CHARACTERISATION AND EFFECTIVENESS EVALUATION OF MICROBIAL BIOSURFACTANTS FOR THEIR USE IN OIL SPILL RESPONSE

Christina N. Nikolova

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Heriot-Watt University

School of Engineering and Physical Sciences

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ABSTRACT

Surfactants are a group of amphiphilic chemical compounds (i.e. having both hydrophobic and hydrophilic domains) that form an indispensable component in almost every sector of modern industry. Their significance is evidenced from the enormous diversity of applications they are used in, ranging from food and beverage, agriculture, public health, healthcare/medicine, textiles, oil & gas, and bioremediation. This PhD aimed to investigate two hydrocarbon-degrading bacterial strains, *Halomonas* sp. stain TGOS-10 and *Pseudomonas* sp. strain MCTG214(3b1), for their ability to produce biosurfactants and their usefulness for oil spill response. For this, three strategies were developed. First, the two strains were screened for production of surface-active compounds using sustainable substrates such as glucose and sunflower oil. Surface-active compounds were extracted, purified and their chemical structure was characterised with carbohydrate and amino acid assays, and NMR. Results revealed that both stains produced surface-active compound, TGOS-10 strain produced both an emulsifier and surfactant when grown on different substrates, whereas MCTG214(3b1) strain produced only surfactant. Second, the extracted and purified surfactants were tested for dispersion effectiveness at different concentrations and three oil types by utilising a standard baffled flask test. Both biosurfactants dispersed the crude oil at varying efficiencies depending on concentration and oil type but generally TGOS-10 showed better dispersing results than MCTG214(3b1). Lastly, in a case study from the northeast Atlantic, Illumina MiSeq sequencing was used to determine the response of the natural microbial community when exposed to either chemically-dispersed crude oil (commercial dispersant Finasol) or biosurfactant-dispersed oil (rhamnolipid from *P. aeruginosa*). In addition, parallel microcosms to determine hydrocarbon degradation were performed and analysed with Gas-Chromatography coupled with Flame Ionization Detection (GC-FID). During incubation for 4 weeks in roller-bottle microcosms, members of psychrophilic oil-degrading *Colwellia* and *Oleispira* initially dominated the microbial community in both the rhamnolipid and Finasol treatments. Thereafter, the community structure of these treatments significantly delineated. The microbial diversity was significantly greater in the treatment amended with rhamnolipid compared to that in the dispersant-amended treatment. GC-FID/MS analysis revealed that oil biodegradation was markedly enhanced in the Finasol-amended treatment. However, the “better-performing” qualities of the chemical dispersant Finasol may be in part, at least, conferred by other components that

constituent its formulation, and biosurfactants, such as rhamnolipid, could potentially be developed into dispersant formulations with much improved qualities. Ecological null models were also used to better understand and quantify the relative importance of ecological processes in the assembly of microbial communities.

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Nikolova, C., Ijaz, U.Z. and Gutierrez, T. IN REVIEW 'Use of null models to compare marine bacterial metacommunity assembly in the presence of crude oil and either chemical dispersant or biosurfactant', *Ecology and Evolution*.

Other publications

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Nikolova, C., Angelova, A. and Ijaz, U.Z. IN PREP "Meta-analysis reveal distinctive bacterial response to crude oil and dispersants based on geography and temperature'.

Nikolova, C. and Gutierrez, T. (2020) 'Use of microorganisms in the recovery of oil from recalcitrant oil reservoirs: current state of knowledge, technological advances and future perspectives' *Frontiers in Microbiology, 10:2996.* doi: 10.3389/fmicb.2019.02996.

Nikolova, C., Angelova, A. and Gutierrez, T. (2017) 'Enhanced Oil Recovery (EOR) – Use of microbes for the recovery of oil from older/recalcitrant reservoirs', Joint commission report for the Oil and Gas Innovation Centre (OGIC) and Industry Biotechnology Innovation Centre (IBioIC)

CHAPTER 1

INTRODUCTION

1.1 Overview

This chapter details the current scientific knowledge on the different types of surface-active compounds, their characteristics, origin and industry applications. A version of this chapter has been accepted as a literature review in the *Frontiers in Bioengineering and Biotechnology* journal.

1.2 Rationale and research aims

1.2.1 Rationale

Crude oil spills in the marine environment occur either naturally via oil seeps or due to anthropogenic activities such as oil and gas extraction, production, storage and transport. Natural oil seeps account for ~47% or estimated 600,000 metric tons annually of the oil entering the marine environment, whereas the majority (53%) of crude oil spills are the result of incidents with oil and gas infrastructure (i.e. pipeline failure, well blowouts, compromised oil storage facilities, transportation around the world, and utilisation) (NRC, 2003). A recent study estimated that in the Gulf of Mexico alone, a well-known region with natural oil seeps, between 25,100 and 94,300 metric tonnes of oil flow into the Gulf naturally (MacDonald et al., 2015). The vast majority of the offshore oil spills in history happened due to oil tanker incidents, in fact that is over 10,000 oil spills from 1970 onwards (ITOPF, 2020). Historic incidents include the Torrey Canyon that occurred in 1967 (119,000 tonnes), Amoco Cadiz of 1978 (223,000 tonnes), Atlantic Empress in 1979 (287,000 tonnes), Castillo de Bellver in 1983 (252,000 tonnes), Exxon Valdez in 1989 (37,000 tonnes), Sea Empress in 1996 (72,000 tonnes), and Prestige in 2002 (63,000 tonnes) among many others that collectively resulted in 5.86 million tonnes of crude oil lost (ITOPF, 2020). However, the number of oil tanker incidents and the amount of oil spilled have decreased by 95% since the 1970s due to building double-hulled tankers, improved health and safety standards of operation, and introduction of major international maritime safety conventions and legislations. In contrast, in the last few decades the offshore oil extraction technology has significantly advanced to enable profitable (increased) recovery (factor) of crude oil from challenging environments, such as deep sea (>1,000 m) and arctic regions. Such technology advancement, however, have increased the risk of major oil spill incidents as demonstrated by one of the largest oil spill disasters in history, the Deepwater Horizon oil spill that occurred in the Gulf of Mexico in 2010, caused by a deep-sea (~1,500m) well blowout event which took 3

months to contain. As a result, approximately 700,000 tonnes of Louisiana light crude oil were released in total into the Gulf of Mexico and caused an ecological crisis with devastating consequences for local ecosystems and economy (Camilli et al., 2012; McNutt et al., 2012). Prior to the Deepwater Horizon event, another well blowout incident, Ixtoc I that occurred in the Bay of Campeche, Mexico, released nearly 480,000 tonnes of oil and took roughly 10 months to stop the leak (Jernelöv and Linden, 1981)e. Marine oil spills are today relatively rare, but both these incidents (Deepwater Horizon and Ixtoc I) highlight the magnitude and complexity of managing such events (Johansen et al., 2003; Jernelöv, 2010).

Different techniques are used to combat oil spills in the marine environment. Some of these include skimming, booms, controlled *in-situ* burning, and application of chemical dispersants. Chemical dispersants are generally the primary response option in case of large-scale offshore oil spills in many countries, including the UK (EMSA, 2016). Dispersants are a complex formulation of different ingredients, mainly comprising amphiphilic compounds that consist of a hydrophilic and hydrophobic moiety that serves as the principal component for reducing the surface tension between immiscible liquid phases such as oil-water interfaces. When applied to oil spills, dispersants act to break down the oil into small droplets (< 70 μm in diameter) which become neutrally buoyant and more likely to stay in suspension in the water column (Camilli et al., 2010; Beyer et al., 2016). As a result, the mobility and concentration of the oil increases in the water column instead of floating as a bulk or broken slick on the sea surface (Belore et al., 2009). The advantage of this is that the oil becomes more bioavailable to natural communities of hydrocarbon-degrading microorganisms in the water column (i.e., biodegradation process is speeded up). A major reason for using dispersants by oil spill response authorities is to prevent the oil from beaching on shorelines where the clean-up process could be extremely difficult, destructive and hazardous, not to mention expensive. Furthermore, the fine oil droplets can facilitate the transportation and sedimentation of substantial amounts of the oil via the formation of marine oil snow (MOS) flocks, that eventually sediment to the seafloor through the process of Marine Oil Snow Sedimentation and Flocculent Accumulation (MOSSFA) (Fu et al., 2014; Valentine et al., 2014; Yan et al., 2016; Francis and Passow, 2020). Chemical dispersants are proven effective in dispersing crude oil, although their effectiveness varies with oil composition, oceanographic dynamics, temperature and salinity (National Research Council, 2005;

Kuhl et al., 2013), but whether they stimulate microbial biodegradation remains debatable.

The current generation of chemical dispersants (including Corexit and other commonly used dispersants) are considered non-toxic to most aquatic animals (National Research Council, 2005; ITOPF, 2011b). However, numerous studies have found that exposure to medium and high concentrations of dispersant Corexit 9500 causes significant decrease of larval settlement and survival of two common coral species, *Porites asteroides* and *Montastraea faveolata*, in the Key West of the USA (Goodbody-Gringley et al., 2013) and deep-sea corals *Leiopathes glaberrima*, *Paramuricea* type B3, and *Callogorgia delta* (DeLeo et al., 2016). Although some types of dispersants contain food-grade biodegradable surfactants derived from saccharides (e.g. Span 80, Tween 80 and 85), substantial amounts of hazardous compounds such as the surfactant dioctylsulfosuccinate (DOSS), organic sulfonic acid salt and propylene glycol are also found in dispersant formulations (Kleindienst et al., 2015a; John et al., 2016; Place et al., 2016a). DOSS can persist in the environment for at least 42 days or even years in cold waters, increasing the risk of further and chronic contamination (Kujawinski et al., 2011; Campo et al., 2013). In terms of microbial biodegradation, different studies have reported conflicting results. A few studies comparing the biodegradation rates of chemically dispersed (i.e. oil treated with dispersant) and undispersed crude oil concluded that even though biodegradation occurred in treatments with and without dispersant, it was clearly faster in the dispersed oil than in oil only treatments (McFarlin et al., 2014, 2018; Prince and Butler, 2014; Brakstad et al., 2015b, 2018; Prince et al., 2017; Techtmann et al., 2017; Ribicic et al., 2018). Other studies, however, have revealed that chemical dispersants may actually inhibit the natural microbial oil degradation rather than stimulating it, and this may be attributed either by the dispersant components, the increased concentrations of dispersed toxic hydrocarbon compounds, or a combination of two (Kleindienst et al., 2015b, 2015a; Seidel et al., 2016). In addition, the chemical dispersant Corexit was shown to suppress the rates of hydrocarbon oxidation, bacterial protein production, and exoenzyme activities of prominent hydrocarbon-degrading bacteria such as *Acinetobacter*, *Marinobacter*, *Rhodococcus* (Hamdan and Fulmer, 2011; Kleindienst et al., 2015b; Rahsepar et al., 2016). During marine oil spills, a typical oil-degrading bacterial community consists of many species, some of which specialise in the degradation of different petroleum compounds, or which collectively and synergistically share hydrocarbon-degrading genes and pathways, and which express a unique response

to the application of a synthetic dispersant (Kostka et al., 2011; Bookstaver et al., 2015; Rodriguez-R et al., 2015; Overholt et al., 2016).

Growing awareness among society regarding the environmental hazards associated with the use of chemical dispersants has led to increased interest towards the use of naturally-derived, biological dispersing products (i.e. biosurfactants) which are commonly associated with low toxicity, high biodegradability, better environmental compatibility, and are sustainably sourced compared to their counterparts (i.e. surfactants) that are produced via organo-chemical synthesis in a laboratory or industrial chemical plant (Desai and Banat, 1997). Over the past decades, numerous marine microorganisms, especially bacteria, have been identified that are able to degrade hydrocarbons by producing effective biosurfactants (Al-Wahaibi et al., 2014; Cai et al., 2014; Chandankere et al., 2014). However, the current knowledge on microbial biosurfactants has been limited to only a few compounds produced by a small number of bacteria and yeast species, such as *Pseudomonas*, *Bacillus*, *Candida* and *Acinetobacter* (Ruggeri et al., 2009). These organisms, and their produced biosurfactants, have potential promise for application in offshore oil spill response, enhanced oil recovery and soil washing treatment of petroleum-contaminated sites (Banat et al., 2010; De Almeida et al., 2016). However, it is important to find other promising biosurfactant-producing bacteria and/or yeast (and other fungi) in order to increase the variability of these biomolecules available for large-scale production and also to decrease the dependence on some of these microbial genera which have species of known human pathogens (e.g. *Pseudomonas aeruginosa, Candida* and *Bacillus*). A highly promising source for discovering novel biosurfactant-producing microorganisms is the marine environment as it harbours an extensive and largely untapped microbial biodiversity, which has shown itself as a proven repository of powerful molecules currently used for pharmacological, food and cosmetics applications (Kennedy et al., 2011; Gudiña et al., 2016; Perfumo et al., 2018).

1.2.2 Aims of the research

This research aims to improve the understanding of three marine hydrocarbon-degrading bacterial strains, namely *Pseudomonas* sp. strain MCTG214(3b1) and *Halomonas* sp. strain TGOS-10, their ability to produce surface-active compounds and suitability to be used in oil spill response. In this thesis, a range of chemical and molecular biology analyses were used to investigate the chemical nature of the surface-active compounds that are produced by the two strains, their efficiency to disperse crude oil, and

what would be the effect of biosurfactants with or without crude oil on the natural seawater microbial community – a case study from the Faroe-Shetland Channel. Understanding the effect of biosurfactants on the *in-situ* microbial community would improve future decision-making process when evaluating applicable oil spill response tools.

In addition, in this thesis it was anticipated to include a so called "herding" test that would determine whether the microbial surface-active compounds produced by the two strains would also "herd" the spilled crude oil. Herding means that a surface-active compound has the ability to push or collect the oil slick into a smaller surface area (opposite to chemical dispersants) with increased thickness. In certain situations (e.g. oil spill in the Arctic) conventional oil spill response options such as mechanical recovery (skimmers, booms, pumps), chemical dispersants, and surface containments cannot not be used or would be inefficient. The rationale behind herding oil is that it can then be burnt *in-situ*. Different oil spill options will be discussed in more detail later in this chapter so that the context is made clear for which this study is set up. The testing was meant to be completed in the US National Oil Testing Facility Ohmsett in Leonardo, New Jersey by Dr Paul Panetta. Unfortunately, due to the global pandemic COVID-19, the testing has been repeatedly postponed and, as such, not completed in time to be included in this thesis.

1.3 Types of biological surface-active agents

Biological surface-active agents are amphiphilic compounds produced by microorganisms, plants or animals. Generally, microorganisms such as bacteria, yeast, and archaea are the most commercially promising and sustainable source of surface-active compounds owing to their enormous genomic diversity. These compounds can form part of microorganism's cell wall, or are excreted extracellularly out of the cell (Shahaliyan et al., 2015). Because of their amphiphilic nature, biosurfactants can dissolve in both polar and non-polar solvents (Chen et al., 2015). The effectiveness of a surfactant is determined by its ability to lower the surface tension (ST) and interfacial tension (IFT) between two immiscible phases (e.g., air/liquid and non-polar/polar liquids, respectively). The ST is a measure of the energy (per unit area) required to increase the surface area of a liquid due to intermolecular forces. When a surfactant is present, less work is required to bring a molecule to the surface and consequently the ST is reduced. It is accepted that a good biosurfactant can lower the ST of water from 72 to less than 35 mN/m, and the

IFT for water against *n*-hexadecane from 40 to 1 mN/m (Mulligan, 2005). An efficient biosurfactant is one that has a low critical micelle concentration (CMC). Low CMC means that less biosurfactant is necessary to reduce the ST or IFT. By definition, the CMC is the minimum concentration required to initiate micelle formation and generally correlates with the ST and IFT (Figure 1-1) (Mulligan, 2009).

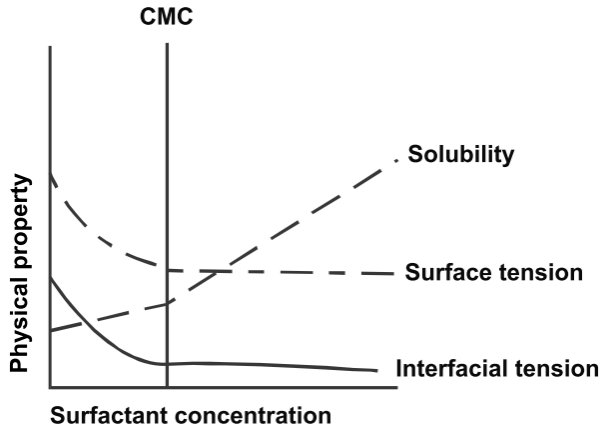


Figure 1-1. Biosurfactant critical micelle concentration (CMC) correlation with surface tension, interfacial tension and solubility. Source: Mulligan (2005).

The chemical composition of biosurfactants varies greatly between different species of microorganisms and broadly can be classified based on their molecular weight or chemical charge. Based on their molecular weight, surface-active compounds are classified as either low-molecular-weight (LMW) surfactants, which reduce surface tension between two immiscible liquids, or high-molecular-weight (HMW) emulsifiers, which enable the formation of oil-in-water or water-in-oil emulsions and which are also referred to as polymeric surfactants (or bioemulsifiers) and commonly composed of exopolysaccharides (EPS). Surface-active compounds can be categorised into four groups, based on their chemical charge: anionic, cationic, non-ionic, and zwitterionic. The anionic and non-ionic surfactants are the most commonly used in industrial applications.

1.3.1 LMW biosurfactants

The main chemical structures of LMW biosurfactants are glycolipids, phospholipids and fatty acids, lipopeptide, and lipoproteins. These structures can form biosurfactant as single macromolecules, polymers, and/or particulate structures (Banat, 1995; Makkar and Rockne, 2003; Satpute et al., 2010b). Glycolipids are the most studied

biosurfactants and consist of different sugars linked to ß-hydroxy fatty acids (carbohydrate head and a lipid tail) while lipopeptides, in contrast, consist of cycloheptapeptides with amino acids linked to fatty acids of different chain lengths (Figure 1-2) (Uzoigwe et al., 2015).

1.3.1.1 Glycolipids

Glycolipids are a class of non-ionic surfactants that exhibit excellent functional properties (i.e., reduction of surface and interfacial tension). Glycolipids produced from renewable resources through natural fermentation processes are now common and preferred choice for industry. The best explored groups of glycolipids include rhamnolipids, sophorolipids, trehalolipids and mannosylerythritol lipids (MELs).

1.3.1.1.1 Rhamnolipid

Rhamnolipids are the most commercially available biosurfactants finding many industrial applications, including bioremediation, agriculture, cosmetics and pharmaceuticals (Irorere et al., 2017). As the name suggests, rhamnolipids consists of rhamnose monosaccharide/s linked to 3-hydroxyl fatty acid unit via ß-glycosidic bond. Furthermore, different types of rhamnolipids can be differentiated into various congeners based on the number of rhamnose units and the composition of the fatty acid units within the molecule (e.g. mono-rhamnolipids and di-rhamnolipids) (Abdel-Mawgoud et al., 2011). Rhamnolipids have powerful surface-active properties – reducing the ST of water from 72 mN/m to around 30 mN/m and below, which is comparable with some types of synthetic industrial surfactants such as Tween 80 (Mulligan, 2009). In addition, pure rhamnolipids have been shown to lower the IFT against n-hexadecane to about 1 mN/m at concentration (CMC) as low as 10 mg/L (Lang and Wullbrandt, 1999). Traditionally, rhamnolipids were mainly produced by the well-known opportunistic human pathogen *Pseudomonas aeruginosa* (Chong and Li, 2017). The pathogenic nature of *P. aeruginosa* has raised concerns regarding the health hazards during production and the safety of the end product. Recent reports have shown rhamnolipid production by non-pathogenic species of *Pseudomonas* isolated from the marine environment (Kristoffersen et al., 2018; Twigg et al., 2018), but also from other bacterial species including *Burkholderia thailandensis* E264 (Funston et al., 2016), *Acinetobacter calcoaceticus* and *Enterobacter asburiae* (Hošková et al., 2015) and more recently from *Marinobacter* (Tripathi et al., 2019).

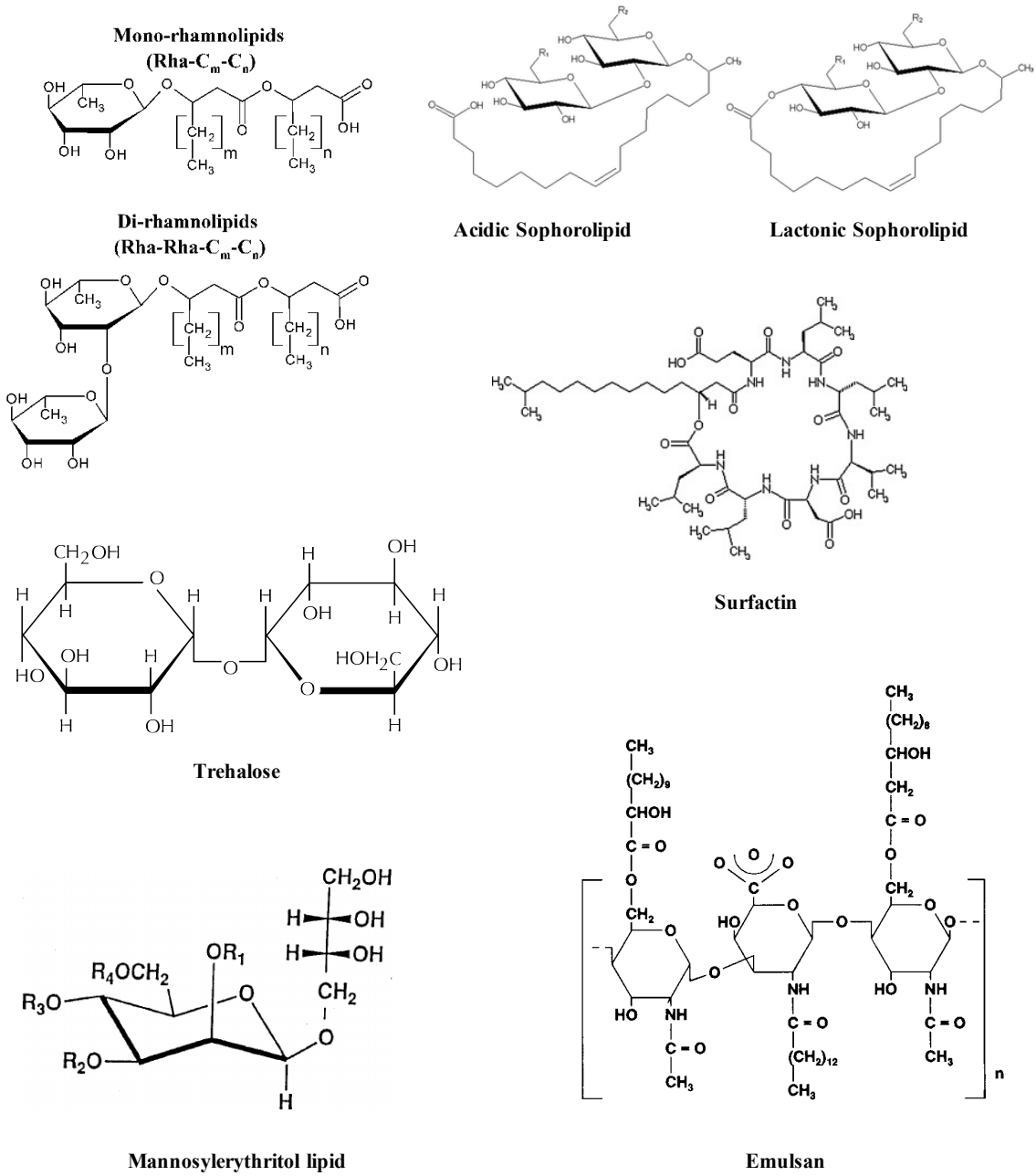


Figure 1-2. Illustration showing the chemical structure of the best-known biosurfactants (from Banat et al. 2010). Rhamnolipids, sophorolipids, and trehalose are glycolipids; surfactin is a lipopeptide and emulsan is an exopolysaccharide.

1.3.1.1.2 Sophorolipid

Sophorolipids are the second most common biosurfactants after rhamnolipids and are produced by non-pathogenic yeast species from the genus *Candida* (Kurtzman et al., 2010). Sophorolipids consist of the dimeric sugar sophorose head linked to a long chain hydroxy fatty acid tail (Figure 1-2). Sophorose is a glucose disaccharide that can be acetylated on the 6'- and/or 6"-positions. The chemical structure of sophorolipds can vary

depending on how and where the hydroxylic fatty acid is linked to the sophorose molecule. Whether the carboxylic end of this fatty acid is free or internally esterified at the 4" or at the 6'- or 6"-position would determine if the sophorolipid molecule has acidic or lactonic pattern, respectively (Van Bogaert et al., 2007). The acidic and lactonic sophorolipid forms have different biological and physicochemical properties. The degree of lactone formation, for example, strongly influences the foam formation capacity, hydrophilic/lipophilic balance (HLB) and antimicrobial properties of the sophorolipid molecule. Lactonic sophorolipids are characterised by better surface-tension reduction and antimicrobial activity, whereas the acidic form shows better foam formation capacity and solubility (Van Bogaert et al., 2007). Sophorolipid biosurfactants lower the ST of water from 72 mN/m to between 40 to 30 mN/m, and for this have found application in cosmetic and personal care industries. Acidic sophorolipids are more effective in lowering the IFT between *n*-hexadecane and water (1-2 mN/m) than lactonic sophorolipids (5 mN/m) (Rosenberg and Ron, 1999). The HLB ranges between 10 to 13, which means that sophorolipids could be useful as detergents or as stabilisers for oil-in-water emulsions. The main microorganism used on industrial scale for production and application of sophorolipids is the yeast species *Candida bombicola* which has been reported to produce a yield of over 400 g/L depending on fermentation conditions (Van Bogaert et al., 2007). A recent study demonstrated that *Candida lipolytica* produced a sophorolipid that exhibited 80% oil spreading efficiency and low toxicity, making it potentially suitable for oil spill remediation (Santos et al., 2017).

1.3.1.1.3 Trehalose lipids

Trehalose lipids are composed of trehalose as the sugar moiety linked to fatty acids which are highly variable among the producing microorganisms with very long chain length from C20 to C90. The chemical structures between congeners are further complicated by numerous branches and substitutions (Perfumo et al., 2010b). In contrast to other glycolipids, trehalose biosurfactants are cell-bound and usually require laborious extraction and purification processes resulting in very small amounts of recoverable product compared to other glycolipids. Despite low yields, trehalose lipids are known to have low CMC (approx. 2 mg/L). Trehalose biosurfactants are mainly synthesised by *Rhodococcus erythropolis* (Peng et al., 2007).

1.3.1.1.4 Mannosylerythritol lipids

Mannosylerythritol lipids (MELs) are molecules that consist of long-chain fatty acids linked to a mannopyranosyl-meso-erythritol hydrophilic head group, and are synthesised in various congeners, mostly diacetylated (MEL-A) and monoacetylated (MEL-B and MEL-C) and several other minor variants. The differences in the chemical structure can greatly affect the biochemical and biological properties, and thus have a high versatility in terms of applications and uses (Morita et al., 2013). In addition, they can be produced at high yields (up to 100 g/L) from vegetable oils by intermittent feeding, which makes them attractive for industrial applications (Konishi et al., 2007; Morita et al., 2009, 2013). The yeast *Candida antarctica* was able to produce up to 28g/L of MEL with lowering the ST to 35 mN/m (Adamczak and Bednarski, 2000). The ST at CMC of sucrose-derived MEL-A produced by *Pseudozyma antarctica* JCM 10317 was 25.3 mN/m (Morita et al., 2009). *Psedozyma aphidis* ZJUDM34 on waste cooking oil as the sole carbon source produced 55 g/L MEL compared to 61 g/L from soybean oil (Faria et al., 2014; Niu et al., 2019)**.** This organisms is now known as *Moesziomyces antarcticus* and was first isolated from the Lake Vanda in the Wright Valley, Antarctica (Goto et al., 1968).

1.3.1.2 Lipopeptides

The second major group of LMW biosurfactants is represented by the lipopeptides. All lipopeptides consist of a short cyclic or linear oligopeptide linked to a fatty acid tail but they can greatly vary in the number, type and order of the amino acid residues as well as the length and branching of the chain. Lipopeptides are known for their exceptional ability to interact with the cell membrane making them powerful antimicrobial agents. However, in the presence of hydrophobic compounds they act as potent biosurfactants with a wide range of physio-chemical properties (Perfumo et al., 2017). Lipopeptides are mainly produced by *Bacillus*, but other species including *Pseudomonas* (Bonnichsen et al., 2015), *Streptomyces* (Yan et al., 2014), *Aspergillus* (Kiran et al., 2010), and *Serratia* (Nalini and Parthasarathi, 2014) are also known as lipopeptide producers. However, the *Bacillus* derived lipopeptides are the best known and include surfactin, lichenysin, viscosin, fengycin, and iturin, as discussed below.

1.3.1.2.1 Surfactin

Surfactin is one of the most powerful biosurfactants on the market. It is classified as cyclic anionic lipopeptide and consists of long hydroxyl fatty acid chain and hydrophobic amino acid ring (Chen et al., 2015). Surfactin's chemical structure makes it poorly soluble in water which limits its applications in water systems but highly effective in soil bioremediation and enhanced oil recovery. Surfactin tends to stay at the hydrophilic/hydrophobic interfaces which greatly affects the adsorption of hydrophobic molecules. Surfactin can reduce the ST to approximately 26 mN/m of oil-water interfaces (Al-Wahaibi et al., 2014) and the IFT of *n*-hexadecane to water from 43 to 1 mN/m at concentration as low as 0.005% (Mulligan, 2005), and it has been shown to enhance oil recovery from packed sand columns up to 90% (Makkar and Cameotra, 1997). In addition to the strong surface activity, surfactin has excellent foaming properties. The main producer of surfactin is *B. subtilis*, however, other members of the genus *Bacillus* can produce surfactins that are structurally different analogues, even producing them simultaneously in culture (Liu et al., 2015). In particular, *B. subtillis* has been the most studied surfactin producer (Inès and Dhouha, 2015) (Table 1).

1.3.1.2.2 Lichenysin

Lichenysin is a cyclic lipopeptide produced by *Bacillus licheniformis* (Yakimov et al., 1995) and *Bacillus mojavensis*, and is similar to surfactin in terms of chemical structure and biosynthesis (Grangemard et al., 2001). It is considered the most potent anionic biosurfactant due to being particularly effective in reducing IFT to less than 1 mN/m (Joshi et al., 2015), and reducing the ST to 24 mN/m with CMC of 15 mg/L (Coronel-León et al., 2016). Unlike other biosurfactant-producing bacteria, *Bacillus licheniformis* produces lichenysin only when grown on non-hydrocarbon substrates. There are different variants of lichenysin, known as A, B, C, D, E and G, the difference between them is caused by the species-specific variation in their fatty acid composition. Lichenysin A expresse surface activity at very low concentrations, reaching CMC of 12 mg/L (Yakimov et al., 1995). The lichenysin produced by *B. licheniformis* JF-2 has been patented for applications in microbial enhanced oil recovery (MEOR) due to its stability within a wide range of temperatures, salinities and pH (Shete et al., 2006) while recovering up to 40% of residual oil from sandstone core-flood experiments (McInerney et al., 1990; Joshi et al., 2016).

1.3.1.2.3 Viscosin, iturin and fengycin

Viscosin, iturin and fengycin are the lesser studied lipopeptide surfactants. All three are cyclic lipopeptides. Viscosin is produced by antagonistic plant-associated *Pseudomonas fluorescens* (De Bruijn and Raaijmakers, 2009) and has found applications mainly as a plant pest control agent due to its powerful antibacterial and antifungal properties (De Bruijn et al., 2007; Alsohim et al., 2014). Viscosin is composed of nine amino acids coupled to a 3-hydroxydecanoic acid (Raaijmakers et al., 2010). In addition, viscosin has excellent surface active properties, with the ability of lowering the ST to 27 mN/m with approximately 4-9 mg/L required to reach CMC (Laycock et al., 1991).

Iturin and fengycin are both produced by *B. subtilis*. Their variants have similar structure with the same peptide length, but with different amino acid residues at specific position (Ongena and Jacques, 2008). The iturin family includes seven isomers, including mycosubtilin and bacillomycin, which are heptapeptides linked from C14 to C17 to a ß-amino fatty acid chain (Raaijmakers et al., 2010). Iturin A was the first discovered compound of this group, isolated from a petroleum soil sludge *B. subtilis* strain C-1(Vater et al., 2002). The fengycin family consists of fengycin A and B which are decapeptides linked to a ß-hydroxyl fatty acid chain (C14 – C18). Fengycins are known for their antimicrobial and antifungal properties attributed to their ability to interfere with membrane and cell wall functions of different fungi and bacterial species (Vanittanakom et al., 1986).

1.3.2 HMW emulsifiers

Bioemulsifiers are more complex than biosurfactants and consist of mixtures of heteropolysaccharides, lipopolysaccharides, lipoproteins and proteins. They are also known as exopolysaccharides (EPS). Similar to low-molecular weight biosurfactants, EPS molecules can efficiently emulsify two immiscible liquids (e.g oil and water), but in contrast are less effective at ST reduction. In oil polluted environments, EPS molecules bind tightly to dispersed hydrocarbons preventing the oil droplets from coalescing and ‘bursting’ open. This process is known as stabilisation of emulsion and has been attributed to the large number of reactive groups exposed in their structures (Uzoigwe et al., 2015). The best-studied microbial EPS are emulsan, alasan, liposan, sphingan, and xanthan gum.

1.3.2.1 Emulsan

Emulsan is a potent polyanionic heteropolysaccharide bioemulsifier synthesised by *Acinetobacter calcoaceticus* RAG-1 when grown on hydrocarbon substrates, producing powerful emulsions at very low concentrations of 0.001 to 0.01% (Rosenberg and Ron, 1999; Uzoigwe et al., 2015). It is a noncovalently linked complex of a lipoheteropolysaccharide (referred to as apoemulsan) and a protein. The major sugar components of the apoemulsan are D-galactosamine, D-galactosaminuronic acid, and diaminodideoxy glucosamine (Navon-Venezia et al., 1995). Emulsan from *A. calcoaceticus* stain BD4 is capsular polysaccharide consisting of four alpha-rhamnose units, one alpha-mannose, one beta-glucose and one beta-glucuronic acid. Interestingly, BD4 excretes only 10% of the total production of exopolysaccharide (202-593 mg/L) when grown on glucose-minimal medium (Kaplan et al., 1985). BD4 total exopolysaccharide production per cell was 2.5 $x10^{-13}$ g, and represented 31% of the dry cell weight, with 87% of it being cell-associated. Kaplan and Rosenberg (1982) noted that under certain growth conditions, the capsular EPS can be released together with the cell-bound protein, producing a highly active emulsifier complex.

1.3.2.2 Alasan

Alasan is an extracellular bioemulsifier produced by *Acinetobacter radioresistens* KA53 that is significantly different from RAG-1's emulsan and other biopolymers. It is a complex polymer formed by anionic polysaccharides rich in alanine and proteins with high molecular mass of 1000 kDa (Navon-Venezia et al., 1995). Alasan has been reported to effectively stabilise (up to 10 days) highly dispersed oil-in-water emulsions of C_{10}-C_{18} alkanes either alone or in mixture with some two-ringed aromatic hydrocarbons at concentration as low as 0.005% (Barkay et al., 1999). For example, the emulsification activity of alasan with 1:1 mixture of *n*-hexadecane and methylnaphthalene was 100% whereas RAG-1 and BD4 emulsans were not able to emulsify both pure aliphatics and aromatics (Navon-Venezia et al., 1995). In addition, alasan increased its emulsifying activity by 30% when heat- or alkali-treated (100°C or 0.1 N NaOH) and remained stable for at least 3 months (Toren et al., 2001). In contrast, emulsan form RAG-1 strain completely loses its activity when treated with alkali. The active component (emulsifying activity) of alasan's structure is the protein, whereas the polysaccharide contributes to emulsion stability (Toren et al., 2001). Alasan's ability to solubilise polycyclic aromatic hydrocarbons (PAH) was evaluated by Barkay et al. (1999). The concentration of soluble

PAH was on average 17 times higher than the control treatment without alasan. The yield of alasan was reported at 4.6 g/L (Toren et al., 2002).

1.3.2.3 Liposan

Liposan is a type of water-soluble EPS composed of approximately 83% heteropolysaccharides (mainly galactose, galactosamine and galacturonic acid) and 17% protein (Cirigliano and Carman, 1985) and are synthesised in high concentrations (up to 400 g/L) by non-pathological yeast *Candida lipolytica* (Van Bogaert et al., 2007). Liposan is produced by growing *Candida* on hydrocarbons or other water-immiscible carbon substrates and has been commercially used in pharmaceutical, food, and cosmetics industries due to its ability to stabilise oil-in-water emulsions (Cirigliano and Carman, 1985; Campos et al., 2013a).

1.3.2.4 Sphingan

Sphingans are a family of structurally closely related bacterial exopolysaccharides synthesised by members the genus *Sphingomonas*. Most sphingans have the same linear repeating tetrasaccharide backbone structure (glucose-glucuronic acid-glucose-rhamnose or mannose) to which glucosyl, rhamnosyl, mannosyl or acetyl side chains are attached (Schultheis et al., 2008). The known sphingans include gellan (glycetyl and acetyl substituents without sugar side chains), welan (acetyl and L-rhamnosyl or L-mannosyl as side chains), rhamsan and diutan. The structural variations have an influence on the physiochemical properties and commercial applications of different sphingans. Novel sphingans have excellent rheological properties such as high viscosity and good thermal stability, making them suitable in the food industry as thickening agents (Schultheis et al., 2008; Prajapati et al., 2013) as well as in other industries such as oil and gas, textile, and pharmaceuticals (Kaur et al., 2014). Welan gum production is usually 20 g/L but optimising the fermentation process and using mutant producing strains can increase the yield as has been shown for strain HT-1 which was able to produce 26 g/L (Zhu et al., 2014). *Sphingomonas* sp. CS101 produced up to 17 g/L of a new type of sphingan under optimal conditions (Seo et al., 2004). The marine *Sphingomonas* sp. WG was recently reported to reach a yield of 33 g/L when fed on glucose and the viscosity of the fermentation broth was measured at over 70,000 mPa/s (jelly-like viscosity) (Li et al., 2016).

1.3.2.5 Xanthan gum

Xanthan gum is one of the most commercially produced microbial EPS and is sourced from the plant pathogenic bacterium *Xanthomonas campestris* (de Mello Luvielmo et al., 2016). It has been widely used in the food industry as an authorised food additive (according to Annex II and III to EU Regulation No 1333/2008) due to its thickening, stabilising, emulsifying, antioxidant, and high viscosity properties (Palaniraj and Jayaraman, 2011). Xanthan gum is composed of a backbone of repeating sub-units that consists of 3 to 8 monosaccharides including glycose, mannose (with pyruvic acid and acetyl moieties), and glucuronic acid in molar ratio of 2:2:1 (Lopes et al., 2015; Kang et al., 2019). Higher pyruvic acid content in the xanthan gum would increase its viscosity and thermal stability making it an versatile EPS in wider applications including oil drilling where it is used to thicken the drilling mud (Kuppuswami, 2014).

1.4 Marine biosurfactant-producing bacteria

1.4.1 Pseudomonas

Members of the genus *Pseudomonas*, of the class Gammaproteobacteria, can colonise diverse habitats and produce different biosurfactant molecules including glycolipids (rhamnolipids) and lipopeptides (e.g., viscosin, amphisin, tolaasin and syringomycin). The majority of isolated *Pseudomonas* species have been derived from terrestrial habitats, but members of this genus are common in marine environments (Baumann et al., 1983; Bollinger et al., 2020). The most commonly studied biosurfactant producer is *Pseudomonas aeruginosa*. It grows well on various hydrocarbon and non-hydrocarbon substrates and produces rhamnolipids that can form stable emulsions with crude oil and kerosene (Shahaliyan et al., 2015). Another study observed that *P. aeruginosa* had a high affinity for crude oil (93% cell adhesion to crude oil) which is also an indication of biosurfactant production (Thavasi et al., 2011). *P. aeruginosa* DQ8 strain was shown to decrease the surface tension from 63 to 38 mN/m of culture broth in the presence of various crude oil fractions including PAHs (Zhang et al., 2011). In addition, non-hydrocarbon substrates such as soybean oil, fish oil, mannitol, and glycerol, can be utilised by *Pseudomonas aeruginosa* to produce non-toxic biosurfactant which could be useful in oil spill bioremediation as an alternative to chemical dispersants or as substitute of synthetic surfactants in commercial dispersant formulations (Coelho et al., 2003; Prieto et al., 2008; Das et al., 2014). Strains of *P. aeruginosa* grown on glycerol produced

rhamnolipids (3.8 g/L; CMC 50 mg/L) which reduced the ST to 29 mN/m and emulsified petrol (EI_{24} 70%) and diesel (EI_{24} 80%), further indicating its potential application in oil recovery and bioremediation (Das et al., 2014). A species of *Pseudomonas putida*, stain BD2, was isolated from Arctic soil that was able to grow on glucose and produce rhamnolipid and sophorolipid simultaneously; the rhamnolipid reduced ST to 31 mN/m and emulsified veg oil at 70% efficiency (Janek et al., 2013).

1.4.2 Bacillus

Members of the genus *Bacillus* have been predominantly isolated from oil reservoirs or oil contaminated soils and shown to be particularly efficient biosurfactant producers with applications in MEOR. *Bacillus methylotrophicus* USTBa, for example, was isolated from a petroleum reservoir and grew well on crude oil in aqueous medium. After 12 days of incubation, *B. methylotrophicus* removed more than 90% of the crude oil. The ST of the culture medium was measured at 28 mN/m indicating that the bacteria produced a strong glycolipid-type biosurfactant (Chandankere et al., 2014). In addition, the biosurfactant was stable under various pH values and high temperatures (up to 100°C) suggesting its potential application as an oil spill treatment agent in marine environments and in MEOR processes where high salinity and temperatures are common (Chandankere et al., 2014). *B. subtilis* strain A1 was able to achieve 78% emulsification activity by the production of lipopeptide biosurfactant when grown on crude oil as a sole source of carbon (Parthipan *et al.*, 2017). This strain completely degraded a range of the low-molecular weight alkanes ($C_{10} – C_{14}$) and up to 97% of the high-molecular weight alkanes ($C_{15} – C_{19}$) after 7 days of incubation at 40°C. These results suggest that *B. subtilis* A1 strain could be used in oil spill remediation where light crude oils (high proportion of alkanes) have been spilled. A non-pathogenic *Bacillus licheniformis* R2 was studied for its potential use in MEOR in laboratory conditions. It produced a low-yield lipopeptide biosurfactant (1 g/L) that lowered the ST to 28 mN/m and the IFT between heavy crude oil and formation water-brine used in core flooding to 0.53 mN/m (Joshi et al., 2015). When treated with temperature of 85°C, the R2 biosurfactant recovered 37% heavy crude oil recovery over residual oil saturation and retained 88% activity for 90 days (Joshi et al., 2015).

