte *et al*., 2020).

Although not considered part of the OHCB genus due to not being reliant on hydrocarbon substrates for growth, *Marinobacter* is still worth briefly mentioning as one species has been described as an obligate hydrocarbon-degrading bacteria, *Marinobacter hydrocarbonoclasticus*. Described as a gram-negative, Aerobic rod-shaped bacterium this species optimally grows at 32^{o}C and is capable of utilising a variety of cyclic and non-cyclic hydrocarbons including tetradecane, hexadecane and pristane (Gauthier *et al*., 1992).

Degradation pathways

Due to hydrocarbons being ubiquitous in marine environments, microbial communities have evolved to utilise a wide variety of hydrocarbons as a source of carbon due to the presence of oil within the marine environment through natural seeps (Prince *et al*., 2019). This capability has occurred through the evolution of various

metabolic pathways to which microbes have access to and include both aerobic and anaerobic pathways dependent on environmental conditions and microbial species.

Aerobic hydrocarbon degradation

Before hydrocarbons can be catabolised, they must be oxygenated if they contain two or more carbons. The enzymes involved in oxygenation are known as oxygenase's and incorporate O_2 into organic compounds, in this case, hydrocarbons (Xu *et al.*, 2018). There are two types of oxygenase, known as monooxygenase and dioxygenase. Monooxygenase catalyses the transfer of one of the two oxygen molecules to the hydrocarbon, whilst the second is reduced to H_2O and tend to require an electron doner of NADH or NADPH. Dioxygenase however catalyses the transfer of both oxygen molecules (Olajire and Essien, 2014).

Under aerobic conditions four metabolic pathways have been described for n-alkane hydrocarbons, the first of which is monoterminal (Terminal) oxidation being very common amongst hydrocarbon-degrading bacteria. This process is initialised by monooxygenase oxidising at the terminal methyl group to yield a primary alcohol. Further oxidation takes place producing aldehyde (initialised by alcohol dehydrogenases) and then into fatty acids via aldehyde dehydrogenases. The fatty acids then enter β-oxidation, in which they are subsequently metabolised to acetyl-CoA which move through the citric acid cycle or are used to make new cell material. During β-oxidation, NADH forms and is oxidised for energy conservation purposes. The process of β-oxidation is then repeated releasing another acetyl-CoA molecule. Biterminal oxidation is the second pathway to be described, in which oxidation to the corresponding fatty acid occurs without rupturing the carbon chain. During this process fatty acids are produced through the terminal pathway, however, undergo ω-hydroxylation, which yields a ω-hydroxy fatty acid. This is further catalysed to a

dicarboxylic acid and sent through the β-oxidation process. The third pathway is known as subterminal oxidation, in which alkanes are oxidised at the subterminal methyl group producing a primary and secondary alcohol. The fourth pathway to be described is the Finnerty pathway, which uses dioxygenase to catalyse n-alkanes into hydroperoxides, which are then converted to their corresponding aldehyde for further conversion into fatty acids (Figure 1.8) (Van Beilen *et* al., 2003; Ji *et al*., 2013; Abbasian *et al*., 2015). Terminal and sub-terminal pathways tend to specialise in the oxidation of short-chained alkanes, whilst the Finnerty pathway focuses more on longer chained alkanes (Van Beilen *et al.*, 2003).

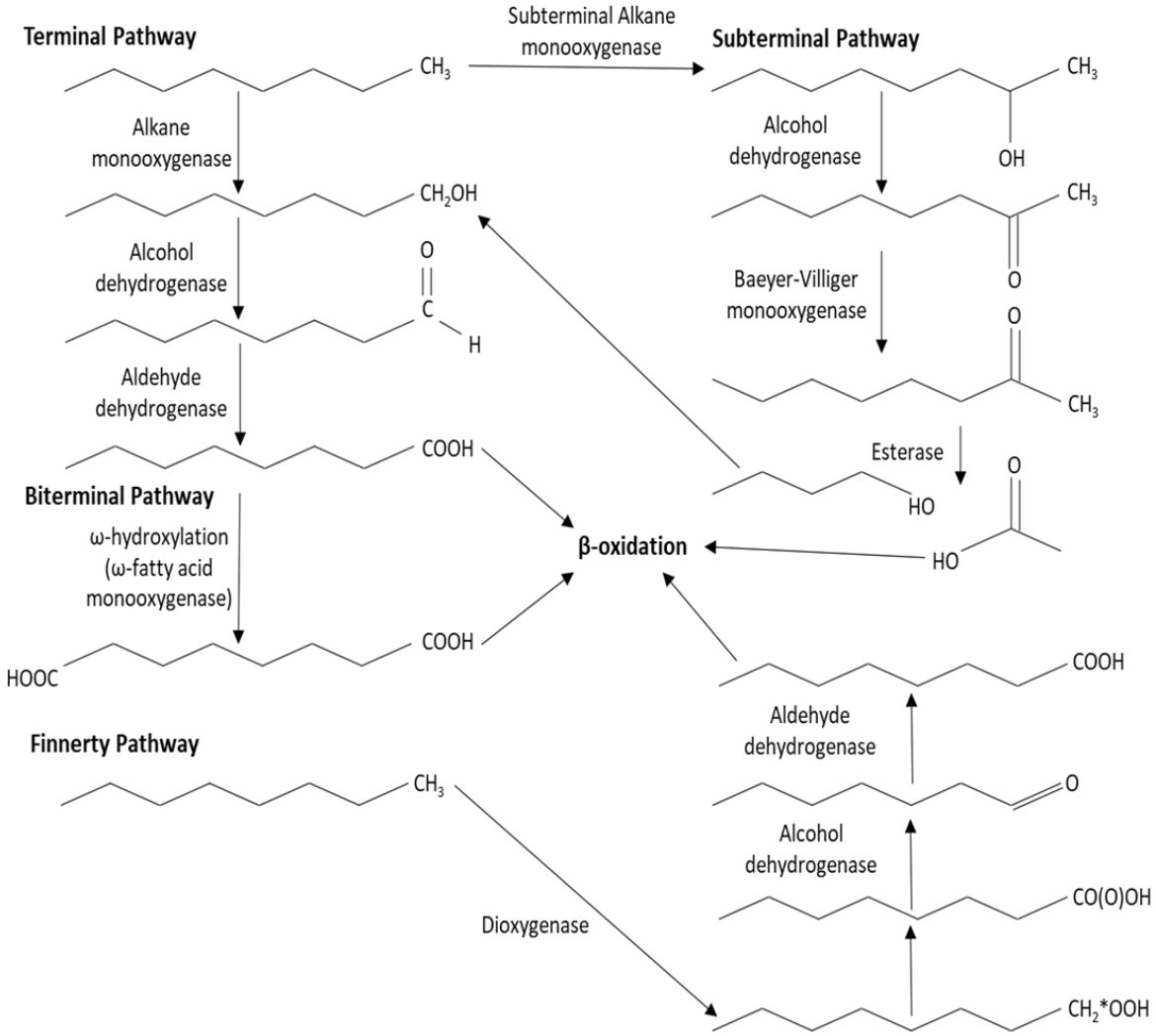


Figure 1.8: Schematic of the four aerobic alkane metabolic pathways. Oxidation via terminal pathway producing fatty acids for β-oxidation, or further oxidation via ω-fatty acid monooxygenases through Biterminal pathways, subterminal pathways or the Finnerty pathway initialised by dioxygenase (Adapted from Ji *et al*, 2013).

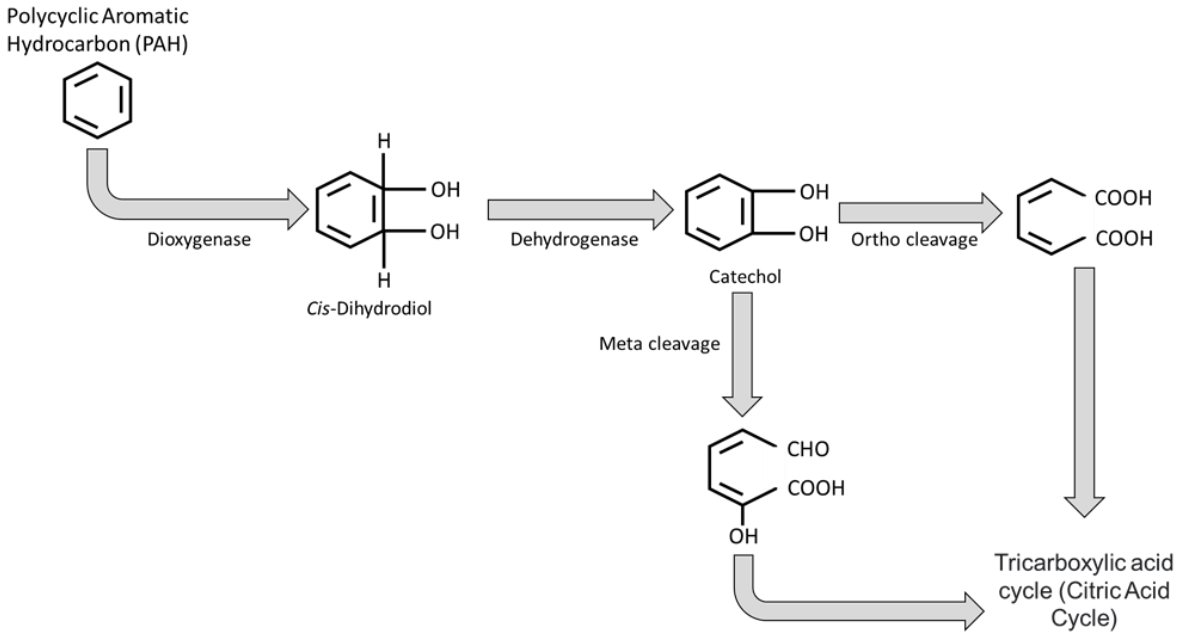


Figure 1.9: Schematic representation of the general polycyclic aromatic hydrocarbon aerobic metabolic pathway.

During aromatic hydrocarbon degradation, dioxygenase enzymes are typically responsible for initiating oxidation, in which both oxygen molecules are incorporated into the hydroxyl group of the hydrocarbon and thus oxidise the aromatic ring. Cis-dihydrodiols are then formed which are further oxidised to aromatic dihydroxy compounds known as catechol's. Catechol's are further reduced through ortho or meta cleavage pathways through ring cleaving dioxygenase, in which both O_2 atoms are catalysed and incorporated to catechol derivatives resulting in "ring-open" semialdehyde adducts. Ortho cleavage (intradiol) use intradiol enzymes to cleave the aromatic ring between two hydroxyl groups, whereas meta cleavage (extradiol) use extradiol enzymes to cleave between a hydroxylated carbon and adjacent non-hydroxylated carbon. These are then readily degraded to TCA (Tricarboxylic acid) cycle metabolites for entry into the citric acid cycle (Figure 1.9) (Cerniglia, 1993; Lipscomb, 2008; Kumar *et al.*, 2011). The degradation of aromatics tends to be carried out by this scheme, however, due to differences in complexity between aromatic compounds slight alterations to the pathway and the type of reaction produced vary

(Kumar *et al*., 2011). As previously mentioned RHD's (Ring hydroxylating dioxygenases are the key enzyme in aromatic hydrocarbon degradation.

Anaerobic hydrocarbon degradation

In the absence of oxygen anaerobic degradation can occur with the terminal electron acceptor generally being replaced with nitrate, sulphate, or iron. Due to this anaerobic hydrocarbon degraders tend to be denitrifying, iron-reducing, or sulphate-reducing microbes (Heider *et al.*, 1998). Dependent on the hydrocarbon and microbial community there are a variety of degradation pathways available that have been described (Madigan *et al*., 2018). Two observed n-alkane anaerobic pathways consist of the fumarate addition and carboxylation pathway, which use nitrate and sulphate as electron acceptors respectively (Ji *et al*., 2013). These pathways have been observed thoroughly within sulphate-reducing bacteria (strain AK-01) and denitrifying bacteria (strain HxN1). The fumarate addition pathway proceeds through the sub-terminal pathway adding fumarate to the hydrocarbon at the second carbon, this results in the formation of an alkylsuccinate. This is followed by further degradation via "carbon skeleton rearrangement" and β-oxidation to form a fatty acid. Another example of this pathway has been suggested to occur in aromatics (toluene and naphthalene), which produces benzylsuccinate, which undergoes further degradation to benzoyl-CoA via a modified β-oxidation process (Heider *et al*., 1998; Aitken *et al*., 2004). Carboxylation, also known as "biological Kolbe-Schmitt reaction" has been suggested to occur in monoaromatic and non-substituted Polyaromatic hydrocarbons (Coates *et al.*, 2002). Various other pathways have also been identified as Phosphorylation, O_2 – independent hydroxylation and Methylation (Ladino-Orjuela *et al.*, 2016). However many of these pathways remain incompletely understood, especially O_2 independent hydroxylation (Pelosi *et al*., 2019).

Remediation and bioremediation technology and strategies

Within the “oil response” industry, there are a variety of remediation/bioremediation tools available. Whilst these tools and practices are consistently used during spill events, they are, however, constantly under review for their efficiency and safety as to maintain the best possible response when applied. A wide selection of variables determine what response is chosen and tend to differ between governments and organisations. These include the personal health and safety of responders, “Window of opportunity” available for the remediation tool, environmental and oceanographic conditions, geographical location, proximity to sensitive regions and the political restrictions that may be in place (NOAA, 2010). Overall, the goal of using these remediation tools is to mitigate the impact of oil pollution on the natural environment and reduce the economic loss that may occur. These goals are what drive the spill impact mitigation assessment (SIMA) (IPIECA, 2017). Within open water, remediation strategies such as booming, skimming, burning and dispersant use are all typically used during a spill event (NOAA, 2010; Fingas, 2011a; ITOPF, 2011b; ITOPF, 2012; Cormack, 103; Ndimele *et al.*, 2018). Other bioremediation strategies consist of biostimulation and bioaugmentation, used to describe tools which accelerate the natural biodegradation process of oil, either through anthropogenic intervention via thre application of additional nutrients and/or indigenous and in some cases non-indigenous microorganisms capable of hydrocarbon degradation in the affected area (Bragg *et al*., 1994; Jiménez *et al.*, 2007; Dave and Ghaly, 2011; Adams *et al*., 2015; Sayed *et al*., 2021). The efficiency of such intervention is however entirely dependent on the environmental conditions and the microbes being introduced, as for this approach to be successful, the microbial consortium must be capable of degrading the majority of oil components within the

spill, maintain genetic viability (ability of a population to survive and reproduce) and stability during storage and effectively compete with indigenous microbes' whilst adapting to potentially hostile environments (Adams *et al*., 2015). For the purpose of this thesis, chemical dispersant use will be the main focus being considerable topic of debate on its effectiveness and ecological impact to microbial communities and wider environments.

Dispersants and their application

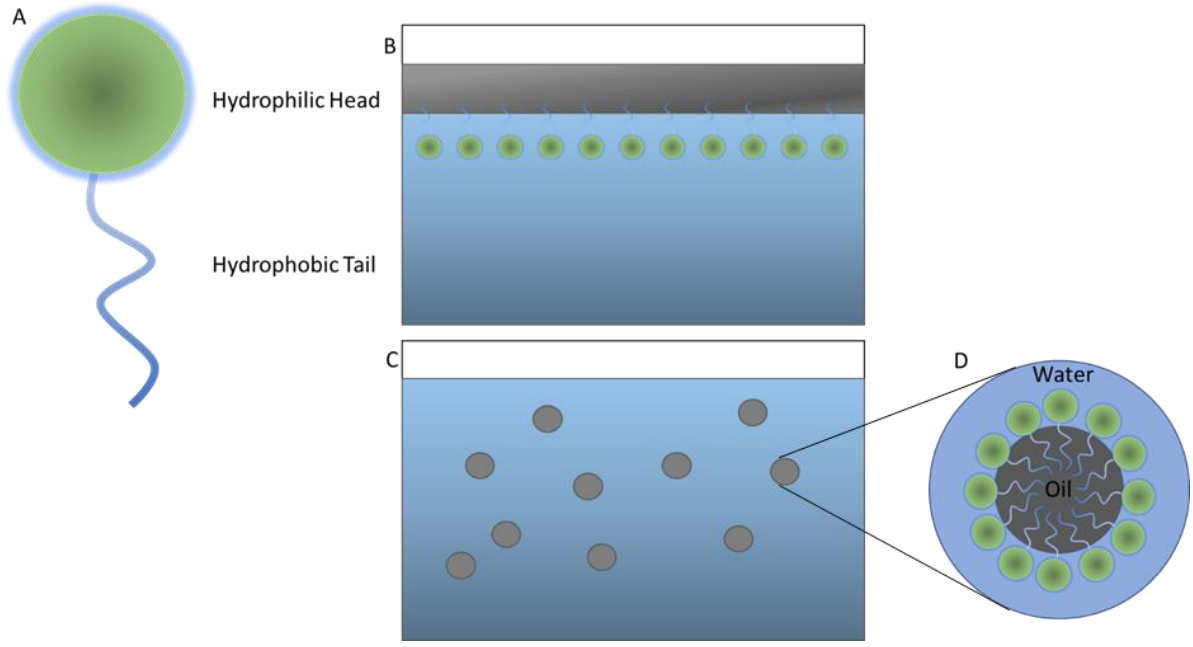


Figure 1.10: Schematic of Surfactant molecule and dispersant process during an oil spill event. A) Surfactant molecule (Hydrophilic head and Hydrophobic tail), B) As surfactant is added to the oil slick it becomes saturated and starts to sink forming Micelles, C/D) Oil droplets form being contained by the micelle containers formed by the surfactant molecules.

The use of chemical dispersants for the treatment of oil spills first started in 1967 during the Torrey Canyon spill but the dispersant used was found to be to toxic, leading to the reformulation of said dispersants to be more environmentally sensitive. Nowadays chemical dispersants consist of surfactants (surface active agents), dissolved into solvents and a stabiliser (Dave and Ghaly, 2011). Surfactants are the main component of commercial dispersants and consist of two parts, a hydrophilic head (with an attraction to water) and a hydrophobic tail (Figure 1.10A) ((Karlapudi *et*

al., 2018). The surfactants' role is to reduce the formation of oil in water emulsion, by reducing the interfacial tension between oil and water by forming strong interactions with both (Rahsepar *et al.*, 2016). Once the surface is saturated the surfactant molecules start to sink and link together to form micelle. These micelles then form "containers" (Figure 1.10B-D), creating a suitable environment to hold the oil. This allows small droplets to break away from the oil slicks and become suspended in the water column. In turn this increases the surface area in which crude oil degrading microbial communities can utilise to metabolise the hydrocarbons, thus potentially increasing the rate of natural biodegradation processes (Zhang *et al.*, 2018). These containers consist of weak bonds, forming a malleable structure. This enables these containers to expand taking on oil molecules of varied sizes. The solvents are introduced to reduce the viscosity of the surfactants, allowing the surfactant to travel through the oil to the oil/water interface, whilst also depressing the freezing point and optimising the concentration of the dispersant. These solvents can include hydrocarbons such as kerosene and heavy aromatics with boiling points above 150^{o}C. Stabilisers such as polycarboxylates are incorporated to adjust pH levels, colour, stop corrosion and increase the stability of the dispersant (Dave and Ghaly, 2011).

Chemical dispersants can be applied through a variety of methods depending on the circumstances of the spill. For example, small vessels/aircraft and helicopters are suitable for application for smaller nearshore spills, whereas larger aircraft are best equipped to handle larger offshore spills. When applying dispersants many considerations must be made as to whether success is likely, including the viscosity of oil, environmental conditions (temperature, wind speed and sea conditions) and potential environmental impacts (Nomack and Cleveland, 2010). During spill events, some oil types are more readily dispersible. In dispersant use trials at sea conducted

in the United Kingdom, it was found that IFO180 (intermediate fuel oil, maximum viscosity of 180 centistokes) was more readily dispersible than IFO380 (Intermediate fuel oil with a maximum viscosity of 380 centistokes) due to being more viscous, meaning the delivery of surfactant to the oil-water interface was much more difficult (Chapman *et al.*, 2007). Although some oils are naturally very viscous, the weathering process means most oils will increase in viscosity at the later stages of a spill event, therefore the window of opportunity is small for dispersant application to be effective enough to be considered. Environmental considerations that may need to be addressed include application to shallow nearshore sites, as in the past, observations have alluded to chemical dispersants being responsible for fouling of shorelines and the contamination of drinking water sources (NRC, 1989). The 2000 NATUNA SEA incident in the Singapore Strait serves as a reminder as to the importance of taking into consideration the environmental conditions and oil properties during a spill. Approximately 7000 tonnes of Nile Blend crude oil entered the marine environment and part of the remediation efforts was the application of dispersants. However, the temperature was 3^{o}C below the oil's pour point and the viscosity was observed to be >50,000 mPa, well beyond effective dispersion conditions (<2000 mPa for optimal dispersion). This with little wave action at the sea surface resulted in the addition of chemical dispersants to be ineffective (Chapman *et al*., 2007). Even so, under the correct conditions, chemical dispersants have proven to be a viable remediation tool in increasing the rate of oil dispersion. Davies *et al*. (1998) reported that 50-75% of the No. 5 bunker oil slick was dispersed with the application of Corexit 9500 dispersant. In contrast to the aforementioned NATUNA SEA incident, an IFO380 leak from container vessels was successfully treated with dispersant due to the oil's lighter

composition and warmer waters, reducing viscosity and allowing the surfactant to penetrate to the oil-water interface (Chapman *et al*., 2007).

Impact on microbial biodegradation

With the increase in surface area available to hydrocarbon-degrading microbial communities brought on by increased dispersion, microbes can expend more energy on growth and less on producing natural biosurfactants (e.g. rhamnolipid, trehalolipid, sophorolipid) for dispersion. This then expedites hydrocarbon biodegradation, leading to the idea that chemical dispersants may indirectly accelerate natural biodegradation processes of crude oil (Thomas *et al.*, 2021). Throughout the literature, there is a lot of evidence pointing towards the enhancement of the biodegradation of hydrocarbons. Prince *et al*. (2016b) assessed three commonly used dispersants (Corexit 9500, FinasolOSR52 and Dasic Slickgone). A significant increase in biodegradation using each of these dispersants was found when compared to oil slicks with no added dispersants. On average biodegradation increased with the application of dispersants from approximately 32% to 65% after seven days and approximately 48% to 72% after 62 days. This included the complete degradation of pristane and phytane and a >90% reduction in phenanthrene and methylphenanthrenes within seven days. Various other studies have also observed similar trends in biodegradation enhancement (Brakstad *et al.,* 2015; Hazen *et al*., 2016; Techtmann *et al*., 2017). One such experiment aimed to observe the biodegradation of different oil droplet sizes, noting that the biodegradation of *n*-alkanes and aromatic hydrocarbons within smaller droplets of 10 µm was significantly faster when compared to droplets of 30 µm. There it was concluded that dispersant treatments that would reduce oil droplet size increased the biodegradation rates of crude oil (Brakstad *et al*., 2015).

However, other studies contradict these observations, either showing chemical dispersants use to be ineffective in increasing biodegradation (McFarlin *et al*., 2014; Louvado *et al*., 2019) or even inhibiting biodegradation. One study by Kleindienst *et al*. (2015) observed that Corexit 9500 suppressed biodegradation, in direct contradiction to previous studies (Baelum *et al*., 2012; Techtmann *et al*., 2017; Thomas et al., 2021). However, this was criticised for the use of a WAF (Water accommodated fraction) and CWAF (Chemically enhanced water-accommodated-fraction) methodology (Prince *et al*., 2016b), which mechanically mixes the oil into water before the addition of dispersants, removing volatile and insoluble fractions of oil that can be degraded by microbes, thus may not be directly representative of surface spill events as no sufficient comparisons between a floating oil slick and a slick that has been chemically dispersed can be made. (Passow 2016; Prince *et al*., 2016; Passow *et al*., 2017; McFarlin and Prince, 2021)

Many of these studies however have received criticism in experimental design and viability when comparing findings to natural environmental conditions. Researchers addressing this topic tend to design their experiments without taking into consideration real-world scenarios. Many past experiments use such high concentrations of oil that conditions are not relevant to an *in-situ* dispersed oil scenario, whilst others use such a low concentration that dispersion occurs regardless of the addition of chemical dispersants (Prince *et al*., 2016b). Review papers have also suggested experiments such as this must be able to replicate the natural environment as closely as possible or collect samples during a real-world oil spill event, however, many legislative, economic, and technical issues make such an experiment difficult to undertake (Gregson *et al*., 2021). Dispersants have been designed to operate across a large scale at sea, whereas this is somewhat misunderstood in a lab environment

leading to conflict and criticism within the literature. Notably when dispersants are used *in-situ* concentrations of oil disperse to as low as 1-10 ppm within 24 hours (lee *et al*., 2013). Many prior studies have not taken this into consideration, thus making it difficult to extrapolate results to large scale *in-situ* spills. An overarching issue with *ex-situ* experimentation is the number of potential biases individual investigation can create, namely in micro/mesocosms wherein these containment practices are believed to decrease oil dispersal and dilution, which *in-situ* would dilute to sub-ppm within four hours (Thomas *et al*., 2021). To combat these issues in-situ experimentation is a viable option. However, there are still many difficulties in reproducibility and sample collection. If these limitations can be overcome*, in-situ* experiments will be vital to future observations on the fate and impact of crude oil within a marine environment.

Dispersant toxicity and impact on the natural environment

Any substance that is applied to the marine environment purposefully must be tested rigorously to understand its toxicity and the potential risk it may pose to vulnerable ecosystems, biota and the services they provide. These tests must also be done to protect the response units tasked with dispersant application and those in the immediate range of the dispersant application. Each country that uses dispersants tends to have their own regulations and guidelines in place on the use of these products. This means there is no set global regulations for dispersant use and can become an environmental issue if regulations and guidelines are not stringent enough. In the UK this includes the approval of commercial dispersants Slickgone NS, Superdispersant 25 and Finasol OSR 52 for use as oil remediation tools.

Historically, the potential impact chemical dispersants may have on the environment has been highlighted. During the Torrey Canyon spill in 1967, the earliest oil dispersant application was recorded. During which, the dispersant used contained

a solvent component with a high proportion of aromatic hydrocarbons. These are now known to be highly soluble in water and highly toxic to marine life (Shelton, 1971). 10,000 tonnes of this dispersant was sprayed directly to coastal impacted sites and during the aftermath subsequent studies later discovered this dispersant caused the majority of mortalities in marine organisms such as limpets/barnacles and algae within treated areas (Southward and Southward, 1978). Since 1967, several dispersants have been further developed to be much less toxic such as Corexit 9500 and are highly regulated. Even so, these newer dispersants still come under scrutiny and the correct guidelines and regulations must be upheld in their use. Prior studies on these newer dispersants such as Corexit9500A looked into the potential impacts of dispersant use on pelagic species C*oryphaena hippurus* (Mahi Mahi) larval development (Greer *et al.*, 2019). Using CEWAF methods, exposure at an *in-situ* concentrations of 2.5, 5 and 10 mg/L did not induce acute mortality or malformations to morphology within developing *Coryphaena hippurus* embryos. Differential expression and gene ontology pathways across the three treatments were similar, thus indicating that increases in chemical dispersion did not impact molecular outcomes within the test range. Studies focused on planktonic copepods species *Acartia tonsa, Temora turbinate* and *Parvocalanus crassirostris*, found the use of chemical dispersants in remediation to significantly impact egg production rates (45-54% reduction) and egg hatching rates (11-31% reduction), and was found to be 1.6 times more toxic to these species than crude oil alone (Almeda *et al.*, 2014). Conversely, additional studies using Slickgone NS have been shown to not increase the toxicity of the original hydrocarbon sources tested (Alexander *et al.*, 2017). Comparatively concentrations of dispersant use during real world spill events are negligible compared to crude oil concentrations at an oil to dispersant ratio of 20:1.

Overall, the ecological impact of dispersants may depend almost entirely upon the circumstances under which they are being used. Even with the use of historically highly toxic dispersants, damage to marine fauna across open ocean environments tends to be small, and despite significant toxicity to planktonic species caused by dispersants used the ecological damage was relatively small (Shelton, 1971). The considerable amount of water in which dilution can occur and the evaporative loss of aromatic solvents would help to minimise impacts across these environments. However, across shallow waters and shoreline environments, the use of dispersants has been shown to cause significant mortalities within marine life. In recent years, however, the use of chemical dispersants associated with a phenomenon known as marine oil snow (MOS) has come to prominence as an ecological issue in the debate as to whether dispersants should be used as a remediation tool.

Marine oil snow, sedimentation, and flocculent accumulation

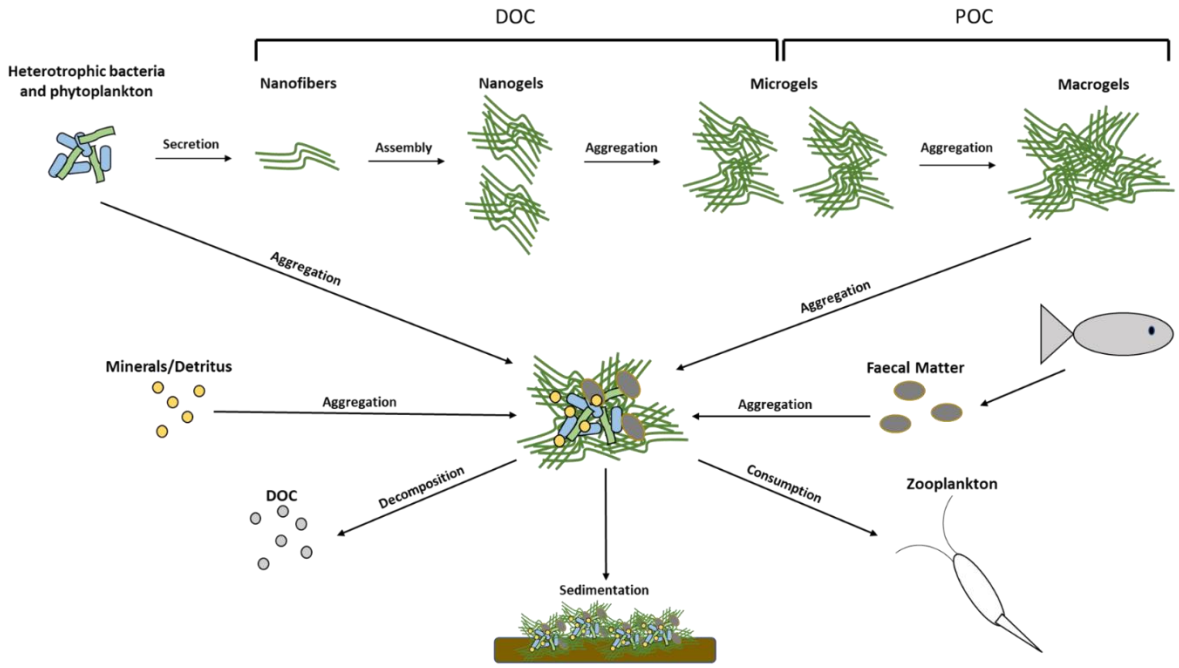


Figure 1.11: Schematic of marine snow (MS) formation and sedimentation: As nanofibers are secreted by microbial communities, they assemble together to form nanogels, which aggregate forming microfibers. During this process they make the transition between dissolved organic matter to particulate organic matter and further aggregate forming macrogels. These in turn aggregate with various other components including other minerals and detritus (phytoplankton, microbes etc) and faecal matter forming MS. This then sinks to the benthos, is decomposed into DOC, or consumed by deep sea zooplankton.

Marine snow (MS) can be defined as ≥ 500 μm diameter aggregates that sink from the euphotic zone (0-200 metres) into the mesopelagic zone and further to the seafloor (Gregson *et al*., 2021). Various organic and inorganic particles make up MS, including, microalgae, archaea, bacteria, microzooplankton, phytoplankton, fungi, faecal matter, and detritus, all bound together by biopolymers (Passow *et al*., 2012; Bochdansky *et al*., 2017). The process of MS formation occurs when dissolved organic matter (DOM), self-assembles to form microgels; three dimensional polymers suspended in seawater, which then aggregates into particulate organic matter (POM) which makes up a large proportion MS. A major component of total DOM count in the ocean are extracellular polymeric substances (EPS), which consist of polysaccharides (up to 90%), lipids, proteins, glycoproteins, and nucleic acids and are secreted by heterotrophic microbes and phytoplankton (Gregson *et al*., 2021). Polysaccharide-rich EPS are known as transparent exopolymer particles (TEP). Due to their sticky nature, they are considered significant in MS formation, providing a matrix for aggregates to form (Daly *et al.*, 2016). Once secreted EPS nanofibers (5-50 nm) congregate, these form nanogels (100-200 nm), which in turn stabilise and aggregate further to form microgels (3-6 μm) (three-dimensional polymer network) that become suspended in the water column. These continue to collide and eventually form macrogels of several 100 micrometres (Verdugo, 2012) (Figure 1.11). These macrogel aggregates then act as a structure for MS to form with TEP acting as the biological glue sticking other organic and inorganic particles together. Several studies suggest increases in TEP concentration enhance the formation of MS and consequent sinking too deep-sea environments (Passow *et al*., 1994; Gärdes *et al*, 2011). Various hydrodynamic processes such as upwellings, eddies, currents and benthic resuspension can alter the formation and distribution of MS (Daly *et al.*, 2020). Once MS has been formed, it

can then transport microbes and nutrients from the surface to deep-sea environments providing much needed nutrient sources and food to organisms living within these regions. MS also supports the organic carbon pump, which sequesters atmospheric $C0_2$ into the deep ocean and benthos (Turner, 2015).

Like MS, MOS particles consist of a mixture of organic matter (e.g., heterotrophic microbes, faecal matter, phytoplankton) and inorganic matter, but also include dispersed weathered crude oil stuck together within a matrix of EPS (Daly et al., 2016). MOS has been considered to form in two ways, either through the physical aggregation of oil particles with naturally occurring MS or through biofilm growth via microbial communities using the oil as a nucleus for floc formation (creating bacteria – oil aggregates, BOA) (Gregson *et al*., 2021). It is suggested these mechanisms are not mutually exclusive and it is very likely they both function together to form MOS. Within these formations, EPS acts as a skeletal matrix holding everything together, whilst the remaining particles would act as a ballast allowing the less dense oil to either sink or become suspended within the water column. During the 2010 Deepwater Horizon (MC-252) oil spill, which saw approximately 640 million litres of oil enter the marine environment, significant marine oil snow (MOS) formation and marine oil snow, sedimentation, flocculation, and accumulation (MOSSFA) event was observed. These events had been recorded during previous spills; however, it was during this event that led to a significant rise in interest in the importance of MOS and its fate as it sinks through the water column (Burd *et al*., 2020). This in turn led to the idea that MOS may act as a vehicle for the transport of oil into deep-sea environments. This assumption was further backed up during analysis of MOS captured using sediment traps which contained approximately 217,700 to 229,900 barrels (representing 6.8 to 7.2% of crude oil that was spilt but not recovered) of biodegraded oil residue in the form of

MOS following the MC-252 spill over an area of approximately 7,600km^2 (Stout and German, 2018). Due to the nature of MOS particles, they are a metabolic hot spot for microbial activity, in some cases being observed to have a concentration of microbes 3 to 4 orders of magnitude greater than the surrounding seawater (Silver *et al.*, 1978; Ziervogel *et al.*, 2012). This in turn can constantly alter the composition of the particle through colonisation and microbial degradation causing further aggregation and fragmentation within MOS, continuously altering the sinking velocity. Other means such as consumption via zooplankton can also cause further fragmentation of MOS (Dilling and Alldredge, 2000; Quigg *et al*., 2020). If these particles reach the benthos in a large abundance, they may accumulate causing a MOSSFA event, causing oil to be deposited into the deep-sea sediment.

Environmental impact of marine oil snow and MOSSFA events

During the MC-252 spill, it is not fully known what percentage of MOS was deposited on the seafloor or the fate of the mesopelagic species during and after the event. Even so, sediment core samples collected after the spill event indicated a decline in the density of 80-93% in benthic foraminiferal species (*Bulimina* spp., *Uvigerina* spp. and *Chibicidoides* spp. Thought to be due to an increase in the accumulation of polycyclic aromatic hydrocarbons (PAH's) within the sediment through MOS and MOSSFA (Schwing *et al.*, 2015). Further observations also noted an impact to macrofauna within the benthos due to oil sedimentation (Washburn *et al*., 2017), noting impoverished diversity, species richness and evenness within 1 km of the deepwater horizon wellhead. Even so, it is uncertain to what extent MOSSFA events may impact marine ecosystems, especially within deep-sea regions. It is believed the introduction of toxic oil components and increased anoxic conditions due to sedimentation associated with the transport of oil through MOS and subsequent

MOSSFA events are the main drivers of such changes and impacts to fragile deep-sea benthic environments (Van Eenennaam *et al*., 2018; Hastings *et al., 2020*). In the case of the MC-252 spill, the full extent of these impacts are currently unknown due to a lack of baseline data of important food web interactions and environmental processes within the impacted area (Daly *et al*., 2016). Since the MC-252 spill, various mesocosm experiments have been undertaken to investigate the impact oil constituents may have on deep-sea biota if they were to be transported and deposited to these fragile ecosystems. One such experiment aimed to investigate the impact of MOS on benthic macroinvertebrates through a set of mesocosm experiments (Van Eenennaam *et al*., 2018). During this experiment, surrogate Macondo oil, representative of oil during the MC-252 spill was weathered over a 24-hour period and subjected to biodegradation during the experiment to simulate natural in-situ processes. During such experiments, weathering is an important factor as this reduces the toxicity of crude oil over time (Di Toro *et al*., 2007). Notably, it was observed that *Corophium voluntator* and *Macona balthica* survival rates significantly reduced by 80 and 79% respectively in the presence of MOS sedimentation. *Hydrobia ulva* survival rates reduced by 40%, however, this was not found to be significant, although it is believed this was due to *Hydrobia ulva* residing on the sides of the tanks. This led to the conclusion that survival rates in *Hydrobia ulva* reduced due to the consumption of MOS and its toxic components. They concluded that the main drivers of mortality via MOS were increased hypoxia and toxicity through feeding and suggested such events would have adverse consequences for the benthic-pelagic environment and food web interactions. Further studies confirmed a reduction in survivability in amphipods and gastropod organisms, although showing these declines to be dose-dependent on oil concentration. They also observed mortality to be trait dependent as highly motile

organisms had the potential to escape low oxygen conditions caused by MOS and MOSSFA events (Van Eenennaam *et al*., 2019). However, the oil used, even though slightly weathered, would not have been representative of what would be deposited to the seafloor, which would be severely degraded and weathered lacking a majority of the more volatile and readily available components of fresh oil such as BTEX compounds; benzene, toluene, ethylbenzene, xylene and lower weight Polyaromatic hydrocarbons such as naphthalene (Stout and Payne, 2016). During a more recent investigation (Rohal *et al*., 2020), mesocosms were used again to assess the impact of oil residue transported via MOS on the meiofaunal abundance of nematode and copepod organisms under different oil concentrations (3 and $10mg^{-2}$, realistically simulate low and high concs of oil *in-situ*). Meiofaunal abundance was found to be 1.7 times higher in the presence of MOS, copepod abundance 24.3 times higher within MOS treated mesocosms and nematode abundance 1.7 times lower in a higher concentration of MOS. These results suggested an enrichment response to the presence of MOS, causing an increase in abundance and decrease in diversity.

The impact of chemical dispersants on MOS formation

Since the Deepwater Horizon event, due to a large amount of marine snow present and the subsequent MOSSFA event, chemical dispersant use has been a significant topic in its potential involvement in MOS formation with debates as to whether it inhibits or enhances the rate of formation. Chemical dispersants alter the size distribution of oil droplets, resulting in smaller droplets in larger quantities by reducing the interfacial tension between oil and seawater (Lessard and Demarco, 2000; Fu *et al*., 2014). This in turn would increase the collision rate between droplets and other marine particles, therefore, theoretically increasing the rate of MOS formation (Burd *et al*., 2020). However, past studies have shown dispersants to affect

MOS formation in a variety of contradictory ways, causing discrepancies between literature. For example, various studies found the addition of dispersants to stimulate flocculation of MOS (Doyle *et al*., 2018; Bretherton *et al*., 2019). Using a roller bottle, Fu *et al*. (2014) also found the addition of chemical dispersants to greatly promote the formation of MOS by enhancing bacterial growth and EPS contents, resulting in increased flocculation and formation of MOS as big as 1.6 – 2.1mm. Another roller bottle experiment observed much larger, stable MOS aggregates with the addition of dispersant Corexit after four days (Wirth *et al.*, 2018). In Oil + Corexit treatments it appeared most MOS particles were incorporated into one significant larger aggregate as opposed to the control and oil treatments which saw several to hundreds of smaller MOS particles present. However, other studies contradict these observations, suggesting the use of dispersant Corexit9500A inhibits MOS formation. One such roller bottle experiment investigated dispersant impact on MOS formation using both a high and low concentration of dispersant at a dispersant:oil ratio of 1:1 and 1:100 (Passow *et al*., 2016). Observations indicated that MOS formation in the presence of Corexit at low concentration was reduced or completely inhibited compared to treatments without Corexit. At higher concentrations, this investigation observed large aggregates form, mostly of Corexit due to self-aggregation, potentially due to the overexposure of dispersants to the environment. Later studies, using similar roller bottle techniques, assessed particulate organic carbon (POC) as a means of determining aggregate formation against two concentrations of Corexit (10 and 30 µL) (Passow *et al*., 2017). Here the addition of dispersants visibly decreased aggregate abundance and size when compared to treatments without dispersants. 10 and 30 µl Corexit treatments, contained only 8 and 47% POC, a 40-80% decrease in POC that aggregated when compared to treatments without the dispersant. Further

observations however indicated treatments with dispersants resulting in higher incorporations of oil into the aggregates, being 6.43 ± 0.66 mg oil-carbon tank^{-1} as opposed to treatments without dispersants at 0.82 ± 0.05 mg oil-carbon tank^{-1}, concluding that Corexit application resulted in fewer aggregates but mediated a higher rate of sedimentation. Interestingly one study using roller bottles containing a WAF and CWAF treatment found MOS formation to depend on the addition of chemical dispersants as no MOS formation occurred within the WAF treatment (Suja *et al.*, 2019).

Dispersant use can also have indirect effects on MOS formation, although our current understanding of these processes is less clear. It has been observed that dissolved and dispersed hydrocarbons could have the potential of impacting the hydrophobic interactions of natural colloidal organic matter, by altering their effective surface area for hydrophobic binding reactions that impact the potential for aggregate formation (Santschi *et al.*, 2020). However, more research must be done to further assess these interactive processes.

Environmental and experimental considerations

As can be seen from previous literature our understanding of how dispersants may be associated with MOS formation has improved, however, whether the addition of chemical dispersants inhibits or enhances the formation of MOS is still somewhat inconclusive. This is most likely due to studies investigating this not being comparable with one another and *in-situ conditions*, using a variety of methodologies, different dispersants/oil and different laboratory conditions, causing a variety of discrepancies. One of the main focuses of these discrepancies is the difference in methodology between roller bottle and WAF (Water accommodated fractions) experiments. Many of the previously mentioned experiments used WAF to some degree, most likely as a

means of avoiding oil adhering to the walls and lids of roller bottle experiments. Although this can be avoided through pre - treatments of glassware, slower roller bottle rotation and the use of hydrophobic seals (Brakstad *et al*., 2015; Prince, 2015; Netzer *et al*., 2018; Henry *et al*., 2021). Originally this method was developed as a toxicological method to assess true toxicity from physical smothering (Gregson *et al*., 2021). The oil is directly stirred via a magnetic stirrer into the water for a set amount of time. It is then allowed to settle, allowing the particles to rise to the surface before the WAF is removed. Whilst being a good method for preparing samples for toxicity tests, WAF and CEWAF have been argued to not be suitable for the use in biodegradation studies. The composition of hydrocarbons varies dependent on the geometry of the container, the energy implemented by the stirrer, the stirring/settling time and the oil:water:air ratio during formation. The oil:water:air ratio can determine the difference between volatility and solubility of hydrocarbon components within the WAF and the amount of oil used can impact the overall concentration of soluble compounds, thus the chemical composition cannot be readily predicted even between WAF of a similar oil concentration (Prince *et* al., 2016a). In comparison to roller bottle experiments using an oil-in-water dispersion approach, the chemical composition within the container is readily known. The dispersion effect is also produced much more naturally creating variable droplet sizes dependent on energy generating the dispersion. Another particular issue with the WAF method is that CEWAF contains a much higher hydrocarbon concentration per volume than WAF, thus without dilution to an equivalent concentration it would be impossible to determine any effect the presence of dispersant may have over that of the presence of a higher concentration of oil. Therefore, when performing *ex-situ* MOS formation experiments a roller bottle methodology can be recommended.

As well as not being comparable with one another, a major issue facing these studies is that they are far removed from *in-situ* conditions found at sea and the conditions of a real-life oil spill scenario. Particularly there are discrepancies within the concentration of oil and dispersants used. As stated by Brakstad *et al*. (2018) many of these papers providing insight into the impact of dispersant used substantially high concentrations at a wide range of 600 mg/L – 10,000 mg/L (0.06 - 1%), not taking into consideration the experimental conditions of their investigation. During a spill event, dispersant use is recommended at a dispersant to oil ratio (DOR) of 1:20, however studies have either investigated at higher (1:1, 1:10; 1:17) or lower (1:30; 1:100) DOR, making them unsuitable for comparison to in-situ spill events (Passow *et al*., 2016, 2017; Wirth *et al*., 2018). Although, the reasoning behind these varied dispersant concentrations may be that dispersant use during oil spill response is not always accurate at a ratio of 1:20, therefore, leaving room for some error. This may also be caused by studies following different recommended dosage procedures.

Throughout the literature environmental factors such as light intensity and temperature are all but ignored. When MOS forms, EPS is secreted as a microbial defence mechanism against harmful/stressful conditions. Sun *et al*. (2018) found that in the presence of oil, natural sunlight stimulated EPS secretion, along with the formation of larger aggregates. As well as this, photooxidation of the oil via sunlight can change its chemical composition enhancing hydrocarbon solubility, dispersibility and its toxicity influencing microbial communities within MOS which may directly impact MOS formation and structure (Wang and Fingas, 2003; Shankar *et al*., 2015; Daly *et al*., 2016). During previous studies, experiments have been conducted in either no light conditions (Ziervogel *et* al., 2012; Passow *et al*., 2017; Wirth *et al*., 2018) or ambient laboratory light conditions (Fu *et al*., 2014). One particular study did consider

a 12:12 h light:dark cycle, however a gradual change in light intensity was not implemented during the 24 hours and was measured at <30 µmol $m^{-2}s^{-1}$ on average (Passow *et al*., 2016). Another study implemented a 12:12 hr light:dark cycle at <50 µmol $m^{-2}s^{-1}$ (Doyle *et al*., 2018). These measurements are not comparable to *in-situ* conditions For example, across the North Sea, *in-situ* light conditions vary between a maximum of 2,590.11 µmol m^{-2} s^{-1} and a minimum of 17.76 µmol $m^{-2}s^{-1}$ dependent on the time of year of the spill (Dring *et al.*, 2001). Overall a spectrum of natural light conditions should be taken into account as light intensity and photoperiod can also influence phototrophic communities and the metabolite they produce, aswell as the photooxidation of crude oil, impacting heterotrophic communities, which have been shown to influence MOS formation and structure (Bacosa *et al*., 2015b).

Catering experimental design to better emulate *in-situ conditions* of surface water spills (e.g. taking into account light intensity, temperature, wave action and dispersant/oil concentrations) is paramount to better understanding the impact of dispersant use on processes such as biodegradation and environmental processes such as marine snow formation and their associated microbial communities as this will better informing response teams when using such remediation tools.

Overall aim and objectives

A vast array of knowledge is currently available associated with marine oil pollution, its impacts on the natural environment, microbial communities associated with oil pollution and the remediation tools used. However, the effectiveness of chemical dispersants in mitigating marine oil pollution as a remediation tool is still heavily debated, partly due to the difficulty of extrapolating past results to natural in-situ conditions. Therefore, the main aim of this thesis is to investigate the effectiveness of chemical dispersants in mitigating the impacts of marine oil pollution under relevant

conditions, which simulate the natural environment. In turn, this will aim to provide further guidance to oil-spill response teams and improve on established oil-remediation protocols to better protect the marine environment from oil pollution. As MOS formation has been linked to the use of chemical dispersants and attributed to the transport of oil constituents to deeper benthic environments. Concerns are that this may cause unseen impacts to these fragile ecosystems. Therefore, there will be a focus on MOS formation associated with chemical dispersants. As previous studies tend to focus on conditions similar to the Gulf of Mexico, this investigation aims to simulate North Sea conditions across a seasonal spectrum. Due to the large presence of oil exploration and removal, this area can be considered a priority in understanding the impacts of chemical dispersant use under these conditions. An assessment of the biodegradation rates of oil within MOS will also be undertaken, alongside an assessment of the microbial communities within MOS in comparison to the surrounding seawater, to better understand the biodegradation processes that occur when MOS forms and. Extensive analysis of microbial communities associated with oil spills will also contribute to improving post-monitoring models such as the ecological index of hydrocarbon exposure via microbial biomonitoring, by assessing the functionality of microbial consortia when exposed to hydrocarbon contamination.

Chapter Two

The effect of crude oil concentration on hydrocarbon degradation and associated bacterial communities under experimental conditions

Abstract

Chemical dispersants are an important tool for mitigating the environmental impact of marine oil pollution, yet current studies show contrasting results on their efficiency causing considerable debate on their use. Crude oil concentration used in experiments to study the efficacy of dispersants have been shown to significantly impact oil biodegradation rates, which may explain contradictory conclusions between studies, potentially due to oversaturation of hydrocarbons being introduced and the introduction of increased toxicity from intermediate degradation products over time causing an inhibiting factor for bacterial growth of hydrocarbon degraders. Using high crude oil concentration could be particularly problematic when extrapolating laboratory results to the field, as dispersed oil in the open sea would rapidly dilute to sub-ppm concentrations. This study aimed to quantify the impact of crude oil concentrations ranging from 1 to 1000 mg L^{-1} on biodegradation and microbial community composition in dispersed versus non-dispersed crude oil. Biodegradation rates increased linearly as crude oil concentrations within microcosms increased from 1 to 100 mg L^{-1} but plateaued at a concentration of 1000 mg L^{-1}. However, with the addition of chemical dispersant, biodegradation rates continued to increase linearly at concentrations of 1000 mg L^{-1} and rates were significantly greater than non-dispersed concentrations of 1 mg L^{-1} (81% increase), 10 mg L^{-1} (80%), 100 mg L^{-1} (64%) and 1000 mg L^{-1} (388%). Based on 16S rRNA gene sequence analysis, *Alteromonadaceae* and *Cellulophaga* were more dominant at lower oil concentrations, potentially being inhibited or outcompeted at higher concentrations. Even so, the use of dispersants resulted in

more rapid successional changes in community composition brought on by increased degradation rates. These results further our understanding of the importance of initial oil concentrations during laboratory experiments, having a significant impact on and the abundance of key hydrocarbon degraders and bacterial successional changes, causing a significant decline in degradation at initial concentrations of 1000 mg L-1. Therefore standardising the initial concentration of crude oil use will remove the contradictions that we have seen within prior literature thus far.

Introduction

With the rise in transport and production of crude oil, remediation tools to combat marine oil pollution are still needed. Chemical dispersants are valuable for oil-spill response as they disperse oil slicks into small droplets in the water column, enhancing biodegradation (Prince *et al*.,2013; Mu *et al*., 2014), and often prevent oil from reaching sensitive shorelines (Chapman *et al*., 2007; Prince, 2015). However, the efficacy of dispersants has come into question (Kleindienst *et al*., 2015a; Kleindienst *et al*., 2016; Prince *et al*., 2016a), and previous studies have documented contrasting results, either showing significant increases in oil biodegradation (Baelum *et al*., 2012; Techtmann *et al*., 2017; Thomas et al., 2021) or inhibition of biodegradation when chemically dispersed (Hamdan and Fulmer, 2011; Kleindienst *et al*., 2015b). These contradictory outcomes are potentially explained by differences in experimental design, notably the oil and/or nutrient concentrations (Techtmann *et al*., 2017; McFarlin and Prince, 2021). The use of water accommodated fractions (WAF) and chemically enhanced water accommodated fractions (CEWAF) in experiments (Kleindienst *et al*., 2015b), may also be unsuitable for biodegradation studies, as they remove most oil droplets and insoluble hydrocarbons, which are a major fraction of dispersed oil that can be biodegraded by microbes (McFarlin and Prince, 2021).

Therefore, adding crude oil directly to seawater microcosms, then dispersing with chemical dispersant at the industry standard oil:dispersant ratio (20:1), and directly comparing to non-dispersed floating oil, is a more valid comparison for extrapolation to open water spill scenarios.

Oil concentration also has a significant impact on the biodegradation rates of hydrocarbons and can significantly increase the half-life of various hydrocarbons at higher concentrations, particularly as nutrient limitation becomes more likely, coupled with reduced bioavailability as a consequence of oil forming larger droplets, adhering to the container, or floating to the surface (Prince *et al*, 2017, McFarlin and Prince, 2021). When oil is spilt and chemically dispersed at sea, its concentration can rapidly decrease to approximately 100 mg L^{-1}, with further dilution occurring over 24 hours to approximately 1-10 mg L^{-1} (Lee *et al*., 2013). Therefore, studies using high concentrations (e.g. 200–2500 mg L^{-1}), may be far less relevant to field scenarios. The present study compared biodegradation of crude oil with and without chemical dispersants over a range of oil concentrations (1, 10, 100 and 1000 mg L^{-1}) to test the effects that oil concentration has on both the biodegradation of hydrocarbons and the composition of bacterial communities over time. It was hypothesised that increasing the initial concentration of crude oil would inhibit biodegradation but that the use of chemical dispersants would increase overall degradation rates.

Experimental design

Microcosm design

Surface seawater was sampled from the North Sea (7ºC, 51.7397° N, 1.0221° E on March 3, 2022). Microcosms were created in sterile 40 ml glass vials with PTFE-lined silicon septa, containing 20 ml of seawater (naturally containing 46 µmol L^{-1} nitrate and 1 µmol L^{-1} phosphate), and were sampled over a time series of 0, 1, 3, 6

and 14 days (*n* = 3). Norwegian Geochemical Standard North Sea Oil (NGS NSO-1, previously weathered at 60^{o}C over 24 hours) was added at initial concentrations of 1, 10, 100 or 1000 mg L^{-1} (0.0001% v/v to 0.1% v/v) with or without Slickgone NS dispersant (DASIC International Ltd) at a 20:1 oil:dispersant ratio. Killed controls using mercurous chloride (1mM) (Supplementary material: Figure S1) were set up to determine any abiotic loss of hydrocarbons. Microcosms were incubated in the dark at 12^{o}C on an orbital shaker at 100 rpm.

Hydrocarbon degradation analysis

Hydrocarbons were extracted from the microcosms using previously described methods (Coulon *et al*., 2007). Deuterated alkanes and polyaromatic hydrocarbon (PAH) internal standards were added to the solvent prior to extraction at 0.004, 0.04, 0.4 and 4 µg mL^{-1}, for extraction of the 1, 10, 100 and 1000 mg L^{-1} treatments, respectively. Extractions of 1000 mg L^{-1} treatments were diluted 10-fold in hexane:DCM 1:1, whilst 1 and 10 mg L^{-1} samples were concentrated 100 and 10-fold respectively in a TurboVap evaporator (Biotage), standardising all extracts to 100 mg L^{-1} treatment equivalents with final deuterated standard concentrations of 0.4 µg mL^{-1}. Quantification of hydrocarbons (Alkanes C_{10-38}, Pristane and phytane, and a suite of two-six ring PAHs) was performed using a Nexis GC-2030/CMS-TQ8040 NX GC-QqQ-MS/MS (Shimadzu). External standard calibrations were produced with Alkane (C_8-C_{40}) and PAH (QTM PAH Mix) (Sigma) standard mixes over a 1–5 µg mL^{-1} range.

DNA Extraction and qPCR

Microcosms were filtered via 0.22 µm Sterivex filters (Millipore) and DNA extracted using a DNeasy PowerWater Kit (Qiagen), following the manufacturer's instructions. Filtrate was reserved for nutrient analysis. qPCR of the bacterial 16S rRNA gene was performed, acting as a proxy for monitoring bacterial growth within

treatments, using primers 341F: 5'CCTACGGGNGGCWGCAG3' and 805R: 5'GACTACHVGGGTATCTAATCC3' (Klindworth *et al*., 2013) on a CFX Opus 384 (Bio-Rad) as previously described (McKew and Smith, 2017).

Nutrient Analysis

NH_4^+, NO_3^- and PO_4^{3-} concentrations were measured as previously described by Thomas *et al*., (2021).

16S rRNA gene amplicon library preparation and bioinformatics

Amplicon library sequencing preparation was performed via a 30 – cycle PCR using the Veriti 96 well thermal cycler, (Applied biosystems) following the 16S Metagenomic Sequencing Library preparation protocol (Illumina, 2013). PCR primers used were the same as the qPCR primers, with the addition of Illumina adapter overhang nucleotide sequences. PCR products were then cleaned using AMPure XP beads (Beckman Coulter). Using the Nextera XT Index kit (Illumina) a unique combination of index primers were added to the PCR products from each sample via an 8-cycle PCR. A second clean-up of the library PCR product was performed, again using AMPure XP beads. At the end of each step gel electrophoresis was performed for quality control before each subsequent step. Following this, amplicons were quantified using the Quant-T PicoGreen dsDNA Assay kit protocol using the NanoDrop 3300 Flourospectrometer (Thermo Scientific), 16S rRNA gene quantification was performed on all index PCR products. and pooled together at equimolar concentrations. The final pooled library was then quantified using both the Quant-IT PicoGreen dsDNA Assay kit and the NEBNext Library Quant kit for illumina (New England Biolabs, UK), prior to sequencing. Sequencing was then provided by Novogene (Cambridge, UK) using Illumina NovaSeq PE250. Sequences have been submitted to the NCBI SRA archive under the accession number PRJNA1220651.

Sequencing output from the NovaSeq PE250 was processed using the Divisive Amplicon Denoising Algorithm (DADA2) (Callahan *et al*., 2022). Following this pipeline, forward and reverse reads were quality checked and trimmed, forward and reserve primer sequences were also removed. Via the core sample inference algorithm, error correction was performed, and chimeric sequences removed (Callahan *et al*., 2016). Forward and reverse reads were then merged to obtain the full sequence per read, these were then placed into an amplicon sequence variant (ASV) table Taxonomic assignment was conducted using the Ribosomal Project (RDP) Classifier (rdp trainset 18/release 11.5) (Callahan, 2020; Wang et al., 2007) and blastN (Altschul *et al*., 1990). Singleton and doubleton ASVs were discarded, along with sequences below 400 base pairs and non-target organisms (Archaea and algal chloroplast sequences). Homopolymers above 8 b.p. were also discarded.

Statistical analysis

Prior to diversity and community composition analysis of ASVs, sequence data was rarefied to the lowest read depth (3010). Residuals were tested for normality (Shapiro-Wilk test) and tested for significance using ANOVAs (normally distributed) or generalised linear models (non-normally distributed).

Results

Total measured alkanes (C_{10}-C_{38}, pristane and phytane) in non-dispersed oil at 1000 mg L^{-1} did not significantly decrease in concentration over 14 days, whereas total alkanes in non-dispersed oil at 1, 10 and 100 mg L^{-1} did significantly decrease (25-30% degradation; $t = 7.3\text{-}8.1$, $p < 0.05$) (Figure 2.1A). Chemical dispersion significantly enhanced alkane biodegradation at all treatments of oil (24-71% degradation; $t = 7.0\text{-}18$, $p < 0.05$), over the 14 days, with significantly lower concentrations observed in all dispersed microcosms compared to non-dispersed microcosms ($t = 2.8$, $p < 0.05$).

This was especially noticeable at 1 and 10 mg L^{-1}, where alkanes were significantly more degraded by 41% and 17% respectfully (t = 3.2-6.0, $p < 0.05$). A similar pattern was observed with PAHs, whereby at day 14, a significant decrease in PAH concentration was seen within microcosms treated with non-dispersed oil at 1, 10 and 100 mg L^{-1} (31-59% degradation; t = 3.0-11.3, p < 0.053) (Figure 2.1B) but not at 1000 mg L^{-1} (13% degradation, p = ns). The addition of dispersant resulted in a significant reduction in PAH concentration across all oil treatments (46-70% degradation; t = 5.5-10.2, $p < 0.05$) and by day 14, PAH concentrations were significantly lower within dispersed oil at 1, 100 and 1000 mg L^{-1} when compared to non-dispersed microcosms (t = 2.6-4.5, p < 0.05; 18-33% further degradation). In dispersed microcosms at 1 mg L^{-1}, a significant reduction in PAH concentration was seen as early as day 1 when compared to non-dispersed microcosms (t = 2.69, $p < 0.05$).

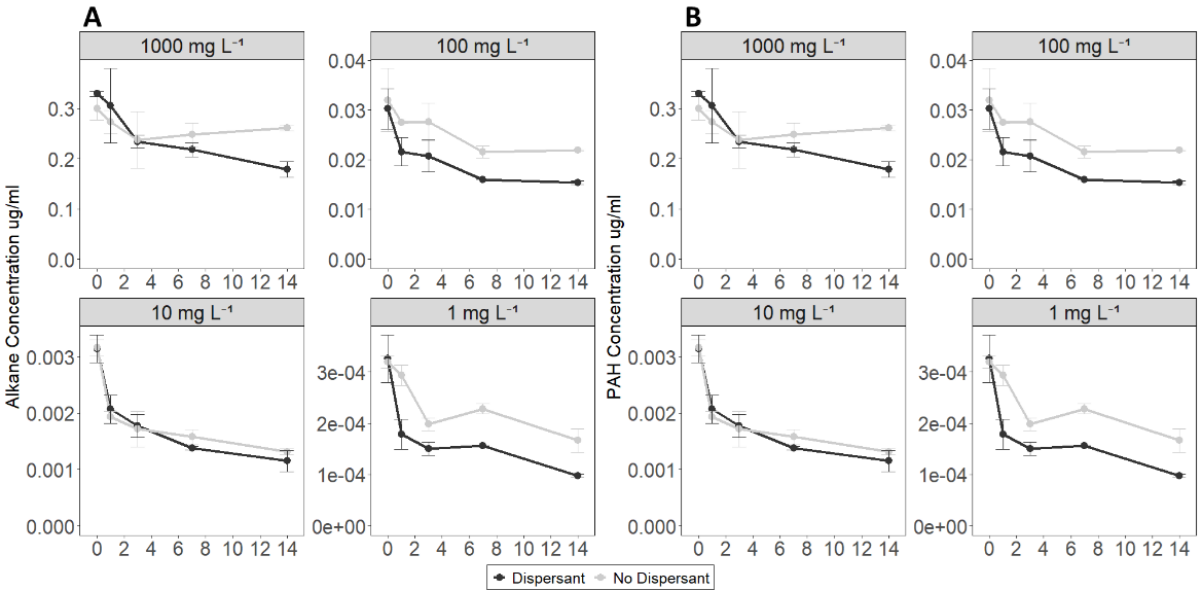


Figure 2.1 (A) Total alkane and (B) polyaromatic hydrocarbon concentration collected from 20 ml microcosms of North Sea seawater collected of the Mersea Coast, Essex, UK, exposed to Norwegian Geochemical Standard North Sea Oil at a range of concentrations (1 – 1000 mg L-1) (NGS NSO-1), with and without the presence of chemical dispersants (Slickgone NS), (± SE, n = 3).

A significantly greater percentage degradation of smaller *n*-Alkanes (C_{11}-C_{20}) occurred within oil concentrations of 1, 10 and 100 mg L^{-1} (dispersed and non-dispersed) when compared to 1000 mg L^{-1} (Figure 2.2). The branched alkanes

pristane and phytane were significantly more degraded in percentage terms in oil treatments of 1 mg L^{-1} (dispersed and non-dispersed) when compared to treatments of 1000 mg L^{-1}. Even larger *n*-alkanes in the size range, C_{35}-C_{38}, were significantly degraded in oil treatments of 1 mg L^{-1} (dispersed and non-dispersed) in contrast to higher concentrations where no significant degradation occurred. A targeted analysis of certain PAH analytes was also caried out to compare degradation across a range of different structures and sizes of PAHs.

Naphthalene, fluorene, phenanthrene, anthracene and pyrene were significantly more degraded in percentage terms in 1 mg L^{-1} treatments, compared to 1000 mg L^{-1} (dispersed and non-dispersed) (Figure 2.2). In 10 mg L^{-1} treatments, naphthalene, fluorene, phenanthrene and pyrene all showed significantly more percentage biodegradation when compared to 100 mg L^{-1} treatments, and naphthalenes and fluorenes had significantly more percentage degradation when compared to 1000 mg L^{-1}. The percentage degradation of larger PAHs (4-rings and above) was much lower across all oil concentrations, however at 1 mg L^{-1} these larger PAH compounds were significantly more degraded when compared to 1000 mg L^{-1}.

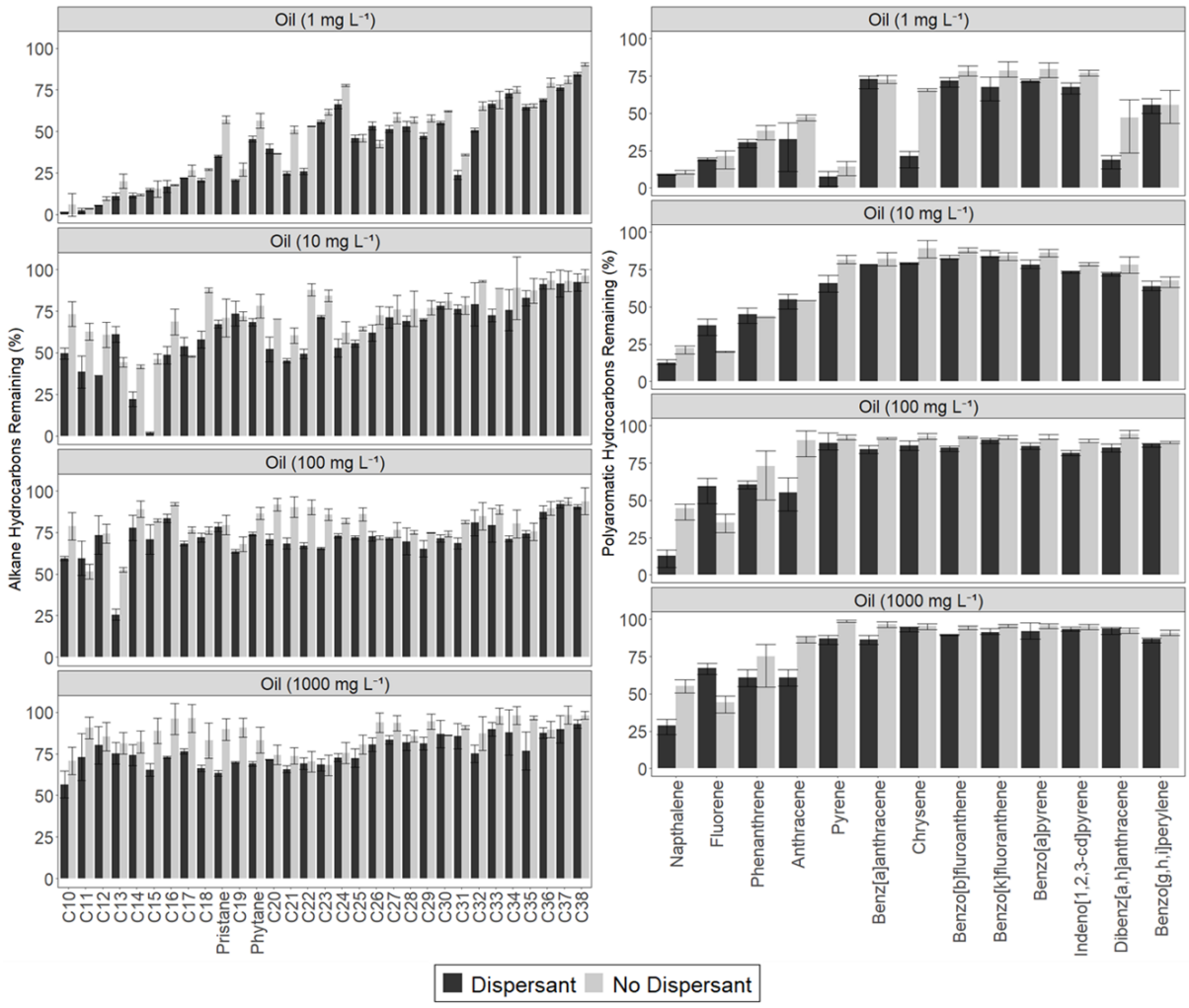


Figure 2.2: Percentage remaining alkane hydrocarbon and polyaromatic hydrocarbon compounds within 20 ml North Sea seawater microcosms, with and without chemical dispersants (Slickgone NS) at varying Norwegian Geochemical Standard North Sea Oil (NGS NSO-1) concentrations (1– 1000 mg L^{-1}) by day 14 (± SE, n = 3).

Whilst experiments with higher initial oil concentrations may show minimal hydrocarbon biodegradation in percentage terms, the actual bulk hydrocarbon content degraded, and rates of hydrocarbon degradation may of course be significantly higher. With an increase in initial concentration, the degradation rates of dispersed oil increased linearly from 1 – 1000 mg L^{-1}, in contrast to non-dispersed oil where degradation rates were only linear within treatments 1 to 100 mg L^{-1}, plateauing at 1000 mg L^{-1} (Figure 2.3). A similar response was also observed with PAH degradation rates.

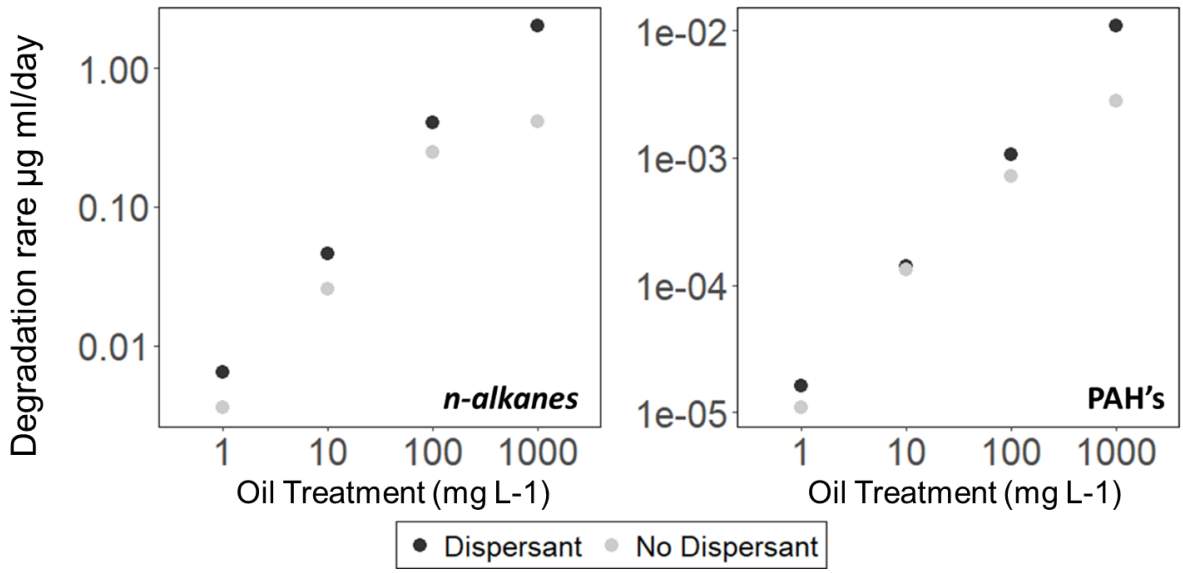


Figure 2.3: Alkane (left) and polyaromatic hydrocarbon (Right) degradation rates in natural North Sea Seawater over 14 days across a range of Norwegian Geochemical Standard North Sea Oil (NGS NSO-1) treatments (1 – 1000 mg L^{-1}) with and without chemical dispersant (Slickgone NS) treatment (± SE, n = 3).

There was no significant increase in the abundance of the bacterial 16S rRNA gene in treatments with or without dispersant between day 0 and 14 (p = 0.07; data not shown). By day 7, there was significant dissimilarity in the community composition between dispersed and non-dispersed microcosms (F = 2.68, p = 0.023). However by day 14, no difference was observed and there was no significant dissimilarity in communities associated with different oil concentrations by day 14 (Figure 2.4).

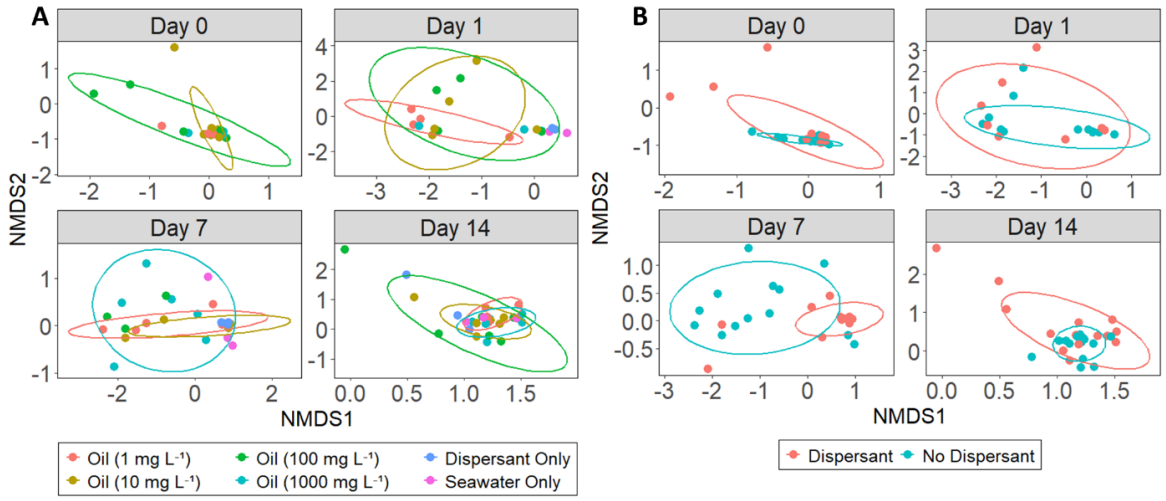


Figure 2.4 nMDS (non-metric multidimensional scaling) ordination using Bray-Curtis dissimilarity (Stress Value = 0.136), based on ASV (amplicon sequence variants) of bacterial 16S rRNA genes at varying concentrations of Norwegian Geochemical Standard North Sea Oil (1 - 1000 mg L^{-1}) with and without dispersants (Slickgone NS) over a period of 14 days, within microcosms using seawater samples collected off the coast of Mersea Island, Essex, UK. A) Community dissimilarity between treatments, B) Community dissimilarity between dispersant use (± SE, n = 3).

Colwellia was the most abundant genus in the seawater (approximately 75% of all reads), the relative abundance significantly declined by day 1 in all oiled treatments except at 1000 mg L^{-1} in the non-dispersed microcosms ($F = 24.575$, $p < 0.001$) and seawater controls until day 7 (Figure 2.5). By day 14, *Pacificibacter*, *Aliiroseovarius* and *Roseovarius* became dominant (approximately 24%, 11% and 9% of the community), significantly increasing in abundance across all treatments ($F = 21.8$-32.4, $p < 0.001$).

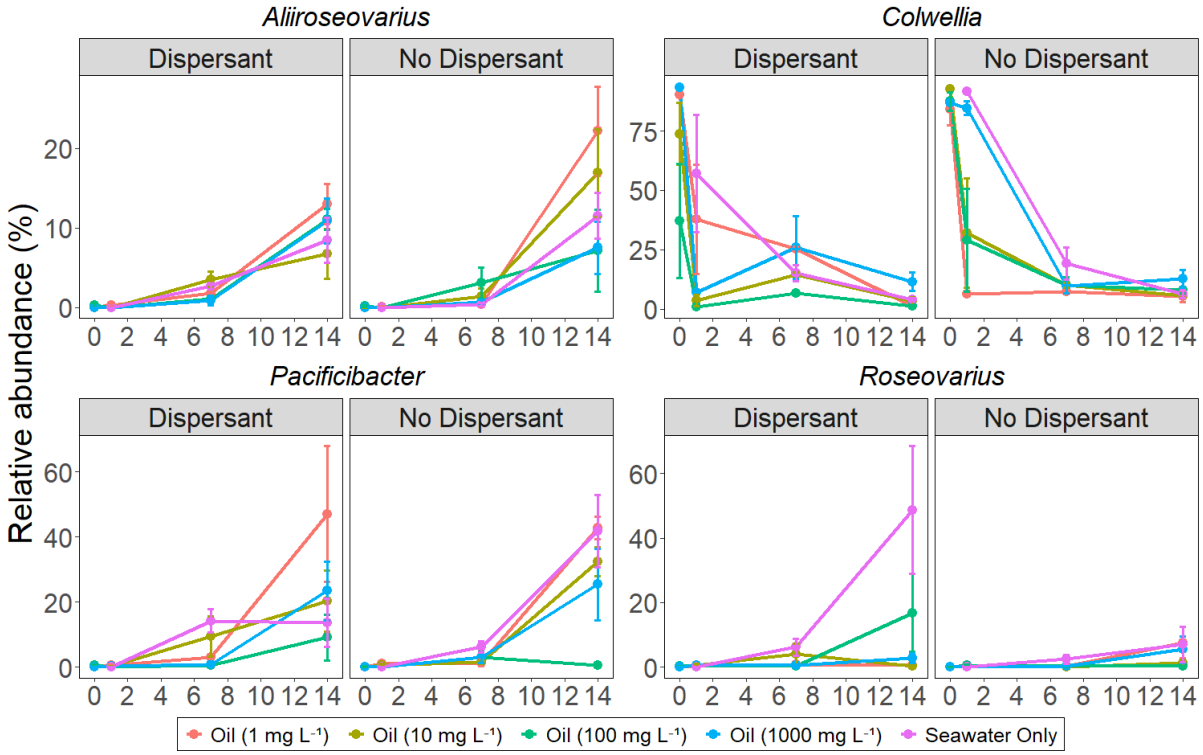


Figure 2.5 Highest relative abundance genera Bacterial 16S rRNA ASVs associated with marine oil pollution at varying concentrations of Norwegian Geochemical Standard North Sea Oil (1 – 1000 mg L^{-1}), with and without chemical dispersant (Slickgone NS). Collected over a 14-day period from microcosms containing natural seawater collected off the Mersea Coast, Essex, UK (± SE, n = 3).

The relative abundance of 16S rRNA genes from known obligate hydrocarbonoclastic bacteria (a group of marine bacteria often associated with oil pollution that are highly specialist hydrocarbon degraders) (Yakimov et al 2007) varied between replicates, and generally small increases were seen in non-dispersed oil treatments compared with dispersed oil. *Alcanivorax* remained at low relative abundance, increasing (non-significantly) by day 14 in the 10 and 100 mg L^{-1} oil

treatments (F = 0.62-0.8 *p* = ns) (Figure 2.6). *Cycloclasticus* significantly increased in relative abundance over time in non-dispersed treatments of 100 mg L^{-1} concentrations (z = 3.3, *p* < 0.001), from undetectable to 4.9% relative abundance. *Thalassolituus* increased in relative abundance by day 14 in 100 and 1000 mg L^{-1} concentrations (z = 0.001-.9, *p* = ns). *Oleibacter* (sequences assigned to *Oleibacter* in this study, represent ASV sequences highly similar to *Oleibacter marinus* strains, that were recently transferred to the genus *Thalassolitus* under the new name *T. maritimus* (Dong et al. 2022)) increased in relative abundance by day 14 in treatments of 100 mg L^{-1} concentration, at 11.9% of the community (z = 3.95, *p* < 0.001).

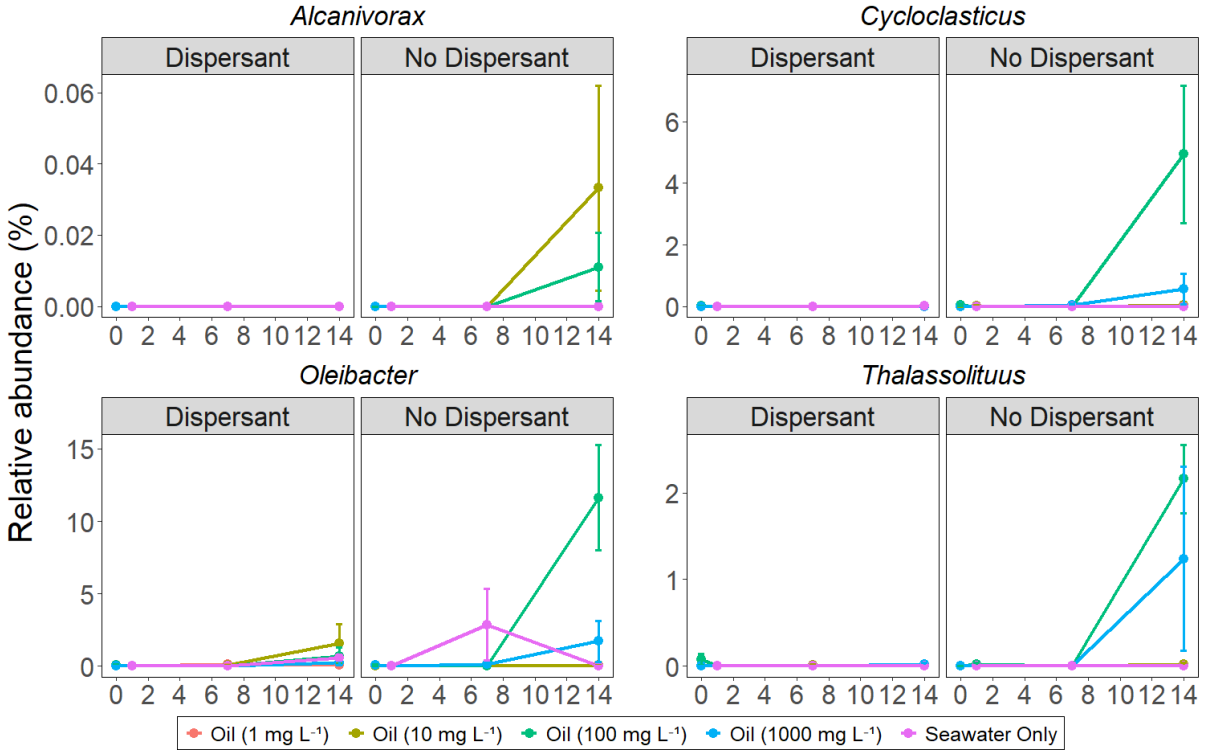


Figure 2.6 Relative abundance of genera that are known hydrocarbon degraders based on Bacterial 16S rRNA ASVs after the addition to seawater of Norwegian Geochemical Standard North Sea Oil (1 – 1000 mg L^{-1}), with and without the presence of chemical dispersant (Slickgone NS). Collected over a 14-day period from microcosms containing natural seawater collected off the Mersea Coast, Essex, UK (± SE, n = 3).

At day 7, the family *Alteromonadaceae* significantly increased in relative abundance in dispersed oil at 1 and 10 mg L^{-1} and dispersant only treatments when compared to 100 and 1000 mg L^{-1} (z = 2.5-3, *p* < 0.05) (Figure 2.7) and by day 14 this family was significantly more abundant in all dispersed oil concentrations (z = 2.68-

5.01, $p < 0.05$). The family *Oceanospirillaceae* significantly increased in relative abundance by day 14 in seawater only and 10 mg L^{-1} treatments ($z = 7.56$, $p < 0.05$). When oil was dispersed, a significant increase in *Oceanospirillaceae* relative abundance was also observed in treatments of 1, 10 and 100 mg L^{-1} ($z = 19.5$-25.2, $p < 0.05$), with no increase in the 1000 mg L^{-1} treatment and seawater controls. *Cellulophaga* had significantly higher relative abundance by day 7 and 14 in microcosm treated with dispersants at 1 and 10 mg L^{-1} oil ($z = 3.6$-4.0, $p < 0.05$) (Figure 7). *Oceanicola* significantly increased in relative abundance across all oil treatments ($p < 0.05$) and particularly with dispersant addition at 1, 10 and 100 mg L^{-1} oil ($z = 2.7$-3.1, $p < 0.05$). *Sulfitobacter* increased in relative abundance over time across all treatments, however this was not significant.

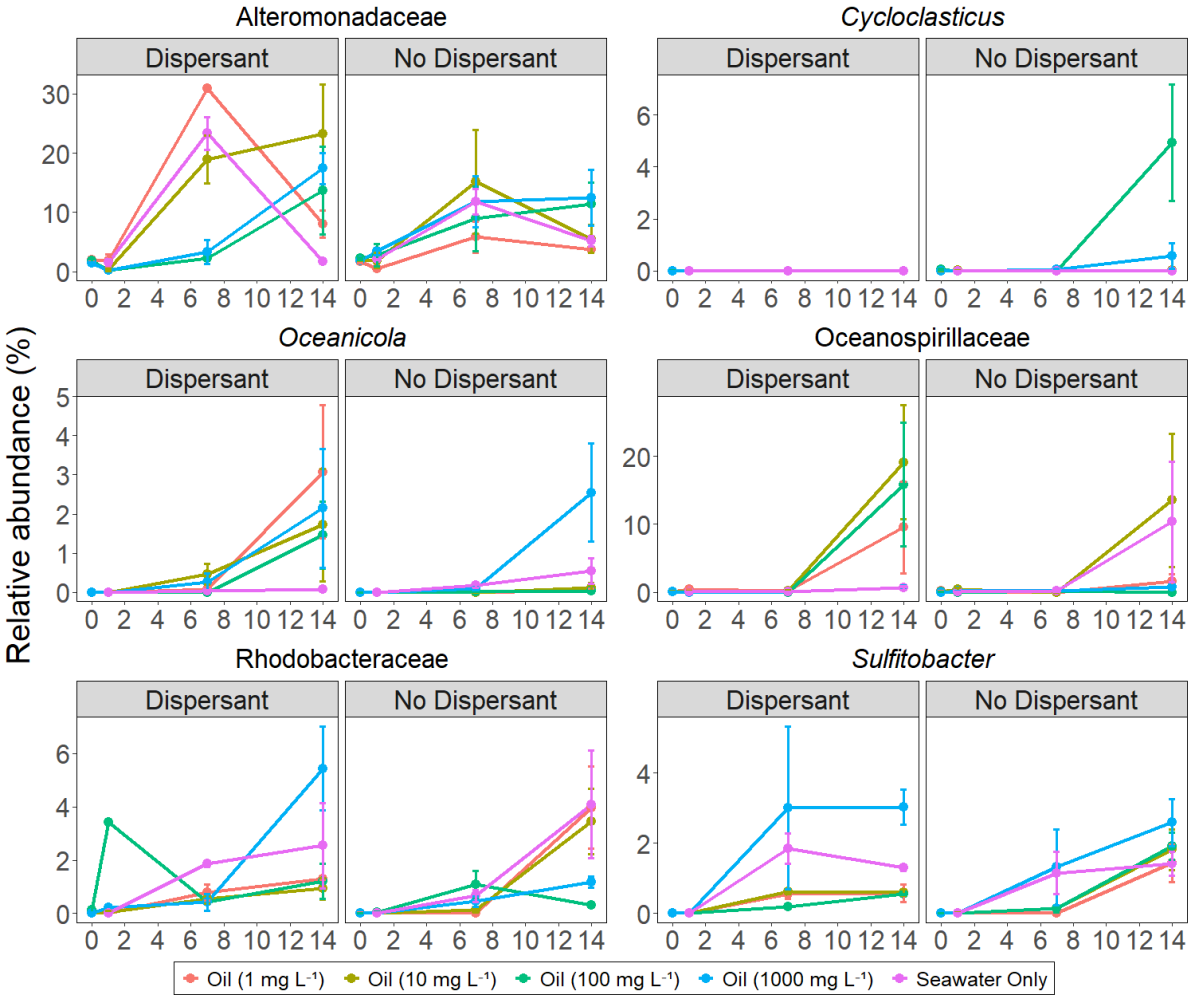


Figure 2.7 Relative abundance of other notable taxa Bacterial 16S rRNA ASVs at varying oil concentrations of Norwegian Geochemical Standard North Sea Oil (1 – 1000 mg L^{-1}), with and without the presence of chemical dispersant (Slickgone NS). Collected over a 14-day period from microcosms containing natural seawater collected off the Mersea Coast, Essex, UK (± SE, n = 3).

Discussion

Initial oil concentration had a clear impact on the proportion of oil degraded over 14 days, with a higher percentage of measured alkanes and PAHs being degraded in the lower concentrations of 1 and 10 mg L^{-1} compared to 100 and 1000 mg L^{-1}. However, when considering the mass of hydrocarbons degraded over time, the degradation rates of both alkanes and PAHs increased linearly from concentrations of 1 to 100 mg L^{-1}, in all treatments. There was clear inhibition of the rate of both alkane and PAH degradation at 1000 mg L^{-1} when oil was not dispersed, but the rate increases remained linear when dispersant was added. Observations made by Prince *et al*. (2017) at concentrations 2.5, 25, 250 and 2500 mg L^{-1} indicated a similar inhibition of degradation rates at the concentrations of 2500 mg L^{-1}, suggesting that oil may inhibit degradation rates at concentrations above 1000 mg L^{-1}, or even lower when oil is not dispersed. Therefore, oil concentration must be accounted for when using closed-system degradation experiments, as measurements in open water slicks and dispersed oil plumes would typically be well below these concentrations (Lee *et al*., 2013). The addition of dispersants had an effect at the earliest sampling point of day 1, increasing the biodegradation of alkanes in initial oil concentrations of 1 and 10 mg L^{-1}, and PAHs in treatments of 1 mg L^{-1} in comparison to the non-dispersed floating oil. By day 14, the addition of dispersant had a positive effect on biodegradation in oil treatments 100 and 1000 mg L^{-1} highlighting the benefits to bacteria when oil is in small droplets with an increased surface area to volume ratio caused by the addition of dispersants and biosurfactants that are produced by hydrocarbon degrading bacteria (McGenity *et al*., 2012; Godfrin *et* al., 2018; McGenity and Laissue, 2023).

Prior studies using concentrations of 100-1000 mg L^{-1} found similar results (Baelum *et al*., 2012, Tremblay *et al*., 2017, Thomas *et al*., 2021). Although biodegradation of dispersed oil at concentrations of 1000 mg L^{-1} showed a delayed response compared to lower concentrations, degradation was still significantly enhanced compared to that of non-dispersed oil. With the addition of dispersant, the concentration of oil entering the seawater as droplets would increase, thus causing this increase in biodegradation rates. This enhancement with dispersant use has also been observed at higher concentrations of 2000 mg L^{-1}, (Zahed *et al*., 2010). However percentage loss of hydrocarbons between dispersed and non-dispersed oil was lower than that of the present study, being 7% (day 45), whereas by day 14 during this study, hydrocarbon loss increased by 41% and 17% when dispersed at 1 and 10 mg L^{-1}, respectively. This may have been due to the higher starting nitrate concentration in the present study. Biodegradation was enhanced by dispersant use to a greater extent at lower oil concentrations of 1 mg L^{-1} than previously observed (Baelum *et al*., 2012).

Bacterial communities need nutrients such as nitrogen (N) and phosphorous (P) to support growth, being fundamental components of biomolecules such as proteins and nucleic acids. Compared to terrestrial environments, open water marine systems are typically N and P limited, especially during a spill event, where the high carbon content of hydrocarbons, significantly raises the C:N:P ratio , and it is well documented that biodegradation of oil is severely inhibited by inorganic nutrient limitations (Vergeynst *et al*., 2018; Imron *et al*., 2020). During this study, initial nutrient concentrations were 50 µM nitrate and 1 µM phosphate (which were the main sources of inorganic N and P in the seawater), and microcosms rapidly became P-limited by day 1 and N-limited by day 14. By day 3 ammonium had become the main inorganic N source, likely released via decomposing organic matter (e.g. from dead algae and

bacteria within the dark-incubated microcosms). The ammonium then rapidly depleted by day 14. Previously, exponential growth of microbial communities was observed in oil contaminated seawater at concentrations of 30 μM (N) and 1.8 μM (P) and above, whilst growth became limited at 5.5 μM (N) and 0.13 μM (P) (Strynar *et al*., 1999). Here, bacterial abundance was consistent over 14 days. This aligns with previous findings as nutrient concentrations were not high enough to support exponential growth, whilst the community composition changed dramatically (Strynar *et al*., 1999). Significant degradation of hydrocarbons occurred across all treatments by day 7 and only ceased once the microcosms became deficient in inorganic N and P. During this study, even at high concentrations of oil (100, 1000 mg L^{-1}) with the addition of dispersants, nutrient concentrations as low as <5 μM (N) and <0.1 μM (P) were capable of facilitating hydrocarbon biodegradation.

Percentage degradation of individual *n*-alkane compounds declined as the chain length increased (*n*-C_{10-18}) within oil treatment 1 mg L^{-1}, in which *n*-C_{10-12} were almost completely degraded (98%, 95% and 88% degradation). This highlights the clear impact hydrocarbon chain length to have an impact on degradation rates (Setti *et al*., 1995). This percentage degradation of *n*-C_{10-18} decreased with an increase in initial oil concentration. There was very little degradation of *n*-C_{35-38} regardless of the initial oil treatment. It has been well documented that the structural property of individual hydrocarbon constituents, determines the degree and rate of degradation, showing an inverse relationship between degradability and molecular weight, with higher complexity and solubility reducing degradability (Setti *et al*., 1993; Mohanty and Mukherji, 2008). The reduction in degradability of higher weight hydrocarbons may coincide with the limiting nutrient resources. Lower molecular weight *n*-alkanes (*n*-C_{11-13}) were significantly more prone to biodegradation than larger *n*-alkanes and with

limiting nutrient resources hydrocarbon-degrading bacteria cannot 'move up' the chain lengths, (as seen at 1 mg L^{-1}) as they run out of N and P resource (Ławniczak *et al*., 2020). This becomes a greater issue in the higher concentrations as C:N and C:P increases 10-fold with each increase in oil concentration. Therefore, at higher oil concentrations, biodegradation is largely limited to a few of the smaller hydrocarbons as there is a greater mass of the more readily degradable fractions. This was also observed in PAHs, with naphthalene being considerably more degraded regardless of oil concentrations, whilst larger PAHs showed little degradation. Similar results were observed by Bacosa *et al*. (2021) who saw biodegradation of individual alkanes decrease with an increased oil concentration in a nutrient limited environment N < 1 µM and P < 0.6 µM by day 12.

Colwellia dominated communities, averaging 79% across all treatments. These generally psychrophilic or psychrotolerant bacteria are ubiquitous in cold marine environments, between -6°C and 19°C (Bauvois *et* al., 2013). Some *Colwellia* species are capable of hydrocarbon degradation, (Methé *et al*., 2005), although *Colwellia psychrerythraea* (26% of *Colwellia* ASVs) is currently not reported to be a hydrocarbon degrading species (Mason *et al*., 2014). The initial dominance of *Colwellia* may be due to the initially nutrient rich, cold waters (7°C). By day 14, *Pacificibacter*, *Roseovarius* and *Aliiroseovarius* became three dominating genera, potentially coinciding with a decrease in nutrient availability, being capable of thriving under the nutrient limiting environment seen by day 6 due to their potential copiotrophic and oligotrophic capacity (Zhao *et al*., 2024). This may further explain the distinct similarities between crude oil treatments and seawater controls, with nutrient availability having the most noticeable impact on community composition in the case of this study (Nõlvak *et al*., 2021). OHCBs *Alcanivorax, Cycloclasticus, Oleibacter* and *Thalassolituus* did not show

exponential growth even in the presence of hydrocarbons. Even so, OHCB's were detectable at higher relative abundances in non – dispersed treatments, notably due to their capacity to produce biosurfactants, increasing hydrocarbon availability naturally (Ron and Rosenberg, 2002). This is in contrast to previous work and real-world spills that saw significant growth in OHCB's (Dubinsky *et al*., 2013; Cappello *et al*., 2007; Thomas *et al*., 2020). However, additional nutrient amendments were added/available in the aforementioned studies influencing microbial growth/succession via biostimulation (Yu *et al*., 2011). Other experiments however, found *Pseudoalteromonas* to significantly increase in relative abundance in North Sea mesocosms when oiled, which may have dominated over OHCBs (Chronopoulou *et al*.,2015). During the present study, *Alteromonadaceae* was dominant at day 7 (average 15%), which was quickly succeeded by *Oceanospirillaceae*, by day 14 (average 7%). These two families have been reported to exponential increase in relative abundance, in the presence of marine oil pollution, namely during the Deepwater Horizon spill (Dubinsky *et al*., 2013; Bacosa *et al*., 2018; Miller *et al*., 2019). The majority of *Alteromonadaceae* sequence reads (ASV 1 making up 98% of the *Alteromonadaceae* reads), were made up of *Glaciecola amylolytica* (99.06% identity) (Xiao *et al*., 2019). Another *Glaciecola* strain (NS 168) (93.91% identity to ASV 1) has been recorded to be capable of hydrocarbon degradation with a preference to alkane degradation (Chronopoulou *et al*., 2015). However, current knowledge of *Glaciecola amylolytica* has yet to address the specifics of their degradation potential. The majority of unassigned *Oceanospirillaceae* (99%), was made up of *Thalassolituus* ASVs (95.6-99.7% identity), suggesting their involvement in aliphatic hydrocarbon degradation between day 7 and 14. *Cellulophaga* increased in relative abundance at day 7 in oil concentrations of 1 and 10 mg L^{-1} when dispersed. Previously shown to be present

around contaminated sites, they are prolific producers of biosurfactants, thus facilitating the availability of hydrocarbons within contaminated communities (Chhatre *et al*., 1996; Rizzo *et al*., 2013).

Overall different oil concentrations (1, 10, 100 and 1000 mg L^{-1}) and the addition of dispersants did not have a major impact to bacterial community composition. The subtle differences in communities are likely related to a combination of factors, such as the susceptibility of certain bacteria to increasing hydrocarbon concentrations, nutrient availability stalling successional changes at higher concentrations, and the changing profile of available hydrocarbons. Similar results were noted in Jung *et al*. (2010), in which impacts to heterotrophic bacterial communities were only noted at concentrations over 1000 mg L^{-1} (sublethal levels). This suggests that initial oil concentrations introduced to a system will not have a major impact to bacterial communities below "sublethal levels". Even so, inhibitory effects were noted in *Alteromonadaceae* and *Cellulophaga* at day 7 and *Oceanospirillaceae* at day 14, which exhibited high relative abundances at concentrations of 1 and 10 mg L^{-1} and no noticeable presence at concentrations of 1000 mg L^{-1} when dispersed. It has previously been shown that the production of intermediate metabolic products during biodegradation in high concentrations can potentially inhibit the growth of hydrocarbon degraders such as *Alcanivorax* and *Polaromonas naphthalenivorans* (Pumphrey and Madsen, 2007; Bacosa *et al*., 2021) with the possibility of inhibiting other genera. *Alteromonadaceae and Oceanospirillaceae* increased in the presence of dispersants. *Alteromonadaceae* being relatively more abundant at day 7 at concentrations of 1 and 10 mg L^{-1}, whereas *Oceanospirillaceae* increased in relative abundance at concentrations 1, 10 and 100 mg L^{-1} by day 14. Previous work has also noted similar patterns in both *Alteromonadaceae* and *Oceanospirillaceae* in relative abundance with

dispersant use by day 15 (Bacosa *et al*., 2018). This may also indicate a delayed response in bacterial succession associated with initial oil concentrations, as by day 14, the relative abundance of *Alteromonadaceae* at concentrations of 100 and 1000 mg L^{-1} increased, whilst in contrast they had already increased in relative abundance at concentrations of 1 and 10 mg L^{-1} by day 7. Microbial community succession has been shown to be influenced by dispersants, as availability of oil increases, the rate of biodegradation increases, thus the rate of succession increases (Baelum *et al*., 2012; Thomas *et al*., 2021). However, initial concentrations of oil within a system may also influence succession due to potential inhibitory effects of higher hydrocarbon concentrations (Bacosa *et al*., 2021).

Conclusion

Higher oil concentrations were shown to inhibit the growth of potential hydrocarbon degrading bacteria, which slowed bacterial succession. This in turn inhibited biodegradation at higher concentrations of oil and the efficiency of dispersant use on the enhancement of hydrocarbon degradation. Even so significant degradation occurred across all oil concentrations. This study also highlights that significant degradation is still possible at *in-situ* open water nutrient concentrations, even at higher oil concentrations with the addition of dispersants, although this does become limiting as the system becomes N and P deficient. This is noted as a limitation of this and many closed system studies. Overall initial oil concentrations should be considered when performing closed system biodegradation experiments. Concentrations of 10 mg L^{-1} and lower are recommended to accurately depict concentrations found *in-situ* when oil is dispersed. Future experiments should consider simulating dilution of oil over time to more closely mimic what happens to dispersed oil at sea, where the concentration would continue to dilute over time with the daily

addition of further artificial seawater and nutrients to the microcosm, with each oil droplet, a hotspot of bacterial hydrocarbon-degrading activity, being exposed to further nutrient enrichment as they move through the water column.

Supplementary Material

Table S1: Experimental design of 18 treatments, including concentrations and measurements of seawater, crude oil, Dispersant and kill control (mercurous chloride)

Treatment	Seawater	Oil (NOS-1)	Dispersant (Slickgone)	Hg_2Cl_2
Seawater only (No oil control)	20ml	-	-	-
Dispersant only (No oil control)	20ml	-	-	-
Oil conc 1	20ml	1000ppm (0.1% v/v) 20ul	0.005% v/v 1ul	-
Oil conc 2	20ml	100ppm (0.01% v/v) 2ul	0.0005% v/v 0.1ul	-
Oil conc 3	20ml	10ppm (0.001% v/v) 0.2ul	0.00005% v/v 0.01ul	-
Oil conc 4	20ml	1ppm (0.0001% v/v) 0.02ul	0.000005% v/v 0.001ul	-
Oil conc 1 + Dispersant	20ml	1000ppm (0.1% v/v) 20ul	0.005% v/v 1ul	-
Oil conc 2 + Dispersant	20ml	100ppm (0.01% v/v) 2ul	0.0005% v/v 0.1ul	-
Oil conc 3 + Dispersant	20ml	10ppm (0.001% v/v) 0.2ul	0.00005% v/v 0.01ul	-
Oil conc 4 + Dispersant	20ml	1ppm (0.0001% v/v) 0.02ul	0.000005% v/v 0.001ul	-
Killed control + Oil conc 1	20ml	1000ppm (0.1% v/v) 20ul	-	1mM
Killed control + Oil conc 2	20ml	100ppm (0.01% v/v) 2ul	-	1mM
Killed control + Oil conc 3	20ml	10ppm (0.001% v/v) 0.2ul	-	1mM
Killed control + Oil conc 4	20ml	1ppm (0.0001% v/v) 0.02ul	-	1mM
Killed control + Oil conc 1 + Dispersant	20ml	1000ppm (0.1% v/v) 20ul	0.005% v/v 1ul	1mM
Killed control + Oil conc 2 + Dispersant	20ml	100ppm (0.01% v/v) 2ul	0.0005% v/v 0.1ul	1mM
Killed control + Oil conc 3 + Dispersant	20ml	10ppm (0.001% v/v) 0.2ul	0.00005% v/v 0.01ul	1mM
Killed control + Oil conc 4 + Dispersant	20ml	1ppm (0.0001% v/v) 0.02ul	0.000005% v/v 0.001ul	1Mm

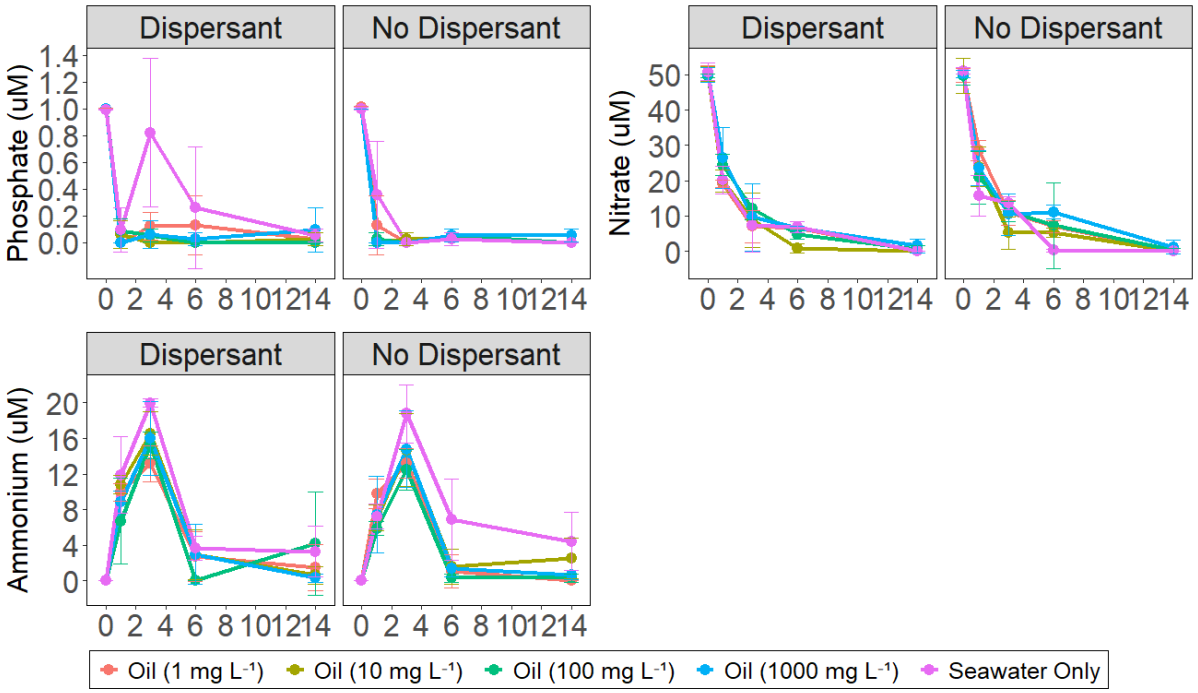


Figure S1: Nutrient concentrations of 20ml seawater microcosms with a range of oil concentrations (1000 – 1ppm), with and without the presence of chemical dispersants (mean ± SE, n = 3) over a 14-day period. Seawater collected off the Mersea coast, Essex, UK.

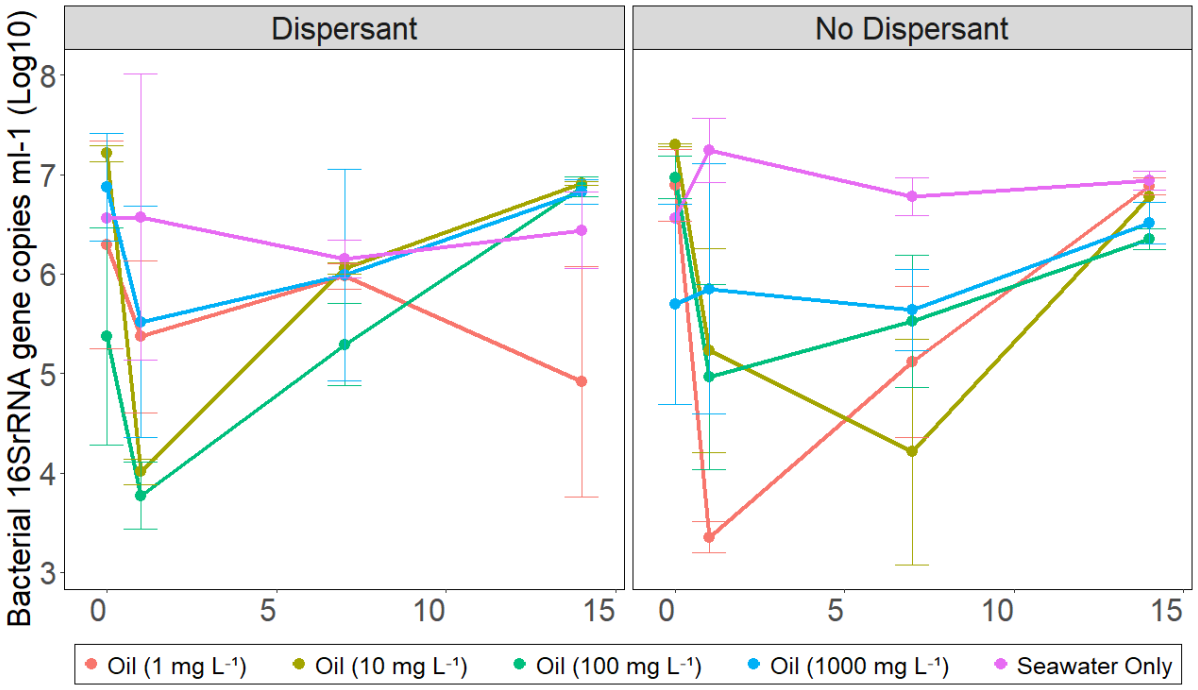


Figure S2: Bacterial 16S rRNA gene abundance, within treatments of varying oil concentration (1000-1ppm), with and without the presence of dispersants (mean ± SE, n=3) over a 14-day period from microcosms containing natural seawater collected off the Mersea Coast, Essex, UK.

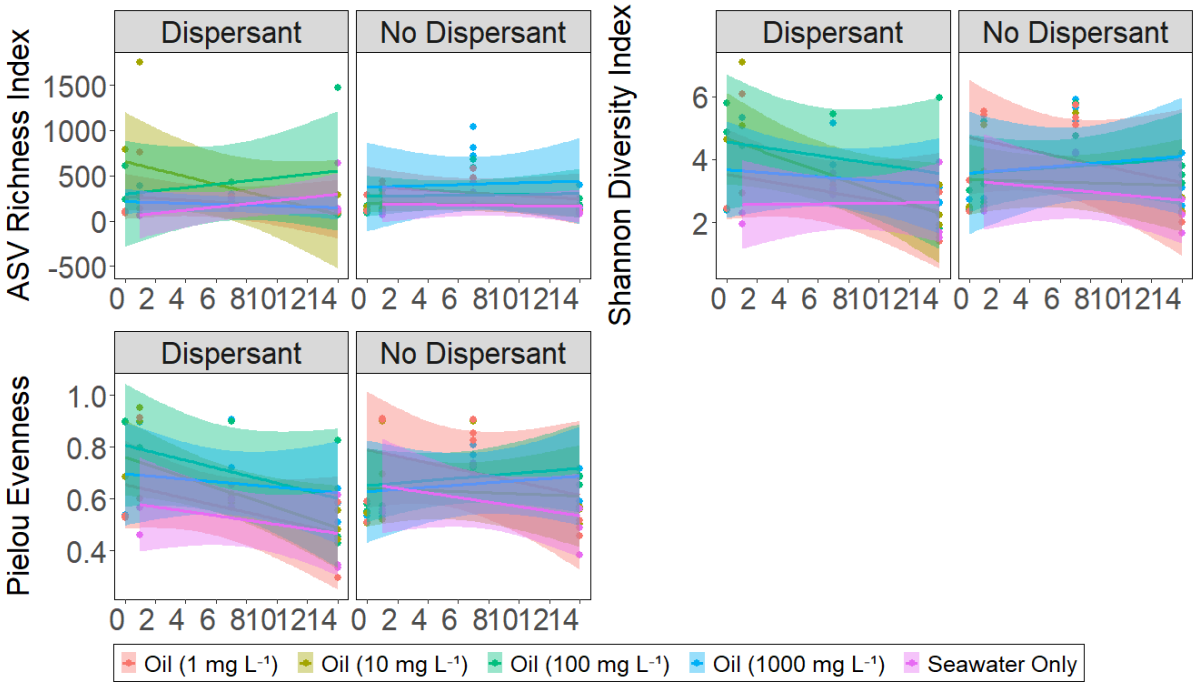


Figure S3: Diversity indices (ASV richness, Shannon index and Simpsons index) of microbial communities at varying oil concentrations (1000 – 1 ppm), with and without the presence of chemical dispersants over a 14 day period from seawater samples collected off the Mersea Coast, Essex, UK

Table S2: GC/MS compound table of Norwegian Geochemical Standard North Sea Oil (NGS NSO-1) n-alkane and polyaromatic hydrocarbons compounds and internal standards, with retention time and molecular weight.

Target Compound	Carbon Number	Molecular Weight (g/mol)	Quantitative ion (m/z)	Qualifier ion (m/z)	Internal Standard
Alkanes					
C10	10	142	57	71	Decane d-22
C11	11	156	57	71	Decane d-22
C12	12	170	57	71	Decane d-22
C13	13	184	57	71	Decane d-22
C14	14	198	57	71	Decane d-22
C15	15	212	57	71	Decane d-22
C16	16	226	57	71	Decane d-22
C17	17	240	57	71	Decane d-22
Pristane	19	268	71	71	Decane d-22
C18	18	254	57	71	Decane d-22
Phytane	20	282	71	71	Decane d-22
C19	19	268	57	71	Nonadecane d-40
C20	20	282	57	71	Nonadecane d-40
C21	21	296	57	71	Nonadecane d-40
C22	22	310	57	71	Nonadecane d-40
C23	23	324	57	71	Nonadecane d-40
C24	24	338	57	71	Nonadecane d-40
C25	25	352	57	71	Nonadecane d-40
C26	26	366	57	71	Nonadecane d-40
C27	27	380	57	71	Nonadecane d-40
C28	28	394	57	71	Nonadecane d-40
C29	29	408	57	71	Nonadecane d-40
C30	30	422	57	71	Triacontane d-62
C31	31	436	57	71	Triacontane d-62
C32	32	450	57	71	Triacontane d-62
C33	33	464	57	71	Triacontane d-62
C34	34	478	57	71	Triacontane d-62
C35	35	492	57	71	Triacontane d-62
C36	36	506	57	71	Triacontane d-62
C37	37	520	57	71	Triacontane d-62
C38	38	534	57	71	Triacontane d-62

Table S2: Continued

Target Compound	Ring Number	Molecular Weight (g/mol)	Quantitative ion (m/z)	Qualifier ion (m/z)	Internal Standard
PAHs					
Naphthalene	2	128	128	127	Naphthalene d-8
Fluorene	3	166	166	165	Naphthalene d-8
Phenanthrene	3	178	178	176	Naphthalene d-8
Anthracene	3	192	178	176	Anthracene d-10
Pyrene	4	202	202	200	Anthracene d-10
Benz[a]Anthracene	4	228	228	226	Anthracene d-10
Chrysene	4	228	228	226	Chrysene d-12
Benzo[b]fluoranthene	5	252	252	250	Chrysene d-12
Benzo[k]fluoranthene	5	252	252	250	Chrysene d-12
Benzo[a]pyrene	5	252	252	250	Chrysene d-12
Indeno[1,2,3-cd]pyrene	6	276	276	277	Perylene d-12
Dibenz[a,h]anthracene	6	278	276	279	Perylene d-12
Benzo[g,h,i]perylene	6	276	276	277	Perylene d-12
Internal Standards					
Decane d-22			66	50	
Nonadecane d-40			66	82	
Triacontane d-62			66	82	
Naphthalene d-8			136	134	
Anthracene d-10			188	184	
Chrysene d-12			240	236	
Perylene d-12			264	260	

Chapter Three

Marine oil snow formation in response to light and chemical dispersant use under *in-situ* North Sea conditions

Abstract

The impact of chemical dispersant use on marine oil snow (MOS) formation has been a significant point of debate over the past decade. Much of our knowledge relates to subsurface oil spill conditions, whereas the majority of dispersant use occurs during surface spills, limiting our understanding, of floc formation and microbial composition. Light is an important factor during spill events, as components of crude oil undergo photooxidation, whilst light intensity, spectrum and photoperiod influence microbial communities associated with MOS. Therefore, this study aimed to quantify hydrocarbon biodegradation and the development of microbial communities during MOS formation under a natural sea-surface light regime both with and without dispersant. Stereomicroscope Imaging techniques were developed to measure size and number of flocs over time and changes in community composition were determined by 16S rRNA amplicon sequencing. Overall dispersant use was shown to have little impact on physical floc formation. Bacterial communities within flocs were highly diverse compared to the surrounding seawater communities, potentially attributed to a greater diversity of energy sources and redox conditions taking place within floc microhabitats. This diverse bacterial activity supported high alkane (87.8% total degradation) and polyaromatic hydrocarbon (70.4% total degradation) biodegradation after 14 days. The use of chemical dispersant and presence of light enhanced this degradation and selected for a wide range of putative hydrocarbon degraders highlighting flocs to be areas of high potential hydrocarbon degradation, whilst having minimal impact to floc formation.

Introduction

Oil exploration, extraction and transportation have continued to grow over the past decade, with global production and consumption rates of crude oil forecast to reach 105 million barrels per day by the end of 2025 (U.S. Energy Information Administration, 2025). Although only making up a small proportion of anthropogenic oil pollution, tanker spills and oil well blowouts are a significant source of oil entering the marine environment, and a major threat to marine ecosystems due to the large quantities being introduced within a small spatial and temporal scale (National Academies of Science, 2022). With an increased need for transportation, the risk of major oil spills occurring is still prevalent, as seen in recent examples such as the 2017 Agia Zoni II spill in Greece (>700 tonnes) and the 2018 Sanchi spill, off Shanghai, China (113,00 tonnes; 9th largest oil spill since 1967) (ITOPF, 2020).

When a spill occurs, remediation strategies are implemented to combat and lessen the impact of oil pollution to the surrounding environment, with tools such as booms and skimmers used to mechanically collect and remove the oil directly from the affected area (ITOPF, 2012; McNutt *et al*., 2012). However in some cases this is not possible due to adverse weather conditions which inhibit these methods (Cormack, 2013). In this case, responders may turn to remediation methods such as chemical dispersants. Dispersants consist of surfactants (surface active agents), dissolved into solvents and a stabiliser (Dave and Ghaly, 2011). Surfactants are made up of a hydrophobic tail and hydrophilic head, which helps the formation of an oil in water emulsion by reducing the interfacial tension between water and oil (Fingas, 2011). This breakdowns oil into small, dispersed droplets which increases the surface area for microbial attachment and enhances bioavailability to hydrocarbon degrading microorganisms, thus accelerating biodegradation processes (Prince *et al*., 2013).

However, over the past decade the use of chemical dispersants became associated with a phenomenon known as marine oil snow (MOS), generating debate over the ecological impact chemical dispersants may have as a remediation tool (Passow *et al*., 2012; Fu *et al*., 2014). Marine snow (MS) is made up of both organic and inorganic particulate (microalgae, archaea, bacteria, zooplankton, phytoplankton, faecal matter, etc.), stuck together via an extracellular polymeric substance (EPS) skeletal matrix (Alldredge and Silver, 1988; Simon *et al*., 2002; Bochdansky *et al*., 2017). Once formed, MS can transport nutrients from the surface to deep-sea benthic environments and support the organic carbon pump, sequestering atmospheric CO_2 into the benthos (Turner, 2015). During oil spill events it has been observed that oil can accumulate within MS forming MOS, either through physical aggregation of oil particles with naturally occurring MS, or through biofilm growth via microbial communities using oil particles as a nucleus (bacteria – oil aggregates, BOA) (Gregson *et al*., 2021; Passow and Overton, 2021). This has led to assumptions that MOS may act as a vehicle for the transportation of oil to deep sea benthic environments. This was further speculated after the subsea Deepwater Horizon (DWH) Macondo prospect Mississippi Canyon block 252 wellhead blowout (MC-252), in which large MOS formations occurred (Passow *et al*., 2012), where sediment trap analysis estimated that between 217,700 to 229,000 barrels of biodegraded oil residue from MOS had sedimented over an area of approximately 7,600 km^2 (Stout and German, 2018) representing 6.8 to 7.2% of crude oil that was spilt but not recovered during the event).

During the MC-252 event, approximately 50,000 barrels of dispersant were applied via subsurface injection as part of the remediation strategy, currently being the only subsurface spill to be chemically dispersed to date. During the spill, a large

amount of MOS accumulated, and subsequently a Marine Oil Snow Sedimentation and Flocculant Accumulation (MOSSFA) event occurred. The use of chemical dispersants thus became a significant point of discussion, notably on how dispersants may impact the formation of MOS flocs within the impacted site. The effect of dispersant use on MOS formation has become a point of contention (Gregson *et al*., 2021). Prior studies had reported that the addition of dispersant stimulated MOS formation (Van Eenennaam *et al*., 2016; Doyle *et al*., 2018; Bretherton *et al*., 2019, Suja *et al*., 2017, 2019), whilst other studies suggest dispersants inhibited MOS formation (Passow, 2016; Passow *et al*., 2017). These contradictions may be explained by differences in environmental conditions such as seawater used (includes different microbial communities and changes in capacity for EPS production) and type of oil present but may also partly be explained by differences in experimental design (chemical dispersant used incubation methods) and analysis. For example, Suja *et al*. (2017, 2019) and Sun *et al*. (2018) used both different dispersant types and incubation methods e.g. water accommodated fractions (WAF) and chemically enhanced WAF (CEWAF) in roller table experiments. Notably the production of WAF removes volatile and insoluble hydrocarbons, leaving only a fraction of dissolved hydrocarbons. The comparison between WAF and CEWAF compares between mechanically and chemically dispersed oil rather than comparing between undispersed oil and chemically dispersed oil (Passow 2016; Prince *et al*., 2016; Passow *et al*., 2017), potentially causing differences in study outcomes that not be directly representative of surface spill events. Floc formation analysis has also been a point of debate, prior studies have used both physical visual measurements of floc count and size and measurements of EPS concentration as a proxy for floc formation (Passow and Alldredge, 1995; Passow, 2016; Suja *et al*., 2017). Previously the use of dispersants

such as COREXIT 9500A has been shown to create artifacts within EPS concentration analysis making it difficult to quantify any potential changes in floc formation, thus making it difficult to compare between studies and extrapolate to real world spill events (Passow *et al*., 2017). As well as these contradictions, a bias toward the conditions of the MC-252, DWH incident is observed throughout the literature, whereas results may vary under different environmental conditions. For example, as this incident remains the only example of sub-sea dispersant addition, the majority of experiments have been performed under complete darkness to emulate the conditions of this particular spill event (Passow, 2016; Passow *et al.*, 2017; Wirth *et al*., 2018; Suja *et al*., 2017, 2019). Thus, there may be an argument against extrapolating results from past studies focusing on DWH to a sea surface environment, which is where the majority of chemical dispersants are used (Chapman *et al*., 2007). Currently, one study has investigated the impact of sunlight on EPS production a key component of MOS formation (again using the WAF:CEWAF method). Seawater was prepared over 36 h under a 12:12 light:dark cycle and the WAF produced was irradiated for a further 40 mins (Sun *et al*., 2018). With MOS experiments generally being incubated in complete darkness, we understand very little on how light conditions may influence MOS formation at the sea surface and thus should be investigated, particularly as light is involved in photooxidation and phototroph-heterotroph interactions. For example, in sunlight, crude oil can undergo various abiotic chemical reactions, when photooxidation is initiated by sunlight absorbing chromophores in the oil (D'Auria *et al*., 2008; Shankar *et al*., 2015) and can result in the physico-chemical properties of oil being altered, such as increases in water solubility, dispersibility and toxicity (Wang and Fingas, 2003; Shankar *et al*., 2015; Daly *et al*., 2016). Light intensity, spectrum and photoperiod also influence phytoplanktonic communities and the metabolites they

produce, which leads to changes to associated heterotrophic communities, thus impacting the community composition of hydrocarbon degrading bacteria associated with MOS (Bacosa *et al*., 2015b). Therefore, the aim of this study was to assess the impact of light and chemical dispersant use under surface spill conditions on floc formation and their associated bacterial communities, which will better inform the spill mitigation impact assessment (SIMA) used in oil spill response decision making. We hypothesised that there would a significant difference in bacterial community composition between light and dark treatments and that light would significantly impact the formation of marine snow would, highlighting the importance of light as an environmental factor during marine snow experiments.

Experimental design

Sample site

Seawater was sampled from the North Sea , approximately 1 mile off of Mersea Island, Essex, UK (51.7509° N, 1.0001° E) on the 2nd September 2023 from the sea surface (first 10 cm layer) using a 20 litre carboy bottle. Approximate sea surface temperature was (17ºC).

Microcosm design

Microcosms were created using sterile acid washed (10% HCl) 500 ml glass jars with PTFE-lined lids (9.9 cm height, 8.6 cm diameter), containing 400 ml of natural North Sea seawater, leaving air space to further allow for simulated wave action and a small level of turbulence at the water surface. Replicate treatments (n=4) included Seawater Controls (400 ml Seawater Only), Dispersant (400 ml Seawater plus 0.0005% v/v COREXIT™ EC9500A), Oil (400 ml Seawater with 0.01% v/v crude oil) and Oil plus Dispersant (400 ml Seawater with 0.01% v/v crude oil and 0.0005% v/v COREXIT™ EC9500A) and were incubated horizontally on two roller tables at

approximately 26 rpm (mimicking sea surface turbulence) for 6 days, submerged in seawater at a constant temperature of 17-19^{o}C (matching the *in-situ* sea surface temperature of the sampled seawater). One roller table was illuminated (45 cm distance from light) under a DYNA LED grow light (Heliospectra) set to simulate a natural September 16:8 day:night cycle (Supplementary Material: Figure S1) with photosynthetically active radiation (PAR) increasing from 0 to 1000 µmol $m^{-2}s^{-1}$ within the microcosms, staying at the maximum PAR for 10 minutes before decreasing to mimic natural light intensities *in-situ* (Dring et al., 2001). The second roller table was kept in darkness for the 6 days . The crude oil used was Norwegian Geochemical Standard North Sea Oil (NGS NSO-1; previously weathered at 60^{o}C over 24 hours) at a concentration of 100 ppm (0.01% v/v), simulating typical initial *in-situ* oil concentrations during an oil spill event (Lee *et al*., 2013). The dispersant used was COREXIT™ EC9500A (Nalco Holding Company) at the industry standard of 20:1 (Oil:Dispersant ratio) (Fingas, 2011). After 6 days surrounding seawater (50 ml) was sampled from each microcosm using 0.22 µM pore size Sterivex filter unit (Millipore), avoiding flocs by first allowing them to settle at the bottom or top of the microcosm for 30 seconds. Flocs were then via filter using a vacuum pump and WhatmanTM Glass microfiber filters (1.5 µM) to sample flocs that formed within each microcosm. The filtered flocs were then freeze-dried in an ALPHA 1-4 LD plus (Martin Christ Freeze Dryers) and stored at -20^{o}C for further microbial community composition analysis.

Another set of microcosms following the same conditions were created using seawater samples collected on the 18th September 2024 and were incubated over a 14 day period for the purpose of floc image analysis and hydrocarbon degradation analysis.

DNA extraction and qPCR bacterial 16S rRNA

DNA was then extracted from the seawater sample using a DNeasy PowerWater Kit (Qiagen), following the manufacturer's instructions and extracted from flocs (17.5 – 43.2 mg) using the DNeasy PowerSoil Pro Kit (Qiagen), following the manufacturer's instructions. Bacterial 16S rRNA gene abundance was quantified via qPCR with primers 341f: 5'CCTACGGGNGGCWGCAG3' and 805R: 5'GACTACHVGGGTATCTAATCC3' (Klindworth et al., 2013) using the SensiFAST SYBR no-ROX kit (Bioline) on a CFX Opus 384 Real-time PCR Detection System (Biorad), using an absolute quantification methods against a standard calibration curve of the 16S rRNA gene from 10^2 – 10^9 copies in 20ul reactions described in (McKew and Smith, 2017).

Amplicon library preparation and bioinformatics

Amplicon library sequencing preparation was performed via a 30 – cycle PCR using a Veriti 96 well thermal cycler, (Applied biosystems) following the 16S Metagenomic Sequencing Library preparation protocol (Illumina, 2013). PCR primers used were the same as the qPCR primers, with the addition of Illumina adapter overhang nucleotide sequences. PCR products were then cleaned using AMPure XP beads (Beckman Coulter). Using the Nextera XT Index kit (Illumina) a unique combination of index primers were added to the PCR products from each sample via an 8-cycle PCR. A second clean-up of the library PCR product was performed, again using AMPure XP beads. At the end of each step gel electrophoresis was performed for quality control before each subsequent step. Following this, amplicons were quantified using the Quant-T PicoGreen dsDNA Assay kit protocol using the NanoDrop 3300 Flourospectrometer (Thermo Scientific), 16S rRNA gene quantification was performed on all indexed PCR products and then pooled together at equimolar

concentrations. The final pooled library was then quantified using both the Quant-IT PicoGreen dsDNA Assay kit (Thermo Scientific) and the NEBNext Library Quant kit for illumina (New England Biolabs, UK), prior to sequencing. Sequencing was then provided by Novogene (Cambridge, UK) using Illumina NovaSeq PE250. Sequences have been submitted to the NCBI SRA archive under the accession number PRJNA1226146.

Sequencing output from the NovaSeq PE250 was processed using the Divisive Amplicon Denoising Algorithm (DADA2) (Callahan *et al*., 2022). Following this pipeline, forward and reverse reads were quality checked and trimmed, forward and reserve primer sequences were also removed. Via the core sample inference algorithm, error correction was performed, and chimeric sequences removed (Callahan *et al*., 2016). Forward and reverse reads were then merged to obtain the full sequence per read; these were then placed into an amplicon sequence variant (ASV) table. Singleton and doubleton ASVs were discarded, along with sequences below 400 base pairs and non-target organisms (Archaea and algal chloroplast sequences). Homopolymers above 8 b.p. were also discarded. Taxonomic assignment was conducted using the Ribosomal Project (RDP) Classifier (rdp trainset 18/release 11.5) (Callahan, 2020; Wang et al., 2007) and blastN (Altschul *et al*., 1990).

Floc imaging

Floc formation was analysed via imaging each microcosm at Day 0, 2, 4, 6, 8, 12 and 14. The microcosms were turned vertically and flocs were allowed to settle for 30 seconds before using a Nikon SMZ25 stereomicroscope; two focal points targeting the bottom and surface of the microcosms were imaged to capture both sinking and floating flocs (Supplementary Material: Figure S2). Raw images were then processed using Fiji ImageJ (Schindelin *et al*., 2012). Firstly the background was subtracted from

the raw image, then using a Huang threshold (Huang and Wang, 1995) the individual flocs were separated to allow for the quantification of the number of flocs, percentage cover (percentage of the image consisting of flocs), min, max and average floc size. Minimum detection size of flocs was 0.01mm^2 (Figure 3.1A).

Hydrocarbon concentration analysis

Hydrocarbons were extracted from microcosms at day 0 and day 14 by adding 20 ml of solvent (1:1 ratio, Hexane: Dichloromethane) and rigorously shaking for a few minutes. The solvent layer was removed via a glass pipette. This process was repeated three times and combined for all microcosms into clean glass vial. Extracts were dried with the addition of 2 g of sodium sulfate, transferred to clean glass vials and concentrated down to 20 ml in a TurboVap evaporator (Biotage). Using 17α(H)21β(H)hopane as an internal non-degradable biomarker (Prince *et al*., 1994), the percentage degradation was quantified using the Nexis GC-2030/CMS-TQ8040 NX GC-QqQ-MS/MS (Shimadzu), targeting Alkanes *n*-$C_{10-38,}$ pristane and phytane and a selection of two-six ring PAHs and their derivatives (Supplementary Material: Table S1). Alkane calibration standard (Merck; contains *n*-C_8 – *n*-C_{38} + pristane and phytane), and QTM PAH mix (Sigma) standard with a range of other two-six ring PAHs and their derivatives were used to determine retention times of hydrocarbon targets. (Supplementary Material: Table S1). A blank was analysed every 25 samples for quality control.

Statistical Analysis

ASV sequence data was rarefied to the lowest read depth (54,234 bp). Diversity indices and community composition analysis were then conducted using vegan (Oksanen et al., 2024). Data was further analysed using multivariate generalised linear modelling through R (R Core Team, 2022). A SIMPER analysis (Clarke, 1993) within

the vegan package was used to identify taxa causing the most significant dissimilarity between communities.

Results

Floc formation

Over the 14 day incubation period, the percentage cover of sinking flocs increased across all treatments ($t = 2.32 – 3.23$, $p < 0.05$), attributed to both an increase in floc size and number of flocs formed (Figure 3.1B). Sinking flocs that formed in the seawater controls under light had the highest percentage cover compared to all other treatments ($t = -5.99 – -4.49$, $p < 0.001$) being 0.8-fold higher by day 14 (Figure 3.1B), attributed by the higher number of flocs formed; ranging from 554 - 2342 individual flocs (Supplementary material: Figure S3). Under dark conditions, the percentage cover was approximately 0.5 – 0.6-fold lower in seawater controls compared to the oil plus dispersant treatment and dispersant control by day 14 ($t = 1.99 – 3.12$, $p < 0.05$), attributed to the higher average and maximum floc size within the oil and the oil plus dispersant treatment (Supplementary Material: Figure S5, S6). Although the number of flocs formed was higher in seawater controls, flocs that formed in the oil treatment reached larger floc sizes up to a maximum floc size of 23.58 mm^2, although with the use of dispersants maximum floc size of flocs was reduced to a maximum of 5.872 mm^2 (Supplementary Material: Figure S5). Formation of floating flocs only occurred under light in the oil and oil plus dispersant treatment and were absent in the seawater controls and dispersant controls ($z = 2.54 – 3.34$, $p < 0.05$). By Day 14 no floating flocs were present in any treatment. Minimum floc size across all treatments did not significantly change over time after initial floc formation. (Supplementary Material: Figure S4). High resolution imaging of an individual floc sampled from oil and oil plus dispersant treatments was undertaken, highlighting

physical differences visually (Figure 3.1C). The floc treated with oil was observed to be much more compact, compared to the oil plus dispersant floc with a potential increase in the flocs microscale rugosity (Anderson *et al.*, 2024). Even so this cannot yet be confirmed as common as only a single floc from each treatment was imaged under high resolution.

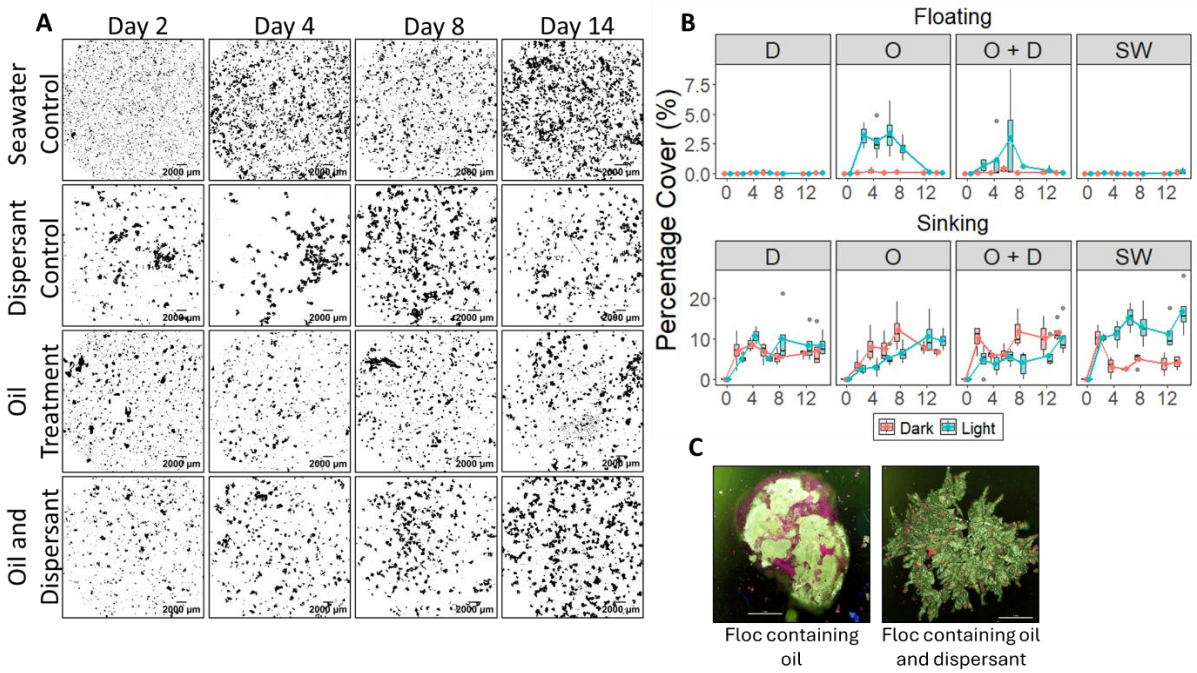


Figure 3.1: A. Processed Images of floc formations incubated over 14 days in 500 ml microcosms containing 400 ml natural seawater (Minimum detection size: 0.01 mm^2). Treatments include Seawater Control, Dispersant Control, Oil, Oil and Dispersant. Oil used: Norwegian Geochemical Standard North Sea Oil (NGS NSO-1), dispersant used :COREXIT™ EC9500A, Scale = 2000 µm (2 mm). Image processing was carried out using Fiji ImageJ (Schindelin *et al.*, 2012). **B.** Percentage Cover (%) of flocs formed **C.** High resolution images of single flocs containing oil only and oil plus dispersant. Oil used: Norwegian Geochemical Standard North Sea Oil (NGS NSO-1), dispersant used :COREXIT™ EC9500A, Scale = 1 mm, autofluorescence: Magenta = Chlorophyll, Green = Microbial biofilm.

Hydrocarbon degradation

After 14 Days substantial Alkane degradation took place within microcosms across all treatments containing oil (Oil (Dark), 86.3%; Oil (Light), 85.1%; Oil plus Dispersant (Dark), 86.6% and Oil plus Dispersant (Light), 93.21% total average *n*-alkane and branched alkane degradation) (Figure 3.2). However, total measured alkanes were 7.6% more degraded within oil plus dispersant treatments incubated under light compared to oil plus dispersant treatments incubated in the dark (t = 2.948,

$p < 0.005$) (Figure 2B), caused by pristane and the larger *n*-alkanes, *n*-C_{29} – *n*-C_{37}, being significantly more degraded under light (z = 2.05 – 7.16, $p < 0.05$). The oil plus dispersant treatment was also more degraded compared to the oil treatment when incubated under light (9.53% more degraded) (t = 3.488, $p < 0.001$), with pristane, phytane, *n*-C_{11}, *n*-C_{12}, *n*-C_{14}, *n*-C_{30} – *n*-C_{32}, *n*-C_{34} and *n*-C_{36} – *n*-C_{38} being significantly more degraded (z = 2.37 – 5.69, $p < 0.05$). There was no significant difference in the oil only treatments between light and dark conditions (Figure 3.2A).

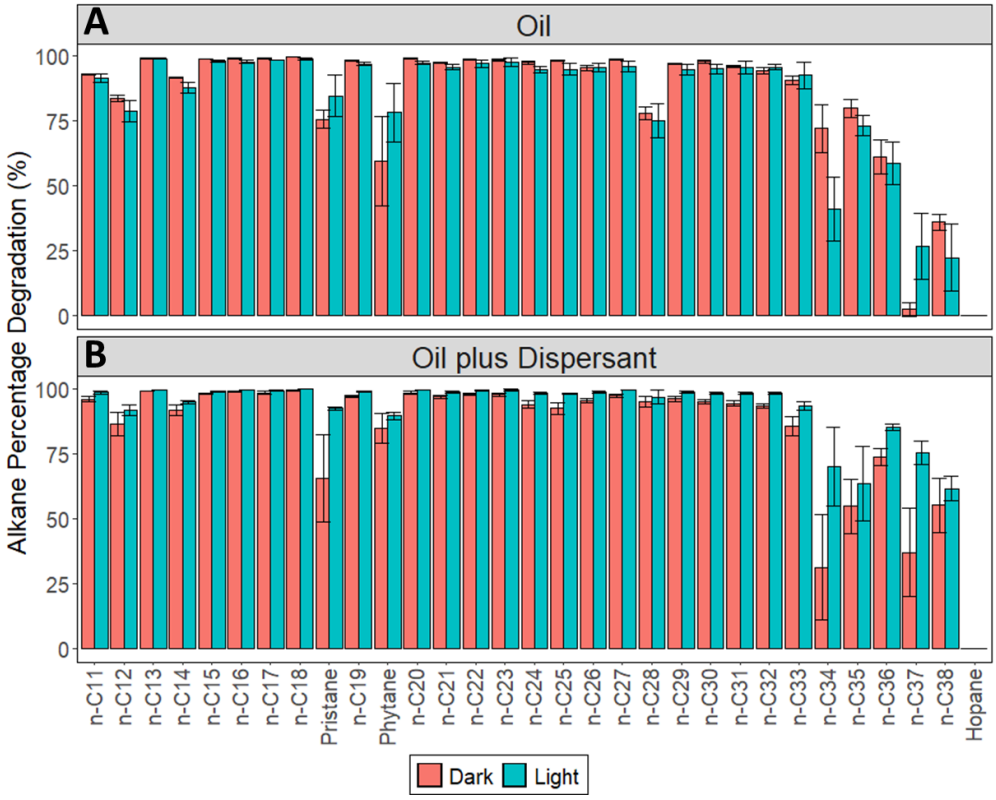


Figure 3.2: Alkane (*n*-C_{10-38}, pristane and phytane) percentage degradation after 14 days in 500 ml microcosms containing 400 ml natural North Sea seawater treated with **A.** Oil and **B.** Oil plus Dispersant incubated under dark conditions and light conditions (16:8 day:night cycle (Supplementary Material: Figure S1) with photosynthetically active radiation (PAR) increasing from 0 to 1000 µmol $m^{-2}s^{-1}$). Oil used: Norwegian Geochemical Standard North Sea Oil (NGS NSO-1) (100 ppm (0.01% v/v), dispersant used :COREXIT™ EC9500A, at an industry standard of 20:1 Oil:Dispersant.

After 14 Days, measured 2-6 Ring PAH's, (Supplementary Material: Table S1) were also substantially degraded within all treatments (Oil (Dark), 65%; Oil (Light), 64.3%; Oil and Dispersant (Dark), 74.2%; Oil and Dispersant (Light), 78.2% total average PAH degradation) (Figure 3.3). In both the oil and the oil plus dispersant

treatment, no difference in total percentage degradation was observed between light and dark. However, C2 Phenanthrenes/Anthracenes, benzo(a) anthracene, C1 Dibenzothiophene, C2 Dibenzothiophene, chrysene, Indeno [123-cd]pyrene were shown to be significantly more degraded in the oil plus dispersant treatments incubated in the light compared to oil plus dispersant treatments incubated in the dark ($z = 2.74 – 11.23$, $p < 0.05$) (Figure 3.3B). Total PAH percentage degradation was also significantly higher in the oil plus dispersant treatments compared to the oil only treatments in both light (13.9% more degraded) and dark (9.2% more degraded) ($z = 2.145 – 3.437$, $p < 0.05$). Specifically PAHs C1 Naphthalene, C2 Naphthalene, C2 Fluorene, C1 Dibenzothiophene, C2 Dibenzothiophene, C1 Phenanthrene/Anthracene, C2 Phenanthrene/Anthracene, Chrysene, Benzo(b)Fluoranthene, Benzo(k)Fluoranthene and Benzo[ghi]perylene were more degraded in oil plus dispersant treatments compared to oil treatments incubated in the dark ($z = 2.46 – 15.23$, $p < 0.05$), whilst C1 Naphthalene, C2 Naphthalene, Acenaphthene C2 Fluorene, C1 Dibenzothiophene, C2 Dibenzothiophene C2 Phenanthrene/Anthracene, Benzo(a)Anthracene, Benzo(b)Fluoranthene, Benzo(k)Fluoranthene, Ideno[123-cd]pyrene and Benzo[ghi]perylene were significantly more degraded in oil plus dispersant treatments compared to oil treatments incubated in the light ($z = 2.31 – 16.58$, $p < 0.05$).

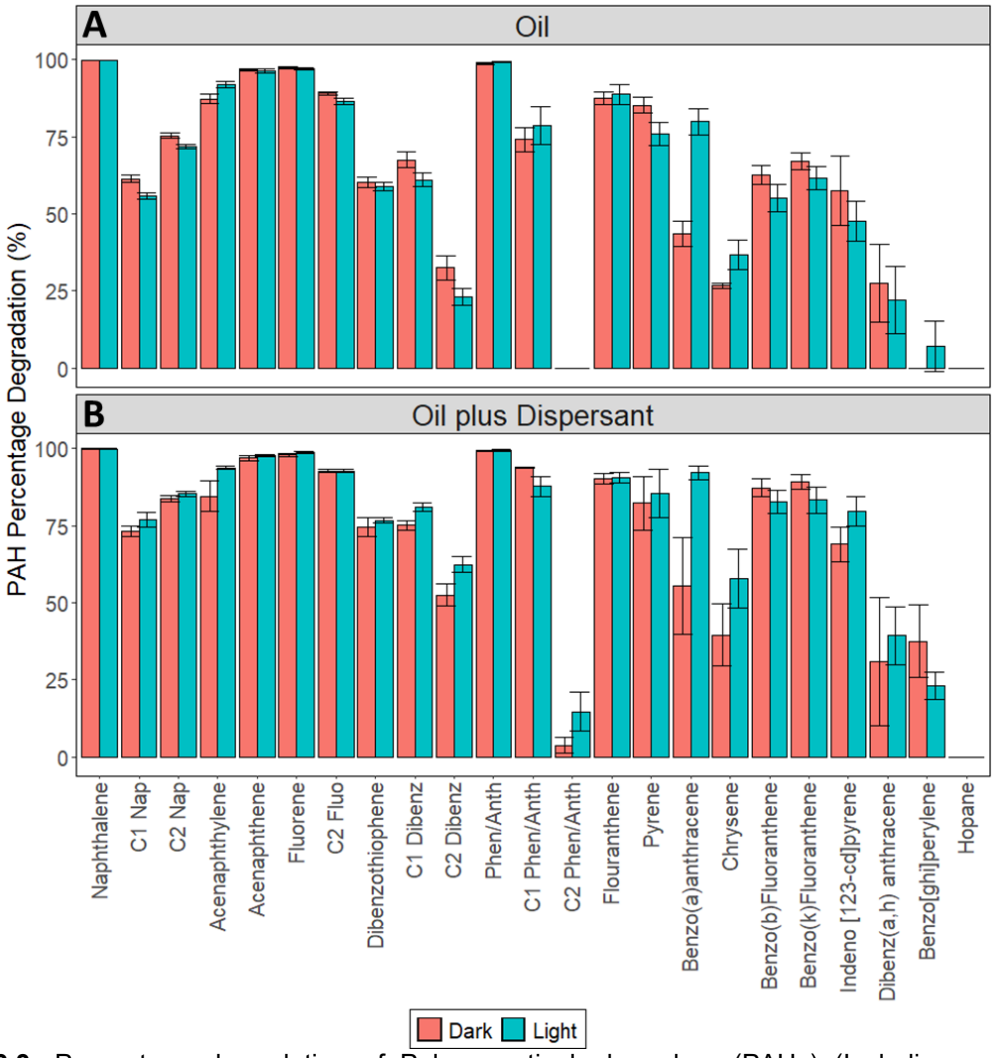


Figure 3.3: Percentage degradation of Polyaromatic hydrocarbon (PAHs) (Including parent and subsituted derivatives) in 500 ml microcosms containing 400 ml natural seawater treated with Oil and Oil and Dispersant incubated under dark conditions and light conditions (16:8 day:night cycle (Supplementary Material: Figure S1) with photosynthetically active radiation (PAR) increasing from 0 to 1000 µmol $m^{-2}s^{-1}$). Oil used: Norwegian Geochemical Standard North Sea Oil (NGS NSO-1) (100 ppm (0.01% v/v), dispersant used :COREXIT™ EC9500A, at an industry standard of 20:1 Oil:Dispersant.

Bacterial diversity and community composition

The bacterial 16S rRNA gene was found to be highly abundant within both Marine Snow (MS) flocs and the Surrounding Seawater (SSW) (Figure 3.4). Bacterial abundance was found to be approximately between 3 – 10-fold higher within flocs from the dispersant control, oil and oil plus dispersant treatment compared to the seawater controls ($z = 3.26$, $p < 0.05$), whilst being significantly higher under light in the seawater control compared to the dark ($z = 1.02$, $p < 0.05$) (Figure 3.4A). No significant

difference in bacterial abundance was observed between any of the oil, oil plus dispersant, dispersant control and seawater control treatments either in the light or dark was found within the SSW (Figure 3.4B).

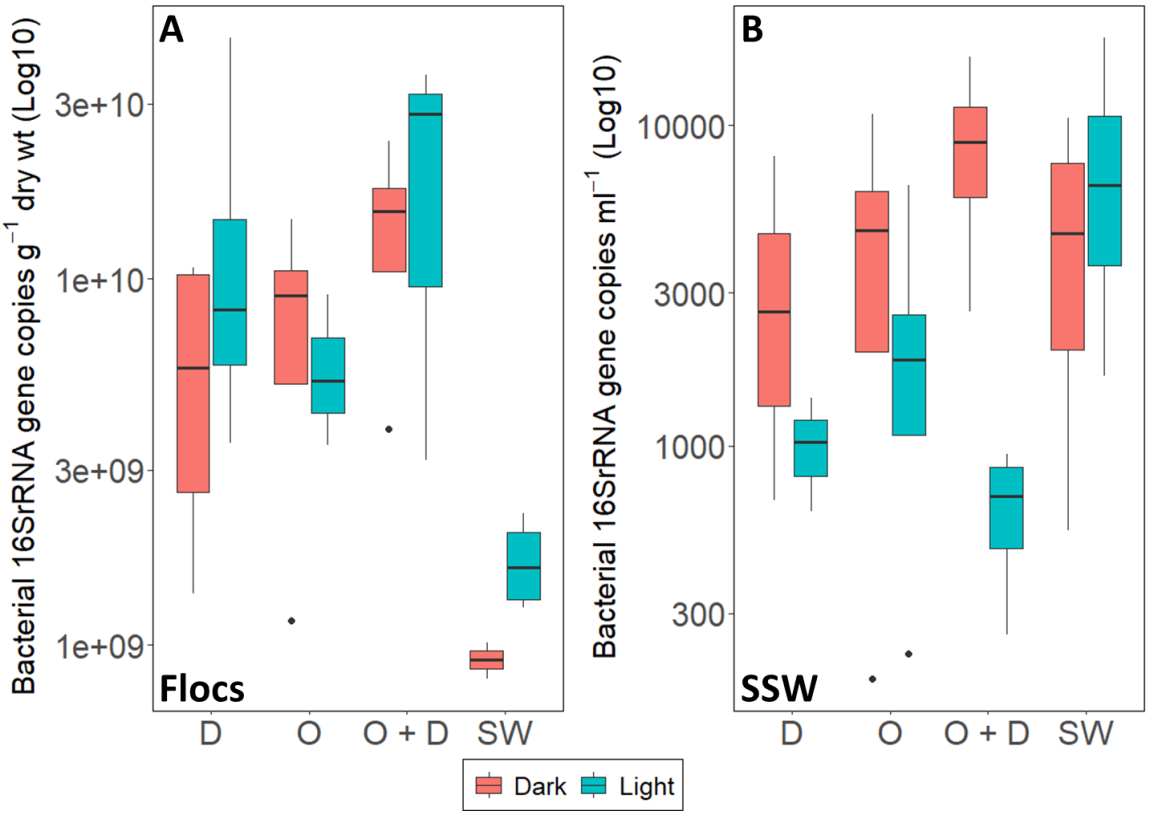


Figure 3.4: Bacterial 16S rRNA gene abundance from **A.** Marine snow (MS), Marine Oil Snow (MOS) flocs and **B.** Surrounding Seawater (SSW), collected after 6 days of incubation incubated under dark conditions and light conditions (16:8 day:night cycle (Supplementary Material: Figure S1) with photosynthetically active radiation (PAR) increasing from 0 to 1000 µmol $m^{-2}s^{-1}$). Oil used: Norwegian Geochemical Standard North Sea Oil (NGS NSO-1) (100 ppm (0.01% v/v), dispersant used :COREXIT™ EC9500A, at an industry standard of 20:1 Oil:Dispersant. D = Dispersant Control, O = Oil, O + D = Oil plus Dispersant, SW = Seawater Control.

Amplicon sequencing of bacterial 16S rRNA resulted in an average of 514,310 sequence reads (range, 14,775 – 6,521,823). Although Shannon diversity index and Pielou's evenness did not significantly change between floc, SSW and treatments (Supplementary Material: Figure S7, ASV richness was approximately 1 – 2.5-fold higher within flocs compared to SSW across all treatments ($t = 3.03$, $p < 0.05$) (Figure 3.5). Notably ASV richness was approximately 0.4-fold lower within flocs from the Oil plus Dispersant treatment compared to dispersant control and Seawater control treatments under light ($t = 2.48$, $p < 0.05$).

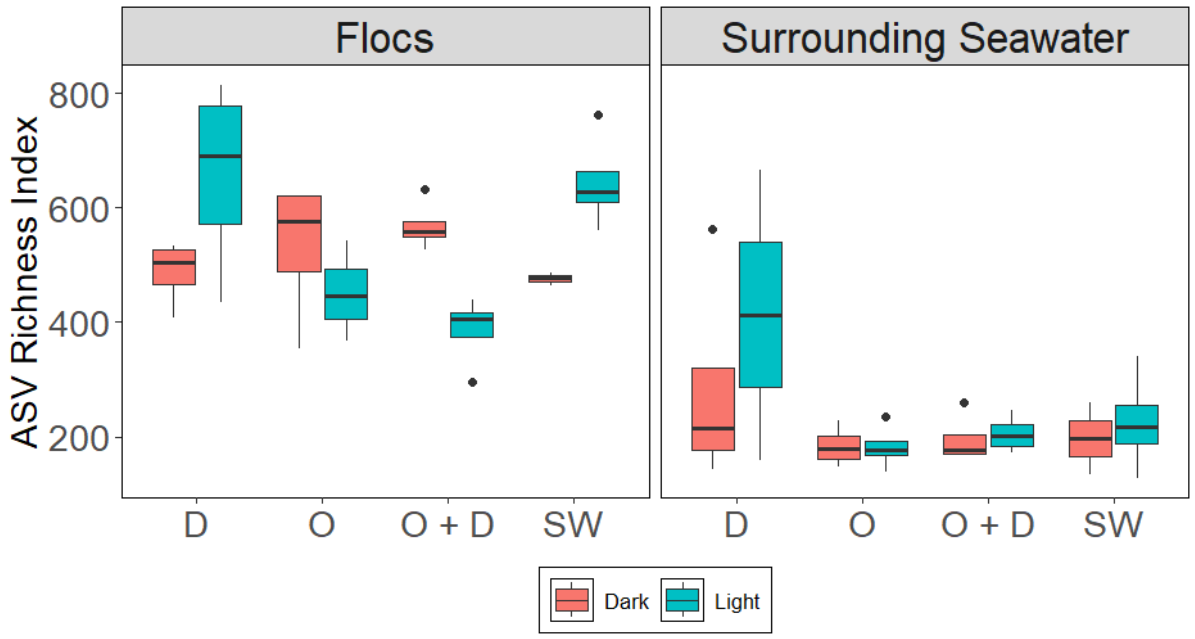


Figure 3.5: Diversity indices (ASV richness, Shannon index and Pielou's Evenness) of microbial communities of Marine snow (MS), Marine Oil Snow (MOS) floc and Surrounding Seawater (SSW) Extracts collected after 6 days of incubation incubated under dark conditions and light conditions (16:8 day:night cycle (Supplementary Material: Figure S1) with photosynthetically active radiation (PAR) increasing from 0 to 1000 µmol $m^{-2}s^{-1}$). Oil used: Norwegian Geochemical Standard North Sea Oil (NGS NSO-1) (100 ppm (0.01% v/v), dispersant used :COREXIT™ EC9500A, at an industry standard of 20:1 Oil:Dispersant. D = Dispersant Control, O = Oil, O + D = Oil plus Dispersant, SW = Seawater Control.

Bacterial 16S rRNA community composition was analysed using nMDS (Non – metric multidimensional scaling) and permutational analysis of variance (PERMANOVA) of dissimilarity between all treatments. Bacterial community composition was highly distinct between flocs and SSW communities across all treatments ($F = 20.77$, $p < 0.05$) (Figure 3.6A). Notably SSW communities showed higher variation in community composition compared to floc communities. Further observations highlighted significant dissimilarity in composition of floc communities between light and dark conditions throughout all treatments ($F = 2.54 – 4.47$, $p < 0.05$) (Figure 3.6B). There were also significant community composition differences between all floc treatments within the light, whilst only seawater control flocs were dissimilar compared to the oil, oil plus dispersant and dispersant control treatment in the dark ($F = 3.22 – 7.01$, $p<0.05$).

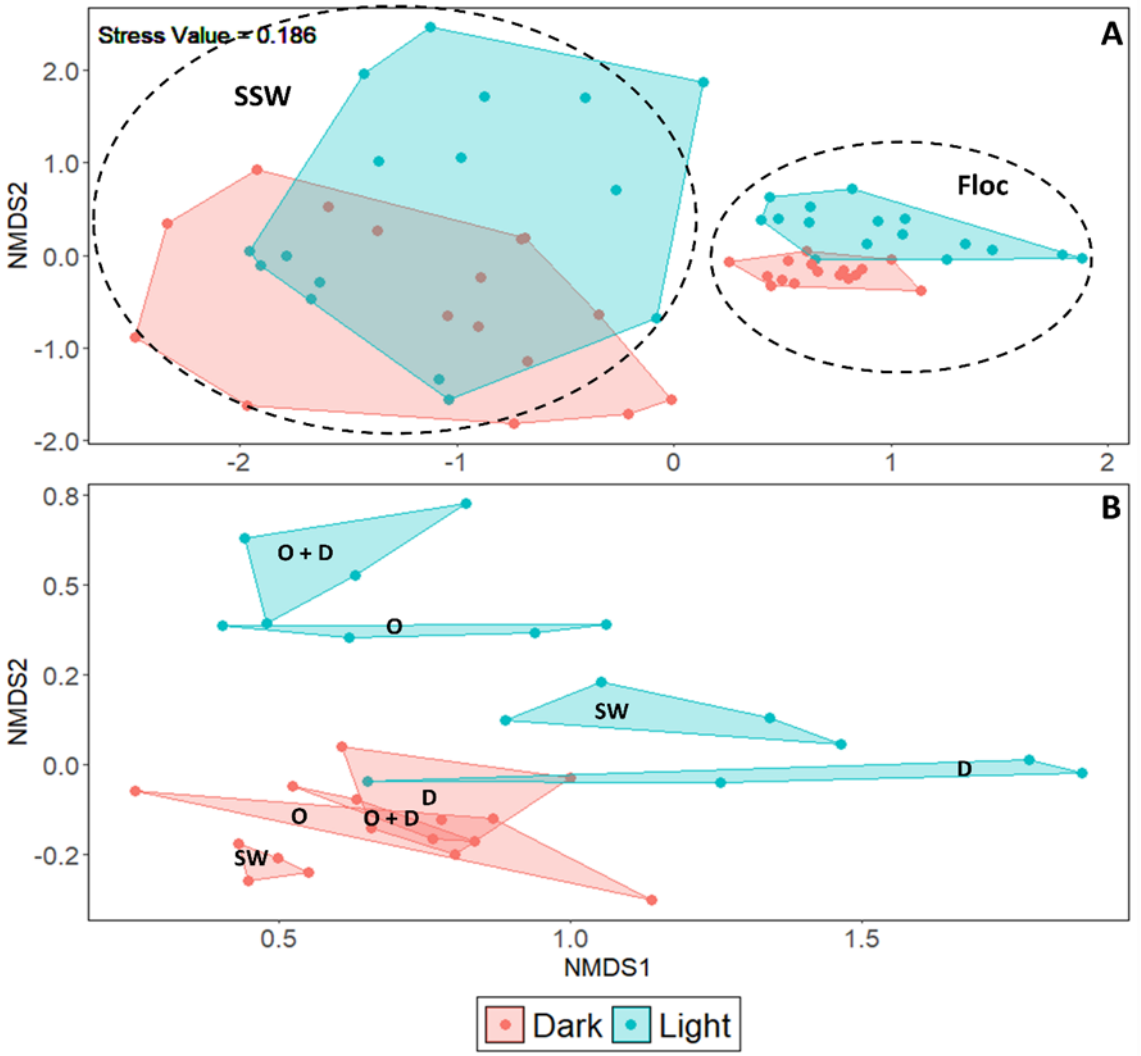


Figure 3.6: A. nMDS (non-metric multidimensional scaling ordination using Bray-Curtis dissimilarity (Stress Value = 0.186), based on ASV amplicon sequence variance of bacterial 16S rRNA communities of Marine snow and Marine Oil Snow (Floc) and Surrounding Seawater (SSW) Extracts, collected after six days of incubation under dark conditions and light conditions (16:8 day:night cycle (Supplementary Material: Figure S1) with photosynthetically active radiation (PAR) increasing from 0 to 1000 µmol $m^{-2}s^{-1}$). Oil used: Norwegian Geochemical Standard North Sea Oil (NGS NSO-1) (100 ppm (0.01% v/v), dispersant used :COREXIT™ EC9500A, at an industry standard of 20:1 Oil:Dispersant. **B.** nMDS using Bray-Curtis dissimilarity based on ASV amplicon sequence variance of bacterial 16S rRNA communities of Marine snow and Marine Oil Snow Extracts. D = Dispersant Control, O = Oil, O + D = Oil plus Dispersant, SW = Seawater Control.

The taxa with highest relative abundance within floc communities consisted of *Alteromonas* (65 ASVs) (Average 15%), *Marivita* (34 ASVs) (Average 8.4%) and *Pseudomonadaceae* (92 ASVs) (Average 26%) (Figure 3.7B, C, D). *Microbacteriaceae* (73 ASVs) (Average 18.9%) and *Rhodobacteraceae* (590 ASVs) (Average 7.4%) were the most abundant in SSW (Figure 3.7E, F). In most cases the relative abundance of these taxa did not significantly differ between light and dark

conditions, however *Acinetobacter* (52 ASVs) was 2-4 fold more abundant within flocs when incubated in the dark, whilst M*icrobacteriaceae* was only abundant in the SSW, notably when incubated in the dark (z = -5.35 – -2.401 , p < 0.05) (Figure 3.7A, E). *Alteromonas* was 1.5 – 3-fold higher in relative abundance within floc communities incubated in oil plus dispersant treatments and dispersant controls, compared to seawater controls (t = 1.3 – 4, *p* < 0.05) (Figure 3.7B).