1.4.3 Acinetobacter

Acinetobacter is a genus of gram-negative Gammaproteobacteria, strictly aerobic bacteria belonging to the order of *Pseudomonadales*. *Acinetobacter* is ubiquitous in

nature and commonly found in marine environments. Many species from this genus are known hydrocarbon-degraders that produce extracellular EPS (Pines and Gutnick, 1986; Barkay et al., 1999; Hassanshahian et al., 2012). As mentioned earlier, *A. calcoaceticus* and *A. radioresitensis* synthesise emulsan and alasan, respectively, well-known HMW bioemulsifiers (Kaplan et al., 1985; Navon-Venezia et al., 1995). *A. calcoaceticus* was also able to synthesise rhamnolipids with CMC of 15 mg/L (Hošková et al., 2015). *A. radioresitensis* can produce a yield of 4.6 g/L of EPS when grown on ethanol as the sole carbon and energy source (Navon-Venezia et al., 1995). Strains of *A. calcoaceticus*, and *A. oleivorans* isolated from the Canadian North Atlantic have been shown to produce bioemulsifiers when grown on petroleum hydrocarbons as the sole carbon source ($E_{24\%} >$ 50%) (Cai et al., 2014). Given that the stains were isolated and hence adapted to the cold marine environment of the Northern Atlantic, the bioemulsifiers they produce could be effective under low temperature and harsh conditions in offshore oil spills remediation.

1.4.4 Antarctobacter

Antarctobacter is a genus of gram-negative bacteria (order Rhodobacterales), that are strictly aerobic bacteria. Only one species, *Antarctobacter heliothermus*, has been validly taxonomically described which was isolated from Antarctica (Labrenz et al., 1998). *Antarctobacter* sp. strain TG22 was isolated from seawater and was found to produce an extracellular water-soluble glycoprotein-type polymer (designated AE22) which formed stable emulsions with different vegetable oils at concentration as low as 0.02% (Gutiérrez *et al.*, 2007b). The strain was grown on marine broth supplemented with 1% glucose and was able to produce an average dry-weight yield of 21 mg/L. The carbohydrate content (total of 15%) of AE22 was dominated by glucosamine, glucuronic acid, fucose and mannose. The protein content represented 5% of the polymer and lipids were not detected, leaving the rest of the polymer content (80%) unidentified (Gutiérrez *et al.*, 2007b). The emulsifying activity of *Antarctobacter* TG22 polymer was comparable to that of xanthan gum which could be considerably useful in applications for healthcare and food additives industries.

1.4.5 Rhodococcus

The genus *Rhodococcus* includes metabolically diverse species that are capable to thrive in different habitats (Finnerty, 1992). Members of the genus have been studied mainly for their ability to degrade hydrocarbons and pollutants from different environments (Whyte et al., 2002; Kuhn et al., 2009; Wang et al., 2014). *Rhodococcus*

erythropolis, *Rhodococcus aurantiacus*, and *Rhodococcus ruber* are among the best known biosurfactant producers of the genus (Bicca *et al.*, 1999; Peng *et al.*, 2007). *Rhodococcus erythropolis* 3C-9 has been shown to grow and produce biosurfactant (CMC of 50 mg/L) only on *n*-alkanes as the sole carbon source, whereas glucose could not enhance its productivity. The 3C-9 biosurfactant contained fatty acids with lengths from C_{10} to C_{22} (docosenoic acid being the most prevalent followed by hexadecenoic acid) and two glycolipids (each dominated by glucose and trehalose monosaccharides). In addition, the 3C-9 biosurfactant significantly enhanced the solubility of PAH substrates (Peng et al., 2007). *Rhodococcus ruber* stain AC 239 produced a small amount of cell-bound glycolipid-type biosurfactant when grown on 1% diesel (v/v). The AC 239 biosurfactant did not reduce the ST as observed for other glycolipid biosurfactants but it emulsified different hydrocarbons with better success (20 to 50% greater EI_{24}) when free cells were present in the culture (Bicca *et al.*, 1999). *Rhodococcus fascians* extracted from Antarctic soil produced a glycolipid with rhamnose sugars which is not typical for *Rhodococcus* which usually produces trehalose biosurfactants (Gesheva et al., 2010).

1.4.6 Halomonas

Halomonas is a ubiquitous genus of the order Altreromonadales. There organisms are found in diverse habitats of both marine (Hassanshahian et al., 2012; Cai et al., 2014) and terrestrial environments, including hypersaline lakes (Poli et al., 2004), soils (Arias et al., 2003; Mata et al., 2006; Llamas et al., 2012; Amjres et al., 2015), and hot springs (Chikkanna et al., 2018). Members of *Halomonas* are known to respond to hydrocarbon enrichment (Calvo et al., 2002; Gutierrez et al., 2013b; Cai et al., 2014) and produce EPS (Gutiérrez et al., 2007b; Gutierrez et al., 2009, 2020) with versatile properties. A thermophilic *H. nitroreducens* strain WB1 isolated from a hot spring produced an EPS that was effective at emulsifying different vegetable oils (68-85%) and aliphatic hydrocarbons (56-65%) in addition to binding metals. The monosaccharide composition of the WB1's EPS was predominantly composed of glucose, mannose and galactose, and traces of uronic acids (Chikkanna et al., 2018). The EPS from *Halomonas eurihalina* strain H96, isolated from saline soil in Spain, has been characterised to contain high amount of uronic acids (Béjar et al., 1998) similarly to some marine-derived strains (Gutierrez et al., 2020). In addition to emulsifying activity, several species of halophilic *Halomonas* have been shown to produce highly sulphated exopolysaccharides (Calvo et al., 2002; Amjres et al., 2015) with anticancer activity. For example, halophilic *H. stenophila* strain B100 exerted a selective proapoptotic effect in T cells from acute

lymphoblastic leukaemia (Ruiz-Ruiz et al., 2011). EPS form *H. halocynthiae* KMM 1376 had inhibitory effect on human cancer cell line MDA-MB-231 at concentrations of 50-100 μg/ml (Kokoulin et al., 2020).

1.4.7 Alcanivorax

Alcanirorax is gram-negative genus of the Gammaproteobacteria (order Oceanospirillales) of strictly aerobic marine obligate hydrocarbonoclasic bacteria (OHCB) utilising predominantly alkanes up to C_{32} and branched aliphatics (Head et al., 2006; Yakimov et al., 2007; Olivera et al., 2009). The best known species of the genera is *Alcanivorax borkumensis* which produces a low molecular weight anionic glycolipid biosurfactant when grown on hydrocarbons (Schneiker et al., 2006). This particular glycolipid consists a glucose sugar linked to a tetrameric chain of fatty acids of C_6 - C_{10} length and can be either cell-bound or extracellular (Abraham et al., 1998). Marine isolate *Alcanivorax borkumensis* SK2 grown on crude oil produced twice more biosurfactant than in the absence of hydrocarbons. In fact, when heavy hydrocarbon fractions were used as the sole carbon source, biosurfactant was the highest (~70 mg/L) and comparable with when crude oil was used (50 ± 20 mg/L). However, the purification of the biosurfactant was easier when the culture was fed with heavy oil fraction as it remained on the surface at all times and consequently there were no substrate impurities (Antoniou et al., 2015). Another species, *Alcanivorax dieselolei* strain B-5, is the second in the genus that has been reported to produce biosurfactant with good surface-active properties (lower ST to 32 mN/m and emulsify *n*-hexadecane at 75%). The chemical analysis of the biosurfactant revealed that it is a linear lipopeptide with CMC value of 40 mg/L which is comparable to that of rhamnolipids and surfactin (Qiao and Shao, 2010). These characteristics make the B-5 lipopeptide an attractive alternative for enhanced oil recovery and bioremediation applications.

1.4.8 Pseudoalteromonas

Pseudoalteromonas is a genus of the order Alteromonadales, members of which are commonly found in sea ice and cold waters and well-known producers of glycolipid-type EPS with a wide-range of biological activities and chemical composition (Holmström and Kjelleberg, 1999). *Pseudoalteromonas* sp. strain SM20310 isolated from Arctic sea ice produced EPS (yield of 567 mg/L) with mannose and glucose being the dominant carbohydrates. The ecological role of the EPS was determined to improve the high-salinity and low-temperature tolerance of the strain (Liu et al., 2013). Another study found

that marine *Pseudoalteromonas* (isolated from Antarctica) produced EPS that contained 40% protein with mannose, glucose and galacturonic acid representing the dominant monosaccharides (Nichols et al., 2005). The carbohydrate content of EPS from *Pseudoalteromonas agarivorans* stain Hao 2018 (yield 4.5 g/L) isolated from Yellow Sea of China contained 90% glucose and 6% mannose. The main biological activity of this strain was moisture retention and absorption of free radicals (i.e. antioxidant) with potential applications in food and cosmetics industries (Hao et al., 2019). *Pseudoalteromonas* sp. strain MD12-642 (isolated from Madeira) produced EPS with a particularly high content of uronic acids (up to 68%) and which might find potential applications in biomedical industry as active ingredients for anti-thrombotic and anti-arthritic drugs (Roca et al., 2016). *Pseudoalteromonas* sp. strain TG12 (isolated from West Scotland) produced EPS that was able to effectively emulsify *n*-hexadecane (EI_{24} of 60%) and some vegetable oils. This strain also contained high levels of uronic acids (~29%) in addition to xylose (27%), glucosamine (25%) and was effective in desorption of sediment-adsorbed metals (e.g. Al^{3+}, $Fe^{2+/3+}$, K^{+}, Mg^{2+}, Na^{+}, and Si^{4+}) (Gutierrez et al., 2008).

1.4.9 Marinobacter

Marinobacter is a genus within the order Alteromonadales. Members of the genus, such as *M. hydrocarbonoclasticus* and *M. algicola* are commonly isolated from oil-enriched marine environments (Gauthier et al., 1992; Gutierrez et al., 2013b). Although *Marinobacter* can use hydrocarbons as carbon source, various studies demonstrated that it can also grow and produce EPS on other carbon sources such as glucose. *Marinobacter* species have been shown to produce exopolysaccharide polymers with excellent emulsifying activity against hydrocarbons that were superior to commercial synthetic surfactants like Tween 80 (Caruso et al., 2019). *Marinobacter* sp. W1-16 from Antarctic surface seawater produced EPS (molecular weight of 260 kDa) with varying yields, strongly depending on the sugar substrate used to grow the strain and the incubation temperature. The highest yield was produced when the culture was grown at 15°C and in the presence of 2% glucose. However, the strain was able to synthesise EPS, even at 4°C, albeit in lower quantities, suggesting that the EPS might have cryoprotective functions (Caruso et al., 2019). In addition, *Marinobacter* sp. MCTG107b was able to produce a glycolipid-type biosurfactant (grown on glucose) with di-rhamnolipid congeners present that was able to lower the ST to 30 mN/m (Tripathi et al., 2019). Marine sediment isolates belonging to *Marinobacter* genus produced (when grown on glucose or soybean oil)

powerful emulsifiers with activity against hexane and toluene in the range of 45-64% and 33-75%, respectively, with some strains producing stable emulsions at 4°C and after high-temperature treatment for up to 18 months (Raddadi et al., 2017).

1.4.10 Others

Other identified biosurfactant-producing bacteria from marine origin are summarised at end of this chapter in Table 1-2**.**

1.5 Ecology and environment

Biosurfactant-producing bacteria can be found in a wide range of habitats, from aquatic (fresh and sea water, and groundwater) to terrestrial (soil, sediment, and sludge) environments. Extreme environments, characterised by extremes of high/low temperature, salinities, pH, and/or pressure, are also commonplace where biosurfactant-producing microbes can be found due to the ecological role biosurfactants play for the producing organisms in those environments, such as in cell adhesion to surfaces and potential food sources, retention of water and concentration of nutrients, production of biofilms etc. (Nicolaus et al., 2010). The environment can have a direct influence on the type of biosurfactants that microorganisms produce. For instance, marine microbial EPS contain higher levels of uronic acids which makes them polyanionic and relatively highly reactive (Gutiérrez et al., 2007; Decho and Gutierrez, 2017) compared to non-marine microbial surfactants. With respect to cold-adapted bacteria, the monosaccharides in the EPS are usually characterised by the presence of mannose and galactosamine (Nichols et al., 2004, 2005). Examples of extreme environments from which biosurfactant-producing microbes have been isolated and cultured under laboratory conditions include oil reservoirs (Arora et al., 2019; references in Nikolova and Gutierrez, 2020), cold environments (e.g. polar regions) (Gesheva et al., 2010; Malavenda et al., 2015; Casillo et al., 2018; Perfumo et al., 2018), salt lakes (Béjar et al., 1998; Amjres et al., 2015), and hydrothermal vents (Raguénès et al., 1996; Rougeaux et al., 1998). Moreover, the most obvious place to search for marine biosurfactant-producing microorganisms is in hydrocarbon-polluted areas since biosurfactants play an important part in the process of microbial biodegradation of hydrocarbons (Chandankere et al., 2014).

Most biosurfactant-producing microorganisms can survive and even thrive in a wide range of temperatures, pH and salinity and therefore exhibit a wide range of metabolic processes. The majority of isolated and cultured microorganisms are aerobic

because they are relatively easy to be sampled and handled in laboratory conditions. However, the number of biosurfactant-producing microorganisms, largely comprising anaerobes, that are being discovered and successfully cultured in *ex-situ* conditions is steadily growing (VanFossen et al., 2008). Anaerobic microorganisms, however, are also able to produce biosurfactants and have typically been found in oil reservoirs where anaerobic hydrocarbon biodegradation processes occur through methanogenesis (Head et al., 2003; Jones et al., 2008). *Bacillus lichenformis*, for example, can grow and produce biosurfactants under both aerobic and anaerobic conditions (Javaheri et al., 1985; Al-Sayegh et al., 2015), however, the biomass and surfactant yields under such conditions can significantly lower compared to under conditions (Yakimov et al., 1995).

1.6 Growth requirements

Oxygen transport, adequate availability and origin (organic or inorganic) of nutrients and trace elements, temperature, pH, and culture medium are important parameters for fermentation process, principally for optimization and scaling-up of production of microbial biosurfactants (Chen *et al.*, 2015). Marine microorganisms, for example, require between 3-15% (w/v) NaCl for optimal growth (Tripathi et al., 2018). In particular, temperature, dissolved oxygen, and nutrients origin (eps. carbon and nitrogen) will be discussed briefly below.

1.6.1 Temperature

The optimal temperature for biosurfactant-producing microorganisms would vary greatly depending on the environment from which they were isolated. However, in many cases the optimal temperature for culturing biosurfactant-producing microorganisms has been reported to range between 25 to 30°C (Lopes et al., 2015). Higher temperatures can cause an increase in growth and, thus biomass, but lower consumption of the carbon source, and consequently lower biosurfactant yield. Exception are thermophilic microorganisms isolated from extreme environments, which require higher than normal temperatures (>30°C) to thrive. For example, thermophilic *Bacillus* species, isolated from oil reservoirs where temperatures often are in the range of 45-70°C, grow and synthesise biosurfactants only in temperatures as high as 40-55°C under laboratory conditions (Joshi et al., 2008; Al-Wahaibi et al., 2014; Daryasafar et al., 2016). In contrast, some strains isolated from low-temperature environments are reported to tolerate wider temperature ranges. For example, isolates from the Antarctic (<5°C) have been shown to grow and

produce biosurfactants even at temperatures above 25°C under laboratory conditions (Janek et al., 2013; Malavenda et al., 2015; Perfumo et al., 2018; Hao et al., 2019).

1.6.2 Dissolved oxygen

Strictly aerobic biosurfactant-producing microorganisms require oxygen for growth and for production of biosurfactants as both processes are energy dependent. Constant aeration of the liquid medium throughout the fermentation is usually applied by agitation. Dissolved oxygen and mass transfer efficiency can critically affect biosurfactant production, as has been shown for surfactin from *B. subtilis* (Sheppard and Cooper, 1990; Guez et al., 2008). Theoretically, higher concentrations of dissolved oxygen would result in faster nutrient consumption, an increase of the cell biomass and biosurfactant production but also lead for faster cell death caused by nutrient depletion by the end of the fermentation (Fonseca et al., 2007). Higher substrate intake was achieved by increasing the agitation speed as it was observed for *B. subtilis* which utilised almost 100% of growing substrate under 150 and 250 rpm compared to 78% at 50 rpm (Fonseca et al., 2007). Therefore, the fermentation can be significantly shortened and ended before nutrient depletion occurs. Agitation speed of 250 rpm was reported to be optimal for rhamnolipid production by *Pseudomonas aeruginosa* S2 which yielded 5.3 g/L from glucose-based medium (Chen et al., 2007) and speed of 200 rpm for some bacterial EPS (Liu et al., 2013; Hao et al., 2019). The highest quantities of sophorolipids from *Candida* was produced (75 g/L) with much higher aeration of ~350 rpm (Kurtzman et al., 2010). Agitation speed higher than 350 rpm, however, can result in hydrodynamic stress (shear) and consequently cell damage, and potentially also result in the accumulation of copious amounts of foam which, in turn, can affect biosurfactant yield (Chen et al., 2007, 2015).

1.6.3 Carbon and nitrogen source

The carbon source composition also has a strong influence on the production yield of crude biosurfactants. Biosurfactant-producing microbes can utilise both water-soluble (hydrophilic) and water-insoluble (hydrophobic) carbon sources. Hydrophobic carbon substrates, such as oils, are especially effective at promoting the production of biosurfactants. Hydrophilic carbon sources, on the other hand, promote the production of polymeric bioemulsifiers. Typically, hydrocarbons have been used as growth substrates to produce surface-active compounds, mainly biosurfactants and less so for bioemulsifiers, because the organisms isolated by researchers for producing these biomolecules have often been found to be hydrocarbon-degrading bacteria, such as the

OHCB. For instance, hydrocarbon substrates stimulated higher biosurfactant production from *Alcanivorax*, which is an OHCB, compared to a non-hydrocarbon substrate (Qiao and Shao, 2010; Antoniou et al., 2015). In recent years, cheaper and sustainable carbon sources have come into more favour in order to reduce production costs and increase biocompatibility of surfactant molecules for human applications (Banat et al., 2014). Most commonly, glucose, sucrose or starches are used as a hydrophilic carbon source (Zhang and Chen, 2010; Joshi et al., 2016; Raddadi et al., 2017), and vegetable oils, fatty acids, or alkanes as hydrophobic carbon sources (Desai and Banat, 1997; Rahman et al., 2002; Sahoo et al., 2011; Das et al., 2014).

Vegetable oils can be classified as the most economical and profitable carbon substrate for large-scale biosurfactant production. Oils that contain fatty acids with chain length of less than C_{10} (e.g. olive, sunflower) incorporate easier into surface-active biomolecules (Banat et al., 2014). Smaller scale studies have demonstrated that waste vegetable oils are promising substrates to produce biosurfactants from species *Pseudomonas* (Soares da Silva et al., 2019), and *Candida* (Pinto et al., 2018; Niu et al., 2019) with yields of 40.5 g/L and 61 g/L, respectively. In some cases, a combination of hydrophilic and hydrophobic carbon sources can significantly increase the biosurfactant yield as demonstrated for sophorolipids (Rau et al., 1996). It was reported that for sophorolipid production from *C. bombicola* ATCC22214, for example, a 1:1 mixture of glucose and corn oil resulted in impressive yield of over 400 g/L (Pekin et al., 2005). Industrial wastes from the food and agriculture industries (e.g. molasses, corn steep liquor, peanut oil cake, cassava waste, waste cooking oil, mill waste etc.) have also been particularly popular as low-cost substrates with great potential for creating economically competitive sustainable products, while at the same time providing an effective solution for the current global waste crisis (Moya Ramírez et al., 2016; Satpute et al., 2017; Singh et al., 2018).

After carbon, nitrogen is the next most important limiting nutrient, with the carbon-to-nitrogen ratio (C:N) being critical. In general, a high C:N ratio is preferred for high-yield production of rhamnolipids (Prieto et al., 2008; Moya Ramírez et al., 2016), lipopeptides (Cubitto et al., 2004; Fonseca et al., 2007; Parthipan et al., 2017) and most EPS (Becker et al., 1998; Hao et al., 2019). Nitrogen can be provided either as an organic or as an inorganic compound. Popular nitrogen sources used in fermentations of biosurfactant-producing bacterial cultures are urea, uric acid, yeast extract, peptone, ammonium nitrate, and corn steep liquor (Palaniraj and Jayaraman, 2011). For example,

urea and yeast extract were the optimal nitrogen source for lipopeptide production from *B. subtilis*, resulting in the lowest ST and the highest amount of biosurfactant (Makkar and Cameotra, 1997; Parthipan et al., 2017). High nitrogen levels in the early stages of fermentation support rapid cell growth but also yields a purer product.

1.7 Current exploitation of biosurfactants

Biosurfactants are becoming important biotechnology products for many industrial applications including in food, cosmetics and cleaning products, pharmaceuticals and medicine, and oil and gas. The global market revenues generated by biosurfactants exceeded USD 1.5 billion in 2019 and is projected to grow at over 5.5% CARG[1] between 2020 and 2026 (Ahuja and Singh, 2020). Household detergents are the largest application market, followed by cosmetics and personal care, and the food industry (Singh et al., 2018). Key manufacturers of biosurfactants include Ecover, Jeneil Biotech, Evonik, and Biotensidon among others (Table 1-1). Europe has over half of the market share followed by the United States and Asia (Singh et al., 2018). The increasing global interest in biosurfactants is due to their low toxicity, biodegradability, low environmental footprint and impact (Desai and Banat, 1997).

1.7.1 Food ingredients

Bacterial biosurfactants and bioemulsifiers (e.g. surface-active EPS) find many applications as food additives with emulsifying, antioxidant, antiadhesive, and emulsion-stabilising properties (Campos et al., 2013a). At the industrial scale, sphingans and xanthan gum (EPS) are the most commonly used, finding usage in many types of foods, from soft baked goods (e.g. for binding water and improving texture), beverages (to enhance mouth feel by maintaining suspension of fruit pulp particles), dairy products (to stabilise emulsions, such as in ice-creams, soft cheeses, yogurts etc.), salad dressings (for long-term emulsion stability, and provide ease of pourability and good cling), pet food (to homogenise gelled products), soups (for good temperature stability) and others (Palaniraj and Jayaraman, 2011). Xanthan can thicken liquids without materially changing the colour or flavour of foods or beverages. Adding a small quantity of the gum can produce a large increase in the viscosity of a liquid, so in most foods is used at concentrations of only 0.5%.

[1] Cumulative annual growth rate.

1.7.2 Cosmetics and cleaning products

Sophorolipids and rhamnolipids are commonly used in household cleaning and cosmetic products due to their excellent surface-active properties. Several companies in France, Japan and Korea, for example, produce sophorolipids to make dishwasher formulations and vegetable wash. Saraya Co. Ltd. manufactures sophorolipids using the basidiomycetous yeast *Pseudozyma* with palm oil as the main fermentation substrate. Ecover markets some household cleaning products containing sophorolipids, whereas the Korean company MG Intobio uses it in some of its soap products for acne treatment. The French company Soliance also produces sophorolipids in skin care with antibacterial and sebum regulator activities. The Spanish company Lipotec S.A.U produces exopolysaccharides from marine derived *Pseudoalteromonas antarctica* NF3 with high content of hyaluronic acids for use in advance skin care products for cosmetic brands such as The Ordinary, The White Company, Tarte and Lumene. Xanthan gum is also widely used in cosmetics and personal care products as emulsion stabiliser, binder, film formers, viscosity-increasing agents in creams, lotions, toothpaste, shampoo and make-up.

1.7.3 Biomedical sciences

Owing to their antimicrobial and antifungal properties, some biosurfactants find a wide range of applications in biomedical and pharmaceutical industries (Singh and Cameotra, 2004). For example, surfactin biosurfactants synthesised by *B.subtilis* and rhamnolipid from *Pseudomonas* sp. stain IMP67, have shown exceptional antibiotic and antifungal activity (Stein, 2005; Das et al., 2014). In addition, anticancer properties of surfactin have been known for several decades and recently demonstrated in suppressing TPA[2]-induced human breast cancer (Park et al., 2013; Duarte et al., 2014) and several others including colon, leukaemia, and pancreatic by inhibiting cancer growth and metastasis (Wu et al., 2017). Recently, lipopeptides (surfactin, iturin, lichenysin) isolated from vaginal probiotic lactobacilli showed strong anti-biofilm (up to 74% reduction) and antagonistic properties against common human pathogenic yeast *Candida albicans* which causes urinary tract, genital, oral, and skin infections (Nelson et al., 2020). Glycolipid MELs have been shown to possess several antimicrobial, immunological and neurological properties demonstrated by stimulating expression of cancer cell differentiation (Wakamatsu et al., 2001). Microbial exopolysaccharides have also been

[2] A.k.a 12-*O*-tetradecanoylphorbol-13-acetate (tetradecanoylphorbol acetate). It is an inflammatory cytokine that promotes tumour cell proliferation.

shown to have promising anticancer properties, especially over-sulphated polysaccharides from *Halomonas* spp. (Ruiz-Ruiz et al., 2011; Raveendran et al., 2013; Kokoulin et al., 2020) which act similarly to heparin[3]. In general, marine-derived microbial EPS could be useful for biomedical applications because of the relatively high number of therapeutic compounds such as glycosamineglycans (e.g., hyaluronic acids and heparin) present in their composition. Hyaluronic acid, in particular, has strong visco-elastic properties that find application in osteoarthritis treatment, wound healing and as wrinkle filler.

[3] Heparin is a polyanionic molecule with anticoagulant, anti-thrombotic, anti-inflammatory, antiviral and anticancer properties extracted from pig intestines and widely used in medicine.

Table 1-1. Biosurfactant and EPS producing companies. Adapted from Randhawa and Rahman (2014).

Company (website)	Location	Products	Industry application
AGAE Technologies LLC (www.agaetech.com)	USA	Rhamnolipids	Pharmaceuticals, cosmetics, personal care, bioremediation, EOR
Jeneil Biotech (www.jeneilbiotech.com)	USA	Rhamnolipids	Cleaning products, EOR
Biotensidon (www.biotensidon.com)	Germany	Rhamnolipids	Agriculture (plant pest control), cosmetics, cleaning products, EOR
Frauhofer IGB (www.igb.fraunhofer.de)	Germany	Glycolipids, MELs	Cleansing products (shower gels, shampoos, washing-up liquids)
Saraya Co. Ltd (worldwide.saraya.com)	Japan	Sophorolipids	Cleaning products, cosmetics, hygiene products
Ecover (www.ecover.com)	Belgium	Sophorolipids	Cleaning products, cosmetics, bioremediation, pest control, pharmaceuticals
Groupe Soliance (www.soliance.com)	France	Sophorolipids	Cosmetics
MG Intobio Co. Ltd	South Korea	Sophorolipids	Beauty and personal care
Evonik (corporate.evonik.com)	Germany	Sophorolipids	Cleansing products
Lipotec S.A.U (www.lipotec.com)	Spain	Marine EPS	Cosmetics, personal care
Biopolymer International (www.biopolymer-international.com)	Belgium	Xanthan and gellan gums	Food, personal care, pharmaceuticals, oil drilling, animal feed

1.7.4 Oil and gas industry

1.7.4.1 Soil bioremediation

Hydrocarbon soil contamination, such as from drilling, leaking pipelines, storage tanks, transportation etc., is a widespread problem with long lasting environmental impacts. Being highly hydrophobic, particularly when adsorbed onto soil particles, hydrocarbons, and heavy metals are very resistant to removal. Typically, a variety of physical and chemical treatments, such as removal, incineration, soil washing, and solvent extraction have been used successfully in the past. However, such techniques are deeply damaging to the soil structure and the autochthonous biodiversity, as well as cost-

prohibitive. As such, bioremediation is the preferred soil treatment due to its efficiency, lower environmental impact and cost-effectiveness. Bioremediation involves naturally occurring soil microorganisms which convert petroleum hydrocarbons into carbon dioxide, water and cell biomass. There are many factors that influence the rate and extent of hydrocarbon degradation in soils, such as moisture content, aeration, pH, temperature, the biological condition of the soil (aged vs fertile soils; nutrient content and bioavailability), and the concentration, molecular structure and bioavailability of the hydrocarbon contaminants (Venosa and Zhu, 2003; Huesemann, 2004). The optimisation of these environmental factors is critical for the bioremediation success.

Soil bioremediation can be conducted either in place (i.e. *in-situ*), or the contaminated soil is upended and, transported to be subsequently treated elsewhere (*ex-situ*). *In-situ* bioremediation involves, generally, the treating of only the top 30-cm layer of the soil with fertilisers to stimulate indigenous soil microorganisms to break down the hydrocarbons (Atlas and Hazen, 2011). This treatment is the preferred method of choice, but the risk of contaminating underlying aquifers with dissolved hydrocarbons must be considered. Partially purified biosurfactants have been used *in-situ* to increase the solubility and bioavailability of hydrocarbons, and other hydrophobic contaminants, by increasing their surface area (Ron and Rosenberg, 2002; Bustamante et al., 2012). A field trial on LaTouche Island in Alaska demonstrated that a biologically derived surfactant, PES-51, could remove 30% of semi-volatile petroleum hydrocarbons from a subsurface beach material (Tumeo et al., 1994). However, the majority of bioremediation studies with biosurfactants are under laboratory conditions. A study from Argentina demonstrated that surfactin from *B. subtilis* strain O9 contributed to significantly more removal of crude oil from sandy loam soil than in soil without surfactin within a period of 300 days (Cubitto et al., 2004). The addition of rhamnolipid from *Pseudomonas aeruginosa* strain SSC2 to crude oil-contaminated soil sludge resulted in 98% degradation after four weeks compared to the non-rhamnolipid control treatment (67%). The effect was enhanced by adding nutrients to the treatments (Cameotra and Singh, 2008). An *in-situ* experiment of soil bioremediation conducted near oil production facility in Pakistan demonstrated that higher crude oil degradation (up to 77%) was achieved in soil treated with a combination of a specialised bacterial consortium, rhamnolipids and nutrients (Tahseen et al., 2016). The efficiency of MELs produced by *Candida antarctica* SY16 to degrade crude oil in soil was investigated by Baek *et al.* (2007). The authors compared different bioremediation techniques (i.e., natural attenuation, biostimulation,

bioaugmentation, biosurfactant addition, and a combination of all) and concluded that the combined treatment of biostimulation, bioaugmentation with oil degrading *Nocardia* sp. H17-1 and with MELs caused the highest total petroleum hydrocarbon degradation rate during the first 4 weeks of treatment. However, at the end of the experiment (100 days) the amount of residual hydrocarbons was similar for all treatments (Baek et al., 2007).

1.7.4.2 Marine bioremediation

Crude oil is highly hydrophobic and hence has a very low water solubility, although it is composed of thousands of hydrocarbon and non-hydrocarbon species and metals, each with their respective aqueous solubilities. When an oil is introduced into a water phase, the oil will float on top of the water phase due to the action of ST, the force of attraction or repulsion between the molecules of oil and water at their interface. Together with viscosity, surface tension is an indication of how rapidly and to what extent an oil spreads on water. The lower the interfacial tension with water, the greater is the extend of spreading (Fingas, 2011). To increase the solubility of oil in water (i.e., to decrease the surface tension between oil and water), chemicals are applied to an oil slick (Brakstad et al., 2015). Dispersed oil is usually in the form of fine neutrally buoyant droplets with higher surface area-to-volume ratio (diameter size 1-70μm) compared to non-dispersed oil, thus making the oil available for biodegradation by hydrocarbon-degrading bacteria (ITOPF, 2011). The natural fate of crude oil biodegradation (biological oxidation by microorganisms) in the marine environment has been extensively described (Atlas and Hazen, 2011; Kostka et al., 2011; Wade et al., 2011; Campo et al., 2013; Prince et al., 2013; Hazen et al., 2016; Joye et al., 2016b; Seidel et al., 2016).

Marine bioremediation research has largely been limited to application of fertilisers and/or seed cultures of highly efficient oil-degrading microorganisms, though with conflicting results (Prince, 2010). The limitation of marine oil remediation when relying solely on indigenous microorganisms is that the concentrations of cells in oil-polluted open water systems is never high enough to effectively emulsify oil (Ron and Rosenberg, 2002). The addition of surfactants (biogenic or synthetically produced) aims to disperse/emulsify the oil and, in turn, speed up the biodegradation process. Biosurfactants, have been shown to be effective in dispersing crude oil and enhancing the biodegradation process only under laboratory conditions due to logistical, financial and regulatory limitations of conducting large-scale field trials. In a laboratory study, a bacterial consortium containing oil degrading strains of *Ochrobactrum* and *Brevibacillus*

with/without rhamnolipids were added to crude oil in 1L water tanks to stimulate marine oil spill bioremediation. The results showed that the removal efficiency of oil by the bacterial consortium alone was lower by 6% compared to the combination of consortium and biosurfactant (Chen et al., 2013). The authors also noted that the presence of rhamnolipid enhanced the biodegradation of alkanes of chain length > nC$_{15}$, but, interestingly, had the opposite effect on shorter chain alkanes (nC$_{13}$-C$_{15}$) by reducing their solubility and increasing their stability. However, the overall removal efficiency of *n*-alkanes by the bacterial consortium and rhamnolipid was higher than the control. Similar trend was observed for PAH and biomarkers (Chen et al., 2013). These results were more or less consistent with another study which also used rhamnolipids in combination with a pre-adapted bacterial consortium (Nikolopoulou et al., 2013a), and which were more pronounced when nutrients were added to the treatments. The average specific degradation rate was reported to be 23, 20 and 10 times higher than the control for nC$_{15}$, nC$_{20}$ and nC$_{25}$, respectively. The rhamnolipid also stimulated the growth of hydrocarbon degraders within five days, which were able to utilise 50% of the crude oil saturated fraction. In addition, LMW PAHs and, notably, the biomarkers pristane and phytane were also significantly degraded in the presence of rhamnolipid (Nikolopoulou et al., 2013a). The origin of nutrients (i.e. organic lipophilic or water-soluble) that are added together with rhamnolipids to the treatments can further enhance the degradation of crude oil in seawater and sediment environments (McKew et al., 2007; Nikolopoulou and Kalogerakis, 2008; Nikolopoulou et al., 2013b).

All of these studies, however, focused entirely on the degradation rate of crude oil on a handful of selected oil-degrading bacteria without investigating the indigenous marine microbial community response as a whole. Understanding how the natural microbial communities is affected by the crude oil with/without presence of biosurfactants is crucial for advancing the rationale for further research into their suitability for oil spill response. Chapter 4 of this thesis contains an exploration of this topic.

1.7.4.3 Microbial enhanced oil recovery (MEOR)

MEOR is a process in which microorganism and/or their metabolic by-products are injected into mature oil reservoirs for the recovery of residual crude oil that was not extracted during the initial and secondary extraction processes. The idea behind MEOR is that when favourable conditions are present in the reservoir, the introduced microbes

grow exponentially and their metabolic products would mobilise the residual oil (Gao and Zekri, 2011). MEOR bares with it its advantages and limitations, and the various processes of its application have been described extensively in the literature and recently summarised by Nikolova and Gutierrez (2020).

MEOR is based on two fundamental principles. Firstly, oil movement through porous media (rock formation) is facilitated by altering the interfacial properties of the oil-water-minerals displacement efficiency (i.e., decrease in IFT to increase the permeability of media), driving force (reservoir pressure), fluidity (miscible flooding; viscosity reduction), and sweep efficiency (selective plugging; mobility control). The second principle constitutes the degradation but also the removal of sulphur and heavy metals from heavy oils, by microbial activity (Shibulal et al., 2014). In the majority of MEOR field trials, injection of indigenous (or other MEOR suitable) pre-cultured bacteria or a consortium of bacteria along with nutrients (e.g. oxygen, nitrogen) has been the preferred choice of method because bacteria can produce biosurfactant *in-situ* which in turn significantly reduces the operational costs (Lazar et al., 2007; Geetha et al., 2018). However, biosurfactants produced *ex-situ* can also be used to enhance the microbial growth in oil reservoirs. In addition, when poor oil recovery from an oil well is due to low permeability of the rock formation, or to the high viscosity of the crude oil, the ability of biosurfactants to reduce IFT between the flowing aqueous phase and the residual oil saturation can improve the recovery process (Brown, 2010). Reduction of IFT by biosurfactants can also reduce the capillary forces that prevent the oil from moving through rock pores, however, the decrease in IFT must be at least two orders of magnitude to achieve mobilisation of the oil. Typically, IFT between hydrocarbons and water is between 30 and 40 mN/m. For biosurfactants to have any effect in MEOR, they must reduce the IFT to 10^{-3} mN/m (Gray et al., 2008). To our knowledge such values have not yet been reported for known biosurfactants, and hence the effectiveness of IFT reduction may be limited in practice. Moreover, considering the type of oil reservoir (sandstone, carbonates etc.), residual oil saturation and the incremental oil recovery, the volume of biosurfactant that needs to be injected to achieve 30-60% oil recovery rate could be quite substantial and hence not practical or economical (Kowalewski et al., 2006; Gray et al., 2008; Afrapoli et al., 2011). In addition to reduction of IFT, biosurfactants can alter the wettability of rock formations, emulsify the crude oil, and contribute to the microbial metabolism of viscous oil (Sen, 2010).

Nevertheless, there are some promising results reported from different research groups that investigate biosurfactants for MEOR applications. Of all known biosurfactants, lipopeptides were mostly used in laboratory-based MEOR studies due to their ability to reduce the IFT to below 0.1 mN/m (Youssef et al., 2007; Gudiña et al., 2012; Pereira et al., 2013). Both bench-scale and *in-situ* lipopeptide production by stains of *Bacillus* spp. have proven successful in improving the oil recovery, including from wells close to their production limits (Al-Wahaibi et al., 2014; Al-Sayegh et al., 2015). Surfactins have been shown to maintain activities under a wide range of temperature, pH, and salinity while able to recover sand trapped oil. For example, *B. subtilis* produced surfactin at high temperature which could emulsify diesel with 90% efficiency and recover over 60% of oil entrapped in sand core (Makkar and Cameotra, 1997). Surfactin was recently shown to alter the wettability of CO_2 injected in a subsurface rock formation demonstrating its potential suitability in carbon capture and storage application (Park et al., 2017). Lichenysin was reported to reduce the IFT to values of less than 10^{-2} mN/m (even at low concentrations of 10-60 mg/L) and to have exceptional stability under temperatures as high as 140°C, a pH ranges from 6 to 10, at salinities up to 10% NaCl, and at calcium (as $CaCl_2$) concentrations up to 340 mg/L. In core flooding experiments, partially purified lichenysin recovered up to 40% of residual oil from sandstone cores compared to 10% recovery when chemical surfactants were applied (McInerney et al., 1990). Addition of biosurfactants during chemical surfactant flooding can improve the flooding performance in general. In the presence of rhamnolipids, the adsorption to sandstone of the surfactant alkylbenzene sulfonate was reduced by 25-30% and the quality of oil recovery increased by 7%. It has been suggested that rhamnolipids act as sacrificial agents by preferably adsorbing to the oil sands, making the surfactant more available for displacement activity and resulting in altering the wettability of porous media (Perfumo et al., 2010a). Macromolecular biopolymers such as emulsan were shown to remove up to 98% of pre-adsorbed crude oil to limestone core samples, even at low concentration of 0.5 mg/ml (Gutnik et al., 1983). Recently, another biopolymer produced by *Rhizobium viscosum* CECT908 showed better efficiency than xanthan gum in the recovery of heavy oil (Couto et al., 2019).

1.8 Thesis layout

This thesis is split into six chapters. Chapter 1 (herein) provides an overview of the thesis and literature review on different types of biosurfactants and the microorganisms

that produce them, as well as their application by the oil industry. In this thesis, two bacterial strains were investigated for production of biosurfactants. After initial screening the two stains, namely *Pseudomonas* sp. strain MCTG214(3b1) and *Halomonas* sp. strain TGOS-10, were able to produce biosurfactants. In addition, *Halomonas* TGOS-10 produced bioemulsifier too. Chapter 2 presents the results of an investigation of the chemical composition of the bioemulsifier produced by *Halomonas* sp. strain TGOS-10, and its suitability to emulsify hydrocarbon compounds. Chapter 3 investigates the characteristics of the biosurfactants produced by *Halomonas* sp. strain TGOS-10 and *Pseudomonas* sp. strain MCTG214(3b1) and their effectiveness and efficiency in dispersing crude oil. Chapter 4 follows a case study from the Faroe-Shetland Channel where *in-situ* surface seawater was used to investigate the microbial response to crude oil amended with either biosurfactant or chemical dispersant. For this Illumina MiSeq and GC-MS/FID analyses of the crude oil were used to assess the microbial response and their oil biodegradation activities as a function of amending the seawater with and without the biosurfactant or synthetic chemical dispersant. Advanced bioinformatics and statistics were also used to analyse the results, which were used for further *in-silico* ecological analysis in Chapter 5. Chapter 5 looks at the use of null models to better understand the ecological drivers in the microbial communities amended with crude oil, biosurfactant and a synthetic chemical dispersant. Final conclusions and further considerations are provided in Chapter 6.

Table 1-2. Biosurfactant producing bacteria from marine, hydrocarbon-polluted soil, oil reservoir, or other origin. Empty fields mean that these parameters were not determined by the source. Table continues on next page.