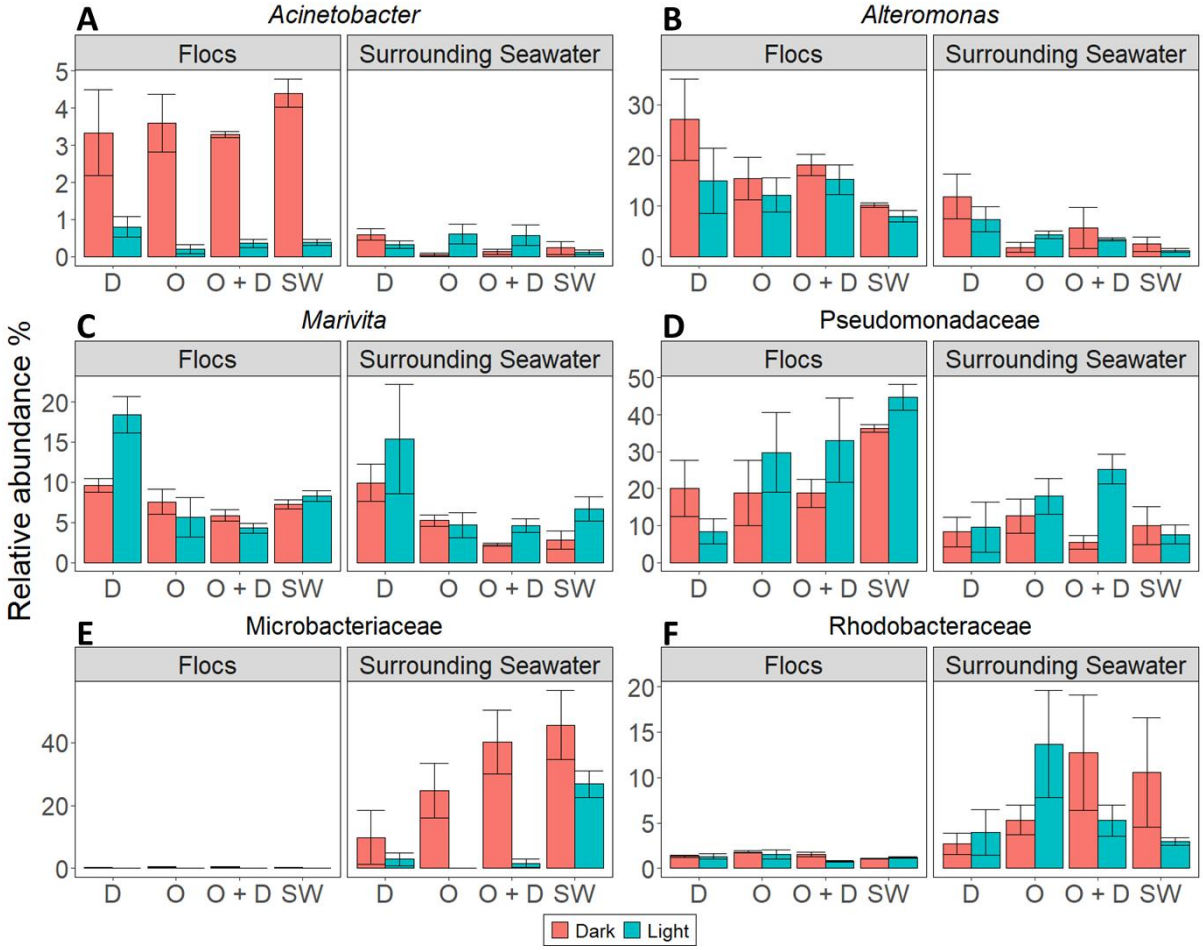


Figure 3.7: Highest abundant bacterial taxa within 16S rRNA bacterial communities of Flocs and Surrounding Seawater (SSW), collected after 6 days of incubation under dark conditions and light conditions (16:8 day:night cycle (Supplementary Material: Figure S1) with photosynthetically active radiation (PAR) increasing from 0 to 1000 µmol $m^{-2}s^{-1}$). Oil used: Norwegian Geochemical Standard North Sea Oil (NGS NSO-1) (100 ppm (0.01% v/v), dispersant used :COREXIT™ EC9500A, at an industry standard of 20:1 Oil:Dispersant. D = Dispersant Control, O = Oil, O + D = Oil plus Dispersant, SW = Seawater Control. O = Oil, O + D = Oil and Dispersant, D = Dispersant Only, S = Seawater Only.

The relative abundance of obligate hydrocarbonoclastic bacteria (OHCB's) was shown to be relatively low within floc and SSW communities (Figure 3.8). *Alcanivorax* (Represented by ASV 669 and ASV 916) and *Oleiphilus* (represented by ASV 2153

and 3061) were shown to favour flocs (Figure 3.8A, C) *Cycloclasticus* (represented by ASV 850) was higher in relative abundance in the SSW (z = 2.26, $p < 0.05$) (Figure 3.8B). *Thalassolituus* (Represented by ASV 87 and ASV 215) was higher in relative abundance within flocs from the oil plus dispersant treatment incubated in light when compared to oil plus dispersant treated flocs in the dark (z = 7.124 – 33.38 p < 0.05) (Figure 3.8F). *Alcanivorax* and *Marinobacter* were also significantly higher in relative abundance in all floc treatments compared to seawater controls (z = 2.14 – 3.74, $p < 0.05$), whilst *Thalassolituus* was relatively higher in relative abundance within the oil plus dispersant floc treatment compared to the seawater controls (-2.58 – -2.32, $p < 0.05$).

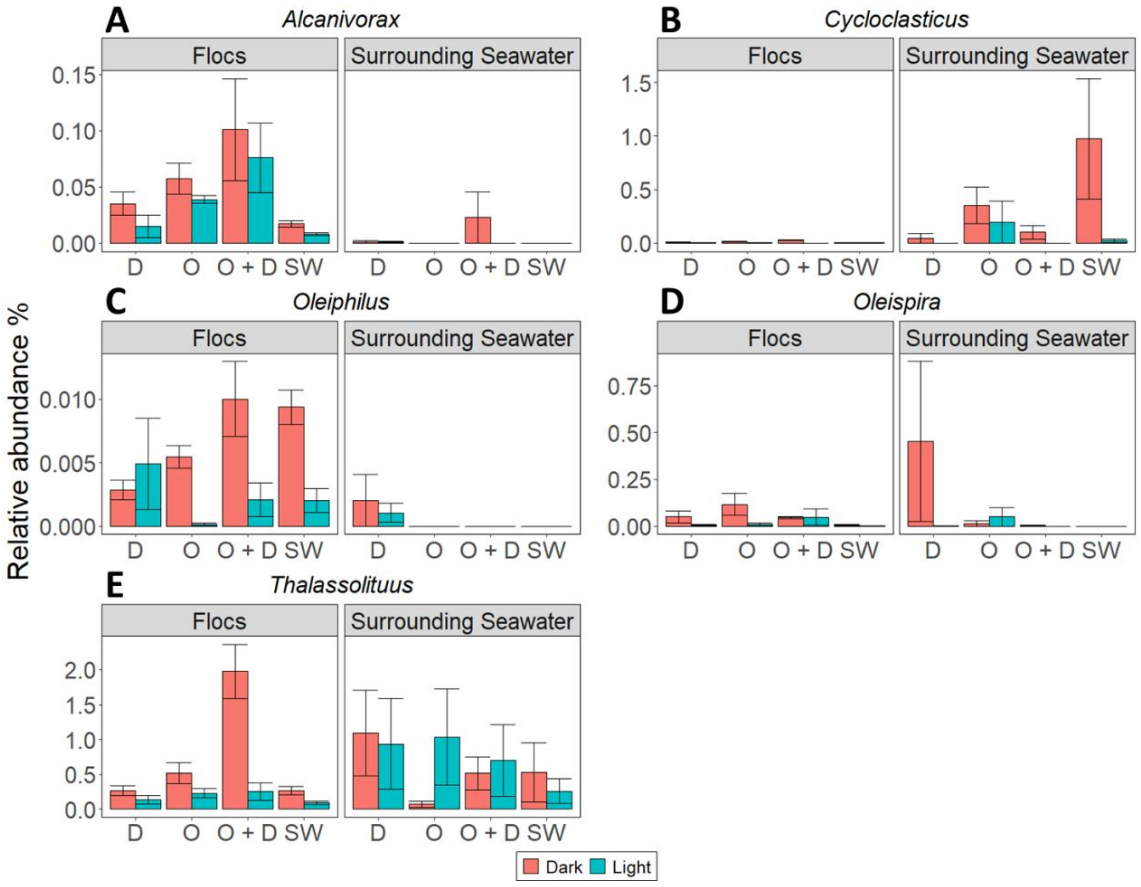


Figure 3.8: Average relative abundance of Obligate Hydrocarbonoclastic Bacteria (OHBCs) within 16S rRNA bacterial communities of Flocs and Surrounding Seawater (SSW), collected after 6 days of incubation under dark conditions and light conditions (16:8 day:night cycle (Supplementary Material: Figure S1) with photosynthetically active radiation (PAR) increasing from 0 to 1000 µmol $m^{-2}s^{-1}$). Oil used: Norwegian Geochemical Standard North Sea Oil (NGS NSO-1) (100 ppm (0.01% v/v), dispersant used :COREXIT™ EC9500A, at an industry standard of 20:1 Oil:Dispersant. D = Dispersant Control, O = Oil, O + D = Oil plus Dispersant, SW = Seawater Control. O = Oil, O + D = Oil and Dispersant, D = Dispersant Only, S = Seawater Only.

SIMPER analysis was used to highlight other taxa that were contributing most to the community dissimilarity between floc and SSW communities and between treatments (Figure 3.9). *Alkanindiges* (Represented by ASV 19), *Comamonas* (Represented by ASV 58, 60 and 148), *Thalassomonas* (Represented by ASV 194 and 238) and *Thalassospira* (Represented by ASV 249, 317 and 366) were shown to predominantly favour floc communities (z = -4.91 – -2.11, $p < 0.05$) (Figure 3.9A, B, G, H). *Alkanindiges* was approximately 1 – 2-fold higher in relative abundance within the light (z = 5.85, p < 0.05), whilst *Comamonas* and *Thalassomonas* were approximately 1 – 3-folds higher in relative abundance within the dark (z = -6.41 – -2.71, $p < 0.05$). *Marinobacter* (Represented by ASV 161 and 175 *Marinomonas* (Represented by ASV 129, 138 and 234), *Halioglobus* (Represented by ASV 102, 172 and 230), *Pseudoaltermonas* (Represented by ASV 45, 50 and 53), *Thalassospira* and *Winogradskyella* (Represented by ASV 30, 33 and 77) were 1 – 6-fold higher in relative abundance within floc communities incubated in oil and oil plus dispersant treatments compared to seawater controls (z = 1.09 – 3.68, $p < 0.05$) (Figure 3.9C, D, E, F, G, I). *Winogradskyealla* relative abundance was 3 – 6-fold higher in oil plus dispersant treatments under light compared to all other treatments. Under dark conditions *Thalassomonas* was approximately 2.5-folds higher in floc communities incubated in oil plus dispersant and dispersant controls compared to seawater controls (z – 2.71, $p < 0.05$).

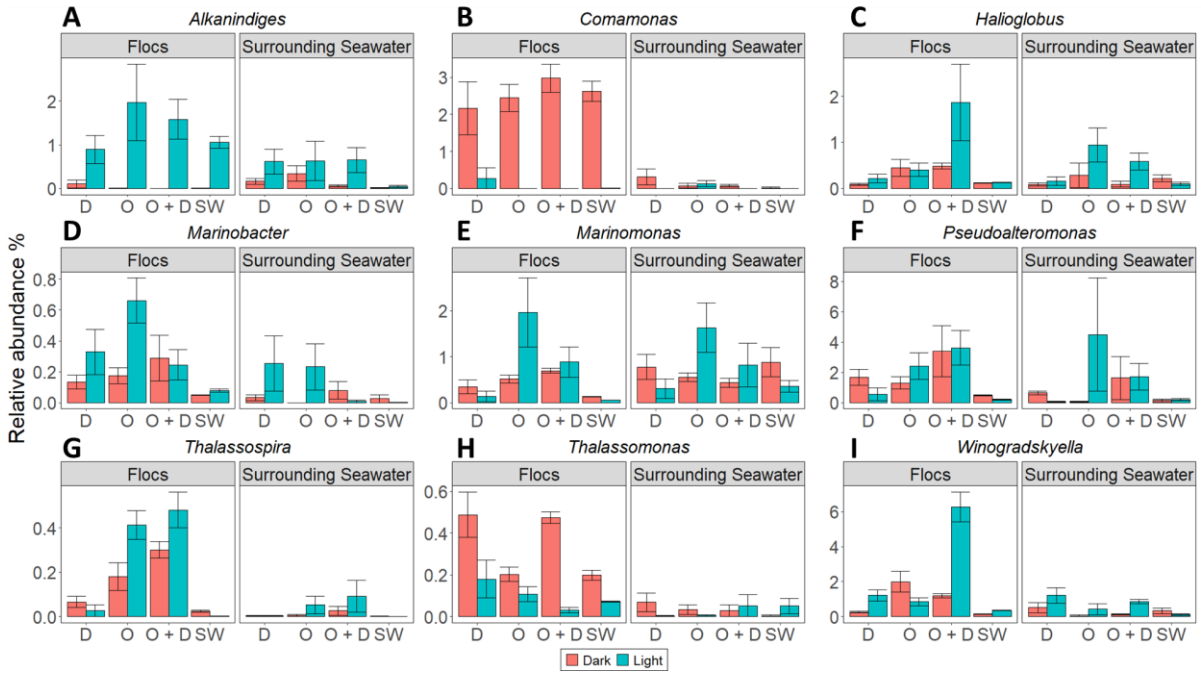


Figure 3.9: Average Relative abundance of other prevalent potential hydrocarbon degraders within 16S rRNA bacterial communities of Flocs and Surrounding Seawater (SSW), collected after 6 days of incubation incubated under dark conditions and light conditions (16:8 day:night cycle (Supplementary Material: Figure S1) with photosynthetically active radiation (PAR) increasing from 0 to 1000 µmol $m^{-2}s^{-1}$). Oil used: Norwegian Geochemical Standard North Sea Oil (NGS NSO-1) (100 ppm (0.01% v/v), dispersant used :COREXIT™ EC9500A, at an industry standard of 20:1 Oil:Dispersant. D = Dispersant Control, O = Oil, O + D = Oil plus Dispersant, SW = Seawater Control.

Discussion

Floc formation with changes in light conditions and dispersant use

Floc formation was observed over a 14 day period, quantifying number of flocs formed, percentage cover of flocs and the minimum, maximum and average sizes of the flocs, observing both floating flocs and sinking flocs. Chemical dispersant use (either alone or combined with oil) did not significantly enhance the number of flocs formed over time, nor the percentage cover of flocs within the microcosms, whilst having minimal impact to maximum and average floc under both dark and light conditions. These results are supported by prior studies which also saw no impact to floc formation with the presence of dispersant (Passow, 2016; Passow *et al*., 2017), following the roller table experimental design used during this study. Other past studies have suggested otherwise, indicating that the presence of dispersant can enhance MOS formation. Notably many past studies have used TEP (transparent exopolymer

particles) and EPS (extracellular polymeric substances) as proxies for MOS formation, as they are a large component of the skeletal matrix of flocs(van Eenennaam *et al*., 2016; Passow *et al*., 2017; Suja,*et al*., 2019). During such experiments, COREXIT 9500A has been shown to create artifacts during TEP analysis via Alcian Blue staining (Passow and Alldredge, 1995), and currently there has been no updated method to account for this issue, therefore, making EPS and TEP concentrations difficult to use as a proxy for physical floc formation. The use of high resolution image analysis during this current study has been proven to be an effective method in quantifying the size of flocs and their abundance within microcosms, removing the ambiguity of TEP/EPS analyses as a proxy and human error via visual video and photography used in other studies (Passow *et al*., 2012; Fu *et al*., 2014; Passow, 2016), allowing for a better representation of physical formation. To date, studies of MOS have focused on the Deep Water Horizon (DWH) incident, often incubating samples in darkness using water accommodated fraction (WAF) (Suja *et al*., 2017, 2019; Sun *et al*., 2018; Wozniak *et al*., 2019). This removes much of the volatile and insoluble hydrocarbon compounds (Valentine *et al*., 2014; McFarlin and Prince, 2021), which does not extrapolate well to surface spills where dispersant use is the most common. The current study, therefore, better represents surface level spill environments over that of the DWH subsurface incident. Whilst light had minimal impact on the formation of flocs across all treatments, by day 12 maximum size of floc within oil treatments under light was larger than seawater controls by 345.75% at 23.58mm^2, whilst with the addition of chemical dispersant use, maximum floc size was massively reduced by 75.10% at 5.872mm^2 compared to flocs incubated in undispersed oil. Prior studies have shown the formation of larger aggregates in the presence of oil after short term irradiation from sunlight (Sun *et al*., 2018). It was suggested that the toxicity of oil can be

enhanced by elevated oxidation stress via sunlight, causing changes in components such as EPS, polysaccharide production and cellular debris. Polysaccharides were shown to accumulate much more under light conditions compared to dark, as bacterial cells were secreting more protective polysaccharides and aggregating via hydrogen-bonding and/or cationic (Ca^{2+}) complexing (Chin *et al*., 1998; Zhou *et al*., 1998). As these aggregates act as the building blocks of MOS, we can assume sunlight would play a significant role in the growth of flocs over time. Observations have also been made suggesting the presence of Corexit may disrupt these aggregate formations, in turn disrupting floc formation over an extended period of time (Chiu *et al*., 2017). Flocs in oiled treatments, both with and without dispersant, were also shown to be more prone to floating, whilst all other unoiled flocs sunk. Due to the buoyancy of crude oil it is believed when oil integrates with flocs it will become positively buoyant (Omotoso *et al*., 2002). Over time MOS became negatively buoyant, through the biodegradation of crude oil compounds which increase in density over time as many of the lighter fractions are degraded first (Ławniczak *et al*., 2020).

Hydrocarbon degradation in the presence of marine oil snow

After 14 days, substantial hydrocarbon degradation took place across all oiled microcosms (Oil (Dark), 86.3% average degradation; Oil (Light), 85.1% average degradation; Oil and Dispersant (Dark), 86.6% average degradation and Oil and Dispersant (Light), 93.21% average degradation). During the formation of flocs, the majority of crude oil integrated with these flocs. Oil aggregates have previously been shown to be sites of high bacterial activity, notably oil-degrading activity, suggesting enhanced bacterial degradation compared to the surrounding environment (Ziervogel *et al*., 2012; Daly *et al*., 2016). This is potentially due to a natural increase in surface area availability to bacterial biofilms once the oil integrates with these aggregates. With

the addition of chemical dispersants, biodegradation was further enhanced, with a larger amount of degradation occurring within higher weight alkanes and PAH's. The use of chemical dispersants has previously been shown to enhance biodegradation (Baelum et al., 2012; Prince et al.,2013; Mu et al., 2014; Bacosa *et al*., 2015a; Techtmann et al., 2017; Thomas et al., 2021; Smallbone *et al*., 2024), by increasing the surface area of oil, thus increasing the bioavailability of oil to hydrocarbon degraders. With the increased dispersion of oil, the formation of MOS via bacteria-oil aggregation (BOA's), represented by biofilms forming at the oil-water interface, with oil droplets acting as a nuclei (Burd *et al*., 2020; Passow and Overton, 2021), may provide a greater surface area of crude oil, compared to untreated oil in which larger clumps of a slick would be integrated with pre formed marine snow (Gregson *et al*., 2021), Even so, once crude oil is integrated within flocs, the surface area available to bacteria would significantly increase even without dispersant use, leading to the substantial degradation that was observed across all microcosms. It was proposed that ectoenzyme activity associated detritus degradation may further increase surface area of particles over time for bacterial/biofilm growth, via an increase in microscale rugosity, hence the substantial bacterial activity for hydrocarbon degradation (Anderson *et al*., 2024). This suggests the presence of MS flocs may enhance biodegradation. Under light conditions, further degradation of larger *n*-alkanes (*n*-C29 – C37) and some larger weight PAH's (Benzo(a)anthracene, Chrysene, Indeno[123-cd]pyrene and various derivatives) occurred. Prior to this, it has been suggested that the susceptibility of various hydrocarbon compounds to biodegradation can be enhanced via photooxidation, via oxidation of hydrocarbon compounds making them more accessible for degradation (Dutta *et al*., 2000). Photooxidation itself has been shown to weather crude oil over time (Prince *et al*., 2003). In regard to this study, no

light/dark difference in degradation was observed within the oil only treatment, thus photooxidation was not a key player in enhancing the susceptibility of hydrocarbons to degradation. In regard to this, the lighting set up provided a majority of wavelengths associated photooxidation, however, UV-B (280-315 nm) was not provided as a limitation of the set up used. Even so, with the addition of dispersants key PAHs and their derivatives such as C2-Dibenzothithene, C2 Phenanthrene/Anthracene, Chrysene and Indeno(1,2,3-cd)pyrene were observed to be more degraded under light, being more susceptible to photooxidation (Aeppli *et al*., 2022). Prior studies have shown the use of dispersants has enhanced the photooxidation of parent and alkylated PAHs such as anthracene within seawater (Cai *et al*., 2017), notably by promoting the formation of $^1O_2$ playing a significant role in hydrocarbon degradation within floc communities.

Bacterial communities of marine snow in comparison to surrounding seawater

A significant change in bacterial composition was observed between communities associated with flocs and the surrounding seawater. Prior to this study this has consistently been observed across a variety of environments (DeLong *et al*., 1993; Acinas *et al*., 1999; Crump *et al*., 1999). Previously it was stated that flocs were shown to be sites of high bacterial activity, notably oil-degrading activity and potentially enzymatic activity associated with polysaccharide degradation, with exopolymeric substances (EPS) being a major component of floc formation. Arnosti *et al*. (2016) stated that the composition of bacterial communities associated with these aggregates were strongly selected for oil degradation and/or polysaccharide degradation due to the increased access to these hydrocarbon and EPS nutrient resources within floc structures compared to surrounding seawater, thus creating an observable change in community composition between floc and surrounding seawater communities. This

can be observed with the increased relative abundance of known potential hydrocarbon degraders *Alcanivorax*, *Alkanindiges*, *Thalassospira, Thalassomonas, Marinobacter*, *Marivita, Oleiphilus* and *Halioglobus* within oiled flocs (Prince *et al*., 2019; Kahla *et* al., 2021; Charalampous *et al*., 2024; Chen *et al*., 2024). This consortium of bacteria have been known to degrade a multitude of alkanes and aromatics, suggesting a high level of hydrocarbon degradation potential (Gauthier *et al*., 1992; Yakimov *et al*., 1998; Meyer *et al*., 1999; Golyshin *et al*., 2002; Bogan *et al*., 2003; Kodama *et al*., 2008). *Winogradskyella*, a chemoorganotrophic polysaccharide degrader (Nedashkovskaya *et al*., 2005; Alejandre-Colomo *et al*., 2020), was another prevalent genus present within flocs. Shown to be a prolific producer of EPS, this genus may have had a role in the formation of these flocs and the utilisation of various complex macromolecules associated with floc structures. *Winogradskyella* strains have previously been isolated from algal specimens (Kurilenko *et al*., 2019), suggesting in bacterial/algal relationship. This also aligned with the proportion of 16S algal reads (Supplementary material: Figure S9) found in floc samples during this study further suggesting a bacterial/algal relationship. In the presence of hydrocarbons, *Winogradskyella* have also been shown to elevate emulsifying activities. Alongside the additional dispersion effect of chemical dispersants within the current study (in which the highest relative abundance of *Winogradskyella* was observed), they may also play a significant role in enhancing biodegradation by increasing their water solubility (Kelkar *et al*., 2007; Calvo *et al*., 2009; Caruso *et al*., 2018). Many of these potential hydrocarbon degraders were also present within the surrounding seawater highlighting the hydrocarbon degradation potential of the whole microcosm system. This was further highlighted by the Ecological Index of Hydrocarbon Exposure (Lozada *et al*., 2014) (Supplementary material: Figure S8)

which showed approximately 18 – 40% of the floc communities and 10-20% of the surrounding seawater communities as potential hydrocarbon degraders. This also suggests flocs are microhabitats of heightened hydrocarbon degrading potential compared to their surrounding seawater environment.

Bacterial community changes of marine snow with light and dispersant use

Within the flocs the different light conditions had a major impact to bacterial assemblages. *Alkanindiges*, *Halioglobus*, *Marinomonas*, *Thalassospira* and *Winogradskyella* were noted to be selected under simulated natural light conditions, whilst *Acinetobacter*, *Alteromonas, Thalassolituus*, *Comamonas* and *Thalassomonas* were more abundant under dark conditions. During previous studies, observation on the impact of light availability and natural light have indicated significant shifts in bacterial community composition associated with biofilm communities and oil-degrading communities. Wagner *et al*. (2015) observed a similar shift with no major change in alpha diversity, similar to what was observed within floc communities in this study. They highlighted that during the investigation, heterotrophic communities relied heavily on phototroph derived organic matter under high light conditions, whilst switching to more complex polyphenolic compounds under decreasing light levels. This suggests that changes in phototrophic communities and the metabolites they produce may have a direct impact on associated heterotrophic communities (Bacosa *et al*., 2015b). During the present study algal growth (proportion of 16S algal reads) was influenced by the presence of light (Supplementary Material: Figure S9). Algal growth was higher within the surrounding seawater incubated under light across all the treatments and was higher in floc communities under light, incubated in seawater and dispersant controls. This suggests higher algal activity, thus a higher production of phototrophic derived organic matter, influencing the heterotrophic community of

marine snow. However, within oiled flocs algal growth was shown to be inhibited, potentially by the presence of localised hydrocarbon contamination (Othman *et al*., 2023), in which with the proportion of algal reads declined further in the light. Potentially the production of photooxidation products may have enhanced the toxicity of crude oil under the light, inhibiting algal growth (Daly *et al*., 2016). However, prior studies have highlighted the ability of algal isolates such as *Closterium* to possess a greater ability to degrade crude oil in the absence of sunlight, thus explaining a higher proportion of algal read within oiled flocs within the dark (Uzoh *et al*., 2015). Overall the presence of light was shown to significantly influence phototrophs, thus indirectly impacting heterotrophic floc communtiies. As well as influencing algal communities, photooxidation via light can directly impact bacterial communities by altering crude oil compositions contributing to an increase in the bioavailability of heavier weight hydrocarbons, notably within aromatics that was more susceptible to photic transformations (da Silva *et al*., 2021), thus supporting the growth of bacteria that may have a preference to these higher weight hydrocarbons as a carbon source (Wu *et al*., 2018; Mullaeva *et al*., 2022). Genera such as *Thalassospira* and *Marinomonas* were also uplifted within light conditions. Being potential degraders of PAHs such as pyrene and phenanthrene (Melcher *et al*., 2002; Kodama *et al*., 2008), photooxidation processes may have enhanced the bioavailability of these aromatics during the study, enhancing the uplift of these genera (Dutta and Harayama, 2000; Maki *et al*., 2001).

The impact of crude oil pollution and the use of chemical dispersants on bacterial communities has been well established in literature for decades, highlighting clear changes to bacterial community composition, notably selecting for hydrocarbon degrading bacteria (Gerdes *et al*., 2005; Jung *et al*., 2010; Kostka *et al*., 2011; Cabral *et al*., 2022). The present study highlights these same changes with genera

Alcanivorax, *Marinobacter*, *Thalassospira*, *Marinomonas*, *Alkanindiges*, *Halioglobus*, *Alteromonas* and *Winogradskyella* relative abundances increasing in the presence of hydrocarbon contamination. Further increases in relative abundance with the presence of dispersant were noted in *Halioglobus*, *Alcanivorax* and *Winogradskyella* specifically. Previously stated these genera are known potential hydrocarbon degraders. Notably, many of the hydrocarbon degrading bacteria associated with these flocs were not further uplifted with the presence of dispersant use, this is potentially explained by the process of MOS formation, in that MOS may develop with oil acting as a nucleus for microbial biofilm growth (Passow and Overton, 2021; Gregson *et al*., 2021). In this instance any oil that becomes entrapped within MOS would increase in surface area, thus increasing bioavailability to hydrocarbon degrading bacteria without the use of dispersants. While dispersant use may increase the chance of oil droplets becoming entrapped in flocs it would not necessarily increase the hydrocarbons bioavailability once entrapped. Further suggestions for this may be due to flocs being collected from a single time point during the current study due to experimental design limitations. Many degraders can dominate a community very early on during a spill event, initially targeting very low weight hydrocarbons, whilst other bacteria may appear much later on (Dubinsky *et al*., 2013), for example *Thomas et* al. (2021) observed an initial increase in *Marinomonas*, *Oleispira* and *Pseudoalteromonas* which were rapidly succeeded by *Cycloclasticus* and *Alcanivorax.* Thus, bacterial successions should also be considered, especially with dispersant use which has proven to influence succession over time.

Conclusion

Whilst dispersant use was shown to have minimal impacts to physical floc formations, crude oil and light were shown to have a considerable impact. Prior studies

would suggest the interaction between hydrocarbons and light may influence EPS and polysaccharide production, being a vital component of MOS formation. Further work into single floc structures should consider how the chemical composition of MOS changes over time in the presence of anthropogenic pollutants under multiple light conditions and how this links with their physical properties. It was also noted that oiled flocs were prone to positive buoyancy, only becoming less buoyant and prone to sedimentation over time as biodegradation occurs. As a high abundance of potential hydrocarbon degrading bacteria was observed within these flocs, degradation potential was shown to been high, with the potential to target a multitude of alkanes, aromatics and their derivatives, shown by an increase in hydrocarbon degrading bacteria abundance and the ecological index of hydrocarbon exposure being approximately 2-fold higher within floc communities compared to the surrounding seawater (Supplementary Material: Figure S8). This suggests degradation potential within floc communities is much greater that of the surrounding seawater. The majority of hydrocarbons had been heavily degraded across all treatments by the end of the study period, suggesting that the impact of changing light conditions and dispersant use, although positive, were minimal. This further highlights these microhabitats to be highly productive systems under both light and dark environments, conditions that these communities would face as they sink to the benthos. By the time they reach the benthos a substantial amount of degradation would have occurred leaving less volatile, less toxic, higher molecular weight compounds.

Supplementary Material

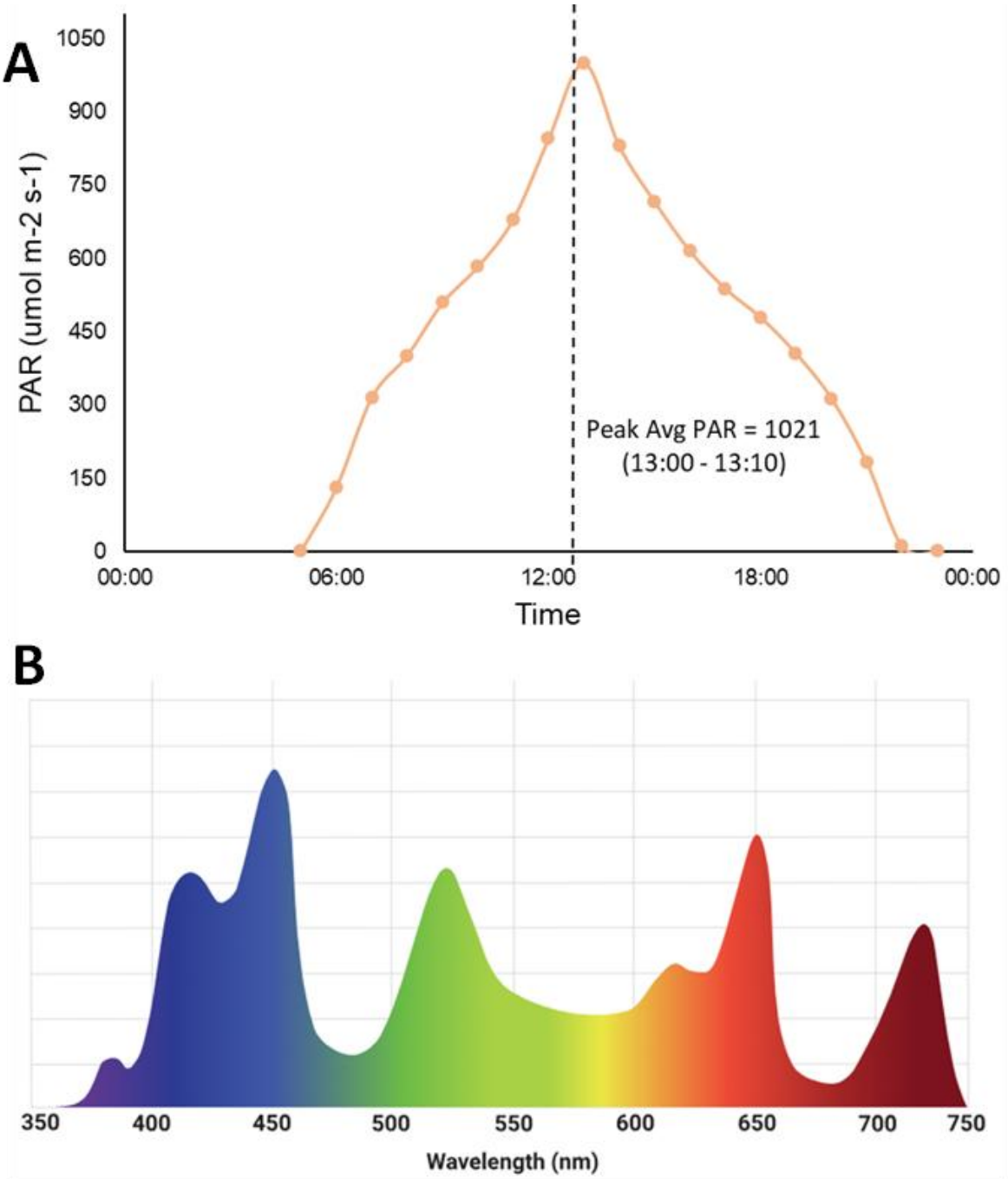


Figure S1: A. Simulated natural September 16:8 day:night cycle with photosynthetically active radiation (PAR) increasing from 0 to 1000 µmol $m^{-2}s^{-1}$ in the water column, staying at the maximum PAR for 10 minutes before decreasing to mimic natural light intensities *in-situ* (Dring et al., 2001), Peak average PAR = 1021 µmol $m^{-2}s^{-1}$. B. Wavelength (nm) emitted by DYNA Heliospectra range from 380 – 750 nm.

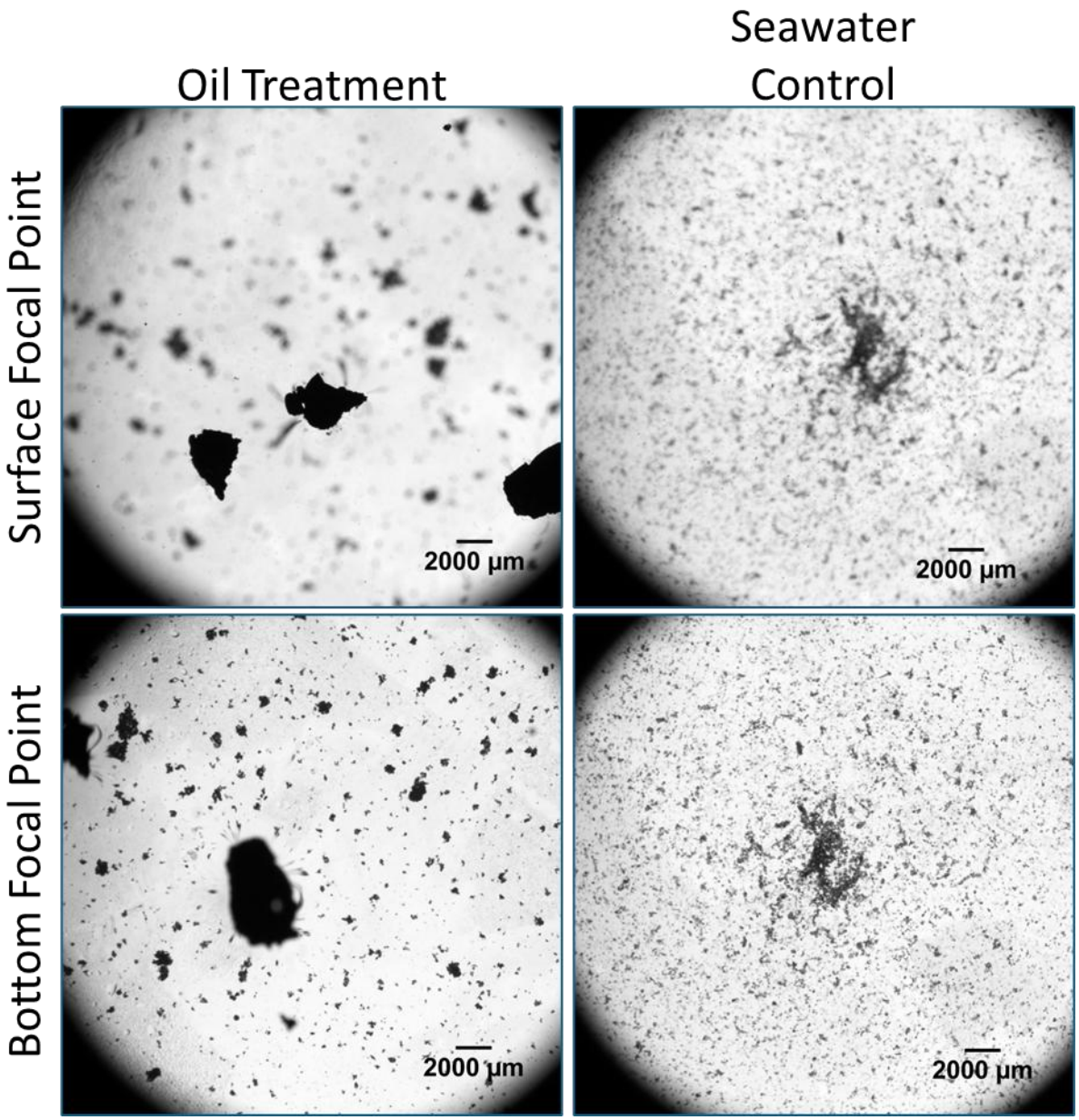


Figure S2: Raw images of Marine Oil Snow (MOS) and Marine Snow (MS) formations incubated over 14 days in 500 ml microcosms containing 400 ml natural seawater. Treatments shown include Seawater Control, and Oiled microcosm. Two focal points were imaged including the surface and bottom of the microcosms as to capture both negatively and positively buoyant MOS and MS flocs (Minimum detection size: 0.01mm2). Oil used: Norwegian Geochemical Standard North Sea Oil (NGS NSO-1), dispersant used :COREXIT™ EC9500A, Scale = 2000 µm (2mm).

Table S1: Table of Polyaromatic hydrocarbon compounds used in GC MS hydrocarbon concentration analysis. Compounds with an asterisk * were contained in the QTM PAH Mix (Sigma), all other parent compounds and their derivatives were added to the standard mix separately.

PAH compound	Ring Number	Mass	Quantitative ion (*m*/*z*)	Qualifier ion (*m*/*z*)
Naphthalene *	2	128	128	127
Methyl naphthalenes	2	142	142	141
Dimethyl naphthalenes	2	156	156	141
Acenaphthylene *	3	152	152	151
Acenaphthene *	3	154	154	153
Fluorene	3	166	166	165
Methyl fluorene *	3	180	180	165
Dimethyl fluorene *	3	194	194	179
Dibenzothiophene *	3	184	184	152
Methyl dibenzothiophenes *	3	198	198	
Dimethyl dibenzothiophenes *	3	212	212	
Phenanthrene	3	178	178	176
Anthracene	3	178	178	176
Methyl phenanthrene/anthracene *	3	192	192	191
Dimethyl phenanthrene/anthracene *	3	206	206	191
Fluoranthene	4	202	202	101
Pyrene	4	202	202	101
Benzo[a] anthracene	4	228	228	229
Chrysene	4	228	228	226
Benzo[b] fluoranthene	5	252	252	253
Benzo[k] fluoranthene *	5	252	252	250
Benzo[e] pyrene *	5	252	252	250
Perylene *	5	252	252	253
Indeno[123-cd] pyrene	6	276	276	277
Dibenz(a,h) anthracene	6	278	278	279
Benzo[ghi] perylene	6	276	276	277

* Contents of QTM PAH Mix

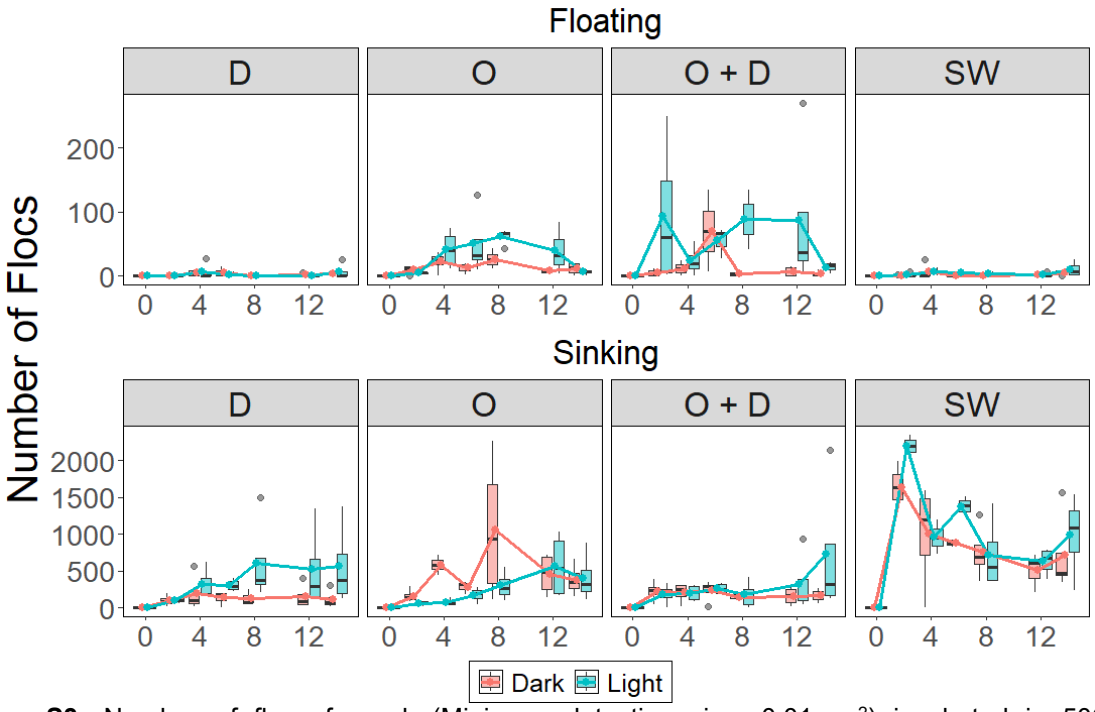


Figure S3: Number of flocs formed, (Minimum detection size: 0.01mm^2) incubated in 500 ml microcosms containing 400 ml natural seawater. Treatments include D = Dispersant Control, O = Oil, O + D = Oil and Dispersant, SW = Seawater Control (mean ± SE, n=3). Bottom = negativly bouyant flocs on the bottom of the microcosm, surface = positivly bouyant flocs on the surface of the microcosm. Oil used: Norwegian Geochemical Standard North Sea Oil (NGS NSO-1), dispersant used :COREXIT™ EC9500A.

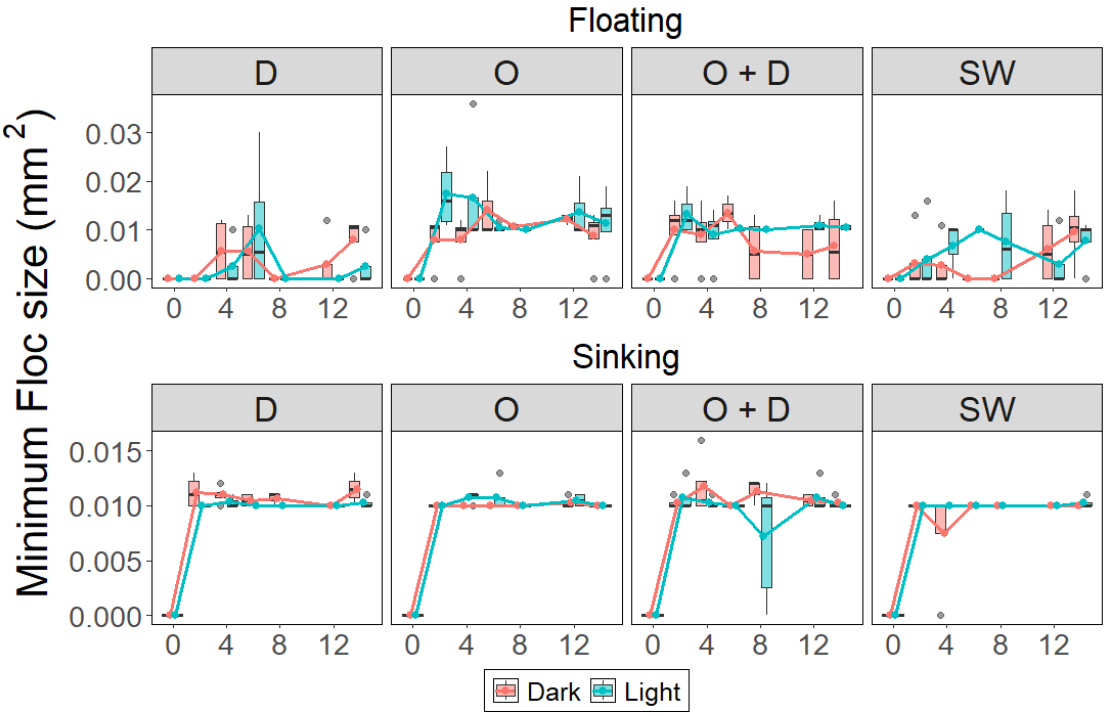


Figure S4: Minimum floc size (mm^2) (Minimum detection size: 0.01mm^2) incubated in 500 ml microcosms containing 400 ml natural seawater. Treatments include D = Dispersant Control, O = Oil, O + D = Oil and Dispersant, SW = Seawater Control (mean ± SE, n=3). Bottom = negativly bouyant flocs on the bottom of the microcosm, surface = positivly bouyant flocs on the surface of the microcosm. Oil used: Norwegian Geochemical Standard North Sea Oil (NGS NSO-1), dispersant used :COREXIT™ EC9500A.

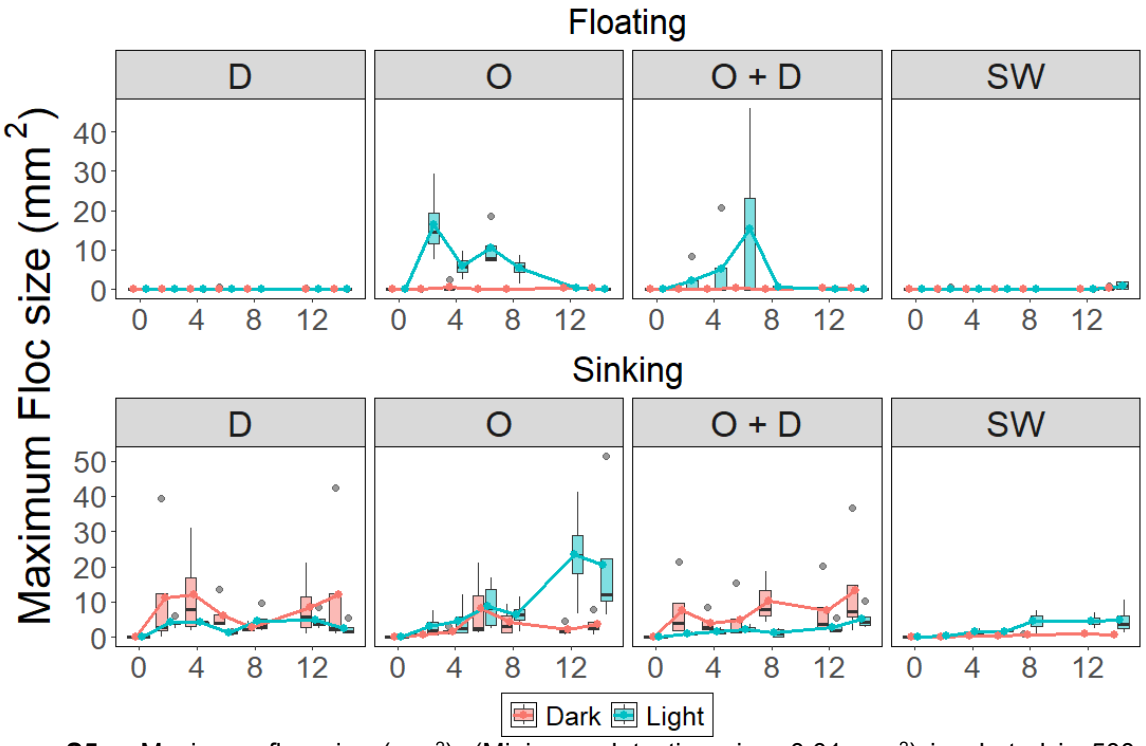


Figure S5: : Maximum floc size (mm²), (Minimum detection size: 0.01 mm²) incubated in 500 ml microcosms containing 400 ml natural seawater. Treatments include D = Dispersant Control, O = Oil, O + D = Oil and Dispersant, SW = Seawater Control (mean ± SE, n=3). Bottom = negativly bouyant flocs on the bottom of the microcosm, surface = positivly bouyant flocs on the surface of the microcosm. Oil used: Norwegian Geochemical Standard North Sea Oil (NGS NSO-1), dispersant used :COREXIT™ EC9500A.

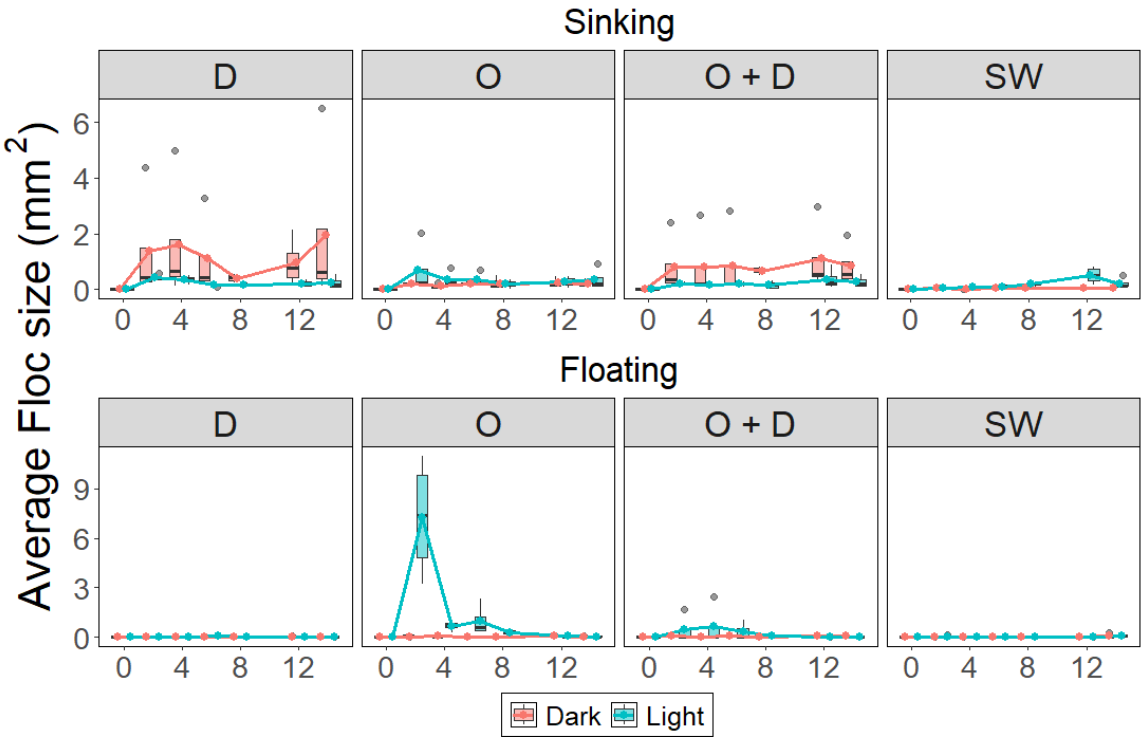


Figure S6: Average floc size (mm²) (Minimum detection size: 0.01 mm²) incubated in 500 ml microcosms containing 400 ml natural seawater. Treatments include D = Dispersant Control, O = Oil, O + D = Oil and Dispersant, SW = Seawater Control (mean ± SE, n=3). Bottom = negativly bouyant flocs on the bottom of the microcosm, surface = positivly bouyant flocs on the surface of the microcosm.

Oil used: Norwegian Geochemical Standard North Sea Oil (NGS NSO-1), dispersant used :COREXIT™ EC9500A.

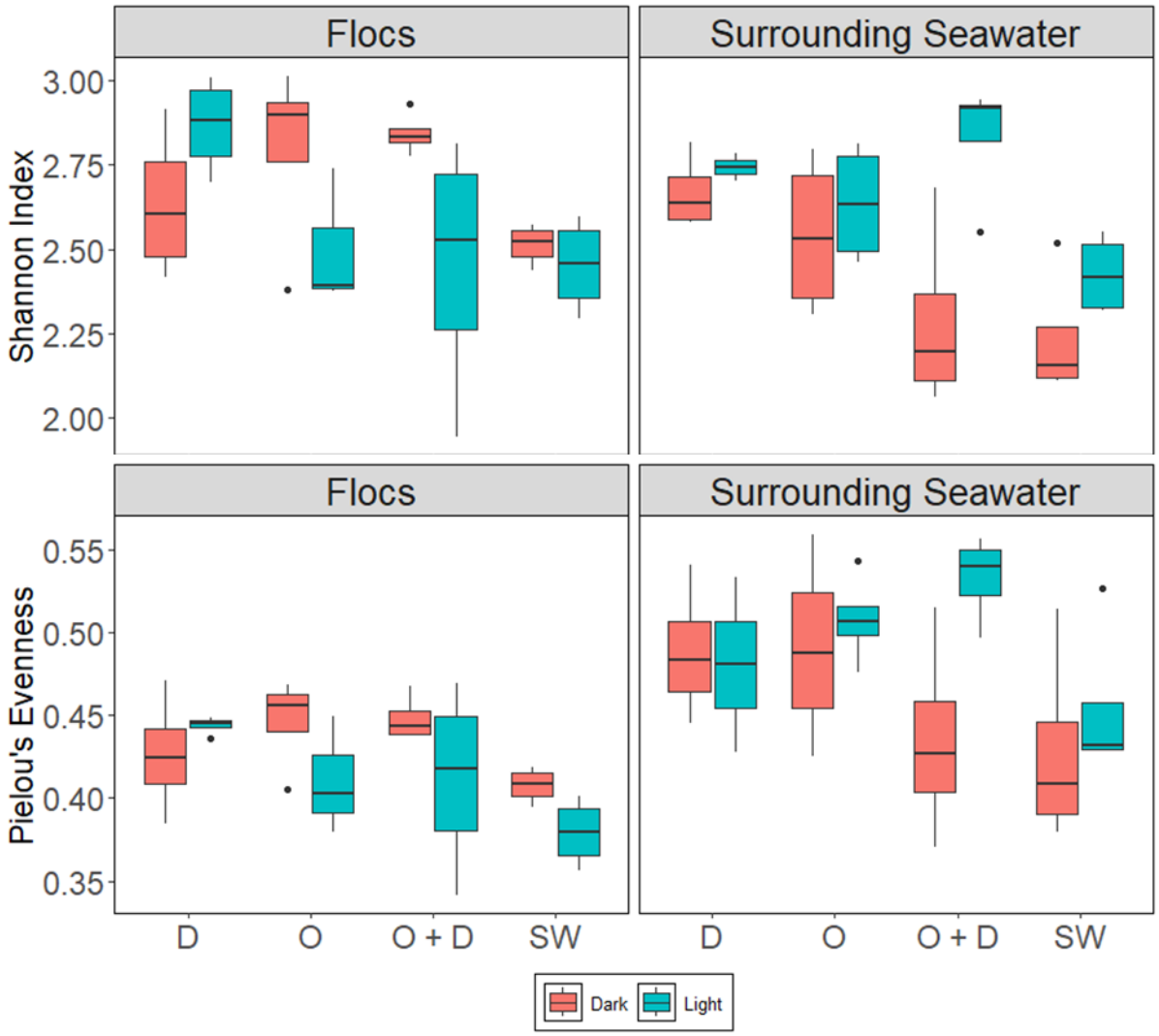


Figure S7: Diversity indices (Shannon index and Pielou's Evenness) of microbial communities of flocs and Surrounding Seawater (SSW) Extracts treated with and without the presence of Norwegian Geochemical Standard North Sea Oil (NGS NSO-1) and COREXIT™ EC9500A, collected after 6 days of incubation under complete darkness and day:night lighting cycle.

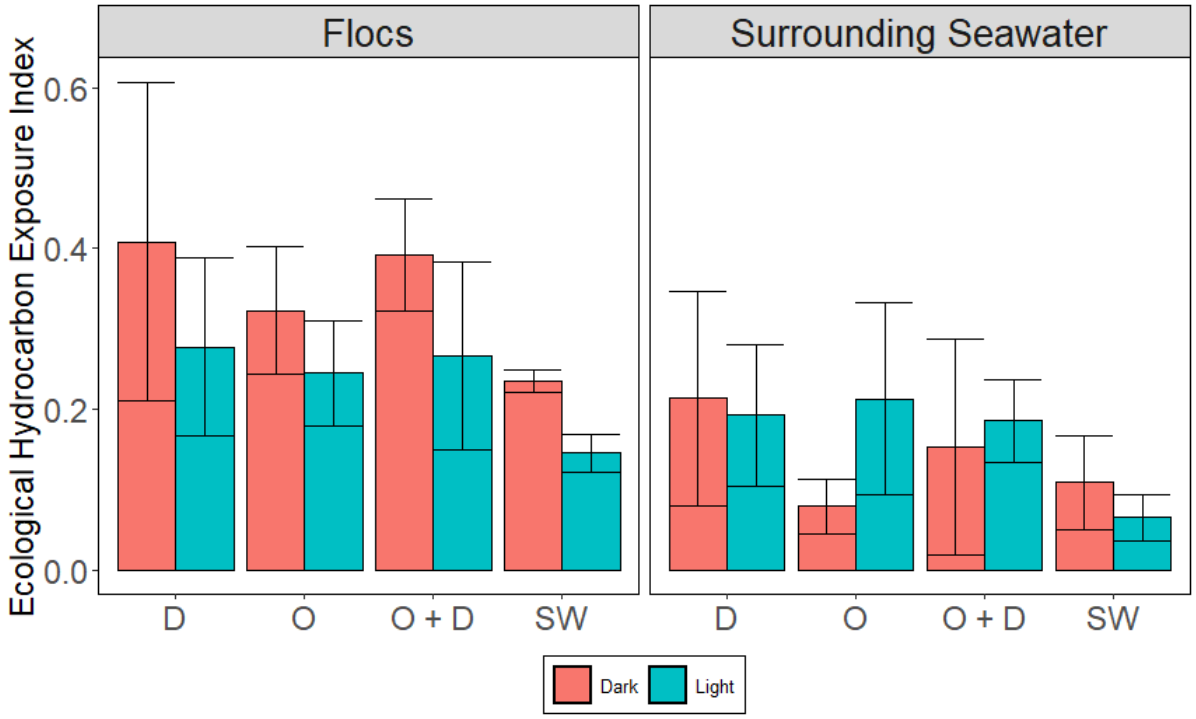


Figure S8: The Ecological Index of Hydrocarbon Exposure (EIHE; Lozado et al., 2014) of 16srRNA bacterial communities within flocs and surrounding seawater (SSW), incubated in 500 ml microcosms containing 400 ml natural North Sea seawater. Treatments include Dispersant Control, Oil, Oil and Dispersant and Seawater Controls (mean ± SE, n=3) 100 ppm concentrations of Norwegian Geochemical Standard North Sea Oil and dispersants (COREXIT™ EC9500A) were used at a 20:1 ratio, flocs collected 6 days after exposure under complete darkness and day:night lighting cycle.

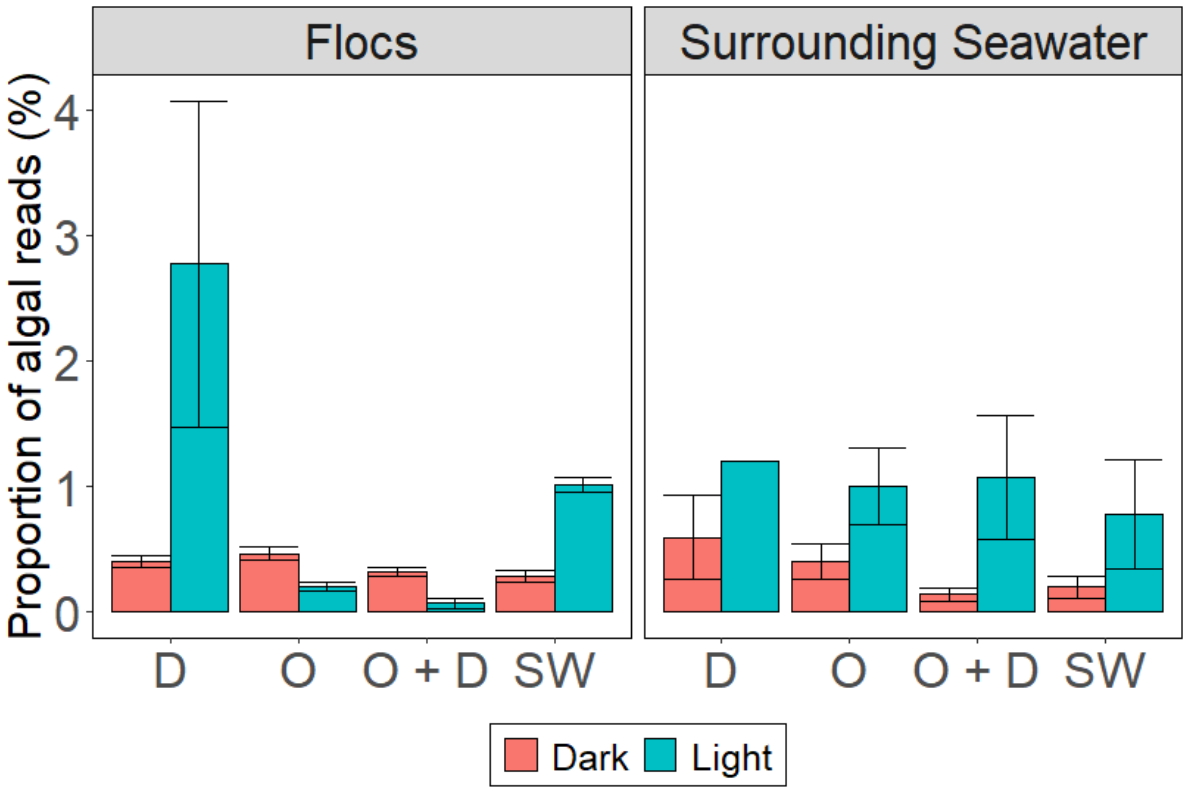


Figure S9: Proportion of Bacterial 16S rRNA amplicon sequence variants taxonomically classified as algae (chloroplasts), from communities within flocs and surrounding seawater (SSW), incubated in 500 ml microcosms containing 400 ml natural North Sea seawater. Treatments include Dispersant Control, Oil, Oil and Dispersant and Seawater Controls (mean ± SE, n=3) 100 ppm concentrations of Norwegian Geochemical Standard North Sea Oil and dispersants (COREXIT™ EC9500A) were used at a 20:1 ratio, flocs collected 6 days after exposure under complete darkness and day:night lighting cycle.

Chapter Four

The impact of crude oil and chemical dispersant use on the successional change of microbial communities within floating and sinking floc formations

Abstract

Marine snow (MS) flocs are sites of high levels of biological and enzymatic processes due to their nutrient rich, complex porous microstructures and rich microbial community. With the integration of crude oil to these structures during an oil spill event, marine oil snow (MOS) has been suggested to be a hotspot of hydrocarbon degradation, however, little is known about the succession of MOS microbial communities and their potential for hydrocarbon degradation. Therefore the aim of this study was to explore the impact of crude oil contamination and chemical dispersant use on the succession of prokaryote and eukaryote communities over 14 days. The ecological index of hydrocarbon exposure suggested that single MOS flocs had a high capacity for oil degradation, as they contained communities that consisted of 20-80% of 16S rRNA sequences from genera associated with hydrocarbon degradation. Successional changes of prokaryotes and eukaryotes over time was clear, notably being impacted by the presence of oil, selecting for a succession of obligate hydrocarbonoclastic bacteria such as *Oleispira, Alcanivorax* and *Cycloclasticus*, alongside other generalistic potential hydrocarbon degraders. The use of chemical dispersants caused a faster increase in the abundance of hydrocarbon degraders suggesting an increase in the bioavailability of oil within flocs. Eukaryotic relative abundance was reduced and algal community within flocs were highly impacted by the presence of oil, notably with diatoms such as *Entomoneis* and *Amphora* dominating oiled flocs, whilst protists dominated unoiled flocs, thus suggesting the use of eukaryotes as biomarkers may be beneficial during spill monitoring models. Overall,

MOS floc communities have been highlighted as sites of high hydrocarbon degrading activity and may be a viable source for exploring hydrocarbon degradation pathways further within both prokaryotes and eukaryotes.

Introduction

Marine snow (MS) is defined as suspended aggregates ≥500 μm in diameter that are made up of detritus, organic and inorganic matter, microalgae, bacteria, zooplankton, fungi and faecal matter, all bound together via an extracellular polymeric substance matrix acting as a skeletal structure (Alldredge *et al*., 1993; Daly *et al*., 2016; Passow and Ziervogel, 2016; Brakstad *et al*., 2018). These aggregates act as a microhabitat, containing a rich microbial community in which a variety of biological and chemical processes occur, such as photosynthesis, decomposition and nutrient cycling (Alldredge and Silver, 1988). Their relatively large surface-area to volume ratio due to the complex porous microstructure, which may also increase over time as ectoenzymes act on the surfaces of particles (Anderson *et al*., 2024) elevates these processes, making MS particularly effective in the transformation of organic matter and nutrients (Azam and Long, 2001; Mestre *et al*., 2018). MS has also been shown to be a significant driver within the biological carbon pump, as sinking particles export carbon and energy to the aphotic zone and sequester atmospheric CO_2 within deep sea benthic sediments (DeVrie *et al*., 2012). Following an oil spill, marine oil snow (MOS) may form when crude oil physically aggregates with naturally occurring MS, or via biofilm growth with microbial communities using oil particles as the aggregate nuclei (Bacteria–oil aggregates, BOA) (Passow and Overton, 2021; Gregson *et al*., 2021). During the 2010 Macondo spill (MC-252), MOS came to prominence as a potential vehicle for the transportation of crude oil to the deep sea benthos, during which it was estimated that 217,700 – 229,900 barrels (7% of estimated 3.19 million

barrels spilt) of extensively biodegraded crude oil residue was deposited in the form of MOS over approximately 7,600 km^2 via marine oil snow sedimentation and flocculent accumulation (MOSSFA) (Barbier, 2015; Stout and German, 2018). Notably, concerns arose around the use of chemical dispersants during the MC-252 spill, sparking debate as to their impact on MOS formation. It has since been suggested that MOS also formed and sedimented during other major spills in the past , such as the 1979 Ixtoc I and 1969 Santa Barbara blowouts , suggesting MOSSFA events to be more widespread and significant in crude oil transporation through the water column (Vonk *et al*., 2015; Lincoln *et al*., 2020).

Since the MC-252 spill, there has been considerable research around understanding MOS formation and microbial activity in association with chemical dispersant use (Passow, 2016; Passow *et al*., 2017; Wirth *et al*., 2018; Passow and Overton, 2021). Notably, MOS has been shown to harbour a high abundance of oil degrading bacteria, suggesting high levels of hydrocarbon biodegradation (Ziervogel *et al*., 2012; Daly *et al*., 2016; Suja *et al*., 2017). However many of these studies are specifically relevant to the conditions of the MC-252 spill which was a subsea wellhead spill at approximately 1600 metres and currently the only spill in which dispersants have been used subsurface. It is therefore difficult to extrapolate results from such studies to surface spill scenarios, where dispersant use is more common. Notably, prior studies have been carried out using water accommodated fraction (WAF) methods, which remove much of the volatile and insoluble fractions of oil (particulate oil) during the long mixing and settling time, retaining dissolved hydrocarbons (Suja *et al*., 2017; 2019). With the removal of these major fractions of oil, this neglects to take into consideration the influence of the majority of hydrocarbons on the system and limits the opportunity for certain oil degraders to attach to these particulate oil droplets,

thus may directly influence community composition (McFarlin and Prince, 2021). Therefore adding crude oil to microcosms as a slick and dispersing it to the industry standard of 20:1 (Oil: dispersant) may be a more suitable method for simulating an open water spill scenario.

Studies focused on the impact of hydrocarbon pollution and dispersant use on MOS floc communities and formation are somewhat limited and have typically been incubated under dark conditions or ambient lab light conditions (<30 μmol $m^{-2}s^{-1}$) simulating the low-light environmental conditions of deep water events such as the MC-252 spill (Passow, 2016; Passow *et al*., 2017; Suja *et al*., 2017; Wirth *et al*., 2018; Suja *et al*., 2019). Light availability, photo period and spectrum have all been shown to significantly influence microbial communities associated with biofilms and floc aggregates, notably in oil-degrading communities (Wagner *et al*., 2015; Bacosa *et al*., 2015), as these heterotrophic communities rely heavily on phototrophs and the metabolites they produce. Thus realistic lighting conditions should be considered to further understand MOS communities that initially form during sea surface spill events which are subjected to sunlight .

Furthermore, during the formation of MOS, the presence of crude oil influences mechanisms such as the buoyancy and sinking rate of flocs, as oiled flocs have been shown to be more prone to positive buoyancy (Omotoso *et al*., 2002). Changes in buoyancy may indicate the presence of oil integrated within flocs, as well as differences within other biological processes, such as algal and biofilm growth over time. Thus with changing buoyancy, differences in microbial communities may be observable when considering these factors.

As of 2025 the world liquid fuel production and consumption has reached approximately 104 million barrels per day, with forecasts estimating an increase to

between 105 – 107 million barrels per year by 2026 (U.S. Energy Information Administration, 2025). This increase therefore requires increases in crude oil exploration and the need for transportation via subsea pipeline and shuttle tankers in areas of limited pipeline infrastructure (Rystad Energy, 2022). Although only making up a small proportion of crude oil entering marine environments, tankers and oil exploration (well head blowouts) remains a significant source of anthropogenic pollution and threat to marine ecosystems due to the large quantities introduced over a small temporal and spatial scale (Academies of Science, 2022). With the prevalent risk of oil spills, understanding how hydrocarbon pollutants and oil spill dispersants impact marine ecosystems under surface spill conditions is paramount to informing spill response, which includes understanding the influence that oil and dispersants have on MOS formation, the microbial activity occurring within floc communities and how these may change over time throughout the water column. Therefore, this study aims to comparatively assess the succession of prokaryotic and eukaryotic communities within single marine snow flocs under relevant environmental conditions, to assess potential for hydrocarbon degradation with and without dispersant use.

Experimental design

Sample site

Surface seawater (16ºC). was sampled from the North Sea on the 18th September 2024, approximately 1 mile off of Mersea Island, Essex, UK (51.7509° N, 1.0001° E).

Microcosm design and floc sampling

Using 500 ml glass jars (9.9 cm Height, 8.6 cm Diameter) (Acid Washed 10% HCl) with PTFE-lined lids, microcosms were produced containing 400 ml of natural North Sea seawater, leaving air space to simulate wave action and minor turbulence

at the water surface when rolling. Microcosms included four treatments Seawater Controls (400 ml Seawater Only), Dispersant (400 ml Seawater plus 0.0005% v/v COREXIT™ EC9500A), Oil (400 ml Seawater with 0.01% v/v crude oil) and Oil plus Dispersant (400 ml Seawater with 0.01% v/v crude oil and 0.0005% v/v COREXIT™ EC9500A) in replicates of four. Norwegian Geochemical Standard North Sea Oil (NGS NSO-1) that was previously weathered at 60°C over 24 hours, was used at a concentration of 100 ppm (0.01% v/v), simulating initial concentrations often found during marine oil spill events (Lee *et al*., 2013). The dispersant COREXIT™ EC9500A (Nalco Holding Company) was used at the industry standard Oil:Dispersant ratio (20:1) (Fingas, 2011). Microcosms were placed horizontally in a bespoke roller table tank at approximately 26 rpm and were incubated over a period of 14 Days under a DYNA Heliospectra lighting system to emulate a natural 16:8 day:night cycle providing photosynthetically active radiation (PAR) increasing slowly from 0 to approximately 1000 µmol $m^{-2}s^{-1}$ within the microcosms, staying at the maximum PAR for 10 minutes before decreasing back to 0 µmol $m^{-2}s^{-1}$, mimicking surface PAR found across the North Sea (Dring *et al*., 2001). The bottles were partly submerged in seawater (the tank water being at the same level as the surface of the water within microcosms) at a constant surface temperature of 17-19°C to avoid the microcosms overheating and to provide more natural lighting from the surface. Single flocs were sampled from each microcosm at days 2, 4, 7, 9 and 14 using a 1000 µl pipette and moved to individual micro centrifuge tubes and frozen at -20^{0}C until processed for microbial community analysis. The microcosms were turned vertically and flocs were allowed to settle for 30 seconds so that both a positively buoyant (floating) and negatively buoyant floc (sinking) were sampled at each time point within all treatments when possible (n = 4)

(1 single floc from each microcosm). Floating flocs were only observed in the oil and oil plus dispersant treatments.

DNA extraction and qPCR analysis

DNA was extracted from each individual MOS floc using the DNeasy PowerSoil Pro Kit (Qiagen), following the manufacturer's instructions. DNA extracts were then frozen at -20^{o}C for further analysis. Quantitative real–time PCR (qPCR) was performed on a CFX Opus 384 Real-time PCR Detection System (Biorad), using the SensiFAST SYBR no-ROX kit (Bioline). Universal Primers 515F: 5'GTGYCAGCMGCCGCGGTAA3' and 926R: 5'CCGYCAATTYMTTTRAGTTT3' (Quince *et al*., 2011; Parada *et al*., 2016) were used to target the prokaryotic 16S rRNA and eukaryotic 18S rRNA genes. Gene abundance was quantified against a standard calibration curve of the 16S rRNA gene from 10^2 – 10^9 copies in 20 µl reactions as previously described (McKew and Smith, 2017).

Amplicon library preparation and bioinformatics

Amplicon library preparation was conducted following the *16S Metagenomic Sequencing Library Preparation* (Illumina, 2013) protocol, using a Veriti 96 well thermal cycler (Thermo Fisher). Primers used were the same as used in the qPCR analysis, with the addition of Illumina adapter overhang nucleotide sequences. AMPure XP beads (Beckman Coulter) were used to clean PCR products. At each PCR and clean-up step gel electrophoresis was performed for quality control. Final cleaned amplicons were quantified using the Quant-IT PicoGreen dsDNA Assay Kit (Thermo Scientific) on a FLUOstar Omega microplate reader (BMG LABTECH). Samples were then pooled together in a final library at equimolar concentrations. The final library was quantified using the NanoDrop 3300 Flourospectrometer (Thermo Scientific), following the Quant-IT PicoGreen dsDNA Assay kit protocol (Thermo Scientific). Amplicon

sequencing was provided by Novogene (Cambridge, UK) using the NovaSeq PE250 service. Sequences have been submitted to the NCBI SRA archive under the accession number PRJNA1226152.

Raw sequence data was processed following an in house bioinformatics script via a sun-grid engine (SGE) high performance cluster (HPC). Firstly, Cutadapt was used to trim the primers away from the sequences and to remove any sequences that did not contain both the forward and reverse primers (Maritn, 2011). Fastp (Chen *et al*., 2018) was used for quality control and sequencing filtering, removing any sequences if greater than 20% of bases are lower than q20. Following this, Usearch was used to merge forward and reverse pair-end reads (Edgar, 2010). At this stage only Prokaryotic 16S rRNA gene sequences were merged, whilst eukaryotic 18S rRNA gene sequences were moved to a separate working directory for further processing. Vsearch was used to then dereplicate sequences and remove singletons (Rognes *et al*., 2016). Unoise (USEARCH function) was then used to denoise sequences, producing amplicon variant sequences (ASVs), followed by using vsearch to map reads to ASV at a 99% similarity threshold. Taxonomy was assigned using DADA2 in R, using the silva_nr99_v138.1 train set to produce a Prokaryotic 16S rRNA ASV table for further analysis (Callahan *et al*., 2016; Mclaren and Callahan, 2021). The eukaryotic sequence reads were further processed using seqkit to convert the reverse reads to fastq (Shen *et al*., 2016). This cut the reads at 230bp, filtered any reads shorter than 230 and added an N to the end of each read. Forward reads were cut at 231bp and any reads lower than 230 were removed. Seqkit was further used to merge the forward and reverse eukaryotic reads. Further denoising and dereplication was performed using VSEARCH and USEARCH producing ASVs. Reads were then mapped to the ASVS at a 99% similarity threshold. Taxonomy assignment was

performed via dada2 using the silva_132.18s.99_rep_set to produce a Eukaryotic 18S rRNA ASV table for further analysis (Morien and Parfrey, 2018).

Statistical Analysis

Prokaryotic 16S rRNA and Eukaryotic 18S rRNA sequence data was rarefied to the lowest read depth (10,931 and 10,540 respectively), Diversity indices and community composition analysis were conducted using vegan (Oksanen *et al*., 2024). Further analysis included a SIMPER (Similarity Percentage) (Clarke, 1993) analysis within the vegan package to highlight genera causing Bray-Curtis dissimilarity (Bray and Curtis, 1957) between communities over time and between treatments. Multivariate generalised linear modelling through R was used to determine any significant differences between means (R Core Team, 2023).

Results

qPCR microbial abundance analysis

Microbial small subunit rRNA gene abundance within flocs was analysed with a single primer set that amplifies both prokaryotic (Bacterial and Archaeal) 16S rRNA genes and eukaryotic 18S rRNA genes to assess differences in gene abundance between treatments and the positively buoyant (floating) vs negatively buoyant (sinking) flocs (Figure 4.1). The proportions of 16S rRNA vs 18S rRNA genes copies analysed via qPCR were estimated from the relative number of reads assigned to prokaryotes (Bacteria and Archaea) or eukaryotes respectively within the amplicon libraries. Prokaryotic 16S rRNA gene abundance (99.99% Bacteria, 0.01% Archaea; based on estimates from the relative number of reads assigned to each within the amplicon libraries) was observed to be approximately 100-fold lower in sinking flocs incubated in the oil plus dispersant treatment compared to all other treatments at day 2 ($t = -2.37$, $p < 0.05$) but by day 7 gene abundance was similar across all treatments.

Sinking flocs incubated in oil, seawater control and dispersant control treatments experienced a 10-fold increase by day 14 ($z = 2.39 – 9.35$, $p < 0.001$) (Figure 4.1A). Prokaryotic 16S rRNA gene abundance was on average 10-fold higher by day 9 within sinking flocs compared to floating flocs ($z = -3.60$, $p < 0.001$) (Floating flocs were only present in the oil and oil plus dispersant treatments) (Figure 4.1A,B). Eukaryotic 18S rRNA gene abundance within flocs increased approximately 100 – 10000-fold by day 14 within all treatments($z = 2.39 – 9.35$, $p < 0.001$) (Figure 4.1C,D). The 18S rRNA gene abundance was higher in seawater only controls throughout the study period, being approximately 100 – 1000-fold higher by day 9, compared to all other treatments ($t = -5.14 – -2.63$, $p < 0.01$) (Figure 4.1C). 18S rRNA gene abundance was also shown to be approximately 10-fold higher throughout the study period in sinking flocs compared to floating flocs ($z = -6.43$, $p < 0.001$) (Figure 4.1C,D). Further analysis of 16S and 18S rRNA gene abundance calculated the prokaryote/eukaryote gene abundance ration (P/E ratio) to highlight changes over time and between treatments. The P/E ratio significantly declined across all treatments by approximately 90% ($t = -6.297$, $p < 0.001$) (Figure 4.1E,F). Further observations showed the P/E ratio was significantly lower in seawater controls compared to all other treatments ($t = 6.13 – 6.45$, $p < 0.001$), notably between day 4 – 9, being approximately 20-fold lower (Figure 4.1E).