Environment	Organism	Carbon source	ST (mN/m)	EI_{24} (%)	Chemical composition of surfactant	Reference
Marine	*Alcanivorax borkumensis*	Alkanes	29		Glucose-lipid	Yakimov et al. (1998)
Marine	*Alcanivorax dieselolei*	Alkanes	35	51	Glycolipid	Hassanshahian et al. (2012)
Marine hydrothermal vent	*Alteromonas infernus*	Glucose			Acidic EPS	Raguénès et al. (1997b)
Marine hydrothermal vent	*Alteromonas macleodii*	Glucose			Sulphated EPS	Raguénès et al. (1996) Rougeaux et al. (1998)
Marine	*Arthrobacter sp. EK1*	Alkanes			Trehalose lipid	Passeri et al. (1991)
Oiled soil	*Bacillus cereus IAF 346*	Sucrose	28	60	EPS	Cooper and Goldenberg (1987)
Oil reservoir	*Bacillus methylotrophicus USTBa*	Alkanes, PAH	28		Glycolipid	Chandankere et al. (2014)
Oiled soil	*Bacillus pumilus 2IR*	Crude oil/glucose	32	>60	Lipopeptide	Fooladi et al. (2016)
Oiled sludge	*Brevibacillus sp. PDM-3*	Phenanthrene	47	57	Glycolipid	Reddy et al. (2010)
Other	*Burkholderia thailandensis*	Glycerol	32	42	Rhamnolipid	Díaz De Rienzo et al. (2016)
Oil reservoir	*Clostridium sp. N-4*	Sucrose	32		Glycoprotein	Arora et al. (2019)
Marine	*Cobetia sp. MM1IDA2H-1*	PAH	33	44	Lipidic surfactant	Ibacache-Quiroga et al. (2013)
Marine	*Colwellia psychrerythraea 34H*				Capsular EPS	Casillo, Ståhle, et al. (2017)
Marine	*Corynebacterium kutscheri*	Motor oil; Peanut oil			Glycolipopeptide	Thavasi et al. (2007)
Oil field	*Corynebacterium lepus*	Kerosene	30		Lipopeptide	Cooper et al. (1979)
Marine	*Enterobacter cloaceae 71a*			>60	EPS	Iyer et al. (2006)
Oiled soil	*Escherichia fergusonii KLU01*	Diesel oil	32		Lipopeptide	Sriram et al. (2011)
Marine	*Flexibacter sp. TG382*	Glucose	67	>60	Glycoprotein EPS	Gutiérrez et al. (2009)
Marine	*Gordonia amicalis LH3*[*]	Paraffins, crude oil			Rhamnolipid	Hao et al. (2008)

Marine	*Halomonas eurihalina*	Glucose, PAH	-	78	Sulfated heteropolysaccharide	Calvo et al. (2002)
Marine	*Idiomarina sp. 185*	Tetradecane	29	30	Glycolipid	Malavenda et al. (2015)
Marine	*Pantoea sp. A-13*	Paraffins, kerosene	30		Glycolipid	Vasileva-Tonkova and Gesheva (2007)
Marine sediment	*Pseudoaltermonas sp. 93*	Tetradecane	63	55	Glycolipid EPS	Malavenda et al. (2015)
Oil reservoir	*Pseudomonas aeruginosa WJ-1*	Alkanes, Veg oils	24	100	Rhamnolipid	Xia et al. (2013)
Other	*Pseudomonas cepacian CCT6659*	Veg oil	27-29	90-100	Rhamnolipid	Soares da Silva et al. (2019)
Marine hydrothermal vent	*Vibrio diabolicus HE800*	Glucose			EPS	Raguénès et al. (1997a)
Oiled soil; marine	*Virgibacillus salanis*[*]	Glucose	30	80	Lipopeptide	Elazzazy et al. (2015)

CHAPTER 2

EXTRACTION AND ANALYSIS OF BIOEMULSIFIER PRODUCED BY *HALOMONAS* SP. STRAIN TGOS-10

2.1 Overview

This chapter aims at understanding the type of bioemulsifier produced by *Halomonas* sp. strain TGOS-10 and its ability to emulsify different hydrophobic substances. A version of this chapters is currently being prepared for the *Applied Microbiology and Biotechnology* journal.

2.2 Introduction

Bioemulsifiers are characterised by their ability to emulsify hydrophobic compounds to form stable emulsions in a hydrophilic phase. Bioemulsifiers are complex polymeric compounds consisting of mixtures of heteropolysaccharides, lipopolysaccharides, lipoproteins and/or proteins, and usually are known to have high molecular weight (HMW) and a diverse set of properties (Decho and Gutierrez, 2017). Their importance as valuable sources of natural products for different industries (e.g. cosmetics, food, pharmaceuticals, bioremediation, and in oil and gas) has been highlighted in numerous studies and reviews (Satpute et al., 2010a; Campos et al., 2013b; Banat et al., 2014; Sałek and Gutierrez, 2016; Geetha et al., 2018; Nikolova and Gutierrez, 2020).

In the oil and gas industry, bioemulsifiers have been predominantly promoted in oil bioremediation. Hydrocarbon contamination of soil and water often is common where oil production, processing and transport occur. Once released in the environment and left untreated, hydrocarbons can seriously alter the local biodiversity and remain in the environment for a long time, prolonging the exposure to contamination and ecological recovery of the site (Atlas and Hazen, 2011). In both soil and water systems, microorganisms are protagonist in the natural attenuation or bioremediation process for removing hydrocarbon contaminants (Head et al., 2006). In some areas, however, bioremediation can be limited by the lack of nutrients or unfavourable conditions in general (i.e. temperature, oxygen, light etc.) (Joye et al., 2016b; Perfumo et al., 2018). In such cases, chemical treatments, usually that are synthetically derived, are applied along with nutrients to speed up the breakdown of oil by indigenous communities of microorganisms. Although highly effective, as briefly discussed in Chapter 1, synthetic chemicals have their limitations in terms of environmental compatibility and toxicity (Kleindienst et al., 2015a; Joye et al., 2016a). Therefore, naturally derived biological compounds, such as bioemulsifiers, have gained significant interest in recent years as they

can be similarly effective in oil bioremediation and do without the negative environmental impacts associated with their synthetic counterparts (Banat, 1995).

In liquid systems, bioemulsifiers increase the surface area of the oil by breaking it up into small droplets and keeping it as an emulsion (i.e., preventing it from coalescing), and as a result increasing its bioavailability to oil-degrading microorganisms. In soil, hydrocarbons bind strongly to soil particles which makes the oil removal and degradation difficult. The addition of emulsifiers can increase the displacement of oil from the soil particles due to the high number of reactive groups in the structure of these surface-active macromolecules which facilitate the adhesion to oil droplets during emulsification (Rosenberg and Ron, 1999). Alasan, a bioemulsifier produced by *Acinetobacter radioresistens*, has been demonstrated to be effective in increasing the solubility of three-ringed PAH phenanthrene (Barkay et al., 1999; Toren et al., 2002). In soil microcosms, exopolysaccharide substances (EPS) from *Ochrobactrum anthropic* strain AD2 enhanced an indigenous microbial population and the hydrocarbon efficiency removal (~80%) after 21 days of treatment (Calvo et al., 2008).

Members of the genus *Halomonas* are ubiquitous bacteria and, of which many species are able to degrade hydrocarbons, but more importantly they are widely recognised for their ability to produce EPS with powerful surface-active properties (discussed in Chapter 1). For this reason, halomonids have received increasing interest to understanding the chemical structure and functionality of their produced EPS. Although there are numerous studies describing the EPS produced by *Halomonas* sp., these are predominantly from hypersaline soils (Béjar et al., 1998; Arias et al., 2003; Mata et al., 2006; Amjres et al., 2015). For example, *Halomonas eurihalina* H-28 was isolated from saline soil and found able to produce EPS when grown on glucose and some medium chain-length alkanes and LMW PAHs as the sole carbon source, but produced the largest amount of EPS on glucose and *n*-hexadecane (Martínez-Checa et al., 2002). The strain's EPS was also capable of emulsifying crude oil at an efficiency of 80% compared to control treatments (50%) where no EPS was added and treatments with the synthetically-derived surfactants Tween 20 (15%), Tween 80 (16%), and Triton X-100 (11%) (Martínez-Checa et al., 2002). Marine-derived halomonids, are often detected in oil-contaminated marine environments and form an important part of the natural oil-degrading microbial community (Hazen et al., 2010; Gutierrez et al., 2013b). Members of the genus *Halomonas* have been shown to degrade various hydrocarbons, such as

PAHs, mostly facilitated via their production of EPS which has been shown to increase the bioavailability of these poorly soluble substrates (Gutierrez et al., 2013a, 2013b). *Halomonas* sp. strain TG39, isolated from a laboratory culture of unclassified marine Chrysophyte (Gutiérrez et al., 2007b), was found to produced HMW EPS that was able to increase (4-fold) the solubilisation of phenanthrene within 8 days of incubation (Gutierrez et al., 2013a). Recently, *Halomonas* sp. strain MCTG39a was demonstrated to produce stable oil-in-water and water-in-oil emulsions with *n*-hexadecane and some PAH with emulsifying activity varying between 30 and 100% (Gutierrez et al., 2020).

While the genes and pathways involved in LMW biosurfactants biosynthesis, such as rhamnolipid, are well described (Ochsner et al., 1994; Rudden et al., 2015), there remains a paucity of knowledge and understanding on the expression of genes involved in the biosynthesis of HMW surface-active compounds (biopolymer/EPS), and especially in marine bacteria. Due to the enormous genetic diversity of marine microorganisms, genetic and regulatory pathways are not necessarily universal in different biosurfactant producers. Moreover, different species from the same genus can produce biosurfactants with varying structure and chemistries, which in turn can translate to different functional properties (Rosenberg and Ron, 1999). Better understanding of the chemical structure of each EPS would not only reveal what the EPS are made of, but also how this confers their functionality. There are a number of ways to determine the chemical and structural analysis of HMW bioemulsifiers, from colorimetric assays to advanced mass spectrometry (MS) and nuclear magnetic resonance (NMR). Colorimetric assays, such as Bradford protein assay and the phenol-sulphuric acid method for carbohydrate determination are commonly used for determination of the relative content of protein and carbohydrates, respectively (Smyth et al., 2010b).

In this chapter, the chemical and physical properties of a novel HMW glycoprotein emulsifier produced by *Halomonas* sp. strain TGOS-10 were investigated. This organism was isolated during the active phase of the Deepwater Horizon oil spill from sea surface oil slicks in the Gulf of Mexico, and its produced exopolymer was found to be involved in the formation of marine oil snow (MOS) and enhance the biodegradation of hydrocarbons (Gutierrez et al., 2013a). The produced emulsifier was also tested for its ability to emulsify different hydrocarbon compounds, demonstrating its potential application in oil bioremediation.

2.3 Materials and Methods

2.3.1 Bacterial strain

Halomonas sp. stain TGOS-10 was obtained from Dr Tony Gutierrez's culture collection and was isolated from surface seawater contaminated with crude oil in the Gulf of Mexico (discussed above). *Halomonas* sp. strain TGOS-10 produced both bioemulsifier and biosurfactant. In this chapter, the extraction of the bioemulsifier and its emulsifying properties are discussed.

2.3.2 Growth conditions

Strain TGOS-10 was routinely cultured at 28ºC on Zobell's Marine agar and broth (ZM/1) (Zobell, 1946). The agar plates were used to isolate single colonies that were then cultured in broth medium for seeding. ZM/1 broth was composed of 5 g/L Bacto-peptone, 1 g/L yeast extract dissolved in ¼ distilled water and ¾ filtered natural seawater. The medium was autoclaved at 121ºC for 15 min and then supplemented with sterile 100x marine supplements (0.5% v/v) (Zobell, 1941), 0.5 % v/v (final concentration) glucose and 1% v/v (final concentration) seed culture. Glucose was used as growing substrate for the production of HMW bioemulsifier, whereas sunflower oil was used for the production of LMW biosurfactant (discussed in Chapter 3). Measurements for bacterial growth were taken periodically in triplicates and determined spectrophotometrically at 600 nm (Genesys 6; Thermo Fisher, USA).

For measurement of growth, ST and emulsification index (EI), CMC, stability, and ionic activity, triplicate 2 L Erlenmeyer flasks containing 750 ml of ZM/1 medium were supplemented in the same way as above and incubated at 28ºC for up to 96 hours. Measurements were taken as described below.

2.3.3 Emulsification assay

The emulsification capability of the bacterial strain was measured using the method described by Cooper and Goldenberg (1987) with some modifications. Sample of the culture broth (2ml) was taken periodically during growth and centrifuged at 13,000 g for 10 min to remove bacterial cells. The cell-free broth was mixed with *n*-hexadecane in ratio 1:1 v/v (i.e., 2 ml of broth to 2 ml *n*-hexadecane) in acid-washed (0.1 M HCl) screw-cap glass tubes (100 × 13 mm). The mixtures were manually shaken for 10 sec and

vortexed at full speed for 10 sec, and this procedure repeated four times. The mixtures were then left to stand for 10 min undisturbed at room temperature. The emulsion stability and type (oil-in-water or water-in-oil) was determined after 24 h by calculating the emulsification index (EI_{24}), which was performed by dividing the height of emulsified layer by the total height of the mixture, multiplied by 100.

The same assay was used to measure the EI_{24} produced by solutions of the extracted emulsifier (in deionised water) at different concentrations against the following hydrophobic compounds (ratio 1:1 v/v): *n*-hexadecane, diesel, and motor oil.

2.3.4 Extraction of biopolymer

The method for biopolymer (EPS) extraction was adopted from Gutiérrez et al. (2007). Pure culture of strain TGOS-10 was inoculated in 6 ml ZM/1 medium (Zobell, 1946) supplemented with glucose solution to a final concentration of 0.5% (w/v). The inoculum was set up in acid-washed glass tubes incubated with shaking (80-120 rpm) at 28°C until exponential growth was reached. The growth was observed by measuring the optical density (600 nm) with a spectrophotometer. After the exponential growth was reached, the inoculum was transferred to two 2-litre Erlenmeyer flasks (3 ml inoculum in each flask) each containing 750 ml of ZM/1 medium amended with the same concentration of glucose as previously. The flasks were incubated with shaking (80-120 rpm) at 28°C in darkness. When the exponential growth rate was reached, the culture was centrifuged for 20 min at 13,000 g to remove biomass. The supernatant was filtered through 0.2 μm flow membrane to remove residual cells. The emulsifier was precipitated with KCl (7.5% w/v) added to the filtered supernatant and two volumes of ice-cold ethanol. The mixture was left to mix for 30 min until all of the KCl was dissolved and then to precipitate at 4°C for 24 h. The precipitated EPS was centrifuged for 16 min at 10,000 g and transferred into 1 kDa molecular-weight cut-off Spectra/Por® Biotech cellulose ester membranes (Cole-Palmer, UK) and was extensively dialyzed against distilled water for 4-5 days and then freeze-dried. The freeze-dried product was weighted to determine the yield of crude EPS per litre of culture medium.

2.3.5 Bradford protein assay

The protein content in the dried polymer was determined by the Bradford protein assay based on the binding of Coomassie Brilliant Blue dye to protein molecules under

acidic conditions that causes a colour change from brown to blue which can then be quantified spectrophotometrically (Bradford, 1976). For this, protein standards were prepared with Bovine Serum Albumin (BSA) diluted in distilled water to final concentrations varying from 0 to 1.2 mg/ml. In clean acid-washed glass test tubes, 100 µl of each of the standards and dried polymer (dissolved in PBS at concentration of 1 mg/ml) are mixed with 2 ml of Bradford dye reagent (final concentration of 0.01% (w/v) Coomassie brilliant Blue G-250, 4.7% (w/v) ethanol, and 8.5% (w/v) phosphoric acid). Mixtures were vortexed for 10 sec and incubated at room temperature for 5 min. The absorbance of each standard and polymer sample was measured in triplicates at 590 nm using polystyrene cuvettes in a spectrophotometer. Blanks were prepared with 100 µl of distilled water and 2 ml Bradford dye reagent. A standard curve was constructed with the absorbance readings corresponding to the BSA standard concentrations. The equation of the standard curve was used to calculate the concentration of protein in a solution of the TGOS-10 EPS of known concentrations (0.25, 0.5, and 1 mg/ml).

2.3.6 Carbohydrate assay

The carbohydrate content in the dried TGOS-10 polymer was determined by the method of Dubois et al. (1956). First, glucose standards were prepared in the following concentrations: 0, 20, 40, 60, 80, 100 µg/ml. In clean acid-washed glass test tubes, 200 µl of each standard and TGOS-10 EPS (dissolved in PBS at concentration of 1 mg/ml) was mixed to 200 µl of 5% phenol. Then 1 ml of concentrated H_2SO_4 was added directly to the mixture's surface to produce good mixing and even heat distribution. The mixtures were left to stand for 15 min at room temperature, vortexed for 10 sec, and then left to stand for another 30 min. The absorbance of each standard and polymer sample was measured in triplicates at 490 nm using polystyrene cuvettes. A sample containing no glucose was used as a blank. Similarly to the protein assay, a standard curve was constructed with the absorbance readings corresponding to the glucose standards and was used to calculate the concentration of carbohydrate in a solution of the TGOS-10 EPS of known concentrations (100, 250, 500 and 1000 µg/ml).

2.3.7 Amino acid, monosaccharide and molecular weight analyses

Total amino acids content was determined by an external facility. For this, acid hydrolysis was performed on 3 mg of the polymer sample which was dissolved in 2 ml of 6M HCl and hydrolysed for 24h under vacuum and then dehydrated and diluted in

0.1M NCl. Analysis was performed using a Waters 2695 Separations Module, a 2487 Dual Absorbance Detector and a 1515 Isocratic high-performance liquid chromatography pump equipped with a 300 x 3.5 mm Laborsevide 7-micron resin cation exchange column. Quantification was performed using a Sigma Acid Standard (AAS18) external standard. The total protein content was calculated from the individual amounts of amino acids.

Total monosaccharides and molecular weight analysis were performed by Dr Gordon Morris at the University of Huddersfield. To determine the monosaccharide composition, TGOS-10 biopolymer (2 mg) was dissolved in 1 mL of 4 M trifluoroacetic acid and hydrolysed at 121 °C for 2 h (Denman and Morris, 2015). The samples were then prepared for analysis by high-performance anion exchange chromatography coupled with pulsed amperometric detection (HPAEC-Pad) using a Dionex Carbopac PA-20 column on a Dionex ICS-3000 Ion Chromatography System (Dionex Corp. Sunnyvale, USA) and eluted with 0.01 M NaOH at a flow rate of 0.3 ml/min for 20 minutes to elute neutral sugars and then for a further 20 minutes with 1 M NaOAc in 0.15 M NaOH to elute uronic acid residues. The monosaccharide composition was then quantified in duplicate using external standards (fucose, rhamnose, galactosamine, arabinose, glucosamine, galactose, glucose, xylose, mannose, galacturonic acid and glucuronic acid).

The molecular weights and molecular weight distributions of TGOS-10 biopolymer were absolutely measured using an analytical system with on-line light scattering. The analysis was performed on size exclusion chromatography coupled to multi-angle light scattering and refractive index (SEC-MALS-RI) system composed from Agilent 1200 Infinity Series Analytical LC System (1200 Vacuum Degasser, 1260 Infinity Binary Pump and Auto-sampler, Agilent Technologies LDA UK Limited, Stockport, UK), connected in-line to a DAWN 8+ MALS and Optilab T-REX RI (Wyatt Technology Corporation, Santa Barbara, USA). The TSK G6000 PW (7.5 mm × 30 cm), TSK G5000 PW (7.5 mm × 30 cm) and TSK G4000 PW (7.5 mm × 30 cm) size-exclusion chromatography columns were connected in series and protected by TSK SWXL Guard column (6 mm × 4 cm) (Tosoh Bioscience, Tokyo, Japan). The columns were eluted with a 0.1 M NH_4OAc + 0.05% Na_3N mobile phase at flow rate of 0.6 ml/min and temperature of 30 °C. The injection volume was set to 100 µl. Each sample was filtered via 0.45 µm regenerated cellulose (RC) syringe filter (17 mm, Target2™, Thermo Fisher Scientific,

Paisley, UK) and sample concentration equalled 5 mg/ml. Before each sequence of samples, the normalization procedure was performed with a BSA standard (2 mg/ml), prepared in the same solvent. The chromatograms were recorded with Astra software v. 6.1.5 (Wyatt Technology Corporation, Santa Barbara, USA) using a refractive index increment (*dn/dc*) of 0.150 ml/g (Harding et al., 1991; Theisen et al., 2000).

2.3.8 Proteolytic digestion

Proteolytic digestion of the emulsifier was attempted in order to digest the protein portion of the EPS into smaller peptides by enzymatic digestion and, therefore, obtain clearer NMR spectra of the polysaccharide peaks. For this, 20 mg of the dried emulsifier was dissolved in 2.5 ml Proteinase K solution containing 5 mg Proteinase K (Fisher Scientific, UK) in Proteinase buffer (50 mM TrisHCl buffer pH 7.5; 1 mM CaCl2). The mixture was incubated at 37°C for minimum 16 h and then placed in boiling water for 5 minutes to inactivate the Proteinase K. The solution was left to cool down before centrifuging it at 10,000 g for 10 min to precipitate down the protein. The supernatant containing the polysaccharides was gently transferred into 10 kDa molecular-weight cut-off Spectra/Por® Biotech cellulose ester membrane (Cole Palmer, UK) and dialysed in 1 L distilled water at room temperature until the product was fully dialysed (2-3 days). The distilled water was periodically changed during dialysis to ensure efficient removal of unwanted molecules.

2.3.9 Nuclear magnetic resonance (NMR)

The NMR analysis was performed and analysed with the assistance of Dr David Ellis (Heriot-Watt University) and Dr Barbara Mulloy (Imperial College London). For this, the dried polymer was dissolved in D_2O (to 0.7 ml) containing 1 µl of 2% acetone in D2O as an internal reference. Spectra were acquired at 60°C on a Bruker 800MHz Neo four-channel ultra-stabilised spectrometer. One dimensional spectra were acquired using the Bruker water-suppression sequence, 'noesygppr1d'. The number of scans was set at 32, the acquisition time was 2.03 sec, and a line-broadening factor of 0.30 Hz was applied to the data prior to processing. COSY spectra were acquired using the Bruker pulse program 'cosygpprqf', featuring a pre-saturation sequence. TD(1) was set to 2048 W and TD(2) to 512 W. TOCSY spectra were acquired using the Bruker sequence 'dipsi2gppphzspr.3.du' featuring a pre-saturation sequence. TD(1) was to 4096 W and TD(2) to 512 W. The mixing time was 60 msec. HSQC spectra were acquired using the

Bruker pulse program 'hsqcedetgpsp.3', TD(1) was set to 2048 W and TD(2) to 256 W. HSQC-TOCSY spectra were acquired using the Bruker pulse program 'hsqcdietgpsisp.2', TD(1) was set to 2048 W and TD(2) to 512 W. The mixing time was 100 msec.

2.4 Results

2.4.1 Growth

The growth of TGOS-10 in ZM/1 broth amended with glucose was rapid and reached the stationary phase in the first 24 h (Figure 2-1). The highest emulsification value (63% ± 2.1%) was measured in the cell-free culture broth at 48 h, already in the stationary phase. Following this, the emulsification index steadily decreased to 40% ± 1% at the end of the incubation period. However, the emulsions remained completely stable when left to stand unshaken at room temperature for two weeks (results not shown). The growth and emulsification measurements were repeated twice more on separate occasions to ensure the optimal time for extraction of the emulsifier. Based on these results, the emulsifier was extracted after 48 h of incubation as this was when the emulsification index was the highest on all occasions. The final yield of extracted dried polymer was 1.23 g/L.

2.4.2 Emulsification of different hydrocarbons

The extracted and freeze-dried emulsifier was also tested for emulsification of different hydrocarbons to ensure the emulsification properties of the polymer remained after extraction and long-term storage (~ 1 year at 4℃). The freeze-dried TGOS-10 polymer was dissolved in deionised water at concentrations of 0.25, 0.5 and 1 g/L, and the solution was used to perform the emulsification assay. In general, the polymer emulsified all of the three tested hydrocarbons (*n*-hexadecane, synthetic motor oil and diesel) but emulsification values varied with concentration and hydrocarbon type (Figure 2-2). From the three hydrocarbon types, the *n*-hexadecane showed the greatest variation in emulsification, whist motor oil and diesel showed relative consistency in emulsification regardless of concentration. At the lowest concentration (0.25 g/L), the polymer emulsified the motor oil and diesel to values of around 60%, while with *n*-hexadecane the emulsification values were substantially less – at 10% (Table 2-1). Doubling the polymer concentration (0.5 g/L) did not increase the emulsification index significantly for any of the hydrocarbon types. Unsurprisingly, the polymer emulsified

the three hydrocarbon types the most at 1 g/L. In fact, the diesel oil did not show a high variation in the emulsification values across the three concentrations. However, the emulsification of *n*-hexadecane increased 3-fold from 14% ± 3.85% at an EPS concentration of 0.5 g/L to 65% ± 1.77% at 1 g/L. For motor oil, the increase in emulsification between 0.5 to 1 g/L was 30% (Table 2-1). The emulsions with diesel and motor oil remained stable for at least two weeks at room temperature.

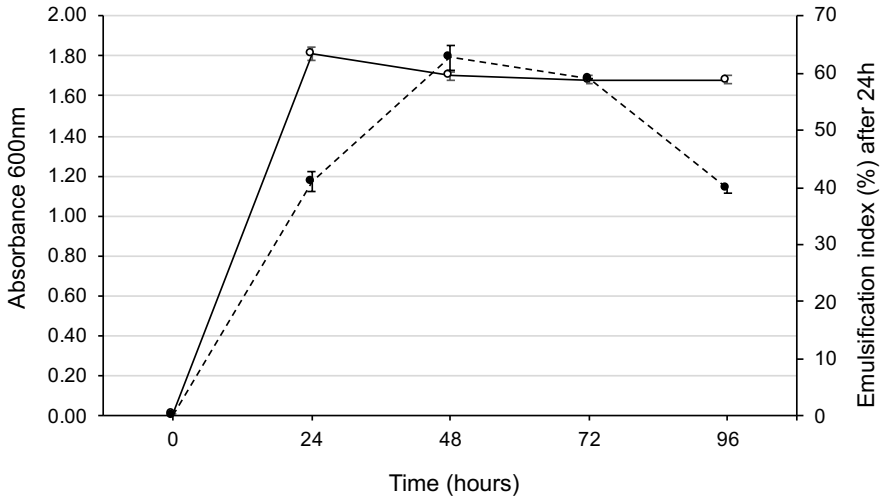


Figure 2-1. Growth measured by absorbance at 600 nm (solid line and empty circles) and emulsification index (separated line and black circles) of TGOS-10 cell-free supernatant bioemulsifier against hexadecane. Values are the mean of three independent replicates and error bars are standard deviation from the mean.

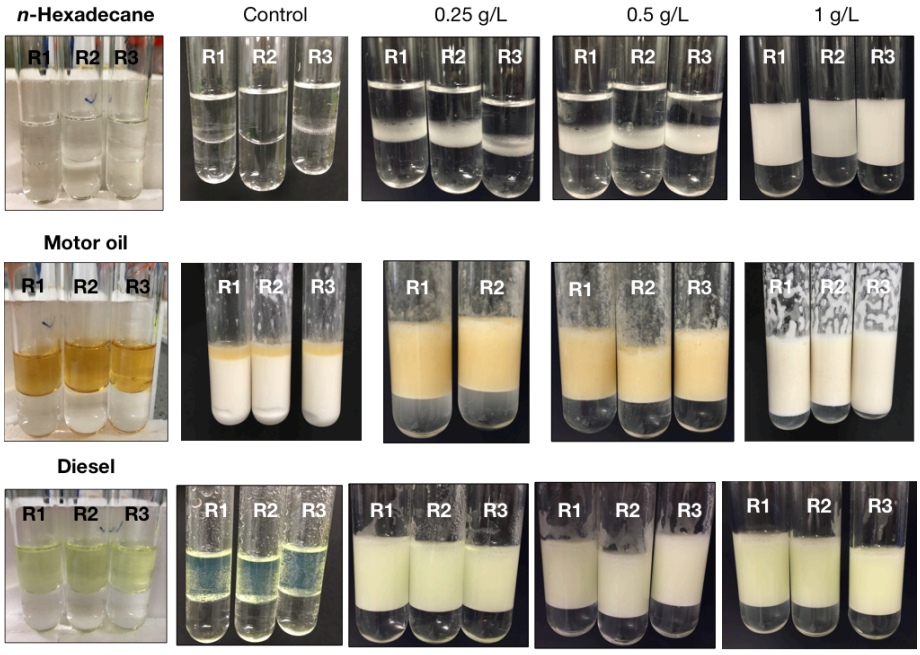


Figure 2-2. Emulsification of three different hydrocarbon compounds by the extracted, freeze-dried EPS from *Halomonas* strain TGOS-10 at three concentrations and compared to the control using MilliQ water. Pictures on far left show the treatments before commencing the emulsification test. Pictures on the right show the respective emulsifications after 24h at room temperature. Replicate tubes are labelled with R1, R2, and R3.

Table 2-1. Emulsification index (EI_{24}) of dried TGOS-10 EPS extract at three concentrations tested against *n*-hexadecane, motor oil or diesel. Values are the mean from three independent replicates ± standard deviation.

EPS concentration (g/L)	*n*-Hexadecane	Motor oil	Diesel
	Average % (± SD)	**Average % (± SD)**	**Average % (± SD)**
0	0 ± 0	87 ±	0 ± 0
0.25	10 ± 3.22	60 ± 0.51	62 ± 2.69
0.5	14 ± 3.85	64 ± 5.18	63 ± 0.64
1	65 ± 1.77	82 ± 5.88	64 ± 1.74

2.4.3 Protein and carbohydrate content of the TGOS-10 EPS

Bradford protein assay was performed to determine the concentration of protein in the extracted EPS from TGOS-10. The absorbance readings of the sample with all tested concentration (0.25, 0.5, and 1 mg/ml) were within the range of the standard curve, so the absorbance reading of 1 mg/ml (A = 0.883) concentration was used to calculate the content of the protein via the standard curve equation: y = 1.2765x + 0.1267 (Appendix

A). The results showed that on average the protein concentration was 0.59 ± 0.05 mg/ml of the EPS sample composition. The Dubois method was used to determine the content of carbohydrates in the extracted TGOS-10 EPS of known concentrations (100, 250, 500 and 1000 µg/ml). The absorbance readings of all concentrations were within the range of the standard curve, therefore the absorbance reading of 1000 µg/ml (A = 0.680) concentration was used to calculate the total carbohydrate content via the standard curve equation: y = 0.0117x + 0.0109 (Appendix A). The carbohydrate assay revealed that the carbohydrate content in the EPS was 57 ± 0.09 µg/ml or 0.057 mg/ml. Overall, the dried TGOS-10 polymer consists of 60% protein and 5.7% carbohydrates.

2.4.4 Chemical structure of the TGOS-10 EPS

The carbohydrate content of the TGOS-10 polymer was 1.5% of the total weight of the dried polymer (Table 2-2). Monosaccharide analysis showed that hexoses (fucose, rhamnose, galactose and mannose), traces of amino sugars (galactosamine and glucosamine), uronic acids (galacturonic and glucoronic acid) and the pentose arabinose were present in the polymer. Galactose (72% ± 4%) and mannose (17% ± 1%) were the most abundant, while all other monosaccharides were each present at less than 3%. The total uronic acid content was low at 3%, as contributed mostly by glucoronic acid.

The total amino acids content of TGOS-10 polymer was 46.6% of the total weight of the dried polymer (Table 2-3). Amino acid analysis of hydrolysed samples identified the presence of four major amino acids – aspartic and glutamic acids, glycine and alanine, which in total contributed 61% to the total amino acid content. The percentage contribution of polar amino acids to the total amino acid content was 13.4%, whereas that of hydrophobic non-polar amino acids was 52.7%, of charged acids– 29.3%, and of amphipathic acids – 2.7%.

Table 2-2. Monosaccharide content of the EPS emulsifier produced by *Halomonas* sp. strain TGOS-10.

Monosaccharide	Mol % composition
Fucose	2
Rhamnose	2
Galactosamine	trace
Arabinose	2
Glucosamine	trace
Galactose	72
Mannose	17
Galacturonic acid	trace
Glucoronic acid	3
Total (%) [a]	**1.5**

[a] Total percent values are expressed as the mean percentage of total dry weight of the polymer from triplicate determinations.

Table 2-3. Amino acid composition of the EPS emulsifier produced by *Halomonas* sp. strain TGOS-10.

Amino acid	Mol % composition
Aspartic acid - charged	17.3
Threonine - polar	6.1
Serine - polar	5.1
Glutamic acid - charged	10.1
Proline - hydrophobic	5.2
Glycine - hydrophobic	18.8
Alanine - hydrophobic	14.8
Cysteine - polar	ND
Valine - hydrophobic	4.7
Methionine - amphipathic	0.7
Isoleucine - hydrophobic	2.9
Leucine - hydrophobic	4.5
Tyrosine - amphipathic	2.0
Phenylalanine - hydrophobic	1.8
Histidine - polar	2.2
Lysine - charged	1.9
Arginine – charged	2.0
Total (%) [a]	**46.6**

ND – not detected

[a] Total percent values are expressed as the mean percentage of total dry weight of the polymer from triplicate determinations.

The SEC-MALS-RI analysis of the polymer produced by *Halomonas* sp. strain TGOS-10 showed that it was composed of a molecular-weight average molar mass (Mw)

of 6.44 ± 0.44 x 106 g/mol, with a number-average molar mass (Mn) of 4.93 ± 0.03 x 106 g/mol. The polydispersity index (PDI) was calculated as the ratio of weight average by number average molecular weight (Mw/Mn) and was determined as 1.31 ± 0.01. PDI is measure of the heterogeneity of sizes of molecules of a polymer in solution, and a value close to 1 indicates that the polymer is monodisperse, i.e., all molecules have the same degree of polymerisation or relative molecular mass.

2.4.5 NMR of the TGOS-10 EPS

The ^{1}H NMR spectrum (Figure 2-3 A) of the EPS is complex and overlapping, containing signals attributable to both carbohydrate and peptide moieties. Signals in the aromatic region (6.5-7.5 ppm) indicate the presence of aromatic amino acids, such as Tyr, Phe and Trp; prominent signals between 0.5 and 3.0 ppm originate largely from methylene and methyl groups in the sidechains of aliphatic amino acids. Carbohydrate signals typically occur between 6.0 and 3.0 ppm, overlapping with amino acid alpha hydrogen and some beta hydrogen signals. An informative region in the spectrum lies between 6.0 ppm and about 4.4 ppm, containing signals from the anomeric (H1) protons (Figure 2-3 B). At least six clearly resolved narrow resonances between 4.9 and 5.3 ppm are attributable to alpha-anomers, and are labelled A to F. An envelope of overlapping signals between 4.8 - 4.4 ppm may obscure further anomeric signals due to beta-anomers, among the majority of amino acid alpha hydrogens.

COSY and TOCSY spectra (not shown) include cross-peaks that identify the chemical shifts of H2 resonances corresponding to A-F (Table 2-4). Cross-peaks from the 4.4-4.8 ppm envelope may contain H1-H2 peaks as well as numerous amino acid alpha hydrogen to beta hydrogen cross-peaks, including those from Asp/Asn, Glu/Gln, Ser, Thr, Ala, Val, all identified in the amino acid analysis of this preparation.

An expansion of the heteronuclear ^{1}H-^{13}C HSQC spectrum is shown in Figure 2-3 C. H1-C1 cross-peaks are visible for A-F and also for a further group of signals with 1H shifts between 4.8 and 4.4 ppm. This group (labelled G-K) has both ^{1}H and 13C chemical shifts characteristic of beta-anomeric H1/C1 (and therefore distinguishable from amino acid alpha carbon/proton cross peaks with 13C shifts between 50 and 75 ppm). Unfortunately, 2D NMR spectroscopy (COSY, TOCSY, HSQC-TOCSY) was not able to identify any H2 or further ring proton resonances linked to signals G-K. However, low intensity cross-peaks in the HSQC-TOCSY spectrum due to H1-C2 and C1-H2

correlations allowed the assignment of H2 and C2 for some of the A-F series of spin systems (Table 2-4).

NMR spectra recorded prior to protease digestion are shown in supplementary figures S1 (iD ^{1}H spectrum) and S2 (2D HSQC spectrum expansion). They can be compared with Figure 2-3 A and Figure 2-3 C, respectively. Protease digestion was intended to simplify the NMR analysis by removing peptide signals from the spectra, but the digestion was not effective, and many peptide resonances remain in the post-digestion NMR spectra.

Table 2-4. Chemical shifts in ppm of H1/C1 and H2/C2 carbohydrate signals in the NMR spectrum of TGOS-10 EPS.

Peaks	H1	C1 (HSQC)	H2 (COSY)	C2 (HSQC-TOCSY)
A	5.27	103.3	4.12	80.9
B	5.14	104.9	4.07	72.8
C	5.10	101.2, 99.4	4.03	
D	5.06	104.8		
E	5.05	104.9	4.06	72.6
F	4.90	102.3	4.00	72.7
G	4.69	104.5	3.76	
H	4.62	104.4	3.82	
J	4.61	104.4	3.64	
K	4.50	105.6	3.35	

Taking the NMR data together with monosaccharide analysis, residues giving rise to signals A to F may well be alpha (F may be beta) mannose, as the chemical shifts of the corresponding H2s are at relatively low field (>4.0 ppm). The signals G-K may originate from beta-sugars and are consistent with beta-galactose.

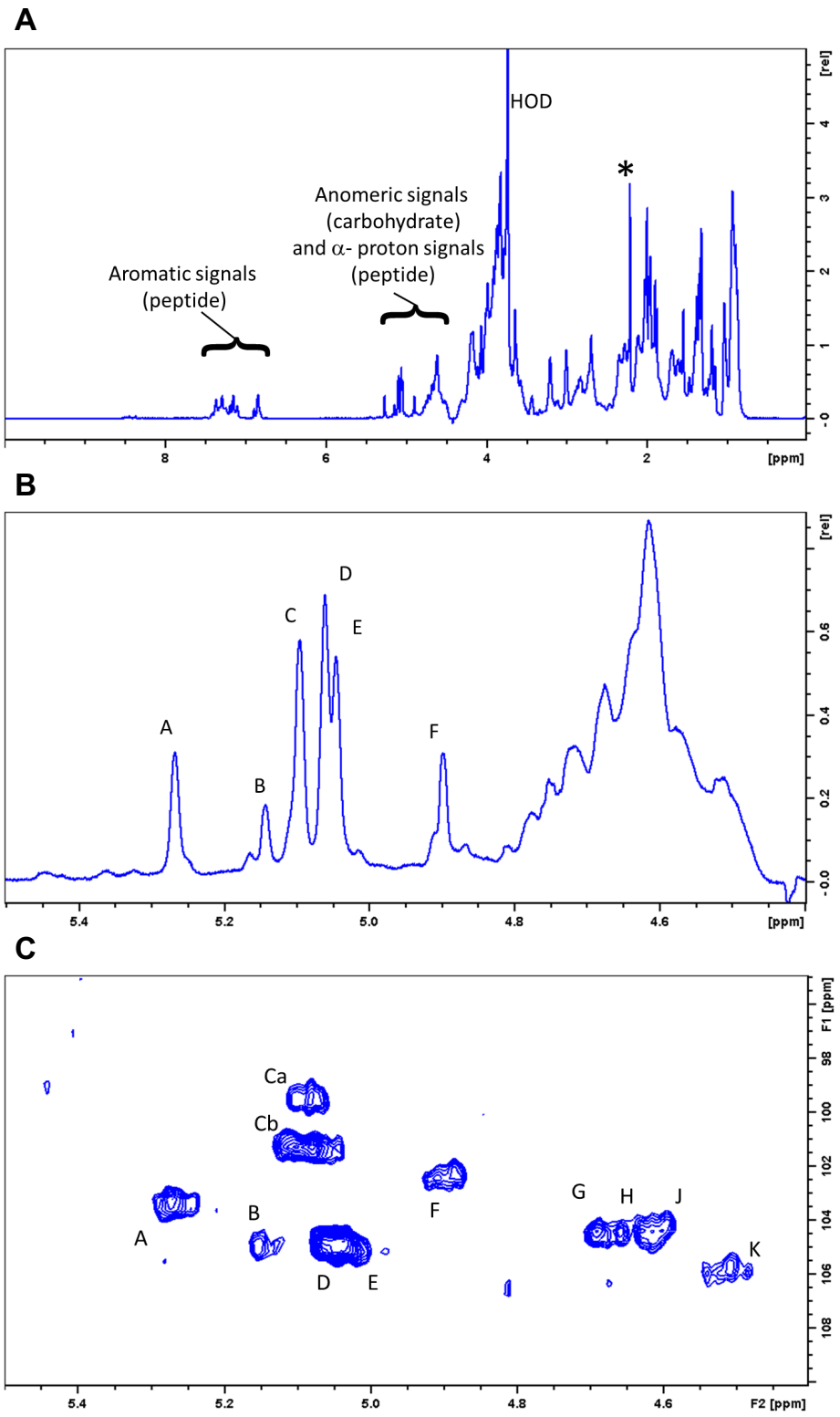


Figure 2-3. ^{1}H NMR spectrum at 800 MHz, 60 ºC of the EPS (after protease treatment) in D2O. **A)** The spectrum includes resonances characteristic of both peptide and carbohydrate components, with well-resolved signals from aromatic amino acids between 6.5 and 7.5 ppm, and several signals between 4.9 and 5.3 ppm characteristic of a-anomeric signals from sugars. The peak marked HOD results from a small amount of H_2O in the D_2O NMR solvent. The peak

denoted with an asterisk comes from acetone, used as an internal chemical shift reference (2.218 ppm). The vertical scale is relative intensity in arbitrary units. **B**) An expansion of the 1H NMR spectrum in A showing the region in which signals from anomeric protons (H1) of each monosaccharide residue in the structure occur. Five clearly resolved signals in the a-anomeric region are labelled A to F. In addition, a complex envelope of signals between 4.3 and 4.8 ppm may include signals from b-anomeric protons in addition to signals from the a-protons of amino acids. **C**) An expansion of the ^{1}H-^{13}C HSQC NMR spectrum of the EPS (after protease treatment) to show H1-C1 cross-peaks, including those corresponding to signals A to F in the 1H spectra shown in A and B. In addition, four further cross-peaks in the ^{1}H 4.7 to 4.5 ppm region, likely to come from b-anomeric positions, are labelled (G, H, J and K). These peaks could not be resolved from amino acid a-protons in the ^{1}H 1D spectrum but are identified in the HSQC by their characteristic ^{13}C chemical shifts.

2.5 Discussion

Halomonas sp. strain TGOS-10 was recently found to share 100% 16S rRNA sequence identity with *Halomonas* strains TG39 and MCTG39a, and 99% sequence identity to *Halomonas titanicae* BH1 (Gutierrez et al., 2020). Despite the genetic similarities and comparable growing conditions between the strains, there were, however, some major differences in emulsification and chemical composition. Previous studies on *Halomonas* sp. strain TG39 (Gutierrez et al., 2009) revealed that the strain was best grown on glucose and produced emulsification of 60% against *n*-hexadecane and relatively high yield (0.66 g/L). During the growth of TGOS-10 in ZM/1 amended with glucose, the emulsification was clearly correlated with intense growth, producing the highest emulsification of n-hexadecane only after 48 h of incubation. The results showed that TGOS-10 produced a yield of 1.3 g/L when grown on glucose and produced powerful emulsions with the three hydrocarbon compounds tested (> 60% emulsification).

Chemical characterisation, using monosaccharide, amino acid and NMR analyses, identified the EPS polymer from TGOS-10 to be a glycoprotein composed glycosylated with mainly galactose and mannose, and with a low uronic acid content. Interestingly, polysaccharides containing predominantly galactose and mannose are the plant galactomannans (Muschin and Yoshida, 2012) and the fungal mannogalactans (Rosado et al., 2003). Both of these polysaccharide types contain alpha-galactose rather than beta, and beta-mannose rather than alpha. As the NMR analysis showed, our alpha monosaccharide residues are not likely to be galactose: the chemical shifts of H1 and H2

are distinctive for mannose, as seen in NMR studies of yeast mannans (Shibata et al., 2007). Initially, the NMR spectrum had unresolved peptide signals that interfered with determining the sugar peaks, we performed proteolytic digestion of the protein component of the EPS in order to simplify the NMR spectra. The proteolytic digestion, however, was not successful and assuming that the protocol and reagents were not faulty, this might indicate protection of a peptide backbone by extensive glycosylation, rather in the manner of mucin glycoconjugates (Variyam and Hoskins, 1983) but this interpretation is speculative and further work will be needed to better resolve the role of protein in the functionality of the TGOS-10 polymer.

The TGOS-10 EPS was constituted of a very low uronic acids content, which was not expected as marine-derived EPS, including from *Halomonas* spp., are usually characterised for having high content of these acids (Gutierrez et al., 2020). TGOS-10's closest relative TG39 had around 30% uronic acids in its carbohydrate composition, and strain TG67 was reported to have up to 60% uronic acids (Gutiérrez et al., 2007b). EPS produced by other marine hydrocarbon-degrading bacteria have also been reported to have a high uronic content, such as from *Pseudoalteromonas* (28%) (Gutierrez et al., 2008), *Antarctobacter* (24%) (Gutiérrez et al., 2007a), and *Alteromonas* (13%) (Gutierrez et al., 2018). This finding appear to be more consistent with polymeric emulsifiers extracted from saline soil derived *Halomonas* sp. (Béjar et al., 1998; Arias et al., 2003; Amjres et al., 2015) which contained significantly lower levels of uronic acids (Chikkanna et al., 2018). In addition, compositional analysis of a glycolipid emulsifier produced by *H. anticariensis* FP35, also derived from saline soil, was not found to contain any detectable levels of uronic acids (Mata et al., 2006). The low amount of uronic acids in TGOS-10's EPS might suggest that this EPS could have high solubility in water as opposed to EPS with high uronic acids (Decho and Gutierrez, 2017). One could also speculate that under different culturing conditions, possibly using a different growth medium and/or amended with a different carbon source, strain TGOS-10 may produce EPS with higher levels of uronic acids.

The total protein content present in the TGOS-10 EPS was first determined with the Bradford protein assay, according to which the total protein content of the dried polymer was almost 60%. More accurate quantification of the protein content was done by acid hydrolysis of the EPS and HPLC analysis. The HPLC analysis revealed that the protein content was 46% of the total weight of the EPS. This was much higher than that

reported for EPS produced by its closest relatives such as *Halomonas* sp. strain MCTG39a, which had 36% protein of the total weight of dried polymer. Even, after proteolytic digestion, the protein content was evidently lower but still not completely removed suggesting that the protein has a structure that is resistant to protease digestion, possibly because of heavy glycosylation. The high protein content could also explain the strong emulsification properties of the TGOS-10 polymer, even at low concentrations. Indeed, proteins can play an essential role in the emulsifying ability of some bacterial exopolysaccharides, including for those produced by different *Halomonas* species (Llamas et al., 2012).

The emulsifying qualities of the TGOS-10 EPS appear comparatively high for all three oil/hydrocarbon compounds tested. The cell-free broth during the growth period showed 63% emulsification activity (EI_{24}) against n-hexadecane, however, the extracted dried EPS reached similar EI_{24} values only at the highest concentration (1 g/L) tested. This result is comparable to emulsification activity of other EPS produced by *Halomonas* spp. (strains TG39 and TG67) against *n*-hexadecane (Gutiérrez et al., 2007b) using the same emulsification assay (Cooper and Goldenberg, 1987). It is worth mentioning that the EPS from TG39 and TG67 achieved an emulsification activity of 60% with EPS concentrations as low as 0.02% w/v. In contrast, the TGOS-10 EPS in the work present here emulsified diesel oil and synthetic motor oil to higher values, even at low concentration of 0.25 g/L. However, with regards to the emulsification results for motor oil, the motor oil control treatment (motor oil and deionised water) showed the highest EI24 compared to the EPS treatments. This can be explained by the fact that detergent additives are commonly added to engine oils to clean and neutralise oil impurities which would normally cause oil deposits on essential engine parts. In fact, detergent additives have both polar and nonpolar ends which causes them to form stable emulsions between hydrophobic (oil) and hydrophilic (water) compound. However, emulsions can seriously compromise engine parts and hence are undesirable in lubricating systems where motor oil is used. The TGOS-10 EPS actually reduced the emulsification of the motor oil compared to the control, which may be beneficial as additive to reduce the affinity of motor oil to form emulsions.

CHAPTER 3

EFFECTIVENESS OF BIOSURFACTANTS FROM *HALOMONAS* SP. STRAIN TGOS-10 AND *PSEUDOMONAS* SP. STRAIN MCTG214(3B1) TO DISPERSE CRUDE OIL

3.1 Overview

The purpose of this chapter is to present the experimental work conducted on *Halomonas* sp. strain TGOS-10 and *Pseudomonas* sp. strain MCTG214(3b1) for their ability to produce biosurfactants and the efficiency of these biomolecules to disperse crude oil.

3.2 Introduction

There are different clean-up methods available in oil spill response, but the use of dispersants is considered to be one of the most effective options in terms of environmental impact on surface seawater and shoreline and reducing the duration and cost of the clean-up operation. The physical dispersion of oil at sea is a complex process that depends on a variety of factors such as type of oil spilled, water temperature, weather conditions, and energy input in the forms of waves, wind, and water currents (Chandrasekar et al., 2006; Chapman et al., 2007). Among those factors, the energy required to break up an oil slick into droplets is crucial. At sea, wind-induced capillary waves or breaking waves provide the necessary energy to break up the oil (Lessard and DeMarco, 2000). The physical dispersion of oil slicks can significantly be enhanced by applying chemical dispersants on the oil slick. Dispersants reduce the IFT of the oil-water interface which results in the oil being broken up and dispersed in the water column as fine neutrally buoyant droplets with size 1-70 μm (Fingas, 2011; Brakstad et al., 2015a). Small droplets increase the surface area-to-volume ratio of the oil and thus making it available to hydrocarbon-degrading bacteria and other marine organisms (ITOPF, 2011a). The advantages of dispersant use are extensively discussed in literature (Lessard and DeMarco, 2000; Prince et al., 2003; National Research Council, 2005; ITOPF, 2011b).

Dispersants consist of surfactants and organic solvents, which facilitate the surface action of the surfactants by "carrying" them to the oil-water interface, and additives that improve the mobility of the surfactants or increase long-term stability of the dispersion (Ufford et al., 2014). The exact composition of commercial dispersants is a propriety secret. In the chemical dispersant Finasol OSR-52, for example, the percentage contribution of non-ionic surfactants is more than 30%, for anionic surfactants it is in the range of 15-30%, DOSS is 15-25%, (2-methomethylethoxy) propanol – 15-20%, with the remainder of the dispersant composition made up by hydrocarbons (*n*-alkanes, isoalkanes, and aromatics) (Total Fluides, 2017). Although the non-ionic surfactants in

dispersant formulations are approved as a food-grade component worldwide and believed to be safe and non-toxic, the anionic surfactant DOSS is considered toxic to aquatic animals in concentrations of 40,000 ng/L (Place et al., 2016b). The Span and Tweens are synthetically derived from saccharides and are considered to be easily biodegraded, while DOSS has been shown to persist in the environment for months or even years (Kujawinski et al., 2011; Campo et al., 2013; White et al., 2014). An indiscriminate application of dispersants can result in significant amounts of dispersant being wasted if the application is not successful or inaccurate, such as when large quantities of the dispersant fail to come into contact with the oil. Exposure to medium and high concentrations of dispersant Corexit 9500 (widely used in the Macondo spill) has been shown to cause significant decrease of larval settlement and survival of shallow and deep-sea coral species (Goodbody-Gringley et al., 2013; DeLeo et al., 2016). Negative impacts on the oil-degrading microbial community have also been highlighted (Kleindienst et al., 2015b; Rahsepar et al., 2016) and discussed in Chapter 4.

The effectiveness of an oil spill dispersant, referred to as the dispersion effectiveness (DE), is the degree to which it disperses oil into the water column compared to the amount that remains on the sea surface. The DE can be influenced by numerous factors such as type of oil, amount of dispersant added, type of surfactants, mixing energy, and salinity (Sorial, 2006). It can be defined and measured by a variety of bench-scale laboratory tests, with the most reliable attempting to stimulate the energy inputs from shearing and mixing action that naturally occurs at sea (Lewis et al., 1985; Holder et al., 2015). Simulating the complex mixing that occurs at sea in laboratory conditions is often difficult and inaccurate. However, sufficient mixing in laboratory conditions can be achieved by using high-speed rotation (150-200 rpm) and specially designed baffled flasks that enhance the mixing (Kaku et al., 2006). The Baffled Flask Test (BFT) has been shown to be the most reliable method and hence has been adopted as a standard effectiveness test by many countries which resort to using dispersants as their primary response tool to treat oil spills, including the UK and the USA (Venosa et al., 2002; Kirby and Law, 2008). The BFT provides consistent and reproducible mixing energy and allows for undisturbed sampling from the flask compared to other tests (i.e. Swirling Flask Test) (Sorial et al., 2004a).

As extensively discussed in Chapter 1, biosurfactants are effective in reducing the ST and IFT of oil-water interfaces and, hence, find many applications in different

industries, particularly as bioremediation and oil spill treating agents (McKew et al., 2007; Nikolopoulou and Kalogerakis, 2008; Cappello et al., 2012; Nikolopoulou et al., 2013b). EPS with bioemulsifying properties produced by *Halomonas* spp. has been known to effectively emulsify different hydrocarbon compounds and light crude oil (e.g., Gutierrez *et al.*, 2009; Gutierrez *et al.*, 2020). Indeed, in Chapter 2 of this thesis *Halomonas* sp. strain TGOS-10 was been shown to produce a powerful glycolipid bioemulsifier when grown in ZM/1 medium with glucose as the main carbon source. Furthermore, other halomonids have been shown to produce EPS with emulsifying qualities in addition to glycolipid-type biosurfactants that both reduce the ST to 30 mN/m and emulsify different hydrocarbons that give EI_{24} values of up to 86% (Donio et al., 2013; Dhasayan et al., 2014; Neifar et al., 2019). *Pseudomonas* spp. are predominantly rhamnolipid producers and their excellent surface activity is discussed in detail in Chapter 1. The other bacterial strain studied in this chapter, *Pseudomonas* stain MCTG214(3b1) was of interest because it is a marine non-pathogenic bacterium that was previously shown to produce rhamnolipids when grown on rapeseed oil as the sole carbon source (Twigg et al., 2018) and has powerful surface activity.

The purpose of the work presented in this chapter was to investigate these two marine oil-degrading bacteria, *Halomonas* sp. strain TGOS-10 and *Pseudomonas* sp. strain MCTG214(3b1), for biosurfactant production and to determine the effectiveness of the produced biosurfactants for dispersing crude oil. Specifically, the stability, ionic activity, and chemical composition of the biosurfactants produced by the strains were determined and also assessed for their DE against different types of crude oil based on the United States Environmental Protection Agency's (EPA) BFT. The effect of biosurfactant-to-oil ratio (BOR) and oil type on DE for each biosurfactant was investigated by measuring the light diffraction from samples and the dispersed oil droplets size and distribution. The results from this chapter provide insights and groundwork further developing these biosurfactants, produced by *Halomonas* sp. strain TGOS-10 and *Pseudomonas* sp. strain MCTG214(3b1) as substitutes for some of the synthetic surfactants that are present in dispersant formulations or as stand-alone oil spill treating agents.

3.3 Materials and Methods

3.3.1 Strains

Halomonas sp. strain TGOS-10 was originally isolated as outlined in Chapter 2. *Pseudomonas* sp. strain MCTG214(3b1) was isolated from a seawater sample from Sarasota Bay in Florida during a bloom of the dinoflagellate *Protoperidinium*, as described in Twigg *et al.* (2018). The selection of these two stains was based on preliminary screening for surface tension and emulsification ability of their biosurfactants.

3.3.2 Growth conditions

Strains *Halomonas* sp. strain TGOS-10 and *Pseudomonas* sp. strain MCTG214(3b1) were routinely cultured at 28ºC on referred to as ZM/1 medium (Zobell, 1946) agar or broth supplemented with sterile 100× marine supplements (0.5% v/v). Single colonies were established on agar, as described in Chapter 2 (section 2.3.2), prior to transferring an isolated colony into ZM/1 broth to prepare fresh inocula for experimentation. Triplicate 2 L Erlenmeyer flasks, each containing 750 ml of ZM/1 medium, were autoclaved at 121ºC for 15 min and then amended with 1% (v/v) sterile sunflower oil and inoculated (1% v/v) with a growing culture of strain TGOS-10 or MCTG214(3b1). The flasks were incubated with constant shaking (180 rpm) at 28ºC for 96 hours. Samples were taken every 12 hours to monitor growth as determined spectrophotometrically (600 nm) and to measure the ST by tensiometry; and the CMC, stability of surface activity, and the ionic activity were assessed at the end of the incubation (96 h) as described below.

3.3.3 Measurement of ST

Samples (2 ml) from each triplicate flask were centrifuged (13,000 × g; for 10 min) to remove cells. The cell-free supernatant was then used to measure the ST (in triplicate per sample) using the Du Noüy ring method with a digital Kibron EZ-Pi tensiometer (Kibron, Finland). ST measurements were reported as the average of the triplicate measurements. Milli-Q water (72 mN/m) was used as a blank control and to calibrate the tensiometer. The ST of a control sample containing non-inoculated ZM/1 medium containing sunflower oil (1% v/v) was also measured to take into account any effect by the oil in reducing the ST of the ZM/1 medium.

3.3.4 Stability test

The performance of biosurfactants under different environmental factors was determined according to the method described in Rocha e Silva et al. (2014). The effect of salinity (2, 4, 6, 8, and 10 % w/v NaCl), temperature (4, 25, 50, 70, and 105°C) and pH (adjusted to 2, 4, 6, 8, 10, and 12) was determined by measuring the ST of cell-free broth from the three independent culture flasks. Each temperature was maintained for 90 min, after which the samples were left to normalise at room temperature for 10 min prior to measuring the ST. The effect of pH on ST was evaluated after adjustment of the test cell-free broth with 6 M NaOH or HCl at room temperature, and the salinity by dissolving NaCl to the required final concentrations of NaCl on the cell-free broth.

3.3.5 Ionic activity test

The ionic activity of the biosurfactants was determined by the method described in Weatherburn (1951). Briefly, 8ml of methylene dye solution (containing methylene blue, concentrated sulphuric acid, anhydrous sodium sulphate dissolved in deionised water) was added to a 5 ml chloroform in clean acetone-rinsed and acid-washed 25 ml test tube. Anionic surfactant solution (0.05% sodium dioctyl sulfosuccinate) was then added drop by drop (4-5 drops) with vigorous shaking between drops until the two layers were equal in colour and intensity. An amount of 2 ml of 0.1% cell-free biosurfactant broth solution in deionised water was mixed with chloroform-based methylene blue dye solution. The difference in colour between the chloroform and aqueous phases translates into the ionic activity of the biosurfactant. For example, deeper colouration of the chloroform (bottom) layer and mostly colourless aqueous phase (top) is an indication of an anionic surfactant. The opposite is true for a cationic surfactant, where the aqueous phase shows a deeper colouration than the chloroform phase. If the two phases are more or less the same colour, then surfactant is very likely non-ionic. If both anionic and non-ionic surfactants are present, the reaction of this test will be anionic positive.

3.3.6 Biosurfactant extraction and purification

The remaining culture broth (~ 700 ml) from each flask at the end of the incubation period from section 3.3.1 was collected for biosurfactant extraction according to the protocol of Smyth *et al.* (2010). The cell broth was centrifuged at 13,000 × g (4°C; 20 min) to remove cell biomass. The supernatant was acidified to pH 2.0 with 1M HCl and

extracted with equal volume of ethyl acetate and repeated at another two times. The organic phase was collected in clean acid-washed round bottom flasks and dried with anhydrous $MgSO_4$. The ethyl acetate phase containing the crude biosurfactant was rotary evaporated under vacuum to around 2 ml, and the resultant residue transferred to clean acid-washed pre-weighed glass vials. The remaining ethyl acetate was blown down with nitrogen gas. The vial was weighed once again and the difference in weight represented was the yield of crude biosurfactant.

The crude biosurfactant was purified using Phenomenex solid phase extraction (SPE) vacuum manifold and Strata C18-E Silica Giga tubes (55 µm, 70 Å) (Phenomenex Inc., UK). First, the silica tubes were washed with a continuous flow of chloroform. The crude biosurfactant was dissolved in 2-3 ml of chloroform and added to the silica tube. The silica tube was continuously washed with chloroform until the surfactant had moved to the silica layer. The silica layer was then eluted with a chloroform:methanol mixture (1:1, v/v) and the purified surfactant collected in clean acid-washed and chloroform-rinsed round bottom flasks. The chloroform:methanol mixture was rotary evaporated until approx. 2 ml of the biosurfactant remained. The biosurfactant was transferred to clean pre-weighted glass vials and the remaining chloroform:methanol was blown down with nitrogen gas. The yield of the purified biosurfactant was determined in the same way as above.

3.3.7 High-performance liquid chromatography (HPLC) analysis

HPLC analysis was performed to identify and quantify the components of the SPE purified extracts. HPLC/MS separation analysis was performed at the University of Ulster. For this, 1mg of each biosurfactant sample was dissolved in 1 ml of HPLC-grade methanol. A sample volume of 5 µl was injected in a spectra system LC P4000 (Thermo Finnigan) coupled to LCQ classic electrospray ion-trap mass spectrometer (Thermo Finnigan) using a 150 × 4.6 mm Kinetex 5 µM F5 100 Å LC column. Pure HPLC-grade water and acetonitrile were used as mobile phase. The spectra were acquired in the negative ionisation mode from *m/z* 110 to 1200.

3.3.8 Crude oils

Three different types of crude oil were used to conduct the dispersant effectiveness of the biosurfactants. Details about the physical properties of each oil and their SARA

analysis are presented in Table 3-1. The crude oils were provided by the Institute of Petroleum Engineering at Heriot-Watt University.

Table 3-1. Physical properties and SARA (saturates, aromatics, resins and asphaltenes) analysis of the three crude oils used in this chapter. The analysis was performed by Intertek (Aberdeen).

	Light crude	**Medium crude**	**Heavy Crude**
Origin	North Sea	Offshore North Africa	Alaska
Physical Properties			
Density at 15°C (g/ml)	0.865	0.920	0.946
API Gravity	32	22	18
Specific Gravity at 60/60°F	0.865	0.921	0.946
SARA			
Saturates (% wt)	47.8	44.5	28.4
Aromatics (% wt)	34.2	36.9	29.7
Resins (% wt)	16.3	18.2	32.2
Asphaltene (% wt)	1.7	0.4	9.7

3.3.9 Analysis of biosurfactant dispersion effectiveness

The baffled-flask test (BFT) was used to determine the dispersion effectiveness (DE) of the extracted biosurfactants, as adapted from the method of Venosa and Holder (2007) with some modifications as described below. The test was performed in the laboratory of Clariant Oil Services based in Aberdeen. For this, clean and acid-washed custom-made 250-ml screwcap trypsinising flasks (Erlenmeyer flasks with baffles) fitted with stopcock at the bottom were used for the BFT (Figure 3-1). The stopcocks facilitated an undisturbed drainage of the oil-water mixture. The flask baffles ensure over-and-under motion of water flow which is somewhat akin to the mixing of seawater on the sea surface caused by breaking waves as sea (Kaku et al., 2006). All flasks contained 120 ml filtered natural seawater from a tap supply in the laboratory and 100 µl of crude oil (Table 3-1) which was carefully dispensed directly onto the surface of the seawater to achieve a standard oil-to-water ratio of 0.1 ml of oil to 120 ml seawater (Venosa and Holder, 2007). Extracted crude biosurfactant from each strain was firstly dissolved in 1 ml of DMSO (dimethyl sulfoxide), because during initial testing the biosurfactant did not dissolve completely in water and dispensed directly onto the center of the oil slick (without touching the water) to achieve a biosurfactant-to-oil ratio (BOR) of 1:10, 1:25, and 1:50 each in triplicate independent flasks. The selected BOR are within the recommended range for commercial dispersants used in real case oil spill response. Additional flasks

were prepared in the same way with commercial chemical dispersant Finasol OSR52 (Total Fluides, France) or commercial rhamnolipid (produced by *Pseudomonas aeruginosa*) for comparison. A control treatment containing only seawater and crude oil was also prepared in triplicate independent flasks. The flasks were then placed on an orbital shaker (200 rpm; at room temperature) for 10 min. The rotation speed provided mixing that is equivalent to an energy dissipation rate of 0.163 W/kg water as expected in typical mixing conditions (e.g. from small breaking waves) at sea (Kaku et al., 2006). At the end of the mixing period, the flasks were allowed to remain stationary for 10 min. A 2 ml sample was then withdrawn and discarded prior to collection of a 20 ml sample for use in measuring the oil-in-water concentration of the mixture. For this, a Turbiscan LAB (Formulaction, France) stability analyser was used which uses static multiple light scattering (SMLS) optical method to characterise concentrated liquid dispersions. The measurements acquired were expressed as the transmission of scattered light (in %) by the sample. The transmission was measured for each biosurfactant extract for the three types of crude oil at three BORs (1:10, 1:25, and 1:50). Higher transmission indicates that less oil is present in the water phase (i.e., more light is passing through the sample), whereas low transmission means that greater number of oil droplets are present in the water phase and therefore, less light passes through.

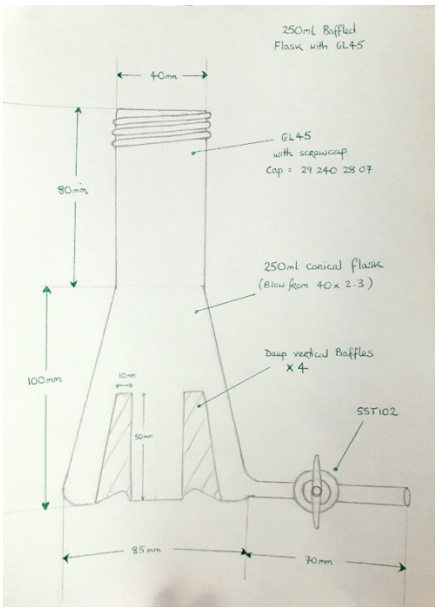


Figure 3-1. A schematic drawing of custom-made baffled flasks.

3.3.10 Measurement of oil droplet size and distribution

The baffled flask test was repeated in the exact same way as described in the above section in order to determine the dispersed oil droplet size and distribution in each treatment. Approximately 60 ml was collected from each flask at the end of the 10 min stationary period and aliquoted in the hydro dispersion unit of a Mastersizer MS 20 Particle Size Analyser (Malvern Instruments Ltd., UK). The Mastersizer uses a laser diffraction method to measure the size of particles by measuring the intensity of light scattered as a Helium neon laser beam (632.8 nm) passes through a dispersed particulate sample. The data output from this is then analysed to calculate the size of the particles that created the scattering pattern, the surface area of droplets, and polydispersity (size range of the oil droplet). An optical lens of 100 mm was used to detect particle sizes of 0.5 – 180 µm in diameter. After each sample was measured, the hydro dispersion unit and optical cell were rinsed with clean deionised water prior to analysing another sample.

3.3.11 Statistical analysis

Statistical analysis of biosurfactant stability, dispersant efficiency, and dispersed oil droplet size and distribution was carried out in statistical software R-studio v. 3.5.3 (R Core Team, 2019). Variance between the two biosurfactants for different treatments and measurements were compared by performing an ANOVA followed by Tukey's post hoc testing. The significance of the results was accepted at $p < 0.05$.

3.4 Results

3.4.1 Growth and ST

The relationship between growth and surface tension reduction for both strains, TGOS-10 and MCTG214(3b1), is shown in Figure 3-2. The growth and ST were measured every 12 h for a total incubation period of 96 h. Both strains were incubated under the same conditions and exponential growth was prominent during the first 48 h without any apparent lag phase, although the 12-hour period between sampling may have missed catching a lag. After 48 h, the growth rate slowed down, with both strains entering the stationary phase by this point; no death phase was reached by the end of the incubation period. The ST of the *Halomonas* sp. strain TGOS-10 culture started to decrease after 12 h from initial values of around 42 mN/m, reaching values of around 32mN/m by 48 h (Figure 3-2 A). The reduction of ST matched the period when the bacterial growth was

at its highest and just before reaching the stationary growth phase. The ST of the TGOS-10 culture was maintained kept the ST to 32mN/m by the end of the incubation period of 96h. For *Pseudomonas* sp. strain MCTG214, on the other hand, the ST gradually decreased from the start and reaching 31mN/m by the end of the incubation (Figure 3-2 B).

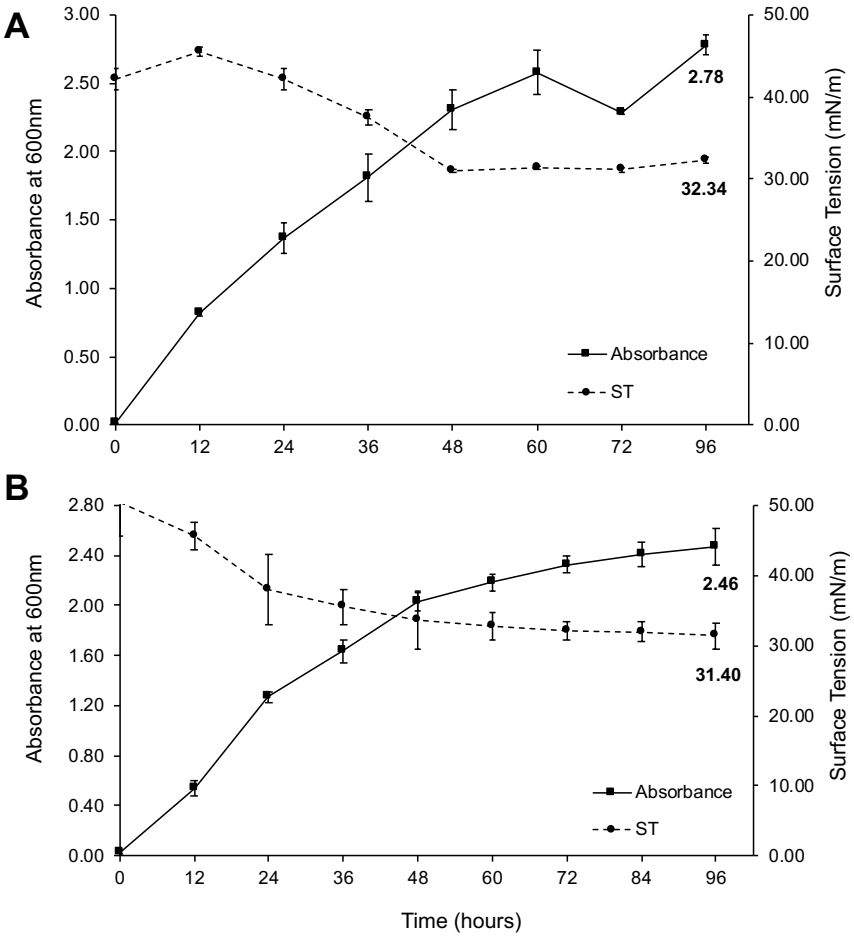


Figure 3-2. Bacterial growth and surface tension relationship for (A) *Halomonas* sp. strain TGOS-10 and (B) *Pseudomonas* sp. strain MCTG214(3b1) incubated at 28ºC and 180 rpm in ZM/1 medium supplemented with 1% v/v sunflower oil and 1% v/v inoculum. Values represent the mean from three independent replicate flask incubations, and error bars represent standard deviation of the mean.

3.4.2 Biosurfactant yield

The yield of extracted biosurfactants was determined gravimetrically. The crude biosurfactant yield for *Halomonas* TGOS-10 was 1.45 g/L, whereas for *Pseudomonas* MCTG124(3b1) it was 1.2 g/L. After purification, however, the yield decreased dramatically. The yield of purified biosurfactant from *Pseudomonas* MCTG214(3b1) was

0.04 g/L, and from *Halomonas* TGOS-10 it was 0.03 g/L. After multiple extractions, the yield was consistently low and for this reason, the ionic activity and stability test were performed with cell-free culture broth. The dispersion efficiency test and oil droplet measurement and distribution measurements were performed with crude biosurfactant extracts. The biosurfactants were purified only for HPLC and NMR analysis.

3.4.3 Stability of biosurfactants and ionic activity

The stability test was performed on cell-free culture broth. The incubation was stopped when the ST reached its lowest value – for *Halomonas* TGOS-10 it was 48 h, whereas it was 48-60 h for *Pseudomonas* MCTG214(3b1). The effect of different temperatures (4, 25, 50, 70, and 105°C), pH range (2 to 12), and NaCl concentrations (2 to 10%) was determined by measuring the ST of the cell-free culture broth from triplicate culture flasks (Figure 3-3). Overall, there was significant difference ($p < 0.001$) in the ST between the three parameters tested (temperature, pH, and salinity). Changes in temperature did not significantly affect the ST performance for neither of the biosurfactants ($p > 0.05$). Increasing temperature caused slight increase in ST of the *Pseudomonas* MCTG214(3b1) biosurfactant, whereas the opposite was observed for *Halomonas* TGOS-10 biosurfactant. Both biosurfactants produced similar ST values of 38 mN/m across the NaCl concentrations tested (Figure 3-2), which was significantly higher ($p < 0.001$) than the recorded values during incubation for both biosurfactants. Changes in pH caused the largest change in ST performance. For both biosurfactants, the lowest ST was achieved with pH of 6, which was the initial pH of the culture broths. Overall, the biosurfactant from *Pseudomonas* MCTG214(3b1) produced slightly lower, albeit not significant ($p = 0.664$) ST values than *Halomonas* TGOS-10 across the different parameters of pH, NaCl concentration, and temperature.

The ionic activity of the cell-free culture broths from the two strains was assessed using the method of Weatherburn (1979) which uses methylene blue and chloroform. Both biosurfactants were shown to be non-ionic in nature as shown in Figure 3-4.

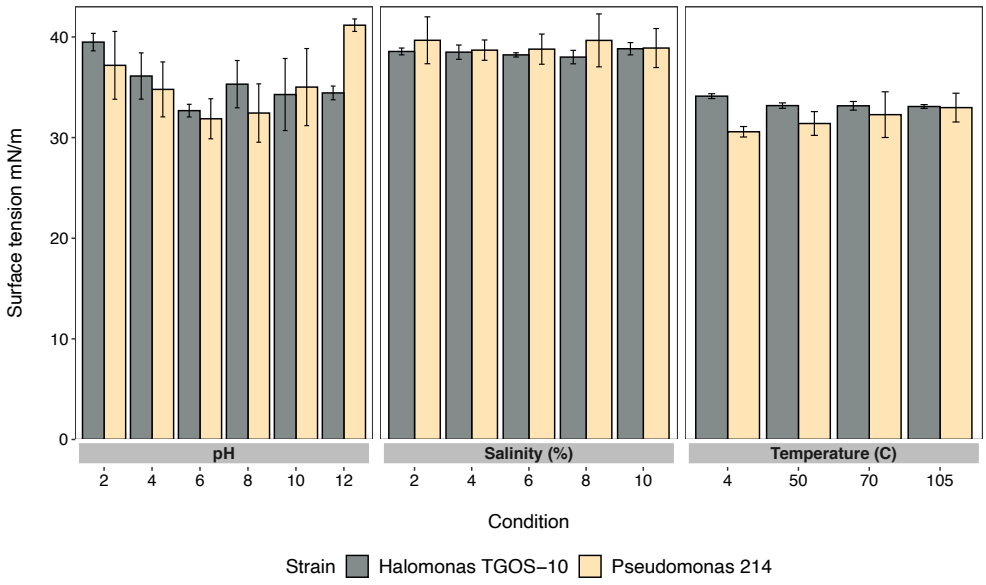


Figure 3-3. Surface tension performance of cell-free broth from *Halomonas* sp. strain TGOS-10 and *Pseudomonas* sp. strain MCTG214(3b1) under different conditions. Values represent the mean of ST measurements from three independent flasks and error bars are the standard deviation of the mean.

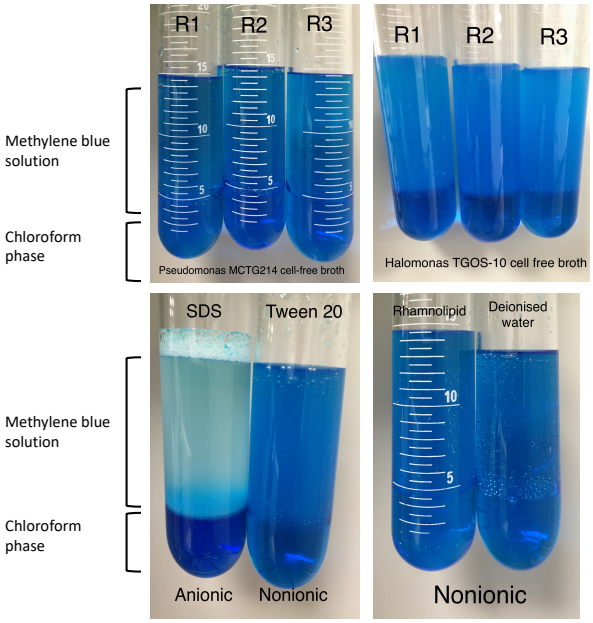


Figure 3-4. Illustration of ionic activity results for biosurfactants from *Pseudomonas* sp. strain MCTG214(3b1) (top left) and *Halomonas* sp. strain TGOS-10 (top right). Known anionic (SDS) and non-ionic (Tween 20) commercial surfactants (bottom left), as well as commercial rhamnolipid and control (deionised water) are shown for comparison. The methylene blue solution and chloroform phase are indicated for visual differentiation of the two phases. Independent triplicates are indicated as R1, R2 and R3.

3.4.4 Chemical structure of biosurfactants

To determine the chemical composition of the biosurfactants, HPLC-MS analysis was carried out on the purified biosurfactants. The identification of glycolipid congeners produced by strains TGOS-10 and MCTG214(3b1) were characterised by HPLC-MS operating in the negative mode. A base peak in the TGOS-10 purified extract with a *m/z* (mass-to-charge ratio) value of 448 was identified which corresponds to mono-rhamno-di-lipidic (Rha-C_8-C_8) congener (Figure 3-5 A). The next most abundant peaks were with *m/z* of 328, 391, and 407, however, did not match any known rhamnolipid congeners. They appear in both extracts but are more abundant in the TGOS-10 extract. There were two other known mono rhamnolipid congeners with *m/z* values of 385 (Rha-$C_{14:2}$) and 557 (Rha-C_{10}-$C_{14:1}$), although they were not as abundanct as the Rha-C_8-C_8 congeners. The mass spectra from the MCTG214(3b1) purified extract showed the most abundant congener to have a *m/z* value of 464 (Figure 3-5 B). It was also found in the TGOS-10 extract, but significantly less abundant compared to the MCTG214(3b1) biosurfactant. This peak did not match any previously identified rhamnolipid congeners. These results confirm that strains TGOS-10 and MCTG214(3b1) synthesise mono rhamnolipids with a C_8 fatty acid chain. In addition, this analysis suggests that the chemical structure of the TGSO-10 biosurfactant extract is more complex than that of the MCTG214(3b1) biosurfactant.

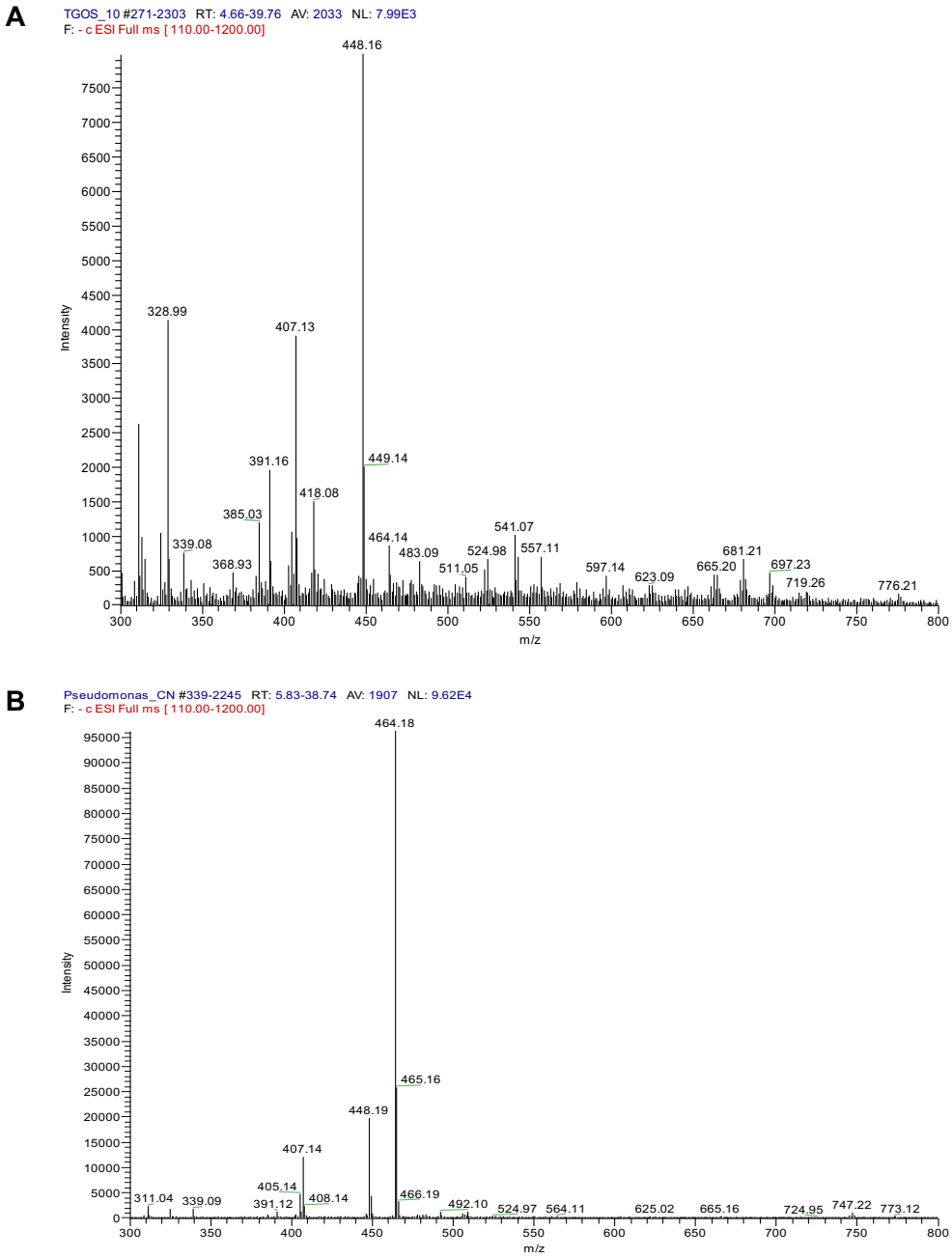


Figure 3-5. HPLC-MS-MS profile of the Phenomenex solid phase extraction (SPE) purified extracts from (A) *Halomonas* sp. strain TGOS-10 and (B) *Pseudomonas* sp. strain MCTG214(3b1).

3.4.5 Dispersion effectiveness (DE)

The DE of the two biosurfactants was assessed with laser diffraction which measures the transmission of light through dispersed particles in the sample. Compared to the control, which contained only seawater and crude oil, both biosurfactants dispersed all three oils significantly better ($p < 0.05$; Figure 3-6). The transmission values between the two biosurfactants were significantly different ($p < 0.001$), with the biosurfactant from

strain TGOS-10 achieved better dispersion as demonstrated by lower transmission values compared to values obtained with the biosurfactant from strain MCTG214(3b1). The light crude oil used in all tested BORs with the TGOS-10 biosurfactant was significantly more dispersed than when the medium ($p < 0.001$) and heavy ($p < 0.001$) crude oils were used in this test. The type of oil was not a significant factor ($p > 0.05$) in the dispersion efficiency achieved by the *Pseudomonas* MCTG214(3b1) biosurfactant, whilst the DOR had a greater effect on the DE (Figure 3-6). Both biosurfactants at DORs of 1:10 ($p < 0.001$) and 1:25 ($p = 0.012$) achieved better dispersion than a DOR of 1:50.

Rhamnolipid produced by *Pseudomonas aeruginosa* and the synthetic chemical dispersant Finasol OSR52 were both also tested for DE, although only at a DOR of 1:25 (Figure 3-6); this was because of limited time allowed to work in Clariant's laboratory. It is evident that Finasol achieved less than 0.2% transmission values (i.e., high dispersion efficiency) than both TGOS-10 and MCTG214(3b1) biosurfactants (10-30%) and the rhamnolipid of *P. aeruginosa* (3%) at a DOR of 1:25 for light and medium crude oils. The heavy oil was less dispersible in all treatments.