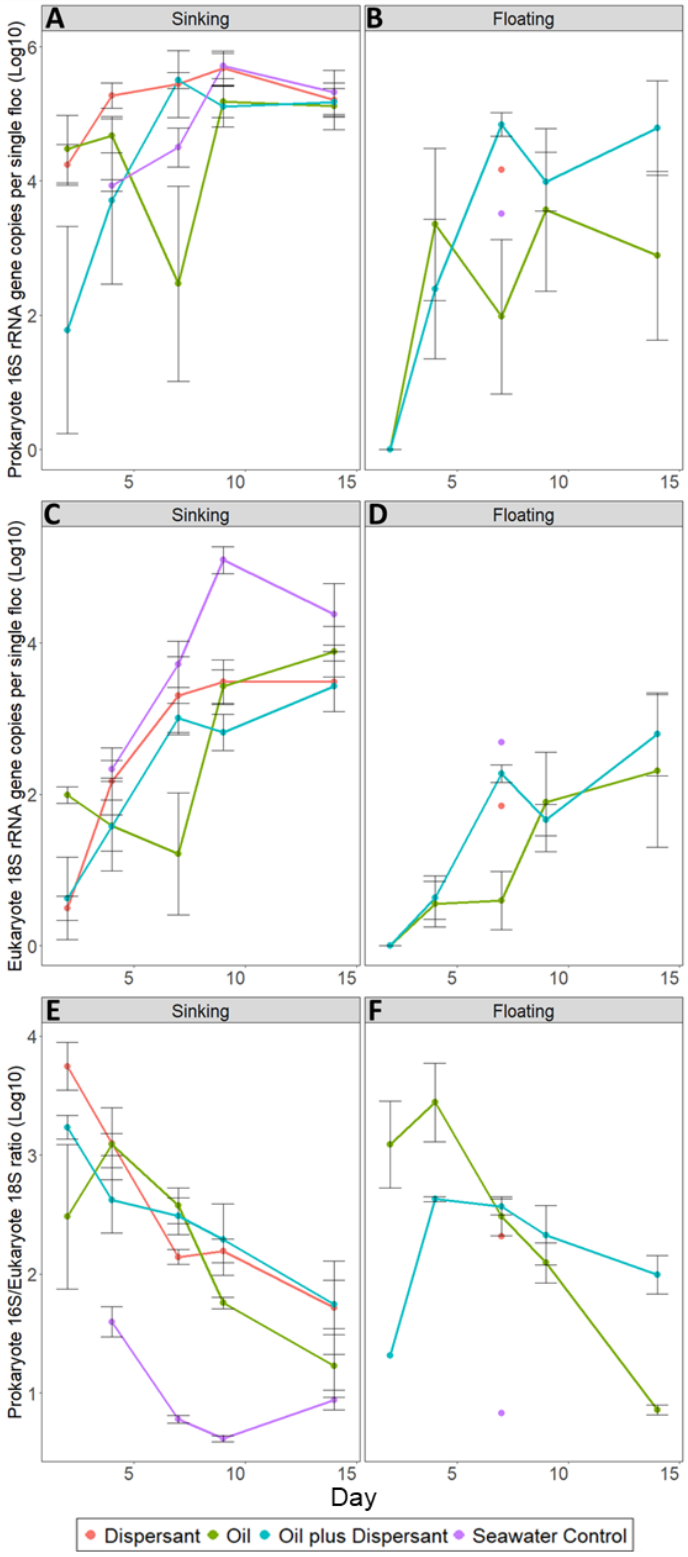


Figure 4.1: 16S/18S rRNA gene (copies per single floc (log10) within of sinking and floating single marine snow (MS) and marine oil snow (MOS) flocs (mean ± SE, n=4) from North Sea microcosm treatments Seawater Controls (400 ml Seawater Only), Dispersant (400 ml Seawater plus 0.0005% v/v COREXIT™ EC9500A), Oil (400 ml Seawater with 0.01% v/v crude oil) and Oil plus Dispersant (400 ml Seawater with 0.01% v/v crude oil and 0.0005% v/v COREXIT™ EC9500A) collected at days 2, 4, 7, 9 and 14. A) Prokaryote 16S rRNA gene abundance, B) Eukaryote 18S rRNA gene abundance, C) Prokaryote 16S rRNA/Eukaryote 18S rRNA abundance ratio. Oil used: Norwegian Geochemical Standard North Sea Oil (NGS NSO-1), dispersant used :COREXIT™ EC9500A.

Prokaryotic community composition analysis

Prokaryotic community composition showed significant dissimilarity between flocs within the seawater controls compared to all other treatments at days 4, 7, 9 and 17. ($F = 9.86$, $p < 0.001$) (Figure 4.2A,B,C,D). Significant dissimilarity in community composition was also observed between the oil plus dispersant and dispersant treatments by day 9 and 14 ($F = 7.45$, $p < 0.001$) (Figure 4.2C,D). By day 14 communities were significantly dissimilar to all other days across all treatments ($F = 1.99 – 2.36$, $p < 0.005$) (Figure 2D). Significant dissimilarity between communities within sinking and floating flocs was also observed ($F = 3.71$, $p < 0.05$), notably at days 7 and 9 in the oil treatment and days 7 and 14 in the oil plus dispersant treatment ($F = 2.89$, $p < 0.005$). (Figure 4.3).

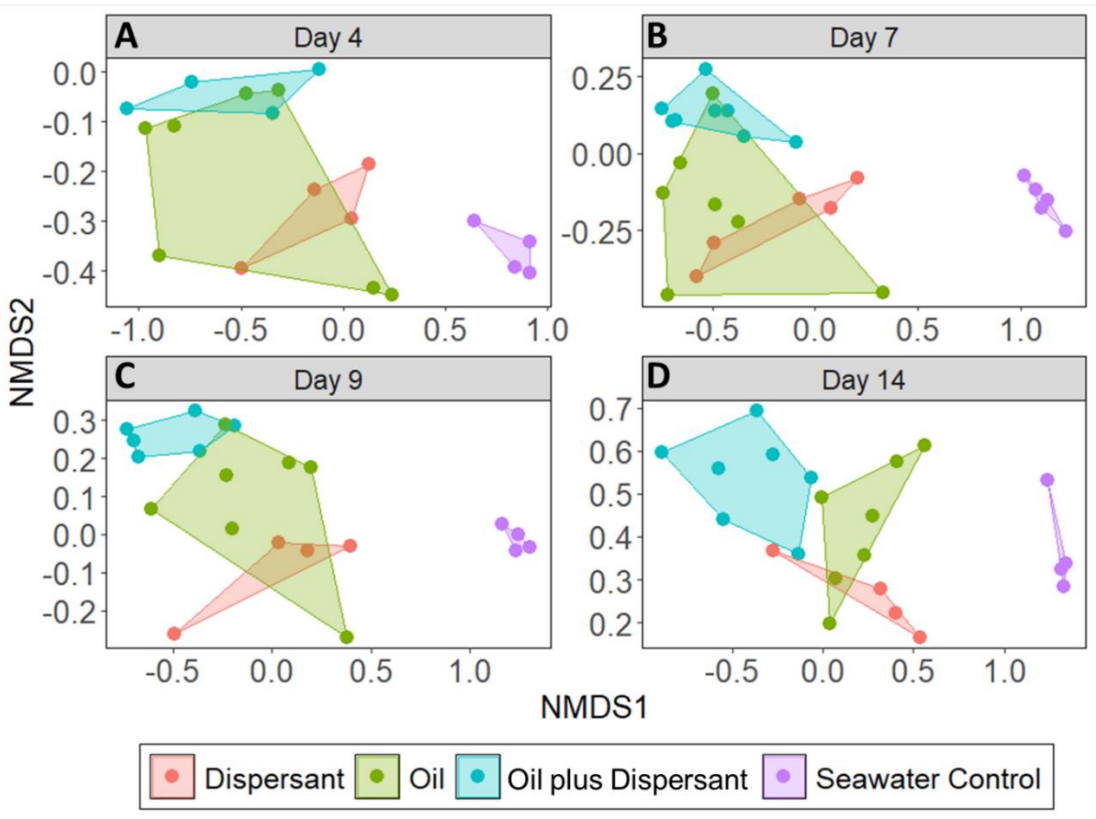


Figure 4.2: nMDS (non-metric multidimensional scaling) ordination using Bray-Curtis dissimilarity, based on ASV (amplicon sequence variants) of prokaryote 16S rRNA genes within single marine snow (MS) and marine oil snow (MOS) flocs incubated in microcosm treatments Seawater Controls (400 ml Seawater Only), Dispersant (400 ml Seawater plus 0.0005% v/v COREXIT™ EC9500A), Oil (400 ml Seawater with 0.01% v/v crude oil) and Oil plus Dispersant (400 ml Seawater with 0.01% v/v crude oil and 0.0005% v/v COREXIT™ EC9500A) over a period of 14 days. Oil used: Norwegian Geochemical Standard North Sea Oil (NGS NSO-1), dispersant used :COREXIT™ EC9500A.

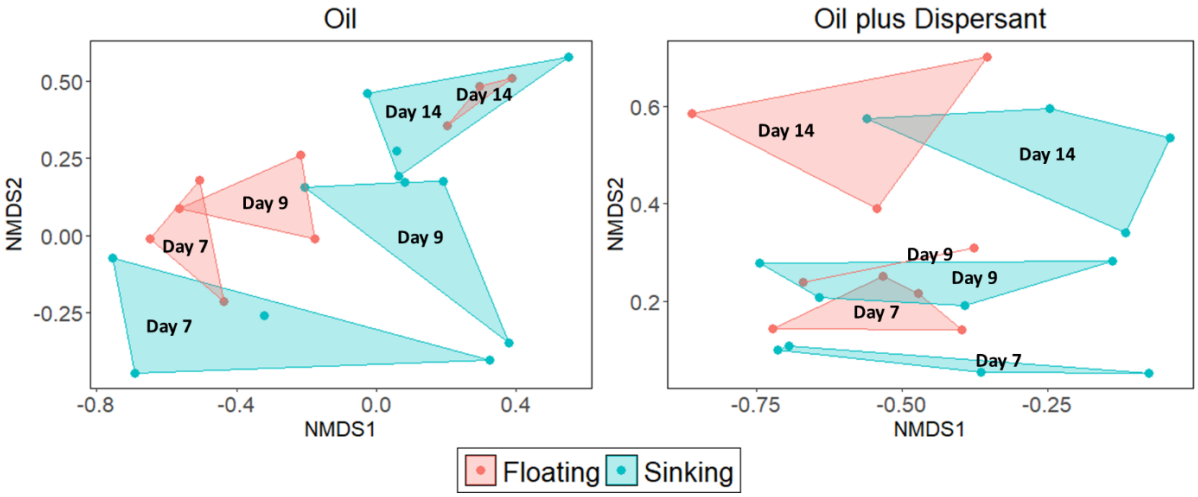


Figure 4.3: nMDS (non-metric multidimensional scaling) ordination using Bray-Curtis dissimilarity, based on ASV (amplicon sequence variants) of prokaryote 16S rRNA genes within sinking and floating single marine snow (MS) and marine oil snow (MOS) flocs incubated in microcosm treatments Oil (400 ml Seawater with 0.01% v/v crude oil) and Oil plus Dispersant (400 ml Seawater with 0.01% v/v crude oil and 0.0005% v/v COREXIT™ EC9500A), collected at days 4, 7 and 9 . Oil used: Norwegian Geochemical Standard North Sea Oil (NGS NSO-1), dispersant used :COREXIT™ EC9500A.

Amplicon sequence variant (ASV) richness of prokaryote communities was shown to be significantly lower in sinking flocs treated with oil plus dispersant compared to all other treatments throughout the study ($t = -3.712$, $p < 0.001$) (Figure 4.4A). In floating flocs ASV richness was significantly lower in the oil plus dispersant treatment compared to the oil treatment ($t = -2.98$, $p < 0.05$) (Figure 4.4B). Over time ASV richness significantly increased by approximatly 1.3-fold, in the dispersant treatments and oil treatment ($t = 5.24 – 5.63$, $p < 0.001$). Within the oil and oil plus dispersant treatment, ASV richness was approximately 1.2-fold higher in sinking flocs compared to floating flocs over the 14 days ($t = -2.1$, $p < 0.05$). The Shannon Index was significantly lower in oil plus dispersant treated flocs compared to seawater controls throughout the study ($t = 5.28$, $p < 0.01$) (Figure 4.4C). No significant change in shannon index was observed between sinking and floating flocs (Figures 4.4C, D). Pielou's evenness in sinking flocs was lower in the oil treatment compared to seawater controls on days 2 - 7, following a 1.2-fold increase to similar levels by day 14 ($t = -3.351$, $p < 0.005$) (Figure 4.4E) and there was no significant difference between sinking and floating flocs (Figure 4.4E, F).

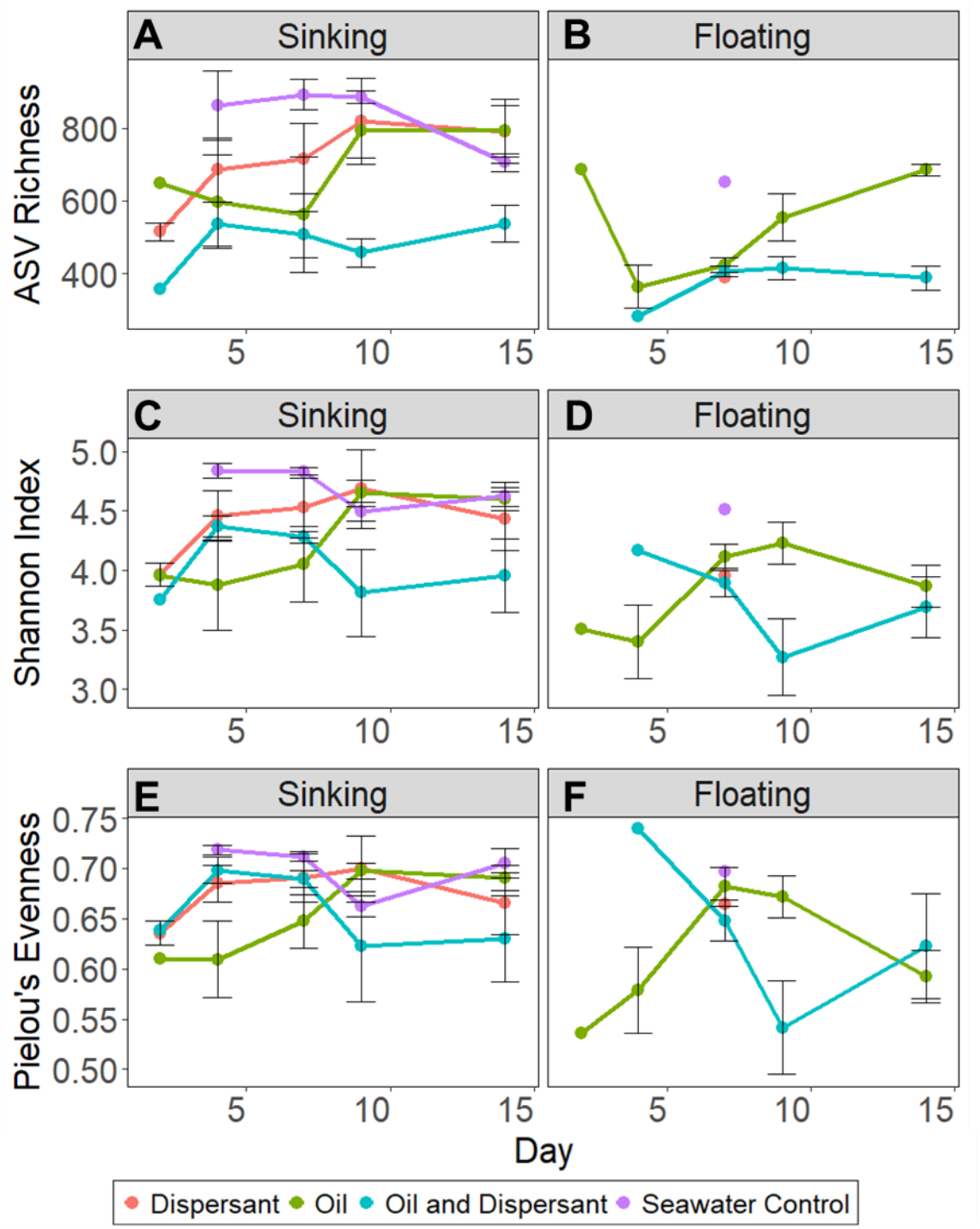


Figure 4.4: Diversity Indices (ASV Richness, Shannon Index, and Pielou's Evenness) of prokaryote 16S rRNA ASVs (Amplicon sequence variants), within sinking and floating single marine snow (MS) and marine oil snow (MOS) flocs (mean ± SE, n=4) incubated in microcosm treatments Seawater Controls (400 ml Seawater Only), Dispersant (400 ml Seawater plus 0.0005% v/v COREXIT™ EC9500A), Oil (400 ml Seawater with 0.01% v/v crude oil) and Oil plus Dispersant (400 ml Seawater with 0.01% v/v crude oil and 0.0005% v/v COREXIT™ EC9500A) collected at days 2, 4, 7, 9 and 14. Oil used: Norwegian Geochemical Standard North Sea Oil (NGS NSO-1), dispersant used :COREXIT™ EC9500A.

Obligate hydrocarbonoclastic bacteria (OHCB) *Alcanivorax, Cycloclasticus, Oleispira,* and *Thalassolituus* were highly abundant genera throughout the study and initial SIMPER analysis further highlighted their contribution to dissimilarity in community composition over time and between treatments (Figure 4.5). Amplicon sequence reads assigned to *Alcanivorax* were significantly higher in the oil plus dispersant treatment compared to all other treatments throughout the study period, peaking at day 9 at around 20% (Sinking flocs) and 40% (floating flocs) relative abundance of the community ($t = 2.17 – 5.09$, $p < 0.05$) (Figure 4.5A). By day 14 *Alcanivorax* was observed to be approximately 2.5-fold more abundant in floating flocs treated with oil plus dispersant ($t = -2.24$, $p < 0.05$). *Cycloclasticus* reads rapidly increased 5-fold in relative abundance by day 4 in both the oil and oil plus dispersant treatments, remaining significantly more abundant than in the seawater controls and dispersant treatment throughout the study period ($z = 2.66 – 2.74$, $p < 0.01$) before declining over time to approximately 1-2% of the community by day 14 (Figure 4.5B). *Oleispira* made up approximately 60% of the reads in floating flocs in the oil treatment on day 2 but then declined by day 4 to approximately 25%. Within sinking flocs in the oil plus dispersant and dispersant treatments, *Oleispira* made up 38% and 21% of the reads respectively, and again also significantly declined by day 4 to less than 1% ($z = -3.842 – -2.389$, $p < 0.05$) and was approximately 19% of the community in the oil treatment by day 4. *Thalassolituus* reads increased to approximately 2.5% by day 7 within flocs in the oil treatment, (due to high variance between replicates the difference was not statistically significant), before decreasing to below 1% by day 9 (Figure 4.5D).

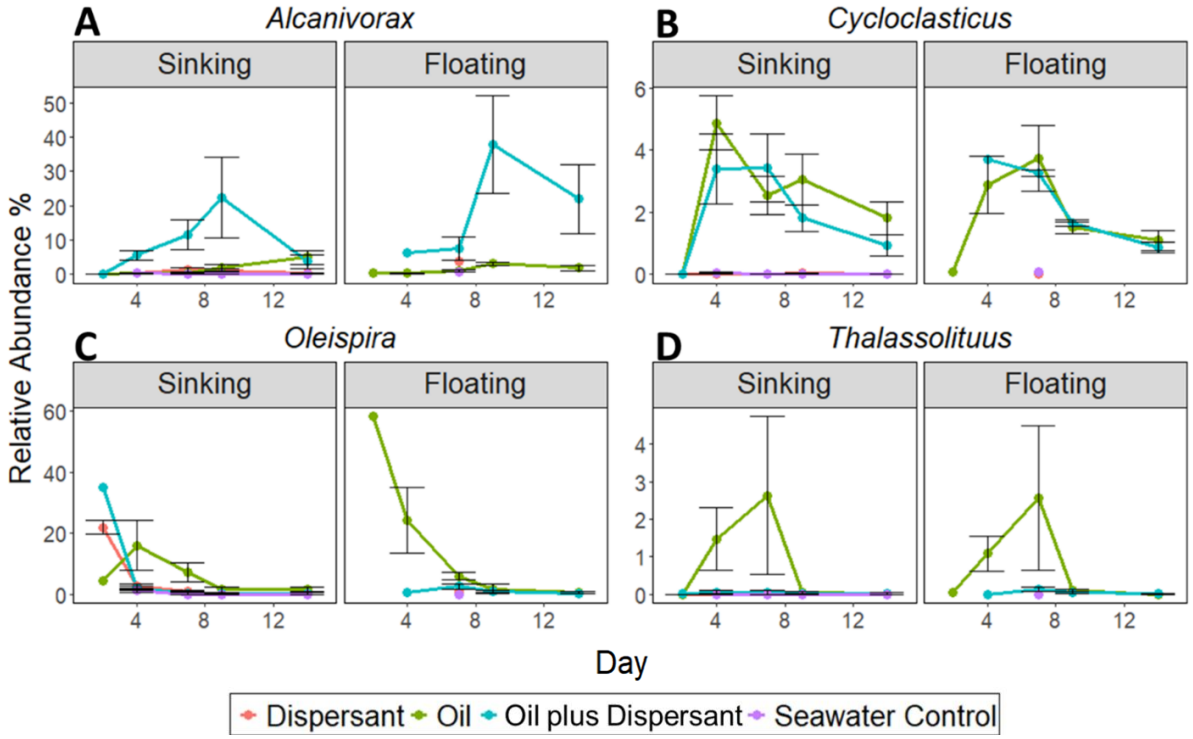


Figure 4.5: Percentage relative abundance of Obligate Hydrocarbon degrading bacteria (OHCBs) within prokaryote communities from sinking and floating single marine snow (MS) and marine oil snow (MOS) flocs (mean ± SE, n=4) revealed by 16S rRNA amplicon sequence variant (ASV) analysis. Microcosm treatments include Seawater Controls (400 ml Seawater Only), Dispersant (400 ml Seawater plus 0.0005% v/v COREXIT™ EC9500A), Oil (400 ml Seawater with 0.01% v/v crude oil) and Oil plus Dispersant (400 ml Seawater with 0.01% v/v crude oil and 0.0005% v/v COREXIT™ EC9500A) collected at days 2, 4, 7, 9 and 14. Oil used: Norwegian Geochemical Standard North Sea Oil (NGS NSO-1), dispersant used :COREXIT™ EC9500A.

Other genera were also highlighted through SIMPER (Figure 4.6). Reads assigned to *Colwellia* and *Psychrobacter* were highly dominant in oil, oil plus dispersant and dispersant treatments (7.5 – 20% relative abundance) at day 2, followed by a 2 – 4-fold decline by day 4 (Figure 4.6F, N), whilst *Cobetia* relative abundance was approximatly 6-fold higher in the sinking flocs within theoil treatment at day 2 (t = -8.81 – 4.05, $p < 0.005$), decreasing 4-fold by day 14 (Figure 4.6E). *Alkanindiges* and *Marinobacterium* were relatively more abundant in the oil and oil plus dispersant treatments at days 4, 7 and 9 (3 – 8% relative abundance) (*Alkanindiges* decreasing to below 1% by day 14) (Figure 4.6B, J), whilst *Aurantivirga* was observed to be approximatly 2-fold higher in relative abundance in floating flocs within the oil and oil plus dispersant treatments at day 7 (t = 2.15 – 5.67, $p < 0.05$)

(Figure 4.6D). By day 14 *Erythrobacter* and *Seohaeicola* were 3.3 – 5-fold more abundant in both sinking and floating flocs incubated in the oil plus dispersant treatment (t = 3.43 – 9.06, $p < 0.001$) (Figure 4.6G, O). *Olleya* and *Alteromonas* assigned reads had increased 4 – 5-fold to aproximately 5 – 20% of the community in sinking flocs within the dispersant treatment by day 2, followed by a reduction to less than 1% of the community by day 14 (t = 2.58 – 7.97, $p < 0.05$) (Figure 6C, K). In the seawater controls, *Loktanella* was was most dominant ay day 4 (approximately 8% of the community), following a steady decline to aproximatly 4% by day 9) (Figure 4.6I). This was followed by a large increase in *Algibacter*, *Lewinella* to approximatly 1.5 and 30% maximum relative abundance respectivly by day 9 , with *Polaribacter* increasing to aproximatly 10% by day 14 (t = -11.51 – -2.68, $p < 0.05$) (Figure 4.6A, H, L).

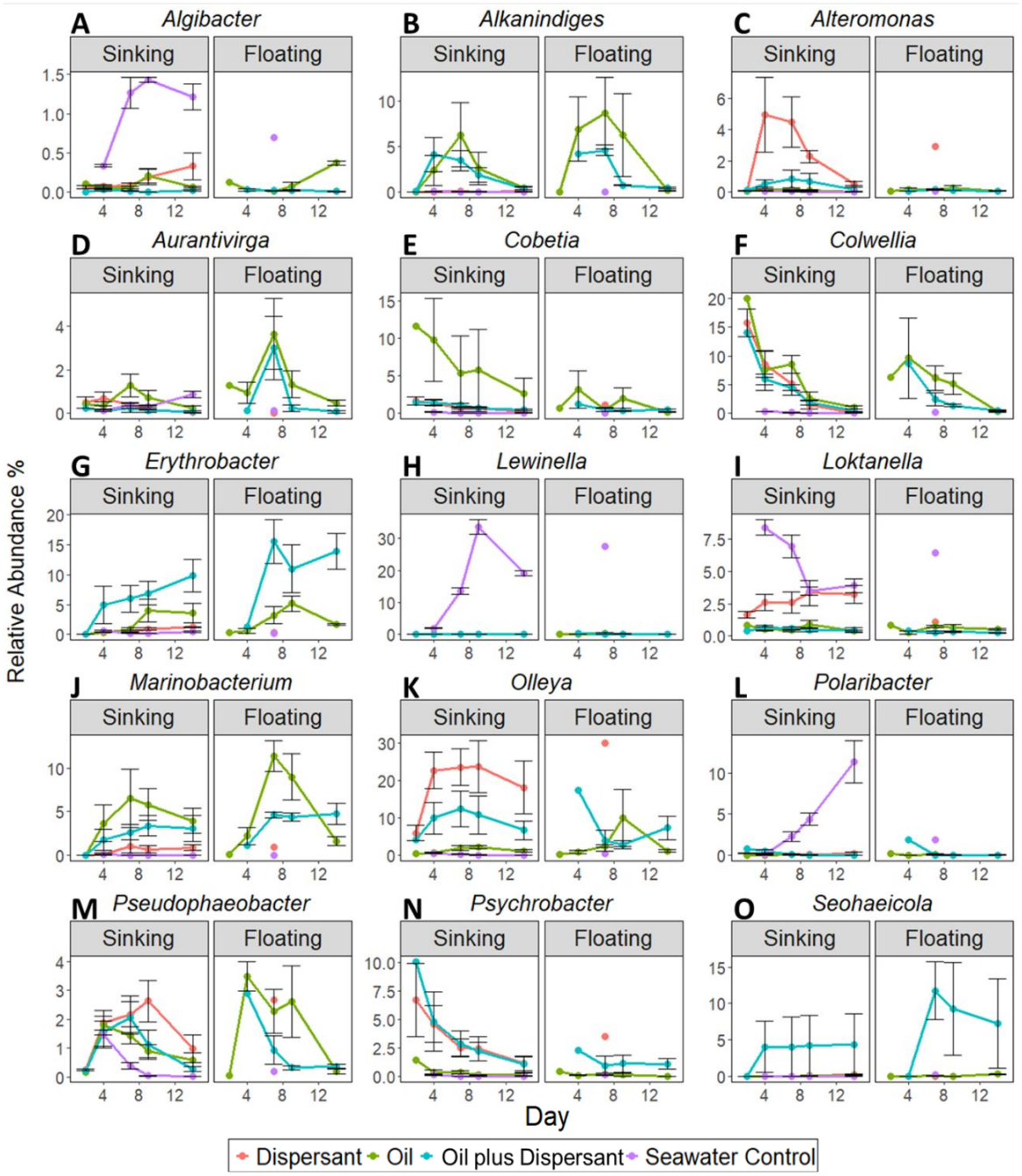


Figure 4.6: Percentage relative abundance of prokaryote genera within prokaryote 16S rRNA communities from sinking and floating single marine snow (MS) and marine oil snow (MOS) flocs (mean ± SE, n=4) incubated in microcosm treatments Seawater Controls (400 ml Seawater Only), Dispersant (400 ml Seawater plus 0.0005% v/v COREXIT™ EC9500A), Oil (400 ml Seawater with 0.01% v/v crude oil) and Oil plus Dispersant (400 ml Seawater with 0.01% v/v crude oil and 0.0005% v/v COREXIT™ EC9500A) collected at days 2, 4, 7, 9 and 14. Oil used: Norwegian Geochemical Standard North Sea Oil (NGS NSO-1), dispersant used :COREXIT™ EC9500A.

Ecological index of hydrocarbon exposure

The Ecological Index of Hydrocarbon Exposure (EIHE), (metric based on the percentage of reads in 16S rRNA sequence libraries assigned to 63 genera that are linked to hydrocarbon degradation (Lozada et al 2014)) was lower in the seawater

control throughout the study period, being approximately 20-fold lower at day 14 (Figure 4.7) (t = 3.62 – 5.12, $p < 0.001$). By day 2 the EIHE was approximately 70% and 40% in floating flocs within the oil, and oil plus dispersant treatments respectively. By day 14 all treatments experienced a significant decline in EIHE to 0.1 – 0.2 (10 – 20%)

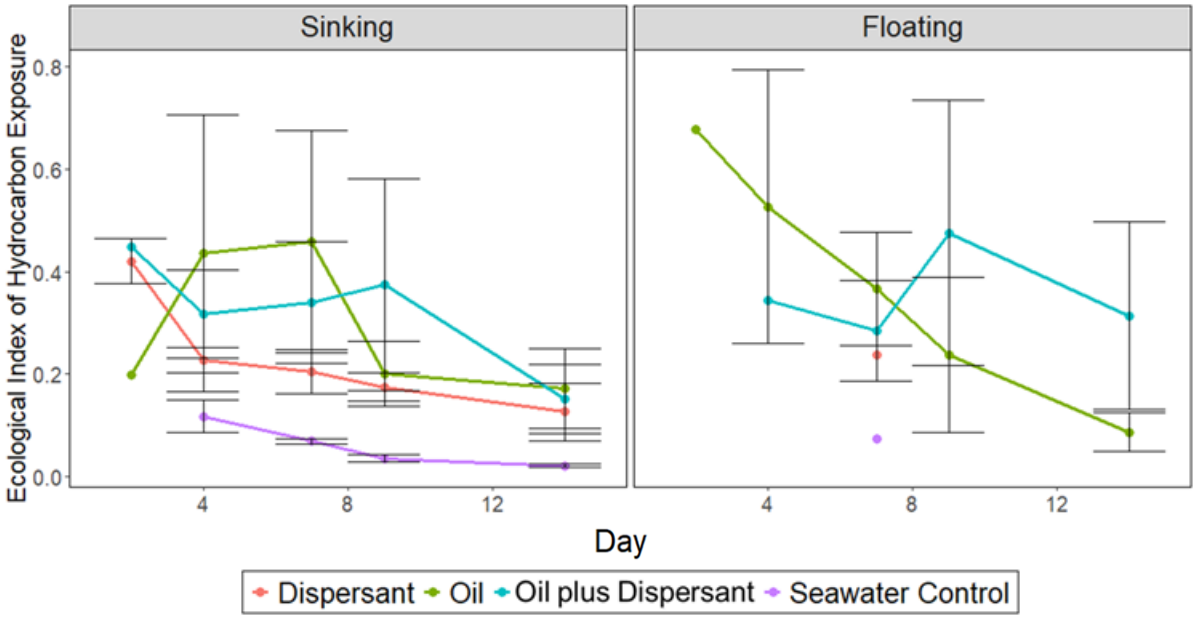


Figure 4.7: Ecological Index of Hydrocarbon Exposure (EiHE) within sinking and floating single marine snow (MS) and marine oil snow (MOS) flocs (mean ± SE, n=4) incubated in microcosm treatments Seawater Controls (400 ml Seawater Only), Dispersant (400 ml Seawater plus 0.0005% v/v COREXIT™ EC9500A), Oil (400 ml Seawater with 0.01% v/v crude oil) and Oil plus Dispersant (400 ml Seawater with 0.01% v/v crude oil and 0.0005% v/v COREXIT™ EC9500A) collected at days 2, 4, 7, 9 and 14. EiHE is a metric based on the percentage of sequence reads assigned to genera with known hydrocarbon degrading species , 0 = 0%, 1 = 100%. Oil used: Norwegian Geochemical Standard North Sea Oil (NGS NSO-1), dispersant used :COREXIT™ EC9500A.

Eukaryotic community composition

Brays-Curtis dissimilarity was calculated on eukaryote sequences and visualised by nMDS (non-metric multidimensional scaling) (Stress value = 0.012). Significant dissimilarity between community composition within the seawater controls and all other treatments was observed at each time point during the study period (F = 7.44 – 9.06, $p < 0.001$) (Figure 4.8A, B, C, D). By day 9 and 14, the eukaryotic communities in all treatments were significantly dissimilar to each other (F = 2.03 – 4.16, $p < 0.001$) (Figure 4.8C, D). Significant dissimilarity in community composition

was also observed in oil treatments between sinking and floating flocs a day 9 and 14 ($F = 1.92 – 2.52$, $p < 0.05$) (Figure 4.9). No significant dissimilarity between sinking and floating flocs was observed in the oil plus dispersant treatment.

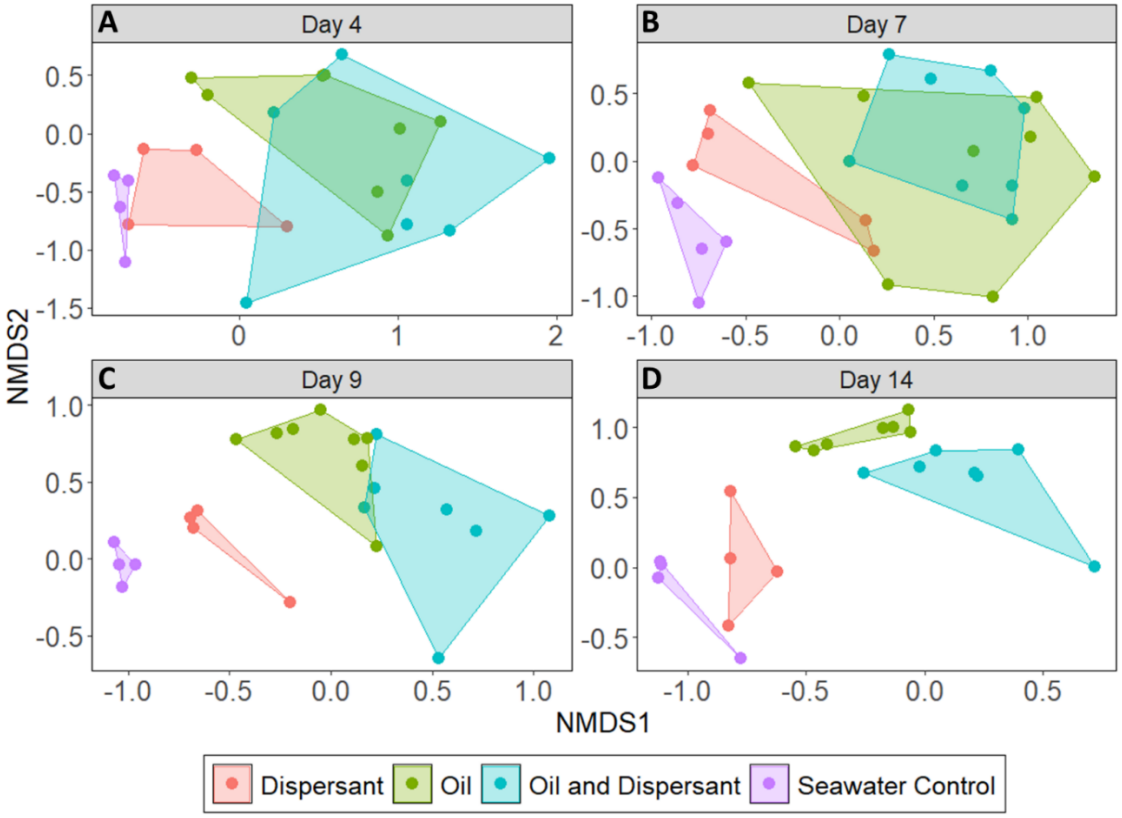


Figure 4.8: nMDS (non-metric multidimensional scaling) ordination using Bray-Curtis dissimilarity, based on ASV (amplicon sequence variants) of eukaryote 18S rRNA genes within single marine snow (MS) and marine oil snow (MOS) flocs incubated in microcosm treatments Seawater Controls (400 ml Seawater Only), Dispersant (400 ml Seawater plus 0.0005% v/v COREXIT™ EC9500A), Oil (400 ml Seawater with 0.01% v/v crude oil) and Oil plus Dispersant (400 ml Seawater with 0.01% v/v crude oil and 0.0005% v/v COREXIT™ EC9500A) over a period of 14 days. Oil used: Norwegian Geochemical Standard North Sea Oil (NGS NSO-1), dispersant used :COREXIT™ EC9500A.

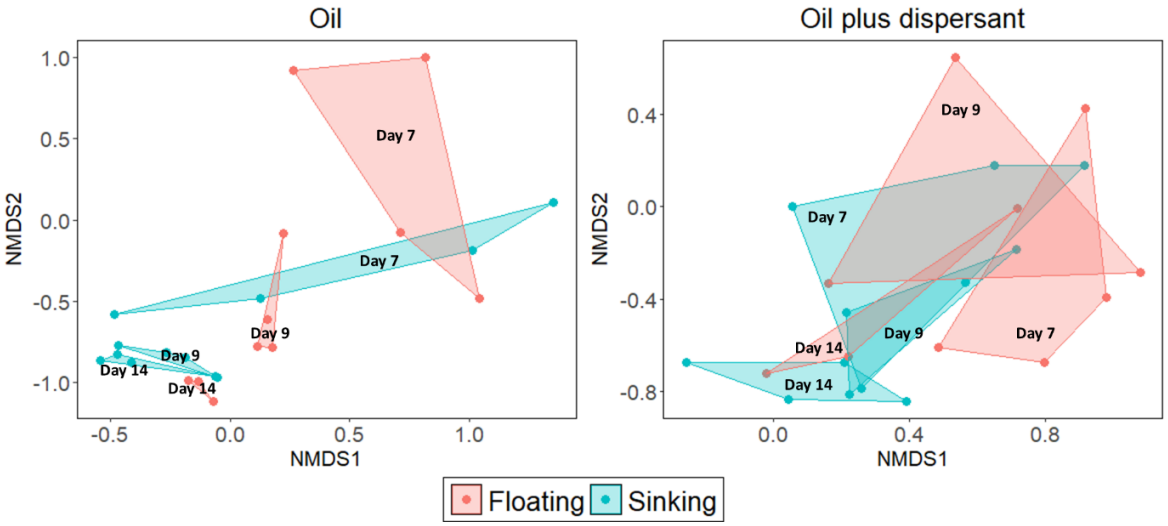


Figure 4.9: nMDS (non-metric multidimensional scaling) ordination using Bray-Curtis dissimilarity, based on ASV (amplicon sequence variants) of eukaryotic 18S rRNA genes within sinking and floating single marine snow (MS) and marine oil snow (MOS) flocs incubated in in microcosm treatments Oil (400 ml Seawater with 0.01% v/v crude oil) and Oil plus Dispersant (400 ml Seawater with 0.01% v/v crude oil and 0.0005% v/v COREXIT™ EC9500A) collected at days 4, 7 and 9. Oil used: Norwegian Geochemical Standard North Sea Oil (NGS NSO-1), dispersant used :COREXIT™ EC9500A.

By day 14, 18S rRNA ASV Richness of eukaryote communities increased across all treatments (t = 3.52 – 5.63, $p < 0.001$) (Figure 4.10A, B). 18S rRNA ASV richness within flocs from the oil plus dispersant treatment were 0.5-fold lower than seawater controls, (t = -3.71, $p < 0.001$). Sinking flocs were observed to have a higher 18S rRNA ASV richness throughout the study period compared to floating flocs (t = -2.10, $p < 0.05$). Shannon index was generally lower in the seawater controls throughout the study compared to all other treatments, (excluding day 4 (t =5.23 – 5.38, $p < 0.001$) (Figure 4.10C, D). No noticeable difference in 18S rRNA richness was observed between sinking and floating flocs in the oil and oil plus dispersant treatments. Pielou's evenness was approximatly 5-fold lower in flocs within the seawater controls by day 7 compared to all other treatments throughout the study period (t = 2.99 – 4.38, $p < 0.005$). All treatments experienced a gradual decline in Pielou's evveness over the 14 days (t = -3.35, $p < 0.005$) (Figure 4.10E, F). No difference in eveness was observed between sinking and floating flocs (t = -0.07 – 2.02, $p > 0.05$).

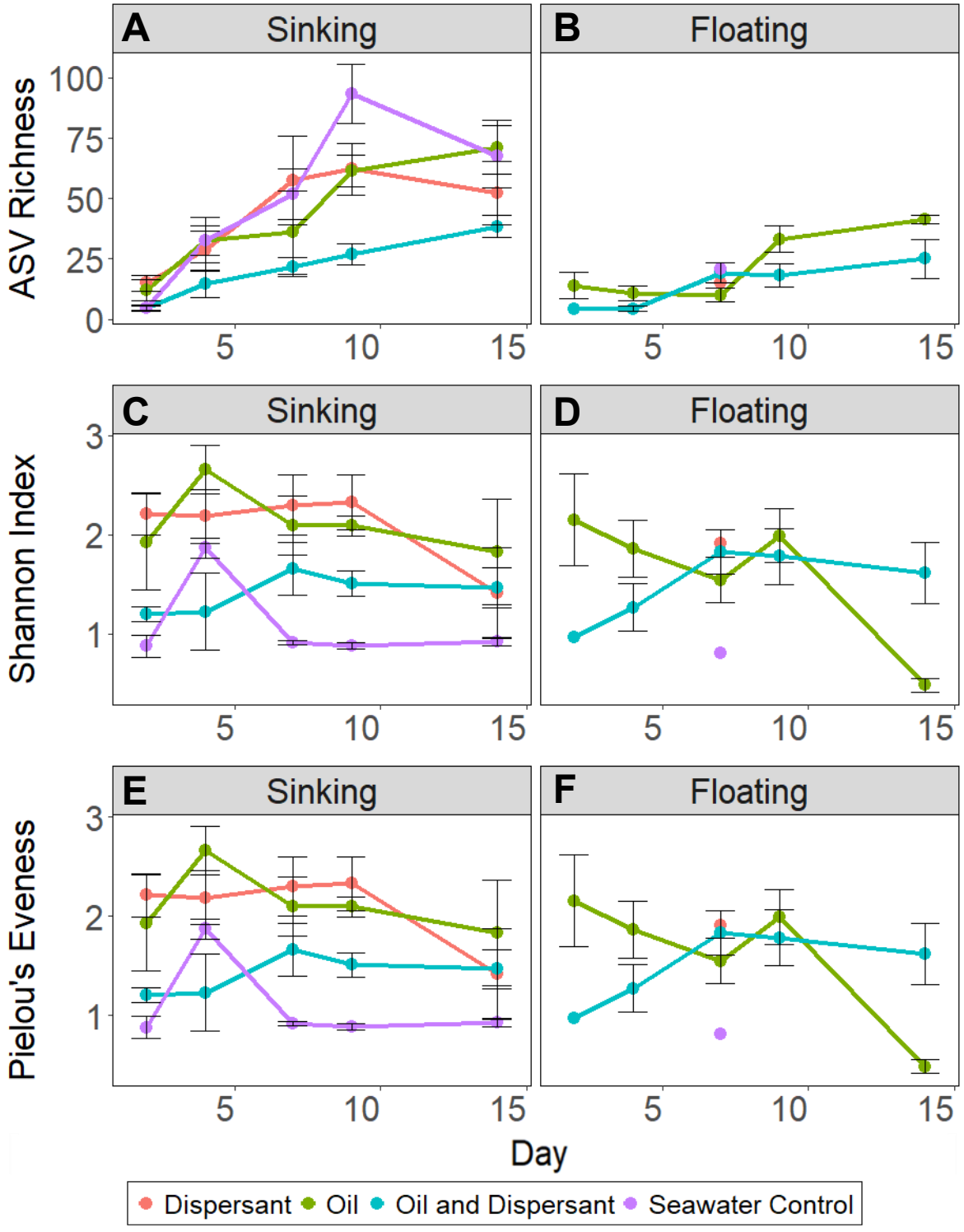


Figure 4.10: Diversity Indices (ASV Richness, Shannon Index, and Pielou's Evenness) of Eukaryotic 18S rRNA ASVs (Amplicon sequence variants), within sinking and floating single marine snow (MS) and marine oil snow (MOS) flocs (mean ± SE, n=4) incubated in microcosm treatments Seawater Controls (400 ml Seawater Only), Dispersant (400 ml Seawater plus 0.0005% v/v COREXIT™ EC9500A), Oil (400 ml Seawater with 0.01% v/v crude oil) and Oil plus Dispersant (400 ml Seawater with 0.01% v/v crude oil and 0.0005% v/v COREXIT™ EC9500A), collected at days 2, 4, 7, 9 and 14. Oil used: Norwegian Geochemical Standard North Sea Oil (NGS NSO-1), dispersant used :COREXIT™ EC9500A.

SIMPER analysis highlighted multiple genera that were causing the majority of dissimilarity in 18S rRNA composition between treatments and over time (Figure 4.11). Reads assigned to the ameboid genera *Parvamoeba* were highly abundant within flocs from all treatments, initially making up between 5 – 15% of eukaryotic reads before decreasing to below 1% by day 14 (Figure 4.11K). *Cymbella* and *Nitzschia* diatom reads were more dominant in floating flocs in the oil treatment at day 2 compared other treatments ($z = 3.05 – 3.34$, $p < 0.05$) (2 and 30% relative abundance). However, by day 4 they both decreased to below 1% relative abundance ($z = -2.41 – -2.64$, $p < 0.05$) (Figure 4.11D, J). This was followed by *Ulva* (chlorophyte) and *Amphora* (diatom) reads were relatively more abundant (approximately 8-10% of the community by day 4) in both sinking and floating flocs within the oil treatment ($z = 3.95 – 4.88$, $p < 0.01$), declining to 0 – 4% by day 14 (Figure 4.11F, G). MAST-3J (unclassified marine Stramenopiles) were shown to rapidly increase by day 14 in relative abundance in floating flocs treated with oil to approximately 90% of the community ($z = -3.57 – -4.43$, $p < 0.001$) (Figure 4.11C). By day 14 *Entomoneis* (diatom) made up between 5-12% of 18S rRNA reads within oil and oil plus dispersant treated sinking flocs and was significantly more abundant than in the dispersant treatment and seawater controls ($z = 3.19 – 4.69$, $p < 0.001$) (Figure 4.11I). *Thalassiosira* (diatom) reads were more abundant in sinking flocs within the dispersant and oil plus dispersant treatment on days 4, 7 and 9 ($z = 5.46 – 6.44$, $p < 0.001$), making up about 20-30% of the reads, followed by a decline to below 5% by day 14 ($2.06 – 6.44$, $p < 0.05$) (Figure 4.11H). *Heterochlorella* (chlorophyte) reads were dominant at day 2 in seawater controls ($z = -3.34 – -2.69$, $p < 0.01$), making up approximately 40% of the reads, followed by a rapid decline by day 4 to below 1% (Figure 4.11B). By day 9, *Aplanochytrium* and

Protaspa reads dominated seawater control floc libraries at approximately 60% and 30% of the reads respectively (t = -8.02 – -5.69, $p < 0.001$) (Figure 4.11A, L).

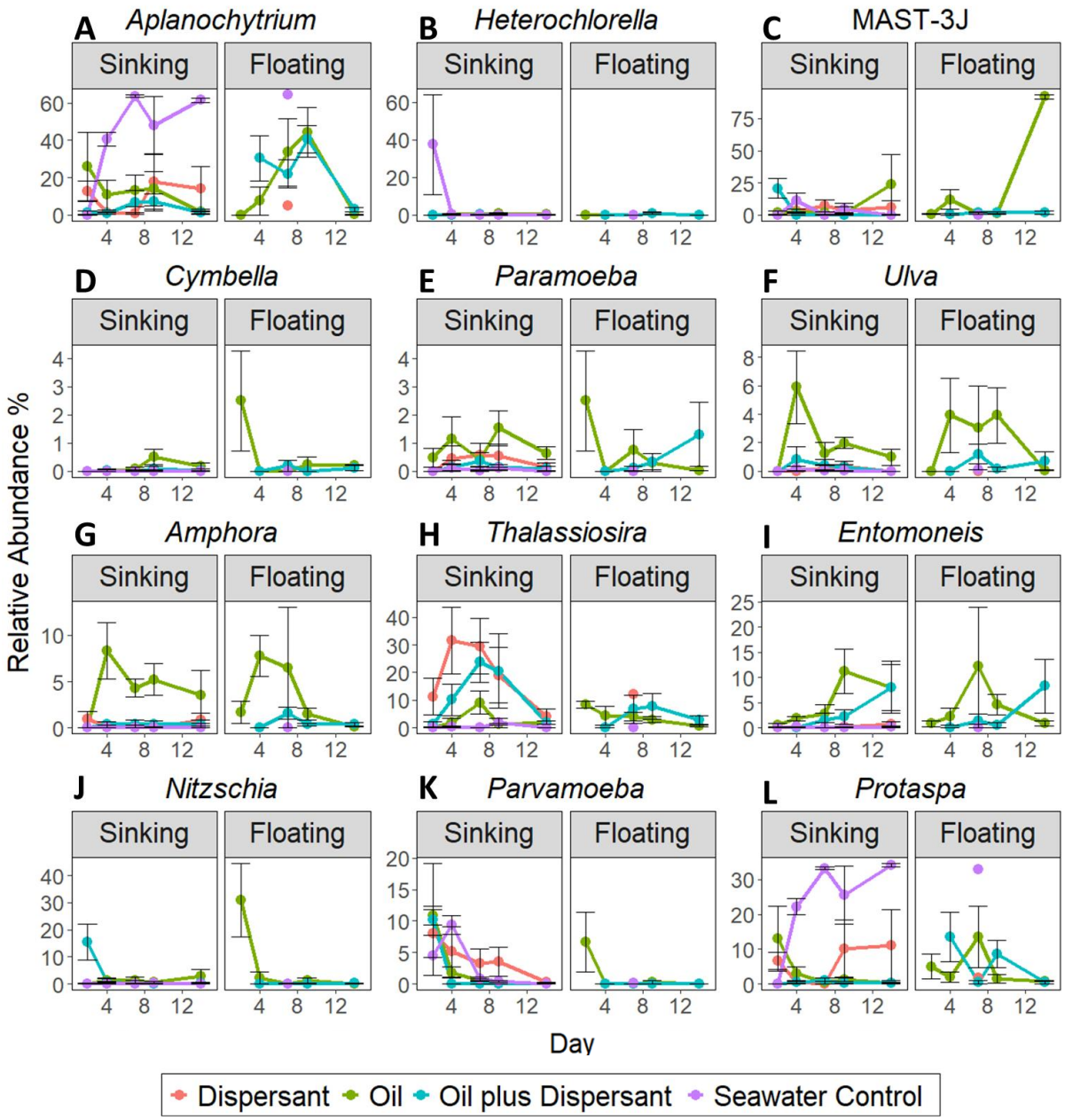


Figure 4.11: Percentage relative abundance of Eukaryote genera within Eukaryotic 18S rRNA communities from sinking and floating single marine snow (MS) and marine oil snow (MOS) flocs (mean ± SE, n=4) incubated in microcosm treatments Seawater Controls (400 ml Seawater Only), Dispersant (400 ml Seawater plus 0.0005% v/v COREXIT™ EC9500A), Oil (400 ml Seawater with 0.01% v/v crude oil) and Oil plus Dispersant (400 ml Seawater with 0.01% v/v crude oil and 0.0005% v/v COREXIT™ EC9500A). Oil used: Norwegian Geochemical Standard North Sea Oil (NGS NSO-1), dispersant used :COREXIT™ EC9500A.

Discussion

Prokaryotic and eukaryotic abundance associated with the formation of flocs

Using qPCR, prokaryotic and eukaryote abundance was observed to increase within flocs over the 14-day study period within all treatments. However, this increase is more than likely attributed to the increase in biomass of single marine oil snow (MOS) flocs collected during DNA extraction, as they increase in size over time (Alldredge and Silver, 1988; Arnosti *et al*., 2016). Throughout the study the prokaryote to eukaryote ratio declined rapidly. This highlights the importance of bacteria during the initial stages of floc formation, notably through EPS production, being an integral component, acting as the skeletal structure of flocs, followed by the integration of phytoplankton, algae and other eukaryotic detritus into these EPS structures during formation (Shanks and Trent, 1979; Tansel, 2018) over time, which may then counteract the positive buoyancy of crude oil that was observed during this study. Prior studies have also shown different phytoplankton to produce various biogenic minerals (calcium carbonate, opal and lithogenic material) known to act as ballasts, highlighting increased eukaryotic incorporation into flocs and their activity may contribute to the rapid sinking of flocs (Iversen *et al*., 2010). They further highlighted that sinking velocity was independent of the aggregate size itself and more related to the biogenic mineral composition of flocs. We can surmise that as eukaryotic abundance increases, the production of these ‘ballast’ minerals increases. This can be observed in the seawater controls in the present study having the highest eukaryote abundance and no indication of floating flocs throughout the collection period. As well as this an increase in diatom abundance may also act as a ballast as they sink rapidly due to their silica frustules being durable and resistant to decomposition (Lavoie *et al*., 2020). The

presence of oil initially reduced the abundance of eukaryotes, whilst making flocs more prone to positive buoyancy (Omotoso *et al*., 2002).

The impact of crude oil and chemical dispersant integration within flocs on bacterial communities

Initial analyses of floc prokaryotic community diversity indicated no change in Shannon index or pielou's evenness between treatments. Even so, Shannon index throughout the study period sat between 4 and 5, whist Pielou's index sat between 0.6 and 0.7. This indicates that these flocs contain highly diverse prokaryotic communities with moderate evenness (Pielou, 1966; Magurran *et al*., 2013). Although ASV richness was lower in flocs incubated in the oil plus dispersant treatment, with clear selection for hydrocarbon degrading bacteria, in contrast to our prior understanding of the impact of oil pollution, the presence of oil integrating with flocs did not decrease overall prokaryotic diversity (Head *et al*., 2006; Yakimov et al., 2005; McGenity et al., 2012). We can surmise that crude oil integrating with individual flocs is in low enough concentrations that diversity is not significantly impacted within these microhabitats, allowing for a consistently diverse community upheld by the nutrient rich flocs (Shanks and Trent, 1979; Rath *et al*., 1998). When discussing diversity however, we must take into consideration the influence of read depth via sequencing when observing increases in diversity indices, especially in closed system experiments. For example, rarer hydrocarbon degrading taxa may initially not be detectable due to insufficient read depth, although will still be present in the community, only being detected as they increase in abundance increasing diversity. This can explain sudden increases in diversity within communities, specifically during these closed system experiments.

With the integration of crude oil and chemical dispersants into floc formations, prokaryotic community compositions rapidly changed, with the conditions of these

microenvironments selecting for a wide range of hydrocarbon degrading genera, including, but not limited to, the obligate hydrocarbonoclastic bacteria *Alcanivorax, Oleispira, Cycloclasticus* and *Thalassolituus* (Dyksterhouse *et al*., 1995; Yakimov *et al*., 1998; Chung *et al*., 2001; Yakimov *et al*., 2004; Prince *et al*., 2019), as well as other more generalistic potential hydrocarbon degraders; *Alkanindiges*, *Alteromonas*, *Colwellia*, *Cobetia*, *Erythrobacter*, *Marinobacterium*, *Olleya* and *Psychrobacter* (Deming *et al*., 1988; Iwabuchi *et al*., 2002; Bogan *et al*., 2003; Vandecandelaere *et al*., 2008; Pham *et al*., 2009; Ibacache-Quiroga *et al*., 2013; Okai *et al*., 2015). This diverse consortium has the capacity to degrade a wide range of short and long-chain alkanes and parent PAHs and their substituted derivatives. Early on, *Oleispira* and *Colwellia* dominated oiled flocs (50-70% relative abundance), which was quickly succeeded by an uplift of *Cycloclasticus* (3 – 6% relative abundance by day 4) and *Alcanivorax* becoming the dominant OHCB by day 9 (20 – 40% relative abundance). Prior studies focused on the succession of free-living bacteria within seawater environments have observed similar responses to hydrocarbon exposure with *Oleispira* outcompeting other hydrocarbon degraders early on during a spill event, followed by *Colwellia, Cycloclasticus* and *Alcanivorax* (Dubinsky *et al*., 2013; Thomas *et al*., 2021). In this instance *Oleispira* sequences represented by ASV14 had a 98.93% 16S rRNA sequence identity to *Oleispira antarctica* strain RB-8 (Yakimov *et al*., 2003). Shown to be psychrophilic, optimally growing at lower temperature of 2 – 4 ^{o}C but with the ability of growing between 1 – 25 ^{o}C, *Oleispira Antarctica* tends to be outcompeted in warmer waters. Thus the perceived high relative abundance of *Oliespira* once floc formation occurred by day 2 may be due to an initial higher abundance *in-situ* providing a priority effect advantage (Thomas *et al*., 2021), followed by their steep decline in favour of better competitors such as *Alcanivorax* and

Cycloclasticus. By day 4 and 7 *Alkanindiges*, *Erythrobacter, Marinobacterium* and *Olleya,* which are more metabolically versatile genera that contain some hydrocarbon-degrading species, were in greater abundance. It has been well documented that the structural properties of hydrocarbon constituents within crude oil determines the degree of degradation, indicated by an inverse relationship between hydrocarbon moelcular weight and degradability; as compound complexity and solubility increases, degradability reduces (Setti *et al*., 1993; Mohanty and Mukherji, 2008). With lower molecular weight hydrocarbons being more prone to biodegradation, hydrocarbon degrading bacteria follow a successional pattern, periodically "moving up" the chain length as degradation occurs (Ławniczak *et al*., 2020). Specifically different genera of bacteria are better competitors for different sized and structured hydrocarbons. For example in many cases *Oleispira* and *Alcanivorax* are found to dominate oil contaminated environments within days of oiling, targeting smaller straight chained and branched alkanes, followed by *Cycloclasticus* which have evolved to use a range of more complex polyaromatic hydrocarbons (Head *et al*., 2006). This explains the patterns seen during this study and prior studies, with *Oliespira* targeting lower molecular weight straight chain *n*-alkanes (*n*-C_{10-24}), followed by *Alcanivorax* with the capacity of targeting a more diverse range of *n*-alkanes up to C_{36} along with branched alkanes and *Cycloclasticus* which can target higher complexity PAH's such as phenanthrene, anthracene, fluorene and dibenzothiophene and their many substituted derivatives (Dyksterhouse *et al*., 1995; Kasai *et al*., 2002; Wang *et al*., 2014; Gregson *et al*., 2019; Gregson *et al*., 2020).Some genera of *Erythrobacter* and *Olleya* which appeared after the initial growth of *Oleispira* and *Alcanivorax* are capable of targeting higher molecular weight PAH's such as pyrene and benzo(A)pyrene (Okai *et al*., 2015; Zhuang *et al*., 2015), further acknowledging this inverse relationship

between degradability, molecular weight and compound complexity. These relationships and quick successional changes can somewhat be explained using the Hutchinsonian niche concept, in which the environmental conditions and resources within multidimensional space define the requirements for an individual to persist (Schoener, 2009). In this case being the specific hydrocarbon degrading bacterial genus targeting the specific resources (hydrocarbon compound) they prefer. Therefore as other higher weight compounds become more readily available whilst lower weight compounds are reduced, we see this rapid shift in genus that may persist due to their surrounding environment and available resources. With these observable patterns of prokaryotic succession, MOS has been shown as a site of high potential hydrocarbon degradation, following similar processes seen during surface open water spills. *Seohaeicola* was highly abundant within flocs incubated in oil and dispersant treatments throughout the study period. Prior work has shown *Seohaeicola* to become enriched in the presence of oil and have been found in groundwater sites containing short chain alkanes (Mishamandani *et al*., 2016; Wilpiszeski *et al*., 2020). However current knowledge does not indicate any hydrocarbon degradation ability within members of this genera, thus should be considered for further study of their potential degradative capacity.

Dispersant use has been well documented to influence prokaryotic community succession, as dispersion increases the availability of oil, thus the rest of biodegradation increases, in turn increasing the rate of succession (Baelum *et al*., 2012; Thomas *et al*., 2021). During the present study we observe minor changes in succession within *Alcanivorax* and *Erythrobacter* being detected as early as day 4 within dispersed oil, whilst only being detectable within undispersed oil by day 9. As MOS floc sites have been shown to be high surface area sites, the initial bioavailability

of hydrocarbons within these systems may be considerable, leading to minimal impacts on succession via dispersant use that we would normally see within the surrounding water column. We do however see that dispersant use specifically selected for *Olleya* and *Seochaeicola* having a significant impact to the succession of these communities, potentially highlighting an increase in the availability of certain hydrocarbon compounds. *Olleya* was observed to be highest in the dispersant treatment, this may indicate *Olleya* growth using specifically the hydrocarbon carrier within the COREXIT^{TM} EC9500A (petroleum distillate; C9 – C16, cycloalkanes, and iso- and *n*-paraffins) (NALCO, 2008; McFarlin *et al*., 2018), contributing to the higher ecological index of hydrocarbon exposure within the dispersant treatment compared to the seawater control.

Communities did not change considerably between sinking and floating flocs partly due to the high variance in abundance of some genera within individual flocs, with the majority of differences being present within in the undispersed oil treatment. However, the EIHE (Ecological Index of Hydrocarbon Exposure) (Lozada *et* al., 2014) highlighted that during initial floc formation approximately 70% of floating floc communities consisted of potential hydrocarbon degraders, whilst only being approximately 20% of sinking flocs. It is suggested that the integration of undispersed oil into single flocs is not evenly distributed, with larger concentrations integrating with individual flocs, enhancing the uplift of hydrocarbon degraders and buoyancy, thus explaining the observed EIHE increase withing floating flocs. With the addition of dispersant it is believed that the dispersion effect allowed for a more even distribution of oil (Abdallah *et al*., 2005) between flocs throughout both sinking and floating flocs, increasing homogeneity between individual flocs, reducing the differences in

communities considerably. Further research should be conducted to understand how dispersant use impacts oil integration within single flocs.

Loktanella was shown to be highly abundant within flocs formed within the seawater controls during the initial formation, making up approximately 8-10% of prokaryotic communities. Notably this genera have been associated with algicidal attributes, acting as crucial regulators of toxic algal blooms, such as toxic dinoflagellates (Bloh *et al*., 2016; Meyer *et al*., 2017; Coyne *et al*., 2022), potentially acting as a regulator of floc health during early formation. Notably the presence of crude oil did inhibit *Loktanella* growth, thus potentially inhibiting this regulatory role during formation. Over time, *Algibacter Lewinella* and *Polaribacter* succeded *Loktanella* dominating floc communities within the seawater control. All of which have been associated with the breakdown of complex organic matter, with a preference toward algal polysaccharides, and can play a significant role in microbial food webs of floc communities, thus may be indicators of important biochemical cycling processes within floc formations (Miranda *et al*., 2013; Sun *et al*., 2016; Nguyen *et al*., 2023; Sajid *et al*., 2024).

The impact of crude oil and chemical dispersant integration within flocs on Eukaryote communities

Eukaryote communities were notably less diverse compared to prokaryote communities, following a similar trend that has been previously observed across a wider gradient (Oladi *et al*., 2022). ASV richness was observed to be lower in the oil plus dispersant treatments, however Shannon index and pielou's evenness of eukaryotic communities were lower in the seawater controls, suggesting particular genera are notably dominant within these communities (Pielou, 1966; Magurran *et al*., 2013). *Heterochlorella* was initially dominant in seawater controls making up 40% of

the community, affecting the low alpha diversity indices. By day 14 *Aplanochytrium* and *Protaspa* dominated seawater control communities making up approximately 60% and 35% of the community indicating low diversity. *Aplanochytrium,* a marine protist, are recognised as prolific decomposers of organic matter ranging from microalgae to diatoms and other forms of detritus (Raghukumar, 2017). As previously stated, flocs are sites high nutrient and organic matter transformation, thus would provide *Aplanochytrium* and large nutrient source to dominate seawater control flocs. Notably, eukaryotes such as *Heterochlorella* (green microalgae) declined during the formation of seawater control flocs communities. Thus it could be surmised that a trophic interaction is occurring with *Aplanochytrium* targeting *Heterochlorella* cells alongside other abundant algal and diatom cells (Hamamoto *et al*., 2019). Even so, little is still known about *Aplanochytrium* feeding and nutrient acquisition, thus more research should be considered. *Protaspa (*heterotrophic flagellates) was also shown to be highly abundant, however research on these protists is highly limited. Other similar protists, however, have been suggested to function in a similar manner as prokaryotic grazers of microalgae, potentially contributing to microalgae predation pressures of *Heterochlorella* and other diatoms (Arndt *et al*., 2000; Hu *et al*., 2021). Although eukaryotic diversity did not differ considerably between sinking and floating flocs, pielou's evenness was also observed to be lower in floating flocs in the oil treatment by day 14. This was attributed to the increase of MAST-3J (Marine Stramenopiles) making up 80-90% of these communities. Being a diverse and uncharacterised clade of protists (Seeleuthner *et al*., 2018) it is difficult to surmise the specific ecological niche's associated with flocs.

Eukaryotes have been highlighted as ideal candidates for biomonitoring, in many cases having a narrow environmental tolerance range (Reid *et al*., 1995;

Stefanidou *et al*., 2018). Shifts in communities brought on by contaminants, notably hydrocarbons has been heavily reported on over a range of environmental conditions, further highlighting the possibility of using eukaryotic community composition data alongside prokaryotic data for biomonitoring purposes (Coulon *et* al., 2012; Polmear *et al*., 2015). Previously stated *Aplanochytrium, Heterochlorella* and *Protaspa* were highly abundant within MS incubated in seawater controls whilst limited growth was observed in the presence of crude oil and dispersant use, therefore could be considered indicators of a healthy eukaryotic community within marine snow aggregates. In other instances marine snow biomass has been shown to be dominated by *labyrinthulomycetes*, similarly to the present study in which *Aplanochytrium* being part of the *labyrinthulomycetes* class was dominant within seawater controls, suggesting eukaryotic taxa such as *Aplanochytrium* may be just as useful as bacterial taxa as indicator species for post monitoring techniques (Bochdansky *et al*., 2017).

The relative abundance of diatoms *Entomoneis* and *Amphora* was considerably higher within oiled treatments from day 4 compared to seawater controls. Prior studies focused on PAH contamination have shown *Entomoneis* to be favoured within communities under PAH exposure (Othman *et al*., 2018). Rather than stimulation of growth from PAH exposure directly, the stimulation in growth of these diatoms may be attributed to an increase in nutrient regeneration, via the breakdown of other sensitive eukaryotes exposed to PAH's and/or the reduction of heterotrophic grazers attributed to predation pressure on phytoplankton (Kelly *et al*., 1999; Hjorth *et al*., 2008; González *et al*., 2009; Othman *et* al., 2018; Othman *et al*., 2023). Even so, some diatoms such as *Nitzichia,* observed in high abundance by day 2 within the oil and oil plus dispersant treated flocs are capable of PAH degradation (Juhasz *et al*., 2000; Hong *et al*., 2008; Paniagua-Michel and Banet, 2024). Notably *Nitzichia* was

succeded by both *Amphora* and *Entomoneis*. However to our current knowledge these diatoms have not been shown to have the capacity for PAH degradation, therefore more research should be considered to fully understand the capacity for eukaryotes to degrade hydrocarbons. *Amphora* has however been shown to be a prolific producer of extracellular polymeric substances (EPS), potentially contributing to the formation of flocs which use EPS as a skeletal structure (Zhang *et al*., 2008; Jin *et al*., 2018).

Conclusion

Throughout the study period, floc habitats were observed to be sites of potential hydrocarbon degradation with the ecological index of hydrocarbon exposure highlighting a high proportion of floc communities treated with oil being associated with potential degraders, as well as clear successional changes in prokaryotic communities similar to communities associated with our prior understanding of open water spill succession. Chemical dispersant use was shown to impact succession with genera such as *Alcanivorax* and *Erythrobacter* increasing in abundance earlier when exposed to hydrocarbon contamination. Even so, due to the increased surface area of flocs, the initial bioavailability of hydrocarbons incorporated within these formation was potentially high enough to encourage the substantial and rapid growth of hydrocarbon degrading floc communities without the addition of dispersant use. More research should be considered into highlighting how crude oil pollution physically integrates with single floc formations to further understand how bioavailability of hydrocarbons are impacted. The presence of crude oil within floc formation may impact nutrient transportation to deep sea benthic environments due to the impact of hydrocarbon contamination to important genera associated with organic matter breakdown and nutrient cycling. Further studies should be considered to assess changes in floc community functionality, highlighting changes to microbial food webs and

biogeochemical cycling to further understand the implications of hydrocarbon pollution integrating with flocs during oil spills. Eukaryote community composition was heavily impacted by hydrocarbon integration with MOS, with diatoms dominating oiled flocs and protists dominating unoiled MS. This suggests changes in trophic interactions between eukaryotes and a possible uplift of diatoms associated with hydrocarbon degradation. Further research should be considered highlighting eukaryote potential for hydrocarbon degradation and biomonitoring purposes due to the distinct effect hydrocarbon presence had within eukaryote floc communities. Overall the current study highlights individual floc communities as being sites of high hydrocarbon degradation potential and overall microbial activity comparatively to surrounding seawater environments, thus MOS habitats may be a viable source for exploring hydrocarbon degradation pathways further within both prokaryotes and eukaryotes.

Chapter Five

Effects of the 2023 Poole Harbour oil spill on sediment microbial communities and ecosystem functioning

Abstract

On the 26th March 2023, approximately 200 barrels of reservoir fluid escaped a burst pipeline beneath Ower Bay creek, entering Poole Harbour (Dorset, UK). Due to our currently limited understanding of *in-situ* oil spill microbial ecology, this provided an excellent opportunity to perform a much needed *in-situ* study on the impacts of hydrocarbon contamination to microbial communities within temperate coastal environments. Our aims were to assess hydrocarbon concentrations throughout the impacted region and to better understand their capacity for biodegradation via a hydrocarbon degrading (HYD) pathways and identify potential microbial biomarkers that can be introduced into post spill monitoring models. Hydrocarbon contamination was minimised to the Spill Site, with minimal impact to the surrounding area. Clear changes in community structure were observed with notable genera such as *Anaerolina, Thiobacillus* and *Dechloromonas* favouring the Spill Site, whilst *Woeseia* was observed as a potential biomarker for uncontaminated sediment environments. A clear switch from Aerobic to anaerobic degradation was observed with a significant uplift of anaerobic degradation genes *AssA* (alkane degradation) and *AbcA* (aromatic degradation) at the Spill Site, Associated with the uplift of *Anaerolina,* Thiobacillus and *Dechloromonas*. Overall 24 HYD gene pathways were identified through the study site, across 48 Metagenomic assembled genomes (MAGs), highlighting the sites capacity for hydrocarbon biodegradation under both aerobic and anaerobic conditions.

Introduction

The amount of oil entering the marine environment globally from pipelines and vessel spills was approximately 7,299,000 kg y^{-1} between 1990 and 1999, with a much lower amount of 588,000 kg y^{-1} between 2010 and 2019 (National Academies of Science, 2022), following improvements in safety standards. However, as the production of crude oil is forecast to grow to above 105 million barrels per day by the end of 2025 (U.S. Energy Information Administration, 2023), the risk of marine oil spills from tankers and pipelines remains. On the 26th of March 2023, approximately 200 barrels (~27 tonnes) of reservoir fluid (comprising 15% light crude oil and 85% water) leaked from a fractured pipeline at Wytch Farm oil field beneath Ower Bay creek, which enters Poole Harbour (Dorset, UK) (PMCC, 2024; Hatch, 2024). The harbour is an important Site of Special Scientific Interest (SSSI) and a Special Protection Area (SPA), and contains numerous islands, including Brownsea Island, which forms part of the Purbeck National Nature Reserve and is recognised as an internationally important area for wildlife. A rapid response to the spill was conducted by Oil Spill Response Ltd (www.osrl.com) and Adler and Allan Environmental Risk Consultancy, in which 653 SCAT (Shoreline Cleanup Assessment Technique) surveys (covering 199 km^2) were performed, along with aerial imagery surveys indicating initial petroleum hydrocarbon contamination throughout Poole Harbour. Absorbent booms were deployed within 24 hours of the spill at the Mid Point of the creek and the End Point of the creek at the entry point into Poole Harbour, with ongoing monitoring of the site. In August 2023, a bubble barrier was also implemented to prevent oil that had migrated to the surface of the sediment from being carried out on high tides and contain the oil to the localised spill area. Further remediation from November 2023 to January 2024,

involved the removal of sediment and dead plant matter to allow for habitat recovery. From the time of the spill up to February 2024, od the multiagency PREMIAM (Pollution Response in Emergencies: Marine Impact Assessment and Monitoring) Monitoring Coordination Cell (PMCC) took action to develop and implement a monitoring plan and issued its final report in March 2024 (PMCC, 2024). Poole harbour is Europe's largest natural harbour and very shallow (average depth = 0.5 m; maximum depth in shipping channels = 7.5 m) with substantial commercial and recreational boating activities as sources of hydrocarbons (Underhill-Day, 2006). Whilst data is limited, sediment polyaromatic hydrocarbons (PAHs) have previously been measured at between 91 – 166 μg kg^{-1} (Law *et al*., 1997; Woodhead *et al*., 1999).

Marine oil pollution can alter sediment microbial community composition, often resulting in decreases in community diversity and selection for hydrocarbon-degrading bacteria (Yakimov *et al*., 2005; McGenity *et al*., 2012)**.** This can include increases in the obligate hydrocarbonoclastic bacteria (OHCB), known to use almost exclusively hydrocarbons and their derivatives as a source of carbon and energy (Yakimov *et al*., 2007), such as alkane-degrading *Alcanivorax* and *Thalassolituus* and polyaromatic hydrocarbon (PAH)-degrading *Cycloclasticus.* (Yakimov *et al*., 1998; McKew *et al*., 2007a; McKew *et al*., 2007b; Rubin-Blum *et al*., 2017). There are also many more metabolically diverse genera that have been evidenced to contain species capable of hydrocarbon degradation, such as *Marinobacter*, *Pseudomonas* and *Colwellia,* resulting in at least 300 bacterial genera that contain some species that can grow on different hydrocarbons as a sole or major carbon source (Prince *et al*., 2019). Following oil spills, such as the 1989 Exxon Valdez, 1996 Sea Empress (Etkin, 1999), 2017 Agia Zoni II (Parinos *et al*., 2019; Thomas *et al*., 2020) and 2018 Sanchi (Chen *et al*., 2020) spills, there has been an emphasis on understanding oil-induced effects

on sediment and seawater microbial community composition and the hydrocarbon biodegradation capabilities of its members (Lamendella *et* al., 2014). However, a potential limitation of many studies is that they are conducted as *ex-situ* laboratory experiments (e.g., Alonso-Gutiérrez *et al*., 2009; Jung *et al*., 2010; Stauffert *et al*., 2013; Morais *et al*., 2016; Engel *et al*., 2017) due to the difficulties of performing *in-situ* research (e.g. access, permissions, logistics, cost), so results are often difficult to extrapolate to the field (Head *et al.,* 2006). When oil-contaminated sediments have been studied *in-situ*, such as after the Agia Zoni Spill, selection for OHCB has been shown, with a higher relative abundance in *Marinobacter hydrocarbonoclasticus, Thalassolituus and Oleiphilus* compared to uncontaminated sites and a legacy of *Alcanivorax* and *Cycloclasticus* many months later, even after hydrocarbon concentrations returned to undetectable levels (Thomas *et al*., 2020). The microbial response can differ between spills. For example, *Acinetobacter* dominated oiled sediments following the 2017 Ennore spill, representing 52% of total bacterial community (Neethu *et al*., 2019). Differences may be attributed to many factors such as the composition of oil, and varying environmental conditions, such as temperature, availability of O_2 (required for aerobic respiration but also the oxidant for oxygenase enzymes that activate hydrocarbons in the initial step of biodegradation), or N and P that often become limiting nutrients following the large influx of carbon from oil (Atlas& Bartha 1972; Head et al., 2006) . The Poole Harbour incident provided a unique opportunity to perform an *in-situ* investigation on the effects of an oil spill on microbial community structure and functional potential in temperate coastal sediments. The aim of this study was to quantify the extent of petroleum hydrocarbon pollution in real time and its impact on the *in-situ* sediment bacterial communities, whilst also investigating

their capacity for biodegradation via shotgun metagenomic analysis and quantification of genes associated with aerobic and anaerobic hydrocarbon degradation pathways.

Survey design

Sample site and sample collection

Surface sediment samples (top 0-5 cm) were collected from Ower Bay creek, located near the Wytch Farm oil field, run by Perenco UK Ltd, which included the Spill Site at the head of the creek, Mid Point (164 m from the Spill Site) and End Point (387 m from the Spill Site) which is the point of entry into Poole Harbour (Figure 1). At the Mid Point and End Point, sampling took place behind the absorbent booms that were deployed as part of the spill response. The northeastern coast of Brownsea Island was also sampled, which had evidence of oil sheens from initial surveys. This sample programme commenced on the 14th June, 80 days after the initial spill, followed by subsequent sampling on the 12th July (108 days after the spill), 30th August (158 days after the spill) and 18th October 2023 (206 days after the spill). Control sites for Brownsea Island (Brownsea Control) and Corfe River (Control Creek) were also sampled for comparison (Figure 1), as these sites had showed no observational evidence of oiling during initial SCAT surveys. All sediment samples were placed in sterile falcon tubes and immediately snap-frozen (-150□C on site) using a CryoShipper (MVE Biological Solutions). Samples were collected in triplicate (1 – 3 m apart) at each site and each time point. Additional sediment samples were taken at a single timepoint (18th April 2023, 24 days post-spill) from a range of sites in the wider Harbour area by CEFAS as part of the Premiam Monitoring Coordination Cell post-spill monitoring (Figure 1A,C) and were frozen at -20^{o}C for further analysis.

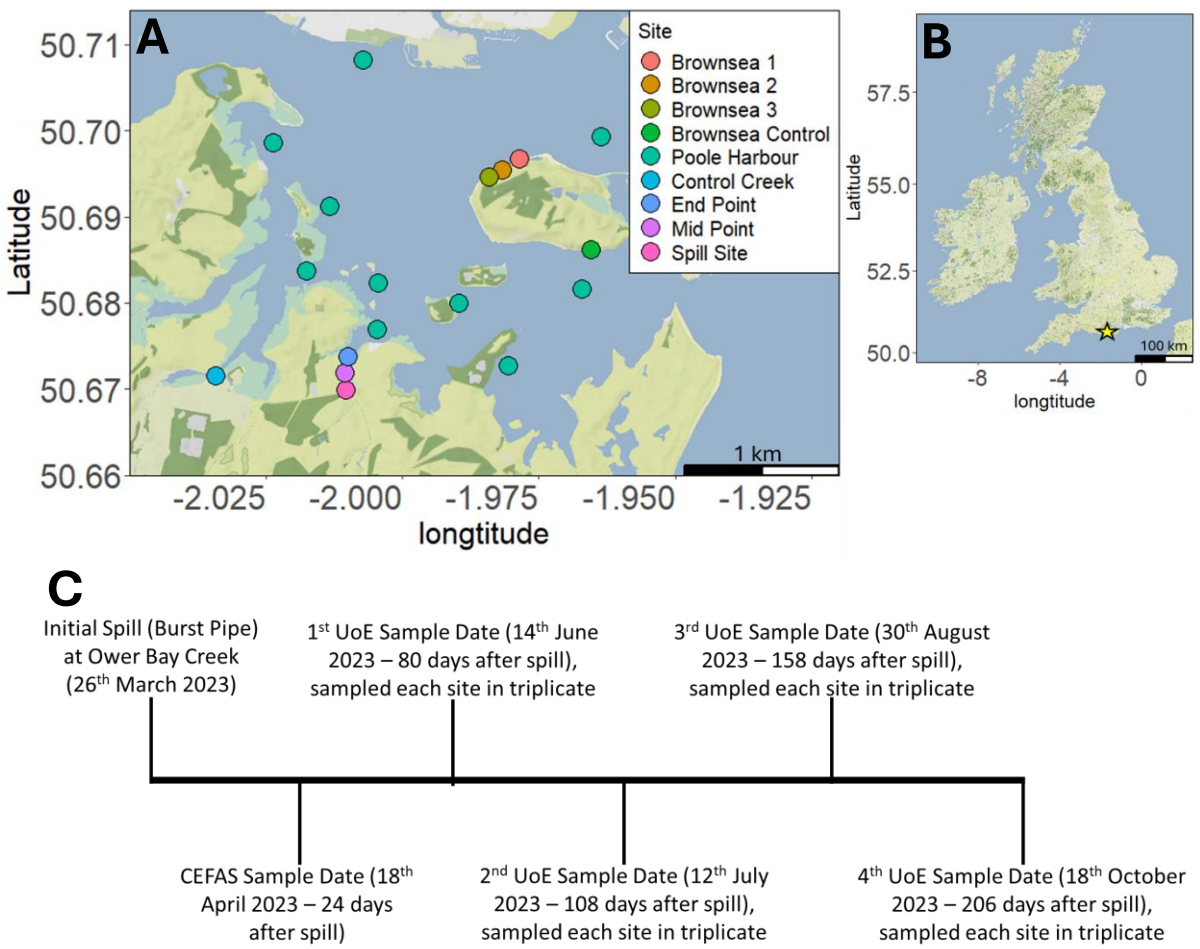


Figure 5.1: A. Sample sites across Poole Harbour, Dorset, United Kingdom (Samples sites include Brownsea Island (Brownsea 1, 2 and 3, Brownsea Control), Poole Harbour (Collected by Cefas, 18th April 2023) Wytch farm Owen Bay Creek (End point, Mid-Point and Spill Site) and the Corfe River Control Creek, each dot represents samples collected in triplicate from each site on the 14th June, 12th July, 30th August and 18th October 2023. **B.** United Kingdom map, yellow star indicates the location of Poole Harbour. **C.** Timeline of initial spill and subsequent sample collection

Hydrocarbon extraction and concentration analysis

Sediment samples (2 g) were dried by mixing with 2 g of sodium sulphate, and hydrocarbons were then extracted by adding 12 ml of extraction solvent (Hexane: Dichloromethane 1:1 ratio) and shaking for 24 hours at 1500 rpm at room temperature on a Multi Reax orbital shaker (Heidolph). Deuterated alkanes and polyaromatic hydrocarbon (PAH) internal standards were added to the solvent prior to extraction at 0.04 µg/mL. Quantification of hydrocarbons was performed using a Nexis GC-2030/CMS-TQ8040 NX GC-QqQ-MS/MS (Shimadzu), targeting n-alkanes n-C10-40, and branched alkanes, pristane and phytane, and a selection of two- to six-ring PAHs

(Supplementary Material: Table S1) as previously described (Coulon et al., 2007). External standard calibration curves (six points over a range of 0 – 5 µg/mL) were produced using C8-C40 Alkanes Calibration Standard (Merck), and QTM PAH Mix (Sigma) calibration standard, mixed with an additional range of two- to six-ring PAHs (Supplementary Material: Table S1). A hexane blank was analysed every 25 samples for quality control.