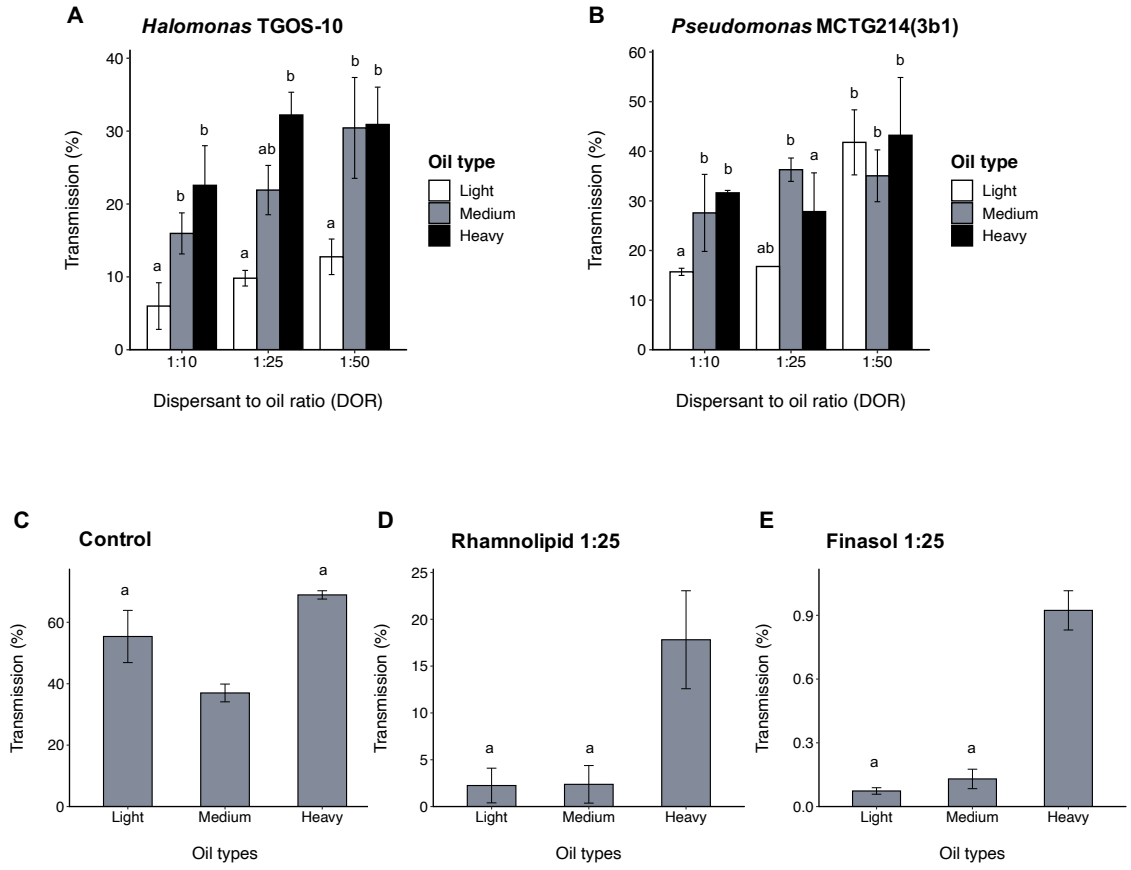


Figure 3-6. Transmission measurements for dispersion of three types of crude oil (light, medium, and heavy) by biosurfactants from (**A**) *Halomonas* sp. strain TGOS-10 and (**B**) *Pseudomonas* sp. strain MCTG214(3b1). Bars that share a letter (a or b) are not significantly different. Transmission measurement for (**C**) a control treatment (seawater and crude oil), crude oil dispersed by (**D**) commercial rhamnolipid biosurfactant and (**E**) commercial synthetic dispersant Finasol OSR52 at DOR 1:25. Error bars represent standard deviation from mean values of three independent replicates.

3.4.6 Oil droplet size and distribution

Using laser diffraction analysis, the dispersed oil droplet size and distribution were measured. The laser obscuration range (range of sample concentration) for the two biosurfactants was found to be between 5-10%, which was low but usable with a good signal-to-background ratio. Although both TGOS-10 and MCTG214(3b1) biosurfactant extracts were able to disperse all three types of crude oil, the concentration of the oil in the samples was low. Due to sudden equipment failure and later access restriction to the laboratory caused by the COVID-19 pandemic national lockdown, it was not possible to complete all anticipated measurements for the heavy oil droplet size and distribution. As such, only the results for light and medium crude oil are presented below.

Oil droplets sizes that were detected in treatments with both crude oils (light and medium) varied from 0.5 to 85 µm for both biosurfactants at the three tested BORs. The maximum size of detected droplets varied with the type of oil and BOR. For examples, in samples with the TGOS-10 extract and light crude oil, the largest oil droplets size was found to be 23, 33 and 30 µm for BORs of 1:10, 1:25 and 1:50, respectively. The droplet size increased when medium crude oil was used – 58 (1:10), 58 (1:25), and 85 µm (1:50). The same trend was observed for samples with the MCTG214 extract and light or medium oil. Overall, increasing the concentration of biosurfactant led to a decrease in the maximum oil droplet size detected in the samples. The oil droplet size distribution was significantly influenced by the type of oil ($p < 0.001$). In addition, the oil droplet distribution was significantly different between the control and the biosurfactants ($p = 0.001$). However, the difference in droplet sizes distribution between the two biosurfactants was insignificant ($p = 0.389$). In samples with the TGSO-10 biosurfactant and light oil, 90% of the oil droplets measured had an average size of 10 µm, and this was regardless of the BOR tested (Table 3-2). The difference in droplet sizes in samples with the TGOS-10 extract and medium oil, however, was more pronounced with decreasing BOR. Droplets with size of 17 µm and 32 µm had 90% of the volume distribution at BOR 1:10 and 1:50, respectively. When the MCTG214(3b1) biosurfactant was used with light oil, the oil droplet sizes were higher compared to that for the TGOS-10 biosurfactant and light oil across the three BORs tested (Table 3-2).

Polydispersity was measured to determine the heterogeneity in sizes of oil droplets in mixtures of the biosurfactants with crude oil. Values close to 1 indicate that mixtures have droplets with the same size or shape (uniform dispersion). The opposite is true for polydispersity values higher than 1. The polydispersity of the samples was mostly affected by the type of oil ($p < 0.001$) used rather than the BOR ($p = 0.087$) or biosurfactant type ($p = 0.910$). The mixtures with either TGOS-10 or MCTG214(3b1) biosurfactants and light crude oil had polydispersity values between 5 and 7, which was lower than the mixtures with medium oil (between 7 and 15). Overall, the polydispersity in the biosurfactant mixtures were significantly higher than of the control (4; $p = 0.017$) and of the rhamnolipid and Finasol mixtures. This is further supported by the difference between 90% and 50% volume distributions (Table 3-2). A big difference in the size of oil droplets in 90% and 50% volume distributions suggest that the size of droplets is inconsistent.

Table 3-2. Oil droplet size measurements and distribution of samples amended with biosurfactants crude extracts of *Halomonas* sp. TGOS-10 and *Pseudomonas* sp. MCTG214(3b1).

Sample	Crude oil type*	BOR**	Span (SD)	Polydispersity (SD)	Surface area (µm) (SD)	Size of oil droplets (µm) (SD)***		
						90%	10%	50%
Control	Light	0	1.99 (± 0.14)	4.15 (± 0.78)	1.64 (± 0.55)	8.14 (± 1.08)	0.66 (± 0.23)	3.79 (± 0.70)
	Medium	0	2.53 (± 0.69)	4.52 (± 0.66)	1.67 (± 0.33)	9.17 (± 1.07)	0.60 (± 0.10)	3.93 (± 0.62)
	Heavy	0	2.41 (± 0.33)	13.18 (± 1.31)	3.50 (± 0.51)	27.13 (± 4.12)	1.50 (± 0.55)	8.29 (± 4.71)
TGOS-10	Light	1:10	2.26 (± 0.11)	5.09 (± 0.60)	1.98 (± 0.36)	10.56 (± 1.43)	0.79 (± 0.23)	4.30 (± 0.42)
		1:25	3.34 (± 0.05)	5.06 (± 0.73)	1.91 (± 0.15)	11.82 (± 1.76)	0.83 (± 0.04)	3.29 (± 0.47)
		1:50	3.11 (± 0.23)	4.59 (± 0.42)	1.87 (± 0.06)	10.46 (± 1.18)	0.83 (± 0.04)	3.09 (± 0.16)
	Medium	1:10	2.73 (± 0.22)	7.88 (± 0.84)	2.21 (± 0.17)	17.59 (± 1.50)	0.81 (± 0.04)	6.20 (± 0.97)
		1:25	2.99 (± 0.22)	10.06 (± 0.93)	2.06 (± 0.37)	23.42 (± 2.42)	0.65 (± 0.19)	7.62 (± 0.77)
		1:50	2.37 (± 0.12)	15.37 (± 2.54)	3.12 (± 0.50)	32.30 (± 4.46)	1.05 (± 0.17)	13.28 (± 2.32)
Pseudomonas 214	Light	1:10	3.62 (± 0.21)	5.16 (± 0.96)	1.89 (± 0.14)	12.31 (± 2.54)	0.82 (± 0.03)	3.15 (± 0.49)
		1:25	3 (± 0.15)	4.20 (± 0.45)	1.81 (± 0.14)	9.42 (± 1.07)	0.81 (± 0.03)	2.87 (± 0.34)
		1:50	3.12 (± 0.50)	7.53 (± 0.88)	2.49 (± 0.26)	17.18 (± 1.37)	1.01 (± 0.08)	5.29 (± 1.12)
	Medium	1:10	3.92 (± 0.66)	8.60 (± 1.17)	2.13 (± 0.24)	21.20 (± 2.27)	0.81 (± 0.07)	5.35 (± 1.36)
		1:25	4.56 (± 0.34)	15.24 (± 8.24)	2.74 (± 0.72)	39.98 (± 22.32)	1 (± 0.22)	8.59 (± 4.72)
		1:50	3.41 (± 0.35)	9.73 (± 1.96)	2.44 (± 0.36)	23.15 (± 4.20)	0.90 (± 0.09)	6.65 (± 1.82)
Finasol	Light	1:25	1.42 (± 0.06)	1.79 (± 0.13)	1.28 (± 0.08)	3.03 (± 0.27)	0.72 (± 0.04)	1.63 (± 0.10)
	Medium	1:25	2.15 (± 0.22)	2.21 (± 0.46)	1.26 (± 0.16)	4.41 (± 1.10)	0.62 (± 0.05)	1.74 (± 0.31)
	Heavy	1:25	2.35 (± 0.18)	5.72 (± 3.11)	1.52 (± 0.30)	10.47 (± 4.00)	0.53 (± 0.06)	4.09 (± 1.44)
Rhamnolipid	Light	1:25	2.24 (± 0.12)	3.86 (± 0.31)	1.86 (± 0.05)	7.90 (± 0.79)	0.84 (± 0.01)	3.15 (± 0.20)
	Medium	1:25	2.70 (± 0.33)	4.46 (± 0.53)	1.57 (± 0.19)	10.25 (± 1.64)	0.60 (± 0.12)	3.56 (± 0.20)
	Heavy	1:25	NA	NA	NA	NA	NA	NA

* Based on API gravity. Light crude oil (32 API); Medium crude oil (22 API); Heavy crude oil (18 API)
** Biosurfactant-to-oil ratio
*** 90%, 10% and 50% of oil droplets are less than this size

3.5 Discussion

This study has shown that *Halomonas* sp. strain TGOS-10 can produce biosurfactant in addition to an EPS bioemulsifier (discussed in Chapter 2). The change of hydrophilic carbon source (glucose) to hydrophobic (sunflower oil) proved successful in influencing what type of surface-active agent the strain would produce. Along with the strong emulsifying properties of the TGOS-10 EPS, the biosurfactant produced by the same strain also exhibited excellent surface-active properties. The ability of the strain to produce both bioemulsifier and biosurfactant when grown on different carbon sources suggests that it can be potentially used to tailor the type of surface-active agents that are in demand for certain applications. Biosurfactant production was also confirmed for *Pseudomonas* sp. strain MCTG214(3b1) when grown on sunflower oil. Previously, MCTG214(3b1) was shown to produce di-rhamnolipids when grown on rapeseed oil. The aim of this study was not to optimize the biosurfactant production but to evaluate the ability of the selected strains to produce biosurfactants grown on sustainable substrates (i.e., sunflower oil) and to assess their DE against crude oil. Production optimisation,

however, would be necessary if this strain is to reveal its true potential for possible industrialisation.

3.5.1 Biosurfactant production

While *Pseudomonas* spp. are known for their production of rhamnolipids, this study also provides a rare insight into the biosurfactant production by a marine hydrocarbon degrading *Halomonas* sp. strain TGOS-10. Both strains, TGOS-10 and MCTG214(3b1) in this study reduced the ST to 31-32 mN/m when grown on sunflower oil as the carbon source, indicating the production of biosurfactants. There are few reports on biosurfactant production by marine *Halomonas*. For example, *Halomonas* strain BOB-3 isolated from the Bay of Bengal produced biosurfactant that exhibited a good anti-biofilm activity against two pathogens *Vibrio cholerae* (99.5%) and *Salmonella typhi* (99.8%) at biosurfactant concentration of 125 µg/ml (Kayanadath et al., 2019). *Halomonas* sp. strain MB-30 isolated from a shallow marine sponge *Callyspongia diffusa* produced maximum biosurfactant (grown on crude oil) after 48 h of incubation with ST of 32 mN/m (Dhasayan et al., 2014). The MB-30 biosurfactant obtained from glucose-amended media was characterised as anionic glycolipid with fatty acid and carbohydrate moieties (^{1}H NMR) and was able to recover 62% of crude oil from sand pack column. Along with excellent surface activity, this biosurfactant exhibited high emulsification activity against crude oil (86.6%), diesel (75.7%), and n-hexadecane (66.6%) (Dhasayan et al., 2014). Terrestrial halophilic *Halomonas* spp. have also been shown to produce biosurfactants. Biosurfactant from *Halomonas* sp. strain BS4 isolated from solar salt works in India was comprised mainly by fatty acids and 1,2-Ethanediamine N, N, N', N'-tetra, 8-Methyl-6-noneamide, and (*Z*)-9-octadecenamide which are polymeric biosurfactants with antitumor, antifungal, and microbicidal activities (Donio et al., 2013). Another halophilic *Halomonas desertis* G11isolated from desert salt-lake sediment has been shown to grow on crude oil as the sole carbon source and produce glycolipid biosurfactant identical to rhamnolipids while reducing the ST to 28 mN/m (Neifar et al., 2019). The TGOS-10 strain shown here produced non-ionic biosurfactant also likely to be a glycolipid-type as supported by the HPLC-MS analysis.

Pseudomonas sp. strain MCTG214 in study produced non-ionic biosurfactant which reduced the ST to 31 mN/m after 48 h of incubation on glucose. The reduction of ST was not far from what has been reported for other *Pseudomonas* species grown on hydrophobic (Oliveira et al., 2009; Raza et al., 2009; Twigg et al., 2018) and hydrophilic

carbon substrates (Prieto et al., 2008; Aparna et al., 2012; Janek et al., 2013; Das et al., 2014). In addition, *Pseudomonas* sp. strain MCTG214 was previously demonstrated to be able to grow on rapeseed oil as the sole carbon source, reduce the ST of water to 30 mN/m, and produce di-rhamnolipid biosurfactant (Twigg et al., 2018). The mean crude yield of both biosurfactant was in the range of 1.2-1.4 g/L, however, after SPE purification a dramatic loss of product yield was observed - less than 0.05 g/L. Other studies that employed the SPE purification method also reported substantial loss of crude product (Twigg et al., 2018; Tripathi et al., 2019) but not to such extend. The low purified yield in this study could be explained by the purification process being inefficient or that the crude products contained a lot of impurities.

3.5.2 Effect of environmental factors on biosurfactant activity

Environmental factors (temperature, salinity and pH) are known to affect the activity of biosurfactants (Rocha e Silva et al., 2014). Therefore, the stability of the biosurfactants produced by strains TGOS-10 and MCTG214 was tested under these changing factors. Due to the low yield of purified product, the stability test was performed with the cell-free broth as done elsewhere (Ibrahim et al., 2013; Pinto et al., 2018). Stability test indicated that both biosurfactants retained their surface activity at all tested temperatures and at pH of 6 and 8, while the salinity treatments reduced the surface activity for both biosurfactants. The stability at low (4ºC) and high (105ºC) temperatures indicate that the biosurfactants can find application in oil spill response in cold environments and even in MEOR where temperature may reach 100ºC, as well as being effective in any temperature within the tested range.

3.5.3 Biosurfactant chemical structure

HPLC-MS analysis was used to identify whether there were rhamnolipids in both biosurfactants. There are over 60 rhamnolipid congeners identified with new ones being reported regularly (Abdel-Mawgoud et al., 2010). Typically, most common mono-rhamnolipid congeners and homologues can be expected to have *m/z* 448 (Rha-C_8-C_8), 503 (Rha-C_{10}-C_{10}), 531 (Rha-C_{12}-C_{10} or Rha-C_{10}-C_{12}), and 559 (Rha-C_{12}-C_{12}) while di-rhamnolipids congeners are expected at *m/z* 479 (Rha-Rha-C_{10}), 621 (Rha-Rha- C_{10}-C_8), 649 (Rha-Rha-C_{10}-C_{10}), and 677 (Rha-Rha-C_{12}-C_{10} or Rha-Rha-C_{10}-C_{12}). Members of the genus *Pseudomonas* are known rhamnolipid producers (Irorere et al., 2017) with different species can show great variation of different rhamnolipid homologues (Aparna et al.,

2012; Pereira et al., 2012; Ndlovu et al., 2017). *Pseudomonas* sp. strain MCTG214 has been previously shown to produce predominantly di-rhamnolipids (Rha-Rha-C_{10} and Rha-Rha-C_{10}-C_{10}) when grown on 1% v/v rapeseed oil as the sole carbon source (Twigg et al., 2018). The authors of the study did not find any rhamnolipids with fatty acid chain longer than C_{12}, this same strain was grown on sunflower oil and the purified extract had a predominant peak at *m/z* 464 which did not match any previously identified congeners. The second most abundant congener, however, matched the known *m/z* for the mono-rhamnolipid Rha-C_8-C_8 (448), which was not found in the extract described in Twigg *et al.* (2018). *Pseudomonas* sp. strain MCTG214(3b1) was taxonomically identified to be closely related to both *Pseudomonas pseudoalcaligenes* and *Pseudomonas oleovorans* (Twigg et al., 2018). From a search in the literature, no studies were found on the chemical characterisation of biosurfactants produced by *Pseudomonas pseudoalcaligenes* and *Pseudomonas oleovorans* biosurfactants. However, both mono- and di-rhamnolipid congeners were identified in *Pseudomonas alcaligenes* when grown on palm oil, with the mono-rhamnolipids (Rha-C_{10}-C_{10} and Rha-C_{12}-C_{10}) found to be the most abundant (Oliveira et al., 2009). It is possible that the type of oil substrate, rather than other factors such as incubation conditions, can be attributed to influence the difference in rhamnolipid congener type and composition, even from the same strain (Vasileva-Tonkova and Gesheva, 2007; Raza et al., 2009). The HPLC mass spectra showed that the most abundant peak found in the *Halomonas* TGOS-10 extract was with *m/z* 448 (also found in the extract produced by MCTG214(3b1) strain). *Halomonas* spp. are known for producing glycolipid bioemulsifiers (see Chapter 2 and discussed in Chapter 1). However, glycolipid biosurfactant production by a halophilic *Halomonas desertis* strain G11 was recently reported, the chemical structure of which was identical to rhamnolipids (Neifar et al., 2019). The marine derived *Halomonas* MB-30 produced a glycolipid-type biosurfactant, although it was not clear whether rhamnolipids were present (Dhasayan et al., 2014). The HPLC results presented here for the biosurfactant produced by *Halomonas* sp. strain TGOS-10 may be the first description of a *Halomonas* shown to produce rhamnolipids.

3.5.4 Effectiveness of the biosurfactants

BFT is used as a standard test for testing the DE of dispersants prior to their approval for use to treat oil spills at sea (Chandrasekar et al., 2005; Venosa and Holder, 2007; Holder et al., 2015; Riehm et al., 2015). Recently, this method, was used to test the

DE of mixtures of soy bean lecithin and Tween 80 surfactants (with ethanol as a solvent) that were proposed as a new sustainable dispersant formulation (Riehm et al., 2015). Crude oil is an extremely complex mixture of hydrocarbon compounds each with its own chemical characteristics, but generally crude oil is characterised into three main types based on their API gravity (i.e., how heavy or light a petroleum liquid is compared to water). Light crude oil has API gravity higher than 31.1°, while medium crude oil is between 23.3° and 31.1°, and heavy oil below 23.3°. Although light crude oil has a higher market price, the global production of oil is not constrained to light crude only. The production of medium and heavy oil has been steadily increasing in recent years due mainly to technological advancements making it now possible to produce them economically. It was, therefore, paramount to assess the effectiveness at which each of the biosurfactant extracts disperse these different types of oil. It was clear from the work presented in this chapter that DE was dependent on oil viscosity and density. The higher the density of the oil (heavy > medium > light), the lower the dispersibility. This outcome was not a surprise since the heavy oil used here contained a higher fraction of resins and asphaltenes than the light and medium oils (Table 3-1). Resins and asphaltenes are the heaviest and most polar crude oil fractions which makes them more stable than non-polar fractions (i.e. saturates and some aromatics) and less likely to disperse (Lessard and DeMarco, 2000). In this study, three biosurfactant-to-oil ratios of 1:10, 1:25, and 1:50 were tested. BOR of 1:10 is the maximum amount of dispersant likely to be applied during an oil spill and it is usually used for emulsified or viscous heavy oils (National Research Council, 2005). In reality, however, field applications of dispersants are likely to be lower, (i.e. 1:20 or 1:50) when highly efficient dispersants are used (National Research Council, 2005).

In the UK, the minimum effectiveness requirements for a UK Type 3 (high effectiveness concentrate applied undiluted) oil spill product to be considered effective is 60%. This threshold means that 60% of the oil must be dispersed in the water column (EMSA, 2016). The biosurfactants in this study were also used undiluted and are considered as proxy for a UK Type 3 product. Accurate determination of the DE (%) can be achieved by liquid/liquid extraction of the oil which is then analysed spectrophotometrically (Venosa et al., 2002; Sorial et al., 2004b). However, laser diffraction method is also a quick and reliable method for measuring the DE (Li et al., 2011). The transmission measurements following the BFT revealed that the effectiveness of the TGOS-10 and MCTG214(3b1) biosurfactants was > 60%, as suggested by

transmission values of 10-15%. Light scattering method does not quantify the exact amount of oil in the water, but it gives a good indication of whether the oil is dispersed or not and to what extent. Although not as effective as the industrially available dispersant Finasol OSR52, both biosurfactants appeared to show they have a potential to be used either as stand-alone dispersants or as substitutes for synthetic surfactants ingredients that are typical of dispersants' formulations.

The presence of a large number of small particles, and high volume concentration of small particles, is highly correlated to effective oil dispersion (Li et al., 2011). Overall, it was clear from the oil droplet size and distribution measurements that the light crude oil was more likely to stay dispersed. On average, the average droplet size of light oil was smaller than the medium oil. This was, generally, observed for both of the TGOS-10 and MCTG214(3b1) biosurfactants. However, when the strain MCTG214(3b1) biosurfactant was used with light oil, the size of the oil droplets was higher compared to that for the TGOS-10 biosurfactant with light oil, indicating that the latter biosurfactant is more effective in dispersing the light crude oil. Smaller droplets of oil(< 100 μm in diameter) in dispersions are desirable because they tend to stay buoyant in the water column and become more rapidly and more bioavailable to microorganisms (Head et al., 2006). Even though both biosurfactants dispersed the oils, the inconsistent droplet size, as indicated by the polydispersity, suggests that the biosurfactant-facilitated dispersions would not be stable, and as such some of the oil droplets may return to the surface oil slick soon after the agitation period ends. For comparison, BFT was performed for the commercial synthetic dispersant Finasol OSR52, which is stockpiled around the world as a response tool to treat oil spills. Finasol is a finished product used undiluted and contains unspecified non-ionic and anionic surfactants (Total Fluides, 2017). These non-ionic surfactants are likely Tweens and Spans (non-disclosed proprietary knowledge) as they are common ingredients in the composition of the dispersant Corexit (Kleindienst et al., 2015a).

Although the crude biosurfactant yields obtained from both strains, *Halomonas* sp. strain TGOS-10 and *Pseudomonas* sp. strain MCTG214, are in accordance with ranges previously reported in the literature, the yields from were not enough to conduct the natural seawater microbial microcosms experiments described in the following chapter (Chapter 4) as initially planned. Instead, a commercially available rhamnolipid biosurfactant, produced by *Pseudomonas aeruginosa*, was used to evaluate its

effectiveness compared to a synthetic chemical dispersant for treating oil in the event of a spillage in a subarctic region of the northeast Atlantic where there is a prominent presence of oil and gas industry activity.

CHAPTER 4

MICROBIAL COMMUNITY RESPONSE TO CRUDE OIL, CHEMICAL DISPERSANT AND RHAMNOLIPID

(A CASE STUDY FROM THE FAROE-SHETLAND CHANNEL)

4.1 Overview

The aim of this chapter was to present the finding of the metagenomic analysis of marine surface seawater microbial community in response to added crude oil, synthetic dispersant and/or biosurfactant. A version of this chapter is peer-review in the *Microbiome* journal.

4.2 Introduction

Extensive tracking of the microbial response to crude oil contamination in the ocean after the Deepwater Horizon oil spill in the Gulf of Mexico in 2010 provided an unprecedented view into feedbacks between environmental chemical signatures and microbial community evolution (Joye and Kostka, 2020). During this historic spill, approximately 700,000 tonnes (4.9 million barrels) of Louisiana light sweet crude oil was discharged into the Gulf from a blown-out wellhead at a depth of ~1,500 m. Because of the scale and nature of the oil spill, synthetic dispersants were the primary response tool employed (National Academies of Sciences, Engineering, 2020). The decision to employ synthetic dispersants during a marine oil spill is driven largely by the desire to keep oil from reaching sensitive coastlines – often the primary goal of dispersant application. This unprecedented dispersant application involved approximately 7 million litres of the synthetic dispersants Corexit 9500 and 9527 to sea surface oil slicks and directly at the discharging wellhead along the seabed (Hamdan and Fulmer, 2011). Prior to the Deepwater Horizon incident, limited knowledge of the effects of synthetic dispersants use on open ocean microbial communities was available. As a consequence, questions were raised about the response of autochthonous populations of hydrocarbon-degrading (hydrocarbonoclastic) bacteria – key players in oil biodegradation – to these dispersants and the need to identify the impact of dispersants on oil bioremediation was highlighted.

Following the Deepwater Horizon incident, a number of studies investigated the effects of Corexit on natural microbial communities; some studies also reported the response of oil biodegradation rates. Corexit appeared to inhibit natural microbial oil biodegradation in some cases, possibly due to the toxicity by one or more of the dispersant ingredients and/or because some microbes that responded to dispersants (e.g. *Colwellia* spp.) preferred to metabolize dispersant constituents more than oil (Kleindienst et al., 2015b; Rahsepar et al., 2016). Some studies have reported that Corexit, and other synthetic dispersants, stimulated oil biodegradation by increasing its bioavailability to

microorganisms (Prince et al., 2013; McFarlin et al., 2014; Brakstad et al., 2018). Although the main components of synthetic dispersants are food-grade surfactants, including Tween 80 and Span 80, other components are hydrocarbon-based solvents that could confer toxicological impacts, whilst others are unknown as they are proprietary knowledge. Furthermore, a commonly used surfactant in dispersant formulations is dioctyl sodium sulfosuccinate (DOSS), a known toxin (Judson et al., 2010), that persists in the environment for months (Kujawinski et al., 2011) to years in cold (deep sea) environments (Campo et al., 2013; White et al., 2014).

Hydrocarbonoclastic bacteria produce biosurfactants (Head et al., 2006) that serve a similar purpose as synthetic dispersants, namely to reduce the surface and interfacial tension between oil droplets and seawater and increase the rate of oil biodegradation (Head et al., 2006). The most commonly studied biosurfactant producer is *Pseudomonas aeruginosa,* a ubiquitous bacterial species that grows on a wide range of hydrocarbon and non-hydrocarbon substrates and is known for its production of the glycolipid surfactant rhamnolipid with excellent surface-active properties (reduces the surface tension of water from 72 mN m^{-1} to less than 30 mN m^{-1} and facilitates formation of stable emulsions of petrol and diesel) (Das et al., 2014). Rhamnolipids have been shown to be effective in dispersing crude oil and enhancing its biodegradation by a pre-selected bacterial consortiums (Nikolopoulou et al., 2013a) some of which containing oil-degrading strains of *Ochrobactrum* sp. and *Brevibacillus* sp. (Chen et al., 2013). However, studies comparing synthetic and bio-based surfactants effects on indigenous marine microbial communities are rare. We are aware of only one, albeit, recent study that compares the effects of a biosurfactant, in this case, a surfactin produced by *Bacillus* sp. strain H2O-1, to a synthetic dispersant, Ultrasperse II, with a natural marine microbial community, including its biodegradation of crude oil (Couto et al., 2016). The surfactin enriched hydrocarbonoclastic bacteria more so than the synthetic dispersant, but no difference in oil biodegradation across treatments was observed.

The effect of rhamnolipid and the synthetic dispersant Finasol OSR52, which like Corexit, is stockpiled worldwide for use in oil spill response, on the composition of a natural marine microbial community and its ability to biodegrade crude oil was investigated. The study site was the Faroe-Shetland Channel (FSC), located on the UK Continental Shelf west of the Shetland Islands. This area has a 20 year history of oil exploration and production, with some fields as deep as 1,500 m (e.g. Lagavulin) (Gallego

et al., 2018). The FSC has complex and dynamic physical circulation characterized by mixing of distinct water masses (Bett, 2001) and the area is remote, cold, complex, and characterized by rough weather conditions for majority of the year, meaning that an oil spill response would be challenging. In addition, the FSC hosts important deep sea biological diversity, such as deep-sea sponges, cold-water coral communities, and a vibrant commercial fishing industry (Bett, 2001) which can be negatively impacted by major oil spills where synthetic surfactants were used.

Laboratory microcosm experiments in this study elucidated and compared the effects of the synthetic dispersant Finasol and the biosurfactant rhamnolipid on bacterial communities in the FSC and on their ability to biodegrade crude oil. A roller-table setup was employed to simulate sea surface conditions under *in situ* temperature, and crude oil was obtained from the Schiehallion oil field in the FSC (Fig. 1). Illumina MiSeq 16S rRNA sequencing was used to track microbial community abundance and dynamics, and the PICRUSt2 algorithm of QIIME2 was used to predict community functional diversity and abundance. Gas chromatography-Flame ionization detection coupled mass spectrometry (GC-FID/MS) was used to track the crude oil biodegradation in the microcosms.

4.3 Materials and Methods

4.3.1 Seawater collection

During a research cruise on MRV *Scotia* in May of 2018, surface seawater (3m depth) was collected from the Faroe-Shetland Channel (FSC), a subarctic region of the northeast Atlantic. The seawater temperature at the time of collection was measured at 9.7°C and the salinity 35.28‰. This sampling site (60°16.36’ N, 04°20.60’ W; Figure 4-1) lies on the Fair Isle-Munken line (Turrell et al., 1999) near the Foinaven oil field development area, approximately 3 and 9.3 nautical miles respectively from the Petrojarl Foinaven and Glen Lion production facilities. Collection of seawater samples was performed using 10 L Niskin water bottles mounted on a CTD (conductivity, temperature, depth) carousel. Immediately after recovery, some of the collected seawater was used to rinse, at least three times, two Nalgene carboys (10 L each; acid-washed, acetone-rinsed and dried) prior to filling, and immediately stored at 10°C onboard the vessel until transport back to the laboratory for the preparation of water-accommodated fractions (WAFs) and for the crude oil and biosurfactant/dispersant enrichment experiments, as

described below. All experiments were setup within two days of returning to the laboratory. Sub-samples were collected for DNA extraction and quantification of the *in-situ* bacterial communities and to account for any changes before initiation of experiments (described below). For this, 3 litres of the sampled water was filtered through polycarbonate membrane filters (1 L per filter; 0.22 µm pore size) and the filters stored onboard at -20°C until return to the laboratory when they were transferred to -80°C for subsequent analysis. Another 120 ml of the collected seawater was fixed on board with 3.7% formaldehyde and stored at 4°C for subsequent microbial cell counts, as described below.

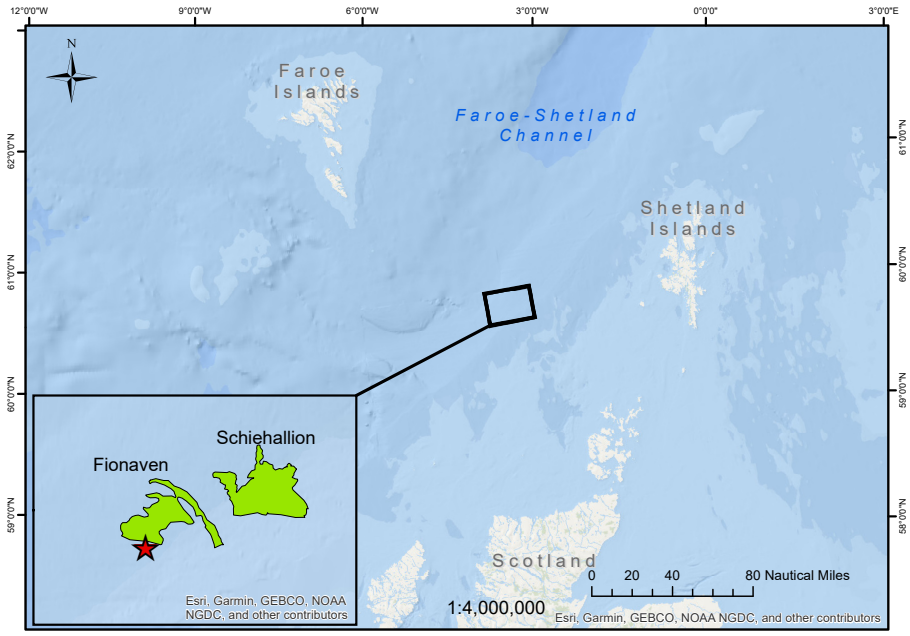


Figure 4-1. Map of the sampling site location (red star) and nearby oil-producing fields (green) in the Faroe-Shetland Channel. The map was created with ArcGIS Map software ver.10.6.1 (ESRI, USA) and freely available data from Oil & Gas UK.

4.3.2 Preparation of water accommodated fractions

Three main water accommodated fractions (WAFs) were prepared in acetone-rinsed, acid-washed and autoclaved 2 L glass aspirator bottles (with an outlet at the bottom) according to previous methods (Aurand and Coelho, 2005; Kleindienst et al., 2015b), though with some modifications as described below. For preparation of the WAFs, the seawater was filtered (0.22 µm; Millipore) in order to avoid the possibility of bacterial growth during the preparation of the WAFs. The first WAF contained seawater and crude oil only and is on referred to as WAF. A Chemically Enhanced WAF (CEWAF)

was prepared with seawater, crude oil and addition of the synthetic dispersant Finasol OSR-52 (Total Fluides, Paris, France) at a dispersant-to-oil (DOR) ratio of 1:20. Biosurfactant Enhanced WAF (BEWAF) was prepared with seawater, crude oil and rhamnolipid (produced by *P. aeruginosa*) at the same DOR as in the CEWAF. All three WAFs contained the same volume of filter-sterilised seawater (1560 ml) and Schiehallion crude oil (120 ml; API 25°; BP) which also originates from the FSC. The working concentration of the rhamnolipid used to prepare the BEWAF was 10 000 mg/L with critical micelle concentration of 27 mN/m. Briefly, each of the three main WAFs (WAF, CEWAF, BEWAF) were prepared by combining the prescribed quantities of seawater, crude oil and synthetic dispersant or biosurfactant in the aspirator bottles and leaving the solutions to mix on a rotary magnetic stirrer (140 rpm; 10°C) for up to 48 hours. In addition, two control WAFs were set up in the same way to assess the microbial community response to the dispersant or biosurfactant alone and in the absence of the crude oil. One of these contained only seawater and Finasol (SWD); the other contained seawater and rhamnolipid (SWBS). Both Finasol and rhamnolipid were used at the same concentration as in the CEWAF and BEWAF, respectively. After mixing for 48 hours, the three WAFs containing crude oil were allowed to stand undisturbed for one hour to allow for any undispersed oil to settle to the surface. The aqueous phase from each of the WAF mixtures was then carefully collected from the bottom outlet of the bottles, avoiding any of the undispersed oil.

4.3.3 Setup and sampling of microcosm treatments

Acetone-rinsed, acid-washed and autoclaved 500 ml glass bottles, with Teflon-lined caps, were used and each treatment was prepared in triplicate. Each treatment contained 66 ml of filtered WAF, CEWAF, BEWAF, SWD, or SWBS added to 234 ml of unfiltered seawater from the FSC to a total volume of 300 ml, leaving 200 ml of head space to ensure aerobic conditions. In addition, untreated control comprising seawater alone with no other additions was setup and run in parallel. All of the bottle treatments were placed on a roller table to maintain constant gentle mixing (15 rpm) and at a temperature of 9.7°C (in situ sea surface temperature at the time of sampling) for 4 weeks in darkness. At the beginning of these incubations (day 0), and then subsequently thereafter at days 3, 7, 14 and 28, each treatment was sub-sampled (5 ml) for total microbial cell counts, and also for DNA extraction (10 ml), following the methods described below. Additional WAF, BEWAF

and CEWAF microcosms (in triplicate) were set up identically to the rest of the microcosms for hydrocarbon biodegradation analysis as described below.

4.3.4 Total microbial cell counts

Samples (5 ml) for quantification of total microbial cells were first fixed with 3.7% formaldehyde. The fixed samples were filtered onto gridded polycarbonate Millipore filters (0.22 μm); the volume filtered was adjusted to achieve 10 to 150 cells per grid. The filters were then each stained with 1 μg/ml DAPI (4',6-diamidino-2-phenylindole) for 15-20 min. The filters were then divided into two and mounted onto glass microscope slides. Cell counts were performed with a Zeiss AxioScope epifluorescence microscope (Carl Zeiss, Germany). For each filter, three random optical field pictures were taken and in each picture a minimum of 10 grid squares were randomly selected for counting of cells (Suzuki et al., 1993). The number of cells counted was calculated using the formula: $N = (n_b / n_{Sq}) \times V_f \times (A/A_{Sq})$, where N is the total number of bacteria per ml, n_b is the number of bacteria counted, n_{Sq} is the number of squares counted, V_f is the volume of sea water filtered, A is the effective filter area, and A_{Sq} is the area of one square of the grid.

4.3.5 DNA extraction

Filters with collected biomass from each treatment (incl. replicates) were each transferred into sterile 1.5 ml Eppendorf tubes and submerged in liquid nitrogen until completely frozen. The frozen filters were carefully crushed into fine particles with sterile pipette tip. DNA extraction was performed according to the method of Tillett & Neilan (2000). This method utilizes chemical cell lysis with potassium xanthogenate buffer (1 M Tris HCl, 0.5 M EDTA, 10% SDS, 7.5 M Ammonium acetate, potassium ethyl, and molecular water). Negative control sample (sterile deionised water) was used to ensure the sterility of the method and used reagents. First, 1% potassium xanthogenate XS buffer of volume of 750 μl was added to Eppendorf tube with biomass pellet and vortexed for 15 sec. The tubes were then incubated at 75°C for up to 50 min, vortexed half-way the incubation and again afterwards. The tubes were then left on ice for 10 min to stop the DNA digestion and stimulate the precipitation of cell debri, which were pelleted by centrifugation at 13,000 rpm and 4°C for 10 min. The supernatant was transferred to a new sterile 1.5 ml Eppendorf tube containing 750 μl of 100% molecular-grade isopropanol and after mixing stored at -20°C for at least one hour. Tubes were once again centrifuged at 13,000 × g and 4°C for 30 min to allow the DNA to concentrate to the

bottom of the tubes. The supernatant was removed, and DNA pellets were washed with 750 µl of ice-cold 70% molecular-grade ethanol to precipitate the DNA (13,000 rpm for 10 min). The ethanol was discarded, and the DNA pellets were left to dry under sterile conditions. Dried DNA pellets were resuspended in 20 µl of 1 mM TE buffer and stored at -20°C until further sequencing analysis. The presence of DNA was detected by gel electrophoresis at 100 V for 35 min with 1% agarose gels made with 1 x TAE buffer.

The 16S rRNA gene was first amplified with universal bacterial primers 8F (5` AGAGTTTGATCCTGGCTCAG 3`) (Galkiewicz and Kellogg, 2008) and 1492R (5` TACGGYTACCTTGTTACGACT 3`) (Chirino et al., 2013) in 25 µl reactions, each containing 2.5 U MyTaq™ Red DNA Polymerase (Bioline Reagents Ltd.), 5 µl 5x MyTaq Red Reaction buffer (containing 5 mM dNTPs and 15 mM $MgCl_2$) (Bioline Reagents Ltd.), 17.5 µl molecular grade water, forward and reverse primer at final concentration of 0.2 µM each, and 1 µl of target DNA. Amplification was carried out on a thermocycler at the following conditions: initial denaturation at 96°C for 5 min, followed by 32 cycles of 96°C for 30 sec, 54°C for 30 sec and 72°C for 30 sec, and a final extension at 72°C for 10 min. The quality and quantity of PCR products were assessed by gel electrophoresis with 1% agarose gels stained with GelRed® nucleic acid gel stain (Biotin) and 2-log DNA ladder. Consequently, PCR reactions were purified with Illustra GFX PCR DNA and Gel Band purification kit (GE Healthcare Life Sciences) according to manufacturer instructions. Purified samples were quantitated with Nanodrop and kept at -20°C until further analysis.

4.3.6 Barcoded amplification of 16S rRNA gene

Two-step amplification procedure was implemented to minimize the heteroduplex formation in mixed-template reactions (Berry et al., 2011). The 16S rRNA gene was first amplified in the same way as described in the section above. The second step of 16S rRNA amplification was performed with barcoded PCR targeting the V4 hypervariable region of the 16S SSU rRNA gene. The purified samples from the first-step PCR were amplified in duplicate 25 µl reactions on a MicroAmp® Optical 96-well reaction plate (Applied Biosystems). Each reaction consisted of the same reagents as in the first PCR step but this time barcoded 515F (5′ GTGYCAGCMGCCGCGGTAA 3′) (Parada et al., 2016) and 806R (5′ GGACTACNVGGGTWTCTAAT 3′) (Apprill et al., 2015) primers were added to the PCR mixture. Both primers had Illumina MiSeq adapters added to the

5′ ends and unique Golay barcodes added to the 515F primer (Table 4-1). Barcoded amplification was carried out on a thermocycler at the following conditions as per the Earth Microbiome project (Caporaso et al., 2011): initial denaturation at 94°C for 3 min, then followed by 35 cycles at 94°C for 45 sec, 50°C for 60 sec and 72°C for 90 sec, and a final extension step at 72°C for 10 min. The amplicon product was expected to be of a size ~390 bp. The final PCR products were once more purified and quantified using a Nanodrop spectrometer and on 1% TAE agarose gel stained with GelRed. The duplicate reactions were pooled together to maximise product yield for sequencing. A final concentration of each PCR amplicon of 240 ng was achieved and all amplicons were pooled together. A 20 µl aliquot of the pool was sent for paired-end Illumina MiSeq sequencing (Illumina 2 x 250 v2 kit) in Edinburgh Genomics Facility (University of Edinburgh).