Nutrient analysis of sediments

Sediment samples (10 g) were freeze dried for 24 hours in an ALPHA 1-4 LD plus (Christ Freeze Dryers), then 6 g of dried sediment was placed in a 50 ml centrifuge tube with 18 ml of MilliQ ultrapure water and shaken on a Multi Reax orbital shaker (Heidolph) for 15 min at 1000 rpm before centrifugation (30 min at 2875 × g. The supernatant was filtered using a 0.2 µm filter (Millipore) and analysed on a AA3 AutoAnalyzer (Seal) (for nitrate, nitrite, ammonia and phosphate concentrations) and a Dionex ICS-6000 HPIC (Thermo Fisher) for anion/cation (e.g. sulphate) concentrations.

DNA extraction and qPCR analysis

DNA was extracted from 250 mg of sediment per sample using the DNeasy PowerSoil Pro Kit (Qiagen), following the manufacturer's instructions. Bacterial 16S rRNA gene abundance was quantified via qPCR with primers 341f: CCTACGGGNGGCWGCAG and 805R: GACTACHVGGGTATCTAATCC (Klindworth et al., 2013) using SensiFAST SYBR No-ROX Kit (Bioline) on a CFX384 Real-Time PCR Detection System (BioRad) using an absolute quantification method against a standard calibration curve (E = 70%; R2 = 0.999; y-int = 44) of the target gene from 102 to 109 copies in 20 µl reactions as previously described (McKew and Smith, 2017).

16S rRNA gene amplicon library preparation and bioinformatics

Amplicon library sequencing preparation was performed following the 16S Metagenomic Sequencing Library Preparation protocol (Illumina, 2013). Primers used were the same as the qPCR primers for bacterial 16S rRNA amplification, with the addition of Illumina adapter overhang nucleotide sequences (Forward Overhang: 5'-TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG, Reverse Overhang: 5'-GTCTCGTGGGCTCGGAGATGT GTATAAGAGACAG). PCR products were cleaned using AMPure XP beads (Beckman Coulter). Nextera XT Index Kits v2 (Illumina) were used to add unique combinations of index primers to each PCR product via an 8-cycle PCR, followed by a second clean-up with AMPure XP beads. Gel electrophoresis was performed after each PCR stage for quality control. Quantification of the 16S rRNA gene amplicons was conducted using a FLUOstar Omega microplate reader (BMG LABTECH), following the Quant-iT PicoGreen dsDNA Assay Kit protocol (Thermo Scientific) and then pooled in equimolar concentrations. Final library quantification was then performed using the NEBNext Library Quant Kit for Illumina (New England Biolabs, UK). Sequencing was performed by Novogene (Cambridge, UK) using Illumina NovaSeq PE250.

Sequence data was processed using the Divisive Amplicon Denoising Algorithm (DADA2) (Callahan et al., 2022). Singleton ASVs, sequences shorter than 400 bp, sequences containing homopolymers of eight or above bp, and non-target organisms (archaea and chloroplast sequences) were all discarded. Taxonomic assignment was conducted using the Ribosomal Database Project (RDP) Classifier (Callahan, 2020; Wang et al., 2007) against the RDP Trainset 19. Amplicon library size averaged 526,267 reads per sample. Amplicon sequences have been submitted to the NCBI Sequence Read Archive under the accession number PRJNA1226135.

Shotgun metagenomic analysis

Shotgun metagenomic sequencing library construction was performed at the Earlham Institute (Norwich Research Park, UK), according to the LITE (Low Input, Transposase Enabled) 2.0 pipeline (Perez-Sepulveda et al., 2021). The DNA library was then sequenced on two lanes of a NovaSeq X Plus PE150, 10B flow cell. Metagenomic sequences have been submitted to the NCBI Sequence Read Archive under the accession number PRJNA1227550.

MetaWRAP (Uritskiy et al., 2018) was used to process raw Illumina sequencing reads. Raw reads were trimmed with Trim-galore v0.4.3 (Krueger, 2015) and any human-derived reads were removed via bmtagger v3.101 (Rotmistrovsky et al., 2011).

Using the k-mer based approach of Kraken 2, metagenomic reads were taxonomically classified (Wood et al., 2019) against a custom Kraken2 database including complete NCBI RefSeq genomes for bacteria, archaea, fungi, protozoa, plasmids, viruses, human, and a collection of known vectors (UniVec_Core). Bracken (Lu et al., 2017) was then also used to overcome issues with Kraken2 underestimating the number of reads at lower taxonomic levels. This approach has been shown to provide the most accurate abundance estimations for short reads (Simon et al., 2019; Seppey et al., 2020).

Using SCycDB (Yu et al., 2021), NCycDB (Tu et al., 2019) and MCycDB (Qian et al., 2022) sulphur-cycling, nitrogen-cycling and methane-cycling genes, were identified from metagenomic reads at 100% sequence identity using DIAMOND v 2.0.4 (Buchfink et al., 2015) with an E-value cut-off of 0.0001. Forward and reverse reads were merged and randomly subsampled down to the lowest sample read count.

Co-assembly of the trimmed reads from all samples was then performed with MEGAHIT v1.2.9 (Li et al., 2015). The assembly was formatted to include scaffold

length and k-mer depth, sorted by length, and contigs shorter than 1000 bp were removed.

Prokka v1.14.6 (Seemann, 2014) was used to functionally annotate contigs. To map processed reads for each sample against the contigs, Bowtie2 was used (Langmead and Salzberg, 2012). The output Sequence Alignment/Map (SAM) file was converted to Binary Alignment Map (BAM) file format and sorted by leftmost alignment coordinate using SAMtools (Li et al., 2009). The MarkDuplicates function in Picard v2.23.3 was used to remove duplicated reads (http://broadinstitute.github.io/picard/). An in-house script was used to search for gene regions within the General Feature Format (GFF) file generated by Prokka. The 'HTSeq' library htseq-count tool was used to calculate raw counts per gene (Anders et al., 2015). The CANT-HYD database was then used to extract and characterise functional genes associated with hydrocarbon degradation pathways from the Prokka annotated contigs within all samples, hits with an e-value of ≥ 0.0001 were retained (Khot et al., 2022).

Assembled contigs were binned into three different bin sets within metaWRAP's Binning module (Uritskiy et al., 2016) using MetaBAT (Kang et al., 2015), MetaBAT2 (Kang et al., 2919) and CONCOCT (Alneberg et al., 2014), which were then consolidated into a final bin set using the Bin-refinement module. This resulted in 221 Metagenome-Assembled Genomes (MAGs) estimated to be >50% complete with <10% contamination (Uritskiy et al., 2016) (Supplementary Material: Table S2). MAG abundance was quantified using Salmon (Patro et al., 2017). MAGs were taxonomically classified by Taxator-tk (Droge et al., 2015) against the NCBI database (Sayers et al., 2022). To further refine the consensus taxonomy assigned by Taxator-tk, 16S, 5S and 18S rRNA sequences were extracted from MAGs using Barrnap (Seemann, 2015). Extracted sequences were compared to those present in the NCBI

nr database using the Basic Local Alignment Search Tool (BLAST) (Altschul et al., 1990). CANT-HYD was again used to determine which MAGs contained hydrocarbon degradation genes, retaining hits with an e-value of ≥ 0.0001 (Khot et al., 2022), highlighting 48 out of the 221 MAGs as containing sequences classified as coding for hydrocarbon degradation gene pathways.

Statistical analysis

ASV sequence data was rarefied to the lowest read depth (30,405). Diversity indices and community composition analysis were then conducted using vegan (Oksanen *et al*., 2024). Data was further analysed using multivariate generalised linear modelling through R via the glmmTMB, MASS and mgcv packages (Venables and Ripley, 2002; Brooks *et al*., 2017; Woods, 2017). A SIMPER analysis (Clarke, 1993) within the vegan package was used to identify genera causing the most significant dissimilarity between communities and differences in functional gene abundance between sample sites.

Results

The spill impact on hydrocarbon concentrations across Poole Harbour

All measured alkanes (*n*-alkanes, pristane and phytane) and PAHs within sediment samples followed a broadly similar pattern (Figure 2 and 3), being significantly higher in the Spill Site compared to all other locations in June (80 days after the spill) and increasing to a maximum in late August (158 days after the spill), before returning to background levels by mid-October (206 days after the spill). Specifically, *n*-alkane concentrations were approximately 8 – 12-fold higher at the Spill Site compared to all other sites in June, July and August ($Z = 3.41 – 9.67$, $p < 0.01$) (Figure 2A, B, C). The branched alkanes, pristane and phytane (Figure 2D) followed a very similar pattern to the *n*-alkanes at the Spill Site ($Z = 2.57 – 3.41$, $p < 0.05$),

although concentrations still remained significantly 8-fold higher than at all other sites at the final sampling time in October. Similarly, 2- to 5-ringed PAHs were also approximately 8 – 14-fold higher in concentration at the Spill Site compared to all other sites in June, July and August ($Z = 2.78 – 12.26$, $p < 0.05$) (Figure 3A, B, C, D) before declining significantly by October ($Z = -2.95 – -5.12$, $p < 0.01$). The 2- and 3-ring PAHs were in much higher concentrations than the 4- to 5-ringed PAHs, and these larger PAHs had returned to background levels found across all other sites including the non-oiled control sites by October. In August, 4-ringed PAHs were also significantly higher by approximately 10-fold at the Mid Point site compared to Brownsea Island, the End Point and control site ($Z = 3.495$, $p < 0.001$). *n*-alkane concentrations and Brownsea Island showed no elevated levels at any time in comparison to the control site. Concentration of 6-ringed PAHs were below the limit of detection at all sites, with no detectable change in concentration over time throughout the study site

To distinguish the source of PAHs between pyrogenic (from natural geological sources e.g. crude oils) or petrogenic origin (hydrocarbons produced by combustion e.g. boating activity), the Pyrogenic Index (Wang *et al*., 1999) and the proportion of low molecular weight and alkylated PAHs to total PAHs (Law *et al*., 1999) was calculated. Both indexes confirmed that the PAHs at the Spill Site were petrogenic, whilst the much lower levels of PAHs at all other sites were predicted to be of pyrogenic origin. (Supplementary Material: Figure S1.

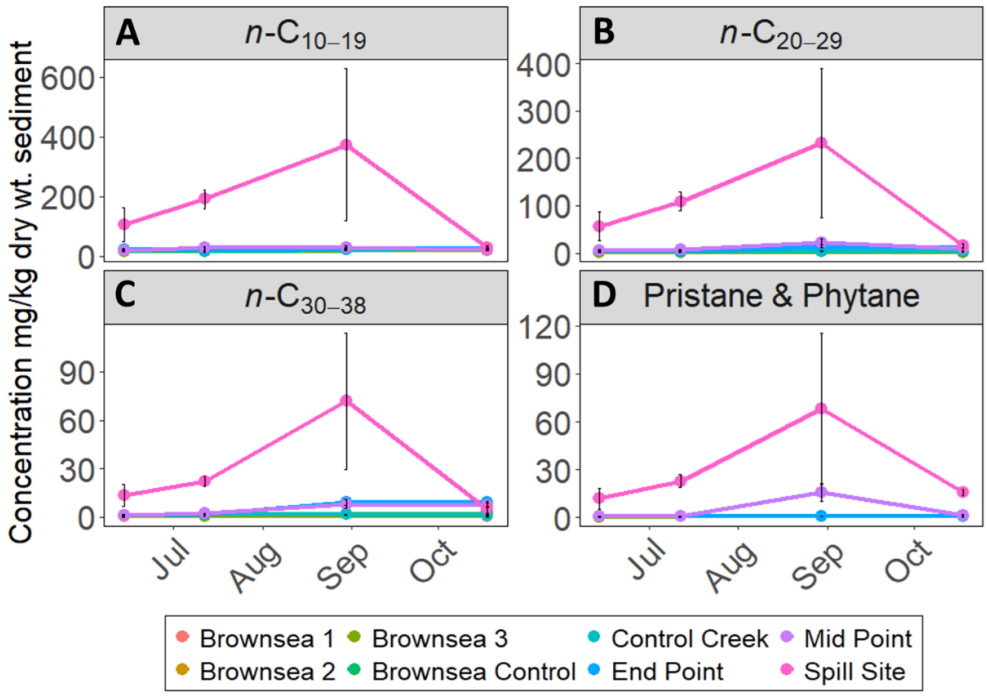


Figure 5.2: Alkane (**A.** *n*-C_{10-19}, **B.** *n*-C_{20-29}, **C.** *n*-C_{30-38} and **D.** pristane and phytane) concentrations (mg/kg dry wt. sediment) from surface sediments (mean ± SE, n = 3) across Brownsea Island and Ower Bay Creek, Dorset, UK over June – October 2023, following an oil pipline spill on 26th March,in comparison to uncontaminated Control sites. Sampling dataes (14th June, 12th July, 30th August and 18th October).

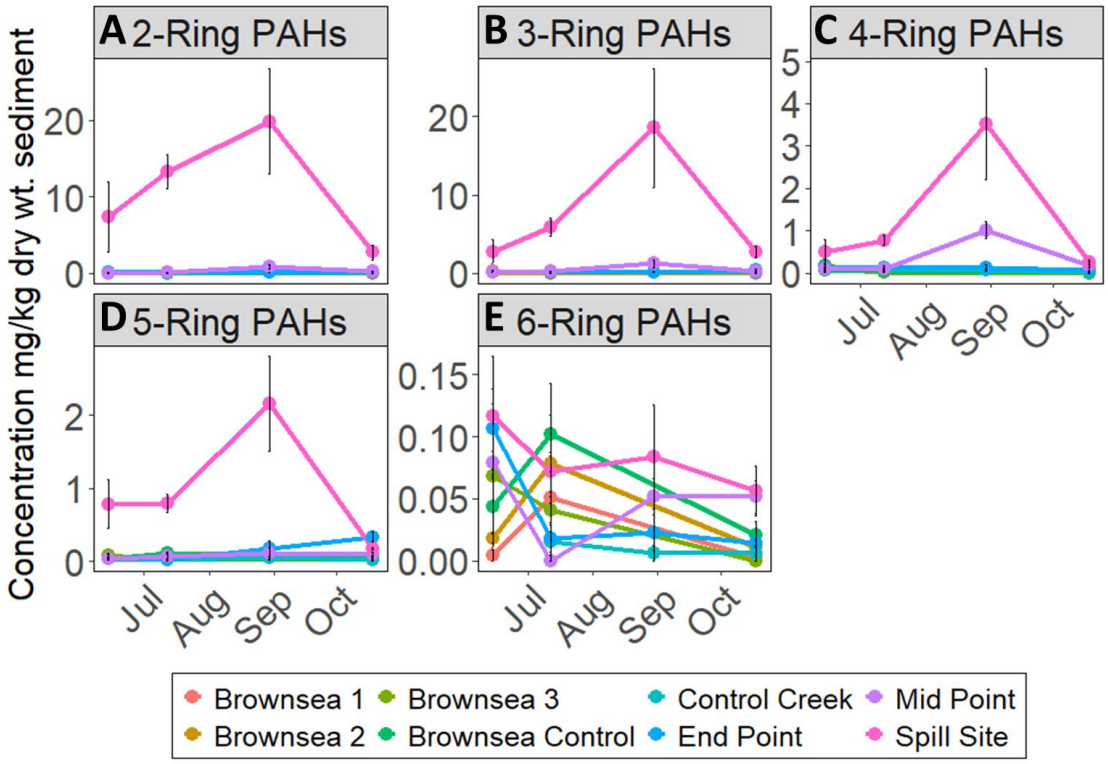


Figure 5.3: Polyaromatic hydrocarbon (PAH) concentration (mg/kg dry wt.), grouped via number of benzene rings, **A**. 2-Ring PAHs , **B.** 3-Ring PAHs, **C.** 4-Ring, **D.** 5-Ring PAHs, **E.** 6-Ring PAHs, (inlcudes Parent and subsituted derivatives; see Supplementary Material: Table S1), from surface sediment samples (mean ± SE, n =3) across Brownsea Island and Ower Bay Creek, Dorset, UK over June – October 2023, following an oil pipline spill on 26th March 2023, in comparison to uncontaminated Control sites. Sampling dates (14th June, 12th July, 30th August and 18th October 2023).

The impact of hydrocarbon pollution on microbial communities

qPCR highlighted that in June bacterial 16S rRNA gene abundance (Bacterial 16S rRNA gene copies per g dry wt.) was significantly lower, by approximately an order of magnitude, at the spill site compared to the Mid Point, End Point and Control Creek ($Z = 2.94 – 3.38$, $p < 0.01$), however by July, gene abundance was not significantly different thereafter. Compared to the muddy fine sediments of the Ower Bay creek and control creek sites, bacterial 16S rRNA gene abundance was significantly less abundant by approximately two orders of magnitude within the sandy coarse sediment of the Brownsea Island sites ($Z = -4.39 – -2.00$, $p < 0.05$) but there were no significant differences between any of the Brownsea sites including the control site (Supplementary Material: Figure S4).

Considering samples by site (i.e. amalgamating time of sampling) 16S rRNA amplicon sequencing showed that the bacterial community composition was significantly dissimilar between Brownsea Island, the harbour and creek sites (PERMANOVA, $F = 6.24 – 16.28$, $p < 0.001$) (Figure 5.4A). Within the creek sites a significant dissimilarity in community composition was observed between the spill site and control creek ($F = 8.53 – 11.24$, $p < 0.001$), whereas in the mid and end point sites, composition was shown to be highly similar to the control creek. Across all time points the spill site community remained significantly dissimilar to all other creek sites ($F = 4.98 – 6.46$, $p < 0.005$). As both the hydrocarbon analaysis and 16S rRNA amplicon analysis showed no impact of the oil spill on sediment hydrocarbon concentrations or community composition at any Brownsea Island sites, we targeted the metagenomic analysis towards only the Ower Bay Creek sites, control creek and harbour. Community composition based on Kraken2 metagenomic taxonomic classifications was significantly dissimilar between the spill site and all other creek

sites and the surrounding Poole Harbour region ($F = 2.31 – 3.33$, $p < 0.05$) (Figure 4B) and EnvFit analysis indicated that higher hydrocarbon and organic carbon (AFDW) concentrations, and lower S and P (resulting in a much higher N:P ratio) concentrations were key predictors in community structure changes at the spill site (Figs 5.4B and 5.4C)

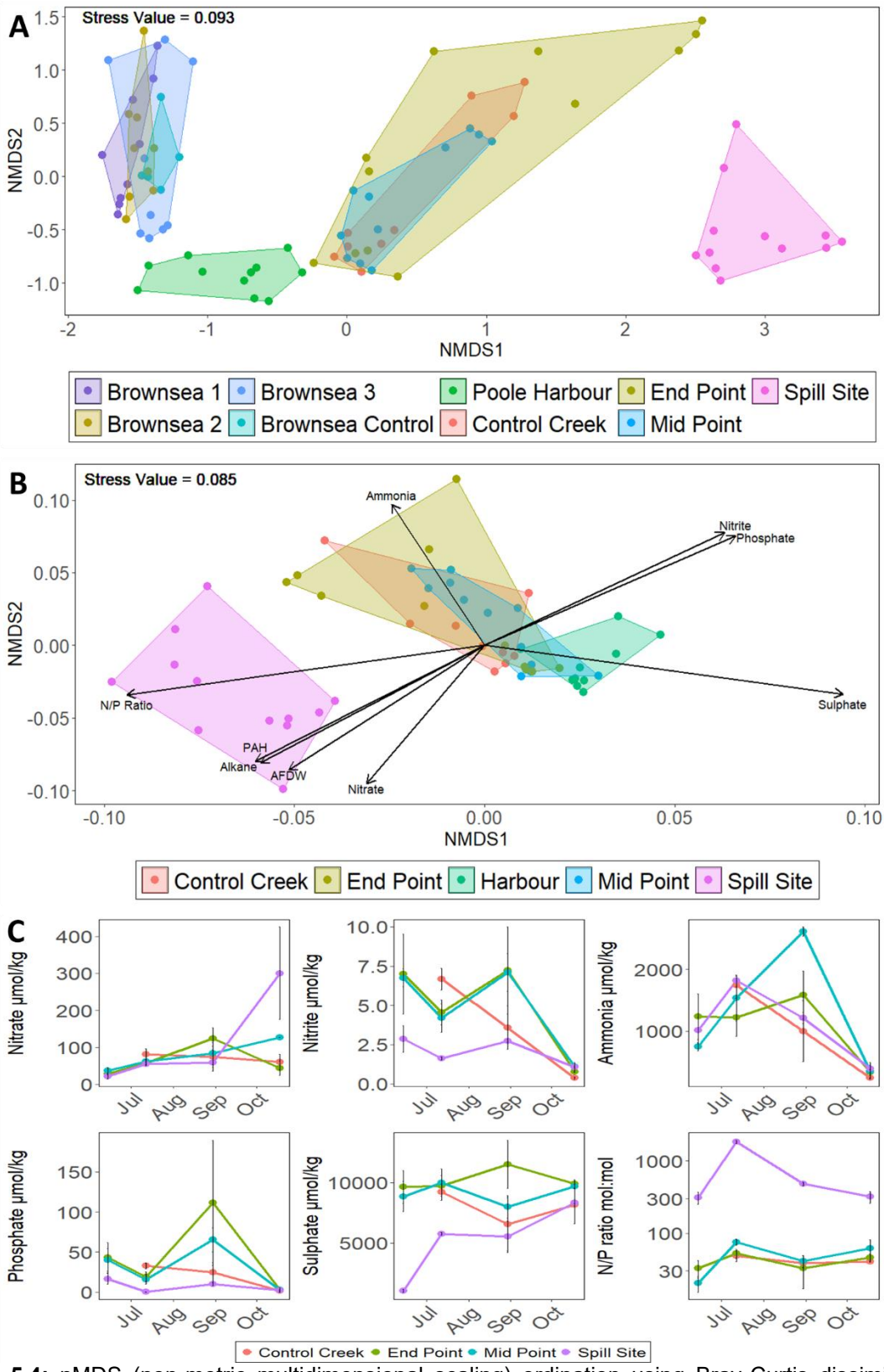


Figure 5.4: nMDS (non-metric multidimensional scaling) ordination using Bray-Curtis dissimilarity, based on **A.** ASV (amplicon sequence variants) of bacterial 16S rRNA genes, and **B.** metagenomic bacterial sequence reads classified using the *k*-mer "Kraken2" approach paired with Bracken including nutrient and hydrocarbon vectors and **C.** nutrient concentrations (µmol/kg) and molar N:P ratio between surface sediment samples (Replications =3) across Brownsea Island and Ower Bay Creek, Dorset, UK over June – October 2023, following an oil pipline spill on 26th March, in comparison to uncontaminated Control sites. Sampling dates (14th June, 12th July, 30th August and 18th October)

SIMPER analysis of bacterial metagenomic reads taxonomically assigned to genus level, revealed that *Anaerolina* (0.1% contribution), *Dechloromonas* (0.5% contirbution), *Thiobacillus* (0.8% contribution), *Hydrogenophaga* (1.5% contribution), *Acidovorax* (1.1% contribution), *Ilumatobacter* (0.7% contribution), *Methyloceanibacter* (1.1% contribution), Woeseia (0.2% contribution) and *Marinobacter* (0.5% contribution) were contributing most significantly to community dissimilairty between the Spill Site and all other sites (Figure 5). *Anaerolina* reads were in significantly higher relative abundance at the Spill Site from June to Oct, being approximatly five-fold more abundant compared to all other sites at its peak in June ($t = -5.34 – -3.01$, $p < 0.001$) (Figure 5A). Both *Dechloromonas* and *Thiobacillus* reads were also significantly more abundant (approximately five- to 10-fold greater) at the Spill Site compared to all other sites, as were *Hydrogenophaga* and *Acidovorax* (approximately two-fold greater) ($z = -7.71 – -3.78$, $p < 0.001$) (Figure 5B, C, D, E). Other taxa increased in relative abundance much later after the spill. For example, *Marinobacter* reads were significantly higher at the Spill Site (three-fold more abundant) and End Point site (five-fold more abundant) compared to the Control Creek and Mid Point site in August ($z = -2.14 – -1.72$, $p < 0.05$) (Figure 5F). Similarly, reads assigned to *Alcanivorax*, *Cycloclasticus* and *Thalassolituus* (known Obligate Hydrocarbonoclastic Bacteria (OHCB)) were all significantly higher in relative abundance at or near the Spill Site in later months ($t = 2.63 – 4.08$, $p < 0.05$) (Figure 5G, H, I). *Alcanivorax* reads peaked significantly (relative to control site) in August at the Spill Site and End Point site ($z = -2.04$, $p < 0.05$), whilst *Cycloclasticus* reads were significantly higher also at the End Point, Mid Point and Spill Site in August and *Thalassolituus* reads were significantly higher compared to the control site in August (20-fold at the End Point and Mid Point and 5-fold at the Spill Site; $z = -2.77$, $p < 0.01$).

Numerous other genera became significantly less abundant at the Spill Site. For example, the relative abundance of *Ilumatobacter, Methyloceanibacter* and *Woeseia* were all significantly lower (2.47-9.55, $p < 0.05$) at the Spill Site compared to all other sites, for most of the study period (Figure 5J, K, L).

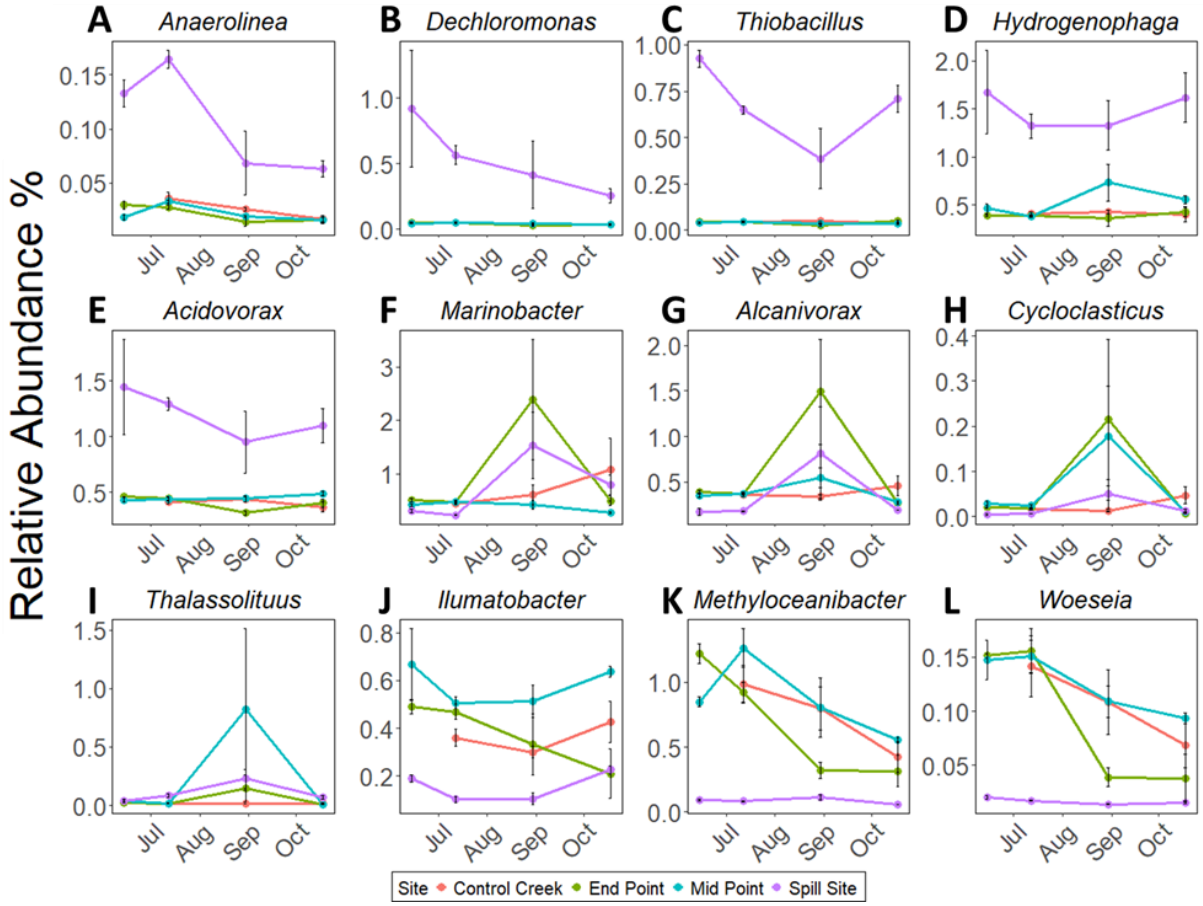


Figure 5.5: Relative abundance (%) of genus showing differential abundance between sites based on Simper analysis within communities based on metagenomic bacterial sequence reads classified using the *k*-mer "Kraken2" approach paired with Bracken from surface sediment samples (mean ± SE, replication = 3) collected from sites across Wytch Farm oil field (Ower Bay Creek), Dorset, UK from June – October 2023, Control Creek site.

Using the CANT-HYD database, genes known to be involved in aerobic or anaerobic alkane and aromatic hydrocarbon degradation were identified from the metagenomic contigs. The abundance counts (Z-score normalised) for each sampling site have been presented and analysed (Figure 6). *assA, assB* (genes coding for: anaerobic alkylsuccinate synthase A and B subunit), *abcA1, abcA2* (anaerobic benzene carboxylase) and *ndoA* (aerobic naphthalene dioxygenase A subunit), were

on average approximately 119%, 187%, 117%, 185% and 75% respectively more abundant at the Spill Site compared to all other sites ($z = -4.95 – -2.03$, $p < 0.05$). *sBmoX, Z* (aerobic butane monooxygenase) and *tmoA_bmoA* (aerobic toluene/benzene monooxygenase) genes were only present at the Spill Site throughout the study period, being on average greater in abundance in August compared to all other times points by 108%, 143% and 200% respectively ($t = 2.13 – 3.03$, $p < 0.05$). In August and October *ahyA* (anaerobic alkane hydroxylase A subunit) and *bssA* (anaerobic benzylsuccinate synthase A subunit) were approximately 200% and 165% more abundant at the Spill Site respectively, compared to all other sites ($z = 3.05 – 3.67$, $p < 0.01$). *cmdA* (anaerobic aromatic dehydrogenase A subunit), CYP153 (cytochrome P450 aerobic alkane hydroxylase), *ladA_Alpha* (aerobic long-chain alkane monooxygenase), *alkB* (aerobic alkane hydroxylase) and *pcaG, H* (aerobic aromatic protocatechuate dioxygenase) were all approximately 145% – 291% more abundant within the Mid Point, End Point and Control Creek compared to the Spill Site ($t = 2.16 – 3.89$, $p < 0.05$). Dependent on the hydrocarbon degrading genes present and their abundance, the Spill Site samples clustered together via Euclidian distance, being highly similar to one another and dissimilar to all other sites.

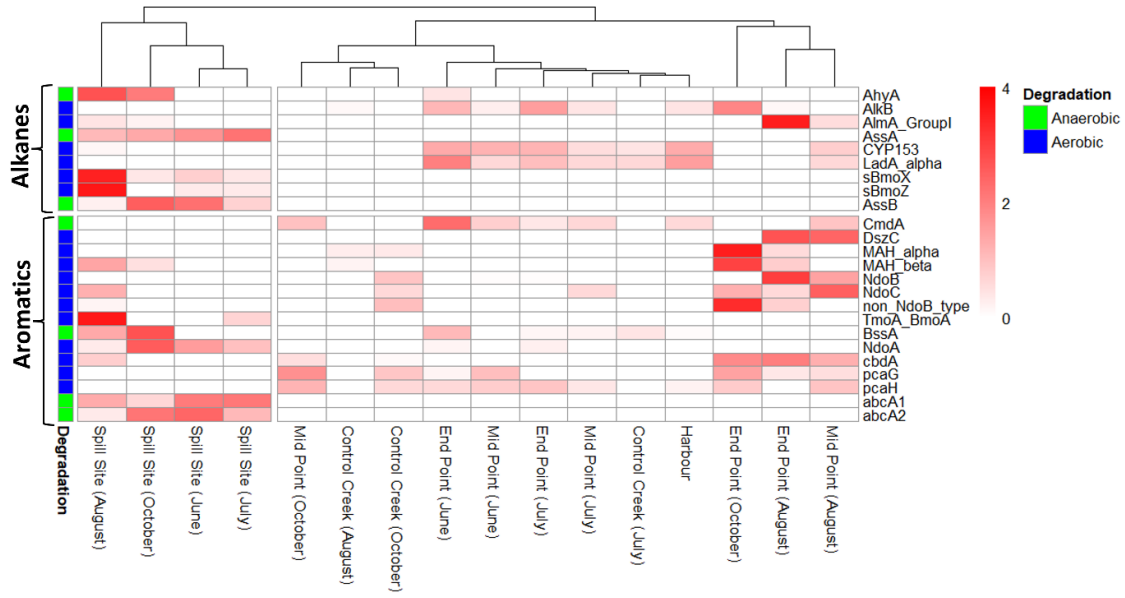


Figure 5.6: Heatmap of functional genes hits using Hidden Markov Model (HMM) associated with hydrocarbon degradation pathways based on sequences (normalised by row using z-score normalisation) pulled out and classified from Contigs, using the CANT-HYD database, from surface sediment samples (mean ± SE, n=3) collected from sites across Wytch Farm oil field (Ower Bay Creek), Dorset, UK from June – October 2023, Control Creek site.

In order to analyse the capacity for hydrocarbon degradation within individual genomes, MAGs (Metagenome-Assembled Genomes) were reconstructed from assembled contigs and their abundance was quantified for each sampling site (Figure 7). Clustering via Euclidian distance reaffirmed much of the findings from the taxonomic classification of metagenomic reads following similar patterns presented in Figure 5, with notable bins 143 (*Anaerolineales*), 99 (*Dechloromonas*) and 84 (*Thiobacillus*) being highly abundant at the Spill Site and absent at other sites ($t = -7.78 – 15.86$, $p < 0.001$), whilst bin 170 (*Woeseia*) and 196 (*Ilumatobacter*) were predominantly abundant across the rest of the creek sites ($t = -6.47 – -2.22$, $p < 0.05$). The CANT-HYD database was further used to identify hydrocarbon degradation genes within the MAGs to explore the contribution these MAGs have towards oil degradation potential (Supplementary Material: Figure S9). Anaerobic hydrocarbon degradation potential within the Spill Site was shown to be considerable. This was confirmed by the presence of multiple anaerobic hydrocarbon degradation genes, including assA and bssA within multiple MAGs that were detected only at the Spill Site (associated

with bin 143 (*Anaerolineales*), 33 (*Desulfosarcina*), 148 (*Desulfobacter*), 138 (*Desulfomonile*) and 136 (*Proteobacteria*), aswell as abcA1/2 (associated with bin.29 (*Betaproteobacteria*), 84 (*Thiobacillus*), 162 (*Caldilinea*) and 11 (*Methanomicrobiaceae*)) and ahyA (associated with bin. 11 (*Methanomicrobiaceae*), 143 (*Anaerolineales*), 148 (*Desulfobacter*), 136 (*Proteobacteria*), 53 (*Porphyrobacter*), 206 (*Bacteroidetes*), 162 (*Caldilinea*), 29 (*Betaproteobacteria*), 164 (*Geobacillus*), 99 (*Dechloromonas*), 84 (*Thiobacillus*), 138 (*Desulfomonile*), 33 (*Desulfosarcina*)). At the Mid Point, End Point and Control Creek sites, further observations indicated a high potential of hydrocarbon degradation through both aerobic degradation pathways and anaerobic degradation pathways highlighted particularly by bins 102 (*Pseudohordobacter*), 113 (*Lamiaceae*), 134 (*Myxoccoccales*) and 16 (*Spongiibacter*).

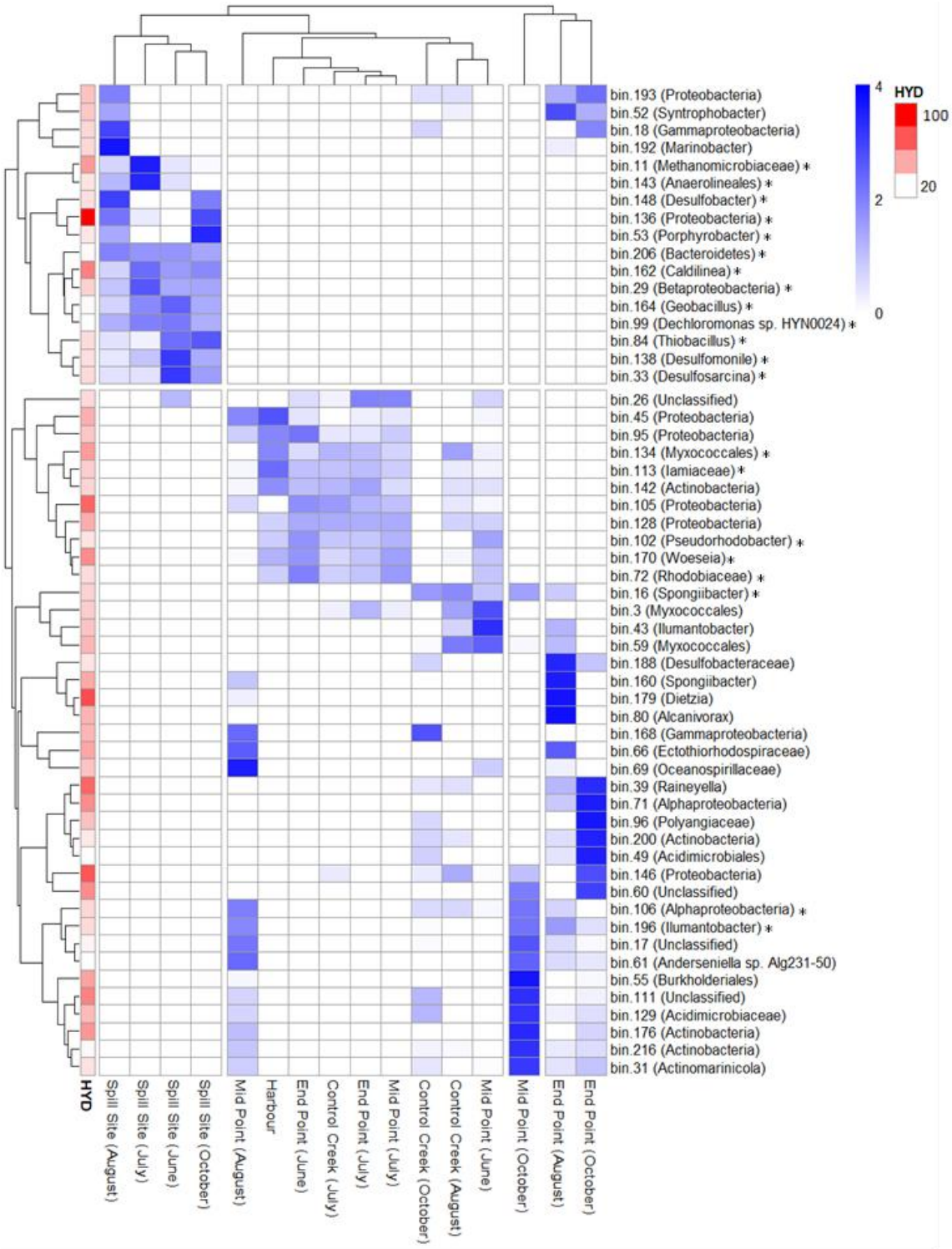


Figure 5.7: Heatmap of MAGs (Metagenome-Assembled Genome's) count (MAG copies per million reads, normalised by row using z-score normalisation) from surface sediment samples (mean ± SE, n=3) collected from sites across Wytch Farm oil field (Ower Bay Creek), Dorset, UK from June – October 2023, Control Creek site. MAGs were taxonomically classified by GTDB-Tk v1.1.0. The number of functional genes associated with hydrocarbon degradation are shown within each MAG, based off matches against the CAN'T-HYD database. HYD = Count of hydrocarbon degrading genes within each MAG. * indicates notable MAG.

Assessment of biogeochemical functional gene pathways throughout Ower Bay Creek

An effect of the oil spill on the abundance of biogeochemical cycling genes associated with the nitrogen, sulphur, and methane cycle was determined using the NCycDB, SCycDB and MCycDB databases. No significant change over time or between sites was observed based on total gene count within each cycle (Supplementary Material: Figure S8). However, nitrogen cycling functional genes *nifD* (nitrogenase molybdenum-iron protein alpha chain) and *nifK* (nitrogenase molybdenum-iron protein beta chain), genes associated with nitrogen fixation were significantly more abundant at the Spill Site compared to all other sites ($t = 3.22 – 3.93$, $p < 0.001$) (Figure 8), whilst no change in the *nifH* gene were observed ($t = 3.22 – 3.93$, $p < 0.001$). *nirK and S* (nitrite reductase) genes, associated with denitrification were also shown to be less abundant at the Spill Site compared to all other sites ($t = -2.43$, $p < 0.05$.

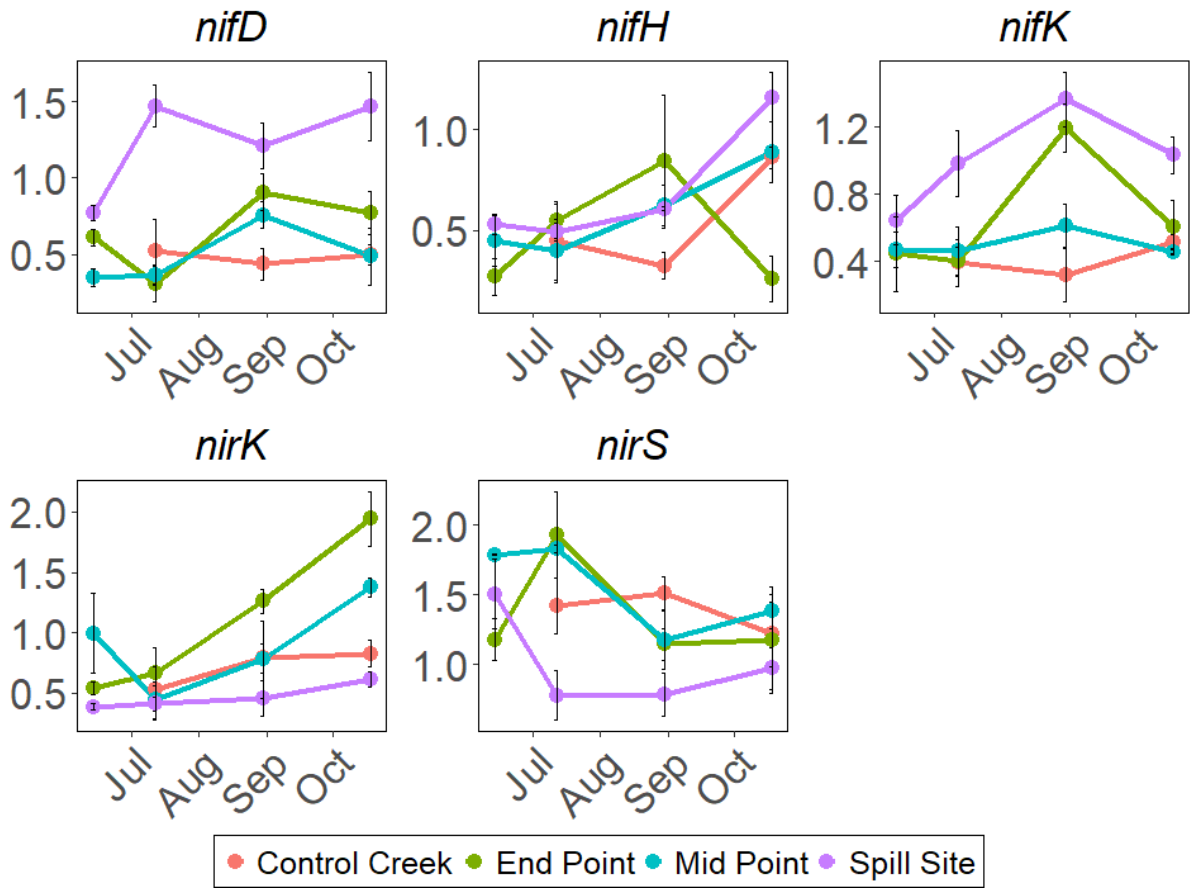


Figure 5.8: Relative abundance (%) of nitrogen cycling genes causing dissimilarity within bacterial communities (Based off SIMPER analysis) based on shotgun metagenomic sequencing reads taxonomically classified using NCycDB sediment samples (mean ± SE, n=3) collected from sites across Wytch Farm oil field (Ower Bay Creek), Dorset, UK from June – October 2023.

The sulphur cycling gene *asrB* (anaerobic sulphite reductase), was found to be more abundant at the Spill Site by approximately 55.5% (t = -3.21, $p < 0.05$) in June (Figure 9), reducing in relative abundance by 44.4% over time between June and October but still remained higher than at all other sites. *asrA* which codes for the alpha subunit of the same enzyme complex was however not significantly different between any of the sites. *cysA, cysU* (sulphate/thiosulphate transporter) genes, associated with the uptake of sulphate/thiosulphate, were approximately 28 and 24% more abundant at the Spill Site compared to all other sites (-3.23 – -2.84, $p < 0.05$), whilst *cysC, I, J* (sulphite reductase) and *dsrA, B* (dissimilatory sulphite reductase) genes did not differ significantly between sites.

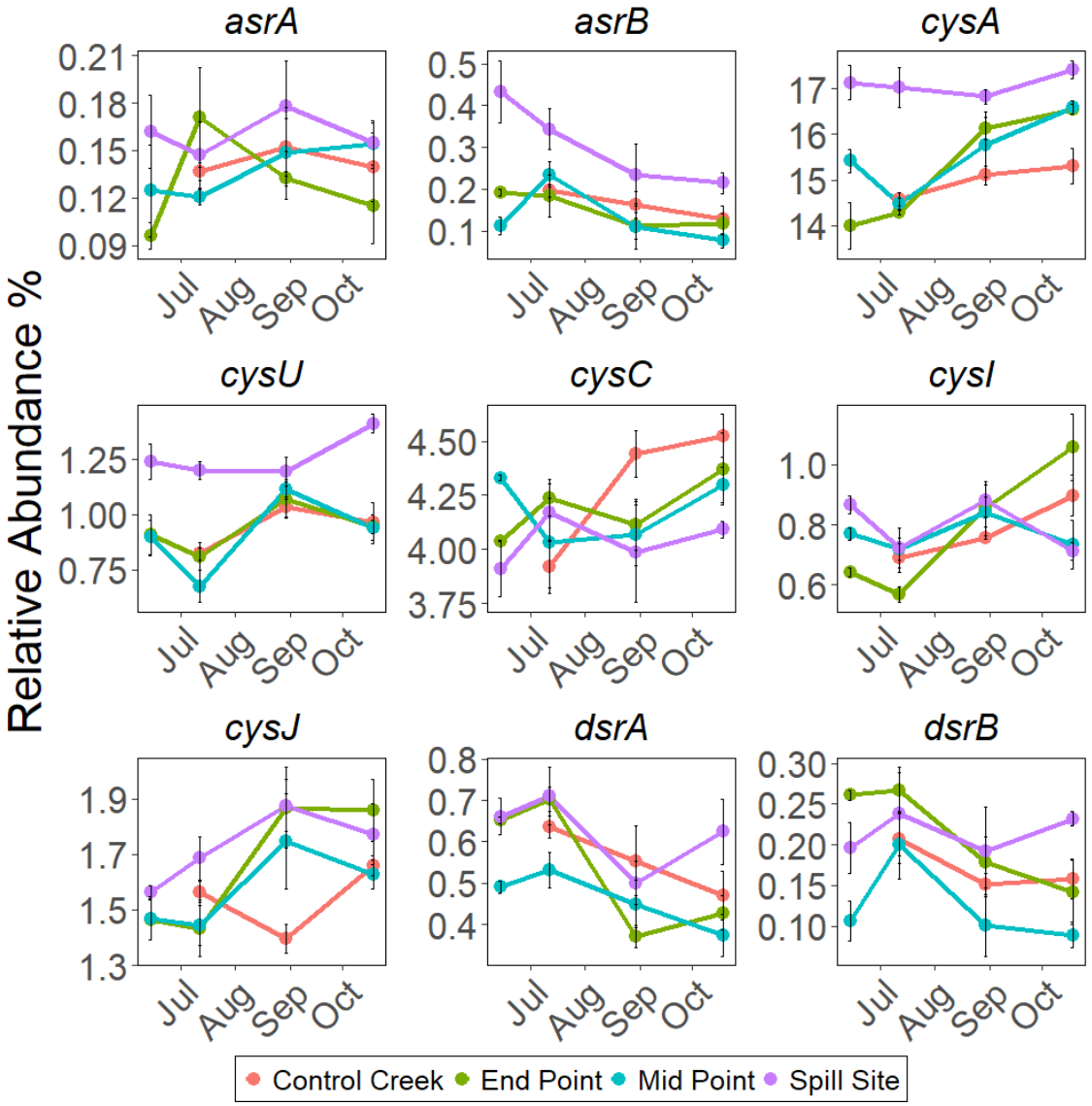


Figure 5.9: Relative abundance (%) of sulphur cycling genes causing dissimilarity within bacterial communities (Based off SIMPER analysis) based on shotgun metagenomic sequencing reads taxonomically classified using SCycDB sediment samples (mean ± SE, n=3) collected from sites across Wytch Farm oil field (Ower Bay Creek), Dorset, UK from June – October 2023.

Within the methane cycle, *mbhJ* (membrane-bound hydrogenase) genes, were approximately 40% more abundant at the Spill Site compared to other sites (-5.43, $p < 0.05$) (Figure 10). *mcrA* and *B* (methyl-coenzyme M reductase) genes were both approximately 80% more abundant at the Spill Site compared to all other sites (-2.54 – -3.23, $p < 0.05$) and were greater in relative abundance by approximately 667%. *mvhA* (F_{420}-non-reducing hydrogenase), associated with the central methanogenesis pathway was approximately 44% more abundant at the Spill Site compared to other sites in June and July (t = -5.47 – -2.74, $p < 0.01$) but declined by August to similar

relative abundances at all other sites. *acdA* (acetate-CoA ligase (ADP-forming)) and *acs* (acetyl-CoA synthetase) genes, associated with acetoclastic methanogenesis, were shown to be 33.3% and 28.6% less abundant at the Spill Site compared to all other sites (t = 2.70 – 5.29, $p < 0.05$).

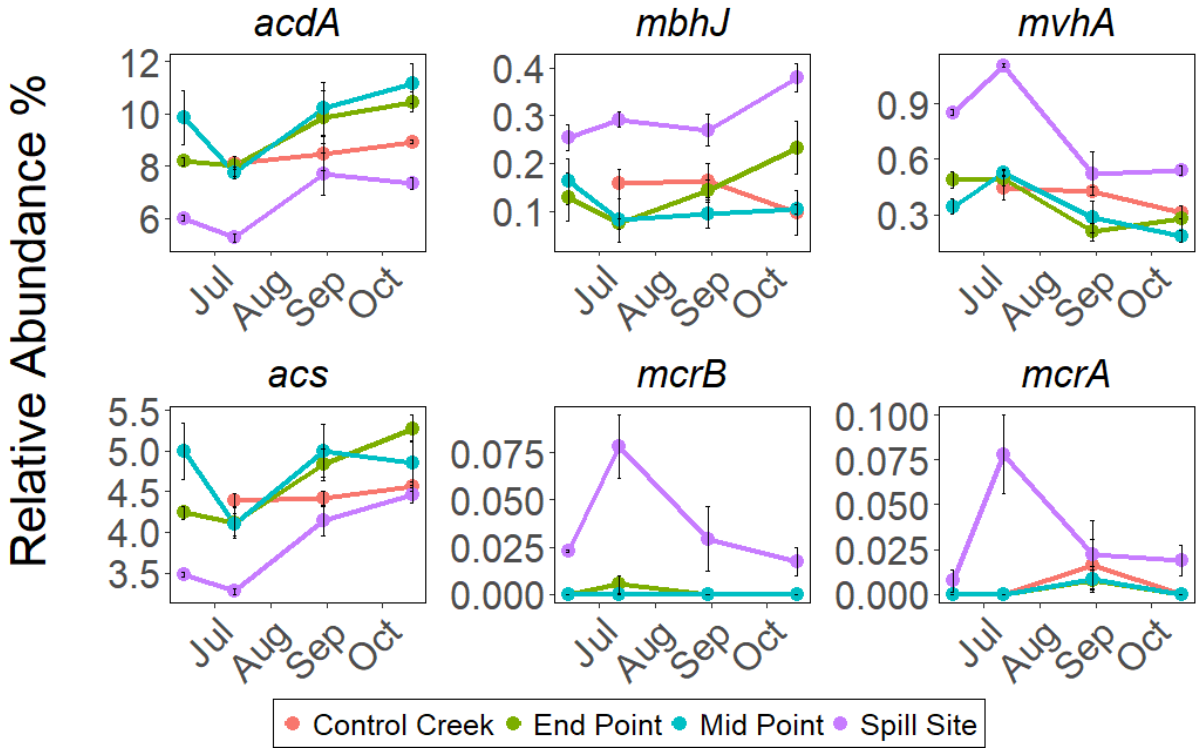


Figure 5.10: Relative abundance (%) of methane cycling genes causing dissimilarity within bacterial communities (Based off SIMPER analysis) based on shotgun metagenomic sequencing reads taxonomically classified using MCycDB sediment samples (mean ± SE, n=3) collected from sites across Wytch Farm oil field (Ower Bay Creek), Dorset, UK from June – October 2023.

Discussion

Hydrocarbon concentration's after initial spill and during clean up

As June 2023 (three months after initial spill), concentrations of alkanes and PAHs, were highest at the Spill Site, whilst the rest of Poole harbour and all other sites were similar to our control sites, being largely unimpacted. This was to be expected as according to response reports (Hatch, 2024), by April 2023 (one month after the initial spill) no further contamination was found outside of the localised Spill Site due to rapid containment by deployment of booms. As Poole Harbour is historically a busy recreational and fisheries port, hydrocarbons would accumulate in sediments from

both anthropogenic and natural sources (Woodhead *et* al., 1999; May and Humphreys, 2005; Underhill-Day, 2006) which would explain the concentrations of hydrocarbons measured outside of the immediate Spill Site, and the PAHs were further highlighted as pyrogenic in origin, meaning they were unlikely to be associated with the pipeline spill. During the investigation, hydrocarbon concentrations outside of the localised Spill Site stayed consistently lower than at the Spill Site, indicating the effectiveness of absorbent booms as a physical barrier in reducing hydrocarbon contamination to the wider harbour area (Dave and Ghaly, 2011; Prendergast and Gschwend, 2014).

By the end of August, alkane and PAH concentrations peaked at the Spill Site. In mid-August, there was also some mobilisation of oil sheens caused by high tides (Hatch, 2024), which may have potentially caused the small increases in hydrocarbon concentration (particularly larger n-alkanes, pristane, phytane and 3- and 4-ring PAHs) that occurred in the adjacent Mid Point zone of the creek. Further response via the deployment of a bubble barrier as an extra safeguard was implemented to avoid further mobilisation of hydrocarbons during high tides (Feng and Zhang, 2023; Hatch, 2024). By October 2023, there was a substantial decline in the concentration of alkanes *n*-$C_{10–38}$ and PAH's at the Spill Site, returning to similar concentrations found across control sites and the surrounding Poole Harbour area. The biodegradation of hydrocarbons via bacteria has been well documented in prior studies and is most likely one of the main processes causing this significant decline (McGenity *et* al., 2012; Liu *et al*., 2020). Larger branched alkanes such as pristane and phytane, along with methylated 2, 3-ringed PAH's persisted within the Spill Site due to their higher molecular weight and complexity reducing their degradability (Setti et al., 1993; Mohanty and Mukherji, 2008). Over time concentrations of these larger complex hydrocarbons may further reduce to similar levels of the surrounding area (Prince and

Lessard, 2004) although in this case, significant amounts of sediment were removed after our final sampling (October 2023) and the site is completely remediated and recovered as observed in March 2025 (Supplementary Material: Figure S10).

Spill impact on sediment bacterial communities

In June 2023 (80 days post-spill), the bacterial 16S rRNA gene abundance was observed to be lowest at the Spill Site. Notably, abundance showed no significant change across all other sites. By July 2023, the bacterial 16S rRNA gene abundance had increased to similar levels found across the rest of the Ower Bay creek area. In this instance, the increased concentration of hydrocarbons in the system may have caused a depletion in oxygen due to an increased oxygen demand via hydrocarbon-degrading bacteria (Shin *et al*., 2000), accounting for the initial drop in bacterial abundance, as well as some bacterial taxa being negitavely impacted by oil toxicity, or being outcompeted by hydrocarbon-degrading taxa for nutrients such as N and P (Sikkema *et al*., 1995; Head et al., 2006). By July 2023, the increase in bacterial abundance to levels similar to the Control Creek, Mid Point and End Point could be attributed to an increase in bacteria better suited to a more anoxic and hydrocarbon contaminated systems, which was clearly observed with the increase in anaerobic bacterial genera and hydrocarbon degradation genes associated with anaerobic degradation pathways found at the Spill Site. Similar switches to anaerobic bacteria has previously been observed in Guo *et al*. (2022), across an increasingly hypoxic region of the Bohai Sea. Bacterial abundance was observed to be much lower within the coarse Brownsea Island sediments, compared to the fine sediment of Ower Bay Creek (Supplementary Material: Figure S4). The different physical and biological properties of these sediments, e.g. sands vs muds respectively, would likely be the

main driver to account for this difference rather than any differences in hydrocarbon contamination (Szava-Kovats, 2008; Xu *et al*., 2021).

Throughout this study *Dechloromonas*, *Anaerolinea*, *Thiobacillus*, *Acidovorax* and *Hydrogenophaga*, were the most prevalent genera at the Spill Site. Outside the Spill Site, *Woeseia* and *Methyloceanibacter* were most prevalent, initially indicating an impact on these genera from hydrocarbon contamination, further altering community structure (Yakimov *et al*., 2005; McGenity *et al*., 2012). *Dechloromonas,* which was only detected at the Spill Site, has been highlighted as a potential anaerobic hydrocarbon degrader, notably in the degradation of PAHs (Salinero *et al*., 2009; Yan *et al*., 2017; Zhang *et al*., 2021) and benzene under nitrate and sulphate reducing conditions (Chakraborty *et al*., 2005). During this study, *Dechloromonas* was shown to have the potential of both anaerobic alkane and aromatic degradation via the *abcA* (anaerobic benzene carboxylase)*, cmdA* (anaerobic aromatic dehydrogenase A) and *ahyA* gene (anaerobic alkane hydroxylase A) pathways, in which sulphate/sulphite may be the primary electron acceptor during fumarate addition aromatic biodegradation (Zhang *et al*., 2010). The presence of dissimilatory sulphite reductase encoding genes such as *dsr* and *asr* throughout the creek along with the large reduction in sulphate further suggests the use of sulphate as an electron acceptor supporting anaerobic biodegradation at the Spill Site. *Anaerolinea* was observed to be more abundant at the Spill Site and has been shown to thrive under anaerobic conditions. Recent studies have suggested *Anaerolinea* may be an important sulphate reducer with genes associated with both assimilatory and dissimilatory sulphate reduction being present in numerous MAGs (Payne *et al*., 2024) but may also have a role to play in anaerobic alkane degradation via *assA*-like genes that were observed within MAGs taxonomically classified as *Anaerolineales*. According to prior studies,

assA genes have not currently been identified in MAGs assigned to *Anaerolineales*, however it has been heavily suggested that they do likely contain these genes, encoding for alkylsuccinate synthase allowing for the initial activation for long-chain *n*-alkane biodegradation (Liang *et al*., 2016; Wu *et al*., 2024). Further classification highlighted these *assA*-like genes as glycl radical proteins (Backman *et al*., 2017) associated with *Anaerolinea* (89% sequence identity), being a part of the *Anaerolineaceae* family that has the potential for anaerobic alkane degradation.

Thiobacillus has been previously documented to be present within hydrocarbon polluted areas (Kellermann *et* al., 2009; Tian *et al*., 2017), which was observed during the present study to be highly abundant at the Spill Site, in contrast to all other sites where its relative abundance was very low. *Thiobacillus* itself is a sulphur oxidiser, capable of oxidising elemental sulphur, hydrogen sulphide and thiosulphate to sulphate (Sublette and Sylvester, 1987; Zhi-Hui *et al*., 2010). Notably this tends to occur in aerobic conditions, however, it also occurs anaerobically, in which thiosulphate is split by rhodanese to sulphite and elemental sulphur, which is further oxidised to sulphate (Schedel and Trüper, 1980). The increase of *Thiobacillus* at the Spill Site suggests *Thiobacillus* to be the dominant sulphur oxidiser involved in hydrocarbon degradation. It's adaptability to both oxic and anoxic conditions, and both alkane and aromatic hydrocarbon degradation, is highlighted by the wide range of aerobic and anaerobic hydrocarbon degradation genes identified within the *Thiobacillus* MAGs (e.g. *abcA*, *ahyA*, *alkB*, *ndoB* and *almA*) (Supplementary Material: Figure S9). Outside of the Spill Site, sequencing indicated that *Woeseia*, another sulphur oxidiser, similarly capable of anaerobic sulphur oxidation (Buongiorno *et al*., 2020; Hoffmann *et al*., 2020) was prevalent throughout all other creek sites, with much lower relative abundance at the Spill Site. Therefore, it is suggested that *Woeseia* may

act as a potential biomarker for system health. Prior studies have also suggested other potential biomarkers of system health, such as *Thioprofundum,* which has previously been shown to decline in hydrocarbon contaminated sediments (Godoy-Lozano *et al*., 2018; Suárez-Moo *et al*., 2020), as well as a broader range of bacteria such as nitrifiers that have been shown to be highly sensitive to hydrocarbon toxicity (Urakawa *et al*., 2019). Particularly, this highlights the importance of understanding genera that are either absent or reduce in abundance at oil Spill Sites, as well as those that increased due to their involvement in hydrocarbon degradation, in understanding the full impacts of hydrocarbon contaminants on microbial community structure and function. Both *Woeseia* and *Thioballicus* are shown to play similar roles within the sulphur cycle, therefore, due to this functional redundancy, oil pollution does not necessarily negatively impact sulphur oxidation, but does however impact the community composition, by selecting for sulphur oxidising bacteria with the capacity to deal with the presence of hydrocarbon contamination and changing environmental conditions (Badmadashiev *et al*., 2023).

Sequences assigned to aerobic *Ilumatobacter* (Matsumoto *et al*., 2009) and *Methyloceanibacter* were much lower in relative abundance at the Spill Site, suggesting they may potentially be outcompeted by anaerobes, such as *Dechloromonas* and *Anaerolinea* (Achenbach *et al*., 2001). Prior studies have highlighted the presence of *Ilumatobacter* within marine polluted sites, suggesting a potential for aerobic alkane and aromatic degradation (Ellis *et al*., 2022; Ashade *et al*., 2024). However, little is known about what hydrocarbons some *Ilumatobacter* species may successfully grow on. Even so, as seen in the present study with the presence of alkane degradation pathway genes *CYP153*, *ladB* and *almA* and aromatic degradation pathway gene *ndoB* within MAGs taxonomically classified as *Ilumatobacter*, their

possible capacity for aerobic hydrocarbon degradation has been further highlighted. Interestingly, the putative anaerobic alkane pathway gene *ahyA* was also found within *Ilumatobacter* MAGs suggesting the possibility for alkane degradation under anaerobic conditions. However, prior studies have shown this genus to be outcompeted by Anaerolineaceae under anaerobic conditions, with the environment selecting for anaerobes (Zhao *et al*., 2023; Gao *et al*., 2024). Thus, in the context of this study *Ilumatobacter* may have been outcompeted by *Anaerolinea, Dechloromonas and Thiobacillus* at the Spill Site as dominant hydrocarbon degrading anaerobes. The lower relative abundance of another aerobe, *Methyloceanibacter,* may further indicate the anoxic nature of the Spill Site compared to other sites with the creek (Takeuchi *et al*., 2014).

The capacity of hydrocarbon degradation with saltmarsh creek environments

CANT-HYD identified 24 hydrocarbon degrading genes with 17 being associated with aerobic degradation and seven being associated anaerobic degradation throughout the study sites (Khot *et al*., 2022). The Spill Site was observed to be dominated by anaerobic hydrocarbon degrading genes, whilst all other sites outside of the Spill Site were dominated by various aerobic HYDs. This highlights a high capacity for hydrocarbon degradation and the capacity to change from aerobic to anaerobic hydrocarbon degradation under anoxic conditions. Notably, *assA, B, abcA1, 2* and *bssA* were the most prevalent anaerobic HYD genes at the Spill Site. *assA and B* are involved in anaerobic alkane degradation, catalysing the initial steps via fumarate addition (Callaghan *et al*., 2010). Analysis of MAGs highlighted the presence of *assA*-like genes, notably in bin.143 (*Anaerolineales*), bin.148 *(Desulfobacter*) *and* bin.136 (Proteobacteria). Further exploration of the *assA* genes within bin.136

(Proteobacteria), identified them as glycl radical proteins associated with Desulfobulbaceae (90.26 Identity).

Genes *abcA and bssA* have previously been shown to be associated with PAH degradation, by the direct carboxylation of benzene and toluene via fumarate addition (Luo *et al*., 2014 Bacosa *et al*., 2018; Toth *et al*., 2021; van Leeuwen *et al*., 2022). Notably, MAGs including bin.99 (*Dechloromonas*), bin.84 (*Thiobacillus*) and bin.11 (Methanomicrobiaceae) contained putative *abcA*-like genes that were identified as coding for UbiD family decarboxylases (93.79 – 98.63% identity), highlighting potential involvement in anaerobic aromatic degradation at the Spill Site (Kim *et al*., 2003; Chakraborty *et al*., 2005). The UbiD enzyme family is however highly widespread, with considerable diversity, with many of them catalysing aromatic decarboxylation, suggesting this may be a possibility that should be considered for further investigation (Marshall *et al*., 2021). Methanomicrobiaceae *abcA* genes were shown to be similar to that of *abcA*-like genes within *Methanoregula* (97.92% identity), indicating the potential for direct anaerobic aromatic degradation within methanogens. Even so it must be highlighted, that the sequences classified as *abcA,* within bin.11 *(Methanomicrobiaceae)* was based off homology to only two available putative *abcA Clostridia* bacterium sequences (Abu laben *et al*., 2010) within the CANT-HYD database, with a 21.95% identity, thus the potential for degradation cannot be directly confirmed.

By August an uplift in the presence of aerobic degradation genes *ndoA*, *ndoC*, CYP153 P450, *sBmoX*, *Z* and *tmoA*/*bmoA* was observed. *ndoA* and *ndoC* have been shown to be associated with aerobic PAH degradation via the hydroxylated gentian acid pathway, specifically being upregulated in the presence of naphthalene, playing a significant role in initiating the degradation of lower-molecular-weight PAHs (Ribeiro

et al., 2018; Li et al., 2023). Cytochrome P450 CYP153 alkane hydroxylases catalyse the hydroxylation of aliphatic and alicyclic alkanes (van Beilen et al., 2005; Funhoff et al., 2007). During monitoring and clean-up of the site some further mobilisation of hydrocarbons occurred due to high tides during the August sampling time period, potentially mobilising more oil to the oxygenated surface which may explain the increase in aerobic hydrocarbon degradation genes at this time. Overall, these communities highlight high hydrocarbon degrading potential within the creek system under both oxic and anoxic conditions and a high level of adaptability within the community, rapidly selecting for genera capable of aerobic and anaerobic hydrocarbon degradation.

The biogeochemical functionality of Ower Bay Creek

Through the analysis of sediment nutrient concentrations and functional genes associated with the nitrogen-cycle, sulphur-cycle, and methane-cycle, any impact of the oil spill on macronutrient cycling was addressed. At the Spill Site specifically, a greater relative abundance of *nifD and nifK,* genes associated with nitrogen fixation were present compared to other creek sites. This process converts atmospheric dinitrogen gas (N_2) to NH_3, potentially contributing to NH_3 input at the Spill Site (Zehr and Capone, 2020), but it is also worth noting that the further inputs of NH_3 at the site may also come from the ammonification of biomass (e.g. reed material killed off during the initial spill event). Prior studies have shown that various hydrocarbonoclastic bacteria (82 potential species) may also be diazotrophic, thus may explain the uplift of genes associated with nitrogen fixation within the Spill Site (Taketani *et al*., 2009; Dashti *et al*., 2015). Although differences in gene relative abundance of *nifD,K* and *nirK,S* are prominent between the Spill Site and the rest of the creek; nitrate, nitrite and ammonia concentrations stayed fairly consistent. However, declines in the *nirK*

and *nirS* nitrite reductase genes, may be attributed with the increase in sulphate reduction from sulphate reducing hydrocarbon degrading bacteria that became dominant the Spill Site (Widdel *et al*., 2007; Sherry *et al*., 2013). At the Spill Site, the system became increasingly phosphate limited compared to the other creek sites, notably, due to the heavy demand from hydrocarbon degraders following the influx of oil as a rich carbon source (Engelhardt, 1985).

During the study sulphate concentrations were observed to be considerably lower at the Spill Site compared to all other sites, suggesting an increased level of sulphate reduction compared to all other sites. As previously stated, *Anaerolinea* was more abundant at the Spill Site, notably due their capacity for anaerobic hydrocarbon degradation, but also their potential importance in sulphate reduction (Sherry *et al*., 2013). This along with the presence of the *cysA and cysU* genes, that are subunits of the sulphate transport system and the *asrB* dissimilatory sulphate reduction pathway (Laudenbach and Grossman, 1991; Kawano *et al*., 2018) indicates a high level of sulphate reduction at the Spill Site. Due to the increase in sulphate reduction it can be suggested an increase in the production of hydrogen sulphide is released into the environment (Muyzer and Stams, 2008). Hydrogen sulphide has been shown to cause various negative impacts, including oxidative damage to RNA and DNA in invertebrates and reduced bacterial growth (Reis *et al*., 1992; Joyner-Matos *et al*., 2010). This would suggest during the initial spill and potential increase in hydrogen sulphide to the system, the surrounding area may suffer many of these negative impacts. However sulphate concentrations at the spill increased back to similar levels seen throughout the creek, suggesting rapid recovery of the system, which may partly be attributed to increase in sulphide oxidisers such as *Thiobacillus* that was

significantly more abundant at the Spill Site and capable of hydrogen sulphide oxidation to sulphate (Cline and Richards, 1969; Sublette and Sylvester, 1987).

The *mcrA, mcrB and mbhJ* genes involved in the central methanogenic pathway (Qian *et al*., 2022) were in higher relative abundance at the Spill Site suggesting an increase in methanogenesis. Notably, a Methanomicrobiaceae MAG (with 95 - 98% identity to *Methanoregula*) was highlighted as a prominent archaeal taxa at the spill, accounting for the increase in relative abundance of the methanogenic gene pathways. It has previously been suggested that within sites of crude oil contamination, enhanced methanogenesis can take place, via hydrogenotrophic methanogenesis, in which Yang *et al*, (2018) observed an 8% increase from 35% to 43% in the proportion of hydrogenotrophic methanogens within the oil contaminated soils, highlighting hydrogenotrophic methanogenesis to be significantly more dominant that that of acetoclastic methanogenesis in contaminated sites. This was also observed during the current study as outside of the Spill Site a larger relative abundance of *acdA* and *acs* genes were present being associated with acetoclastic methanogenesis (Qian *et al*., 2022), suggesting a switch from hydrogenotrophic methanogenesis to acetoclastic methanogenesis. Further comparisons of metagenomic reads classified as *mcr* via the NCBI nr database using the Basic Local Alignment Search Tool (BLAST), confirmed many of these genes to be associated with a variety of methanogenic archaea, notably *Methanosarcina* (94.5-100% identity) and *Methanoregula* (95-98% identity) (Alvarado *et al*., 2014).

Conclusion

By October 2023, 7 months after the spill, the majority of hydrocarbons within the immediate Spill Site had decreased back to the background levels, measured at the outer areas of the spill creek, Brownsea Island and both control sites, highlighting

the effectiveness of the combined effects of natural attenuation by aerobic and anaerobic bacteria and the response effort, and further site visits in March 2025 showed complete restoration of the Spill Site (Supplementary Material: Figure 10). Throughout the spill creek, the presence of multiple functional genes associated with both alkane and PAH degradation indicates the high capacity for both aerobic and anaerobic biodegradation, also highlighting a variety of genera capable of aerobic/anaerobic hydrocarbon degradation and potential candidates as biomarkers for healthy vs. polluted systems.

Overall, this study highlights the potential of coastal microbial communities to naturally attenuate hydrocarbon pollution when coupled with effective remediation response and furthers our understanding of hydrocarbon/bacterial interactions and the degradation pathways highlighted within this system. Further research should be conducted to understand the succession of hydrocarbon degrading functional genes expressed during a spill event and their potential as biomarkers for hydrocarbon exposure (Reid *et al*., 2020). Most post spill monitoring of microbial communities, is still typically carried out only by 16S rRNA sequence analysis, which significantly limits predictions of an environment's capacity to attenuate hydrocarbon pollution, or the wider effects on other ecosystem processes, but metagenomic sequencing coupled with specific metagenomic tools such as CANT-HYD (Khot *et al*., 2022) and HADEG (Rojas-Vargas and Pardo-López, 2023) have much greater predictive power and should be more routinely used as tools with the aim of informing the Net Environment Benefit Analysis (NEBA) and post oil spill monitoring (Kirby *et al*., 2018).

Supplementary Material

Table S1: Table of Polyaromatic hydrocarbon compounds used in GC MS hydrocarbon concentration analysis. Compounds with an asterisk * were contained in the QTM PAH Mix (Sigma), all other parent compounds and their derivatives were added to the standard mix separately.

PAH compound	Ring Number	Mass	Quantitative ion (*m*/*z*)	Qualifier ion (*m*/*z*)	Retention time
Naphthalene *	2	128	128	127	6.384
Methyl naphthalenes	2	142	142	141	8.735
Dimethyl naphthalenes	2	156	156	141	10.714
Acenaphthylene *	3	152	152	151	11.441
Acenaphthene *	3	154	154	153	11.982
Fluorene *	3	166	166	165	13.599
Methyl fluorene	3	180	180	165	13.938
Dimethyl fluorene	3	194	194	179	17.078
Dibenzothiophene	3	184	184	152	16.096
Methyl dibenzothiophenes	3	198	198		17.388
Dimethyl dibenzothiophenes	3	212	212		18.606
Phenanthrene *	3	178	178	176	16.459
Anthracene *	3	178	178	176	16.589
Methyl phenanthrene/anthracene	3	192	192	191	18.686
Dimethyl phenanthrene/anthracene	3	206	206	191	20.865
Fluoranthene	4	202	202	101	19.99
Pyrene	4	202	202	101	20.62
Benzo[a] anthracene	4	228	228	229	24.19
Chrysene	4	228	228	226	24.298
Benzo[b] fluoranthene	5	252	252	253	27.57
Benzo[k] fluoranthene *	5	252	252	250	27.645
Benzo[e] pyrene *	5	252	252	250	28.585
Perylene *	5	252	252	253	28.904
Indeno[123-cd] pyrene *	6	276	276	277	32.237
Dibenz(a,h) anthracene *	6	278	278	279	32.386
Benzo[ghi] perylene *	6	276	276	277	32.994

*QTM PAH Mix

Table S2: MAGs (Metagenome – Assembled Genome's) created from binned Contigs using MetaWRAP, SPAdes v3.11 used to improve final bin set, resulting in 221 MAGs, including completeness % and contamination %. Lineage taxonomically classified by GTDB-Tk v1.1.0 with further classification via blasting marker genes extracted via barrnap

MAG	completeness	contamination	GC	Lineage	N50	Size	binner
bin.1	83.53	2.101	0.541	Bacteria	10405	2970879	binsA
bin.2	88.45	3.181	0.537	Bacteria	6311	10168717	binsBC
bin.3	70.39	3.929	0.625	Myxococcales	5933	2753719	binsABC
bin.4	96.42	1.46	0.459	Bacteroidetes	35605	4562901	binsAB
bin.5	93.88	2.256	0.387	Cyanobacteria	10200	7296273	binsA
bin.6	91.4	4.876	0.676	Rhodobacteraceae	30808	3691430	binsABC
bin.7	78.34	4.7	0.635	Rhizobiales	4725	2197862	binsBC
bin.8	93.86	3.611	0.443	Gammaproteobacteria	12635	3541003	binsAB
bin.9	78.01	4.611	0.641	Rhizobiales	3591	2367372	binsB
bin.10	100	4.925	0.619	Bacteria	23412	2500960	binsABC
bin.11	81.27	2.991	0.484	Methanomicrobiaceae	6308	1663264	binsA
bin.12	74.15	4.795	0.655	Bacteria	5346	4016822	binsB
bin.13	70.18	4.029	0.55	Rhizobiales	10786	3104633	binsB
bin.14	82.53	1.919	0.289	Flavobacteriaceae	4449	2149657	binsABC
bin.15	74	2.149	0.744	Actinomycetales	6708	2384746	binsAB
bin.16	78.06	2.931	0.612	Spongiibacter	6328	2736483	binsBC
bin.17	85.35	4.273	0.678	Bacteria	19418	3359403	binsB
bin.18	75.37	2.839	0.542	Gammaproteobacteria	5175	4079018	binsBC
bin.19	74.09	3.818	0.514	Bacteria	7299	3657989	binsAB
bin.20	70.59	2.761	0.64	Gammaproteobacteria	7764	3472754	binsAB
bin.21	97.16	4.232	0.429	algicola	15455	3913794	binsAB
bin.22	77.18	3.91	0.403	Bacteria	6768	3003013	binsAB
bin.23	92.02	0.873	0.436	Cyanobacteria	10448	6378043	binsAB
bin.24	94.82	4.045	0.473	Gammaproteobacteria	11567	3226295	binsA
bin.25	82.06	3.448	0.64	Bacteria	20657	2375326	binsB
bin.26	83.23	3.919	0.533	Bacteria	7363	4830194	binsBC
bin.27	73.64	3.351	0.429	Bacteria	3396	2918492	binsB
bin.28	97.29	3.172	0.654	Gammaproteobacteria	14475	4674301	binsB
bin.29	82.21	2.292	0.618	Betaproteobacteria	9756	2107142	binsAB
bin.30	90.31	2.088	0.66	Gammaproteobacteria	21074	3007254	binsAB
bin.31	98.13	2.991	0.658	Actinomarinicola	63042	3642553	binsA
bin.32	75.35	4.727	0.515	Bacteria	4227	3100610	binsAB
bin.33	82.62	2.473	0.54	Desulfosarcina	5430	3256635	binsA
bin.34	72.22	3.892	0.618	Gammaproteobacteria	3270	2423603	binsAC
bin.35	87.2	3.104	0.633	Betaproteobacteria	5507	2732554	binsAB
bin.36	80.09	1.907	0.569	Gammaproteobacteria	15683	2844907	binsAB
bin.37	87.79	0.532	0.556	Rhodobacteraceae	37136	3136438	binsAB
bin.38	89.48	4.301	0.464	Bacteria	9437	2819380	binsABC
bin.39	93.78	2.297	0.706	Raineyella	15967	3592616	binsAB

bin.40	93.51	2.787	0.564	Gammaproteobacteria	21221	4043700	binsBC
bin.41	74.15	0.711	0.591	Proteobacteria	8067	2695420	binsAB
bin.42	91.87	4.204	0.394	Bacteria	8671	5060919	binsBC
bin.43	87.2	4.7	0.672	Ilumantobacter	14832	3175197	binsAB
bin.44	75.45	2.909	0.535	Bacteria	40477	4256604	binsAB
bin.45	97.57	2.16	0.459	Deltaproteobacteria	24780	5095226	binsBC
bin.46	76.5	0.046	0.482	Deltaproteobacteria	3699	4536190	binsBC
bin.47	91.66	2.7	0.485	Deltaproteobacteria	13471	2553837	binsBC
bin.48	92.7	1.612	0.398	Bacteria	13139	2617921	binsABC
bin.49	82.84	3.133	0.679	Acidimicrobiales	5016	2704043	binsB
bin.50	71.02	2.564	0.614	Bacteria	12801	3819049	binsAB
bin.51	97.47	0.84	0.605	Proteobacteria	46081	1942690	binsAB
bin.52	93.06	3.387	0.493	Syntrophobacter	7827	4670711	binsABC
bin.53	93.05	4.391	0.649	Porphyrobacter	15643	2910155	binsB
bin.54	89.8	2.561	0.383	algicola	10309	3092028	binsBC
bin.55	91.61	4.129	0.669	Burkholderiales	5372	4865761	binsABC
bin.56	78.66	3.319	0.515	Rhizobiales	12263	3163433	binsAB
bin.57	96.07	2.45	0.692	Actinomycetales	19389	2957646	binsB
bin.58	80.53	4.682	0.506	Bacteroidetes	4099	3667974	binsAB
bin.59	77.98	4.731	0.621	Myxococcales	5687	3625786	binsBC
bin.60	80.65	3.447	0.647	Bacteria	4573	2474689	binsA
bin.61	98.26	0.652	0.558	Anderseniella	34902	3763216	binsA
bin.62	88.03	1.555	0.594	Gammaproteobacteria	6016	2224358	binsBC
bin.63	80.64	1.424	0.605	Bacteria	4953	1700972	binsB
bin.64	75.61	4.51	0.612	Bacteria	3643	3380869	binsA
bin.65	88.88	2.991	0.59	Bacteria	20375	2385192	binsBC
bin.66	93.19	4.932	0.632	Ectothiorhodospiraceae	9225	2139631	binsA
bin.67	78.74	2.991	0.644	Bacteria	4419	2003439	binsAB
bin.68	77.16	4.354	0.564	Deltaproteobacteria	5570	2458755	binsBC
bin.69	77.74	1.836	0.537	Oceanospirillaceae	5128	2411007	binsBC
bin.70	85.44	2.197	0.701	Bacteria	6823	3902895	binsABC
bin.71	82.97	3.149	0.581	Alphaproteobacteria	3628	2905080	binsB
bin.72	89.88	4.724	0.595	Rhodobiaceae	8181	3068633	binsAB
bin.73	78.35	2.355	0.545	Cyanobacteria	4109	3189157	binsA
bin.74	90.5	1.24	0.557	Rhodobacteraceae	20064	3633639	binsAB
bin.75	81.03	0	0.36	Bacteria	12923	3262489	binsB
bin.76	87.59	3.548	0.542	Deltaproteobacteria	13499	2490191	binsAB
bin.77	87.44	3.536	0.598	Betaproteobacteria	6448	2448277	binsAB
bin.78	70.29	3.571	0.636	Alphaproteobacteria	3842	2468842	binsAB
bin.79	95.02	4.121	0.4	Bacteria	19174	3202232	binsB
bin.80	79.79	0	0.63	Alcanivorax	16093	3276270	binsAB
bin.81	87.55	3.47	0.605	Gammaproteobacteria	6072	2768074	binsB
bin.82	77.96	2.687	0.614	Bacteria	9944	6418452	binsAB
bin.83	81.13	3.16	0.681	Actinobacteria	3667	1593322	binsB
bin.84	82.27	4.275	0.62	Thiobacillus	7211	2067245	binsABC
bin.85	92.74	3.87	0.519	Deltaproteobacteria	11641	3688243	binsAB

bin.86	72.4	1.216	0.707	Actinomycetales	12853	1772209	binsABC
bin.87	89.66	4.487	0.405	Bacteria	7596	3711919	binsB
bin.88	98.7	1.29	0.569	Deltaproteobacteria	19669	2987070	binsBC
bin.89	83.81	4.723	0.358	Bacteria	7986	2527539	binsAB
bin.90	90.59	4.188	0.653	Bacteria	27137	2643951	binsABC
bin.91	80.95	1.124	0.381	Bacteria	6683	2856043	binsAB
bin.92	74.81	2.743	0.494	Proteobacteria	4319	1713310	binsBC
bin.93	83.49	0.427	0.716	Bacteria	5258	2731263	binsB
bin.94	78.9	3.087	0.466	Deltaproteobacteria	6361	2843285	binsAB
bin.95	84.97	4.413	0.493	Deltaproteobacteria	5338	3023450	binsA
bin.96	92.01	4.516	0.682	Polyangiaceae	25437	7729402	binsBC
bin.97	79.99	1.829	0.476	Proteobacteria	5386	1689821	binsB
bin.98	96.58	0.943	0.459	Cyanobacteria	15055	4652339	binsA
bin.99	70.4	4.686	0.615	Dechloromonas sp.HYN0024	4376	2168840	binsAB
bin.100	95.74	3.019	0.535	Gammaproteobacteria	15560	3071326	binsAB
bin.101	71.09	2.084	0.498	Bacteria	6182	3404728	binsBC
bin.102	94.89	1.96	0.618	Pseudorhodobacter	18373	4149891	binsAB
bin.103	75.4	3.924	0.566	Deltaproteobacteria	4476	2023196	binsABC
bin.104	75.74	4.061	0.556	Gammaproteobacteria	8693	3058966	binsAB
bin.105	74.62	3.571	0.556	Bacteria	7635	4246914	binsAB
bin.106	88.95	3.913	0.611	Alphaproteobacteria	12732	4973151	binsBC
bin.107	70.32	1.557	0.314	Archaea	9164	524319	binsABC
bin.108	86.84	0.938	0.587	Gammaproteobacteria	40521	2743774	binsABC
bin.109	71.26	0	0.553	Bacteria	7124	3664664	binsB
bin.110	93.3	2.745	0.641	Bacteria	10026	4325108	binsAB
bin.111	72.65	1.991	0.597	Bacteria	3550	3562165	binsAB
bin.112	74.31	0	0.581	Bacteria	22336	3009390	binsABC
bin.113	94.58	3.418	0.702	Iamiaceae	26058	3770027	binsB
bin.114	88.42	2.983	0.613	Gammaproteobacteria	38407	3929017	binsBC
bin.115	74.93	2.383	0.392	Bacteria	5562	2407354	binsAB
bin.116	94.97	1.091	0.399	Cyanobacteria	8354	4871663	binsA
bin.117	75.58	1.254	0.441	Bacteria	4388	2334223	binsB
bin.118	73.45	3.832	0.561	Rhizobiales	3983	3327353	binsAB
bin.119	96.76	1.87	0.469	algicola	15693	3495624	binsA
bin.120	78.49	1.163	0.673	Bacteria	3318	3066214	binsA
bin.121	87.32	4.273	0.677	Bacteria	22790	4356287	binsAB
bin.122	95.19	1.637	0.478	Cyanobacteria	17663	7295948	binsAB
bin.123	97.84	2.956	0.4	Bacteria	19564	4800351	binsB
bin.124	92.65	2.04	0.585	Betaproteobacteria	23456	3493725	binsBC
bin.125	88.47	2.448	0.395	algicola	8823	2772169	binsAB
bin.126	95.67	3.561	0.532	Bacteria	16342	6192953	binsAB
bin.127	79.67	0	0.625	Archaea	14022	1246081	binsA
bin.128	85.38	3.709	0.633	Deltaproteobacteria	46545	3403989	binsAB
bin.129	98	1.282	0.621	Acidimicrobiaceae	82852	2769465	binsABC
bin.130	97.33	3.273	0.485	Deltaproteobacteria	25251	4607458	binsB

bin.131	85.83	2.266	0.701	Alphaproteobacteria	8942	3686914	binsABC
bin.132	86.36	4.864	0.695	Actinobacteria	10741	2434352	binsA
bin.133	74.69	2.991	0.684	Bacteria	3655	5170521	binsAC
bin.134	89.55	2.58	0.628	Myxococcales	31150	4067617	binsB
bin.135	81.71	3.845	0.446	algicola	4461	2254085	binsABC
bin.136	75.42	4.53	0.508	Deltaproteobacteria	5574	3395938	binsAB
bin.137	80.34	4.273	0.638	Bacteria	30173	1508328	binsAB
bin.138	95.16	1.129	0.564	Desulfomonile	12528	6066248	binsAB
bin.139	97.22	0.925	0.561	Bacteria	12888	2108485	binsB
bin.140	91.78	3.553	0.682	Alphaproteobacteria	10089	3462400	binsBC
bin.141	94.62	1.792	0.376	Bacteria	8316	2532392	binsAB
bin.142	90.05	1.282	0.705	Actinobacteria	17221	3952407	binsB
bin.143	85.75	3.818	0.54	Anaerolineales	33290	3817844	binsB
bin.144	81.48	4.723	0.684	Gammaproteobacteria	11755	2858875	binsAB
bin.145	73.13	4.595	0.632	Bacteria	3103	3231478	binsAC
bin.146	87.32	3.44	0.499	Deltaproteobacteria	8714	6451842	binsB
bin.147	96.21	1.68	0.604	Proteobacteria	29124	2164315	binsAB
bin.148	98.04	1.29	0.497	Desulfobacter	179609	5749405	binsB
bin.149	75.07	3.232	0.699	Actinobacteria	4405	1403116	binsB
bin.150	72.76	2.212	0.556	Gammaproteobacteria	4721	2247663	binsAB
bin.151	95.36	2.062	0.684	Actinomycetales	18588	2529291	binsB
bin.152	96.86	1.536	0.617	Alphaproteobacteria	23706	3543446	binsB
bin.153	70.45	4.134	0.524	Bacteria	7919	2104713	binsAB
bin.154	95.22	1.404	0.413	Bacteria	16965	3031395	binsAB
bin.155	98.51	3.406	0.505	Deltaproteobacteria	19286	3643618	binsA
bin.156	90.42	1.757	0.648	Actinomycetales	5862	2544560	binsAB
bin.157	80.65	3.608	0.479	algicola	9031	2605450	binsAC
bin.158	72.07	2	0.515	Bacteria	4415	3936508	binsAB
bin.159	92.68	3.826	0.607	Gammaproteobacteria	28198	2944369	binsAC
bin.160	98.09	4.089	0.495	Spongiibacter	12578	3237003	binsAB
bin.161	91.62	3.91	0.334	Bacteria	11260	3914332	binsB
bin.162	93.33	4.545	0.649	Caldilinea	13091	5070274	binsAB
bin.163	95.6	4.395	0.715	Bacteria	19136	3339135	binsB
bin.164	99.14	0.854	0.589	Geobacillus	43446	3748590	binsABC
bin.165	87.42	4.727	0.554	Bacteria	15955	4230791	binsABC
bin.166	76.14	4.926	0.412	Bacteroidetes	4100	3153198	binsB
bin.167	71.97	3.442	0.644	Gammaproteobacteria	3883	2254965	binsAC
bin.168	95.71	2.222	0.452	Gammaproteobacteria	9946	2656631	binsABC
bin.169	71.78	3.284	0.687	Actinomycetales	3769	2307743	binsBC
bin.170	94.11	4.43	0.62	Woeseia	47959	3746231	binsB
bin.171	78.87	3.821	0.66	Actinobacteria	4942	1484091	binsA
bin.172	82.16	0.706	0.529	Bacteria	3908	2008075	binsA
bin.173	87.24	2.851	0.599	Gammaproteobacteria	18270	3346329	binsAB
bin.174	82.73	2.939	0.52	Deltaproteobacteria	6231	2572841	binsAB
bin.175	92.15	0.537	0.481	Bacteria	12015	3844190	binsABC
bin.176	82.81	2.136	0.632	Actinobacteria	13041	3257234	binsBC

bin.177	94.82	1.404	0.677	Gammaproteobacteria	37283	2078866	binsBC
bin.178	94.01	3.902	0.61	Gammaproteobacteria	18342	4842966	binsAB
bin.179	88.29	2.521	0.71	Dietzia	6549	2827985	binsBC
bin.180	75.81	2.197	0.722	Bacteria	6527	2994761	binsBC
bin.181	96.42	0.297	0.54	Deltaproteobacteria	10745	3492774	binsBC
bin.182	92.27	3.879	0.408	Bacteria	8989	4017044	binsB
bin.183	97.63	1.491	0.537	Cyanobacteria	18574	6265835	binsB
bin.184	70.55	1.941	0.451	Archaea	3855	1184134	binsABC
bin.185	77.12	4.032	0.464	Deltaproteobacteria	5026	2599542	binsAB
bin.186	90.33	0.997	0.472	Gammaproteobacteria	8145	4407376	binsA
bin.187	86.72	1.968	0.557	Gammaproteobacteria	9815	2365180	binsAB
bin.188	94.97	4.121	0.526	Desulfobacteraceae	7101	3299923	binsAB
bin.189	93.99	1.811	0.384	algicola	9136	3538040	binsABC
bin.190	90.7	1.532	0.618	Gammaproteobacteria	10192	2117249	binsAB
bin.191	81.44	1.762	0.548	Gammaproteobacteria	5354	1991866	binsAC
bin.192	70.89	3.132	0.583	Gammaproteobacteria	3228	2525600	binsA
bin.193	75	1.785	0.595	Proteobacteria	5453	4645764	binsAB
bin.194	72.15	1.798	0.425	algicola	3500	2632177	binsABC
bin.195	70.49	1.709	0.607	Bacteria	3217	5052534	binsAC
bin.196	87.35	1.709	0.663	Ilumantobacter	26129	3780785	binsB
bin.197	79.66	1.935	0.437	Gammaproteobacteria	4076	2237289	binsA
bin.198	92	4.166	0.648	Actinobacteria	8472	2219763	binsAB
bin.199	93.87	1.888	0.659	Rhodospirillales	20069	3019743	binsB
bin.200	93.89	3.411	0.716	Actinobacteria	19340	3756089	binsB
bin.201	88.41	2.368	0.519	Proteobacteria	5802	1603655	binsA
bin.202	82.94	4.048	0.474	Proteobacteria	4993	2323441	binsABC
bin.203	95.66	3.846	0.698	Bacteria	12591	3699190	binsB
bin.204	87.25	3.759	0.647	Rhodobacteraceae	5383	3006966	binsAB
bin.205	89.74	1.709	0.615	Bacteria	11187	3472679	binsAB
bin.206	90.25	3.492	0.481	Bacteroidetes	8472	2835088	binsA
bin.207	71.83	2.626	0.456	Deltaproteobacteria	3749	1880109	binsABC
bin.208	84.6	1.898	0.616	Gammaproteobacteria	23167	2208236	binsB
bin.209	70.51	4.414	0.622	Bacteria	3162	2596059	binsABC
bin.210	88.38	3.941	0.581	Rhizobiales	6904	3046036	binsAB
bin.211	92.51	3.119	0.452	Deltaproteobacteria	7765	2968882	binsAB
bin.212	86.52	2.761	0.524	Bacteria	6119	2492728	binsAC
bin.213	76.48	4.876	0.594	Rhizobiales	3908	2509966	binsAB
bin.214	81.16	3.623	0.546	Gammaproteobacteria	37917	2379745	binsAB
bin.215	96.63	2.93	0.672	Bacteria	18487	2840661	binsA
bin.216	97.06	2.327	0.695	Actinobacteria	14767	2819247	binsBC
bin.217	84.58	2.521	0.608	Proteobacteria	6221	1839299	binsAB
bin.218	95.88	2.258	0.652	Deltaproteobacteria	26883	2989650	binsAB
bin.219	73.43	3.6	0.415	Euryarchaeota	4346	1357883	binsAB
bin.220	81.34	0.862	0.58	Bacteria	20913	3356238	binsB
bin.221	71.6	4.494	0.587	Bacteria	3339	3782013	binsAB

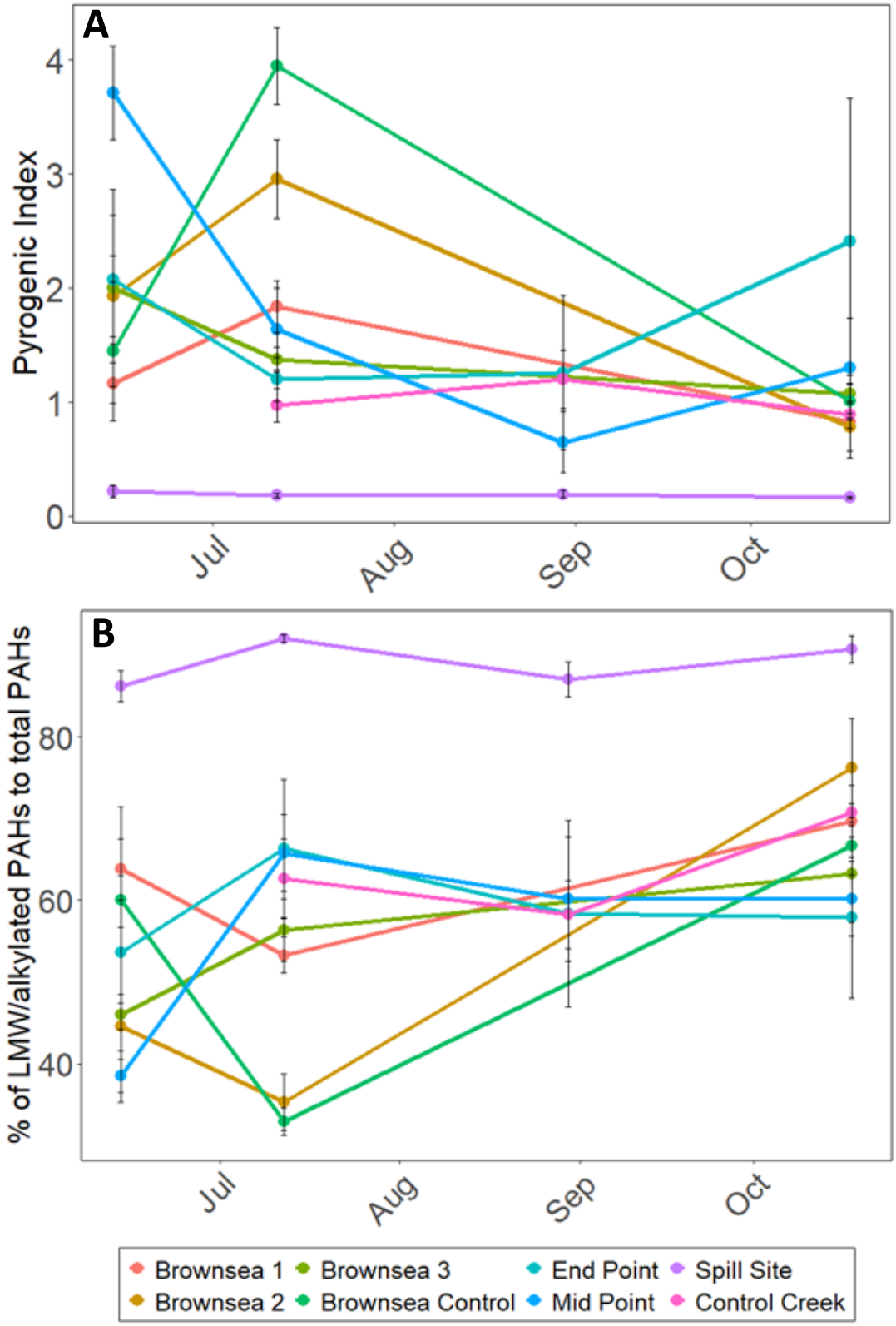


Figure S1: A. Pyrogenic Index [PI: Σ(3− to 6-ring EPA priority PAHs) / Σ(5 alkylated PAH series)] and **B.** LMW/alkylated PAHs to total PAHs) from surface sediments (mean ± SE, replication = 3) across Brownsea Island and Ower Bay Creek, Dorset, UK over June – October 2023, following an oil pipline spill on 26th March,in comparison to uncontaminated Control sites. Sampling dataes (14th June, 12th July, 30th August and 18th October).

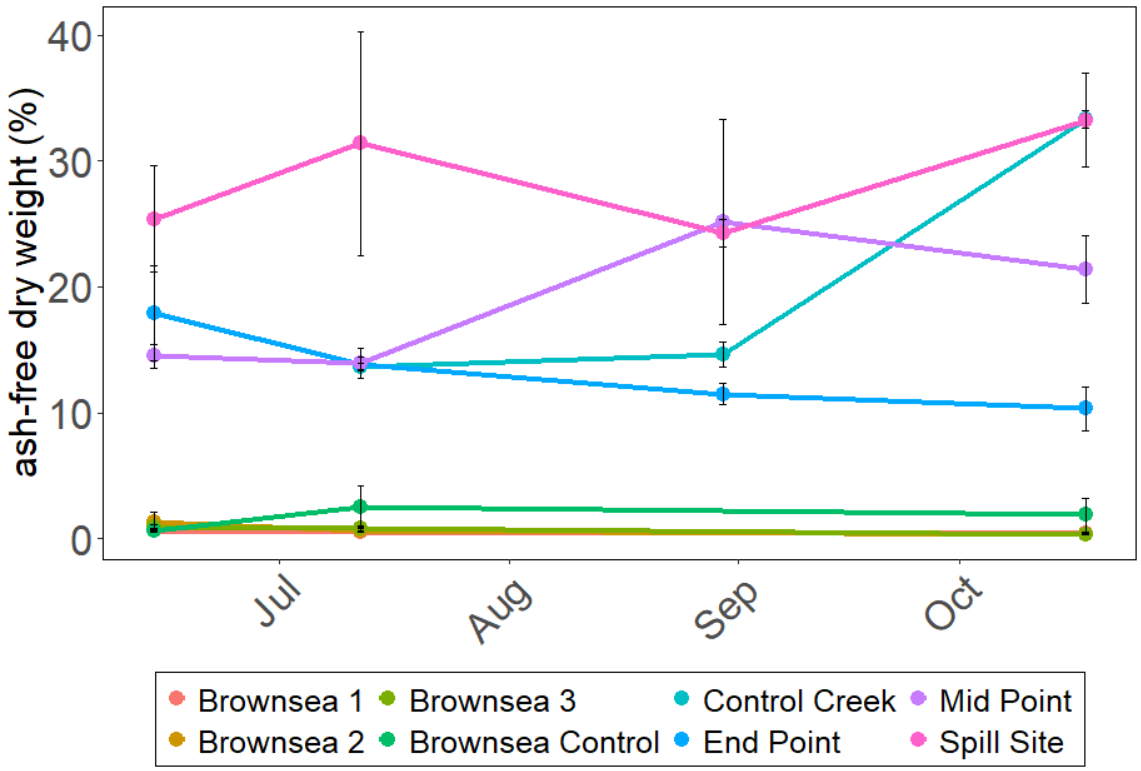


Figure S2: Ash-free dry weight (%) as a proxy for organic carbon content between surface sediment samples (n=3) collected from sites across Brownsea Island and Wytch Farm oil field (Ower Bay Creek), Dorset, UK from June – October 2023, including Brownsea Control and Control Creek sites.

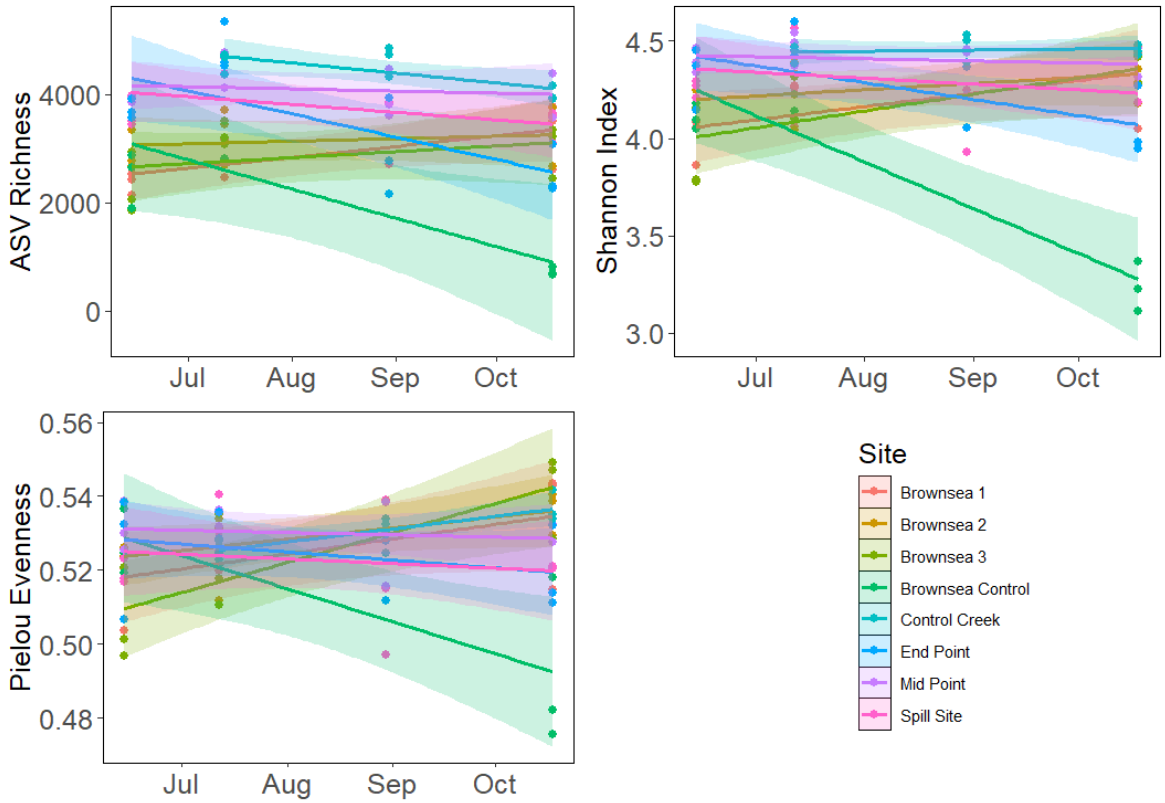


Figure S3: Diversity Indices (ASV Richness, Shannon Index, and Pielou's Evenness) within surface sediment samples (mean ± SE, n=3) collected from sites across Brownsea Island and Wytch Farm oil field (Ower Bay Creek), Dorset, UK from June – October 2023, including Brownsea Control and Control Creek sites.

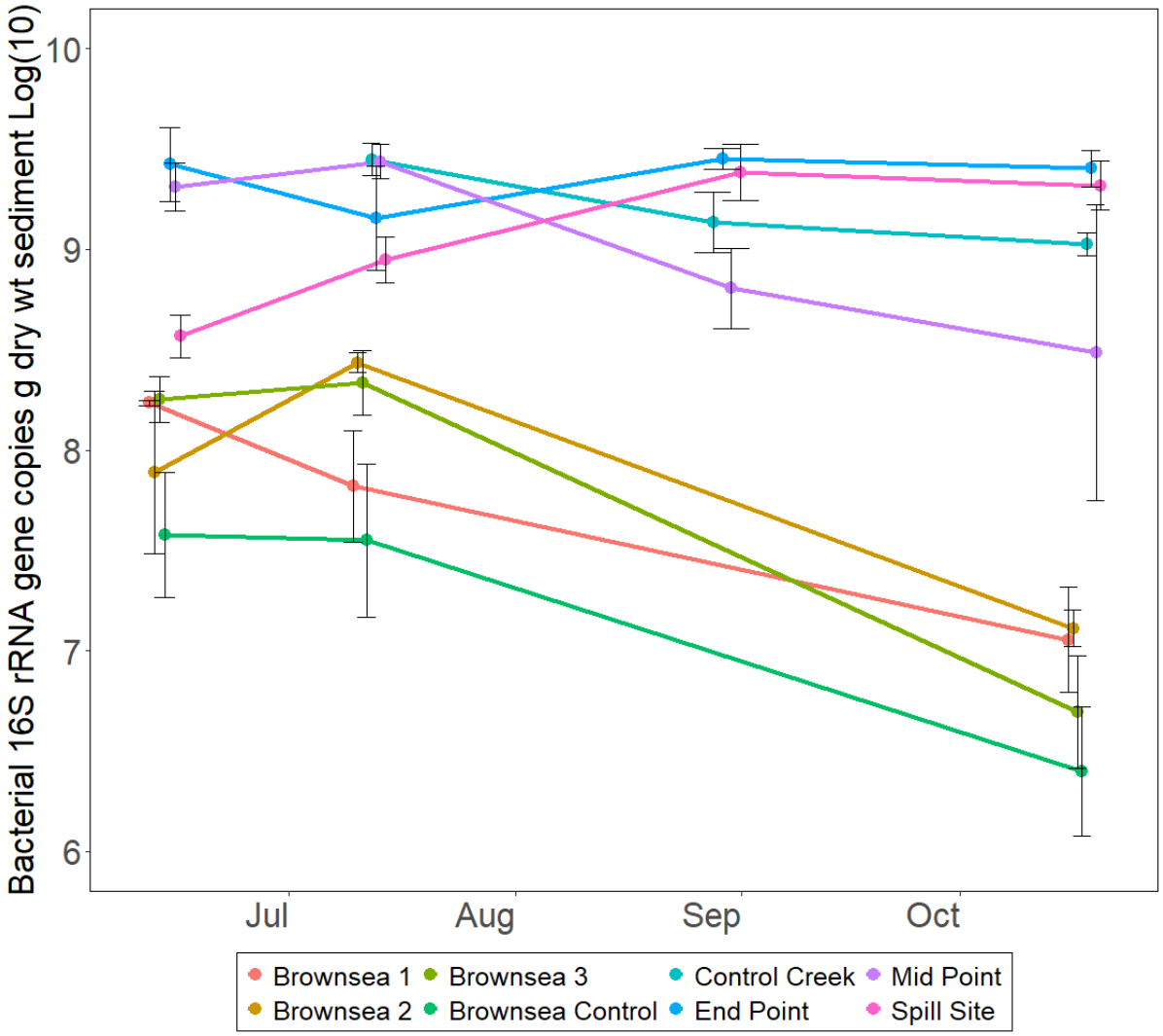


Figure S4: Bacterial 16S rRNA gene abundance (copies per g dry wt. log(10)) from surface sediment samples (mean ± SE, n=3) collected from sites across Brownsea Island and Wytch Farm oil field (Ower Bay Creek), Dorset, UK from June – October 2023, including Brownsea Control and Control Creek sites.

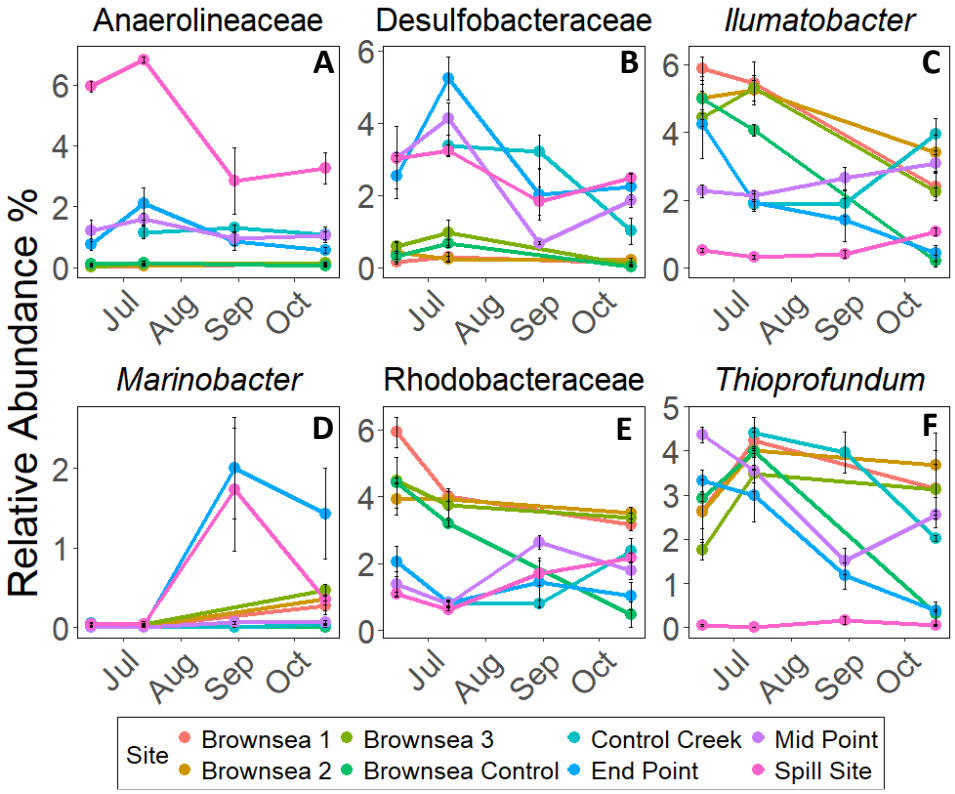


Figure S5: Highest abundant genera within bacterial 16S rRNA communities (Relative Abundance %) from surface sediment samples (mean ± SE, n=3) collected from sites across Brownsea Island and Wytch Farm oil field (Ower Bay Creek), Dorset, UK from June – October 2023, including Brownsea Control and Control Creek sites.

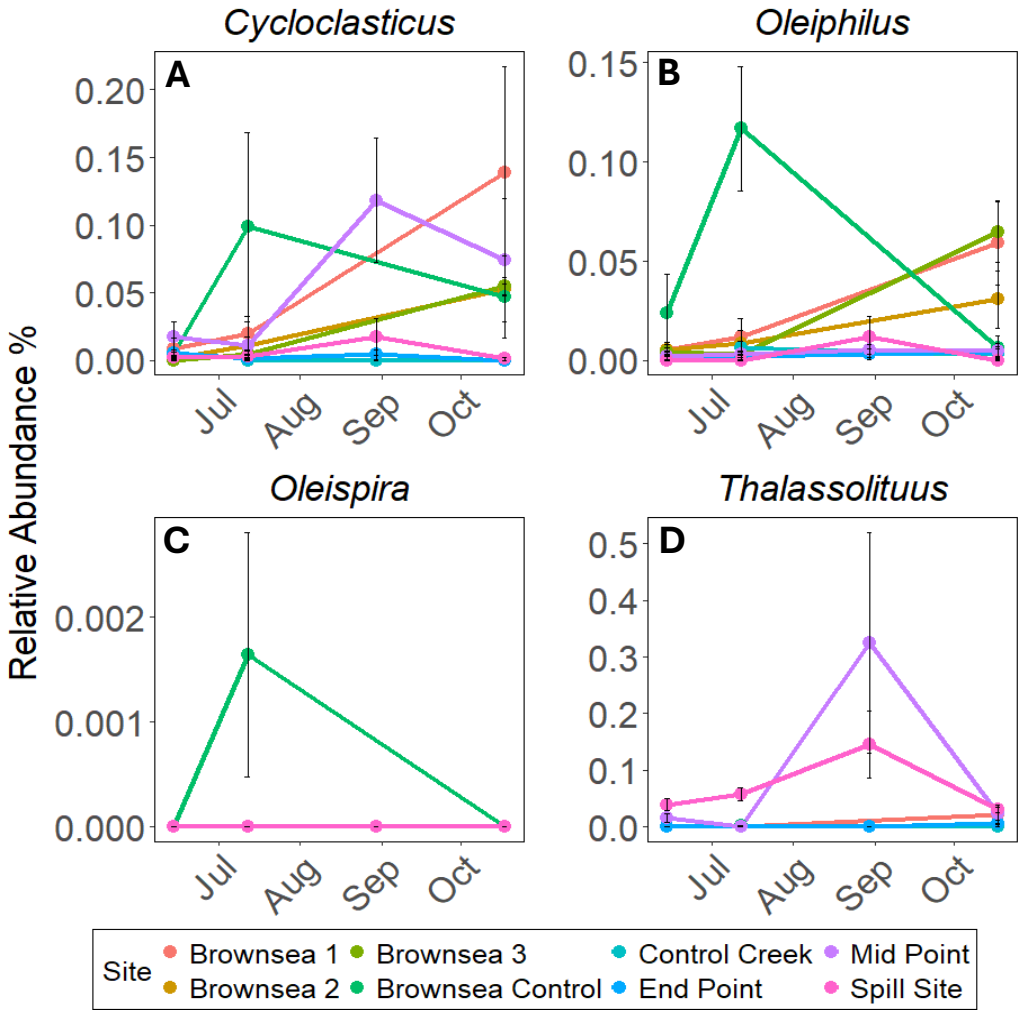


Figure S6: Percentage relative abundance of Obligate Hydrocarbon degrading bacteria (OHCBs) within bacterial 16S rRNA communities from surface sediment samples (mean ± SE, n=3) collected from sites across Brownsea Island and Wytch Farm oil field (Ower Bay Creek), Dorset, UK from June – October 2023, including Brownsea Control and Control Creek sites.

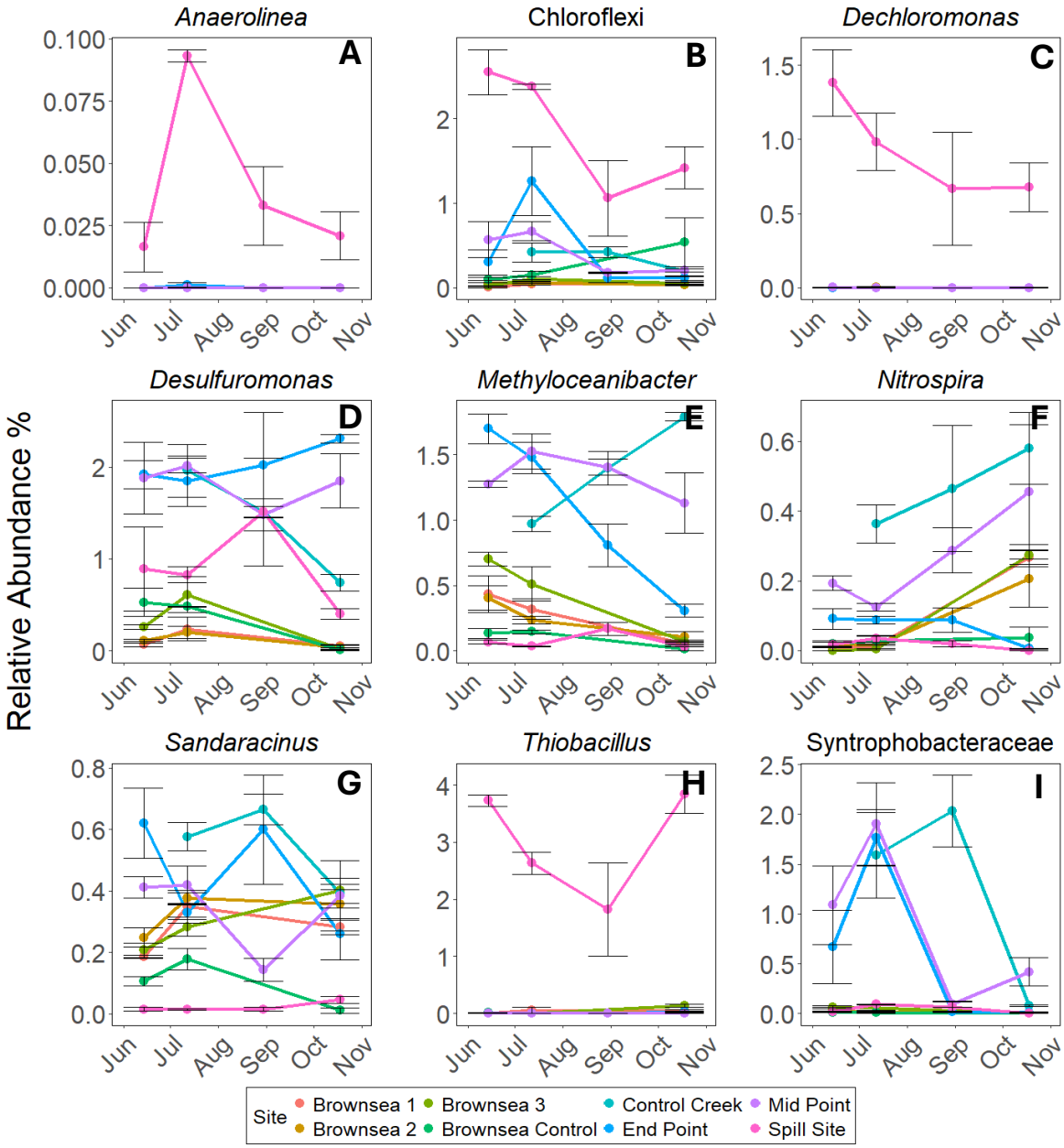


Figure S7: Percentage relative abundance genera of interest within bacterial 16S rRNA communities from surface sediment samples (mean ± SE, n=3) collected from sites across Brownsea Island and Wytch Farm oil field (Ower Bay Creek), Dorset, UK from June – October 2023, including Brownsea Control and Control Creek sites.

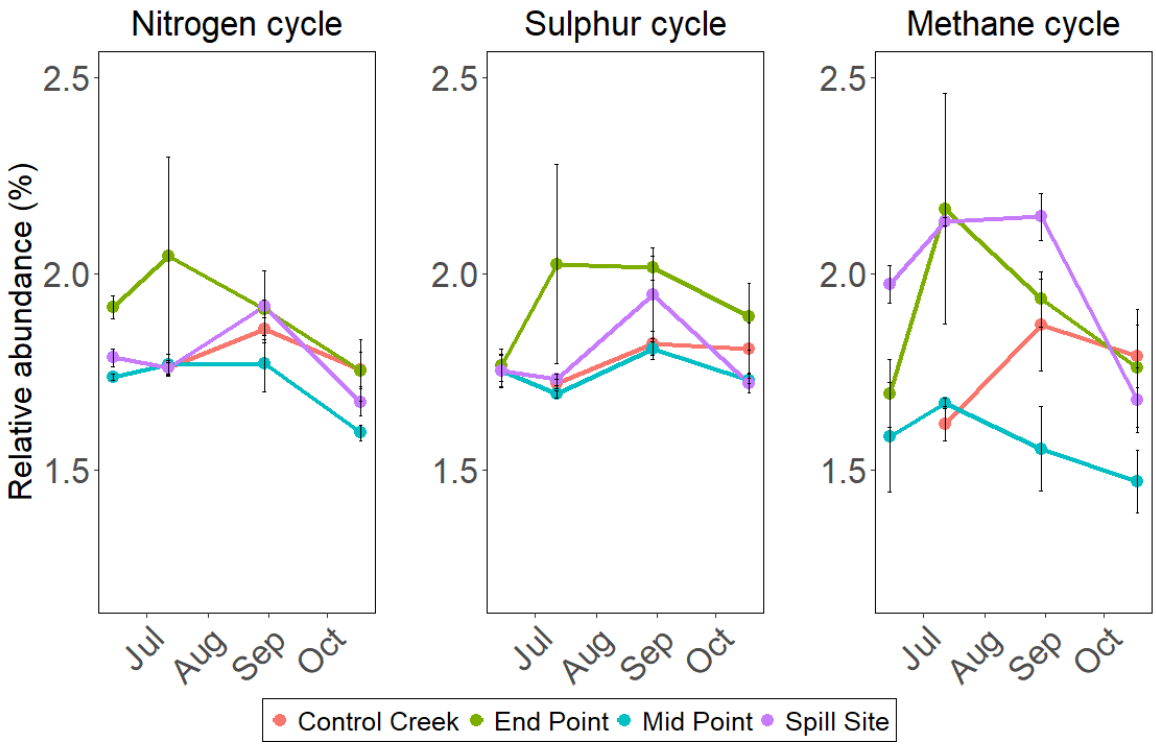


Figure S8: Percentage relative abundance based off the total count of nutrient cycling genes within bacterial communities based on metagenomic bacterial sequence reads classified using the *k*-mer "Kraken2" approach paired with Bracken from surface sediment samples (mean ± SE, n=3) collected from sites across Wytch Farm oil field (Ower Bay Creek), Dorset, UK from June – October 2023, Control Creek site. A) Nitrogen cycling, B) Sulphur cycling, C) Methane cycling.

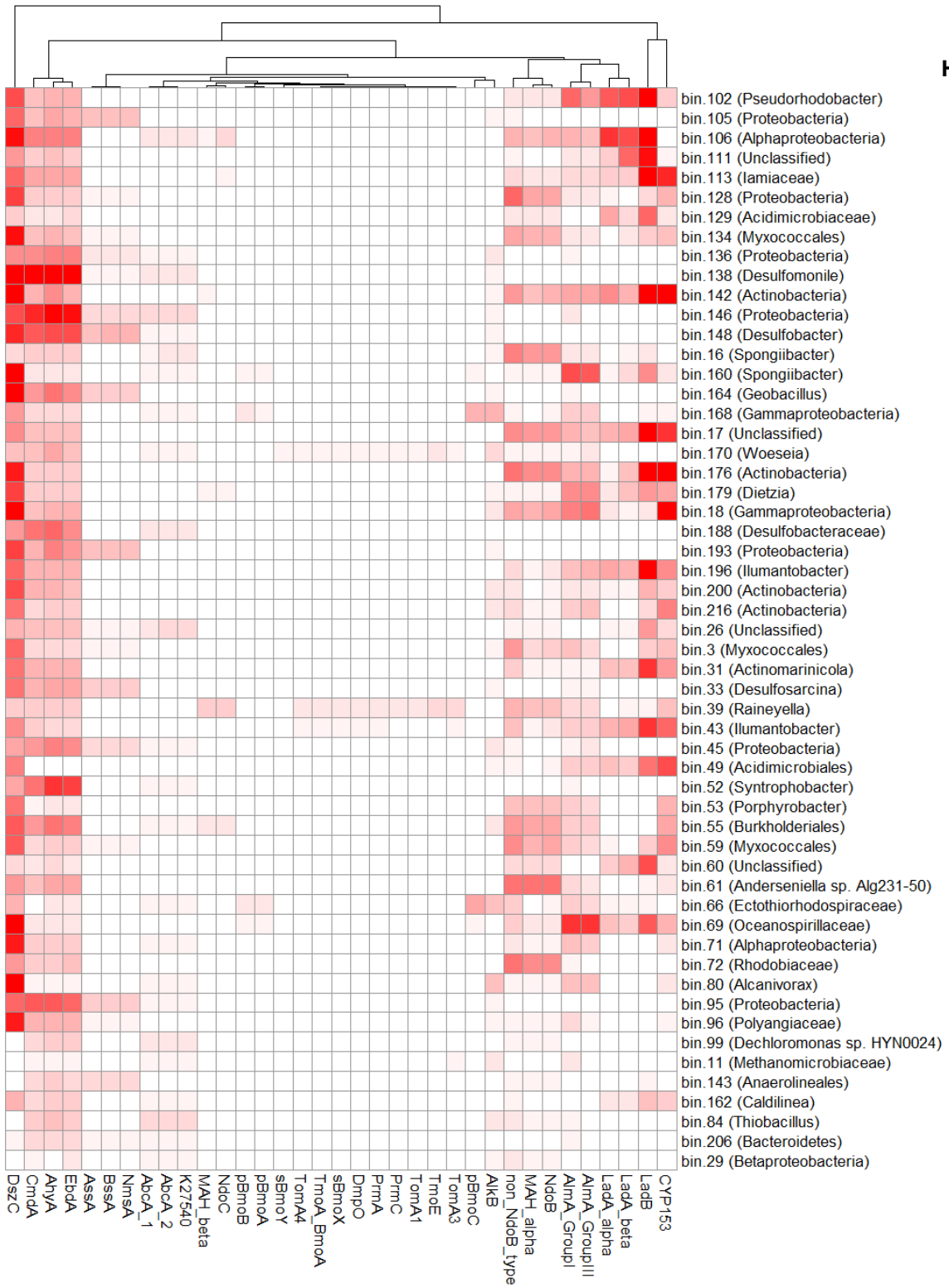
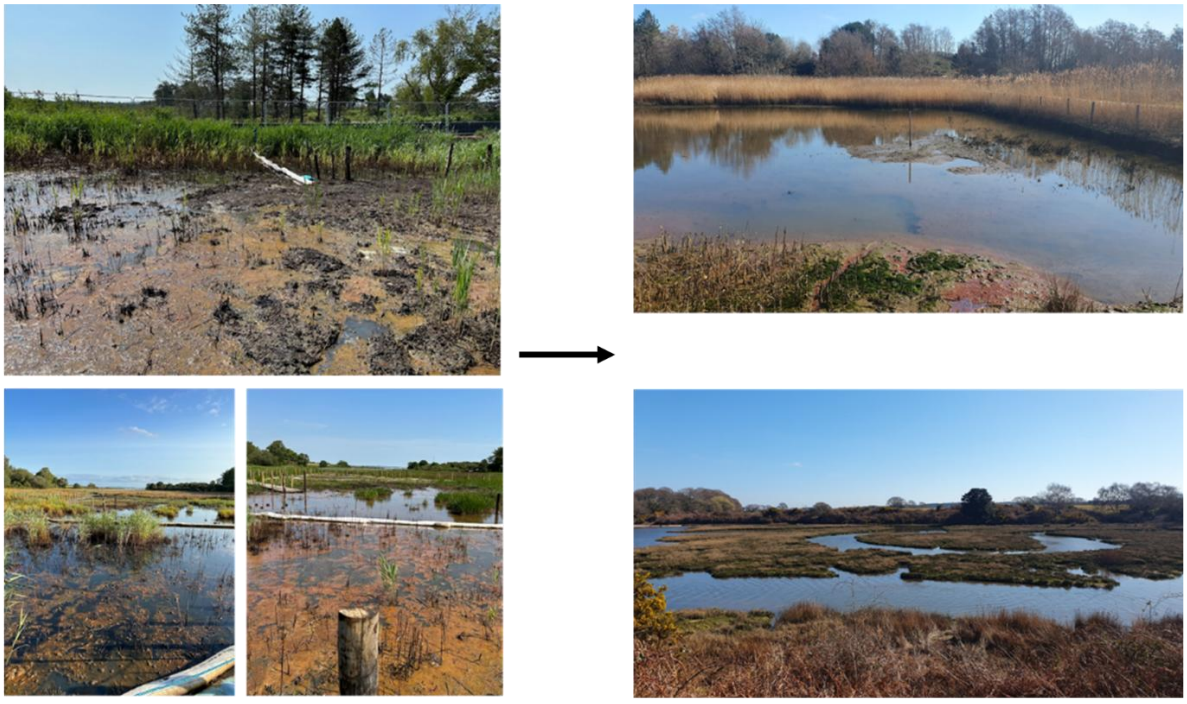


Figure S9: Heatmap of functional genes hits using Hidden Markov Model (HMM) associated with hydrocarbon degradation pathways (HYD) based of sequences pulled from MAGs (Metagenome-Assembled Genome's) and classified using the CANT-HYD database. MAGs produced from samples collected from sites across Wytch Farm oil field (Ower Bay Creek), Dorset, UK from June – October 2023, Control Creek site. MAGs were taxonomically classified by GTDB-Tk v1.1.0.



Directly after the spill event (26th March 2023)

After the completion of the remediation response (20th March 2025)

Figure S10: Images taken at Ower Bay Creek directly after the spill event (26th March 2023) and after the completion of the remediation response (20th March 2025)

Chapter Six

Concluding Remarks

This thesis aimed to explore the impact of marine oil pollution and remediation strategies use on marine environments, microbial community structures, functionality and hydrocarbon biodegradation capability under relevant environmental conditions via both *ex-situ* laboratory experimental conditions and during *in-situ* spill events for the purpose of biomonitoring and to highlight and answer discrepancies within the current literature, to better inform spill response policy through the Spill Impact Mitigation Assessment (SIMA) and its predecessor the Net Environmental Benefit Analysis (NEBA). As a heavily debated remediation tool, chemical dispersants were a main focus of this thesis, exploring its impact on biodegradation, formation of marine oil snow and associated microbial communtiies.

Chapter two highlighted that initial crude oil concentration impacted biodegradation and bacterial communities, thus influenced the outcome of prior studies exploring the effectiveness of dispersant use as a remediation tool. This reinforces the need to consider experimental design and environmental conditions. Importantly, concentrations of 1000 mg L^{-1} were shown to inhibit both alkane and polyaromatic hydrocarbons (PAHs) degradation rates whereas concentrations of 1, 10 and 100 mg L^{-1} did not, indicative of prior studies (Prince *et al*., 2017). As opposed to percentage degradation by day 14 which highlighted reductions in degradation within concentrations of both 100 and 1000 mg L^{-1}, the use of degradation rate was shown to be a more beneficial metric when comparing between different concentrations as this took into consideration the mass of hydrocarbons degraded over time. Degradability and molecular weight have been shown to share an inverse relationship, in which bacteria will initially target lower molecular weight compounds “moving up the

chain length as degradation continues which was clearly observed here (Setti *et al*., 1993; Mohanty and Mukerji, 2008), thus degradation would be perceived as taking longer at these higher concentrations where degradation is limited to lower weight compounds due to the greater mass of readily degradable fractions as oil concentration increased 10-fold. This brings to light the discrepancies throughout the literature, many of which used concentration of crude oil ranging between 2.5 to 2500 mg L^{-1} and failed to report degradation rate, opting for bulk percentage degradation which has shown to produce much more varied outcomes. Furthermore, chemical dispersant use as a remediation strategy was shown to successfully enhance degradation rates across all concentrations, with minimal decreases in efficiency as concentration increased. Even so, oil concentrations of 10 mg L^{-1} or lower should be considered for degradation experiments to better represent *in-situ* concentration when dispersed (Lee *et al*., 2013). To further understand changes in degradation rate over time, further study should consider the addition of a dilution factor of crude oil over time comparable to conditions *in-situ.* Interestingly, observations of bacterial consortiums in the presence of dispersant use and differing oil concentrations highlighted very minimal changes in overall community composition, being dominanted by genera *Roseovarius*, *Aliiroseovarius* and *Pacifibacter*, coinciding with a decrease in nutrient availability. Obligate hydrocarbonoclastic bacteria *Alcanivorax* and *Cycloclasticus* which would generally see an uplift in relative abundance (Dubinsky *et al*., 2013; Cappello *et al*., 2007; Thomas *et al*., 2020) were also outcompeted by more generlistic *Alteromonadaceae*. These distinct similarities within treatments suggests nutrient availability was the dominant factor in shaping community composition compared to oil concentration and dispersant use (Nõlvak *et al*., 2021). Concentration and dispersant use did however influence the bacterial

succession notably between *Alteromonadaceae* and *Oceanospirillaceae* (Bacosa *et al*., 2018; Miller *et al*., 2019). Succession was shown to be delayed at 1000 mg L^{-1}. Similarly to the inverse relationship observed between degradability and hydrocarbon molecular weight, communities will select for genera capable of degrading lower weight compounds, then select for genera as bacteria work up the chain lengths, therefore potentially delaying succession as the concentration of lower weight compounds increases. Intermediate hydrocarbon metabolic products has also been shown to inhibit the growth of certain degraders with may also attribute to delays in succession (Pumphrey and Madsen, 2007; Bacosa *et al*., 2021). However at lower concentrations (1 and 10 mg L^{-1}) dispersant use enhanced succession of these genera by enhancing the bioavailability of hydrocarbons. This reiterates that initial oil concentration needs to be considered during microbial and biodegradation experiments due the impact it has to the outcome of these experiments, thus the rest of this thesis used consistent oil concentrations indicative of *in-situ* spill event concentrations to avoid further discrepancies within the literature.

As a potential vehicle for hydrocarbons entering deep sea benthic environments marine oil snow was another hotly debated topic in the use of chemical dispersant as a remediation tool (Passow *et al*., 2012; Fu *et al*., 2014; Barbier, 2015). Using in house imaging techniques, it was shown that chemical dispersants did not impact the number of flocs formed, percentage cover, nor floc size over time (Passow, 2016; Passow *et al*., 2017). Comparatively, in the past, EPS (extracellular polymeric substances) and TEP (transparent exopolymeric particles) have been used as a proxy for MOS formation, being an important component of marine snow structures (van Eenennaam et al., 2016; Passow et al., 2017; Suja *et al*., 2019). However dispersants and oil have been known to create artifacts during this analysis, thus this high resolution imaging

methodology has provided a simple, yet effective means of quantifying physical floc formation without the risk of under or over estimations (Passow and Alldredge, 1995).

Natural light was another factor implemented during this thesis to better simulate surface level spills, as prior to this much of our understanding of MOS formation came from MOS incubated within dark conditions, indicative of the subsurface Deep Water Horizon 2010 spill which initiated the rise of MOS/dispersant research (Passow et al., 2012; Fu et al., 2014; Suja et al., 2017, 2019). The addition of natural light as an important environmental condition also showed minimal impact on MOS formation throughout. By day 12 maximum floc size within oiled microcosms was significantly larger than seawater controls under natural light conditions, reaching 23.58mm^2. The potential short term irradiation of crude oil under natural light conditions may have elevated oxidation stress, causing changes in polysaccharide production, being a component of EPS (Sun et al., 2018). They have been shown to accumulate more under stressful conditions, with bacterial cells producing more protective polysaccharides (Chin et al., 1998; Zhou et al., 1998), which may act as a building block of MOS. There appears to be an obvious interaction between light and crude oil within MOS driving how the aggregates form, thus further work into floc structures should consider the link between the physical and chemical properties of MOS under a variety of light conditions.

Due to the concerns of marine snow (MS) acting as a vehicle for hydrocarbons entering deep sea benthic environments, further investigations into MS and MOS included an in depth exploration of their associated microbes (Chapter 3 and 4) and how the integration of oil and dispersants may influence these communities. During this exploration MOS was shown to be a site of high bacterial activity, being approximately 6-7 order of magnitudes higher than that of surrounding seawater,

which was further enhanced with the presence of hydrocarbons and dispersants. Oiled aggregates in prior research have also shown this enhanced bacterial activity, suggesting increases in hydrocarbon degradation activity (Ziervogel *et al*., 2012). Using models such as the ecological index of hydrocarbon exposure (EiHE) (Lozada *et al*., 2014) as a proxy for potential hydrocarbon degradation activity, these flocs were further highlighted as sites of high potential hydrocarbon biodegradation compared to surrounding seawater (Chapter 3). Clear changes in floc community composition were observed with the addition of oil and dispersant use, selecting for oil degrading assemblages and EPS production (Arnosti *et al*., 2016), containing a consortium capable of degrading a wide variety of alkanes and aromatics (Dyksterhouse *et al*., 1995; Yakimov *et al*., 1998; Chung *et al*., 2001; Yakimov *et al*., 2004; Prince *et al*., 2019)). Using the community data the EiHE Within Oiled flocs reached between 0.4 – 0.8 (Chapter 4), equating to approximately 40-80% of floc communities consisting of potential hydrocarbon degraders, indicative of communities that have been previously monitored during past spills (Thomas *et al*., 2020). When using the EiHE on its own we still need to be aware of its limitations, as this metric currently does not discern between species within the same genus that can and cannot utilise hydrocarbons. Further improving this model to include species information may alleviate these issues, however producing sequences data to species level is still an ongoing challenge. Even so, the EiHE, coupled with the analysis of hydrocarbon concentration within floc microcosms, still indicates high levels of degradation during this study. Even without the presence of dispersant use, percentage degradation within flocs was shown to be between 85.1 – 86.3% after 14 days. It can be suggested that as oil integrates with floc formations (Burd *et al*., 2020; Passow and Overton, 2021; Gregson *et al*., 2021), surface area; thus bioavailability substantially increases compared to slick oil,

highlighting the presence of MOS as microhabitats during a spill event may enhance biodegradation of crude oil. Dispersant under lighting conditions was shown to have the highest percentage degradation at 93.2%, thus the addition of dispersant use may lead to smaller droplets of oil integrating with MOS with the presence of enhanced photooxidation, making hydrocarbon compounds more susceptible to degradation, further enhanced biodegradation within floc communities (Dutta *et al*., 2000; Prince *et al*., 2013).

Very early on in floc formation *Oleispira* (*Oleispira Antarctica*, 98.93% identity) and *Colwellia* dominated oiled floc communities (50-70% community composition), rapidly succeeded by other obligate hydrocarbonoclastic bacteria *Alcanivorax* and *Cycloclasticus*, followed by more generalistic degraders such as *Erythrobacter* and *Olleya* (Prince *et al*., 2019). These successional changes follow similar patterns to free-living oil spill communities observed during prior studies (Chapter 2) (Dubinsky *et al*., 2013; Thomas *et al*., 2021), following a clear successional pattern of selection of genera with a preference to lower weight hydrocarbon compounds, followed by genera with the capacity of degrade more complex compounds (Ławniczak *et al*., 2020). Notably genera *Loktanella*, *Lewinella* and *Polaribacter* were observed in relative abundance within seawater control MS flocs, possibly acting as regulators of toxic alagal blooms and playing important roles in the breakdown of complex organic matter contributing to the microbial food web. These genera may be indicative of healthy bacterial and algal growth within floc bacterial communities (Miranda *et al*., 2013; Sun *et al*., 2016; Nguyen *et al*., 2023; Sajid *et al*., 2024).

Further exploration of microbial floc communities further highlighted eukaryotes as ideal candidates for biomonitoring, in many cases have a narrow environmental tolerance, shifts in eukaryote composition are highly noticeable (*Reid et al., 1995;*

Stefanidou et al., 2018). Specifically oiled treatments selected for diatom domination, whilst unoiled flocs selected for protists. Thought to be attributed to changes in nutrient regeneration via the breakdown of other sensitive eukaryotes and changes in predation pressure of heterotrophic grazers (*Kelly et al., 1999; Hjorth et al., 2008; González et al., 2009; Othman et al., 2018; Othman et al., 2023*). More research should be considered to highlight eukaryote interactions in the presence of hydrocarbons. Overall MOS floc communities have been highlighted as highly productive sites of substantial potential hydrocarbon degradation and may be a viable source for the further exploration of hydrocarbon degradation pathways and novel hydrocarbon degrading genera via CANT-HYD and HEDAG databases (Khot *et al*., 2022; Rojas-Vargas *et al*., 2023). By the time MOS sinks to the benthos much of the integrated crude oil would have been degraded leaving less volatile, less toxic, heavily degraded compounds, thus future toxicity studies on benthos environments should also take into consideration this heavy degradation when considering crude oil impacts to deep sea benthic systems.

Much of our understanding on microbial community compositions and functionality are based on laboratory experimental conditions, chapter five, thus provided the opportunity to explore community composition and functionality, during an *in-situ* spill event (PMCC, 2024; Hatch, 2024), followed a clear positive impact of effective response and monitoring. As of June (80 days after the spill), much of the hydrocarbon contamination had been isolated to a single site due to the rapid deployment of booms and effective monitoring. 206 days after the spill saw hydrocarbon concentrations at the Spill Site drop back to background levels observed across the control sites and other impacted sites within Poole Harbour and ower bay creek. Comparatively the ower bay creek spill was a small scale event at 200 barrels

worth of reservoir fluid entering the environment, as opposed to other events such as Exxon Valdez (approx 257,000 barrels), The sea Empress (approx 504,000 barrels) and Agia Zoni II (approx 18,300 barrels) (Etkin, 1999 Parinos *et al*., 2019). Notably when using models such as the EiHE, no significant change was observed with all sites ranging between 0.04 and 0.12 (4 – 12% of the community associated with hydrocarbon degradation), in contrast to the EiHE being approximately 0.5 (50% of the community) during the Agia Zoni Spill (Thomas *et al*., 2020) and in the case of this thesis (Chapter 3 and 4) between 0.4-0.8 (40 – 80% of the community) within oiled flocs. This highlights significant variation within these tools dependent on environment and samples, and that analysis conducted with them should be critically assessed. Further development of these biomonitoring metrics is needed, but that also the scale of a spill event/hydrocarbon contamination also needs to be considered when interpreting these models. With metagenomic analysis becoming much more accessible I believe the exploration of metagenomic assembled genomes and their potential functionality, incorporating the presence and absence of hydrocarbon degradation gene pathways will be instrumental in improving these biomonitoring models. Although the event was considered minor, it provided a great opportunity to explore hydrocarbon degradation pathways and applicability of the CANT-HYD and HADEG databases as metagenomic tools (Khot *et al*., 2022; Rojas-Vargas *et al*., 2023), in which a clear switch from predominantly aerobic hydrocarbon degradation was observed outside the Spill Site to anaerobic degradation within the Spill Site, specifically pushed via *AssA* and *AbcA* – like genes associated with the initial stages of anaerobic alkane and aromatic degradation (Callaghan *et al*., 2010; Luo *et al*., 2014 Bacosa *et al*., 2018; Toth *et al*., 2021; van Leeuwen *et al*., 2022). Metagenome-assembled genomes (MAGs), including *Anaerolineales* and *Desulfobacter* harboured

AssA-like genes. Other *Anaerolineales* MAGs prior to this study have shown no indication of these genes, however further exploration highlighted these genes as glycl radical proteins (*Anaerolineae bacterium*) (77.61 – 89.08% identity), *Anaerolineae* being a hydrocarbon degrading genera of *Anaerolineales* and the *AssA* gene being a part of the glycl radical enzyme family (Khot *et al*., 2022). Specifically *AbcA* – like genes were observed within MAGs *Methanomicrobiaceae* as associated with *Methanoregula* sp (96.10 – 97.92% identity). *Methanoregula* have been highlighted as an important archaeal genera within syntrophic interactions between methanogenesis and anaerobic aromatic degradation (Phan *et al*., 2021). This coupled with the potential methanogenic conditions of the Spill Site, supports the involvement of methanogens in anaerobic aromatic degradation *in-situ*. Overall this study highlights the system's capacity for alkane and aromatic degradation under aerobic and anaerobic conditions and showcases the effectiveness of remediation in supporting the natural recovery via biodegradation during *in-situ* spill events.

With databases such as CANT-HYD and HADEG (Khot *et al*., 2022; Rojas-Vargas *et al*., 2023) at our disposal exploration of hydrocarbon degradation pathways *in-situ* has become much more accessible. Even so, much of the contents within these databases are based on putative gene sequences (e.g. *abcA* and *ahyA*) and outputs must be critically assessed when using these databases for gene classification. Adding to these databases will be vital in further understanding these putative genes and improving upon already established models within biomonitoring. The succession of microbial communities has already been well established throughout a variety of environments, as well as within MOS flocs (Chapter 4), thus establishing the succession of expressed functional genes associated with degradation pathways may further improve models such as the EiHE, in which we may track the progression of

contamination through what pathways are being expressed at any given time (Lozada *et al*., 2014).

Overall this thesis aimed to showcase the impact of remediation tools on microbial communtiies and ecological functionality such as biodegradation. Dispersant use following controversy has been highlighted as an effective tool under relevant *in-situ* sea surface conditions, whilst showing minimal impact to processes such as marine snow formation. MOS communities have also been shown to utilise chemical dispersant use to their advantage, further enhancing biodegradation processes within already established sites of high microbial activity. Even so, chemical dispersants are still toxic and have be shown to cause detrimental effects to other higher taxa, notably during embryonic development, thus we should still consider all options before their use to minimise the impact of the spill and dispersant use as best as possible. Many groups are now looking into the production of biological based dispersants in the form of biosurfactants that are produced naturally as a better alternative, which may be an answer to the question of whether we should use dispersants or not.

Spill response outside of chemical dispersant use was also highlighted as very effective in reducing environmental impact, whilst highlighting the natural capacity for hydrocarbon degradation of a spill impacted through effective monitoring and remediation strategies. Although this thesis provides a significant understanding of *in-situ* remediation response via biodegradation processes and MOS formation, this cannot be extrapolated directly towards all environmental conditions. Microbial communities are vastly different throughout the globe due to changes in temperature, salinity, nutrient concentrations and seasonality, which may have a significant impact to their response to oil spillage, MOS formation and remedial strategies. Thus further

study needs to be considered, using the findings of this thesis as a strong framework for the future.

References

Abbasian, F., Lockington, R., Mallavarapu, M. and Naidu, R. 2015. A comprehensive review of aliphatic hydrocarbon biodegradation by bacteria. *Applied biochemistry and biotechnology*. 176: 670-699.

Abdallah, R.I., Mohamed, S.Z. and Ahmed, F.M. 2005. Effect of biological and chemical dispersants on Oil spills. *Petroleum science and technology.* 23, 3-4: 463-474.

Achenbach, L.A., Michaelidou, U., Bruce, R.A., Fryman, J. and Coates, J.D. 2001. *Dechloromonas agitata* gen. nov., sp. nov. and *Dechlorosoma suillum* gen. nov., sp. nov., two novel environmentally dominant (per) chlorate-reducing bacteria and their phylogenetic position. *International journal of systematic and evolutionary microbiology*. 51, 2: 527-533.

Acinas, S.G., Antón, J. and Rodríguez-Valera, F. 1999. Diversity of free-living and attached bacteria in offshore western Mediterranean waters as depicted by analysis of genes encoding 16S rRNA. *Applied and Environmental Microbiology*. 65, 2: 514-522.

Adams, G. O., Fufeyin, P. T., Okoro, S. E. and Ehinomen, I. 2015. Bioremediation, biostimulation and bioaugmention: a review. *International Journal of Environmental Bioremediation & Biodegradation.* 3: 28-39.

Aeppli, C., Mitchell, D.A., Keyes, P., Beirne, E.C., McFarlin, K.M., Roman-Hubers, A.T., Rusyn, I., Prince, R.C., Zhao, L., Parkerton, T.F. and Nedwed, T. 2022. Oil irradiation experiments document changes in oil properties, molecular composition, and dispersant effectiveness associated with oil photo-oxidation. *Environmental science & technology*. 56, 12: 7789-7799.

Aitken, C.M., Jones, D.M., Maguire, M.J., Gray, N.D., Sherry, A., Bowler, B.F., Ditchfield, A.K., Larter, S.R. and Head, I.M. 2013. Evidence that crude oil alkane activation proceeds by different mechanisms under sulfate-reducing and methanogenic conditions. *Geochimica et Cosmochimica Acta*. 109: 162-174.

Alejandre-Colomo, C., Viver, T., Urdiain, M., Francis, B., Harder, J., Kämpfer, P., Amann, R. and Rosselló-Móra, R. 2020. Taxonomic study of nine new *Winogradskyella* species occurring in the shallow waters of Helgoland Roads, North Sea. Proposal of *Winogradskyella schleiferi sp. nov., Winogradskyella costae sp. nov., Winogradskyella helgolandensis sp. nov., Winogradskyella vidalii sp. nov., Winogradskyella forsetii sp. nov., Winogradskyella ludwigii sp. nov., Winogradskyella ursingii sp. nov., Winogradskyella wichelsiae sp. nov.,* and Candidatus *"Winogradskyella atlantica" sp. nov*. *Systematic and Applied Microbiology*. 43, 6: 126128.

Alexander, F. J., King, C. K., Reichelt-Brushett, A. J. and Harrison, P. L. 2017. Fuel oil and dispersant toxicity to the Antarctic Sea urchin (*Sterechinus neumayeri*). *Environmental toxicology and chemistry.* 36: 1563-1571.

Alldredge, A. L. and Silver, M. W. 1988. Characteristics, dynamics and significance of marine snow. *Progress in Oceanography.* 20: 41-82.

Alldredge, A.L., Passow, U. and Logan, B.E. 1993. The abundance and significance of a class of large, transparent organic particles in the ocean. *Deep Sea Research Part I: Oceanographic Research Papers.* 40, 6: 1131-1140.

Al-Majed, A. A., Adebayo, A. R. and Hossain, M. E. 2012. A sustainable approach to controlling oil spills. *Journal of Environmental Management*. 113: 213-227.

Almeda, R., Baca, S., Hyatt, C. and Buskey, E. J. 2014. Ingestion and sublethal effects of physically and chemically dispersed crude oil on marine planktonic copepods. *Ecotoxicology*. 23: 988-1003.

Alonso-Gutiérrez, J., Figueras, A., Albaigés, J., Jiménez, N., Viñas, M., Solanas, A.M., Novoa, B. 2009. Bacterial communities from shoreline environments (Costa da Morte, northwestern Spain) affected by the Prestige oil spill. *Applied Environmental Microbiology*. 75: 11.

Altieri, A. H. and Diaz, R. J. 2019. Dead zones: oxygen depletion in coastal ecosystems. World seas: An environmental evaluation. *Elsevier*.

Altschul, S.F., Gish, W., Miller, W., Myers, E.W. and Lipman, D.J. 1990. Basic local alignment search tool. *Journal of molecular biology*. 215, 3: 403-410.

An, M.M., Shen, L., Liang, R.N., Lu, Y.J. and Zhao, G.Z. 2023. Alcanivorax quisquiliarum sp. nov., isolated from anaerobic fermentation liquid of food waste by high-throughput cultivation. *International Journal of Systematic and Evolutionary Microbiology*. 73, 4: 005764.

Anderson, T.R., Gentleman, W.C., Cael, B.B., Hirschi, J.J.M., Eastwood, R.L. and Mayor, D.J. 2023. Proliferating particle surface area via microbial decay has profound consequences for remineralisation rate: a new approach to modelling the degradation of sinking detritus in the ocean. *Biogeochemistry*. 164, 2: 335-347.

Atlas, R. M. 1975. Effects of temperature and crude oil composition on petroleum biodegradation. *Applied microbiology*. 30: 396-403.

Atlas, R. M. 1981. Microbial degradation of petroleum hydrocarbons: an environmental perspective. *Microbiological reviews.* 45: 180-209.

Arndt, H., Dietrich, D., Auer, B., Cleven, E.J., Gräfenhan, T., Weitere, M. and Mylnikov, A.P. 2000. Functional diversity of heterotrophic flagellates in aquatic ecosystems. *The flagellates: unity, diversity and evolution*. 1: 240-268.

Arnosti, C., Ziervogel, K., Yang, T. and Teske, A. 2016. Oil-derived marine aggregates–hot spots of polysaccharide degradation by specialized bacterial communities. *Deep Sea Research Part II: Topical Studies in Oceanography*. 129: 179-186.

Axiak, V., George, J.J. and Moore, M.N. 1988. Petroleum hydrocarbons in the marine bivalve *Venus verrucosa*: accumulation and cellular responses. *Marine Biology*. 97.

Azam, F. and Long, R.A. 2001. Sea snow microcosms. *Nature.* 414, 6863: 495-498.

Backman, L.R., Funk, M.A., Dawson, C.D. and Drennan, C.L. 2017. New tricks for the glycyl radical enzyme family. *Critical reviews in biochemistry and molecular biology*. 52, 6: 674-695.

Bacosa, H.P., Kang, A., Lu, K. and Liu, Z. 2021. Initial oil concentration affects hydrocarbon biodegradation rates and bacterial community composition in seawater. *Marine Pollution Bulletin*. 162: 111867.

Bacosa, H.P., Erdner, D.L. and Liu, Z. 2015a. Differentiating the roles of photooxidation and biodegradation in the weathering of Light Louisiana Sweet crude oil in surface water from the Deepwater Horizon site. *Marine pollution bulletin*. 95, 1; 265-272.

Bacosa, H.P., Erdner, D.L., Rosenheim, B.E., Shetty, P., Seitz, K.W., Baker, B.J. and Liu, Z. 2018. Hydrocarbon degradation and response of seafloor sediment bacterial community in the northern Gulf of Mexico to light Louisiana sweet crude oil. *The ISME journal*. 12, 10: 2532-2543.

Bacosa, H.P., Liu, Z. and Erdner, D.L. 2015b. Natural sunlight shapes crude oil-degrading bacterial communities in northern Gulf of Mexico surface waters. *Frontiers in microbiology*. 6: 1325.

Badmadashiev, D.V., Stroeva, A.R., Klyukina, A.A., Poludetkina, E.N. and Bonch-Osmolovskaya, E.A. 2023. Phylogenetic diversity of prokaryotic communities of the upper sediment layers of the Kandalaksha Bay, White Sea. *Microbiology.* 92, 6: 807-818.

Bælum, J., Borglin, S., Chakraborty, R., Fortney, J.L., Lamendella, R., Mason, O.U., Auer, M., Zemla, M., Bill, M., Conrad, M.E. and Malfatti, S.A. 2012. Deep-sea bacteria enriched by oil and dispersant from the Deepwater Horizon spill. *Environmental microbiology*. 14, 9; 2405-2416.

Barbier, C. 2015. MDL 2179 oil spill by the oil rig *Deepwater Horizon*.

Basen, M., Krüger, M., Milucka, J., Kuever, J., Kahnt, J., Grundmann, O., Meyerdierks, A., Widdel, F. and Shima, S. 2011. Bacterial enzymes for dissimilatory sulfate reduction in a marine microbial mat (Black Sea) mediating anaerobic oxidation of methane. *Environmental microbiology*. 13, 5: 1370-1379.

Bauvois, C., Huston, A. and Feller, G. 2013. The Cold-Active M1 Aminopeptidase from the Arctic Bacterium *Colwellia psychrerythraea.*

Bloh, A.H., Usup, G. and Ahmad, A. 2016. *Loktanella spp*. Gb03 as an algicidal bacterium, isolated from the culture of Dinoflagellate *Gambierdiscus belizeanus. Veterinary World.* 9, 2: 142.

Bochdansky, A. B., Clouse, M. A. and Herndl, G. J. 2017. Eukaryotic microbes, principally fungi and labyrinthulomycetes, dominate biomass on bathypelagic marine snow. *The ISME Journal*. 11: 362-373.

Bogan, B.W., Sullivan, W.R., Kayser, K.J., Derr, K.D., Aldrich, H.C. and Paterek, J.R. 2003. Alkanindiges illinoisensis gen. nov., sp. nov., an obligately hydrocarbonoclastic, aerobic squalane-degrading bacterium isolated from oilfield soils. *International Journal of Systematic and Evolutionary Microbiology*. 53, 5: 1389-1395.

Boyd, J.A., Jungbluth, S.P., Leu, A.O., Evans, P.N., Woodcroft, B.J., Chadwick, G.L., Orphan, V.J., Amend, J.P., Rappé, M.S. and Tyson, G.W. 2019. Divergent methyl-coenzyme M reductase genes in a deep-subseafloor Archaeoglobi. *The ISME journal*. 13, 5: 1269-1279.

Bragg, J. R., Prince, R. C., Harner, E. J. and Atlas, R. M. 1994. Effectiveness of bioremediation for the *Exxon Valdez* oil spill. *Nature*. 368: 413-418.

Brakstad, O.G., Lewis, A. and Beegle-Krause, C.J. 2018. A critical review of marine snow in the context of oil spills and oil spill dispersant treatment with focus on the Deepwater Horizon oil spill. *Marine pollution bulletin*. 135: 346-356.

Brakstad, O. G., Lofthus, S., Ribicic, D. and Netzer, R. 2017. Biodegradation of petroleum oil in cold marine environments. *Psychrophiles: From Biodiversity to Biotechnology*. Springer.

Brakstad, O. G., Nordtug, T. and Throne-Holst, M. 2015. Biodegradation of dispersed Macondo oil in seawater at low temperature and different oil droplet sizes. *Marine Pollution Bulletin*. 93: 144-152.

Bray, J.R. and Curtis, J.T. 1957. An ordination of the upland forest communities of southern Wisconsin. *Ecological monographs*. 27, 4: 326-349.

Bretherton, L., Kamalanathan, M., Genzer, J., Hillhouse, J., Setta, S., Liang, Y., Brown, C. M., Xu, C., Sweet, J. and Passow, U. 2019. Response of natural

phytoplankton communities exposed to crude oil and chemical dispersants during a mesocosm experiment. *Aquatic Toxicology*. 206: 43-53.

Brooks, M. E., Kristensen, K., van Benthem, K. J., Magnusson, A., Berg, C. W., Nielsen, A., Skaug, H. J., Maechler, M. & Bolker, B. M. 2017. glmmTMB Balances Speed and Flexibility Among Packages for Zero-inflated Generalized Linear Mixed Modeling. The R Journal. 9, 2: 378–400. doi: 10.32614/RJ-2017-066.

Buchfink, B., Xie, C. and Huson, D.H. 2015. Fast and sensitive protein alignment using DIAMOND. *Nature methods*. 12, 1: 59-60.

Burd, A. B., Chanton, J. P., Daly, K. L., Gilbert, S., Passow, U. and Quigg, A. 2020. The science behind marine-oil snow and MOSSFA: past, present, and future. *Progress in Oceanography*. 102398.

Cabral, L., Giovanella, P., Pellizzer, E.P., Teramoto, E.H., Kiang, C.H. and Sette, L.D. 2022. Microbial communities in petroleum-contaminated sites: structure and metabolisms. *Chemosphere*. 286: 131752.

Cafaro, V., Izzo, V., Notomista, E. and Di Donato, A. 2013. Marine hydrocarbonoclastic bacteria. *Marine Enzymes for Biocatalysis*. Elsevier.

Cai, Z., Liu, W., Fu, J., O'Reilly, S.E. and Zhao, D. 2017. Effects of oil dispersants on photodegradation of parent and alkylated anthracene in seawater. *Environmental Pollution*. 229: 272-280.

Calvo, C., Manzanera, M., Silva-Castro, G.A., Uad, I. and González-López, J. 2009. Application of bioemulsifiers in soil oil bioremediation processes. Future prospects. *Science of the total environment*. 407, 12: 3634-3640.