Table 4-1. Illumina MiSeq primer details.

Primer name	MiSeq Adapter Sequence 5′ - 3′	Primer pad	Linker sequence
515F	AATGATACGGCGACCACCGAGATCTACACGCT	TATGGTAATT	GT
806R	CAAGCAGAAGACGGCATACGAGAT	AGTCAGCCAG	CC

4.3.7 Bioinformatics processing

The resulting 16S rRNA gene sequences were processed with the open-source bioinformatics pipeline QIIME2 (Bolyen et al., 2019). Initially, sequences were quality filtered using the DADA2 algorithm as a QIIME plugin (Callahan et al., 2016). DADA2 implements a quality-aware correcting model on amplicon data that denoises, removes chimeras and residual PhiX reads, dereplicate DNA reads, and calls amplicon sequence variants (ASVs) on both forward and reverse reads (Callahan et al., 2017). Next, the quality filtered sequences were taxonomically aligned to the reference alignment database SILVA SSU Ref NR release v132 (Quast et al., 2013).

16S rRNA gene sequencing resulted in 7 703 409 pair-end sequence reads across 90 samples summarised in five enriched seawater treatments (WAF, CEWAF, BEWAF, SWBS, SWD), untreated seawater (SW; control) in triplicates over five sampling points, and one baseline seawater sample (FSC) also in triplicates. The libraries had a median

size of 102 200 reads. After removing unassigned ASVs and sample libraries with less than 5 000 reads, 3 412 ASVs were identified and rarefied to minimum of 18,730 reads for alpha diversity analysis. Rarefaction curves show that variation in the number of ASVs detected per sample was not caused by uneven sequencing depth (Appendix B).

PICRUSt2 algorithm as a QIIME plugin (Douglas et al., 2019) was used on the 16S rRNA gene sequences to predict the functional abundance and diversity (enzymes and metabolic pathways) of the microbial community based on KEGG (Kyoto Encyclopedia of Genes and Genomes) pathway mapping tool and MetaCyc databases (Caspi et al., 2019). Whilst metabolic potential from 16S rRNA studies are often discounted as mere predictions, with the newer version of PICRUSt2 with a comprehensive reference database (> 20,000 genomes covered as opposed to its predecessor which had ~2000 only), and with a very high correlation with matched metagenomics datasets (~0.9), and the fact that majority of the ASVs in our datasets were represented in the reference database, this analysis has a very strong utility to give mechanistic understanding. KEGG orthologs (KO) involved in aliphatic and aromatic hydrocarbon degradation were manually filtered along with biosurfactant-associated enzymes for each treatment. We specifically looked at genes for rhamnolipid (glycolipid) and surfactin (lipopeptide) synthesis as they are the most studied and their metabolic pathways are well known. Because our study focused on marine microbial community, we also selected genes for exopolymers production which are more characteristic for marine bacteria, for example *Alcanivorax* sp. and *Halomonas* sp. It is the *rhl* quorum sensing system in rhamnolipid-producing strains that regulates the production of rhamnolipid biosurfactants.

4.3.8 Statistical analysis

Statistical analysis of the ASVs was performed in statistical software programme R-Studio v. 3.5.3 (R Core Team, 2019). Alpha diversity indices Shannon and Species Richness were calculated on the rarefied abundance of ASVs to investigate within-community diversity of each treatment through time. Variance between treatments at different incubation times for each alpha diversity index were compared by performing ANOVA. Community composition variation between treatments and sampling (incubation) time (Beta diversity) were assessed using pairwise Permutational Multivariate Analysis of Variance (PERMANOVA) using the adonis function in *vegan* package (Oksanen et al., 2019) and visualised with PCoA (Principal Coordinate Analysis) plots based on Bray-Curtis similarity matrix (considers the species abundance count),

Unweighted (considers phylogenetic distance between branch lengths of ASVs observed in different samples without taking into account the abundances) and Weighted UniFrac distance (unweighted UniFrac distance weighted by the abundances of ASVs) matrices calculated from the rarefied and normalised (relative abundance) ASV table. Significance of results was based on high F values and level of significance based on low p-values (<0.05) for a comparison to be considered a significantly different.

To understand multivariate homogeneity of groups variances between multiple conditions (combinations of treatments over time), *vegan*'s betadisper() function was used, in which the distances between objects and group centroids are handled by using a reduced order representation based on distance metrics (Bray-Curtis, Unweighted UniFrac, or Weighted UniFrac) in principal coordinates space and afterwards performing ANOVA on differences of each sample from the mean of the group they belong to. *Vegan*'s adonis() function was used for multivariate analysis of variance (PERMANOVA) among sources of variation (treatment and sampling time) using distance matrices (Bray-Curtis/Unweighted Unifrac/Weighted Unifrac). To determine which ASVs were significantly different between multiple conditions (treatments/days), *DESeq2* package was used (Love et al., 2014).

To show how each sample was markedly different in terms of beta diversity, we have performed Local Contribution to Beta Diversity (LCBD) analysis using *adespatial* package (Dray et al., 2017). The procedure calculates the total beta diversity considering all the samples, and then allocates the proportions based on how different the microbial community structure of a single sample is from the average (with higher LCBD values representing outliers) beta diversity, and also provides a mean to show when the community structure has stabilised in a temporal setting (Ijaz et al., 2018). To characterize the phylogenetic community composition within each sample whether the microbial community structure is driven by competition among taxa (without any extrinsic environmental impact) or being deterministic, we quantified the nearest-taxon-index (NTI; local phylogenetic clustering) and nearest-relative-index (NRI; global phylogenetic clustering). We adopted the approach by Stegen et al.(2012) and used recently in Ijaz et al., (2018).

We performed subset regression of different metrics of microbiome (alpha and beta diversity) by testing all possible combination of the predictor variables (in our case, categorical variables), and then selecting the best model according to some statistical

criteria, with recommendations given in (Kassambara, 2018). The R function regsubsets from *leaps* package (Lumley, 2020) was used to identify different best models of different sizes, by specifying the option nvmax, set to the maximum number of predictors to incorporate in the model. Having obtained the best possible subsets, the k-fold cross-validation consisting of first dividing the data into k subsets. Each subset (10%) served successively as test data set and the remaining subset (90%) as training data. The average cross-validation error was then computed as the model prediction error. This was computed using a custom function utilising R's train function from the *caret* package (Kuhn, 2008) and tab_model function from *sjPlot* package (Lüdecke, 2014).

The R scripts used to generate the analyses are available at http://userweb.eng.gla.ac.uk/umer.ijaz/bioinformatics/ecological.html and as part of R's microbiomeSeq package http://www.github.com/umerijaz/microbiomeSeq.

4.3.9 Crude oil extraction and GC-FID/MS analysis

Additional bottles were similarly set up to the microcosms to determine the hydrocarbon degradation during the length of the experiment. For this, four of the treatments that contained hydrocarbons (WAF, BEWAF and CEWAF) were prepared in the same way as the microcosms in triplicates. Bottles were sacrificed for hydrocarbon extraction at three time points – immediately after setting up (day 0), after 7 days, and at the end of the experiment at day 28.

Hydrocarbons were extracted by solvent extraction with dichloromethane HPLC grade (DCM). Each sample was transferred in a separating funnel with equal parts of DCM. After vigorous shaking, the funnel was left upright to allow the mixture to separate into two layers – aqueous and organic phases. The organic phase, which contained the hydrocarbons dissolved in DCM, was collected in acid-washed, dried and pre-weighted round-bottom flasks. The aqueous phase was re-extracted with DCM twice more or until all hydrocarbons were extracted. The sample bottles were washed twice with 30 ml DCM in order to collect any residual hydrocarbons stuck on the glass. A small amount of $MgSO_4$ anhydrous was added to the collected organic phase to absorb any residual water and filtered through Whatmann paper to get rid of $MgSO_4$ crystals. The paper filter was also washed with DCM to ensure removal of any residual hydrocarbons stuck on the filter. The DCM was then evaporated on a rotary evaporator at 40°C to ~2 ml, which were then transferred to amber glass vials and stored at -20°C until GC-FID/MS analysis.

A known aliquot corresponding to ca. 10 mg of total hydrocarbon content was taken from each sample and transferred to a 10 ml vial and dried down using a gentle stream of nitrogen gas. Sample residues were dissolved in a small volume of hexane (~200 µl). A methodological blank (no crude oil) was used to ensure no contamination from any residual hydrocarbons during the analysis, and a sample of the original Schiehallion crude oil was analysed as a reference. Total hydrocarbon extracts were separated into two complementary polarity-defined fractions (aliphatic and aromatic) by using so-called *flash* open-column chromatography over 0.5 g silica gel (0.060 – 0.200 mm, 60 Å) and 0.5 g alumina (aluminium oxide, 50-200 µm, 60 Å) sorbents. Sorbents were pre-extracted with DCM to minimize organic contaminants and then activated at 120°C prior to use. Sorbents were introduced to open columns as slurries in hexane. Each column was packed with half the alumina, topped with silica gel, and then alumina again. The column was flushed with at least two bed-volumes of hexane (~4 ml) before the sample, which was dissolved in hexane, was applied to the top of the column. The aliphatic fraction of the total petroleum hydrocarbons (TPH) was eluted with 4 ml of hexane into an organics-free 2 ml glass vial. The aromatic fraction of the TPH was eluted with 4 ml DCM into a separate vial. Elution solvents were dried-down under nitrogen, re-dissolved in hexane (aliphatic fraction) and DCM (aromatic fraction) and transferred to organics-free 300 µl GC/MS borosilicate vial inserts. The samples were dried as before with nitrogen gas. The TPH fractions were then analysed by injecting 1 µl of the hydrocarbon fraction diluted in hexane in the autosampler of a Thermo Trace 1310 GC coupled with Thermo ISQ LT MS and fitted with a splitless constant temperature injector (300°C), a flame ionisation detector (FID) at 310°C, and an HP-5MS capillary column (30 m × 0.25 mm × 0.2 µm; Agilent). The column programme was set at 50°C for 2 min and 5°C/min to 310°C for 21 min to a total run time of 75 min. Chromatographic data were acquired and processed with the software Chromeleon (v. 7.2.8; Thermo Fisher). Peak areas of individual C_{12} to C_{30} *n*-alkanes and the isoprenoids pristane and phytane were calculated. The aromatic hydrocarbons were analysed by GC-MS in full scan mode (50-600 amu at 4 min).

Peak areas of aliphatic and aromatic hydrocarbon species/groups that were biodegraded after 7 or 28 days were calculated by subtracting the respective hydrocarbon concentrations measured in the control from those of the treatment incubations. Additionally, ratios of *n*-alkanes to acyclic isoprenoid hydrocarbons (nC_{17}/pristane and nC_{18}/phytane) were used as conventional indicators of biological degradation, due to the recalcitrance imparted by the branched structure of the isoprenoid biomarkers (Dawson

et al., 2013). Similarly, for the aromatic hydrocarbons, four ratios indicative of biodegradation were determined (phenanthrene/9-methylphenanthrene, 3+2-methylphenanthrene/9+1-methylphenanthrene, 3-methylphenanthrene/9-methylphenanthrene) (Dawson et al., 2013). Two-way ANOVA and *post-hoc* Tukey tests were performed to test for significant differences in the degradation of the hydrocarbons analysed between the treatments.

4.4 Results

4.4.1 Total cell counts

To assess the microbial community dynamics across all six treatments, DAPI counts were measured over the 28-day duration of these experiments. At the start of the incubations (day 0), cell numbers were in the range of 6.93×10^7 to 4.16×10^8 cells/ml (Figure 4-2). As expected, cell counts in the untreated control (SW) remained lower than the other treatments throughout the duration of the experiment. In the BEWAF treatment, cell counts decreased from day 0 to day 7, but thereafter increased up until day 14, and then decreased to the end of the experiment. The WAF, CEWAF, SWD and SWBS treatments showed similar trends with initial increases in cell numbers until day 3 (respectively $3.72 \times 10^8 \pm 3.84 \times 10^7$, $6.92 \times 10^8 \pm 1.40 \times 10^8$, $7.45 \times 10^8 \pm 2.48 \times 10^7$ and $3.94 \times 10^8 \pm 1.79 \times 10^8$) and thereafter showing a steady decline until the end of the experiment; the SWD and CEWAF treatments, respectively, showed the lowest and highest cell counts at day 28.

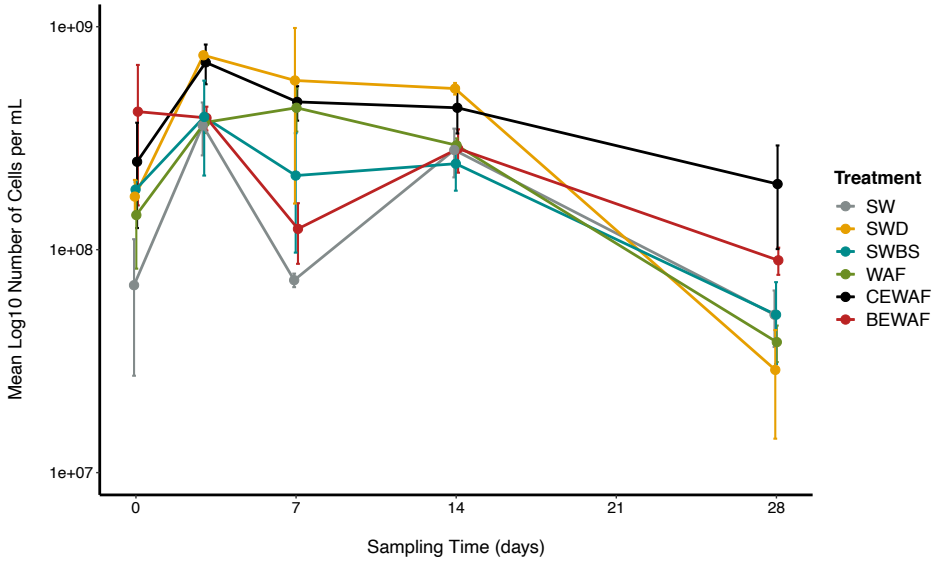


Figure 4-2. Microbial (bacterial and archaea) cell numbers measured by DAPI staining over 28 days in the six different treatments with sea surface water from the Faroe-Shetland Channel supplemented with/without crude oil, Finasol and/or the biosurfactant rhamnolipid. SW – seawater only; SWD – seawater and chemical dispersant; SWBS – seawater and biosurfactant; WAF – seawater and crude oil only; CEWAF – crude oil and chemical dispersant; BEWAF – crude oil and biosurfactant. Values are the mean of three independent replicates +/- standard deviation.

4.4.2 Taxonomical composition and relative abundance of bacterial communities

Members of the Proteobacteria dominated the taxonomic profiles over the 28-day microcosm incubations, ranging between 60-98%. In contrast, the Bacteroidetes decreased from ~40% (initially) to almost undetectable by the end of the experiment. Other phyla were present at <5% relative abundance. At day 0, all treatments showed similar community composition, comparable to the in-situ community. Community profiles were dominated at the family level by *Colwelliaceae*, *Saccharospirillaceae*, *Rhodobacteracea* and *Micavibrionaceae* (>40%; Figure 4-3).

The initial abundance of *Colwellia* varied significantly across treatments. At day 0, *Colwellia* abundance ranged from 15% in the seawater control (SW) to 11% (WAF, BEWAF) to >35% in the dispersant only control (SWD) and CEWAF treatments. In the dispersant only control treatment (SWD), the abundance of *Colwellia* rapidly decreased to 9%, then to 5%, and then 1% on days 3, 7, and 28 respectively. The abundance of *Oleispira* increased to 28% in BEWAF and CEWAF treatments but was negligibly abundant in the oil-only treatment (Figure 4-3). By day 7, members of uncultured

Micavibrionaceae increased in both the BEWAF and WAF treatments, peaking on day 14 (18% and 14%, respectively). On day 14 the community profiles of the WAF and BEWAF treatments were quite distinct, with *Colwellia* having markedly decreased in abundance in both treatments to 6% and 2%, respectively. Across all treatments *Cycloclasticus* and *Alcanivorax* were rare initially (<1%) but had increased by up to 20% by day 14 in the WAF treatment and these abundances were maintained on day 28. In the BEWAF, *Cycloclasticus* and *Alcanivorax* abundances remained below 1% until day 14 but increased to 8% and 4% by day 28, respectively. They were not detected in the seawater only control (SW). Oil degraders, except *Colwellia*, were not enriched in the SW treatment. Similar to the BEWAF and WAF treatments, on day 3 the CEWAF treatment was dominated by *Colwellia* (35%), *Oleispira* (28%), *Sedimentitalea* (8%) and uncultured members of the *Micavibrionaceae* (9%). *Pseudophaeobacter* and *Sedimentitalea* (family *Rhodobacteraceae*) were exclusively enriched in the Finasol-amended treatments (CEWAF and SWD) by the end of incubation. *Alcanivorax* increased in the CEWAF treatment from <1% in the early stages of incubation to 5% by the end.

A strong enrichment in *Vibrio* was observed only in the SWD treatment, with abundance increasing from 2% at day 0 to 26% by day 3, followed by a gradual decrease to 12% (day 14), and then to 2.4% (day 28). In the CEWAF treatment, for comparison, *Vibrio* became only slightly enriched (1-4%) throughout the incubation period. *Pseudomonas* was observed mainly in the CEWAF and SWD treatments where its abundance increased from <1% on day 3, to 6% on day 14 in the CEWAF treatment, and 3% in SWD. In the CEWAF treatment, *Cycloclasticus* was absent.

A phylogenetic tree of top 50 ASVs was also constructed with their abundance changes shown for each treatment (Figure 4-4). In the top 50 ASVs, six ASVs are assigned to the *Colwellia* genus, four to *Oleispira*, three to *Cycloclasticus*, one ASV to *Alcanixorax*, *Pseudomonas*, *Zhongshania*, *Thalassotalea*, *Shimia*, *Sulfitobacter*, and *Amylibacter*. Three of the *Colwellia* ASVs displayed higher abundances in the CEWAF treatment, while the other three had clear enrichment in the WAF. As discussed above, *Cycloclalsticus* ASVs were enriched almost exclusively in the WAF treatment, and *Alcanivorax* dominated in all the oil-amended treatments (WAF, BEWAF, CEWAF). Although *Pseudomonas* had overall low abundance in all treatments, it was mainly enriched in the CEWAF and SWD treatments.

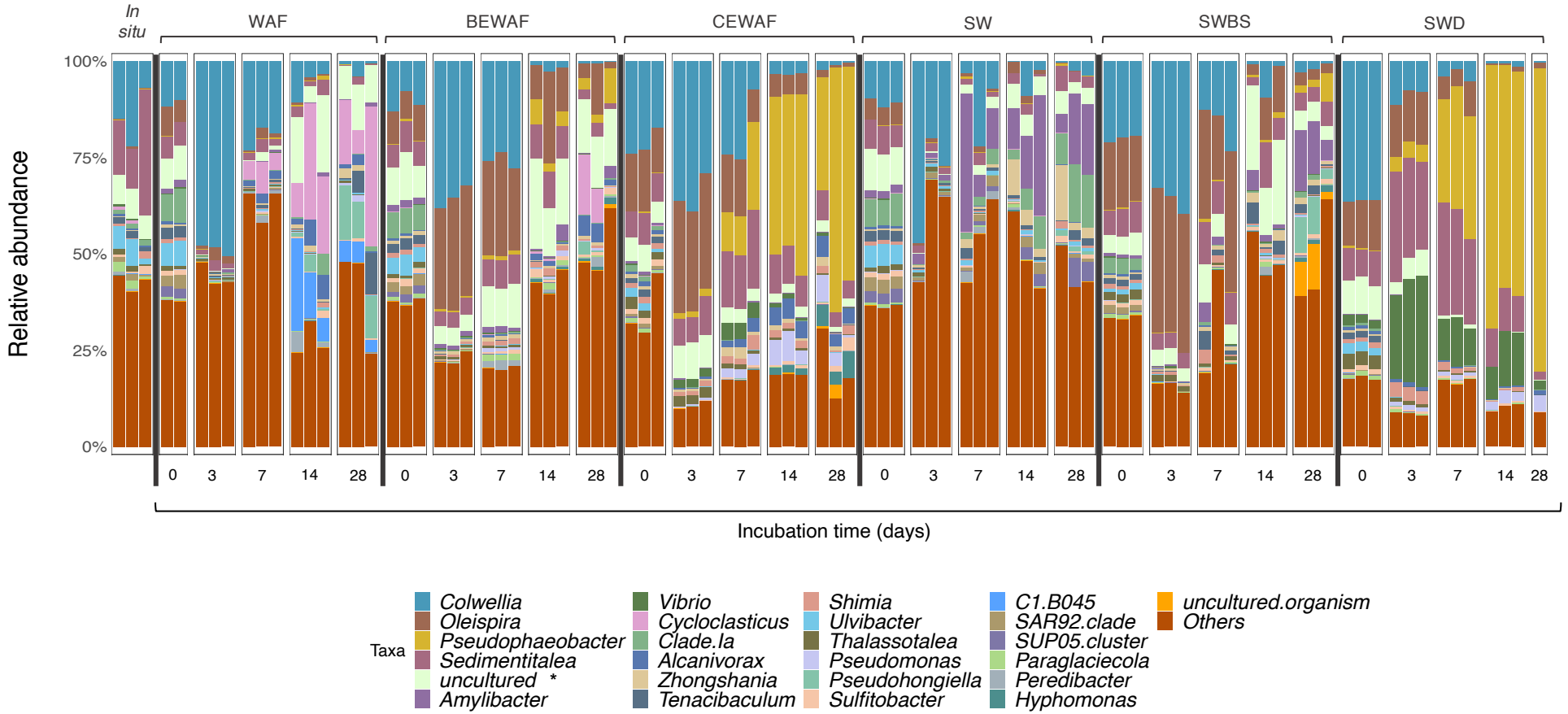


Figure 4-3. Relative abundance of top 25 most abundant taxa shown to genus level. Treatments at different incubation times are shown as independent triplicates where *in-situ* is baseline microbial community at time of seawater sampling (FSC), WAF - seawater and oil only, BEWAF – crude oil and biosurfactant, CEWAF – crude oil and chemical dispersant, SW - seawater only, SWBS - seawater and biosurfactant, and SWD – seawater and chemical dispersant. * represents uncultured bacteria from the *Micavibrionaceae* family. SWD had one replicate on day 28 and WAF had two replicates on day 0.

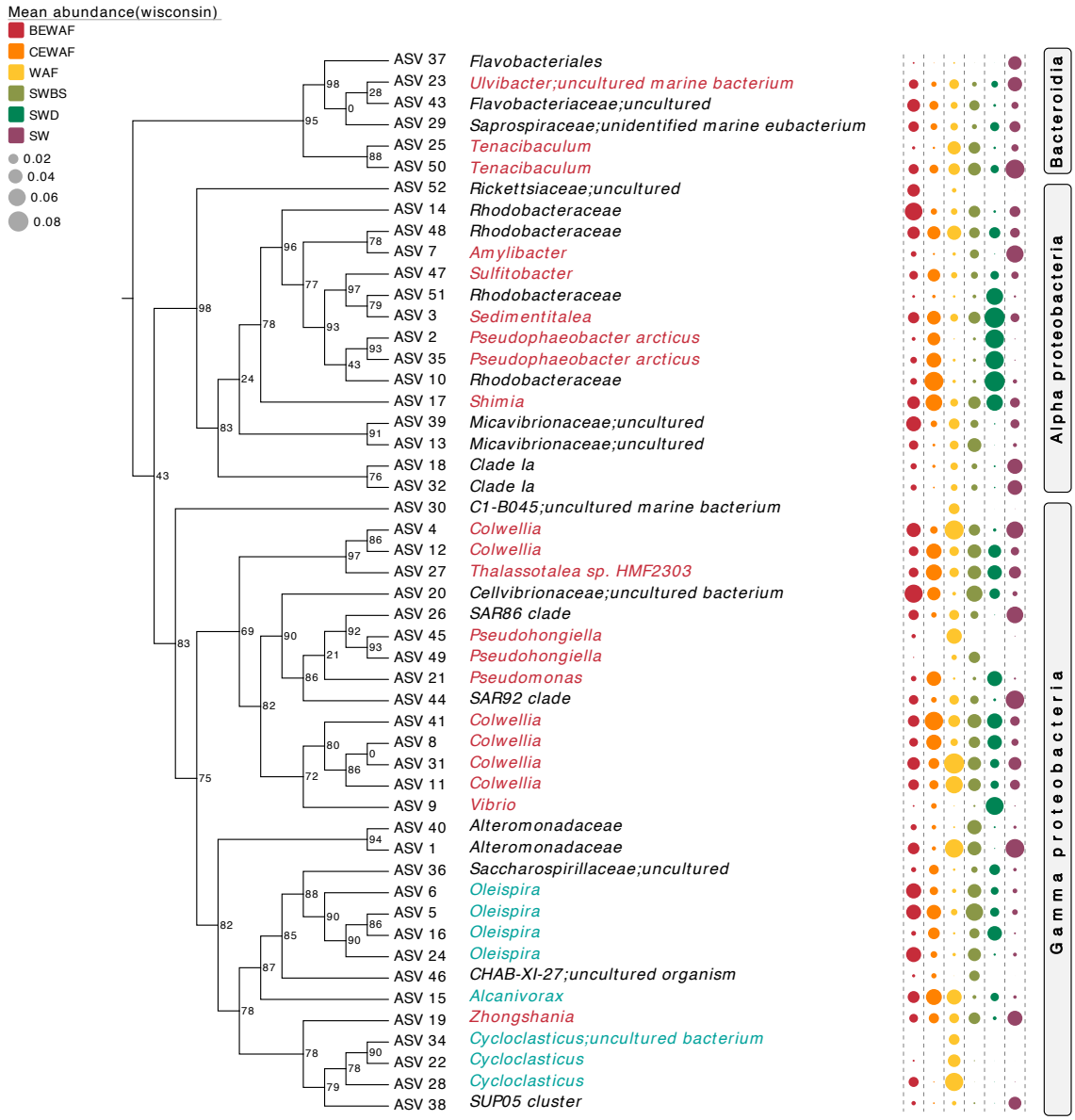


Figure 4-4. Phylogenetic tree showing the top 50 most abundant ASVs which have been taxonomically assigned with SILVA SSU v132 database. ASVs in red font represent known generalist hydrocarbonoclastic bacteria and ASVs in blue font – obligate hydrocarbonoclastic bacteria. Dot plots on the right side of the tree show the mean abundance of each ASV coloured by treatment after performing proportional standardisation using the Wisconsin function. The tree was visualised with freely available web tool Evolview2 (He et al., 2016).

4.4.3 Bacterial diversity

Alpha diversity indices, Species richness and Shannon, were calculated for each treatment at each sampling time point and pair-wise ANOVA was performed to test for significance between treatments. The bacterial diversity was highest for all treatments at

the start of the experiment (day 0), which then dramatically decreased by day 3 where all treatments had similar values for richness (Figure 4-5). The *in situ* FSC community displayed the highest richness and Shannon values and was statistically different from all other treatments. The BEWAF treatment had the second highest species richness among all experimental treatments and significantly different from the CEWAF and SWD treatments. Following day 3, the species richness gradually increased for all treatments, albeit with some small differences, which can be qualified by the community profiles shown in Figure 4-3, where mainly two to three taxa dominated the community on day 3 compared to a greater number that dominated over the course of the incubations.

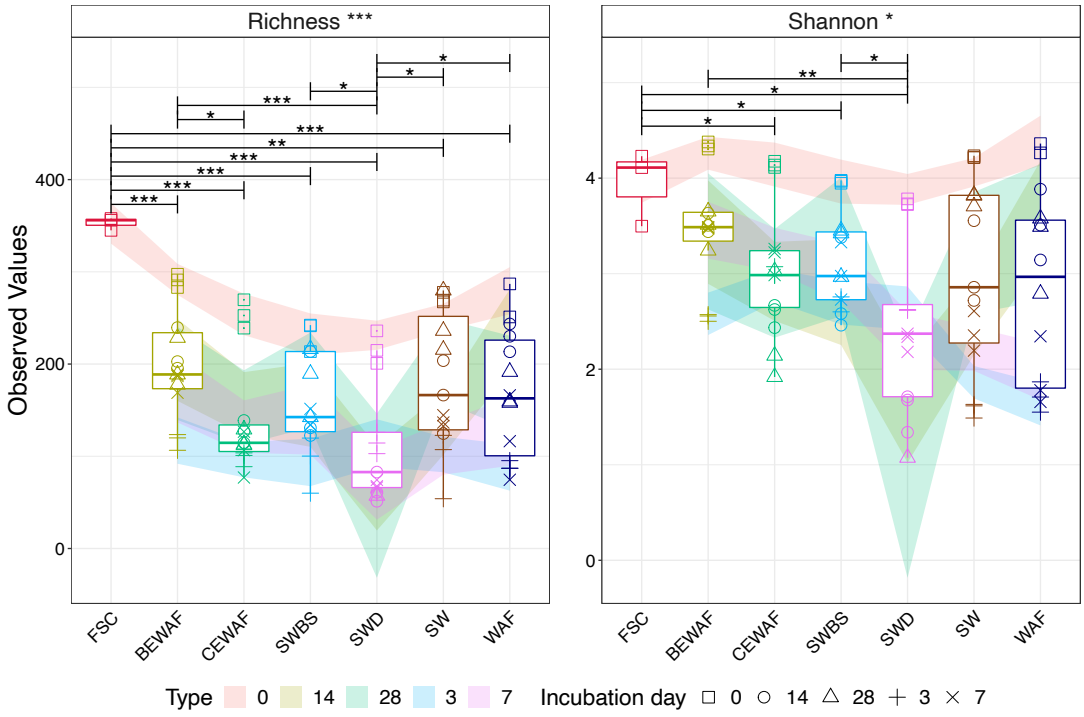


Figure 4-5. Microbial alpha diversity indices according to variances in the 16SrRNA gene of ASVs from across each treatment: FSC- baseline microbial community at time of seawater sampling, BEWAF – crude oil and biosurfactant, CEWAF – crude oil and chemical dispersant, WAF - seawater and oil only, SWD – seawater and chemical dispersant, SW - seawater only, SWBS - seawater and biosurfactant. Shaded regions (using LOESS smoothing) track the diversity shifts over time (red – day 0, blue – day 3, pink – day 7, olive green – day 14, and green - day 28). Statically different treatments (pair-wise ANOVA) are connected by bracket and the level of significance is shown with * ($p < 0.05$), ** ($p < 0.01$), or *** ($p < 0.001$). Colours represent treatments and shapes the incubation time.

In terms of beta diversity, a PCoA demonstrated distinct clustering of some treatments (based on Bray-Curtis dissimilarities), while it showed other treatments to

overlap and cluster close to each other (Weighted UniFrac) indicating a close similarity between them (Figure 4-6). The treatments became more dissimilar from day 3 onwards, whereas treatments clustered closely at day 0, as expected, and close to the *in-situ* FSC community profile (Bray-Curtis). The FSC community clearly separated from the rest of the treatments based on Weighted UniFrac where Dim1 explained 69% of the variation in treatment type. With this grouping, main sources of variation are the distribution of species rather than their phylogenetic relatedness. Treatment type was significant (p = 0.001) in all three measures (Bray-Curtis, Unweighted UniFrac, and Weighted UniFrac) and explains 33% variability in terms of counts alone (Bray-Curtis), 20% in terms of phylogeny (Unweighted UniFrac), and 45% when both combined (Weighted UniFrac) (Figure 4-6). Sampling time, also significant in all measures (p = 0.001), explains 26% of the variability (p = 0.001) in diversity of the microbial communities in terms of Bray-Curtis and Weighted UniFrac, respectively, and 18% in terms of phylogeny alone (Unweighted UniFrac). The technical replicates from each treatment cluster close to one another in all PCoA plots, and this consistency is reflected by the similar ASV diversity of the individual replicates with small exceptions (Figure 4-6).

Complimentary to beta diversity analysis, the Local contribution to beta diversity (LCBD) shows each sample's contribution to the changes in beta diversity that could be derived as a proportion of the total beta diversity. The diversity patterns of samples with high LCBD values suggested that those samples have markedly different microbial communities from the rest of the samples. Based on diversity counts (Bray-Curtis), diversity variation generally increases over time across all treatments and is consistent with beta diversity. Initially at day 0, all treatments show significantly lower diversity (but similar to each other) compared to the *in-situ* FSC community. Expectedly, as time progressed the diversity in each treatment started to vary and at the end of the incubation period the CEWAF and SWD treatments displayed the highest contribution to beta diversity variation, and hence their bacterial communities became significantly different from all other treatments (based on Bray-Curtis and weighted UniFrac). Based on unweighted UniFrac, the community of the SWD treatment was significantly distinct from the other treatments, especially on day 7 which coincided with the emergent dominance of members within the family *Rhodobacteraceae*, such as of *Pseudophaeobacter*, *Sedimentitalea* and *Shimia*. In contrast, BEWAF and WAF treatments were dominated by members of the *Colwelliaceae* and *Saccharospirillaceae* as evident from Figure 4-3.

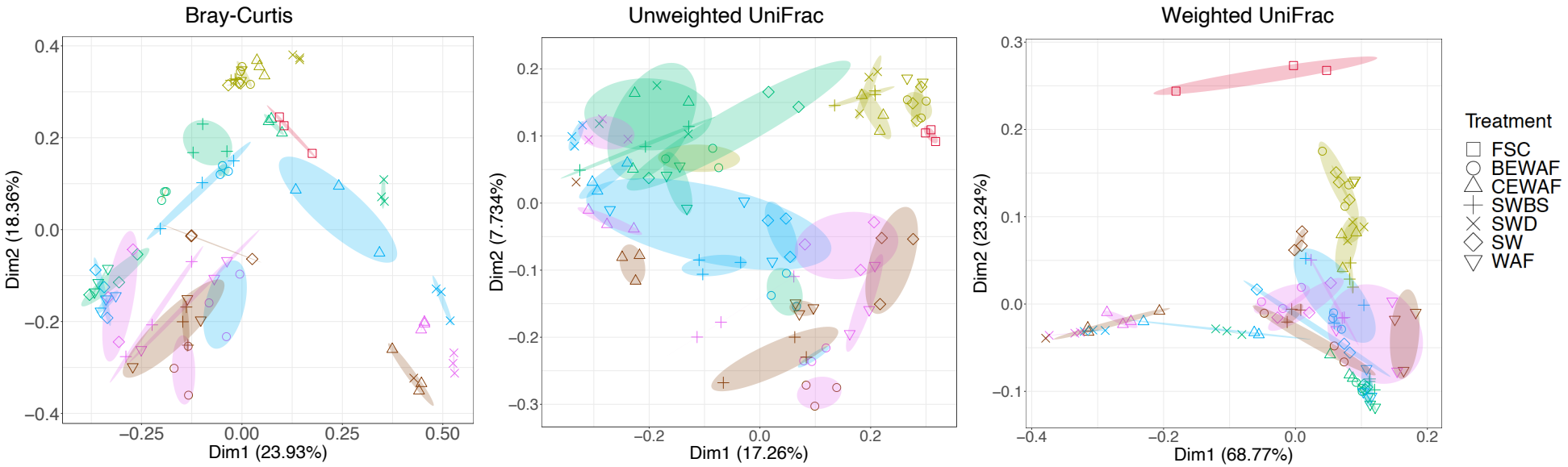


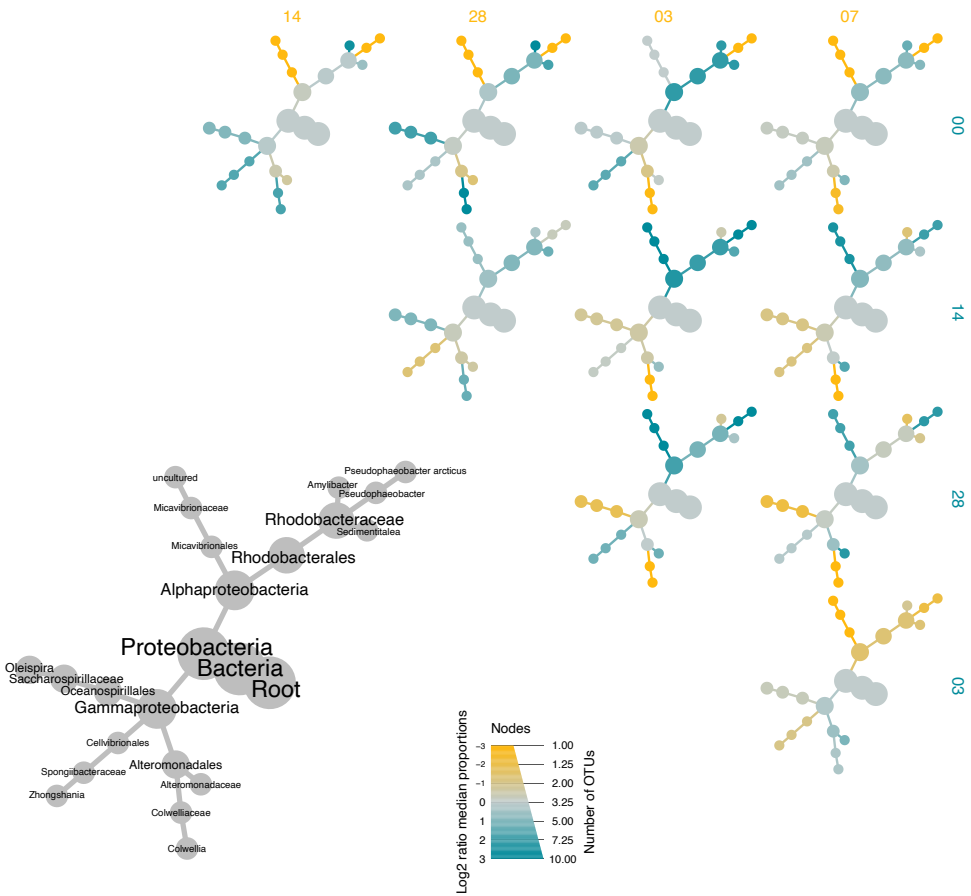
Figure 4-6. Principal Coordinate Analysis (PCoA) using Bray-Curtis, Unweighted Unifrac and Weighted Unifrac distance matrices for the treatments: FSC- baseline microbial community at time of seawater sampling, BEWAF – crude oil and biosurfactant, CEWAF – crude oil and chemical dispersant, WAF - seawater and oil only, SWD – seawater and chemical dispersant, SW - seawater only, SWBS - seawater and biosurfactant. Ellipses represent 95% confidence interval of the standard error of the ordination points of a given grouping. Results from PERMANOVA test for each distance matrix are shown underneath each plot. Colours represent sampling time (red – In-situ seawater at time of collection, olive green – day 0, green – day 3, blue – day 7, pink – day 14, and brown – day 28).

4.4.4 Key taxa representing major shifts in microbial communities

In order to identify key taxa representing major shifts in the communities across the different treatments, differential abundance analysis was performed between BEWAF and CEWAF, BEWAF and WAF, and CEWAF and WAF. The analysis returned ASVs that were an order of magnitude (log_2-fold change) different between treatments. Common oil-degraders from the genera *Marinobacter*, *Oleispira*, and *Pseudomonas* were enriched in all treatments. *Vibrio*, *Oleiphilus,* and *Glaciecola* were enriched exclusively in CEWAF, while *Alcanivorax*, *Colwellia* and *Thalassotalea* (of the family *Colwelliaceae*) were enriched in both CEWAF and WAF. *Cycloclasticus*, *Pseudohongiella* and *Acinetobacter* were enriched in the BEWAF and WAF treatments, whereas *Alteromonas*, *Moritella* and *Paraglaciecola* were exclusively enriched in the BEWAF.

In addition to the differential analysis, we also considered subset analysis where we imploded the full ASV abundance data to the minimum set of significant ASVs responsible for driving the community dynamics for each treatment over time. The resulting reduced-order abundance table correlated highly with the full table by preserving the beta diversity between samples. In the BEWAF treatment, these were *Alteromonadaceae*, *Pseudophaeobacter*, members of the *Rhodobacteraceae* family (*Amylibacter* and *Sedimentitalea*), *Colwellia*, *Oleispira* and *Micavibrionaceae* that significantly produced the shifts in community dynamics over time, and which explained 39% of the variation (PERMANOVA $R^2 = 0.395$, $p = 0.001$ (Figure 4-7). In the CEWAF treatment, only two taxa, unclassified members of the *Alteromonadaceae* family and *Amylibacter*, represented the diversity shift with 0.96 correlation with the full ASV abundance data with incubation time explaining 54% of the variation (PERMANOVA, $R^2 = 0.539$, $p = 0.001$) (Figure 4-8). In the WAF treatment, mainly the oil-degrading taxa *Oleispira* and *Alcanivorax*, and putative oil-degraders *Colwellia* and *Pseudophaeobacter*, that resulted in the shift in community diversity over time. Here, however, time explained 26% of the variation as a significant factor (PERMANOVA, $R^2 = 0.262$, $p = 0.007$) (Figure 4-9). In the SW control treatment, the key taxa that effected the community dynamics were similar to those for the BEWAF treatment, however, only 16% of variation between samples was explained by incubation time (Figure 4-10).

BEWAF



No	Subset	Correlation with full ASV table	PERMANOVA
1	*Alteromonadales_unclassified + Pseudophaeobacter + Sedimentitalea + Colwellia + Oleispira + Amylibacter + Rhodobacteraceae_unclassified + Micavibrionaceae_uncultured + Zhongshania*	0.963	R^2 = 0.395 P = 0.001
2	*Alteromonadales_unclassified + Pseudophaeobacter + Sedimentitalea + Colwellia + Oleispira + Rhodobacteraceae_unclassified + Micavibrionaceae_uncultured + Zhongshania*	0.958	R^2 = 0.395 P = 0.001
3	*Alteromonadales_unclassified + Pseudophaeobacter + Sedimentitalea + Oleispira + Rhodobacteraceae_unclassified + Micavibrionaceae_uncultured + Zhongshania*	0.946	R^2 = 0.459 P = 0.001

Figure 4-7. Differential heat trees showing the key (significant) differential taxa (DESeq2; Wilcoxon p-value test adjusted with multiple comparison) in the BEWAF treatments. The top 3 subsets with the highest correlation with the full ASV table considering Bray-Curtis distance (PERMANOVA) are listed for each treatment. The grey tree is the taxonomy key for the smaller unlabelled coloured trees. The colour of each taxon represents the $\log_2$ ratio of median proportions of reads observed in each treatment. The size of tree nodes shows the number of ASVs (here labelled as OTUs) present in each treatment.

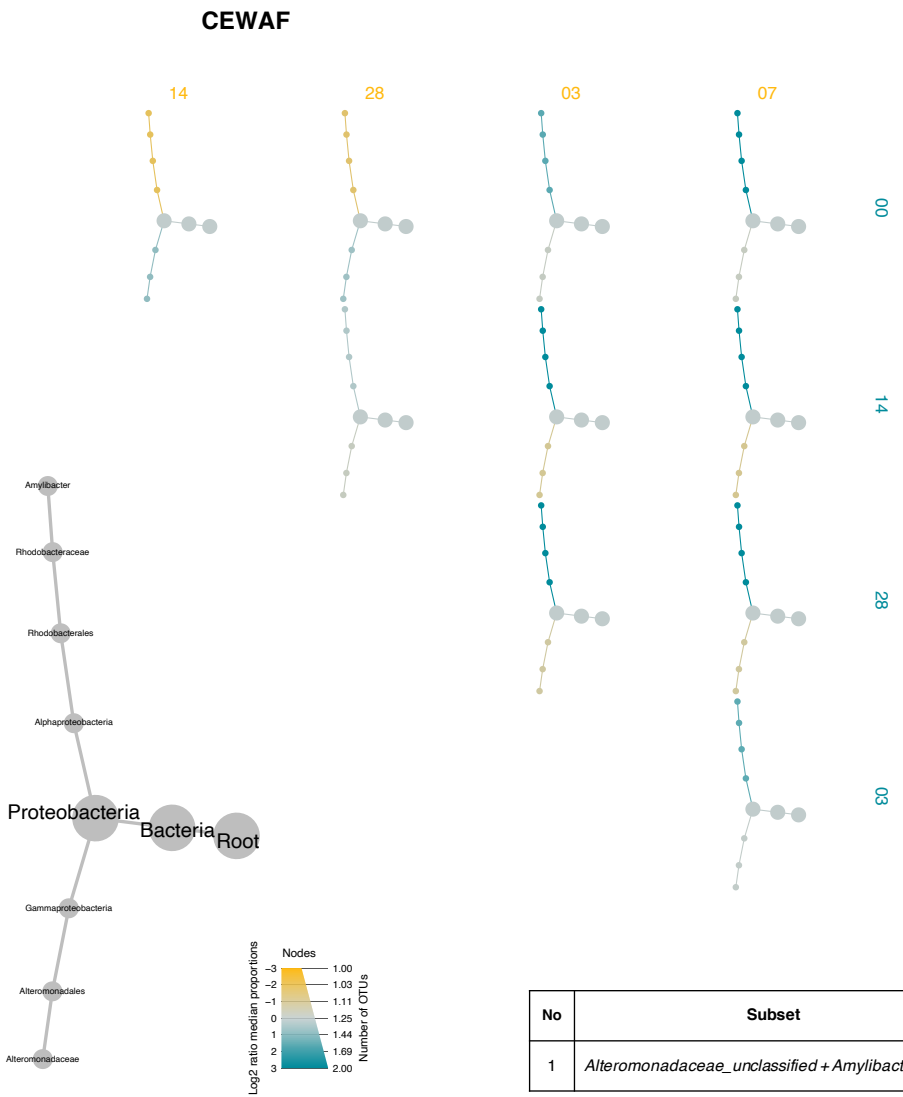
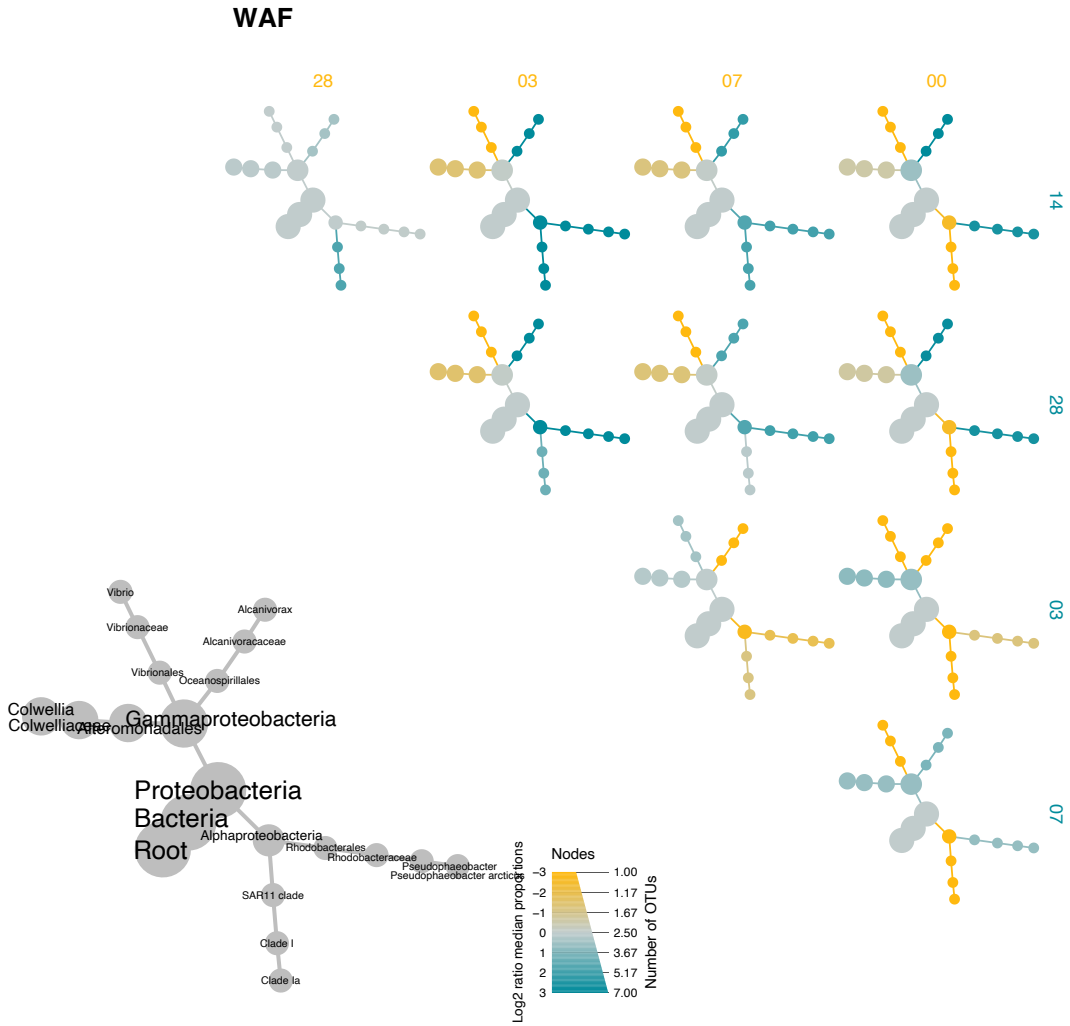


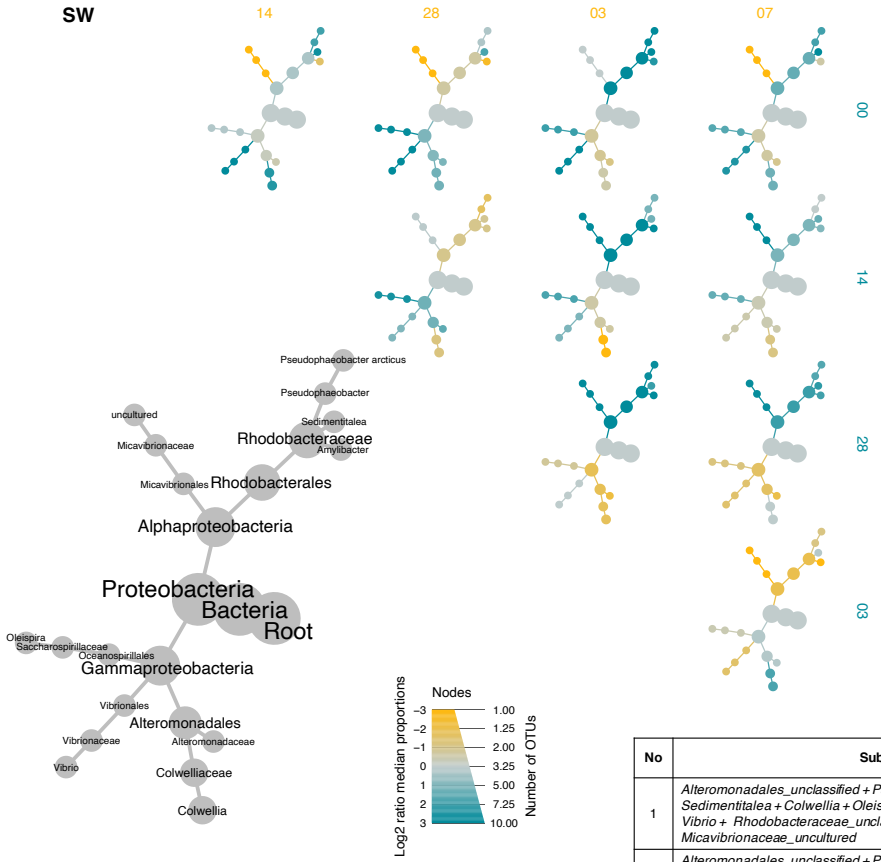
Figure 4-8. See Figure 4-7 caption.

No	Subset	Correlation with full ASV table	PERMANOVA
1	*Alteromonadaceae_unclassified + Amylibacter*	0.958	R^2 = 0.539 P = 0.001



No	Subset	Correlation with full ASV table	PERMANOVA
1	*Pseudophaeobacter + Colwellia + Colwellia + Colwellia + Alcanivorax + Clade Ia*	0.878	R^2 = 0.262 P = 0.007
2	*Pseudophaeobacter + Colwellia + Colwellia + Vibrio + Colwellia + Alcanivorax + Clade Ia*	0.878	R^2 = 0.262 P = 0.007
3	*Pseudophaeobacter + Colwellia + Colwellia + Colwellia*	0.852	R^2 = 0.195 P = 0.037

Figure 4-9. See Figure 4-7 caption.



No	Subset	Correlation with full ASV table	PERMANOVA
1	*Alteromonadales_unclassified + Pseudophaeobacter + Sedimentitalea + Colwellia + Oleispira + Amylibacter + Colwellia + Vibrio + Rhodobacteraceae_unclassified + Micavibrionaceae_uncultured*	0.950	R^2 = 0.162 P = 0.054
2	*Alteromonadales_unclassified + Pseudophaeobacter + Sedimentitalea + Colwellia + Oleispira +Amylibacter + Colwellia + Vibrio + Rhodobacteraceae_unclassified*	0.946	R^2 = 0.159 P = 0.068
3	*Alteromonadales_unclassified + Pseudophaeobacter + Sedimentitalea + Cowellia + Oleispira + Amylibacter + Vibrio + Rhodobacteraceae_unclassified*	0.937	R^2 = 0.145 P = 0.097

Figure 4-10. See Figure 4-7 caption.

4.4.5 Ecological drivers of microbial communities

Ecological drivers that are responsible for changes in microbial communities in each treatment over time were determined by the nearest-taxon-index (NTI) and nearest-relative-index (NRI). NTI and NRI determine whether there is an environmental pressure or competition between taxa in each sample is responsible for community structure. NTI/NRI greater than +2 means that there is a strong phylogenetic clustering driven by environmental filtering. Values less than -2 indicate phylogenetic overdispersion meaning the environment has little or no effect on the community assemblage. It should be noted that NRI reflects the phylogenetic clustering in a broad sense (whole phylogenetic tree) with the negative values representing evenly spread community. The *in-situ* FSC community has the lowest negative NRI value in our data set with -1.59 ± 0.96 and is significantly different from the rest of treatments, indicating that the environment setting has no effect on the community structure, which can be expected (Figure 4-11). Microbial communities of all treatments on day 0 (start of the experiment) have NRI values around 0 with BEWAF and WAF communities having values of -0.08 ± 0.27 and -1.06 ± 0.28, respectively. Over time, NRI increases in all treatments which confirms that environmental pressure (the presence/absence of crude oil, chemical dispersant + crude oil, or biosurfactant + crude oil) determine the response of microbial community and not ecological competition between taxa. NTI focuses more on the tips of the tree and positive values indicate co-occurrence of more closely related species whereas negative values mean that related species do not co-occur. All treatments have NTI values higher than +2 which indicates that they have significantly phylogenetically clustered communities in the terminal clades. The fact that the baseline community (FSC) is significantly different from the seawater used in the incubation (SW) shows that the bacterial communities in the seawater changed during the transition period between collection and start of experiment.

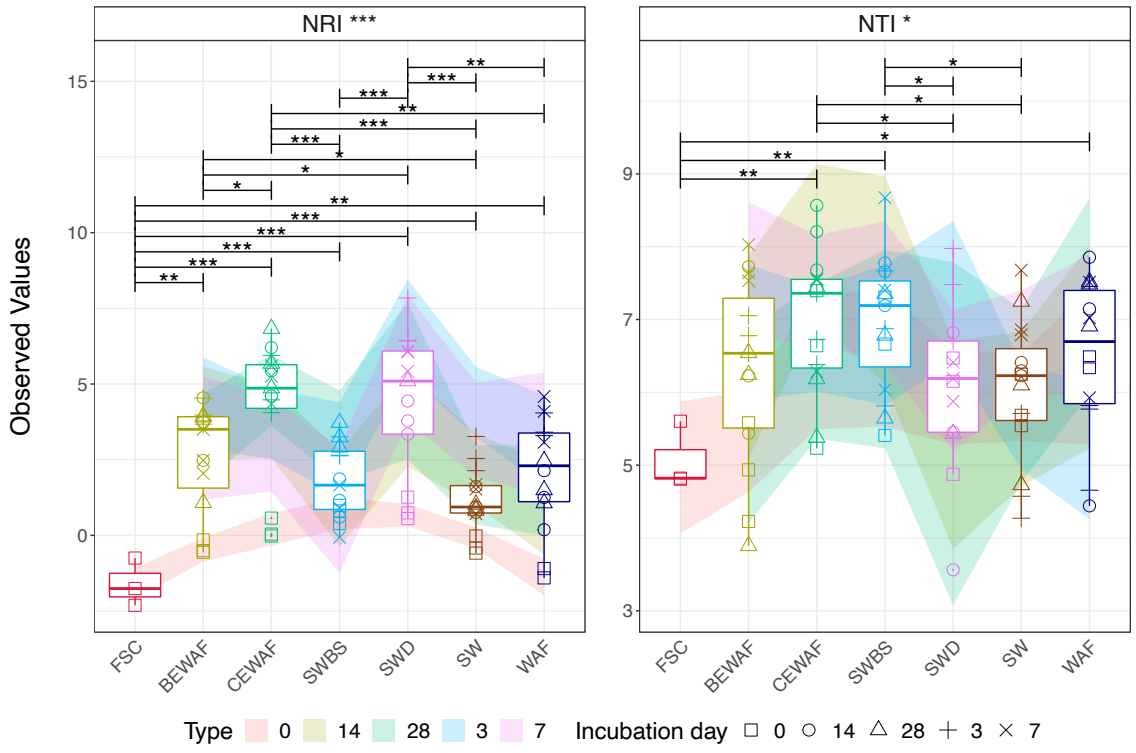


Figure 4-11. Environmental filtering: NRI (net-relatedness-index) and NTI (nearest-taxon index) according to phylogenetic variances in the 16S rRNA gene in sample across each treatment: FSC-baseline microbial community at time of seawater sampling, BEWAF – crude oil and biosurfactant, CEWAF – crude oil and chemical dispersant, WAF - seawater and oil only, SWD – seawater and chemical dispersant, SW - seawater only, SWBS - seawater and biosurfactant. Shaded regions (using LOESS smoothing) track the diversity shifts over time (red – day 0, blue – day 3, pink – day 7, olive green – day 14, and green - day 28). Statistically different treatments (pair-wise ANOVA) are connected by bracket and the level of significance is shown with * ($p <$ 0.05), ** ($p < 0.01$), or *** ($p < 0.001$). Colours represent treatments and shapes the incubation time.

4.4.6 Regression analysis – environmental predictor variables

Regression analysis was used to show which predictor variables best explained the variation of diversity in the dataset. As shown in Figure 4-12, those variables that significantly affected the alpha and beta diversity variation, either positively or negatively in the optimal model (with the lowest prediction error), are highlighted in red and blue, respectively. predictive models for each metric of microbiome analysis – alpha diversity indices (Richness and Shannon), beta diversity (local contribution to beta diversity based on Bray-Curtis, Unweighted and Weighted UniFrac distance matrices), and NTI and NRI

were determined. As expected, the variable that positively affected the species richness and Shannon index was incubation day 0, whilst incubation days 3 and 7, and treatments CEWAF and SWD, negatively affected the variation in alpha diversity. The treatments CEWAF, SWD, WAF and BEWAF, as well as incubation day 3, positively affected the variation in the NRI, indicating that these treatments would increase the clustering of bacterial communities. Conversely, no clustering was expected on incubation day 0. The presence of Finasol in the seawater (SWD treatment) positively influenced (or increased) the variation in beta diversity (LCBD) of the bacterial community. In terms of alpha diversity, Finasol-amended treatments (CEWAF and SWD) reduced the species richness of the bacterial community. This coincided with the dominance of predominantly four taxa, *Colwellia*, *Oleispira*, *Sedimentitalea* and *Vibrio* (Figure 4-3) during the same period. In contrast, the *in situ* FSC community, which was not amended with crude oil, Finasol or the biosurfactant was a positively influencing parameter on alpha and beta diversity. Conversely, the addition of just crude oil to the FSC seawater (WAF treatment) did not significantly influence the variation of diversity in the dataset.

	Richness	**Shannon**	**NRI**	**NTI**	**LCBD** Bray-Curtis	**LCBD** Unweighted UniFrac	**LCBD** Weighted UniFrac
Treatment_CEWAF	***		***		***		***
Treatment_SWD	***	**	***	*	***	***	***
Treatment_BEWAF		**	***				
Treatment_SWBS							
Treatment_SW				*	***	**	
Treatment_WAF			*		***		
Treatment_FSC	***			**	***	***	***
Incubation_day_0	***	***	***		***		
Incubation_day_3	***	**	**			***	
Incubation_day_7	***			**			
Incubation_day_14					***		**
Incubation_day_28					***	***	***

Figure 4-12. Summary of significant predictive parameters (left) determined by regression analysis based on unsupervised machine learning for two alpha diversity measures (richness and Shannon), NTI, NRI, and Local Contribution to Beta Diversity (LCBD). The parameters shown here are from the optimal model for each metric, where blue represent negatively and red – positively influencing variables, respectively.

4.4.7 Functional diversity and abundance

The ASV sequences were further used to characterise the functional diversity as alpha and beta diversity. The predicted functional alpha diversity was calculated using two standard ecological diversity measures – the rarefied genetic functional richness and the Shannon entropy. Pairwise ANOVA on richness and Shannon indicated significant differences in the different treatments (Figure 4-13 A). By days 14 and 28, the richness in the BEWAF and WAF treatments was very similar and close to the maximum number of predicted KO. The SWD treatment displayed the lowest diversity of KO (Figure 4-13 A), especially on day 7 and thereafter. The beta diversity was calculated by Bray-Curtis dissimilarity distance which demonstrated a distinct clustering of all treatments on day 0, and for SWD on days 3, 7, and 14, while the rest of the treatments overlapped (Figure 4-13 B). PERMANOVA analysis revealed that the treatment and incubation time were significant factors (PERMANOVA, $p = 0.001$) and which respectively explained 18% ($R^2 = 0.1814$) and 21% ($R^2 = 0.2064$) of the variability in beta diversity.

To present the predicted functional abundance, we selected specific KO involved in aliphatic and aromatic hydrocarbon degradation pathways, as well as for biosurfacant synthesis. For simplicity, we looked at only the three main treatments containing crude oil – BEWAF, CEWAF and WAF. Samples from CEWAF treatment showed potential enrichment of genes involved in the degradation of medium-chain length alkanes, styrene, fluorobenzoate, PAHs (naphthalene, phenanthrene etc.), chlorocyclohexane, chlorobenzene, and xylene (Figure 4-14). The relative abundance of predicted genes which encode for the degradation of short-chain length (methane monooxygenase) and medium-chain length (alkane 1-monooxygenase and rubredoxin-NAD(+) reductase) alkanes were significantly increased in all three treatments on day 3, and in the CEWAF on days 3 and 7. The BEWAF treatment was potentially enriched with genes involved in the degradation of chloroalkanes, benzoate, bisphenol, furfural, and flurobenzoate, whilst genes involved in the degradation of BTEX (benzene, toluene, ethylbenzene, xylene), dioxin and nitrotoluene were predicted to be more abundant in the WAF treatment.

Genes for different biosurfactants were also predicted in our dataset. Genes involved in rhamnolipid synthesis, namely *rhl*A and *rhl*B (rhamnosyltransferases) were predicted at days 0 and 3 in the BEWAF, CEWAF and WAF treatments, although their

abundance was not high (Figure 4-14). Surfactin synthesis genes were also predicted in the same treatments and time periods, but with relatively higher abundance. Exopolysaccharide production protein ExoY was predicted in all treatments, but at lower abundance than for the rhamnolipid and surfactin genes on days 0 and 3, and it was comparatively higher on day 14 in the BEWAF treatment.

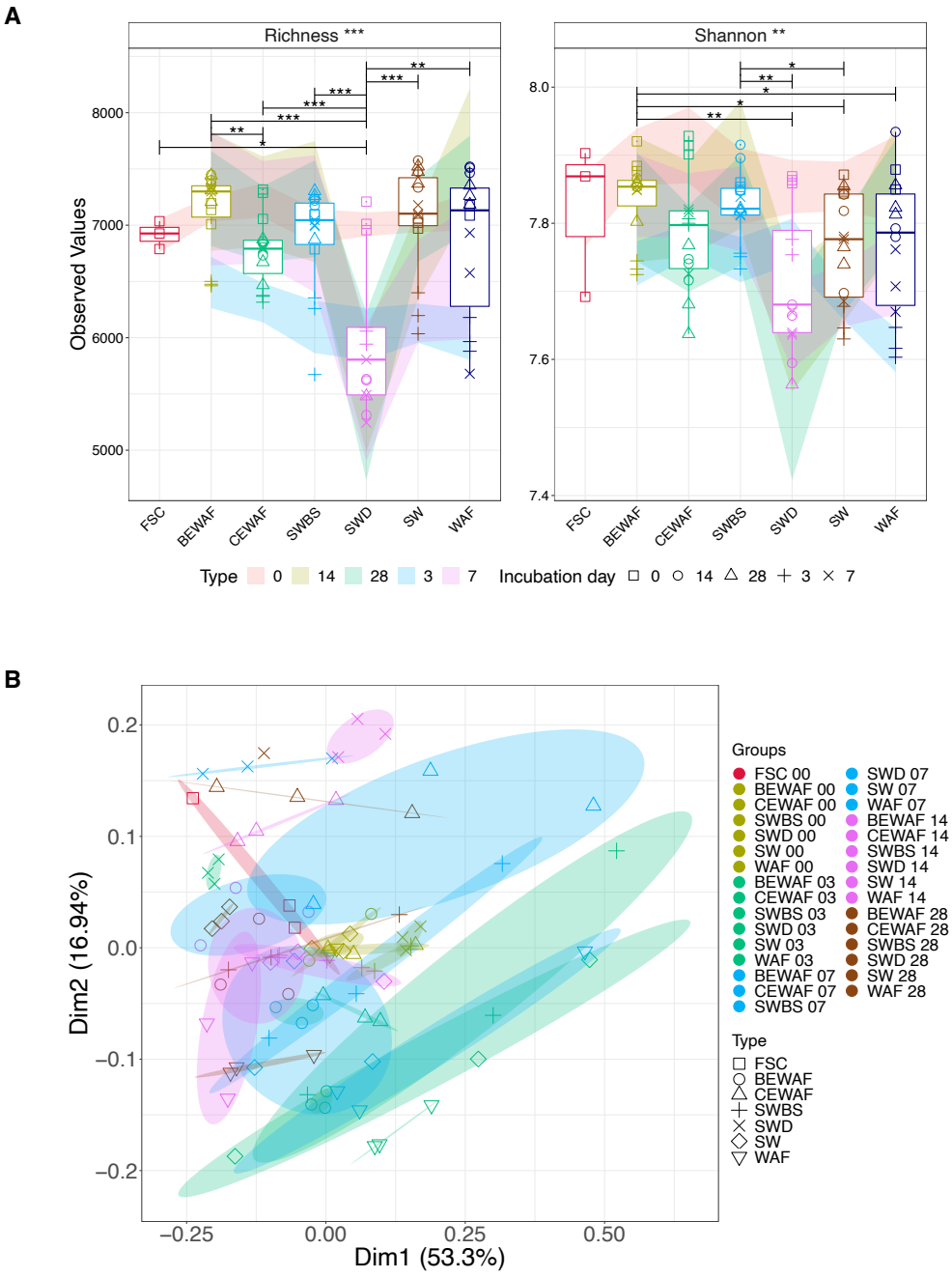


Figure 4-13. (A) Predicted functional alpha diversity of microbial pathways (expressed as number of KEGG orthologs) in treatments: FSC- baseline microbial community at time of seawater sampling, BEWAF – crude oil and biosurfactant, CEWAF – crude oil and chemical dispersant, WAF - seawater and oil only, SWD – seawater and chemical dispersant, SW - seawater only, SWBS - seawater and biosurfactant. Shaded regions (using LOESS smoothing) track the diversity shifts over time (red – day 0, blue – day 3, pink – day 7, olive green – day 14, and green - day 28). Statistically different treatments (pair-wise ANOVA) are connected by bracket and the level of significance is shown with * ($p < 0.05$), ** ($p < 0.01$), or *** ($p < 0.001$). Colours represent treatments and shapes the incubation time. **(B)** Principal coordinate analysis (PCoA)

on beta diversity measured with Bray-Curtis dissimilarity distance matrix. Note: colours and shapes are different in **A** and **B**.

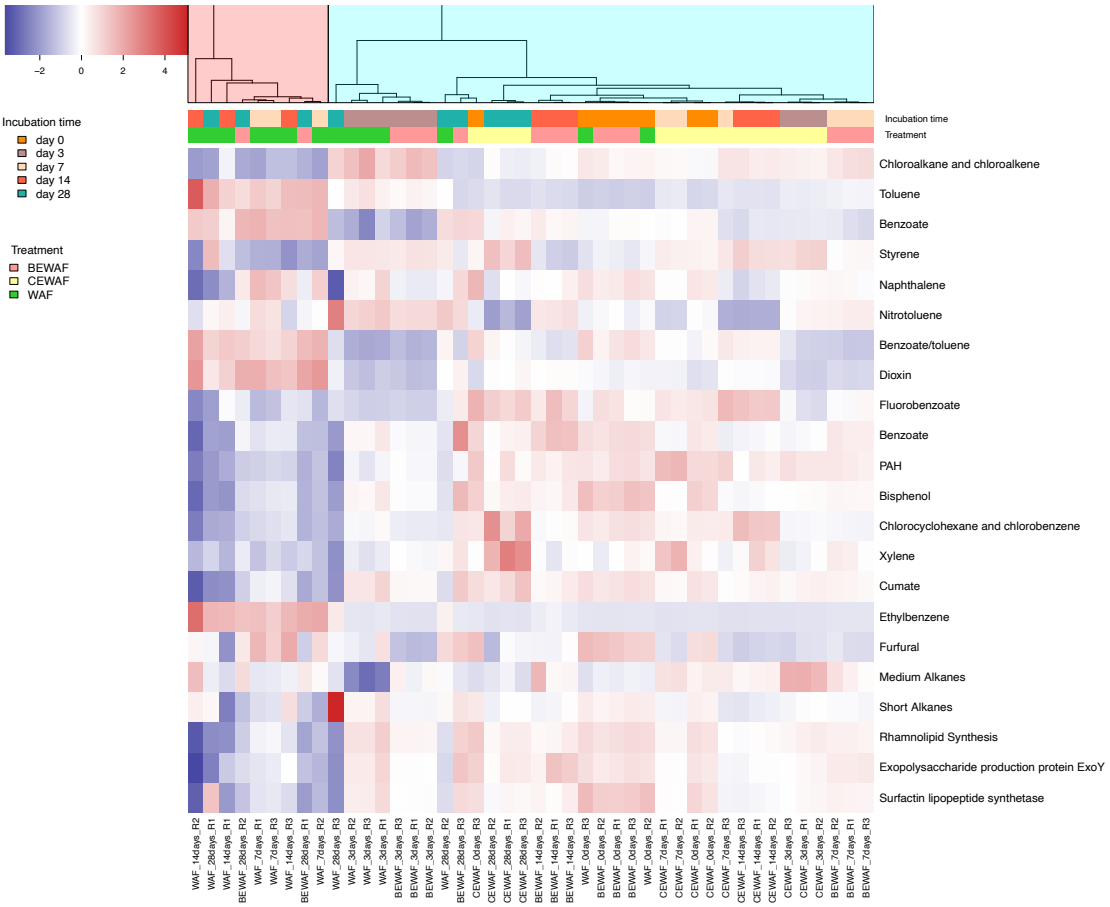


Figure 4-14. Heatmap showing the scaled log abundance (color key on top left) of aliphatic and aromatic degradation, and biosurfactant synthesis pathways. Pathways are shown along the y-axis and BEWAF (crude oil and biosurfactant), CEWAF (crude oil and chemical dispersant), and WAF (seawater and oil only) samples along the x-axis. The two color-coded bars on top of the heatmap indicate their treatments and incubation days status. Hierarchical clustering of the samples (top) is based on the correlation between samples' predicted gene expression.

4.4.8 Crude oil biodegradation

The GC-FID chromatograms of C_{12} to C_{30} *n*-alkanes for each oil-amended treatment on days 0, 7 and 28 were compared in order to assess the extent of degradation over the course of the incubations (Figure 4-15). The higher peak intensity of aliphatic hydrocarbons in the BEWAF treatment was due to some oil inadvertently getting transferred during the microcosm setup. In the BEWAF treatment, the relative intensity

of conventional isoprenoid biomarkers pristane and phytane was reduced approximately 70% by day 7, and then 86% by day 28 compared to day 0. For the CEWAF treatment, visual observation of the GC-FID chromatograms clearly indicated the oil had become substantially degraded by day 7, and analysis of the GC-FID data confirmed that by day 28 most of the lower alkanes including pristane were degraded. The WAF treatment had comparable peak intensities to the CEWAF treatment on day 0, but by day 7 and 28 the pristane and phytane peak intensities were visibly lower in the WAF.

In addition, peak areas for individual C_{12} to C_{30} *n*-alkanes, and two PAHs (phenanthrene, and methylphenanthrene) were obtained for each of the three treatments at days 0, 7 and 28 in order to calculate ratios of specific hydrocarbons that are indicative for biodegradation (Figure 4-15). Two-way ANOVA analysis for variance and *post-hoc* Tukey test confirmed that there were significant differences between the treatments for two of the hydrocarbon ratios, specifically reductions for both nC_{17} and nC_{18} in relation to their isoprenoid counterparts ($F = 5.758$; $p = 0.007$ and $F = 65.14$; $p < 0.001$, respectively). The ratio of nC_{17}/pristane in the WAF treatment was significantly different ($p = 0.009$) from that in the BEWAF, but not the CEWAF treatment, and a Tukey's test showed that sampling time was insignificant. In contrast, the nC_{18}/phytane ratio in the WAF treatment was significantly different to that in the BEWAF and CEWAF treatments on days 0 ($p < 0.001$) and 28 ($p < 0.001$ and $p = 0.003$, respectively). Both nC_{17}/pristane and nC_{18}/phytane ratios were highest for the WAF treatment at the start of the experiments, and this was maintained to the end, showing that biodegradation of alkanes in the WAF treatment took longer than in the CEWAF or BEWAF treatments (Figure 4-16). After just 7 days of incubation, the nC_{17}/pristane and nC_{18}/phytane ratios in the CEWAF treatment decreased by 6-fold and 4.5-fold, respectively, compared to the BEWAF treatment which decreased insignificantly until the termination of the experiment at day 28. With regards to the biodegradation of PAHs, the biomarker ratios were not significantly different between treatments and sampling times, but generally decreased over time in the three oil-amended treatments. However, the phenanthrene/9-methylphenanthrene ratio in the BEWAF treatment decreased the most by the end of the incubation compared to that in the CEWAF and WAF treatments.

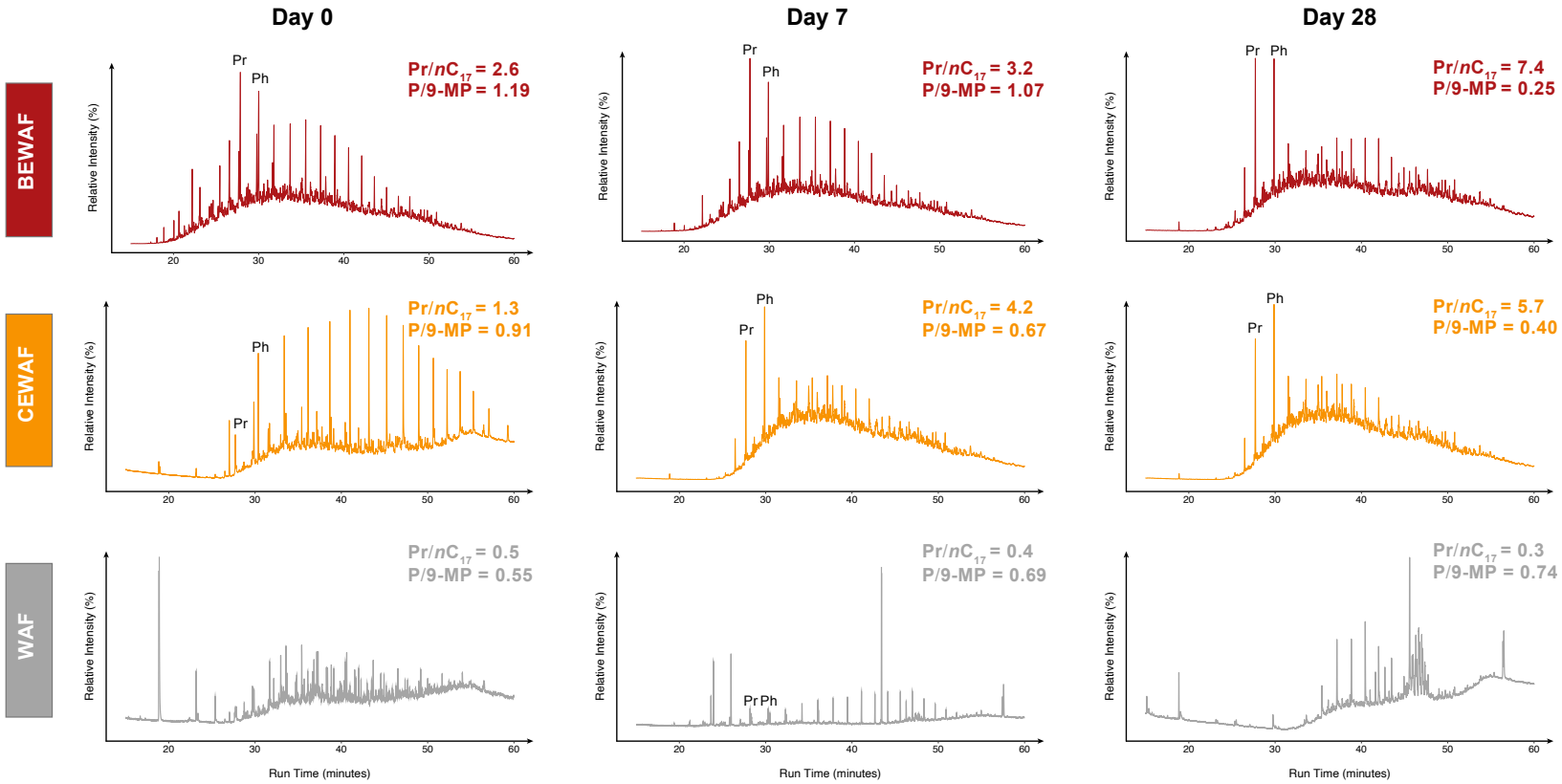


Figure 4-15. Representative flame-ionization chromatograms of the aliphatic hydrocarbon fraction of BEWAF - crude oil and biosurfactant (red), CEWAF - crude oil and chemical dispersant (orange), and WAF - seawater and oil only (grey) through time of incubation (days 0, 7 and 28). Also shown are ratios of pristane versus heptadecane (Pr/*n*C17), which increases with increased biodegradation, and phenanthrene versus 9-methylphenanthrene (P/9-MP), which has an inverse relationship with biodegradation extent. Pristane (Pr) and phytane (Ph) are annotated for reference. Note ordinate axis is displayed in relative abundance.

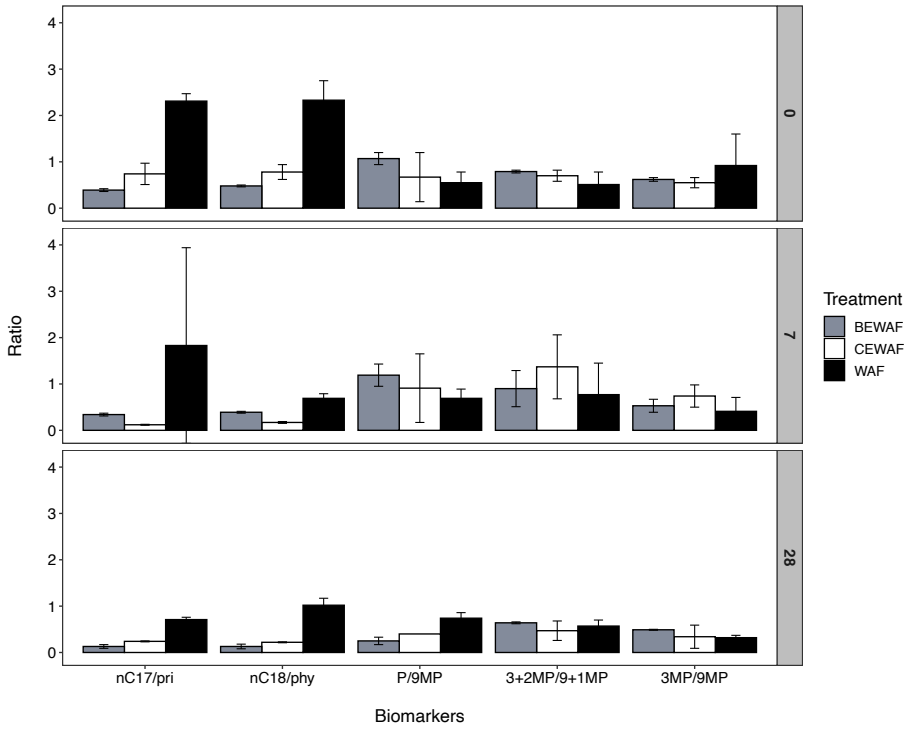


Figure 4-16. Differences in Aliphatic and PAH biomarker ratios of three different treatments: BEWAF - crude oil and biosurfactant, CEWAF - crude oil and chemical dispersant, and WAF - seawater and oil only, over time in days (grey boxes): nC17/pristane (nC17/pri), nC18/phytane (nC18/phy), Phenanthrene/9-methylphenanthrene (P/9MP), (3+2)-methylphenanthrene/(9+1)-mehylphenanthrene (3+2MP/9+1MP), and 3-methylphenanthrene/9-methylphenanthrene (3MP/9MP). Values are the mean of three independent replicates (except for BEWAF day 0 (two replicates) and CEWAF day 28 (one replicate)) +/- standard deviation.

4.5 Discussion

This chapter aimed to better understand the effects of crude oil on the bacterial communities in surface waters of the Faroe-Shetland Channel, with a focus on how the response of the oil-degrading population and its biodegradation of the oil may be influenced by a biosurfactant (rhamnolipid) compared to a government-approved chemical dispersant (Finasol OSR52). The majority of studies describing the response of microorganisms to dispersed crude oil focus on the effect of chemical dispersants which, for many years, have been a primary response tool, particularly in cases of large-scale marine oil spills. The suitability of using biosurfactants for dispersing and enhancing the biodegradation of crude oil and its petrochemical derivatives has been discussed for

decades (Banat, 1995; Desai and Banat, 1997; Rosenberg and Ron, 1999; Banat et al., 2000; Marchant and Banat, 2012; Ron and Rosenberg, 2014), but to the best of our knowledge they have not, hitherto, been compared with chemical dispersants for these purposes. Furthermore, we know of no published reports that have investigated the effect of rhamnolipids on the microbial community response to crude oil from cold marine environments and under *in-situ* simulated experiments. Since there are a number of studies, especially following the Deepwater Horizon spill, reporting conflicting results on the effects of chemical dispersants on natural communities of oil-degrading bacteria, this has highlighted the need to explore alternative products, such as biosurfactants, and to assess these under environmentally relevant conditions. This is especially important in future decisions on dispersant selection and use for combatting marine oil spills.