Callaghan, A.V., Davidova, I.A., Savage-Ashlock, K., Parisi, V.A., Gieg, L.M., Suflita, J.M., Kukor, J.J. and Wawrik, B. 2010. Diversity of benzyl-and alkylsuccinate synthase genes in hydrocarbon-impacted environments and enrichment cultures. *Environmental science & technology*. 44, 19: 7287-7294.

Callahan, B. 2020. RDP taxonomic training data formatted for DADA2 (RDP trainset 18/release 11.5). https://doi.org/10.5281/zenodo.4310151

Callahan, B., McMurdie, P., Holmes, S., Biostrings, I., BiocStyle, S., Rcpp, L. and biocViews Microbiome, S. 2022. Package 'dada2'. Accurate, high-resolution sample inference from amplicon sequencing data.

Callahan, B. J., McMurdie, P. J., Rosen, M. J., Han, A. W., Johnson, A. J. A. and Holmes, S. P. 2016. DADA2: High-resolution sample inference from Illumina amplicon data. *Nature methods*. 13: 581-583.

Camphuysen, C. and Heubeck, M. 2001. Marine oil pollution and beached bird surveys: the development of a sensitive monitoring instrument. *Environmental Pollution*. 112: 443-461.

Cappello, S., Denaro, R., Genovese, M., Giuliano, L. and Yakimov, M.M. 2007. Predominant growth of *Alcanivorax* during experiments on "oil spill bioremediation" in mesocosms. *Microbiological research*. 162: 185-190.

Carpenter, A. 2019. Oil pollution in the North Sea: the impact of governance measures on oil pollution over several decades. *Hydrobiologia*. 845.

Caruso, C., Rizzo, C., Mangano, S., Poli, A., Di Donato, P., Finore, I., Nicolaus, B., Di Marco, G., Michaud, L. and Lo Giudice, A. 2018. Production and biotechnological potential of extracellular polymeric substances from sponge-associated Antarctic bacteria. *Applied and environmental microbiology*. 84, 4: 01624-17.

Catlett, J.L., Ortiz, A.M. and Buan, N.R. 2015. Rerouting cellular electron flux to increase the rate of biological methane production. *Applied and environmental microbiology*. 81, 19: 6528-6537.

Centre for Environment, Fisheries and Aquaculture Science (CEFAS). 2018. PREMIAM: Pollution response in emergencies, Marine impact assessment and monitoring: post-incident monitoring guidelines.

Cerniglia, C. E. 1993. Biodegradation of polycyclic aromatic hydrocarbons. *Current Opinion in Biotechnology*. 4: 331-338.

Chakraborty, R., O'Connor, S.M., Chan, E. and Coates, J.D. 2005. Anaerobic degradation of benzene, toluene, ethylbenzene, and xylene compounds by *Dechloromonas* strain RCB. *Applied and environmental microbiology*. 71, 12: 8649-8655.

Chakraborty, R., Borglin, S. E., Dubinsky, E. A., Andersen, G. L. and Hazen, T. C. 2012. Microbial response to the MC-252 oil and Corexit 9500 in the Gulf of Mexico. *Frontiers in Microbiology*. 3: 357.

Chapman, H., Purnell, K., Law, R. J. and Kirby, M. F. 2007. The use of chemical dispersants to combat oil spills at sea: A review of practice and research needs in Europe. *Marine Pollution Bulletin*. 54: 827-838.

Charalampous, G., Fragkou, E., Kalogerakis, N., Antoniou, E. and Gontikaki, E. 2024. Diversity links to functionality: Unraveling the impact of pressure disruption and culture medium on crude oil-enriched microbial communities from the deep Eastern Mediterranean Sea. *Marine Pollution Bulletin*. 202: 116275.

Chaumeil, P.A., Mussig, A.J., Hugenholtz, P. and Parks, D.H. 2020. GTDB-Tk: a toolkit to classify genomes with the Genome Taxonomy Database.

Chen, J., Di, Z., Shi, J., Shu, Y., Wan, Z., Song, L. and Zhang, W. 2020. Marine oil spill pollution causes and governance: A case study of Sanchi tanker collision and explosion. *Journal of Cleaner Production*. 273: 122978.

Chen, S.C., Musat, F., Richnow, H.H. and Krüger, M. 2024. Microbial diversity and oil biodegradation potential of northern Barents Sea sediments. *Journal of Environmental Sciences*. 146: 283-297.

Chen, Z., Xiang, S., Lu, Y., Lai, Q., Dong, C., Li, J., Li, G. and Shao, Z. 2024. Adaptation mechanisms of Alcanivorax facilitating its predominance in marine environments. *Frontiers in Marine Science*. 11: 1491690.

Chen, J., Zhang, W., Wan, Z., Li, S., Huang, T. and Fei, Y. 2019. Oil spills from global tankers: status review and future governance. *Journal of Cleaner Production*.

Chen, S., Zhou, Y., Chen, Y. and Gu, J. 2018. fastp: an ultra-fast all-in-one FASTQ preprocessor. *Bioinformatics*. 34: i884–i890.

Chhatre, S., Purohit, H., Shanker, R. and Khanna, P. 1996. Bacterial consortia for crude oil spill remediation. *Water Science and Technology*. 34; 187-193.

Chernikova, T.N., Bargiela, R., Toshchakov, S.V., Shivaraman, V., Lunev, E.A., Yakimov, M.M., Thomas, D.N. and Golyshin, P.N. 2020. Hydrocarbon-degrading bacteria Alcanivorax and Marinobacter associated with microalgae Pavlova lutheri and Nannochloropsis oculata. *Frontiers in Microbiology*. 11: 572931.

Chronopoulou, P.M., Sanni, G.O., Silas-Olu, D.I., van der Meer, J.R., Timmis, K.N., Brussaard, C.P. and McGenity, T.J. 2015. Generalist hydrocarbon-degrading bacterial communities in the oil-polluted water column of the North Sea. *Microbial biotechnology*. 8: 434-447.

Chiu, M.H., Garcia, S.G., Hwang, B., Claiche, D., Sanchez, G., Aldayafleh, R., Tsai, S.M., Santschi, P.H., Quigg, A. and Chin, W.C. 2017. Corexit, oil and marine microgels. Marine Pollution Bulletin. 122, 1-2: 376-378.

Chung, W.K. and King, G.M. 2001. Isolation, characterization, and polyaromatic hydrocarbon degradation potential of aerobic bacteria from marine macrofaunal burrow sediments and description of *Lutibacterium anuloederans* gen. nov., sp. nov., and *Cycloclasticus spirillensus* sp. nov. *Applied and environmental microbiology*. 67, 12: 5585-5592.

Clarke, K.R. 1993. Non-parametric multivariate analyses of changes in community structure. *Australian journal of ecology.* 18, 1: 117-143.

Cline, J.D. and Richards, F.A. 1969. Oxygenation of hydrogen sulfide in seawater at constant salinity, temperature and pH. *Environmental Science & Technology*. 3, 9: 838-843.

Cormack, D. 2013. Response to Marine Oil Pollution: Review and Assessment. *Dordrecht:* Springer Netherlands.

Coscolín, C., Bargiela, R., Martínez-Martínez, M., Alonso, S., Bollinger, A., Thies, S., Chernikova, T. N., Hai, T., Golyshina, O. V. and Jaeger, K.-E. 2019. Hydrocarbon-degrading microbes as sources of new biocatalysts. *Taxonomy, Genomics and Ecophysiology of Hydrocarbon-Degrading Microbes*. 353-373.

Coulon, F., McKew, B. A., Osborn, A. M., McGenity, T. J. and Timmis, K. N. 2007. Effects of temperature and biostimulation on oil-degrading microbial communities in temperate estuarine waters. *Environmental Microbiology*. 9: 177-186.

Coulon, F., Chronopoulou, P.M., Fahy, A., Païssé, S., Goñi-Urriza, M., Peperzak, L., Acuña Alvarez, L., McKew, B.A., Brussaard, C.P., Underwood, G.J. and Timmis, K.N. 2012. Central role of dynamic tidal biofilms dominated by aerobic hydrocarbonoclastic bacteria and diatoms in the biodegradation of hydrocarbons in coastal mudflats. *Applied and Environmental Microbiology*. 78, 10: 3638-3648.

Coyne, K.J., Wang, Y. and Johnson, G. 2022. Algicidal bacteria: a review of current knowledge and applications to control harmful algal blooms. *Frontier Microbiology.* 13: 871177

Crump, B.C., Armbrust, E.V. and Baross, J.A. 1999. Phylogenetic analysis of particle-attached and free-living bacterial communities in the Columbia River, its estuary, and the adjacent coastal ocean. *Applied and environmental microbiology.* 65, 7: 3192-3204.

Curson, A.R., Todd, J.D., Sullivan, M.J. and Johnston, A.W. 2011. Catabolism of dimethylsulphoniopropionate: microorganisms, enzymes and genes. *Nature Reviews Microbiology*. 9, 12: 849-859.

da Silva, E.S., Pragana, L.G. and Vasconcelos, U. 2021. Photooxidation vs biodegradation: A short review on fate of heavy hydrocarbons after oil spill in sea water. polycyclic aromatic compounds. 12: 13.

D'Auria, M., Racioppi, R. and Velluzzi, V. 2008. Photodegradation of crude oil: liquid injection and headspace solid-phase microextraction for crude oil analysis by gas chromatography with mass spectrometer detector. *Journal of chromatographic science*. 46, 4: 339-344.

Dalton, H. and Stirling, D. 1982. Co-metabolism. *Philosophical Transactions of the Royal Society of London B: Biological Sciences*. 297: 481-496.

Daly, K. L., Passow, U., Chanton, J. and Hollander, D. 2016. Assessing the impacts of oil-associated marine snow formation and sedimentation during and after the Deepwater Horizon oil spill. *Anthropocene*.

Daly, K. L., Vaz, A. C. and Paris, C. B. 2020. Physical processes influencing the sedimentation and lateral transport of MOSSFA in the NE Gulf of Mexico. *Scenarios and Responses to Future Deep Oil Spills*. Springer.

Dave, D. and Ghaly, A. E. 2011. Remediation technologies for marine oil spills: a critical review and comparative analysis. *American Journal of Environmental Sciences*. 7: 423.

Dashti, N., Ali, N., Eliyas, M., Khanafer, M., Sorkhoh, N.A. and Radwan, S.S. 2015. Most hydrocarbonoclastic bacteria in the total environment are diazotrophic, which highlights their value in the bioremediation of hydrocarbon contaminants. *Microbes and environments*. 30, 1: 70-75.

Denaro, R., Crisafi, F., Russo, D., Genovese, M., Messina, E., Genovese, L., Carbone, M., Ciavatta, M., Ferrer, M. and Golyshin, P. 2014. *Alcanivorax borkumensis* produces an extracellular siderophore in iron-limitation condition maintaining the hydrocarbon-degradation efficiency. *Marine Genomics*. 17: 43-52.

DeLong, E.F., Franks, D.G. and Alldredge, A.L. 1993. Phylogenetic diversity of aggregate-attached vs. free-living marine bacterial assemblages. *Limnology and oceanography*. 38, 5: 924-934.

Deming, J.W., Somers, L.K., Straube, W.L., Swartz, D.G. and Macdonell, M.T. 1988. Isolation of an obligately barophilic bacterium and description of a new genus, Colwellia gen. nov. Systematic and Applied Microbiology. 10, 2: 152-160.

DeVries, T., Primeau, F. and Deutsch, C. 2012. The sequestration efficiency of the biological pump. *Geophysical Research Letters*. 39: 13.

Dibble, J. and Bartha, R. 1976. Effect of iron on the biodegradation of petroleum in seawater. *Applied and Environmental Microbiology*. 31: 544-550.

Dilling, L. and Alldredge, A. L. 2000. Fragmentation of marine snow by swimming macrozooplankton: A new process impacting carbon cycling in the sea. *Deep Sea Research Part I: Oceanographic Research Papers*. 47: 1227-1245.

Di Toro, D. M., McGrath, J. A. and Stubblefield, W. A. 2007. Predicting the toxicity of neat and weathered crude oil: toxic potential and the toxicity of saturated mixtures. *Environmental Toxicology and Chemistry: An International Journal*. 26: 24-36.

Dong, C., Wei, L., Wang, J., Lai, Q., Huang, Z. and Shao, Z. 2022. Genome-based taxonomic rearrangement of *Oceanobacter*-related bacteria including the description of *Thalassolituus hydrocarbonoclasticus* sp. nov. and *Thalassolituus pacificus* sp. nov. and emended description of the genus *Thalassolituus*. *Frontiers in Microbiology*. 13: 1051202.

Doyle, S. M., Whitaker, E. A., De Pascuale, V., Wade, T. L., Knap, A. H., Santschi, P. H., Quigg, A. and Sylvan, J. B. 2018. Rapid formation of microbe-oil aggregates and changes in community composition in coastal surface water following exposure to oil and the dispersant Corexit. *Frontiers in Microbiology*. 9: 689.

Dring, M., Wagner, A., Franklin, L., Kuhlenkamp, R. and Lüning, K. 2001. Seasonal and diurnal variations in ultraviolet-B and ultraviolet-A irradiances at and below the sea surface at Helgoland (North Sea) over a 6-year period. *Helgoland Marine Research*. 55: 3-11.

Du, Z.J., Wang, Z.J., Zhao, J.X. and Chen, G.J. 2016. *Woeseia oceani* gen. nov., sp. nov., a chemoheterotrophic member of the order Chromatiales, and proposal

of Woeseiaceae fam. nov. *International journal of systematic and evolutionary microbiology*. 66, 1: 107-112.

Dubinsky, E.A., Conrad, M.E., Chakraborty, R., Bill, M., Borglin, S.E., Hollibaugh, J.T., Mason, O.U., M. Piceno, Y., Reid, F.C., Stringfellow, W.T. and Tom, L.M. 2013. Succession of hydrocarbon-degrading bacteria in the aftermath of the Deepwater Horizon oil spill in the Gulf of Mexico. Environmental science & technology. 47: 10860-10867.

Dupuis, A. and Ucan-Marin, F. 2015. A literature review on the aquatic toxicology of petroleum oil: an overview of oil properties and effects to aquatic biota. *DFO Canadian Science Advisory Secretariat Research Document*. 2015: 007.

Dutta, T.K. and Harayama, S. 2000. Fate of crude oil by the combination of photooxidation and biodegradation. *Environmental science & technology*. *34*, 8; 1500-1505.

Dyksterhouse, S. E., Gray, J. P., Herwig, R. P., Lara, J. C. and Staley, J. T. 1995. *Cycloclasticus pugetii* gen. nov., sp. nov., an aromatic hydrocarbon-degrading bacterium from marine sediments. *International Journal of Systematic and Evolutionary Microbiology*. 45: 116-123.

Edgar, R.C. 2010. Search and clustering orders of magnitude faster than BLAST. *Bioinformatics*. 26, 19: 2460-2461.

Engelhardt, M., Daly, K., Swannell, R. and Head, I. 2001. Isolation and characterization of a novel hydrocarbon-degrading, Gram-positive bacterium, isolated from intertidal beach sediment, and description of *Planococcus alkanoclasticus* sp. nov. *Journal of Applied Microbiology*. 90: 237-247.

Engel, A.S., Liu, C., Paterson, A.T., Anderson, L.C., Eugene Turner, R., Overton, E.B. 2017. Salt marsh bacterial communities before and after the Deepwater Horizon oil spill. *Applied Environmental Microbiology*. 83.

Etkin, D.S. 1999. March. Historical overview of oil spills from all sources (1960–1998). In *International Oil Spill Conference*. 1999: 1097-1102.

Feng, X. and Zhang, B. 2023. Applications of bubble curtains in marine oil spill containment: Hydrodynamic characteristics, applications, and future perspectives. *Marine Pollution Bulletin*. 194: 115371.

Fingas, M. F. 1995. The evaporation of oil spills. In *Proceedings of the 18th Arctic Marine Oilspill Program Technical Seminar*.

Fingas, M. 2011a. An overview of in-situ burning. In *Oil Spill Science and Technology*. pp. 737–903.

Fingas, M. 2011b. Oil spill dispersants: A technical summary. Oil spill science and technology. Elsevier.

Fu, J., Gong, Y., Zhao, X., O'Reilly, S. and Zhao, D. 2014. Effects of oil and dispersant on formation of marine oil snow and transport of oil hydrocarbons. *Environmental Science & Technology*. 48: 14392-14399.

Fu, X., Lai, Q., Dong, C., Wang, W. and Shao, Z. 2018. Complete genome sequence of *Alcanivorax xenomutans* P40, an alkane-degrading bacterium isolated from deep seawater. *Marine Genomics*. 38: 1-4.

Funhoff, E.G., Salzmann, J., Bauer, U., Witholt, B. and van Beilen, J.B. 2007. Hydroxylation and epoxidation reactions catalyzed by CYP153 enzymes. *Enzyme and microbial technology*. 40, 4: 806-812.

Gad, S. C. 2014. Petroleum hydrocarbons. *Encyclopedia of Toxicology: Third Edition*. 838–840.

Gärdes, A., Iversen, M. H., Grossart, H.-P., Passow, U. and Ullrich, M. S. 2011. Diatom-associated bacteria are required for aggregation of *Thalassiosira weissflogii*. *The ISME Journal*. 5: 436-445.

Gao, M., Li, X., Zhang, Q., Li, S., Wu, S., Wang, Y. and Sun, H. 2024. Spatial distribution of volatile organic compounds in contaminated soil and distinct microbial effect driven by aerobic and anaerobic conditions. *Science of The Total Environment*. 927: 172256.

Gauthier, M.J., Lafay, B., Christen, R., Fernandez, L., Acquaviva, M., Bonin, P. and Bertrand, J.C. 1992. Marinobacter hydrocarbonoclasticus gen. nov., sp. nov., a new, extremely halotolerant, hydrocarbon-degrading marine bacterium. *International Journal of Systematic and Evolutionary Microbiology.* 42, 4: 568-576.

Gerdes, B., Brinkmeyer, R., Dieckmann, G. and Helmke, E. 2005. Influence of crude oil on changes of bacterial communities in Arctic sea-ice. *FEMS Microbiology Ecology*. 53, 1: 129-139.

Godfrin, M.P., Sihlabela, M., Bose, A. and Tripathi, A. 2018. Behavior of marine bacteria in clean environment and oil spill conditions. *Langmuir*. 34: 9047-9053.

Godoy-Lozano, E.E., Escobar-Zepeda, A., Raggi, L., Merino, E., Gutierrez-Rios, R.M., Juarez, K., Segovia, L., Licea-Navarro, A.F., Gracia, A., Sanchez-Flores, A. and Pardo-Lopez, L. 2018. Bacterial diversity and the geochemical landscape in the southwestern Gulf of Mexico. *Frontiers in microbiology*. 9: 2528.

Golyshin, P.N., Chernikova, T.N., Abraham, W.R., Lünsdorf, H., Timmis, K.N. and Yakimov, M.M. 2002. Oleiphilaceae fam. nov., to include *Oleiphilus messinensis* gen. nov., sp. nov., a novel marine bacterium that obligately utilizes hydrocarbons. *International journal of systematic and evolutionary microbiology.* 52, 3: 901-911.

Greer, J. B., Pasparakis, C., Stieglitz, J. D., Benetti, D., Grosell, M. and Schlenk, D. 2019. Effects of corexit 9500A and Corexit-crude oil mixtures on transcriptomic pathways and developmental toxicity in early life stage mahi-mahi (*Coryphaena hippurus*). *Aquatic toxicology*. 212: 233-240.

Gregson, B. H., McKew, B. A., Holland, R. D., Nedwed, T. J., Prince, R. C. and McGenity, T. J. 2021. Marine oil snow, a microbial perspective. *Frontiers in Marine Science.* 8: 11.

Gregson, B. H., Metodieva, G., Metodiev, M. V., Golyshin, P. N. and McKew, B. A. 2020. Protein expression in the obligate hydrocarbon-degrading psychrophile Oleispira antarctica RB-8 during alkane degradation and cold tolerance. *Environmental microbiology.* 22: 1870-1883.

Gregson, B.H., Metodieva, G., Metodiev, M.V. and McKew, B.A. 2019. Differential protein expression during growth on linear versus branched alkanes in the obligate marine hydrocarbon-degrading bacterium Alcanivorax borkumensis SK2T. *Environmental microbiology*. 21, 7: 2347-2359.

Grossi, V., Yakimov, M. M., Al Ali, B., Tapilatu, Y., Cuny, P., Goutx, M., La Cono, V., Giuliano, L. and Tamburini, C. 2010. Hydrostatic pressure affects membrane and storage lipid compositions of the piezotolerant hydrocarbon-degrading Marinobacter hydrocarbonoclasticus strain. 5.

Guigue, C., Tedetti, M., Dang, D.H., Mullot, J.U., Garnier, C. and Goutx, M. 2017. Remobilization of polycyclic aromatic hydrocarbons and organic matter in seawater during sediment resuspension experiments from a polluted coastal

environment: Insights from Toulon Bay (France). *Environmental Pollution*. 229: 627-638.

Gutierrez, T. 2019. Occurrence and roles of the obligate hydrocarbonoclastic bacteria in the ocean when there is no obvious hydrocarbon contamination. *Taxonomy, genomics and ecophysiology of hydrocarbon-degrading microbes*. 337-352.

Gutierrez, T., Green, D. H., Nichols, P. D., Whitman, W. B., Semple, K. T. and Aitken, M. D. 2013. *Polycyclovorans algicola* gen. nov., sp. nov., an aromatic-hydrocarbon-degrading marine bacterium found associated with laboratory cultures of marine phytoplankton. *Applied and Environmental Microbiology.* 79: 205-214.

Gutierrez, T., Green, D. H., Whitman, W. B., Nichols, P. D., Semple, K. T. and Aitken, M. D. 2012. *Algiphilus aromaticivorans* gen. nov., sp. nov., an aromatic hydrocarbon-degrading bacterium isolated from a culture of the marine dinoflagellate *Lingulodinium polyedrum*, and proposal of Algiphilaceae fam. nov. *International journal of systematic and evolutionary microbiology*. 62: 2743-2749.

Guo, X., Song, G., Li, Y., Zhao, L. and Wang, J. 2022. Switch of bacteria community under oxygen depletion in sediment of Bohai Sea. *Frontiers in Marine Science*. 9: 833513.

Hallam, S.J., Girguis, P.R., Preston, C.M., Richardson, P.M. and DeLong, E.F. 2003. Identification of methyl coenzyme M reductase A (mcrA) genes associated with methane-oxidizing archaea. *Applied and environmental microbiology*. 69, 9: 5483-5491.

Hallberg, K.B., Dopson, M. and Lindström, E.B. 1996. Reduced sulfur compound oxidation by Thiobacillus caldus. *Journal of Bacteriology*. 178, 1: 6-11.

Hamam, S. E., Hamoda, M. F., Shaban, H. I. and Kilani, A. S. 1988. Crude oil dissolution in saline water. *Water, air, and soil pollution*. 37: 55-64.

Hamamoto, Y. and Honda, D. 2019. Nutritional intake of *Aplanochytrium* (*Labyrinthulea, Stramenopiles*) from living diatoms revealed by culture experiments suggesting the new prey–predator interactions in the grazing food web of the marine ecosystem. *PLoS One*. 14, 1: 0208941.

Hamdan, L. J. and Fulmer, P. A. 2011. Effects of COREXIT® EC9500A on bacteria from a beach oiled by the Deepwater Horizon spill. *Aquatic microbial ecology*. 63: 101-109.

Han, X., Wang, F., Zhang, D., Feng, T. and Zhang, L. 2021. Nitrate-assisted biodegradation of polycyclic aromatic hydrocarbons (PAHs) in the water-level-fluctuation zone of the three Gorges Reservoir, China: Insights from in situ microbial interaction analyses and a microcosmic experiment. *Environmental Pollution*. 268: 115693.

Harayama, S., Kasai, Y. and Hara, A. 2004. Microbial communities in oil-contaminated seawater. *Current opinion in biotechnology*. 15, 205-214.

Harayama, S., Kishira, H., Kasai, Y. and Shutsubo, K. 1999. Petroleum biodegradation in marine environments. *Journal of molecular microbiology and biotechnology.* 1, 63-70.

Hassanshahian, M. and Cappello, S. 2013. Crude oil biodegradation in the marine environments. *Biodegradation-Engineering and Technology*. 5: 102-135.

Hastings, D. W., Bartlett, T., Brooks, G. R., Larson, R. A., Quinn, K. A., Razionale, D., Schwing, P. T., Bernal, L. H. P., Ruiz-Fernández, A. C. and Sánchez-Cabeza, J.-A. 2020. Changes in redox conditions of surface sediments following the Deepwater Horizon and Ixtoc 1 events. Deep Oil Spills. Springer.

Hatch. 2024. Wytch Farm Oil Spill: Impact Assessment. https://www.phc.co.uk/impact-reports-published-following-oil-spill/

Hawthorne, S.B., Azzolina, N.A., Neuhauser, E.F. and Kreitinger, J.P. 2007. Predicting bioavailability of sediment polycyclic aromatic hydrocarbons to Hyalella azteca using equilibrium partitioning, supercritical fluid extraction, and pore water concentrations. *Environmental science & technology*. 41, 17: 6297-6304.

Hazen, T. C., Prince, R. C. and Mahmoudi, N. 2016. Marine oil biodegradation. ACS Publications.

Head, I. M., Jones, D. M. and Röling, W. F. 2006. Marine microorganisms make a meal of oil. *Nature Reviews Microbiology*. 4: 173-182.

Hedlund, B. P., Geiselbrecht, A. D., Bair, T. J. and Staley, J. T. 1999. Polycyclic aromatic hydrocarbon degradation by a new marine bacterium, *Neptunomonas naphthovorans* gen. nov., sp. nov. *Applied and Environmental Microbiology*. 65: 251-259.

Heider, J., Spormann, A. M., Beller, H. R. and Widdel, F. 1998. Anaerobic bacterial metabolism of hydrocarbons. *FEMS Microbiology Reviews*. 22: 459-473.

Henry, I. A., Netzer, R., Davies, E. and Brakstad, O. G. 2021. The influences of phytoplankton species, mineral particles and concentrations of dispersed oil on the formation and fate of marine oil-related aggregates. *Science of the Total Environment*. 752: 141786.

Hjorth, M., Forbes, V.E. and Dahllöf, I. 2008. Plankton stress responses from PAH exposure and nutrient enrichment. *Marine Ecology Progress Series*. 363: 121-130.

Hong, Y.W., Yuan, D.X., Lin, Q.M. and Yang, T.L., 2008. Accumulation and biodegradation of phenanthrene and fluoranthene by the algae enriched from a mangrove aquatic ecosystem. Marine Pollution Bulletin. 56, 8: 1400-1405.

Hopson, P. 2009. The geology of Poole Harbour & its environs.

Howarth, R.W., Marino, R. and Cole, J.J. 1988. Nitrogen fixation in freshwater, estuarine, and marine ecosystems. 2. Biogeochemical controls. *Limnology and Oceanography*. 33, 4: 688-701.

Hu, S.K., Herrera, E.L., Smith, A.R., Pachiadaki, M.G., Edgcomb, V.P., Sylva, S.P., Chan, E.W., Seewald, J.S., German, C.R. and Huber, J.A. 2021. Protistan grazing impacts microbial communities and carbon cycling at deep-sea hydrothermal vents. *Proceedings of the National Academy of Sciences*. 118, 29: 2102674118.

Huang, L.K. and Wang, M.J.J. 1995. Image thresholding by minimizing the measures of fuzziness. *Pattern recognition*. 28, 1: 41-51.

Ibacache-Quiroga, C., Ojeda, J., Espinoza-Vergara, G., Olivero, P., Cuellar, M. and Dinamarca, M.A. 2013. The hydrocarbon-degrading marine bacterium *C obetia* sp. strain MM1IDA2H-1 produces a biosurfactant that interferes with quorum sensing of fish pathogens by signal hijacking. *Microbial biotechnology*. 6, 4: 394-405.

Illumina .2013. 16S metagenomic sequencing library preparation (Illumina Technical Note 15044223). Illumina. **S**an Diego, CA. http://support.illumina.com/documents/documentation/ chemistry documentation/16s/16s-metagenomic-library-prep-guide-15044223-b.pdf Accessed 14 Oct 2023.

Imron, M.F., Kurniawan, S.B., Ismail, N.I. and Abdullah, S.R.S. 2020. Future challenges in diesel biodegradation by bacteria isolates: a review. *Journal of Cleaner Production*. 251: 119716.

International Tanker Owners Pollution Federation (ITOPF). 2011a. Fate of Marine Oil Spills.

International Tanker Owners Pollution Federation (ITOPF). 2011b. Clean up of oil from shoreline

International Tanker Owners Pollution Federation (ITOPF). 2012. Use of Skimmers in Oil Pollution Response.

International Tanker Owners Pollution Federation (ITOPF). 2020. Oil Tanker Spill Statistics 2020.

IPIECA, I. 2017. Guidelines on implementing spill impact mitigation assessment (SIMA). IPIECA, London, United Kingdom.

Itodo, A., Akeju, T. and Itodo, H. 2019. Polycyclic aromatic hydrocarbons in crude oil contaminated water from Ese-Odo offshore, Nigeria. *Annals Ecol. Environ*. *Sci.* 3: 12-19.

Iversen, M.H. and Ploug, H. 2010. Ballast minerals and the sinking carbon flux in the ocean: carbon-specific respiration rates and sinking velocity of marine snow aggregates. *Biogeosciences*. 7, 9: 2613-2624.

Iwabuchi, N., Sunairi, M., Urai, M., Itoh, C., Anzai, H., Nakajima, M. and Harayama, S. 2002. Extracellular polysaccharides of *Rhodococcus rhodochrous* S-2 stimulate the degradation of aromatic components in crude oil by indigenous marine bacteria. Applied and Environmental Microbiology. 68, 5: 2337-2343.

Jernelv, A. 2010. The threats from oil spills: Now, then, and in the future. *Ambio 39.*

Ji, Y., Mao, G., Wang, Y. and Bartlam, M. 2013. Structural insights into diversity and n-alkane biodegradation mechanisms of alkane hydroxylases. *Frontiers in microbiology*. 4: 58.

Jiao, S., Liu, Z., Lin, Y., Yang, J., Chen, W. and Wei, G. 2016. Bacterial communities in oil contaminated soils: biogeography and co-occurrence patterns. *Soil Biology and Biochemistry*. 98: 64-73.

Jiménez, N., Viñas, M., Bayona, J. M., Albaiges, J. and Solanas, A. M. 2007. The Prestige oil spill: bacterial community dynamics during a field biostimulation assay. *Applied microbiology and biotechnology.* 77: 935-945.

Jin, C., Yu, Z., Peng, S., Feng, K.E., Zhang, L. and Zhou, X. 2018. The characterization and comparison of exopolysaccharides from two benthic diatoms with different biofilm formation abilities. *Anais da Academia Brasileira de Ciências*. 90, 02: 1503-1519.

Joyner-Matos, J., Predmore, B.L., Stein, J.R., Leeuwenburgh, C. and Julian, D. 2010. Hydrogen sulfide induces oxidative damage to RNA and DNA in a sulfide-tolerant marine invertebrate. *Physiological and Biochemical Zoology*. 83, 2: 356-365.

Jormakka, M., Byrne, B. and Iwata, S. 2003. Formate dehydrogenase–a versatile enzyme in changing environments. *Current opinion in structural biology*. 13, 4: 418-423.

Jung, S.W., Park, J.S., Kown, O.Y., Kang, J.H., Shim, W.J. and Kim, Y.O. 2010. Effects of crude oil on marine microbial communities in short term outdoor microcosms. *The Journal of Microbiology.* 48: 594-600.

Juhasz, A.L. and Naidu, R., 2000. Bioremediation of high molecular weight polycyclic aromatic hydrocarbons: a review of the microbial degradation of benzo [a] pyrene. *International biodeterioration & biodegradation*. 45, 1-2: 57-88.

Jurelevicius, D., Alvarez, V.M., Marques, J.M., de Sousa Lima, L.R.F., Dias, F.D.A. and Seldin, L. 2013. Bacterial community response to petroleum hydrocarbon

amendments in freshwater, marine, and hypersaline water-containing microcosms. *Applied and environmental microbiology*. 79, 19: 5927-5935.

Kang, D.D., Froula, J., Egan, R. and Wang, Z. 2015. MetaBAT, an efficient tool for accurately reconstructing single genomes from complex microbial communities. *PeerJ*. 3: 1165.

Kang, D.D., Li, F., Kirton, E., Thomas, A., Egan, R., An, H. and Wang, Z. 2019. MetaBAT 2: an adaptive binning algorithm for robust and efficient genome reconstruction from metagenome assemblies. *PeerJ*. 7: e7359.

Karlapudi, A. P., Venkateswarulu, T., Tammineedi, J., Kanumuri, L., Ravuru, B. K., ramu Dirisala, V. and Kodali, V. P. 2018. Role of biosurfactants in bioremediation of oil pollution-a review. *Petroleum.* 4: 241-249.

Kasai, Y., Kishira, H. and Harayama, S. 2002. Bacteria belonging to the genus Cycloclasticus play a primary role in the degradation of aromatic hydrocarbons released in a marine environment. *Applied and Environmental Microbiology*. 68: 5625-5633.

Kawano, Y., Suzuki, K. and Ohtsu, I. 2018. Current understanding of sulfur assimilation metabolism to biosynthesize L-cysteine and recent progress of its fermentative overproduction in microorganisms. *Applied microbiology and biotechnology*. 102, 19: 8203-8211.

Kelkar, D.S., Kumar, A.R. and Zinjarde, S.S. 2007. Hydrocarbon emulsification and enhanced crude oil degradation by lauroyl glucose ester. *Bioresource technology.* 98, 7: 1505-1508.

Kellermann, C. and Griebler, C. 2009. *Thiobacillus thiophilus* sp. nov., a chemolithoautotrophic, thiosulfate-oxidizing bacterium isolated from contaminated aquifer sediments. *International journal of systematic and evolutionary microbiology*. 59, 3: 583-588.

Kelly, L.D., McGuinness, L.R., Hughes, J.E. and Wainright, S.C. 1999. Effects of phenanthrene on primary production of phytoplankton in two New Jersey estuaries. *Bulletin of environmental contamination and toxicology*. 63, 5: 646-653.

Kessler, J.D., Valentine, D.L., Redmond, M.C., Du, M., Chan, E.W., Mendes, S.D., Quiroz, E.W., Villanueva, C.J., Shusta, S.S., Werra, L.M. and Yvon-Lewis, S.A. 2011. A persistent oxygen anomaly reveals the fate of spilled methane in the deep Gulf of Mexico. *Science*. 331, 6015: 312-315.

Khot, V., Zorz, J., Gittins, D.A., Chakraborty, A., Bell, E., Bautista, M.A., Paquette, A.J., Hawley, A.K., Novotnik, B., Hubert, C.R. and Strous, M. 2022. CANT-HYD: a curated database of phylogeny-derived hidden Markov models for annotation of marker genes involved in hydrocarbon degradation. *Frontiers in Microbiology*. 12: 764058.

Kim, C.W., Park, J.S., Cho, S.K., Oh, K.J., Kim, Y.S. and Kim, D.U. 2003. Removal of hydrogen sulfide, ammonia, and benzene by fluidized bed reactor and biofilter. *Journal of microbiology and biotechnology*. 13, 2: 301-304.

Kleindienst, S., Paul, J. H. and Joye, S. B. 2015a. Using dispersants after oil spills: impacts on the composition and activity of microbial communities. *Nature Reviews Microbiology*. 13: 388-396.

Kleindienst, S., Seidel, M., Ziervogel, K., Grim, S., Loftis, K., Harrison, S., Malkin, S. Y., Perkins, M. J., Field, J. and Sogin, M. L. 2015b. Chemical dispersants can suppress the activity of natural oil-degrading microorganisms. *Proceedings of the National Academy of Sciences*, 112: 14900-14905.

Kleindienst, S., Seidel, M., Ziervogel, K., Grim, S., Loftis, K., Harrison, S., Malkin, S.Y., Perkins, M.J., Field, J., Sogin, M.L. and Dittmar, T. 2016. Reply to Prince *et al*.: Ability of chemical dispersants to reduce oil spill impacts remains unclear. *Proceedings of the National Academy of Sciences*, 113: E1422-E1423.

Klindworth, A., Pruesse, E., Schweer, T., Peplies, J., Quast, C., Horn, M. and Glöckner, F.O. 2013. Evaluation of general 16S ribosomal RNA gene PCR primers for classical and next-generation sequencing-based diversity studies. Nucleic acids research, 4: e1-e1.

Knittel, K. and Boetius, A. 2009. Anaerobic oxidation of methane: progress with an unknown process. *Annual review of microbiology.* 63, 1: 311-334.

Koch, T. and Dahl, C. 2018. A novel bacterial sulfur oxidation pathway provides a new link between the cycles of organic and inorganic sulfur compounds. *The ISME journal*. 12, 10: 2479-2491.

Kodama, Y., Stiknowati, L.I., Ueki, A., Ueki, K. and Watanabe, K. 2008. Thalassospira tepidiphila sp. nov., a polycyclic aromatic hydrocarbon-degrading bacterium isolated from seawater. International journal of systematic and evolutionary microbiology. 58, 3: 711-715.

Kostka, J.E., Prakash, O., Overholt, W.A., Green, S.J., Freyer, G., Canion, A., Delgardio, J., Norton, N., Hazen, T.C. and Huettel, M. 2011. Hydrocarbon-degrading bacteria and the bacterial community response in Gulf of Mexico beach sands impacted by the Deepwater Horizon oil spill. Applied and environmental microbiology. 77, 22: 7962-7974.

Kujawinski, E. B., Kido Soule, M. C., Valentine, D. L., Boysen, A. K., Longnecker, K. and Redmond, M. C. 2011. Fate of dispersants associated with the Deepwater Horizon oil spill. *Environmental science & technology*. 45: 1298-1306.

Kumar, A., Munjal, A. and Sawhney, R. 2011 Crude oil PAH constitution, degradation pathway and associated bioremediation microflora: an overview. *International Journal of Environmental Sciences*. 1: 1420-1439.

Kurilenko, V.V., Romanenko, L.A., Isaeva, M.P., Svetashev, V.I. and Mikhailov, V.V. 2019. *Winogradskyella algae sp. nov*., a marine bacterium isolated from the brown alga. *Antonie van Leeuwenhoek*. 112: 731-739.

Kusk, K.O. 1978. Effects of crude oil and aromatic hydrocarbons on the photosynthesis of the diatom *Nitzschia palea*. *Physiologia plantarum*. 43, 1: 1-6.

Kissin, Y. v. 1990. Catagenesis of light cycloalkanes in petroleum. *Organic Geochemistry 15*.

Krueger, F. 2015. TrimGalore: A wrapper tool around Cutadapt and FastQC to consistently apply quality and adapter trimming to FastQ files. *Babraham Bioinformatics*.

Lamendella, R., Strutt, S., Borglin, S., Chakraborty, R., Tas, N., Mason, O.U., Hultman, J., Prestat, E., Hazen, T.C. and Jansson, J.K. 2014. Assessment of the Deepwater Horizon oil spill impact on Gulf coast microbial communities. *Frontiers in microbiology*. 5: 130.

Langmead, B. and Salzberg, S.L. 2012. Fast gapped-read alignment with Bowtie 2. *Nature methods*. 9: 4, 357-359.

Laudenbach, D.E. and Grossman, A.R. 1991. Characterization and mutagenesis of sulfur-regulated genes in a cyanobacterium: evidence for function in sulfate transport. *Journal of Bacteriology*. 173, 9: 2739-2750.

Latimer, J.S., Davis, W.R. and Keith, D.J. 1999. Mobilization of PAHs and PCBs from in-place contaminated marine sediments during simulated resuspension events. *Estuarine, Coastal and Shelf Science*. 49, 4: 577-595.

Lavoie, M. and Raven, J.A. 2020. How can large-celled diatoms rapidly modulate sinking rates episodically?. *Journal of Experimental Botany*. 71, 12: 3386-3389.

Law, R.J., Dawes, V.J., Woodhead, R.J. and Matthiessen, P. 1997. Polycyclic aromatic hydrocarbons (PAH) in seawater around England and Wales. *Marine pollution bulletin*. 34, 5: 306-322.

Law, R.J., Kelly, C.A. and Nicholson, M.D. 1999. Polycyclic aromatic hydrocarbons (PAH) in shellfish affected by the Sea Empress oil spill in Wales in 1996. *Polycyclic Aromatic Compounds*. 17, 1-4: 229-239.

Ławniczak, Ł., Woźniak-Karczewska, M., Loibner, A.P., Heipieper, H.J. and Chrzanowski, Ł. 2020. Microbial degradation of hydrocarbons—basic principles for bioremediation: a review. *Molecules*. 25: 856.

Leahy, J. G. and Colwell, R. R. 1990. Microbial degradation of hydrocarbons in the environment. Microbiological reviews. 54: 305-315.

Lee, K., Nedwed, T., Prince, R. C. and Palandro, D. 2013. Lab tests on the biodegradation of chemically dispersed oil should consider the rapid dilution that occurs at sea. *Marine Pollution Bulletin*. 73: 314-318.

Lessard, R. R. and DeMarco, G. 2000. The significance of oil spill dispersants. *Spill Science & Technology Bulletin.* 6: 59-68.

Li, D., Liu, C.M., Luo, R., Sadakane, K. and Lam, T.W. 2015. MEGAHIT: an ultra-fast single-node solution for large and complex metagenomics assembly via succinct de Bruijn graph. *Bioinformatics*. 31, 10: 1674-1676.

Li, H., Handsaker, B., Wysoker, A., Fennell, T., Ruan, J., Homer, N., Marth, G., Abecasis, G., Durbin, R. and 1000 Genome Project Data Processing Subgroup. 2009. The sequence alignment/map format and SAMtools. *Bioinformatics*. 25, 16: 2078-2079.

Li, Y.Q., Xin, Y., Li, C., Liu, J. and Huang, T. 2023. Metagenomics-metabolomics analysis of microbial function and metabolism in petroleum-contaminated soil. *Brazilian Journal of Microbiology*. 54, 2: 935-947.

Li, J., Bidigare, R.R. and Laws, E.A. 2017. Effects of Macondo oil on phytoplankton from Grand Isle, Louisiana. J. *Environ. Anal. Toxicol.* 7: 2161-0525.

Liang, B., Wang, L.Y., Zhou, Z., Mbadinga, S.M., Zhou, L., Liu, J.F., Yang, S.Z., Gu, J.D. and Mu, B.Z. 2016. High frequency of *Thermodesulfovibrio spp*. and Anaerolineaceae in association with *Methanoculleus spp*. in a long-term incubation of n-alkanes-degrading methanogenic enrichment culture. *Frontiers in microbiology*. 7: 1431.

Lin, X., Yang, B., Shen, J. and Du, N. 2009. Biodegradation of crude oil by an Arctic psychrotrophic bacterium *Pseudoalteromomas* sp. P29. *Current microbiology.* 59: 341-345.

Liu, J., Zheng, Y., Lin, H., Wang, X., Li, M., Liu, Y., Yu, M., Zhao, M., Pedentchouk, N. and Lea-Smith, D. J. 2019. Proliferation of hydrocarbon-degrading microbes at the bottom of the Mariana Trench. *Microbiome*. 7: 1-13.

Liu, Y., Wan, Y.Y., Wang, C., Ma, Z., Liu, X. and Li, S. 2020. Biodegradation of n-alkanes in crude oil by three identified bacterial strains. *Fuel*. 275: 117897.

Lincoln, S.A., Radović, J.R., Gracia, A., Jaggi, A., Oldenburg, T.B., Larter, S.R. and Freeman, K.H. 2020. Molecular legacy of the 1979 Ixtoc 1 oil spill in deep-sea sediments of the southern Gulf of Mexico. *Deep oil spills: facts, fate, and effects.* 312-327.

Louvado, A., Coelho, F., Oliveira, V., Gomes, H., Cleary, D., Simões, M., Cunha, A. and Gomes, N. 2019. Microcosm evaluation of the impact of oil contamination and chemical dispersant addition on bacterial communities and sediment remediation of an estuarine port environment. *Journal of applied microbiology*. 127: 134-149.

Lozada, M., Marcos, M.S., Commendatore, M.G., Gil, M.N. and Dionisi, H.M. 2014. The bacterial community structure of hydrocarbon-polluted marine environments as the basis for the definition of an ecological index of hydrocarbon exposure. *Microbes and environments*. 29, 3: 269-276.

Lu, J., Breitwieser, F.P., Thielen, P. and Salzberg, S.L. 2017. Bracken: estimating species abundance in metagenomics data. *PeerJ Computer Science*. 3: e104.

Luo, F., Gitiafroz, R., Devine, C.E., Gong, Y., Hug, L.A., Raskin, L. and Edwards, E.A. 2014. Metatranscriptome of an anaerobic benzene-degrading, nitrate-reducing enrichment culture reveals involvement of carboxylation in benzene ring activation. *Applied and environmental microbiology*. 80, 14: 4095-4107.

Madigan, M.T., Bender, K.S., Buckley, D.H., Sattley, W.M. and Stahl, D.A. 2018. Brock biology of microorganisms. 15th Global Edition. *Boston, US: Benjamin Cummins*. 1: 1391-1407.

Magurran, A.E. 2013. Ecological diversity and its measurement. *Springer Science & Business Media*.

Maier, R.M. 2019. Biological Processes Affecting Contaminants Transport and Fate, in: *Environmental and Pollution Science*.

Maki, H., Sasaki, T. and Harayama, S. 2001. Photo-oxidation of biodegraded crude oil and toxicity of the photo-oxidized products. *Chemosphere.* 44, 5: 1145-1151.

Manzetti, S. 2013. Polycyclic aromatic hydrocarbons in the environment: environmental fate and transformation. *Polycyclic Aromatic Compounds.* 33: 311-330.

Margesin, R., Moertelmaier, C. and Mair, J. 2013. Low-temperature biodegradation of petroleum hydrocarbons (n-alkanes, phenol, anthracene, pyrene) by four actinobacterial strains. *International Biodeterioration & Biodegradation*. 84: 185-191.

Martin, M. 2011. Cutadapt removes adapter sequences from high-throughput sequencing reads. EMBnet. Journal. 17, 1: 10-12.

Mason, O.U., Han, J., Woyke, T. and Jansson, J.K. 2014. Single-cell genomics reveals features of a Colwellia species that was dominant during the Deepwater Horizon oil spill. *Frontiers in microbiology.* 5: 332.

Matsumoto, A., Kasai, H., Matsuo, Y., Ōmura, S., Shizuri, Y. and Takahashi, Y. 2009. Ilumatobacter fluminis gen. nov., sp. nov., a novel actinobacterium isolated from the sediment of an estuary. *The Journal of general and applied microbiology*. 55, 3: 201-205.

May, V.J. and Humphreys, J. eds. 2005. *The ecology of Poole harbour*. Elsevier.

Methé, B.A., Nelson, K.E., Deming, J.W., Momen, B., Melamud, E., Zhang, X., Moult, J., Madupu, R., Nelson, W.C., Dodson, R.J. and Brinkac, L.M. 2005. The psychrophilic lifestyle as revealed by the genome sequence of Colwellia psychrerythraea 34H through genomic and proteomic analyses. *Proceedings of the National Academy of Sciences.* 102, 31: 10913-10918.

Morais, D., Pylro, V., Clark, I.M., Hirsch, P.R. and Tótola, M.R. 2016. Responses of microbial community from tropical pristine coastal soil to crude oil contamination. *PeerJ*. 4: 1733.

McFarlin, K.M., Perkins, M.J., Field, J.A. and Leigh, M.B. 2018. Biodegradation of crude oil and Corexit 9500 in Arctic seawater. *Frontiers in Microbiology*. *9*: 1788.

McFarlin, K. M. and Prince, R. C. 2021. Contradictory conclusions surrounding the effects of chemical dispersants on oil biodegradation. *International Oil Spill Conference Proceedings. 2021:* 685852. doi: https://doi.org/10.7901/2169-3358-2021.1.685852

McFarlin, K. M., Prince, R. C., Perkins, R. and Leigh, M. B. 2014. Biodegradation of dispersed oil in arctic seawater at-1 C. *PloS one*. 9: e84297.

McGenity, T. J. 2019. Taxonomy, genomics and ecophysiology of hydrocarbon-degrading microbes, Springer.

McGenity, T. J., Folwell, B. D., McKew, B. A. and Sanni, G. O. 2012. Marine crude-oil biodegradation: a central role for interspecies interactions. *Aquatic Biosystems*. 8: 1-19.

McGenity, T.J. and Laissue, P.P. 2023. Bacteria stretch and bend oil to feed their appetite. *Science*. 381: 728-729.

McLaren, M. R. and Callahan, B. J. 2021. 'Silva 138.1 prokaryotic SSU taxonomic training data formatted for DADA2'. *Zenodo*. doi: 10.5281/zenodo.4587955.

McKew, B.A., Coulon, F., Osborn, A.M., Timmis, K.N., McGenity, T.J. 2007a. Determining the identity and roles of oil-metabolizing marine bacteria from the Thames estuary, UK. *Environmental Microbiology*. 9: 1.

McKew, B. A., Coulon, F., Yakimov, M. M., Denaro, R., Genovese, M., Smith, C. J., Osborn, A. M., Timmis, K. N. and McGenity, T. J. 2007b. Efficacy of intervention strategies for bioremediation of crude oil in marine systems and effects on indigenous hydrocarbonoclastic bacteria. *Environmental microbiology.* 9: 1562-1571.

McKew, B.A. and Smith, C.J. 2017. Real-time PCR approaches for analysis of hydrocarbon-degrading bacterial communities. *Hydrocarbon and Lipid Microbiology Protocols: Microbial Quantitation, Community Profiling and Array Approaches*. 45-64.

McNutt, M. K., Chu, S., Lubchenco, J., Hunter, T., Dreyfus, G., Murawski, S. A. and Kennedy, D. M. 2012. Applications of science and engineering to quantify and control the Deepwater Horizon oil spill. *Proceedings of the National Academy of Sciences.* 109: 20222-20228.

Mestre, M., Ruiz-González, C., Logares, R., Duarte, C.M., Gasol, J.M. and Sala, M.M. 2018. Sinking particles promote vertical connectivity in the ocean microbiome. *Proceedings of the National Academy of Sciences*. 115, 29: E6799-E6807.

Meyer, N., Bigalke, A., Kaulfuß, A. and Pohnert, G. 2017. Strategies and ecological roles of algicidal bacteria. *FEMS Microbiology Reviews*. 41, 6: 880-899.

Meyer, S., Moser, R., Neef, A., Stahl, U. and Kämpfer, P. 1999. Differential detection of key enzymes of polyaromatic-hydrocarbon-degrading bacteria using PCR and gene probes. Microbiology. 145, 7: 1731-1741.

Miller, J.I., Techtmann, S., Fortney, J., Mahmoudi, N., Joyner, D., Liu, J., Olesen, S., Alm, E., Fernandez, A., Gardinali, P. and GaraJayeva, N. 2019. Oil hydrocarbon degradation by Caspian Sea microbial communities. Frontiers in microbiology. 10: 995.

Miri, M., Bambai, B., Tabandeh, F., Sadeghizadeh, M. and Kamali, N. 2010. Production of a recombinant alkane hydroxylase (AlkB2) from *Alcanivorax borkumensis*. *Biotechnology letters*. 32: 497-502.

Miranda, L.N., Hutchison, K., Grossman, A.R. and Brawley, S.H. 2013. Diversity and abundance of the bacterial community of the red macroalga *Porphyra umbilicalis*: did bacterial farmers produce macroalgae?. *PLoS One*. 8, 3: 58269.

Mishamandani, S., Gutierrez, T., Berry, D. and Aitken, M.D. 2016. Response of the bacterial community associated with a cosmopolitan marine diatom to crude oil shows a preference for the biodegradation of aromatic hydrocarbons. *Environmental microbiology.* 18, 6: 1817-1833.

Mohanty, G. and Mukherji, S. 2008. Biodegradation rate of diesel range n-alkanes by bacterial cultures *Exiguobacterium aurantiacum* and *Burkholderia cepacia*. *International Biodeterioration & Biodegradation*. 61: 240-250.

Mori, K., Suzuki, K.I., Urabe, T., Sugihara, M., Tanaka, K., Hamada, M. and Hanada, S. 2011. Thioprofundum hispidum sp. nov., an obligately chemolithoautotrophic sulfur-oxidizing gammaproteobacterium isolated from the hydrothermal field on Suiyo Seamount, and proposal of Thioalkalispiraceae fam. nov. in the order Chromatiales. *International journal of systematic and evolutionary microbiology*. 61, 10: 2412-2418.

Mu, J., Jin, F., Ma, X., Lin, Z. and Wang, J. 2014. Comparative effects of biological and chemical dispersants on the bioavailability and toxicity of crude oil to early life stages of marine medaka (*Oryzias melastigma*). *Environmental toxicology and chemistry*. 33: 2576-2583.

Mullaeva, S.A., Delegan, Y.A., Streletskii, R.A., Sazonova, O.I., Petrikov, K.V., Ivanova, A.A., Dyatlov, I.A., Shemyakin, I.G., Bogun, A.G. and Vetrova, A.A., 2022. *Pseudomonas veronii* strain 7–41 degrading medium-chain n-alkanes and polycyclic aromatic hydrocarbons. *Scientific Reports*. 12, 1: 20527.

Munilla, I., Arcos, J.M., Oro, D., Álvarez, D., Leyenda, P.M. and Velando, A. 2011. Mass mortality of seabirds in the aftermath of the Prestige oil spill. *Ecosphere 2*.

Muyzer, G. and Stams, A.J. 2008. The ecology and biotechnology of sulphate-reducing bacteria. Nature reviews microbiology. 6, 6: 441-454.

NALCO. 2008. Safety Data Sheet for Corexit EC9500A; COREXIT EC9500A. Available at: https://dec.alaska.gov/spar/ppr/docs/dispersant_MSDS/Corexit%209500A%20MSDS.pdf

National Academies of Sciences, Engineering, and Medicine. 2022. Oil in the Sea IV: Inputs, Fates, and Effects.

National Oceanic and Atmospheric Administration. 2010. Characteristics of Response Strategies: A Guide for Spill Response Planning in Marine Environments.

National Research Council (NRC). 2003. Oil in the Sea III: Inputs, Fates, and Effects. Washington, D.C: National Academy Press.

National Research Council (NRC). 1989. Using oil spill dispersants on the sea, *National Academy Press*.

National Toxicology Program (NTP). 2021. Report on Carcinogens, Fifteenth Edition, Research Triangle Park, NC: U.S. Department of Health and Human Services, *Public Health Service.*

National Toxicology Program. 2011. NTP 12th Report on Carcinogens. *Rep Carcinog 12.*

Ndimele, P. E., Saba, A. O., Ojo, D. O., Ndimele, C. C., Anetekhai, M. A. and Erondu, E. S. 2018. Remediation of crude oil spillage. The political ecology of oil and gas activities in the Nigerian aquatic ecosystem. Elsevier.

Nedashkovskaya, O.I., Kim, S.B., Han, S.K., Snauwaert, C., Vancanneyt, M., Swings, J., Kim, K.O., Lysenko, A.M., Rohde, M., Frolova, G.M. and Mikhailov, V.V.

2005. *Winogradskyella thalassocola* gen. nov., sp. nov., Winogradskyella epiphytica sp. nov. and *Winogradskyella eximia* sp. nov., marine bacteria of the family Flavobacteriaceae. *International journal of systematic and evolutionary microbiology*. 55, 1: 49-55.

Neethu, C.S., Saravanakumar, C., Purvaja, R., Robin, R.S. and Ramesh, R. 2019. Oil-spill triggered shift in indigenous microbial structure and functional dynamics in different marine environmental matrices. *Scientific reports*. 9, 1: 1354.

Netzer, R., Henry, I. A., Ribicic, D., Wibberg, D., Brönner, U. and Brakstad, O. G. 2018. Petroleum hydrocarbon and microbial community structure successions in marine oil-related aggregates associated with diatoms relevant for Arctic conditions. *Marine pollution bulletin*. 135: 759-768.

Nguyen, U. T., Lincoln, S. A., Valladares Juárez, A. G., Schedler, M., Macalady, J. L., Müller, R. and Freeman, K. H. 2018. The influence of pressure on crude oil biodegradation in shallow and deep Gulf of Mexico sediments. *PloS one*. 13: e0199784.

Nguyen, T.T., Vuong, T.Q., Han, H.L., Li, Z., Lee, Y.J., Ko, J., Nedashkovskaya, O.I. and Kim, S.G., 2023. Three marine species of the genus *Fulvivirga*, rich sources of carbohydrate-active enzymes degrading alginate, chitin, laminarin, starch, and xylan. *Scientific Reports*. 13, 1: 6301.

Nõlvak, H., Dang, N.P., Truu, M., Peeb, A., Tiirik, K., O'Sadnick, M. and Truu, J. 2021. Microbial community dynamics during biodegradation of crude oil and its response to biostimulation in Svalbard seawater at low temperature. *Microorganisms*. 9: 2425.

Nomack, M. and Cleveland, C. 2010. Oil spill control technologies. *Encyclopedia of Earth.* http://www. eoearth. org/articles/view/158385.

Okai, M., Watanabe, A., Ishida, M. and Urano, N. 2015. Draft genome sequence of a benzo [a] pyrene-degrading bacterium, Olleya sp. strain ITB9. *Genome announcements*. 3, 6: 10-1128.

Oksanen, J., Simpson, G.L., Blanchet, F.G., Kindt, R., Legendre, P., Minchin, P.R., O'Hara, R.B., Solymos, P., Stevens, M.H.H., Szoecs, E., Wagner, H., Barbour, M., Bedward, M., Bolker, B., Borcard, D., Carvalho, G., Chirico, M., De Caceres, M., Durand, S., Evangelista, H.B.A., FitzJohn, R., Friendly, M., Furneaux, B., Hannigan, G., Hill, M.O., Lahti, L., McGlinn, D., Ouellette, M.-H., Ribeiro Cunha, E., Smith, T., Stier, A., Ter Braak, C.J.F., & Weedon, J. 2024. vegan: Community Ecology Package. R package version 2.6-6.1. https://CRAN.R-project.org/package=vegan.

Oladi, M., Leontidou, K., Stoeck, T. and Shokri, M.R. 2022. Environmental DNA-based profiling of benthic bacterial and eukaryote communities along a crude oil spill gradient in a coral reef in the Persian Gulf. *Marine Pollution Bulletin*. 184: 114143.

Olajire, A. and Essien, J. 2014. Aerobic degradation of petroleum components by microbial consortia. *Journal of Petroleum & Environmental Biotechnology*. 5: 1.

Omotoso, O.E., Munoz, V.A. and Mikula, R.J. 2002. Mechanisms of crude oil–mineral interactions. *Spill Science & Technology Bulletin*. *8*, 1: 45-54.

O'Neill, F.G. and Summerbell, K. 2011. The mobilisation of sediment by demersal otter trawls. *Marine Pollution Bulletin*. 62, 5: 1088-1097.

Othman, H.B., Lanouguère, É., Got, P., Hlaili, A.S. and Leboulanger, C. 2018. Structural and functional responses of coastal marine phytoplankton communities to PAH mixtures. *Chemosphere*. 209: 908-919.

Othman, H.B., Pick, F.R., Hlaili, A.S. and Leboulanger, C. 2023. Effects of polycyclic aromatic hydrocarbons on marine and freshwater microalgae–A review. *Journal of Hazardous Materials*. 441: 129869.

Pal, S., Roy, A. and Kazy, S. K. 2019. Exploring microbial diversity and function in petroleum hydrocarbon associated environments through omics approaches. *Microbial Diversity in the Genomic Era*. Elsevier.

Paniagua-Michel, J. and Banat, I.M. 2024. Unravelling diatoms' potential for the bioremediation of oil hydrocarbons in marine environments. *Clean Technologies*. 6, 1: 93-115.

Parada, A.E., Needham, D.M. and Fuhrman, J.A. 2016. Every base matters: assessing small subunit rRNA primers for marine microbiomes with mock communities, time series and global field samples. *Environmental microbiology*. 18, 5: 1403-1414.

Parinos, C., Hatzianestis, I., Chourdaki, S., Plakidi, E. and Gogou, A. 2019. Imprint and short-term fate of the Agia Zoni II tanker oil spill on the marine ecosystem of Saronikos Gulf. *Science of The Total Environment*. 693: 133568.

Parte, A. C., Carbasse, J. S., Meier-Kolthoff, J. P., Reimer, L. C. and Göker, M. 2020. List of Prokaryotic names with Standing in Nomenclature (LPSN) moves to the DSMZ. *International journal of systematic and evolutionary microbiology*: 70, 5607.

Passeri, A., Schmidt, M., Haffner, T., Wray, V., Lang, S. and Wagner, F. 1992. Marine biosurfactants. IV. Production, characterization and biosynthesis of an anionic glucose lipid from the marine bacterial strain MM1. *Applied microbiology and biotechnology*. 37: 281-286.

Passow, U. 2016. Formation of rapidly-sinking, oil-associated marine snow. *Deep Sea Research Part II: Topical Studies in Oceanography*. 129: 232-240.

Passow, U., Alldredge, A. L. and Logan, B. E. 1994. The role of particulate carbohydrate exudates in the flocculation of diatom blooms. *Deep Sea Research Part I: Oceanographic Research Papers*. 41: 335-357.

Passow, U. and Alldredge, A.L. 1995. A dye-binding assay for the spectrophotometric measurement of transparent exopolymer particles (TEP). *Limnology and Oceanography*, *40*(7), pp.1326-1335.

Passow, U. and Overton, E. B. 2021. The complexity of spills: the fate of the Deepwater Horizon oil. *Annual Review of Marine Science*. 13: 109-136.

Passow, U., Sweet, J. and Quigg, A. 2017. How the dispersant Corexit impacts the formation of sinking marine oil snow. *Marine pollution bulletin*. 125: 139-145.

Passow, U., Ziervogel, K., Asper, V. and Diercks, A. 2012. Marine snow formation in the aftermath of the Deepwater Horizon oil spill in the Gulf of Mexico. *Environmental Research Letters.* 7: 035301.

Patro, R., Duggal, G., Love, M.I., Irizarry, R.A. and Kingsford, C. 2017. Salmon provides fast and bias-aware quantification of transcript expression. *Nature methods*. 14, 4: 417-419.

Payne, J.R. and Driskell, W.B. 2005. The importance of distinguishing dissolved-Versus oil-droplet phases in assessing the fate, transport, and toxic effects of marine oil pollution, *International Oil Spill Conference 2005*.

Payne, P.E., Knobbe, L.N., Chanton, P., Zaugg, J., Mortazavi, B. and Mason, O.U. 2024. Uncovering novel functions of the enigmatic, abundant, and active Anaerolineae in a salt marsh ecosystem. *mSystems*. 01162-24.

Perez-Sepulveda, B.M., Heavens, D., Pulford, C.V., Predeus, A.V., Low, R., Webster, H., Dykes, G.F., Schudoma, C., Rowe, W., Lipscombe, J. and Watkins, C. 2021. An accessible, efficient and global approach for the large-scale sequencing of bacterial genomes. *Genome biology*. 22: 1-18.

Pham, V.D., Hnatow, L.L., Zhang, S., Fallon, R.D., Jackson, S.C., Tomb, J.F., DeLong, E.F. and Keeler, S.J. 2009. Characterizing microbial diversity in production water from an Alaskan mesothermic petroleum reservoir with two independent molecular methods. *Environmental microbiology*. 11, 1: 176-187.

Phan, H.V., Kurisu, F., Kiba, K. and Furumai, H. 2021. Optimized cultivation and syntrophic relationship of anaerobic benzene-degrading enrichment cultures under methanogenic conditions. *Microbes and environments*. 36, 3: 21028.

Pielou, E.C. 1966. The measurement of diversity in different types of biological collections. *Journal of theoretical biology*. 13: 131-144.

PMCC. 2024. Wytch Farm Oil Spill: Final Incident Monitoring and Outcomes Summary. https://www.phc.co.uk/impact-reports-published-following-oil-spill/

Polmear, R., Stark, J.S., Roberts, D. and McMinn, A. 2015. The effects of oil pollution on Antarctic benthic diatom communities over 5 years. *Marine pollution bulletin*. 90, 1-2: 33-40.

Prendergast, D.P. and Gschwend, P.M. 2014. Assessing the performance and cost of oil spill remediation technologies. *Journal of cleaner production*. 78: 233-242.

Prince, R. C. 2015a. Introduction: mesocosms and microcosms. *Hydrocarbon and Lipid Microbiology Protocols.* Springer.

Prince, R. C. 2015b. Oil spill dispersants: boon or bane? Environmental science & technology. 49: 6376-6384.

Prince, R. C. 2018. Eukaryotic Hydrocarbon Degraders. In: McGenity, T. J. (ed.) Taxonomy, genomics and ecophysiology of hydrocarbon-degrading microbes. Cham: Springer International Publishing.

Prince, R. C., Amande, T. J. and McGenity, T. J. 2019. Prokaryotic hydrocarbon degraders. Taxonomy, genomics and ecophysiology of hydrocarbon-degrading microbes: 1-39.

Prince, R. C., Butler, J. D., Bragin, G. E., Parkerton, T. F., Redman, A. D., Kelley, B. A. and Letinski, D. J. 2016a. Preparing the hydrocarbon/crude oil. *Hydrocarbon and Lipid Microbiology Protocols.* Springer.

Prince, R. C., Butler, J. D. and Redman, A. D. 2017. The rate of crude oil biodegradation in the sea. Environmental science & technology. 51: 1278-1284.

Prince, R.C. Coolbaugh, T.S. and Parkerton, T.F. 2016a. Oil dispersants do facilitate biodegradation of spilled oil. *Proceedings of the National Academy of Sciences*. 113: E1421-E1421.

Prince, R.C., Elmendorf, D.L., Lute, J.R., Hsu, C.S., Haith, C.E., Senius, J.D., Dechert, G.J., Douglas, G.S. and Butler, E.L. 1994. 17. alpha.(H)-21. beta.(H)-hopane as a conserved internal marker for estimating the biodegradation of crude oil. *Environmental science & technology*. 28, 1: 142-145.

Prince, R., Kelley, B. and Butler, J. 2016b. Three widely-available dispersants substantially increase the biodegradation of otherwise undispersed oil. *J. Marine Sci. Res. Dev.* 6: 183.

Prince, R. C., McFarlin, K. M., Butler, J. D., Febbo, E. J., Wang, F. C. and Nedwed, T. J. 2013. The primary biodegradation of dispersed crude oil in the sea. *Chemosphere*. 90: 521-526.

Prince, R. C., Nash, G. W. and Hill, S. J. 2016c. The biodegradation of crude oil in the deep ocean. *Marine pollution bulletin*: 111, 354-357.

Prince, R.C., Garrett, R.M., Bare, R.E., Grossman, M.J., Townsend, T., Suflita, J.M., Lee, K., Owens, E.H., Sergy, G.A., Braddock, J.F. and Lindstrom, J.E. 2003. The roles of photooxidation and biodegradation in long-term weathering of crude and heavy fuel oils. Spill Science & Technology Bulletin. 8, 2; 145-156.

Prince, R.C. and Lessard, R.R., 2004. Crude oil releases to the environment: Natural fate and remediation options. *Encyclopedia of Energy*, 1(1): 727-736.

Pumphrey, G.M. and Madsen, E.L. 2007. Naphthalene metabolism and growth inhibition by naphthalene in *Polaromonas naphthalenivorans* strain CJ2. *Microbiology*. 153: 3730-3738.

Qiao, N. and Shao, Z. 2010. Isolation and characterization of a novel biosurfactant produced by hydrocarbon-degrading bacterium Alcanivorax dieselolei B-5. *Journal of applied microbiology*. 108: 1207-1216.

Qian, L., Yu, X., Zhou, J., Gu, H., Ding, J., Peng, Y., He, Q., Tian, Y., Liu, J., Wang, S. and Wang, C. 2022. MCycDB: a curated database for comprehensively profiling methane cycling processes of environmental microbiomes. *Molecular Ecology Resources*. 22, 5: 1803-1823.

Quigg, A., Passow, U., Daly, K. L., Burd, A., Hollander, D. J., Schwing, P. T. and Lee, K. 2020. Marine oil snow sedimentation and flocculent accumulation (MOSSFA) events: learning from the past to predict the future. Deep oil spills. Springer.

Quince, C., Lanzen, A., Davenport, R.J. and Turnbaugh, P.J. 2011. Removing noise from pyrosequenced amplicons. *BMC bioinformatics*. 12: 1-18.

R Core Team. 2023. R: A language and environment for statistical computing. *R Foundation for Statistical Computing*, Vienna, Austria. URL https://www.R-project.org/.

Raghukumar, S.,2017. Fungi in coastal and oceanic marine ecosystems (Vol. 378). New York, NY, USA:: Springer.

Rath, J., Wu, K.Y., Herndl, G.J. and DeLong, E.F. 1998. High phylogenetic diversity in a marine-snow-associated bacterial assemblage. Aquatic Microbial Ecology. 14, 3: 261-269.

Rahsepar, S., Smit, M. P., Murk, A. J., Rijnaarts, H. H. and Langenhoff, A. A. 2016. Chemical dispersants: oil biodegradation friend or foe? *Marine pollution bulletin*: 108, 113-119.

Redfield, A.C. 1958. The biological control of chemical factors in the environment. *American Scientist*. 46, 3: 205–221.

Reid, M.A., Tibby, J.C., Penny, D. and Gell, P.A. 1995. The use of diatoms to assess past and present water quality. Australian Journal of Ecology. 20, 1: 57-64.

Reid, T., Droppo, I.G. and Weisener, C.G. 2020. Tracking functional bacterial biomarkers in response to a gradient of contaminant exposure within a river continuum. *Water Research*. 168: 115167.

Reis, M.A.M., Almeida, J.S., Lemos, P.C. and Carrondo, M.J.T. 1992. Effect of hydrogen sulfide on growth of sulfate reducing bacteria. *Biotechnology and bioengineering*. 40, 5: 593-600.

Ribeiro, H., de Sousa, T., Santos, J.P., Sousa, A.G., Teixeira, C., Monteiro, M.R., Salgado, P., Mucha, A.P., Almeida, C.M.R., Torgo, L. and Magalhães, C. 2018. Potential of dissimilatory nitrate reduction pathways in polycyclic aromatic hydrocarbon degradation. *Chemosphere*. 199: 54-67.

Rizzo, C., Malavenda, R., Gerçe, B., Papale, M., Syldatk, C., Hausmann, R., Bruni, V., Michaud, L., Giudice, A. Lo, Amalfitano, S. 2019. Effects of a simulated

acute oil spillage on bacterial communities from arctic and antarctic marine sediments. *Microorganisms*. 7: 12.

Rizzo, C., Michaud, L., Hörmann, B., Gerçe, B., Syldatk, C., Hausmann, R., De Domenico, E. and Giudice, A.L. 2013. Bacteria associated with sabellids (Polychaeta: Annelida) as a novel source of surface active compounds. *Marine pollution bulletin*. 70: 125-133.

Rognes, T., Flouri, T., Nichols, B., Quince, C. and Mahé, F. 2016. VSEARCH: a versatile open-source tool for metagenomics. *PeerJ*. 4, e2584.

Rohal, M., Barrera, N., Van Eenennaam, J. S., Foekema, E. M., Montagna, P. A., Murk, A. J., Pryor, M. and Romero, I. C. 2020. The effects of experimental oil-contaminated marine snow on meiofauna in a microcosm. *Marine pollution bulletin*. 150: 110656.

Rojas-Vargas, J., Castelán-Sánchez, H.G. and Pardo-López, L., 2023. HADEG: a curated hydrocarbon aerobic degradation enzymes and genes database. *Computational Biology and Chemistry*. 107: 107966.

Ron, E.Z. and Rosenberg, E. 2002. Biosurfactants and oil bioremediation. *Current opinion in biotechnology*. 13: 249-252.

Rossmassler, K., Snow, C.D., Taggart, D., Brown, C. and De Long, S.K. 2019. Advancing biomarkers for anaerobic o-xylene biodegradation via metagenomic analysis of a methanogenic consortium. *Applied microbiology and biotechnology*. 103: 4177-4192.

Rotmistrovsky, K. and Agarwala, R. 2011. BMTagger: Best Match Tagger for removing human reads from metagenomics datasets. *NCBI/NLM, National Institutes of Health*.

Rubin-Blum, M., Antony, C.P., Borowski, C., Sayavedra, L., Pape, T., Sahling, H., Bohrmann, G., Kleiner, M., Redmond, M.C., Valentine, D.L. and Dubilier, N. 2017. Short-chain alkanes fuel mussel and sponge Cycloclasticus symbionts from deep-sea gas and oil seeps. *Nature microbiology*. 2, 8: 1-11.

Rystad Energy. 2022. Offshore oil needs more shuttle tankers as volumes requiring transport set to rise by 35% this decade.

Santschi, P. H., Xu, C., Schwehr, K. A., Lin, P., Sun, L., Chin, W.-C., Kamalanathan, M., Bacosa, H. P. and Quigg, A. 2020. Can the protein/carbohydrate (P/C) ratio of exopolymeric substances (EPS) be used as a proxy for their 'stickiness' and aggregation propensity? *Marine Chemistry*. 218: 103734.

Sajid, S., Zhang, G., Zhang, Z., Chen, L., Lu, Y., Fang, J.K.H. and Cai, L. 2024. Comparative analysis of biofilm bacterial communities developed on different artificial reef materials. *Journal of Applied Microbiology*. 135, 11: 268.

Sayed, K., Baloo, L. and Sharma, N. K. 2021. Bioremediation of Total Petroleum Hydrocarbons (TPH) by bioaugmentation and biostimulation in water with floating oil spill containment booms as bioreactor basin. *International Journal of Environmental Research and Public Health.* 18: 2226.

Schedel, M. and Trüper, H.G. 1980. Anaerobic oxidation of thiosulfate and elemental sulfur in *Thiobacillus denitrificans*. *Archives of Microbiology*. 124: 205-210.

Schneiker, S., Dos Santos, V. A. M., Bartels, D., Bekel, T., Brecht, M., Buhrmester, J., Chernikova, T. N., Denaro, R., Ferrer, M. and Gertler, C. 2006. Genome sequence of the ubiquitous hydrocarbon-degrading marine bacterium *Alcanivorax borkumensis*. *Nature biotechnology*. 24: 997-1004.

Schindelin, J., Arganda-Carreras, I., Frise, E., Kaynig, V., Longair, M., Pietzsch, T., Preibisch, S., Rueden, C., Saalfeld, S., Schmid, B. and Tinevez, J.Y. 2012. Fiji:

an open-source platform for biological-image analysis. *Nature methods*. 9, 7: 676-682.

Schoener, T.W. 2009. Ecological niche. The Princeton guide to ecology. 3-13.

Schwarz, J., Walker, J. and Colwell, R. 1975. Deep-sea bacteria: growth and utilization of n-hexadecane at in situ temperature and pressure. *Canadian Journal of Microbiology*. 21: 682-687.

Schwing, P. T., Romero, I. C., Brooks, G. R., Hastings, D. W., Larson, R. A. and Hollander, D. J. 2015. A decline in benthic foraminifera following the Deepwater Horizon event in the northeastern Gulf of Mexico. *PloS one*. 10: e0120565.

Seemann, T. 2014. Prokka: rapid prokaryotic genome annotation. *Bioinformatics*. 30, 14: 2068-2069.

Seemann, T. 2015. Barrnap**:** Bacterial ribosomal RNA predictor. *GitHub repository*. Retrieved from https://github.com/tseemann/barrnap

Seeleuthner, Y., Mondy, S., Lombard, V., Carradec, Q., Pelletier, E., Wessner, M., Leconte, J., Mangot, J.F., Poulain, J., Labadie, K. and Logares, R. 2018. Single-cell genomics of multiple uncultured stramenopiles reveals underestimated functional diversity across oceans. *Nature Communications*. 9, 1: 310.

Seppey, M., Manni, M. and Zdobnov, E.M. 2020. LEMMI: a continuous benchmarking platform for metagenomics classifiers. *Genome research*. 30, 8: 1208-1216.

Setti, L., Lanzarini, G., Pifferi, P.G. and Spagna, G. 1993. Further research into the aerobic degradation of n-alkanes in a heavy oil by a pure culture of a *Pseudomonas sp*. *Chemosphere*. 26: 1151-1157.

Setti, L., Pifferi, P.G. and Lanzarini, G. 1995. Surface tension as a limiting factor for aerobic n-alkane biodegradation. *Journal of Chemical Technology & Biotechnology*. 64: 41-48.

Shankar, R., Shim, W. J., An, J. G. and Yim, U. H. 2015. A practical review on photooxidation of crude oil: Laboratory lamp setup and factors affecting it. *Water Research*. 68: 304-315.

Shanks, A.L. and Trent, J.D. 1979. Marine snow: microscale nutrient patches 1. *Limnology and Oceanography*. 24, 5: 850-854.

Shelton, R. 1971. Effects of oil and oil dispersants on the marine environment. Proceedings of the Royal Society of London. Series B. *Biological Sciences*. 177: 411-422.

Shen, W., Le, S., Li, Y. and Hu, F. 2016. SeqKit: a cross-platform and ultrafast toolkit for FASTA/Q file manipulation. *PloS one*. 11, 10: e0163962.

Sherry, A., Gray, N.D., Ditchfield, A.K., Aitken, C.M., Jones, D.M., Röling, W.F.M., Hallmann, C., Larter, S.R., Bowler, B.F.J. and Head, I.M. 2013. Anaerobic biodegradation of crude oil under sulphate-reducing conditions leads to only modest enrichment of recognized sulphate-reducing taxa. *International Biodeterioration & Biodegradation*. 81: 105-113.

Shigenaka, G. 2014. Twenty-Five Years After the Exxon Valdez Oil Spill: NOAA's Scientific Support, Monitoring, and Research. Twenty-Five Years After the Exxon Valdez Oil Spill: NOAA's Scientific Support, Monitoring, and Research.

Shin, W.S., Pardue, J.H. and Jackson, W.A. 2000. Oxygen demand and sulfate reduction in petroleum hydrocarbon contaminated salt marsh soils. *Water Research*. 34, 4: 1345-1353.

Silberberg, M. 1996. Chemistry: The Molecular Nature of Matter and Change. St. Louis, Missouri, USA: Mosby.

Silver, M. W., Shanks, A. L. and Trent, J. D. 1978. Marine snow: microplankton habitat and source of small-scale patchiness in pelagic populations. *Science.* 201: 371-373.

Simon, M., Grossart, H.-P., Schweitzer, B. and Ploug, H. 2002. Microbial ecology of organic aggregates in aquatic ecosystems. *Aquatic microbial ecology*. 28: 175-211.

Simon, H.Y., Siddle, K.J., Park, D.J. and Sabeti, P.C. 2019. Benchmarking metagenomics tools for taxonomic classification. *Cell*. 178, 4: 779-794.

Smallbone, J.A., McGenity, T.J., Holland, R.D., Thomas, G.E., Coulon, F., Cowley, T. and McKew, B.A. 2024. The effect of crude oil concentration on hydrocarbon degradation and associated bacterial communities under experimental conditions. In *International Oil Spill Conference Proceedings*. 2024: 1.

Southward, A. and Southward, E. C. 1978. Recolonization of rocky shores in Cornwall after use of toxic dispersants to clean up the Torrey Canyon spill. *Journal of the Fisheries Board of Canada*. 35: 682-706.

Speight, J. G. 2017. Environmental organic chemistry for engineers, Butterworth-Heinemann. 43-86.

Stauffert, M., Cravo-Laureau, C., Jezequel, R., Barantal, S., Cuny, P., Gilbert, F., Cagnon, C., Militon, C., Amouroux, D., Mahdaoui, F. and Bouyssiere, B. 2013. Impact of oil on bacterial community structure in bioturbated sediments. *PLoS One*. 8, 6: 65347.

Stefanidou, N., Genitsaris, S., Lopez-Bautista, J., Sommer, U. and Moustaka-Gouni, M. 2018. Unicellular eukaryotic community response to temperature and salinity variation in mesocosm experiments. *Frontiers in Microbiology.* 9: 2444.

Stout, S. A. and German, C. R. 2018. Characterization and flux of marine oil snow settling toward the seafloor in the northern Gulf of Mexico during the Deepwater Horizon incident: Evidence for input from surface oil and impact on shallow shelf sediments. *Marine pollution bulletin.* 129: 695-713.

Stout, S. A. and Payne, J. R. 2016. Macondo oil in deep-sea sediments: Part 1–sub-sea weathering of oil deposited on the seafloor. *Marine pollution bulletin.* 111: 365-380.

Strittmatter, A.W., Liesegang, H., Rabus, R., Decker, I., Amann, J., Andres, S., Henne, A., Fricke, W.F., Martinez-Arias, R., Bartels, D. and Goesmann, A. 2009. Genome sequence of Desulfobacterium autotrophicum HRM2, a marine sulfate reducer oxidizing organic carbon completely to carbon dioxide. *Environmental microbiology*. 11, 5: 1038-1055.

Strynar, M., Sen, D. and Weaver, R.W. 1999. Nitrogen and phosphorus for growth of oil-degrading microorganisms in seawater. *Bioremediation Journal*. 3: 85-91.

Suárez-Moo, P., Lamelas, A., Garcia-Bautista, I., Barahona-Pérez, L.F., Sandoval-Flores, G., Valdes-Lozano, D., Toledano-Thompson, T., Polanco-Lugo, E. and Valdez-Ojeda, R. 2020. Characterization of sediment microbial communities at two sites with low hydrocarbon pollution in the southeast Gulf of Mexico. *PeerJ*, 8: e10339.

Sublette, K.L. and Sylvester, N.D. 1987. Oxidation of hydrogen sulfide by Thiobacillus denitrificans: desulfurization of natural gas. *Biotechnology and Bioengineering*. 29, 2: 249-257.

Suja, L. D., Chen, X., Summers, S., Paterson, D. M. and Gutierrez, T. 2019. Chemical dispersant enhances microbial exopolymer (EPS) production and formation of

marine oil/dispersant snow in surface waters of the subarctic northeast Atlantic. *Frontiers in microbiology*. 10: 553.

Suja, L. D., Summers, S. and Gutierrez, T. 2017. Role of EPS, dispersant and nutrients on the microbial response and MOS formation in the subarctic northeast Atlantic. *Frontiers in microbiology*. 8: 676.

Sun, L., Chiu, M.-H., Xu, C., Lin, P., Schwehr, K. A., Bacosa, H., Kamalanathan, M., Quigg, A., Chin, W.-C. and Santschi, P. H. 2018. The effects of sunlight on the composition of exopolymeric substances and subsequent aggregate formation during oil spills. *Marine Chemistry*. 203: 49-54.

Sun, C., Fu, G.Y., Zhang, C.Y., Hu, J., Xu, L., Wang, R.J., Su, Y., Han, S.B., Yu, X.Y., Cheng, H. and Zhang, X.Q. 2016. Isolation and complete genome sequence of *Algibacter alginolytica* sp. nov., a novel seaweed-degrading Bacteroidetes bacterium with diverse putative polysaccharide utilization loci. *Applied and environmental microbiology*. 82, 10: 2975-2987.

Szava-Kovats, R.C. 2008. Grain-size normalization as a tool to assess contamination in marine sediments: Is the< 63 μm fraction fine enough?. *Marine pollution bulletin*. 56, 4: 629-632.

Takai, K., Miyazaki, M., Hirayama, H., Nakagawa, S., Querellou, J. and Godfroy, A. 2009. Isolation and physiological characterization of two novel, piezophilic, thermophilic chemolithoautotrophs from a deep-sea hydrothermal vent chimney. *Environmental Microbiology*. 11, 8: 1983-1997.

Taketani, R.G., Dos Santos, H.F., van Elsas, J.D. and Rosado, A.S. 2009. Characterisation of the effect of a simulated hydrocarbon spill on diazotrophs in mangrove sediment mesocosm. *Antonie van Leeuwenhoek*. 96: 343-354.

Takeuchi, M., Katayama, T., Yamagishi, T., Hanada, S., Tamaki, H., Kamagata, Y., Oshima, K., Hattori, M., Marumo, K., Nedachi, M. and Maeda, H. 2014. Methyloceanibacter caenitepidi gen. nov., sp. nov., a facultatively methylotrophic bacterium isolated from marine sediments near a hydrothermal vent. *International journal of systematic and evolutionary microbiology*. 64, 2: 462-468.

Takeuchi, M., Ozaki, H., Hiraoka, S., Kamagata, Y., Sakata, S., Yoshioka, H. and Iwasaki, W. 2019. Possible cross-feeding pathway of facultative methylotroph Methyloceanibacter caenitepidi Gela4 on methanotroph *Methylocaldum marinum S8*. *PloS one.* 14, 3: 0213535.

Tansel, B. 2018. Morphology, composition and aggregation mechanisms of soft bioflocs in marine snow and activated sludge: A comparative review. *Journal of Environmental Management*. 205: 231-243.

Techtmann, S. M., Zhuang, M., Campo, P., Holder, E., Elk, M., Hazen, T. C., Conmy, R. and Santo Domingo, J. W. 2017. Corexit 9500 enhances oil biodegradation and changes active bacterial community structure of oil-enriched microcosms. *Applied and Environmental Microbiology*. 83: e03462-16.

Thingstad, T. F. and Rassoulzadegan, F. 1995. Nutrient limitations, microbial food webs and biological C-pumps: suggested interactions in a P-limited Mediterranean. *Marine Ecology Progress Series*. 299-306.

Thomas, G. E., Brant, J. L., Campo, P., Clark, D. R., Coulon, F., Gregson, B. H., McGenity, T. J. and McKew, B. A. 2021. Effects of dispersants and biosurfactants on crude-oil biodegradation and bacterial community succession. *Microorganisms*, 9: 1200.

Thomas, G. E., McGenity, T. J., Zeinstra-Helfrich, M. and McKew, B. A. A Comparison between Chemical and Natural Dispersion of a North Sea Oil-spill. *International Oil Spill Conference*. 2021: 7252525.

Thomas, G.E., Cameron, T.C., Campo, P., Clark, D.R., Coulon, F., Gregson, B.H., Hepburn, L.J., McGenity, T.J., Miliou, A., Whitby, C., McKew, B.A. 2020. Bacterial Community Legacy Effects Following the Agia Zoni II Oil-Spill, Greece. *Frontiers in Microbiology*. 11.

Tian, H., Gao, P., Chen, Z., Li, Y., Li, Y., Wang, Y., Zhou, J., Li, G. and Ma, T. 2017. Compositions and abundances of sulfate-reducing and sulfur-oxidizing microorganisms in water-flooded petroleum reservoirs with different temperatures in China. *Frontiers in Microbiology*. 8: 143.

Tiessen, H. 2008. Phosphorus in the global environment. In The ecophysiology of plant-phosphorus interactions. *Dordrecht: Springer Netherlands*. 1-7.

Timmis, K. N., McGenity, T., Van Der Meer, J. R. and de Lorenzo, V. 2010. Handbook of hydrocarbon and lipid microbiology, Springer Berlin.

Turner, J. T. 2015. Zooplankton fecal pellets, marine snow, phytodetritus and the ocean's biological pump. *Progress in Oceanography.* 130: 205-248.

Tu, Q., Lin, L., Cheng, L., Deng, Y. and He, Z. 2019. NCycDB: a curated integrative database for fast and accurate metagenomic profiling of nitrogen cycling genes. *Bioinformatics*. 35, 6: 1040-1048.

Tremblay, J., Yergeau, E., Fortin, N., Cobanli, S., Elias, M., King, T. L., Lee, K. and Greer, C. W. 2017. Chemical dispersants enhance the activity of oil-and gas condensate-degrading marine bacteria. *The ISME journal*. 11: 2793-2808.

Underhill-Day, J.C. 2006. A condition assessment of Poole Harbour European Marine Site. *Natural England/Footprint Ecology*.

Uritskiy, G.V., DiRuggiero, J. and Taylor, J. 2018. MetaWRAP—a flexible pipeline for genome-resolved metagenomic data analysis. Microbiome. 6: 1-13.

U.S. Energy Information Administration. 2019. The Strait of Hormuz is the world's most important oil transit chokepoint.

U.S. Energy Information Administration. 2025. Short-Term Energy Outlook (STEO).

Valentine, D.L., Fisher, G.B., Bagby, S.C., Nelson, R.K., Reddy, C.M., Sylva, S.P. and Woo, M.A. 2014. Fallout plume of submerged oil from Deepwater Horizon. *Proceedings of the National Academy of Sciences*. *111*, 45; 15906-15911.

Uzoh, C.V., Ifeanyi, V., Okwuwe, C., Oranusi, S.U., Braide, W., Iheukwumere, I.H., Anyanwuocha, C.E. and Ntamzor, B.G. 2015. Effect of light on the biodegradation of crude oil by the algae Closterium species. *J Nat Sci Res*. 5, 22: 112-118.

Van Beilen, J. B., Li, Z., Duetz, W. A., Smits, T. H. and Witholt, B. 2003. Diversity of alkane hydroxylase systems in the environment. *Oil & gas science and technology*. 58: 427-440.

Van Eenennaam, J.S., Wei, Y., Grolle, K.C., Foekema, E.M. and Murk, A.J. 2016. Oil spill dispersants induce formation of marine snow by phytoplankton-associated bacteria. *Marine Pollution Bulletin*. 104, 2: 294-302.

Van Eenennaam, J. S., Rahsepar, S., Radović, J. R., Oldenburg, T. B., Wonink, J., Langenhoff, A. A., Murk, A. J. and Foekema, E. M. 2018. Marine snow increases the adverse effects of oil on benthic invertebrates. *Marine pollution bulletin*. 126: 339-348.

Vandecandelaere, I., Nercessian, O., Segaert, E., Achouak, W., Mollica, A., Faimali, M., De Vos, P. and Vandamme, P. 2008. *Alteromonas genovensis* sp. nov., isolated from a marine electroactive biofilm and emended description of *Alteromonas macleodii* Baumann et al. 1972 (Approved Lists 1980). *International journal of systematic and evolutionary microbiology*. 58, 11: 2589-2596.

Vekeman, B., Kerckhof, F.M., Cremers, G., De Vos, P., Vandamme, P., Boon, N., Op den Camp, H.J. and Heylen, K. 2016. New Methyloceanibacter diversity from North Sea sediments includes methanotroph containing solely the soluble methane monooxygenase. Environmental microbiology. 18, 12: 4523-4536.

Venables, W. N. and Ripley, B. D. 2002. Modern Applied Statistics with S. 4th edn. New York: Springer. ISBN 0-387-95457-0.

Venosa, A. D. and Zhu, X. 2003. Biodegradation of crude oil contaminating marine shorelines and freshwater wetlands. *Spill Science & Technology Bulletin.* 8: 163-178.

Verdugo, P. 2012. Marine microgels. Annual Review of Marine Science. 4: 375-400.

Vergeynst, L., Wegeberg, S., Aamand, J., Lassen, P., Gosewinkel, U., Fritt-Rasmussen, J., Gustavson, K. and Mosbech, A. 2018. Biodegradation of marine oil spills in the Arctic with a Greenland perspective. *Science of the Total Environment*. 626: 1243-1258.

Vonk, S.M., Hollander, D.J. and Murk, A.J. 2015. Was the extreme and wide-spread marine oil-snow sedimentation and flocculent accumulation (MOSSFA) event during the Deepwater Horizon blow-out unique?. *Marine Pollution Bulletin*. 100, 1: 5-12.

Voss, M., Bange, H. W., Dippner, J. W., Middelburg, J. J., Montoya, J. P. and Ward, B. 2013. The marine nitrogen cycle: recent discoveries, uncertainties and the potential relevance of climate change. Philosophical Transactions of the Royal Society B: *Biological Sciences*. 368: 20130121.

Wagner, K., Besemer, K., Burns, N.R., Battin, T.J. and Bengtsson, M.M. 2015. Light availability affects stream biofilm bacterial community composition and function, but not diversity. *Environmental Microbiology*. 17, 12: 5036-5047.

Wang, Q., Garrity, G.M., Tiedje, J.M. and Cole, J.R. 2007. Naive Bayesian classifier for rapid assignment of rRNA sequences into the new bacterial taxonomy. *Applied and environmental microbiology*. 73: 5261-5267.

Wang, W. and Shao, Z. 2012. Diversity of flavin-binding monooxygenase genes (almA) in marine bacteria capable of degradation long-chain alkanes. FEMS microbiology ecology, 80, 523-533.

Wang, W. and Shao, Z. 2014. The long-chain alkane metabolism network of Alcanivorax dieselolei. *Nature communications.* 5: 1-11.

Wang, Y., Yu, M., Austin, B. and Zhang, X.-H. 2012. *Oleispira lenta* sp. nov., a novel marine bacterium isolated from Yellow sea coastal seawater in Qingdao, China. *Antonie van Leeuwenhoek*. 101: 787-794.

Wang, W., Wang, L. and Shao, Z. 2018. Polycyclic aromatic hydrocarbon (PAH) degradation pathways of the obligate marine PAH degrader Cycloclasticus sp. strain P1. *Applied and Environmental Microbiology*. 84: e01261-18.

Wang, Z., Fingas, M., Shu, Y.Y., Sigouin, L., Landriault, M., Lambert, P., Turpin, R., Campagna, P. and Mullin, J. 1999. Quantitative characterization of PAHs in burn residue and soot samples and differentiation of pyrogenic PAHs from

petrogenic PAHs− The 1994 mobile burn study. *Environmental science & technology*. 33, 18: 3100-3109.

Wang, Y., Wegener, G., Ruff, S.E. and Wang, F. 2021. Methyl/alkyl-coenzyme M reductase-based anaerobic alkane oxidation in archaea. *Environmental Microbiology*. 23, 2: 530-541.

Ward, C. P. and Overton, E. B. 2020. How the 2010 Deepwater Horizon spill reshaped our understanding of crude oil photochemical weathering at sea: a past, present, and future perspective. *Environmental Science: Processes & Impacts*. 22: 1125-1138.

Warkhade, Y., Schaerer, L.G., Bigcraft, I., Hazen, T.C. and Techtmann, S.M. 2025. Diversity and Distribution of Hydrocarbon-Degrading Genes in the Cold Seeps from the Mediterranean and Caspian Seas. *Microorganisms*. 13, 2: 222.

Wei, T.T., He, S. and Quan, Z.X., 2022. Thalassolituus alkanivorans sp. nov., a hydrocarbon-utilizing bacterium isolated from the Mariana Trench. *International Journal of Systematic and Evolutionary Microbiology*. 72, 5: 005404.

Whitman, W. B., Rainey, F., Kämpfer, P., Trujillo, M., Chun, J., DeVos, P., Hedlund, B., Dedysh, S. and Nedashkovskaya, O. 2016. Bergey's manual of systematics of Archaea and Bacteria.

Whyte, L. G., Hawari, J., Zhou, E., Bourbonnière, L., Inniss, W. E. and Greer, C. W. 1998. Biodegradation of variable-chain-length alkanes at low temperatures by a psychrotrophic *Rhodococcus* sp. *Applied and Environmental Microbiology*. 64: 2578-2584.

Wilpiszeski, R.L., Sherwood Lollar, B., Warr, O. and House, C.H. 2020. In situ growth of halophilic bacteria in saline fracture fluids from 2.4 km below surface in the deep Canadian Shield. *Life.* 10, 12: 307.

Wirth, M. A., Passow, U., Jeschek, J., Hand, I. and Schulz-Bull, D. E. 2018. Partitioning of oil compounds into marine oil snow: insights into prevailing mechanisms and dispersant effects. *Marine Chemistry.* 206: 62-73.

Wood, D.E., Lu, J. and Langmead, B. 2019. Improved metagenomic analysis with Kraken 2. *Genome biology*, *20*: 1-13

Wood, S. N. 2017. Generalized Additive Models: An Introduction with R. 2nd edn. New York: Chapman & Hall/CRC. doi: 10.1201/9781315370279.

Woodhead, R.J., Law, R.J. and Matthiessen, P. 1999. Polycyclic aromatic hydrocarbons in surface sediments around England and Wales, and their possible biological significance. *Marine Pollution Bulletin*. 38, 9: 773-790.

Wozniak, A.S., Prem, P.M., Obeid, W., Waggoner, D.C., Quigg, A., Xu, C., Santschi, P.H., Schwehr, K.A. and Hatcher, P.G. 2019. Rapid degradation of oil in mesocosm simulations of marine oil snow events. *Environmental science & technology*. 53, 7: 3441-3450.

Wu, M., Li, J., Lai, C.Y., Leu, A.O., Sun, S., Gu, R., Erler, D.V., Liu, L., Li, L., Tyson, G.W. and Yuan, Z. 2024. Nitrate-driven anaerobic oxidation of ethane and butane by bacteria. *The ISME Journal*. 18, 1: 011.

Wu, T., Xu, J., Xie, W., Yao, Z., Yang, H., Sun, C. and Li, X., 2018. *Pseudomonas aeruginosa* L10: a hydrocarbon-degrading, biosurfactant-producing, and plant-growth-promoting endophytic bacterium isolated from a reed (*Phragmites australis*). *Frontiers in microbiology*. 9: 1087.

Xiao, Y.K., Yan, Z.F., Kim, Y., Lee, H.M., Trinh, H., Yang, J.E., Won, K.H., Yi, T.H. and Kook, M. 2019. *Glaciecola amylolytica* sp. nov., an amylase-producing

bacterium isolated from seawater. *International Journal of Systematic and Evolutionary Microbiology*. 69: 957-963.

Xue, C.X., Lin, H., Zhu, X.Y., Liu, J., Zhang, Y., Rowley, G., Todd, J.D., Li, M. and Zhang, X.H. 2021. DiTing: a pipeline to infer and compare biogeochemical pathways from metagenomic and metatranscriptomic data. *Frontiers in microbiology*. 12: 698286.

Xu, X., Shi, R., Lv, C., Liu, H., Yang, W., Qian, S., Fujibayashi, M., Zhi, Y., Wang, G., Nomura, M. and Nishimura, O. 2021. Hydrodynamic-Driven Changes in the Source and Composition of Sedimentary Organic Matter via Grain Size Distribution in Shallow Lakes. *Journal of Geophysical Research: Biogeosciences.* 126, 11: e2021JG006502.

Xu, X., Liu, W., Tian, S., Wang, W., Qi, Q., Jiang, P., Gao, X., Li, F., Li, H. and Yu, H. 2018. Petroleum hydrocarbon-degrading bacteria for the remediation of oil pollution under aerobic conditions: a perspective analysis. *Frontiers in microbiology*. 9: 2885.

Yaghi, B. M. and Al-Bemani, A. 2002. Heavy crude oil viscosity reduction for pipeline transportation. *Energy sources.* 24: 93-102.

Yakimov, M. M., Bargiela, R. and Golyshin, P. N. 2022. Calm and Frenzy: marine obligate hydrocarbonoclastic bacteria sustain ocean wellness. *Current opinion in biotechnology*. 73: 337-345.

Yakimov, M. M., Denaro, R., Genovese, M., Cappello, S., D'Auria, G., Chernikova, T. N., Timmis, K. N., Golyshin, P. N., & Giluliano, L. 2005. Natural microbial diversity in superficial sediments of Milazzo Harbor (Sicily) and community successions during microcosm enrichment with various hydrocarbons. *Environmental Microbiology*. 7: 9.

Yakimov, M. M., Giuliano, L., Denaro, R., Crisafi, E., Chernikova, T. N., Abraham, W.-R., Luensdorf, H., Timmis, K. N. and Golyshin, P. N. 2004. *Thalassolituus oleivorans* gen. nov., sp. nov., a novel marine bacterium that obligately utilizes hydrocarbons. *International journal of systematic and evolutionary microbiology*. 54: 141-148.

Yakimov, M. M., Giuliano, L., Gentile, G., Crisafi, E., Chernikova, T. N., Abraham, W.-R., Lünsdorf, H., Timmis, K. N. and Golyshin, P. N. 2003. *Oleispira antarctica* gen. nov., sp. nov., a novel hydrocarbonoclastic marine bacterium isolated from Antarctic coastal sea water. *International journal of systematic and evolutionary microbiology*. 53: 779-785.

Yakimov, M. M., Golyshin, P. N., Lang, S., Moore, E. R., Abraham, W.-R., Lünsdorf, H. and Timmis, K. N. 1998. *Alcanivorax borkumensis* gen. nov., sp. nov., a new, hydrocarbon-degrading and surfactant-producing marine bacterium. *International journal of systematic and evolutionary microbiology*. 48: 339-348.

Yakimov, M. M., Timmis, K. N. and Golyshin, P. N. 2007. Obligate oil-degrading marine bacteria. *Current opinion in biotechnology.* 18: 257-266.

Yang, J., Li, G., Qian, Y. and Zhang, F. 2018. Increased soil methane emissions and methanogenesis in oil contaminated areas. *Land Degradation & Development*. 29, 3: 563-571.

Yu, X., Zhou, J., Song, W., Xu, M., He, Q., Peng, Y., Tian, Y., Wang, C., Shu, L., Wang, S. and Yan, Q. 2021. SCycDB: a curated functional gene database for metagenomic profiling of sulphur cycling pathways. *Molecular Ecology Resources*. 21: 3, 924-940.

Yu, S., Li, S., Tang, Y. and Wu, X. 2011. Succession of bacterial community along with the removal of heavy crude oil pollutants by multiple biostimulation

treatments in the Yellow River Delta, China. *Journal of Environmental Sciences*. 23: 1533-1543.

Zahed, M. A., Aziz, H. A., Isa, M. H. and Mohajeri, L. 2010. Effect of initial oil concentration and dispersant on crude oil biodegradation in contaminated seawater. *Bulletin of environmental contamination and toxicology*. 84: 438-442.

Zehr, J.P. and Capone, D.G. 2020. Changing perspectives in marine nitrogen fixation. *Science*. 368, 6492: 9514.

Zhang, B., Matchinski, E.J., Chen, B., Ye, X., Jing, L. and Lee, K. 2019a. Marine Oil Spills—Oil Pollution, Sources and Effects. *World Seas: An Environmental Evaluation Volume III: Ecological Issues and Environmental Impacts*. 391–406.

Zhang, S., Hu, Z. and Wang, H. 2019b. Metagenomic analysis exhibited the co-metabolism of polycyclic aromatic hydrocarbons by bacterial community from estuarine sediment. *Environment international.* 129: 308-319.

Zhang, S., Wu, W., Zheng, Q. and Wang, M. 2018. Effect of dispersants on bio-degradation of marine spilled oil. *Journal of Coastal Research*. 364-368.

Zhang, S., Xu, C. and Santschi, P.H. 2008. Chemical composition and 234Th (IV) binding of extracellular polymeric substances (EPS) produced by the marine diatom Amphora sp. *Marine Chemistry*. 112, 1-2: 81-92.

Zhao, X., Li, J., Zhang, D., Jiang, L., Wang, Y., Hu, B., Wang, S., Dai, Y., Luo, C. and Zhang, G. 2023. Unveiling the novel role of ryegrass rhizospheric metabolites in benzo [a] pyrene biodegradation. *Environment International*. 180: 108215.

Zhao, Z., Liu, S., Jiang, S., Zhang, D. and Sha, Z. 2024. Diversity and Potential Metabolic Characteristics of Culturable Copiotrophic Bacteria That Can Grow on Low-Nutrient Medium in Zhenbei Seamount in the South China Sea. *Microbial Ecology*. 87, 1: 157.

Zhi-Hui, Y.A.N.G., Stöven, K., Haneklaus, S., Singh, B.R. and Schnug, E. 2010. Elemental sulfur oxidation by Thiobacillus spp. and aerobic heterotrophic sulfur-oxidizing bacteria. *Pedosphere*. 20, 1: 71-79.

Zhuang, L., Liu, Y., Wang, L., Wang, W. and Shao, Z. 2015. Erythrobacter atlanticus sp. nov., a bacterium from ocean sediment able to degrade polycyclic aromatic hydrocarbons. *International Journal of Systematic and Evolutionary Microbiology*. 65, 10: 3714-3719.

Ziervogel, K., McKay, L., Rhodes, B., Osburn, C. L., Dickson-Brown, J., Arnosti, C. and Teske, A. 2012. Microbial activities and dissolved organic matter dynamics in oil-contaminated surface seawater from the Deepwater Horizon oil spill site. *PloS one.* 7: e34816.

Appendix

Appendix 1: The effect of crude oil concentration on hydrocarbon degradation and associated bacterial communities under experimental conditions

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The effect of crude oil concentration on hydrocarbon degradation and associated bacterial communities under experimental conditions

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ABSTRACT

Chemical dispersants are an important tool for mitigating the environmental impact of marine oil pollution. Crude oil concentration used in experiments to study the efficacy of dispersants has been shown to impact oil biodegradation rates, which may explain contradictory conclusions between studies. Using high crude oil concentration could be particularly problematic when extrapolating laboratory results to the field, as dispersed oil in the open sea would rapidly dilute to sub-ppm concentrations. This study aimed to quantify the impact of crude oil concentrations ranging from 1 to 1000 mg L-1 on biodegradation and microbial community composition in dispersed versus non-dispersed crude oil, Oil biodegradation rates increased linearly from 1 to 100 mg L-1 but plateaued at a concentration of 1000 mg L-1. However, with the addition of chemical dispersant, degradation rates were linear across the full range of concentrations, and significantly greater than non-dispersed treatments at 1 mg L-1 (81% increase), 10 mg L-1 (80%), 100 mg L-1 (64%) and 1000 mg L-1 (388%). Based on 16S rRNA gene sequence analysis, Alteromonadaceae and Cellulophaga were more dominant at lower oil concentrations, potentially being inhibited or outcompeted at higher concentrations. Use of dispersant resulted in more rapid changes in community composition. These results highlight the need to consider oil concentration when performing biodegradation experiments and will inform the Net Environment Benefit Analysis (NEBA).

INTRODUCTION

With the rise in transport and production of crude oil, remediation tools to combat marine oil pollution are still needed. Chemical dispersants are valuable for oil-spill response as they disperse oil slicks into small droplets in the water column, enhancing biodegradation (Prince et al.,2013; Mu et al., 2014), and often prevent oil from reaching sensitive shorelines (Chapman et al., 2007; Prince, 2015). However, the efficacy of dispersants has come into question (Kleindienst et al., 2015a; Kleindienst et al., 2016; Prince et al., 2016), and previous studies have documented contrasting results, either showing significant increases in oil biodegradation (Baelum et al., 2012; Techtmann et al., 2017; Thomas et al., 2021) or inhibition of biodegradation when chemically dispersed (Hamdan and Fulmer, 2011; Kleindienst et al., 2015b). These contradictory outcomes are potentially explained by differences in experimental design, notably the oil and/or nutrient concentrations (Techtmann et al., 2017; McFarlin and Prince, 2021). The use of water accommodated fractions (WAF) and chemically enhanced water accommodated fractions (CEWAF) in experiments (Kleindienst et al., 2015b), may also be unsuitable for biodegradation studies, as they remove most oil droplets and insoluble hydrocarbons, which are a major fraction of dispersed oil that can be biodegraded by microbes (McFarlin and Prince, 2021). Therefore, adding crude oil directly to seawater microcosms, then dispersing with chemical dispersant at the industry standard oil:dispersant ratio (20:1), and directly comparing to non-dispersed floating oil, is a more valid comparison for extrapolation to open water spill scenarios.

Oil concentration also has a significant impact on the biodegradation rates of hydrocarbons and can significantly increase the half-life of various hydrocarbons at higher concentrations, particularly as nutrient limitation becomes more likely, coupled with reduced bioavailability as a consequence of oil forming larger droplets, adhering to the container, or floating to the surface (Prince et al, 2017, McFarlin and Prince, 2021). When oil is spilt and chemically dispersed at sea, its concentration can rapidly decrease to approximately 100 mg L- 1, with further dilution

occurring over 24 hours to approximately 1-10 mg L-1 (Lee et al., 2013). Therefore, studies using high concentrations (e.g. 200–2500 mg L-1), may be far less relevant to field scenarios. The present study compared biodegradation of crude oil with and without chemical dispersants over a range of oil concentrations (1, 10, 100 and 1000 mg L-1) to test the effects that oil concentration has on both the biodegradation of hydrocarbons and the composition of bacterial communities over time. It was hypothesised that the presence of dispersants would increase the rate of biodegradation, that biodegradation in oil concentrations over 100 mg L-1 would be inhibited both with and without dispersant use, and that the growth of hydrocarbon-degrading bacteria would be enhanced with the use of dispersant.

EXPERIMENTAL DESIGN

Microcosm Design: Surface seawater was sampled from the North Sea (7oC, 51.7397° N, 1.0221° E on March 3, 2022). Microcosms were created in sterile 40 ml glass vials with PTFE-lined silicon septa, containing 20 ml of seawater (naturally containing 46 µmol L-1 nitrate and 1 µmol L-1 phosphate), and were sampled over a time series of 0, 1, 3, 6 and 14 days (n = 3). Norwegian Geochemical Standard North Sea Oil (NGS NSO-1, previously weathered at 60oC over 24 hours) was added at concentrations of 1, 10, 100 or 1000 mg L-1 (0.0001% w/v to 0.1% w/v) with or without Slickgone NS dispersant (DASIC International Ltd) at a 20:1 oil:dispersant ratio. Killed controls were set up to determine any abiotic loss of hydrocarbons. Microcosms were incubated in the dark at 12oC on an orbital shaker at 100 rpm.

Hydrocarbon Degradation Analysis: Hydrocarbons were extracted from the microcosms using previously described methods (Coulon et al., 2007). Deuterated alkanes and polyaromatic hydrocarbon (PAH) internal standards were added to the solvent prior to extraction at 0.004, 0.04, 0.4 and 4 µg mL-1, for extraction of the 1, 10, 100 and 1000 mg L-1 oil, respectively. Extractions of 1000 mg L-1 oil were diluted 10-fold in hexane:DCM 1:1, whilst 1 and 10 mg L-1 samples were concentrated 100 and 10-fold respectively in a TurboVap evaporator (Biotage), standardising

all extracts to 100 mg L-1 equivalents with final deuterated standard concentrations of 0.4 µg mL-1. Quantification of hydrocarbons (Alkanes C10-38, Pristane and phytane, and a suite of two-six ring PAHs) was performed using a Nexis GC- 2030/CMS-TQ8040 NX GC-QqQ-MS/MS (Shimadzu). External standard calibrations were produced with Alkane (C8-C40) and PAH (QTM PAH Mix) (Sigma) standard mixes over a 1– 5 µg mL-1 range.

DNA Extraction and qPCR: Microcosms were filtered via 0.22 µm Sterivex filters (Millipore) and DNA extracted using a DNeasy PowerWater Kit (Qiagen), following the manufacturers' instructions. Filtrate was reserved for nutrient analysis. qPCR of the bacterial 16S rRNA gene was performed with primers 341F (CCTACGGGNGGCWGCAG) and 805R (GACTACHVGGGTATCTAATCC) (Klindworth et al., 2013) on a CFX Opus 384 (Bio-Rad) as previously described (McKew and Smith, 2017).

Nutrient Analysis: NH4+, NO3- and PO43- concentrations were measured as previously described by Thomas et al., (2021).

16S rRNA Gene Amplicon Library Preparation and Bioinformatics: Amplicon library preparation for sequencing was performed as previously described (Thomas et al., 2021), and sequenced using NovaSeq PE250 strategy at Novogene (Cambridge UK). Sequencing data was processed using the Divisive Amplicon Denoising Algorithm (DADA2) pipeline (Callahan et al., 2022). Singleton and doubleton ASVs were discarded, along with sequences below 400 base pairs, containing homopolymers above 8 bp, or assigned to non- target organisms. Taxonomic assignment was conducted using the RDP Classifier (Callahan, 2020; Wang et al., 2007) and BlastN (Altschul et al., 1990). The bacterial 16S rRNA amplicon library size averaged 114,120 reads.

Statistical Analysis: Prior to diversity and community composition analysis of ASVs, sequence data was rarefied to the lowest read depth (3010). Data was tested for normality (Shapiro-Wilk test) and tested for significance using ANOVAs (normally distributed) or generalised linear

models (non-normally distributed).

RESULTS

Total measured alkanes (C10-C38, pristane and phytane) in non-dispersed oil at 1000 mg L-1 did not significantly decrease in concentration over 14 days, whereas total Alkanes in non-dispersed oil at 1, 10 and 100 mg L-1 did significantly decrease (25-30% degradation; $t = 7.3- 8.1$, $p < 0.05$) (Figure 1). Chemical dispersion significantly enhanced alkane biodegradation at all concentrations of oil (24-71% degradation; $t = 7.0\text{-}18$, $p < 0.05$), over the 14 days, with significantly lower concentrations observed in all dispersed microcosms compared to non-dispersed microcosms ($t = 2.8$, $p < 0.05$). This was especially noticeable at 1 and 10 mg L-1, where alkanes were significantly more degraded by 41% and 17% respectfully ($t = 3.2\text{-}6.0$, $p < 0.05$). A similar pattern was observed with PAHs, whereby at day 14, a significant decrease in PAH concentration was seen within microcosms treated with non-dispersed oil at concentrations of 1, 10 and 100 mg L-1 (31-59% degradation; $t = 3.0\text{-}11.3$, $p < 0.053$) (Figure 1) but not at 1000 mg L-1 (13% degradation, p = ns). The addition of dispersant resulted in a significant reduction in PAH concentration across all oil concentrations (46-70% degradation; $t = 5.5\text{-}10.2$, $p < 0.05$) and by day 14, PAH concentrations were significantly lower within dispersed oil at 1, 100 and 1000 mg L-1 when compared to non-dispersed microcosms ($t = 2.6- 4.5$, $p < 0.05$; 18-33% further degradation). In dispersed microcosms at 1 mg L-1 oil, a significant reduction in PAH concentration was seen as early as day 1 when compared to non- dispersed microcosms ($t = 2.69$, $p < 0.05$). A significantly greater percentage degradation of smaller n-Alkanes (C11-C20) occurred within oil concentrations of 1, 10 and 100 mg L-1 (dispersed and non-dispersed) when compared to 1000 mg L-1 (Figure 2). The branched alkanes pristane and phytane were significantly more degraded in percentage terms in oil treatments of 1 mg L-1 (dispersed and non-dispersed) when compared to treatments of 1000 mg L-1. Even larger n-alkanes in the size range, C35-C38, were significantly degraded at an oil concentration of 1 mg L-1 (dispersed and non-dispersed) in

contrast to higher concentrations where no significant degradation occurred. A targeted analysis of certain PAH analytes was also caried out to compare degradation across a range of different structures and sizes of PAHs.

Naphthalene, fluorene, phenanthrene, anthracene and pyrene were significantly more degraded in percentage terms at 1 mg L-1 oil, compared to 1000 mg L-1 (dispersed and non-dispersed) (Figure 2). In oil concentrations of 10 mg L-1, naphthalene, fluorene, phenanthrene and pyrene all showed significantly more percentage biodegradation when compared to concentrations of 100 mg L-1, and naphthalenes and fluorenes had significantly more percentage degradation when compared to 1000 mg L-1. The percentage degradation of larger PAHs (4-rings and above) was much lower across all oil concentrations, however at 1 mg L-1 these larger PAH compounds were significantly more degraded when compared to 1000 mg L-1. Whilst experiments with higher oil concentrations may show minimal hydrocarbon biodegradation in percentage terms, the actual bulk hydrocarbon content degraded, and rates of hydrocarbon degradation may of course be significantly higher. With an increase in concentration, the degradation rates of dispersed oil increased linearly from 1 – 1000 mg L-1, in contrast to non-dispersed oil where degradation rates were only linear from 1 to 100 mg L- 1, and then plateaued at 1000 mg L-1 (Figure 3). A similar response was also observed with PAH degradation rates.

There was no significant increase in the abundance of the bacterial 16S rRNA gene in treatments with or without dispersant between day 0 and 14 ($p = 0.07$; data not shown). By day 7, there was significant dissimilarity in the community composition between dispersed and non-dispersed microcosms ($F = 2.68$, $p = 0.023$). However by day 14, no difference was observed and there was no significant dissimilarity in communities associated with different oil concentrations by day 14 (Figure 4).

Colwellia was the most abundant genus in the seawater (approximately 75% of all reads),

the relative abundance significantly declined by day 1 in all oiled treatments except at 1000 mg L-1 in the non-dispersed microcosms ($F = 24.575$, $p < 0.001$) and seawater controls until day 7 (Figure 5). By day 14, Pacificibacter, Aliiroseovarius and Roseovarius became dominant (approximately 24%, 11% and 9% of the community), significantly increasing in abundance across all treatments ($F = 21.8$-32.4, $p < 0.001$). The relative abundance of 16S rRNA genes from known obligate hydrocarbonoclastic bacteria (a group of marine bacteria often associated with oil pollution that are highly specialist hydrocarbon degraders) (Yakimov et al 2007) varied between replicates, and generally small increases were seen in non-dispersed oil treatments compared with dispersed oil. Alcanivorax remained at low relative abundance, increasing (non-significantly) by day 14 in the 10 and 100 mg L-1 oil treatments ($F = 0.62$-0.8 p = ns) (Figure 6). Cycloclasticus significantly increased in relative abundance over time in non-dispersed treatments of 100 mg L-1 concentrations ($Z = 3.3$, $p < 0.001$), from undetectable to 4.9% relative abundance. Thalassolituus increased in relative abundance by day 14 in 100 and 1000 mg L-1 concentrations ($Z = 0.001$-$.9$, p = ns). Oleibacter (sequences assigned to Oleibacter in this study, represent ASV sequences highly similar to Oleibacter marinus strains, that were recently transferred to the genus Thalassolitus under the new name T. maritimus (Dong et al. 2022)) increased in relative abundance by day 14 in treatments of 100 mg L-1 concentration, at 11.9% of the community ($Z = 3.95$, $p < 0.001$).

At day 7, Alteromonadaceae significantly increased in relative abundance in dispersed oil at 1 and 10 mg L-1 and dispersant only treatments when compared to 100 and 1000 mg L-1 ($z = 2.5$-3, $p < 0.05$) (Figure 7) and by day 14 this family was significantly more abundant in all dispersed oil concentrations ($z = 2.68$-5.01, $p < 0.05$). Unassigned Oceanospirillaceae significantly increased in relative abundance at day 14 ($z = 7.56$, $p < 0.05$) and when oil was dispersed, a significant increase in abundance was observed at oil concentrations of 1, 10 and 100 mg L-1 ($z = 19.5$-25.2, $p < 0.05$), with no increase in oil concentrations of 1000 mg L-1 and seawater controls.

Cellulophaga had significantly higher relative abundance by day 7 and 14 in microcosm treated with dispersants at 1 and 10 mg L-1 oil ($z = 3.6\text{-}4.0$, $p < 0.05$) (Figure 7). Oceanicola significantly increased across all oil treatments ($p < 0.05$) and particularly with dispersant addition at 1, 10 and 100 mg L-1 oil ($z = 2.7\text{-}3.1$, $p < 0.05$). Sulfitobacter increased over time across all treatments, however this was not significant.

DISCUSSION AND CONCLUSION

Initial oil concentration had a clear impact on the proportion of oil degraded over 14 days, with a higher percentage of measured alkanes and PAHs being degraded in the lower concentrations of 1 and 10 mg L-1 compared to 100 and 1000 mg L-1. However, when considering the mass of hydrocarbons degraded over time, the degradation rates of both alkanes and PAHs increased linearly from concentrations of 1 to 100 mg L-1, in all treatments. There was clear inhibition of the rate of both alkane and PAH degradation at 1000 mg L-1 when oil was not dispersed, but the rate increases remained linear when dispersant was added. Observations made by Prince et al. (2017) at concentrations 2.5, 25, 250 and 2500 mg L-1 indicated a similar inhibition of degradation rates at the concentrations of 2500 mg L-1, suggesting that oil may inhibit degradation rates at concentrations above 1000 mg L-1, or even lower when oil is not dispersed. Therefore, oil concentration must be accounted for when using closed-system degradation experiments, as measurements in open water slicks and dispersed oil plumes would typically be well below these concentrations (Lee et al., 2013). The addition of dispersants had an effect at the earliest sampling point of day 1, increasing the biodegradation of alkanes in initial oil concentrations of 1 and 10 mg L-1, and PAHs in treatments of 1 mg L-1 in comparison to the non-dispersed floating oil. By day 14, the addition of dispersant had a positive effect on biodegradation in oil treatments 100 and 1000 mg L-1 highlighting the benefits to bacteria when oil is in small droplets with an increased surface area to volume ratio caused by the addition of dispersants and biosurfactants that are produced by hydrocarbon degrading bacteria (McGenity et al., 2012;

Godfrin et al., 2018; McGenity and Laissue, 2023). Prior studies using concentrations of 100-1000 mg L-1 found similar results (Baelum et al., 2012, Tremblay et al., 2017, Thomas et al., 2021). Although biodegradation of dispersed oil at concentrations of 1000 mg L-1 showed a delayed response compared to lower concentrations, degradation was still significantly enhanced compared to that of non-dispersed oil. This enhancement with dispersant use has also been observed at higher concentrations of 2000 mg L-1, (Zahed et al., 2010). However percentage loss of hydrocarbons between dispersed and non-dispersed oil was lower than that of the present study, being 7% (day 45), whereas by day 14 during this study, hydrocarbon loss increased by 41% and 17% when dispersed at 1 and 10 mg L-1, respectively. This may have been due to the higher starting nitrate concentration in the present study. Biodegradation was enhanced by dispersant use to a greater extent at lower oil concentrations of 1 mg L-1 than previously observed (Baelum et al., 2012).

Bacterial communities need nutrients such as nitrogen (N) and phosphorous (P) to support growth, being fundamental components of biomolecules such as proteins and nucleic acids. Compared to terrestrial environments, open water marine systems are typically N and P limited, especially during a spill event, where the high carbon content of hydrocarbons, significantly raises the C:N:P ratio , and it is well documented that biodegradation of oil is severely inhibited by inorganic nutrient limitations (Vergeynst et al., 2018; Imron et al., 2020). During this study, initial nutrient concentrations were 50 μM nitrate and 1 μM phosphate (which were the main sources of inorganic N and P in the seawater), and microcosms rapidly became P-limited by day 1 and N-limited by day 14. By day 3 ammonium had become the main inorganic N source, likely released via decomposing organic matter (e.g. from dead algae and bacteria within the dark-incubated microcosms). This rapidly depleted by day 14. Previously, exponential growth of microbial communities was observed in oil contaminated seawater at concentrations of 30 μM (N) and 1.8 μM (P) and above, whilst growth became limited at 5.5 μM (N) and 0.13 μM (P) (Strynar et al.,

1999). Here, bacterial abundance was consistent over 14 days. This aligns with previous findings as nutrient concentrations were not high enough to support exponential growth, whilst the community composition changed dramatically (Strynar et al., 1999). Significant degradation of hydrocarbons occurred across all treatments by day 7 and only ceased once the microcosms became deficient in inorganic N and P. During this study, even at high concentrations of oil (100, 1000 mg L-1) with the addition of dispersants, nutrient concentrations as low as <5 μM (N) and <0.1 μM (P) were capable of facilitating hydrocarbon biodegradation.

Percentage degradation decreased with an increase in chain length in n-alkanes C10-18 at an oil concentration of 1 mg L-1, in which n-C10-12 were almost completely degraded (98%, 95% and 88% degradation) clearly showing chain length to have an impact on degradation rates (Setti et al., 1995). This percentage degradation of n-C10-18 decreased with an increase in oil concentration. There was very little degradation of n-C35-38 regardless of the initial oil concentration. It has been well documented that the structural property of individual hydrocarbon constituents, determines the degree and rate of degradation, showing an inverse relationship between degradability and molecular weight, with higher complexity and solubility reducing degradability (Setti et al., 1993; Mohanty and Mukherji, 2008). The reduction in degradability of higher weight hydrocarbons may coincide with the limiting nutrient resources. Lower molecular weight n-alkanes (n-C11-13) were significantly more prone to biodegradation than larger n-alkanes and with limiting nutrient resources hydrocarbon- degrading bacteria cannot ‘move up’ the chain lengths, (as seen at 1 mg L-1) as they run out of N and P resource (Ławniczak et al., 2020). This becomes a greater issue in the higher concentrations as C:N and C:P increases 10-fold with each increase in oil concentration. Therefore, at higher oil concentrations, biodegradation is largely limited to a few of the smaller hydrocarbons as there is a greater mass of the more readily degradable fractions. This was also observed in PAHs, with naphthalene being considerably more degraded regardless of oil concentrations, whilst larger PAHs showed little degradation. Similar

results were observed by Bacosa et al. (2021) who saw biodegradation of individual alkanes decrease with an increased oil concentration in a nutrient limited environment N < 1 μM and P < 0.6 μM by day 12.

Colwellia dominated communities, averaging 79% across all treatments. These generally psychrophilic or psychrotolerant bacteria are ubiquitous in cold marine environments, between -6oC and 19oC (Bauvois et al., 2013). Some Colwellia species are capable of hydrocarbon degradation, (Methé et al., 2005), although Colwellia psychrerythraea (26% of Colwellia ASVs) is currently not reported to be a hydrocarbon degrading species (Mason et al., 2014). The initial dominance of Colwellia spp may be due to the initially nutrient rich, cold waters (7oC). By day 14, Pacificibacter, Roseovarius and Aliiroseovarius became three dominating genera, coinciding with a decrease in nutrient availability. This may explain the distinct similarities between crude oil treatments and seawater controls, with nutrient availability having the most noticeable impact on community composition (Nõlvak et al., 2021). OHCBs Alcanivorax, Cycloclasticus, Oleibacter and Thalassolituus did not show exponential growth even in the presence of hydrocarbons. Even so, OHCB's were detectable at higher abundances in non – dispersed treatments, notably due to their capacity to produce biosurfactants, increasing hydrocarbon availability naturally (Ron and Rosenberg, 2002). This is in contrast to previous work and real-world spills that saw significant growth in OHCB's (Dubinsky et al., 2013; Cappello et al., 2007; Thomas et al., 2020). However, additional nutrient amendments were added/available in the aforementioned studies influencing microbial growth/succession via biostimulation (Yu et al., 2011). Other experiments however, found Pseudoalteromonas spp to significantly increase in abundance in North Sea mesocosms when oiled, which may have dominated over OHCBs (Chronopoulou et al.,2015). During the present study, Alteromonadaceae was dominant at day 7 (average 15%), which was quickly succeeded by Oceanospirillaceae, by day 14 (average 7%). These two families have been reported to exponential increase in abundance, in the presence of marine oil pollution, namely during the

Deepwater Horizon spill (Dubinsky et al., 2013; Bacosa et al., 2018; Miller et al., 2019). The majority of Alteromonadaceae (ASV 1) (98%), was made up of Glaciecola amylolytica (99.06% identity) (Xiao et al., 2019). Another Glaciecola Strain (NS 168) (93.91% identity to ASV 1) has been recorded to be capable of hydrocarbon degradation with a preference to alkane degradation (Chronopoulou et al., 2015). However, current knowledge of Glaciecola amylolytica has yet to address the specifics of their degradation potential. The majority of unassigned Oceanospirillaceae (99%), was made up of Thalassolituus ASVs (95.6-99.7% identity), suggesting their involvement in aliphatic hydrocarbon degradation between day 7 and 14. Cellulophaga increased in abundance at day 7 in oil concentrations of 1 and 10 mg L- 1 when dispersed. Previously shown to be present around contaminated sites they are prolific producers of biosurfactants, thus facilitating the availability of hydrocarbons within contaminated communities (Chhatre et al., 1996; Rizzo et al., 2013).

Overall different oil concentrations (1, 10, 100 and 1000 mg L-1) and the addition of dispersants did not have a major impact to bacterial community composition. The subtle differences in communities are likely related to a combination of factors, such as the susceptibility of certain bacteria to increasing hydrocarbon concentrations, nutrient availability stalling successional changes at higher concentrations, and the changing profile of available hydrocarbons. Similar results were noted in Jung et al. (2010), in which impacts to heterotrophic bacterial communities were only noted at concentrations over 1000 mg L-1 (sublethal levels). This suggests that initial oil concentrations introduced to a system will not have a major impact to bacterial communities below “sublethal levels”. Even so, inhibitory effects were noted in Alteromonadaceae and Cellulophaga at day 7 and Oceanospirillaceae at day 14, which exhibited high relative abundances at concentrations of 1 and 10 mg L-1 and no noticeable presence at concentrations of 1000 mg L-1 when dispersed. It has previously been shown that the production of intermediate metabolic products during biodegradation in high concentrations can potentially

inhibit the growth of hydrocarbon degraders such as Alcanivorax and Polaromonas naphthalenivorans (Pumphrey and Madsen, 2007; Bacosa et al., 2021) with the possibility of inhibiting other genera. Alteromonadaceae and Oceanospirillaceae increased in the presence of dispersants. Alteromonadaceae being relatively more abundant at day 7 at concentrations of 1 and 10 mg L-1, whereas Oceanospirillaceae increased in relative abundance at concentrations 1, 10 and 100 mg L-1 by day 14. Previous work has also noted similar patterns in both Alteromonadaceae and Oceanospirillaceae in relative abundance with dispersant use by day 15 (Bacosa et al., 2018). This may also indicate a delayed response in bacterial succession associated with initial oil concentrations, as by day 14, the relative abundance of Alteromonadaceae at concentrations of 100 and 1000 mg L-1 increased, whilst in contrast they had already increased in abundance at concentrations of 1 and 10 mg L-1 by day 7. Microbial community succession has been shown to be influenced by dispersants, as availability of oil increases, the rate of biodegradation increases, thus the rate of succession increases (Baelum et al., 2012; Thomas et al., 2021). However, initial concentrations of oil within a system may also influence succession due to potential inhibitory effects of higher hydrocarbon concentrations (Bacosa et al., 2021).

Higher oil concentrations were shown to inhibit the growth of potential hydrocarbon degrading bacteria, which slowed bacterial succession. This in turn inhibited biodegradation at higher concentrations of oil and the the efficiency of dispersant use on the enhancement of hydrocarbon degradation. Even so significant degradation occurred across all oil concentrations. This study also highlights that significant degradation is still possible at in situ open water nutrient concentrations, even at higher oil concentrations with the addition of dispersants, although this does become limiting as the system becomes N and P deficient. This is noted as a limitation of this and many closed system studies. Overall initial oil concentrations should be considered when performing closed system biodegradation experiments. Concentrations of 10 mg L-1 and lower are recommended to accurately depict concentrations found in situ when oil is dispersed. Future

experiments should consider simulating dilution of oil over time to more closely mimic what happens to dispersed oil at sea, where the concentration would continue to dilute over time, with each oil droplet, a hotspot of bacterial hydrocarbon-degrading activity, being exposed to further nutrient enrichment as they move through the water column.

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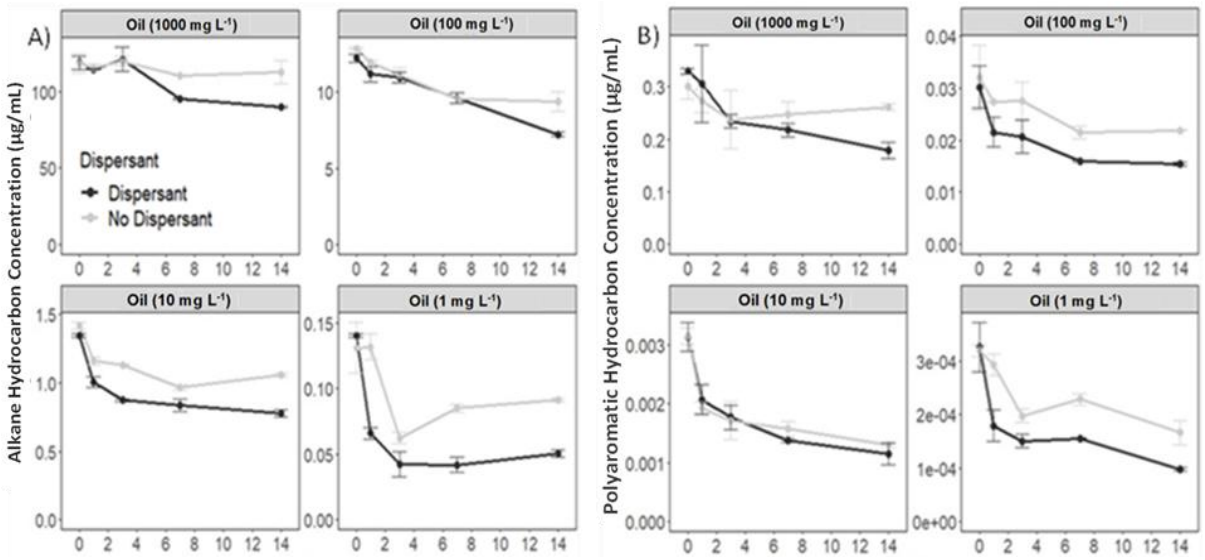

Figure 1 (A) Total alkane and (B) polyaromatic hydrocarbon concentration collected from 20 ml microcosms of North Sea seawater collected of the Mersea Coast, Essex, UK, exposed to Norwegian Geochemical Standard North Sea Oil at a range of concentrations (1 – 1000 mg L^{-1}) (NGS NSO-1), with and without the presence of chemical dispersants (Slickgone NS), (± SE, n = 3).

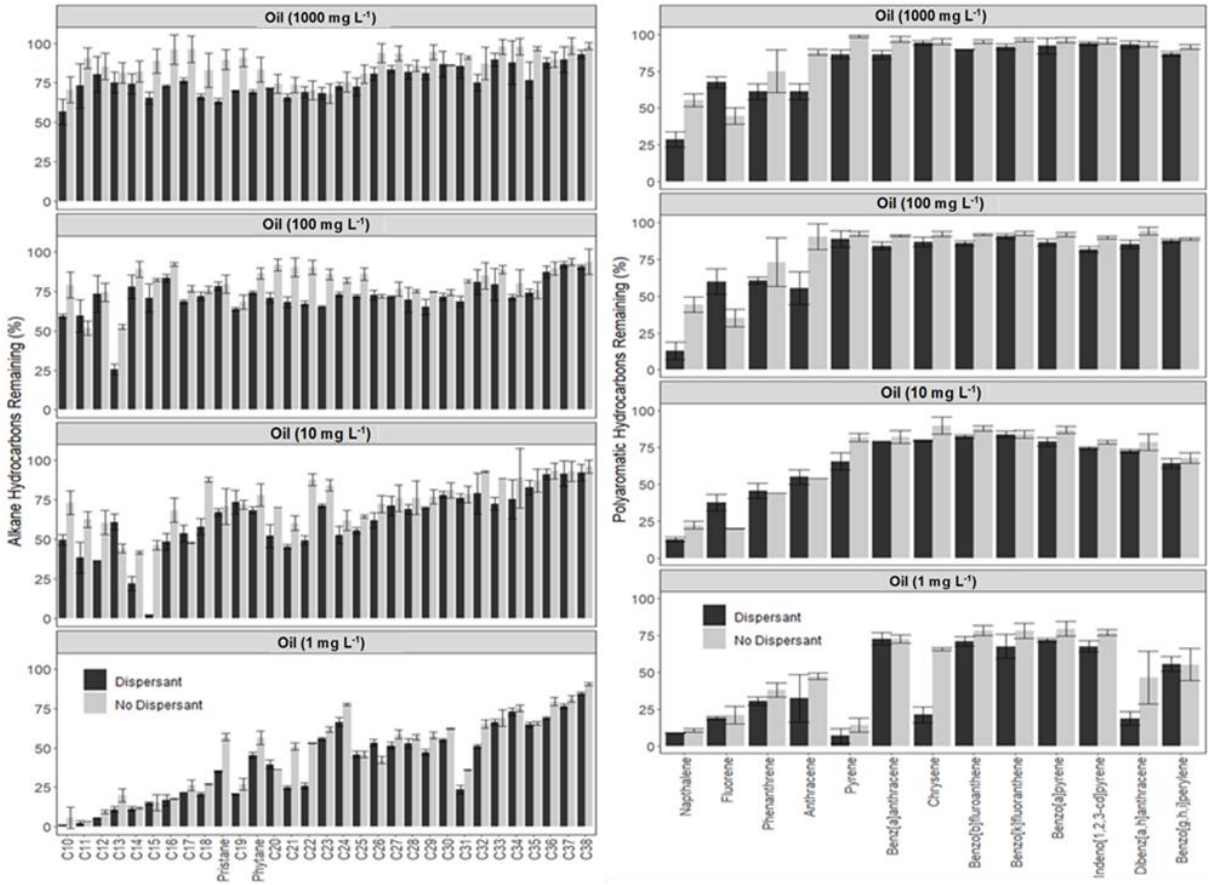

Figure 2: Percentage remaining alkane hydrocarbon and polyaromatic hydrocarbon compounds within 20 ml North Sea seawater microcosms, with and without chemical dispersants (Slickgone NS) at varying Norwegian Geochemical Standard North Sea Oil (NGS NSO-1) concentrations (1– 1000 mg L^{-1}) by day 14 (± SE, n = 3).

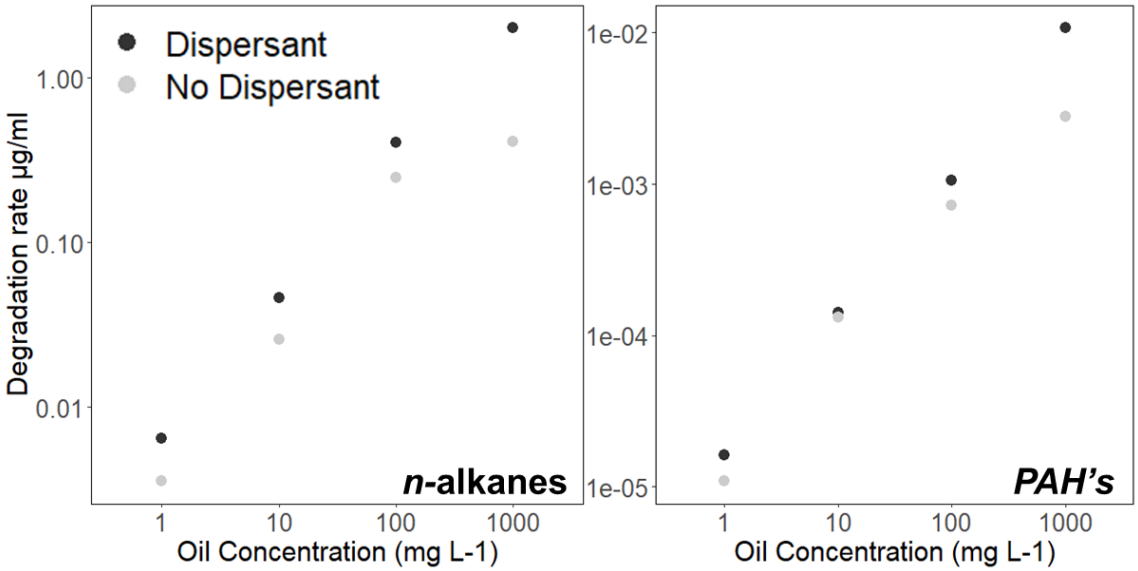

Figure 3: Alkane and polyaromatic hydrocarbon degradation rates in natural North Sea Seawater over 14 days across a range of Norwegian Geochemical Standard North Sea Oil (NGS NSO-1) concentrations (1 – 1000 mg L^{-1}) with and without chemical dispersant (Slickgone NS) treatment (± SE, n = 3).

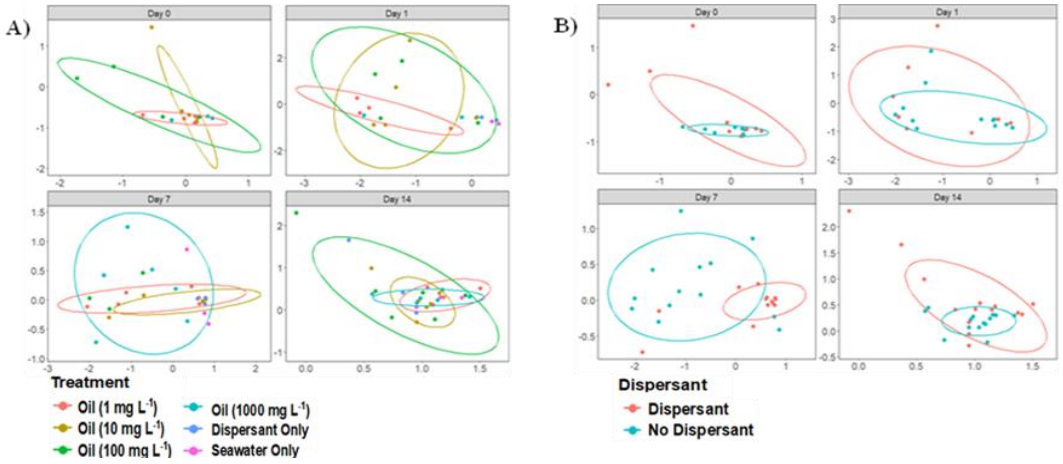

Figure 4 nMDS (non-metric multidimensional scaling) ordination using Bray-Curtis dissimilarity, based on ASV (amplicon sequence variants) of bacterial 16S rRNA genes at varying concentrations of Norwegian Geochemical Standard North Sea Oil (1 - 1000 mg L^{-1}) with and without dispersants (Slickgone NS) over a period of 14 days, within microcosms using seawater samples collected off the coast of Mersea Island, Essex, UK. A) Community dissimilarity between treatments, B) Community dissimilarity between dispersant use (± SE, n = 3).

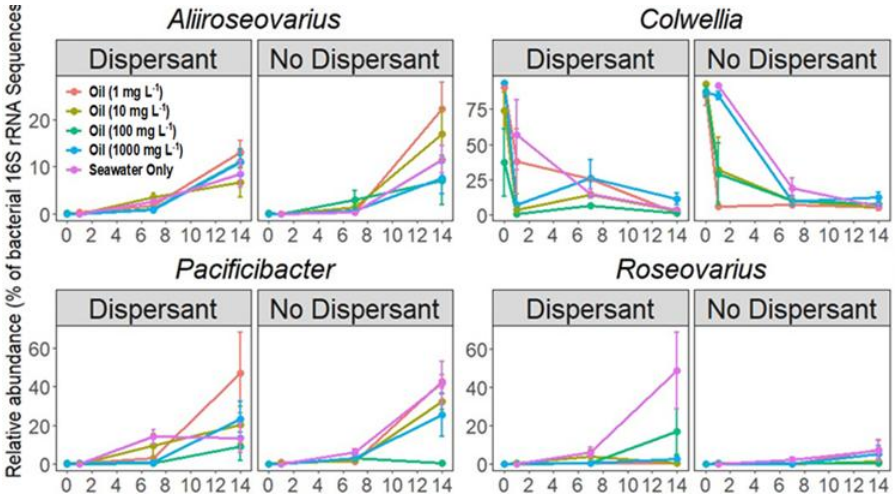

Figure 5 Highest relative abundance genera associated with marine oil pollution at varying concentrations of Norwegian Geochemical Standard North Sea Oil (1 – 1000 mg L^{-1}), with and without chemical dispersant (Slickgone NS). Collected over a 14-day period from microcosms containing natural seawater collected off the Mersea Coast, Essex, UK (± SE, n = 3).

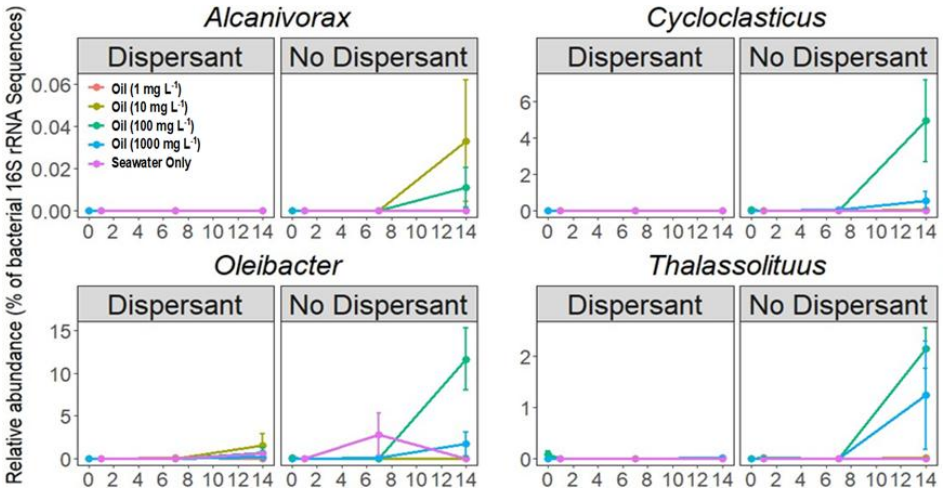

Figure 6 Relative abundance of genera that are known to include hydrocarbon degraders after the addition to seawater of Norwegian Geochemical Standard North Sea Oil (1 – 1000 mg L^{-1}), with and without the presence of chemical dispersant (Slickgone NS). Collected over a 14-day period from microcosms containing natural seawater collected off the Mersea Coast, Essex, UK (± SE, n = 3).

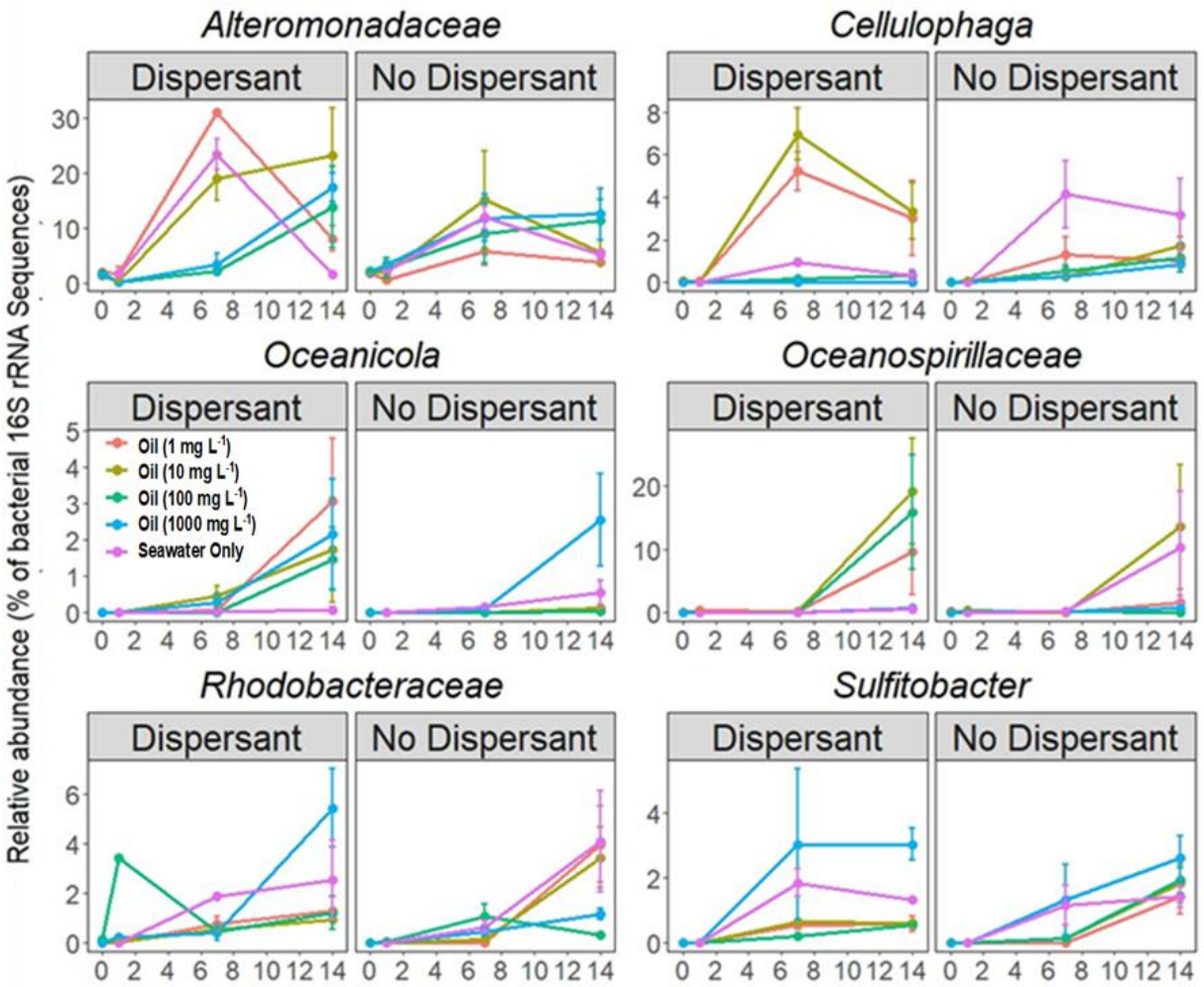

Figure 7 Relative abundance of other notable genera at varying oil concentrations of Norwegian Geochemical Standard North Sea Oil (1 – 1000 mg L^{-1}), with and without the presence of chemical dispersant (Slickgone NS). Collected over a 14-day period from microcosms containing natural seawater collected off the Mersea Coast, Essex, UK (± SE, n = 3).

REFERENCES

Altschul, S.F., Gish, W., Miller, W., Myers, E.W. and Lipman, D.J. 1990. Basic local alignment search tool. *Journal of molecular biology*, 215, 3: 403-410.

Bacosa, H.P., Erdner, D.L., Rosenheim, B.E., Shetty, P., Seitz, K.W., Baker, B.J. and Liu, Z. 2018. Hydrocarbon degradation and response of seafloor sediment bacterial community in the northern Gulf of Mexico to light Louisiana sweet crude oil. *The ISME journal*, 12, 10: 2532-2543.

Bacosa, H.P., Kang, A., Lu, K. and Liu, Z. 2021. Initial oil concentration affects hydrocarbon biodegradation rates and bacterial community composition in seawater. *Marine Pollution Bulletin*, 162: 111867.

Bauvois, C., Huston, A. and Feller, G. 2013. The Cold-Active M1 Aminopeptidase from the Arctic Bacterium Colwellia psychrerythraea.

Callahan, B. 2020. RDP taxonomic training data formatted for DADA2 (RDP trainset 18/release 11.5). https://doi.org/10.5281/zenodo.4310151

Callahan, B., McMurdie, P., Holmes, S., Biostrings, I., BiocStyle, S., Rcpp, L. and biocViews Microbiome, S. 2022. Package 'dada2'. Accurate, high-resolution sample inference from amplicon sequencing data.

Cappello, S., Denaro, R., Genovese, M., Giuliano, L. and Yakimov, M.M. 2007. Predominant growth of Alcanivorax during experiments on "oil spill bioremediation" in mesocosms. *Microbiological research*, 162: 185-190.

Chapman, H., Purnell, K., Law, R. J. and Kirby, M. F. 2007. The use of chemical dispersants to combat oil spills at sea: A review of practice and research needs in Europe. Marine pollution bulletin, 54: 827-838.

Chhatre, S., Purohit, H., Shanker, R. and Khanna, P. 1996. Bacterial consortia for crude oil spill remediation. *Water Science and Technology*, 34; 187-193.

Chronopoulou, P.M., Sanni, G.O., Silas-Olu, D.I., van der Meer, J.R., Timmis, K.N., Brussaard, C.P. and McGenity, T.J. 2015. Generalist hydrocarbon-degrading bacterial communities in the oil-polluted water column of the North Sea. *Microbial biotechnology*, 8: 434-447.

Coulon, F., McKew, B. A., Osborn, A. M., McGenity, T. J., and Timmis, K. N. 2007. Effects of temperature and biostimulation on oil-degrading microbial communities in temperate estuarine waters. *Environmental Microbiology*, 9: 177-186.

Dong, C., Wei, L., Wang, J., Lai, Q., Huang, Z. and Shao, Z. 2022. Genome-based taxonomic rearrangement of Oceanobacter-related bacteria including the description of Thalassolituus hydrocarbonoclasticus sp. nov. and Thalassolituus pacificus sp. nov. and emended description of the genus Thalassolituus. *Frontiers in Microbiology*, 13: 1051202.

Dubinsky, E.A., Conrad, M.E., Chakraborty, R., Bill, M., Borglin, S.E., Hollibaugh, J.T., Mason, O.U., M. Piceno, Y., Reid, F.C., Stringfellow, W.T. and Tom, L.M. 2013. Succession of hydrocarbon-degrading bacteria in the aftermath of the Deepwater Horizon oil spill in the Gulf of Mexico. *Environmental science & technology*, 47: 10860-10867.

Godfrin, M.P., Sihlabela, M., Bose, A. and Tripathi, A. 2018. Behavior of marine bacteria in clean environment and oil spill conditions. *Langmuir*, 34: 9047-9053.

Hamdan, L. J. and Fulmer, P. A. 2011. Effects of COREXIT® EC9500A on bacteria from a beach oiled by the Deepwater Horizon spill. *Aquatic microbial ecology*, 63: 101-109.

Imron, M.F., Kurniawan, S.B., Ismail, N.I. and Abdullah, S.R.S. 2020. Future challenges in diesel biodegradation by bacteria isolates: a review. *Journal of Cleaner Production*, 251: 119716.

Jung, S.W., Park, J.S., Kown, O.Y., Kang, J.H., Shim, W.J. and Kim, Y.O. 2010. Effects of crude oil on marine microbial communities in short term outdoor microcosms. *The Journal of Microbiology*, 48: 594-600.

Kleindienst, S., Paul, J. H. and Joye, S. B. 2015a. Using dispersants after oil spills: impacts on the composition and activity of microbial communities. *Nature Reviews Microbiology*, 13: 388-396.

Kleindienst, S., Seidel, M., Ziervogel, K., Grim, S., Loftis, K., Harrison, S., Malkin, S. Y., Perkins, M. J., Field, J. and Sogin, M. L. 2015b. Chemical dispersants can suppress the activity of natural oil-degrading microorganisms. *Proceedings of the National Academy of Sciences*, 112: 14900-14905.

Kleindienst, S., Seidel, M., Ziervogel, K., Grim, S., Loftis, K., Harrison, S., Malkin, S.Y., Perkins, M.J., Field, J., Sogin, M.L. and Dittmar, T. 2016. Reply to Prince *et al.*: Ability of chemical dispersants to reduce oil spill impacts remains unclear. *Proceedings of the National Academy of Sciences*, 113: E1422-E1423.

Klindworth, A., Pruesse, E., Schweer, T., Peplies, J., Quast, C., Horn, M. and Glöckner, F.O. 2013. Evaluation of general 16S ribosomal RNA gene PCR primers for classical and next-generation sequencing-based diversity studies. Nucleic acids research, 4: e1-e1.

Ławniczak, Ł., Woźniak-Karczewska, M., Loibner, A.P., Heipieper, H.J. and Chrzanowski, Ł, 2020. Microbial degradation of hydrocarbons—basic principles for bioremediation: a review. *Molecules*, *25*: 856.

Lee, K., Nedwed, T., Prince, R. C. and Palandro, D. 2013. Lab tests on the biodegradation of chemically dispersed oil should consider the rapid dilution that occurs at sea. *Marine Pollution Bulletin*, 73: 314-318.

Mason, O.U., Han, J., Woyke, T. and Jansson, J.K. 2014. Single-cell genomics reveals features of a Colwellia species that was dominant during the Deepwater Horizon oil spill. *Frontiers in microbiology*, *5*: 332.

Methé, B.A., Nelson, K.E., Deming, J.W., Momen, B., Melamud, E., Zhang, X., Moult, J., Madupu, R., Nelson, W.C., Dodson, R.J. and Brinkac, L.M. 2005. The psychrophilic lifestyle as revealed by the genome sequence of *Colwellia psychrerythraea* 34H through genomic and proteomic analyses. *Proceedings of the National Academy of Sciences*, 102, 31: 10913-10918.

McFarlin, K. M. and Prince, R. C. 2021. Contradictory conclusions surrounding the effects of chemical dispersants on oil biodegradation. *International Oil Spill Conference Proceedings*: 2021: 685852. doi: https://doi.org/10.7901/2169-3358-2021.1.685852

McGenity, T.J., Folwell, B.D., McKew, B.A. and Sanni, G.O., 2012. Marine crude-oil biodegradation: a central role for interspecies interactions. *Aquatic biosystems*, 8: 1-19.

McGenity, T.J. and Laissue, P.P. 2023. Bacteria stretch and bend oil to feed their appetite. *Science*, 381: 728-729.

McKew, B.A. and Smith, C.J. 2017. Real-time PCR approaches for analysis of hydrocarbon-degrading bacterial communities. Hydrocarbon and Lipid Microbiology Protocols: Microbial Quantitation, Community Profiling and Array Approaches, 45-64.

Miller, J.I., Techtmann, S., Fortney, J., Mahmoudi, N., Joyner, D., Liu, J., Olesen, S., Alm, E., Fernandez, A., Gardinali, P. and GaraJayeva, N. 2019. Oil hydrocarbon degradation by Caspian Sea microbial communities. *Frontiers in microbiology*, 10: 995.

Mohanty, G. and Mukherji, S. 2008. Biodegradation rate of diesel range n-alkanes by bacterial cultures *Exiguobacterium aurantiacum* and *Burkholderia cepacia*. *International Biodeterioration & Biodegradation*, 61: 240-250.

Mu, J., Jin, F., Ma, X., Lin, Z. and Wang, J. 2014. Comparative effects of biological and chemical dispersants on the bioavailability and toxicity of crude oil to early life stages of marine medaka (*Oryzias melastigma*). *Environmental toxicology and chemistry*, 33: 2576-2583.

Nõlvak, H., Dang, N.P., Truu, M., Peeb, A., Tiirik, K., O'Sadnick, M. and Truu, J. 2021. Microbial community dynamics during biodegradation of crude oil and its response to biostimulation in Svalbard seawater at low temperature. *Microorganisms*, 9: 2425.

Prince, R. C. 2015. Oil spill dispersants: boon or bane? *Environmental science & technology,* 49: 6376-6384.

Prince, R. C., Butler, J. D. and Redman, A. D. 2017. The rate of crude oil biodegradation in the sea. *Environmental science & technology*, 51: 1278-1284.

Prince, R.C. Coolbaugh, T.S. and Parkerton, T.F. 2016. Oil dispersants do facilitate biodegradation of spilled oil. *Proceedings of the National Academy of Sciences*, 113: E1421-E1421.

Prince, R. C., McFarlin, K. M., Butler, J. D., Febbo, E. J., Wang, F. C. and Nedwed, T. J. 2013 The primary biodegradation of dispersed crude oil in the sea. *Chemosphere,* 90: 521- 526.

Pumphrey, G.M. and Madsen, E.L. 2007. Naphthalene metabolism and growth inhibition by naphthalene in *Polaromonas naphthalenivorans* strain CJ2. *Microbiology*, 153: 3730- 3738

Rizzo, C., Michaud, L., Hörmann, B., Gerçe, B., Syldatk, C., Hausmann, R., De Domenico, E. and Giudice, A.L. 2013. Bacteria associated with sabellids (Polychaeta: Annelida) as a novel source of surface active compounds. *Marine pollution bulletin*, *70*: 125-133.

Ron, E.Z. and Rosenberg, E. 2002. Biosurfactants and oil bioremediation. *Current opinion in biotechnology*, 13: 249-252.

Setti, L., Lanzarini, G., Pifferi, P.G. and Spagna, G. 1993. Further research into the aerobic degradation of n-alkanes in a heavy oil by a pure culture of a *Pseudomonas sp*. *Chemosphere*, 26: 1151-1157.

Setti, L., Pifferi, P.G. and Lanzarini, G. 1995. Surface tension as a limiting factor for aerobic n-alkane biodegradation. *Journal of Chemical Technology & Biotechnology*, 64: 41-48. Strynar, M., Sen, D. and Weaver, R.W. 1999. Nitrogen and phosphorus for growth of oil-

degrading microorganisms in seawater. *Bioremediation Journal*, *3*: 85-91.

Techtmann, S. M., Zhuang, M., Campo, P., Holder, E., Elk, M., Hazen, T. C., Conmy, R. and Santo Domingo, J. W. 2017. Corexit 9500 enhances oil biodegradation and changes active bacterial community structure of oil-enriched microcosms. Applied and Environmental Microbiology, 83: 10.

Tremblay, J., Yergeau, E., Fortin, N., Cobanli, S., Elias, M., King, T. L., Lee, K. and Greer, C.

W. 2017. Chemical dispersants enhance the activity of oil-and gas condensate- degrading marine bacteria. *The ISME journal*, 11: 2793-2808.

Thomas, G. E., Brant, J. L., Campo, P., Clark, D. R., Coulon, F., Gregson, B. H., McGenity,

T. J. and McKew, B. A. 2021. Effects of dispersants and biosurfactants on crude-oil biodegradation and bacterial community succession. *Microorganisms*, 9: 1200.

Thomas, G.E., Cameron, T.C., Campo, P., Clark, D.R., Coulon, F., Gregson, B.H., Hepburn, L.J., McGenity, T.J., Miliou, A., Whitby, C. and McKew, B.A. 2020. Bacterial community legacy effects following the Agia Zoni II oil-spill, Greece. *Frontiers in Microbiology*, 11: 1706.

Vergeynst, L., Wegeberg, S., Aamand, J., Lassen, P., Gosewinkel, U., Fritt-Rasmussen, J., Gustavson, K. and Mosbech, A. 2018. Biodegradation of marine oil spills in the Arctic with a Greenland perspective. *Science of the Total Environment*, 626: 1243-1258.

Wang, Q., Garrity, G.M., Tiedje, J.M. and Cole, J.R. 2007. Naive Bayesian classifier for rapid assignment of rRNA sequences into the new bacterial taxonomy. *Applied and environmental microbiology*, 73: 5261-5267.

Xiao, Y.K., Yan, Z.F., Kim, Y., Lee, H.M., Trinh, H., Yang, J.E., Won, K.H., Yi, T.H. and Kook, M. 2019. *Glaciecola amylolytica* sp. nov., an amylase-producing bacterium isolated from seawater. *International Journal of Systematic and Evolutionary Microbiology*, 69: 957-963.

Yakimov, M.M., Timmis, K.N. and Golyshin, P.N. 2007. Obligate oil-degrading marine bacteria. *Current opinion in biotechnology*, 18: 257-266.

Yu, S., Li, S., Tang, Y. and Wu, X. 2011. Succession of bacterial community along with the removal of heavy crude oil pollutants by multiple biostimulation treatments in the Yellow River Delta, China. *Journal of Environmental Sciences*, 23: 1533-1543.

Zahed, M. A., Aziz, H. A., Isa, M. H. and Mohajeri, L. 2010. Effect of initial oil concentration and dispersant on crude oil biodegradation in contaminated seawater. *Bulletin of environmental contamination and toxicology*, 84: 438-442.