4.5.1 Bacterial community dynamics

Considering the FSC is a cold subarctic environment (sea surface temperature 9.7°C at the time of our sampling), unsurprisingly the bacterial communities *in situ* were dominated by psychrophilic taxa, including those belonging to recognised oil-degraders such as *Oleispira*, *Colwellia* and *Cycloclasticus* that have similarly been identified in other cold sea surface (Kasai et al., 2002; Yakimov et al., 2003, 2007; Coulon et al., 2007; Brakstad et al., 2018; Ribicic et al., 2018) and subsurface (Kleindienst et al., 2016) waters, and reported in a growing number of studies examining the microbial dynamics in the water column and sediment of the FSC (Ferguson et al., 2017; Suja et al., 2017, 2019; Gontikaki et al., 2018; Perez Calderon et al., 2018; Potts et al., 2018). The relatively high abundance of oil-degrading bacteria and their rapid response following exposure to the crude oil (within 3 days), suggests that these organisms might be already well primed on background concentrations of hydrocarbons, such as from permitted releases of oil-produced waters from oil-producing facilities in the FSC and from adjacent waterways in the North Sea, or from biogenic sources such as cyanobacteria (Lea-Smith et al., 2015; Valentine and Reddy, 2015). Frequent shipping and oil transportation activities in and around the FSC, including from the Sullom Voe which is a major oil terminal on the west coast of Shetland, could also be contributing to a continual release of hydrocarbons into the region. Though no confirmed oil seeps are known along the seabed of the FSC, nor in adjacent water bodies such as the North Sea, evidence from satellite surveys suggests

the presence of subsurface oil seeps on the east and west of Scotland and offshore in the North Sea (Peter Browning-Stamp, pers. comm.). Collectively, a similar situation is well documented in the Gulf of Mexico in potentially supporting a primed community of oil-degrading bacteria (Joye and Kostka, 2020). In the event of a major crude oil spill in these cold subarctic waters, these communities may be well adapted to respond quickly to a large influx of crude oil.

Colwellia are commonly observed in cold surface and deep sea environments (Hazen et al., 2010; Redmond and Valentine, 2012; Suja et al., 2017; Ribicic et al., 2018), and some members of the genus have been reported to utilise a broad range of hydrocarbons, including the short-chain alkanes propane, ethane and butane (Valentine et al., 2010), as well as the PAH phenanthrene (Gutierrez et al., 2013b). This metabolic versatility for hydrocarbon substrates could explain the early bloom of *Colwellia* in the WAF, BEWAF and CEWAF treatments. It may also relate to their atypical high abundance in the *in-situ* baseline community of the FSC, and we posit that this might represent a sub-group of the genus with oil-degrading qualities that is sustained at such elevated levels by background concentrations of hydrocarbons, as explained above. In a recent study, members of *Colwellia* were implicated in degrading Corexit in treatments of deep-sea water from the Gulf of Mexico amended with the dispersant, with their enrichment from initially 1% to 43% after only one week (Kleindienst et al., 2015b). Interestingly, we found *Colwellia* became the dominant organisms in the CEWAF treatment (by day 3), but in the dispersant-only treatment (SWD) the abundance of these organisms markedly decreased by day 3, and thereafter continued to do so to levels of <1% by day 14, subsequently becoming overprinted by members belonging to the families *Rhodobacteracaea* and *Vibrionaceae*; this pattern was similarly reported in another study by our group where we used the chemical dispersant Superdispersant-25 (Suja et al., 2017). Members of both these families were previously linked to utilising the organic matter produced by an earlier bloom of hydrocarbon degraders (Hazen et al., 2016; Hu et al., 2017) or using the dispersant itself as a substrate, as previously shown in other studies which used Corexit (Kleindienst et al., 2015b). We found *Vibrio* became markedly enriched in only the SWD treatment, with highest levels reached by day 3, whereas compared to the CEWAF treatment its abundance was much less, suggesting that these organisms might have a preference for the Finasol over the oil as a carbon and

energy source. With respect to the observed bloom by members of the *Rhodobacteracaea*, mainly *Sedimentitalea, Pseudophaeobacter* and to a lesser extent *Sulfitobacter*, these organisms may likely have contributed to the degradation of the oil components as members of these genera have been reported with hydrocarbon degrading qualities (Hu et al., 2017; Gontikaki et al., 2018).

Whilst *Alcanivorax* was only found in the WAF, BEWAF and CEWAF treatments, it was almost undetectable in the control treatments (SW, SWD and SWBS), indicating that the time it takes to make the WAF for each of these oil-amended treatments is sufficient to result in a partial enrichment in *Alcanivorax* prior to starting the experiments at timepoint day 0. Nonetheless, *Alcanivorax* is recognised for its almost exclusive preference for aliphatic oil hydrocarbons and for commonly being found to bloom in the early days after introduction of oil in the environment (Head et al., 2006; Yakimov et al., 2007; Suja et al., 2017). However, we observed an increase in *Alcanivorax* towards the end of our incubations (days 14 and 28) in the WAF, BEWAF and CEWAF treatments. This was unexpected and may be because these organisms were outcompeted by more resilient earlier bloomers (esp. *Colwellia*, *Oleispira*, *Sedimentitalea*, and uncultured *Microvibrionaceae*), which is reminiscent to the non-enrichment of *Alcanivorax* during the Deepwater Horizon oil spill; 16S rRNA gene sequences for this genus were practically undetected in metagenomic libraries representing the bacterial communities in the water column of the Gulf of Mexico during the active phase of the spill (Hazen et al., 2010; Gutierrez et al., 2013b; Yang et al., 2016). Alternatively, or in addition, *Alcanivorax* in surface waters of the FSC may have the ability to access a greater range of different hydrocarbons when grown on dispersed oil (for example in the CEWAF treatment), as previously observed elsewhere (Overholt et al., 2016), or the *in-situ* cold temperatures (~10°C) and/or nutrient limitation in the FSC could potentially explain the delayed response by *Alcanivorax*.

One of the early bloomers, *Cycloclasticus*, started to appear by day 7 in predominantly the WAF treatment where it increased in abundance until day 28, whereas in the only other treatment it appeared was the BEWAF treatment towards the end of the incubation period at day 28. This late blooming by *Cycloclasticus* is commonly associated with its appetite for predominantly PAHs (including naphthalene, phenanthrene, toluene

and xylenes) (Kasai et al., 2002; Head et al., 2006; Gutierrez et al., 2013b), which are often targeted after the more easily-degradable short-chain alkanes have been consumed by organisms such as *Oleispira* and *Colwellia* and other undefined oil-degrading taxa (Head et al., 2006; Yakimov et al., 2007). This microbial succession, that is largely directed by hydrocarbon substrate degradability, is consistent with a prior study using Norwegian waters and the chemical dispersant Slickgone NS (Brakstad et al., 2018). It was unusual we did not find *Cycloclasticus* in the dispersant-amended treatments, since previous studies have shown these organisms to dominate the microbial communities in chemically-dispersed oil in northeast Atlantic seawater (Suja et al., 2017; Brakstad et al., 2018) and in deep-water oil plumes during the early phase of the Deepwater Horizon spill in the Gulf of Mexico (Hu et al., 2017). Comparing the dynamics of *Cycloclasticus* across the dispersant- and biosurfactant-amended treatments, it was clear that Finasol negatively impacted this taxon, which is profound considering the importance of these organisms in the biodegradation of the aromatic hydrocarbon fraction of oil during a spill (Head et al., 2006).

There was a relatively high abundance of an uncultured member of the family *Micavibrionaceae* in the *in-situ* FSC microbial community (7%) and also initially (day 0) across all treatments (ranging between 5-9%), and a later bloom predominantly in the BEWAF (17%) and SWBS (18%) treatments, and to a lesser extent in the WAF (14%) by days 14 and 28. The order *Micavibrionales* has been assigned to a well-known group of obligate predatory bacteria, called *Bdellovibrio* and like organisms (BALOs) (Davidov et al., 2006). Although species from this order were first described in 1982 (Lambina, V. A et al., 1982), not much is known about this order and their role in natural ecosystems. A recent study, however, from the Lake of Geneva (Ezzedine et al., 2020) demonstrated that *Micavibrionaceae* vary throughout the year, with higher numbers in the spring months and probably linked to phytoplankton dynamics and concentrations, since they may be a possible pray for BALOs; this may have relevance to our detection of these organisms in the FSC as it coincided with a time of year, in the spring, when we sampled and phytoplankton blooms occur in this region. The dynamics of *Micavibrionaceae* observed in our study may be associated with preying on early bloomers of oil-degrading bacteria, as their increased abundance coincided with decreasing numbers of *Colwellia* and *Oleispira* in the WAF, BEWAF and SWBS treatment by days 14 and 28.

4.5.2 Impact on microbial diversity

Treatments with added dispersant (CEWAF and SWD) compared to the BEWAF and WAF treatments had lower species richness. For the CEWAF and SWD treatments, this can be explained by the bloom of a few specialist taxa in the first 7 days of incubation, which had responded directly to the dispersed crude oil or the dispersant itself. To further confirm the main factors which significantly altered the species richness and beta diversity in our treatments, we performed supervised machine learning modelling (regression analysis) (Figure 4-12). The regression analysis showed that the presence of Finasol indeed negatively affected the species richness of the FSC bacterial community, and more so than exposure by the oil or it also with the rhamnolipid. If two biological systems share many characteristics (i.e., temperature, depth, nutrient availability, species etc.), data generated in one system can help inform what changes can be expected in another system for which limited data exist. Whilst conventional analyses on microbiome focus more on the variability of community structure in view of extrinsic environmental parameters, the regression analyses, on the other hand, can offer directionality (positive/negative contribution) to diversity analyses, and are widely useful to delineate diversity changes in multitude of environments, as demonstrated in other studies (Quince et al., 2015; McKenna et al., 2020). To our knowledge, supervised machine learning modelling has rarely been used in marine microbial metagenomic studies, especially in the context of hydrocarbon pollution and chemical dispersant application. However, examples of using regression analyses in other microbial community studies exist (Jones and Hallin, 2010). It was interesting to note that the addition of crude oil alone (WAF) did not affect the alpha and beta diversity significantly, indicating that the microbial community in this treatment maintained a higher diversity over time.

4.5.3 Deterministic processes drive the community assembly

NTI and NRI were determined to better understand what ecological processes govern the composition of the microbial community in each treatment. NTI and NRI specifically reveal whether stochastic (predation, competition, unpredicted disturbance etc.) or deterministic (selection imposed by an external environmental factor, i.e. environmental filtering) processes influence the assembly of the microbial community (Stegen et al., 2012). Ecological processes governing the community compositions have

been studied for subsurface (Stegen et al., 2012) and desert microbiomes (Caruso et al., 2011) but not for marine microcosms amended with crude oil and surfactants. Overall, we found that mean NTI across all communities was significantly greater than +2 providing evidence that deterministic environmental filtering strongly determines local community composition of surface seawater communities when enriched with crude oil in combination with Finasol or rhamnolipid. Therefore, it seems that the crude oil, dispersant and/or rhamnolipid limit community membership whereby closely related (e.g., belonging to the same family) and ecologically similar (e.g., hydrocarbon-degrading) taxa are more likely to coexist than expected if random ecological processes (drift) assembled the composition. The environmental filtering, however, was strongest in the Finasol-amended communities which also displayed the lowest diversity the incubation period. One explanation is that Finasol dispersed the oil to a greater extent and more of the crude oil compounds are dissolved in the microcosms than the rhamnolipid-amended and oil-only treatments, hence prompting a quicker and stronger response of members of the *Rhodobacteracaea* and *Vibrionaceae* families, known opportunistic oil degraders (Head et al., 2006).

4.5.4 Hydrocarbon degradation was enhanced in Finasol-mediated dispersions

Hydrocarbon analysis revealed faster biodegradation of crude oil in the CEWAF treatment, as indicated by the decreased nC$_{17}$/pristane and nC$_{18}$/phytane ratios and chromatogram traces. The dominant taxa in the BEWAF and CEWAF treatments were similar on day 7, with the exception for *Cowellia* and *Oleispira* where their abundance in CEWAF was lower than in BEWAF. This could confer a major portion of the alkane biodegradation to have already occurred by day 3 in the CEWAF treatment and which was still on-going in the BEWAF treatment. On the last day of the experiment (day 28), the high abundance of *Cycloclasticus* in the WAF and BEWAF treatments also supports this likelihood for on-going biodegradation. On the other hand, by day 28 in the CEWAF treatment the community was dominated by members of the *Rhodobacteriaceae* (~55% of the total community) that may have been involved in utilising the 'leftovers' of the earlier alkane-degrading bloom. The persistent presence here of *Alcanivorax* and *Pseudomonas*, albeit at relatively low abundances (respectively 4% and 3.7%), suggests on-going biodegradation until the end of the experiment. The faster biodegradation in

CEWAF might be explained by the better efficiency of Finasol in dispersing the crude oil than that offered by the rhamnolipid, and hence more oil will have been bioavailable to the oil-degrading population when Finasol was used. Our findings support observations of other microbial biodegradation studies where chemical dispersants were also shown to enhance biodegradation (Brakstad et al., 2018). A previous study (Prince and Butler, 2014) found a substantial decrease in the amount (approx. 3 times) of oil in oil plus dispersant mixtures compared to undispersed oil only, even though biodegradation occurred in both treatments. Similar observations were made in Arctic waters of temperatures down to -1°C, where the indigenous Arctic microbial community could completely degrade *n*-hexadecane, *n*-octadecane and some individual PAHs regardless of the presence of Corexit, but where the biodegradation of the oil (2.5 ppm fresh and weathered), in general, was faster in the presence of the dispersant (McFarlin et al., 2014).

CHAPTER 5

USE OF NULL MODELS TO COMPARE BACTERIAL COMMUNITY ASSEMBLY IN THE PRESENCE OF CRUDE OIL WITH EITHER CHEMICAL DISPERSANT OR BIOSURFACTANT

5.1 Overview

An *in-silico* approach was taken in this chapter to better understand the community assembly processes in Chapter 4 for the crude oil enrichment experiments amended in the presence a biogenic or a synthetic chemical surfactant. The work in this chapter has been submitted in the *Ecology and Evolution* journal.

5.2 Introduction

The number of metagenomic studies on the effects of naturally and chemically dispersed crude oil on the natural seawater microbial communities has increased in the last decade with the development of next-generation sequencing and also after major events like Deepwater Horizon oil spill (Hazen et al., 2010; Kostka et al., 2011; Kleindienst et al., 2015b; Rodriguez-R et al., 2015; Hu et al., 2017; Sun et al., 2019). Generally, in these studies the 16S rRNA gene is sequenced from environmental samples or laboratory microcosms to determine the microbial diversity and how community dynamics change with time and/or different environmental factors. Although metagenomics can provide valuable insights into the functional ecology of environmental communities, important ecological questions such as what drives the observed changes in these communities remain largely unexplored. Unravelling the ecological processes that assemble local communities in response to environmental change or perturbation has always been of great research interest (Chase, 2003).

Traditionally, community assembly is thought to be influenced by either stochastic (ecological births, deaths, extinctions, speciation, and colonisation) or deterministic (species traits, interspecies interactions, and environmental conditions) processes. Stochastic neutral theory assumes that species respond to chance colonisation, extinction, and ecological drift not because of their traits or that of their competitors, but rather because of random changes and thus have no interspecific trade-offs (i.e. species are neutral) (Hubbell, 2001). Niche theory, on the other hand, assumes that site-to-site variations in species composition is determined entirely by their specific traits, local habitat conditions, and interspecific relations, which in turn creates different niches that benefit different groups of species (determinism). The work of Chase and co-workers, however, stipulates that the assembly of local communities is simultaneously driven by

both deterministic and stochastic processes (Chase, 2010; Chase and Myers, 2011). Their framework is built around the concept that beta diversity patterns can resolve the relative influence of deterministic from stochastic processes in assembling the community structure along environmental gradients (e.g. space and time) (Chase and Myers, 2011). For this, related but different null model approaches can be applied to determine the relative importance that deterministic and stochastic processes have in generating patterns or variations in biodiversity.

Null models are statistical tests that have been widely used to describe diversity patterns in macroecology and biogeography (Gotelli, 2001). Gotelli and Graves (1996) described null models as "pattern-generating model that is based on the randomisation of ecological data or random sampling from a known or imagined distribution". The null model strategy is designed to intentionally exclude an ecological or evolutionary process of interest and create a beta diversity pattern that would be expected in the absence of this particular process - i.e. the community structure is random in respect to the process being tested (Gotelli, 2001). Recent advancements of bioinformatics and statistical tools have made it possible to apply theoretical macroecological concepts to microbial metagenomics in order to better understand and quantify the mechanisms and patterns controlling the complexity of microbial ecology (Stegen et al., 2013; Zhou et al., 2014; Zhou and Ning, 2017; Vass et al., 2020). There are four fundamental but distinct ecological processes that control community composition – selection, drift, speciation, and dispersal (Vellend, 2010). The relative importance of each process is highly context-dependent and dynamic. It is generally expected that dispersal would not be a limiting factor influencing the community assembly in fluidic ecosystems (e.g. groundwater, lakes, oceans etc.) because they are well connected, and rapid dispersion and population movement occur (Zhou et al., 2014). As speciation can only be observed in the long-term, it is not expected to have a major influence in the microbial community assembly in the study presented in Chapter 4 (experimental time was just 28 days). This leads to the hypothesis that selection (deterministic processes) and/or drift (stochastic processes) will most likely drive the changes in marine microbial community assembly as it was observed for a groundwater bacterial community (Zhou et al., 2014).

In this chapter, seven different but complementary null models were applied to the seawater microbial community data from Chapter 4 in order to better understand and

quantify the relative importance of the different assembly processes on a temporal scale in response to crude oil with either biosurfactant or synthetic chemical dispersant. Some of the null models use incidence-based (presence/absence of microbial species) metrics to calculate the null deviation between the observed and expected beta diversity (Presley et al., 2010; Chase et al., 2011; Ning et al., 2019) with the view that one can reorder the patterns (species and samples) to reveal distinct pattens that have ecological relevance. However, abundance-based data contain more information about species associations and distribution than the incidence-based data, and, therefore, deemed to be more reliable for understanding what are the underlying community assembly processes (Zhou and Ning, 2017). For this reason the latest developed null model frameworks are built on abundance-based metrics (Stegen et al., 2013) with phylogenetic information (Tucker et al., 2016; Lee et al., 2017; Verster and Borenstein, 2018; Darcy et al., 2020).

Randomly assembled communities can be differentiated by those assembled by selection processes, herein by measuring three main elements: coherence, species turnover, and boundary clumping (Leibold and Mikkelson, 2002; Presley et al., 2010). This method is referred to as elements of metacommunity structure (EMS). Coherence refers to the sequence of present species in reordered incident matrix that is not interrupted by any absent species. (Presley et al., 2010). Species turnover reveal the tendency of species to replace each other from site to site, whereas boundary clumping is the degree to which the boundaries of different species' ranges are clustered together (Anderson et al., 2011) as a means to identify subcommunity structures where group of species behave in a similar manner. Analysing these three elements of metacommunity structure can reveal what is the most likely species distribution pattern among sites or treatments that provides the closest fit to real distribution (Leibold and Mikkelson, 2002). Another null model used in this study is the incident-based (Raup-Crick) beta diversity index ($ß_{RC}$) which can distinguish between stochastic and deterministic assembly processes by calculating the dissimilarity among two communities relative to the null expectation (Chase et al., 2011). The Raup-Crick dissimilarity metric provides information on the degree to which pairwise communities are more dissimilar (or more similar) than expected by chance (Raup and Crick, 1979). A statistical framework developed by Ning *et al.* (2019) uses a quantitative index for measuring the relative importance of determinism versus stochasticity in community assembly, called

normalised stochasticity ratio (NST). The authors claim that NST is a better quantitative measure of stochasticity because it is based on magnitude rather than significance of the difference between observed and null expectation (Ning et al., 2019). Furthermore, providing that phylogenetic relatedness is suggestive of shared environmental response traits between species (Gerhold et al., 2015), phylogenetic information can be integrated with abundance-based (Raup-Crick) beta-diversity ($ß_{RCBray}$) to create a null model, called quantitative process estimate (QPE), that can quantitatively estimate the relative importance of selection, drift, dispersal limitation and mass effect (Stegen et al., 2013).

The use of technical jargon to describe ecological processes also varies in scientific literature. Although some studies have been interpreting beta-null deviation values of zero as both, "stochastic" and "neutral" assembly, and deviation values different from zero as both "niche" and "deterministic" (Chase et al., 2011; Stegen et al., 2012; Püttker et al., 2015; Ning et al., 2019), others pointed out that the terms do not represent the same thing (Vellend et al., 2014; Tucker et al., 2016). While "neutrality" implies to a community in which species are equal and so share analogous demographic rates (birth, death, migration, etc.) (Hubbell, 2001), "stochasticity", on the other hand, implies that the variation at which these demographic rates occur is random without implying anything about the mean values of these demographic rates (Vellend et al., 2014). "Niche" entails the differentiation in mean demographic rates between species (Carroli et al., 2011) and "determinisms" is the absence of random variations in species' demographic rates (Tucker et al., 2016). In fact, Tucker et al. (2016) built a new computational framework that allows for more appropriate interpretation of the beta-null deviation measure - i.e., differentiation between neutral and niche community. According to their model, beta-null deviation values close to zero indicate neutral assembly, and values that are different from zero as niche-based assembly. Furthermore, Tucker et al.'s framework has been applied in a study that investigated the response of soil microbial communities to an anthropogenic disturbance (Lee et al., 2017) and in another that explored the role of ecological selection, drift and dispersal in assembling the bacterial communities associated with domesticated and wild wheat species (Hassani et al., 2020).

Ecological process can be used to characterise the distribution of species in the microbial community assembly and identify groups or guilds of phylogenetically related species whose distribution may be governed by strong priority effects (i.e. competitive

lottery model; see work of Sale, 1979). A lottery-based assembly model was developed by Verster and Borenstein (2018) to test to what extent the competitive lottery model applied to the human gut microbiome. The authors postulated that lottery-based assembly model could explain the variation in observed diversity of human gut microbial species between different hosts. This model assumes that there is a "winner" species (hence the name "lottery"), determined randomly, that solely occupies a given niche due to a competitive advantage over other species (Sale, 1979). Although the lotter winner provides information on whether the distribution of phylogenetically related species is governed by priority effects, the order in which new species are recruited in the ecosystem remains to be described. To tackle this, Darcy et al. (2020) developed a null model-based framework to better understand how phylogenetic relationships between microbes influence the order in which microbes are recruited over time, and whether this recruitment occurs slow or fast. The authors applied their model to longitudinal human microbiome data which showed that human microbiome generally follows what they called the "nepotism" hypothesis, i.e., close relatives are more likely to be recruited in a community than distant relatives (also known as phylogenetic underdispersion) (Darcy et al., 2020). This observation is in contrast to the expectation that closely related species would experience strong competition between each other so that they cannot coexist, but hint to growing support that phylogenetic underdispersion may be more common trend in microbial communities (D'Andrea et al., 2019).

To the best of the author's knowledge, this chapter presents the first comprehensive study to apply all seven null models to oil-amended marine microbial communities to better understand the ecological processes behind the community assembly, and if and how the presence of an oil-dispersing agent (i.e., a biosurfactant and/or a synthetic chemical dispersant) had an effect on the assembly. The conclusions from the null models can help predict the changes in microbial biodiversity and ecosystem services in oil polluted environments and therefore assist in making effective decisions with regards to what would be the best oil spill response option for similar environmental conditions.

5.3 Materials and Methods

5.3.1 Elements of metacommunity structure (EMS)

EMS were assessed for each treatment (and time point) following the frameworks developed by Leibold and Mikkelson (2002) and Presley et al. (2010). For this, the ASV matrix was ordinated according to the primary axis via reciprocal averaging and then hierarchically analysed using three tests (coherence, turnover and boundary clumping). The R package *metacom* (Dallas, 2014) was used to detect any pattern the metacommunity type responsible for the assembly structure of the microbial community. The outcome of coherence test, which counts the number of interruptions in species distributions in the ordinated matrix and comparing the empirical value to a null distribution, was the basis for the subsequent statistical analysis (Presley et al., 2010). Metacommunities are randomly structured when -1.96 < coherence z-value > 1.96. Positive significant values (coherence z-value >1.96) indicate that species contribution occurs in response to environmental variation. Significantly negative coherence (coherence z-value < -1.96) indicates competitive exclusion distribution.

5.3.2 Incidence-based beta diversity

The incidence-based beta diversity (Raup-Crick) dissimilarity indices ($ß_{RC}$) were calculated to test whether stochastic or deterministic processes were dominating the assembly of the microbial communities in the different treatments as described in Chase et al. (2011). The function 'raup-crick' within the *vegan* package in R (Oksanen et al., 2019) was used to calculated the $ß_{RC}$. The Raup-Crick metric was standardised to range from -1 to +1 and randomised 999 times. Values of $ß_{RC}$ that were not statistically different from 0 indicate that the community is stochastically assembled. $ß_{RC}$ values close to -1 or +1 indicate deterministically assembled communities that are more similar or dissimilar to each other than expected by chance, respectively.

5.3.3 Normalised stochasticity ratio (NST)

The NST for each treatment was calculated to measure the actual contribution of determinism in relation to stochasticity based on abundance-based similarity Ružička (relative abundance difference of each taxon between two samples) metric using null

model algorithm PF as recommended by Ning et al. (Ning et al., 2019). For this, the 'tNST' function from *NST* package in R was used (Ning et al., 2019). The null model algorithm PF stipulates that the probabilities of taxa occurrence are proportional to the observed occurrence frequencies, and taxon richness in each sample is fixed as observed (Chase et al., 2011). When using abundance-based metric Ružička, null taxa abundances in each sample are calculated as random draw (1000 times) of the observed number of individuals with probability proportional to regional relative abundances of null taxa in the treatments. The microbial community assembly is completely deterministic when NST is 0% and completely stochastic when NST is 100%. Nonparametric permutational multivariate analysis of variance (PERMANOVA) was used to test whether the different treatments differed in their NST. Next, we calculated the NST of the oil-amended seawater (WAF), oil+dispersant amended (CEWAF), oil+biosurfactant (BEWAF), and non-amended seawater (SW) treatments over time to determine whether the proportion of stochasticity changed with time. However, in this case the NST precision is expected to be lower (but not the accuracy) because each treatment for each time point had less than 6 replicates.

5.3.4 Tucker's beta-null model

Beta-null modelling was performed to differentiate between neutral and niche assembly processes by implementing the framework developed by Tucker et al. (2016) and Lee et al. (2017), and, therefore, called Tucker's beta-null model hereafter. Abundance-based (Bray-Curtis) and generalised UniFrac dissimilarity (abundance and phylogenetic information) metrics were used to calculate the beta-null deviation values, which are indicative of the magnitude of deviation between the observed and expected beta-diversity from randomly assembled pair of samples. The number of randomisations was set to 999. The overall beta-null deviation was the mean deviation across all patch pairs. Beta-null deviation values were calculated only for the three oil amended treatments (WAF, CEWAF and BEWAF) as the computation of the results proved computationally heavy and, for this reason, was run on the supercomputer Orion Custer managed by Dr Umer Ijaz's Environmental Omics lab at the University of Glasgow. The beta-null deviation values between treatments for each time point were tested for significance with two-way ANOVA and *post-hoc* Tukey's test. Significance was accepted for p-value of less than 0.05.

5.3.5 Quantitative process estimates (QPE)

QPE require taxa to have different habitat associations (i.e. phylogenetic signal) so that phylogenetic turnover (the evolutionary distance differentiating taxa in one community from taxa in another community) can be estimated (Stegen et al., 2013). To perform QPE, the extent to which the abundance-weighted ß-mean-nearest taxon distance (ßMNTD) deviated from the mean of the null distribution was determined (after 999 randomisations) and the significance was evaluated by the ß-Nearest Taxon Index (ßNTI; difference between observed ßMNTD and the mean of the null distribution in units of SDs). For instance, variable selection assembles the microbial community if the ßNTI value is greater than 2. In contrast, the community is assembled by homogeneous selection when the ßNTI is less than -2. In the case when there is no significant deviation from the null expectation, dispersal limitation, homogenising dispersal (mass effect) or random rift should drive the observed differences in phylogenetic community composition. To determine the relative importance of each of these processes, the abundance-based (Raup-Crick) beta-diversity was calculated using pairwise Bray-Curtis dissimilarity metric ($ß_{RCbray}$) (Stegen et al., 2013). For those communities that were not assembled by selection, the $ß_{RCbray}$ value can differentiate between communities that are assembled by dispersal limitation coupled with drift ($ß_{RCbray} > +0.95$), homogenising dispersal ($ß_{RCbray} < -0.95$) or drift ($ß_{RCbray}$ between -0.95 and +0.95).

5.3.6 Lottery-based assembly model

The lottery-based assembly model was used to characterise the species distribution across treatments amended with crude oil with/without dispersant or rhamnolipid biosurfactant and to identify any microbial guilds whose distribution reflect a competitive lottery schema (Sale, 1979). The detailed protocol developed by Verster and Borenstein (2018) was followed with some exceptions. Briefly, the first step was to quantify how often species distribution within a group/guild includes a lottery winner (i.e., a group member that represent > 90% of the group's abundance). The background expectation of the winner prevalence parameter was determined by implying a null model on the species abundances, which assumes a stick breaking process (Macarthur, 1957; Higgins and Strauss, 2008). Second, a measure of the diversity of lottery winners was calculated by the Shannon diversity of the distribution of winners across samples. This measure is

referred to as the frequency at which each ASV occurs as the lottery winner among all samples/treatments in which lottery winner is observed. ASVs that had less than 5,000 reads, appeared at < 0.05% abundance, and had less than 3 reads were filtered out of the analysis.

5.3.7 Phylogenetic dispersion model

The aim of the phylogenetic dispersion model was to estimate the extent of recruitment of new species in a microbial community over time based on how similar or dissimilar they are from previously recruited species. For this, the null model of Darcy et al. (2020) was applied to the 16S rRNA dataset from Chapter 4. The model characterised the probabilities of detecting new species in a local community over time and then simulated the data 500 times to produce surrogate datasets forward in time, which were then used to evaluate the null polydispersity distribution $D^{\wedge}$ and the amount of polydispersity accumulated over time PD_m. A logistical error model (Darcy et al., 2020) is then performed to generate the dispersion parameter D which determined the extent to which either closely related or distant species were preferentially added to the surrogate community. D value > 0 means that phylogenetically distant species are preferentially recruited in the local community (overdispersion; phylogenetically divergent), whereas $D < 0$ indicate the opposite – phylogenetically similar species are detected in the local community (underdispersion; phylogenetically constrained). If $D = 0$, all species have the same probability of being detected for the first time (neutral).

All null models and visualisations were conducted in R version 3.5.3 (R Core Team, 2019) with adopted scripts developed by Dr Umer Zeeshan Ijaz at the University of Glasgow.

5.4 Results

5.4.1 Elements of metacommunity structure (EMS)

All treatments showed significantly negative coherence z-values indicating that microbial communities in the treatments were shaped by distribution patterns of competitive exclusion - i.e., determinism (Figure 5-1). The crude oil-amended treatments with the addition of either Finasol (CEWAF) or rhamnolipid (BEWAF) did not show

wide variation between each other's coherence z-values but were lower than in the WAF treatment. However, the CEWAF treatment displayed the highest boundary clumping as denoted by the Morisita's index. The *in-situ* FSC community had the lowest coherence z-value with Morisita's index of 1, indicating that the spatial distribution of species in this metacommunity is over-dispersed. In the opposite end was the untreated seawater (SW) control treatment which had the highest coherence z-value but also the highest negative turnover z-value (-8.56). The seawater treatment amended with Finasol only (SWD) also had negative turnover z-value (-5.45) indicating that the species distribution is caused by species loss.

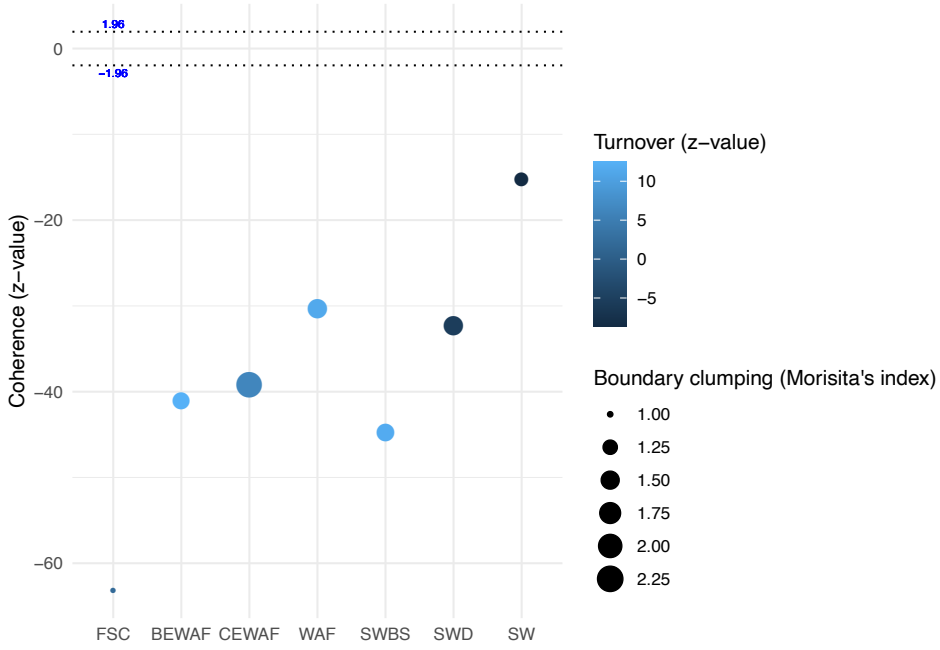


Figure 5-1. Variation of metacommunity types of the bacterial 16S rRNA sequences shown for each treatment: *in-situ* seawater (FSC), crude oil amended seawater (WAF), crude oil + dispersant amended seawater (CEWAF), crude oil + rhamnolipid amended seawater (BEWAF), seawater amended with Finasol (SWD) or rhamnolipid (SWBS), and non-treated seawater (SW; control) treatments. The blue scale bar represents species turnover (z-value; number of observed replacements compared to a null distribution) where positive values indicate species replacements in response to environmental variation and negative values nested species distributions caused by special losses. The size of the circles denotes the Morisita's index (boundary clumping) which shows the degree of spatial distribution of species in a metacommunity where lower numbers indicate over-dispersed boundaries and higher numbers clumped boundaries (analogous to clustering of microbial species).

5.4.2 Incidence-based beta diversity (β_{RC})

The β_{RC} dissimilarity indices were calculated to test whether communities in each treatment were assembled due to stochastic or deterministic processes. In general, all treatments, except the *in-situ* FSC community, varied within a narrow range, not deviating strongly from -0.5, which indicates that the processes responsible for the community assembly in each treatment could be neither strongly stochastic nor deterministic. However, the microbial communities seemed more similar to each other than expected by chance (Figure 5-2). The *in-situ* FSC community did not deviate strongly from the null expectations (β_{RC} = 0.166) and, therefore, the microbial community was more likely to be assembled by stochastic processes.

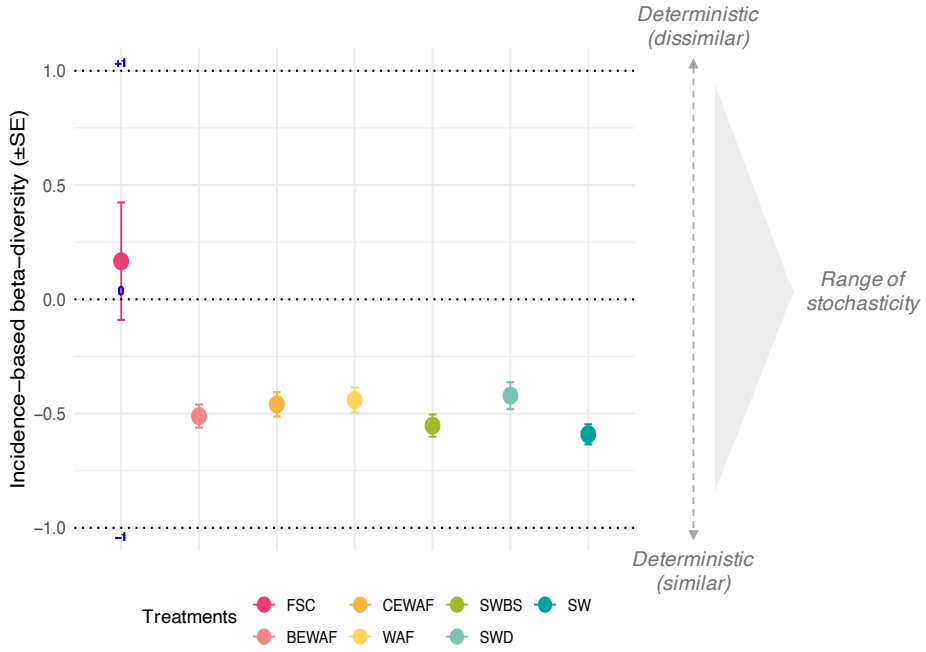


Figure 5-2. Variation of incidence-based (Raup-Crick) beta diversity (β_{RC}) for *in-situ* seawater (FSC), crude oil amended seawater (WAF), crude oil + dispersant amended seawater (CEWAF), crude oil + rhamnolipid amended seawater (BEWAF), seawater amended with Finasol (SWD) or rhamnolipid (SWBS), and non-treated seawater (SW; control) treatments.

5.4.3 Stochastic vs. deterministic assembly processes on a temporal scale

In general, the NST revealed that the microbial community assembly in the studied treatments were neither purely deterministic nor purely stochastic as indicated in Figure 5-3. The *in-situ* FSC community, the crude oil and seawater WAF treatment, and the

seawater only control (SW) had the same NST value of 56%, while the rhamnolipid-amended oiled seawater treatment (BEWAF) had a NST of 51%, suggesting that the communities' assembly in these treatments were driven predominantly by stochastic processes. Communities in the treatments with added chemical dispersant Finasol (CEWAF and SWD), in contrast, were dominated by deterministic processes as their NST was, respectively, 38% and 35%. PERMANOVA analysis revealed that the CEWAF treatment was significantly different from the SW control treatment ($p = 0.019$) and the SWD treatments was significantly different from the BEWAF ($p = 0.038$), WAF ($p = 0.018$), and the SW ($p = 0.002$) treatments. The rest of the treatments were not found to be significantly different from each other (Table 5-1).

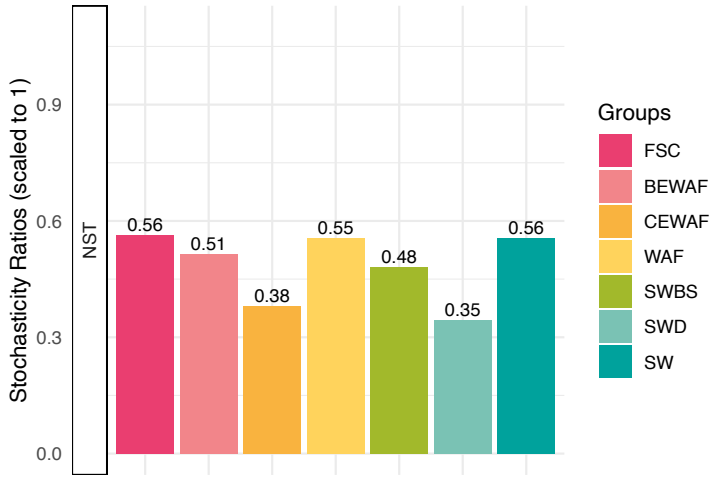


Figure 5-3. Normalised stochasticity ratio (NST) for *in-situ* seawater (FSC), crude oil amended seawater (WAF), crude oil + dispersant amended seawater (CEWAF), crude oil + rhamnolipid amended seawater (BEWAF), seawater amended with Finasol (SWD) or rhamnolipid (SWBS), and non-treated seawater (SW; control) treatments. NST was calculated based on abundance based Ružička metric using null model algorithm PF.

Next, the NST was determined for each treatment in respect to temporal dynamics The NST method becomes less precise when there are less than 6 replicates (Ning et al., 2019), therefore, the results presented in Figure 5-4 should not be taken as absolute true but rather as a guidance. Because of not enough replicates per treatment ($n \leq 3$) at each time point, PERMANOVA was not performed. Overall, stochasticity varied substantially over time in all treatments. On day 0, all presented treatments had similar NST values close to the 50% boundary point (45 - 53%). However, by day 3 the NST dramatically

dropped in all treatments to 16% (WAF), 15% (BEWAF), 9% (CEWAF), and 20% (SW) suggesting that the microbial communities were overwhelmingly driven by deterministic processes and even more so in the CEWAF treatment. From this point forward, the estimated NST started to increase in all treatment but was lowest in the CEWAF treatments until the end of the incubation period. Stochasticity was high at day 28 for the WAF and SW treatment (over 60%) but remained low in the BEWAF and CEWAF treatments. Collectively, these results indicate that stochastic processes could play more important roles in controlling community succession in its early and late phases, while deterministic processes could be more important during the middle phase.

Table 5-1. Output of PERMANOVA test for differences in NST among treatments: *in-situ* seawater (FSC), crude oil amended seawater (WAF), crude oil + dispersant amended seawater (CEWAF), crude oil + rhamnolipid amended seawater (BEWAF), seawater amended with Finasol (SWD) or rhamnolipid (SWBS), and non-treated seawater (SW; control) treatments. P.anova is the *p*-value of parametric ANOVA test; P.panova is the *p*-value of permutational ANOVA (PERMANOVA) test, and P.perm is the p-value of permutational test of the difference. Significantly different PERMANOVA values between treatments are shown in bold.

Treatment 1	Treatment 2	F value	P.anova	P.panova	P.perm
All treatments		9.2718	0.0000	**0.0539**	NA
BEWAF	CEWAF	12.5019	0.0005	0.1209	0.4765
BEWAF	FSC	0.0999	0.7526	0.7293	0.2068
BEWAF	SWBS	0.8930	0.3458	0.6334	0.3541
BEWAF	SWD	20.2497	0.0000	**0.0380**	0.4910
BEWAF	SW	1.6634	0.1986	0.5085	0.1379
BEWAF	WAF	1.0530	0.3061	0.6214	0.1643
CEWAF	FSC	1.2331	0.2693	0.3986	0.1199
CEWAF	SWBS	7.5812	0.0064	0.2747	0.0669
CEWAF	SWD	0.7522	0.3869	0.7872	0.3107
CEWAF	SW	24.9965	0.0000	**0.0190**	0.0080
CEWAF	WAF	17.8384	0.0000	0.0929	0.0275
FSC	SWBS	0.3323	0.5655	0.5245	0.3272
FSC	SWD	2.7104	0.1037	**0.0170**	0.3856
FSC	SW	0.0011	0.9735	0.9750	0.2423
FSC	WAF	0.0019	0.9653	0.9630	0.2333
SWBS	SWD	14.3889	0.0002	0.0919	0.4795
SWBS	SW	5.5818	0.0191	0.2418	0.0714
SWBS	WAF	3.7078	0.0556	0.3836	0.1084
SWD	SW	38.5082	0.0000	**0.0020**	0.0010
SWD	WAF	25.8483	0.0000	**0.0180**	0.0110
SW	WAF	0.0065	0.9357	0.9750	0.2547

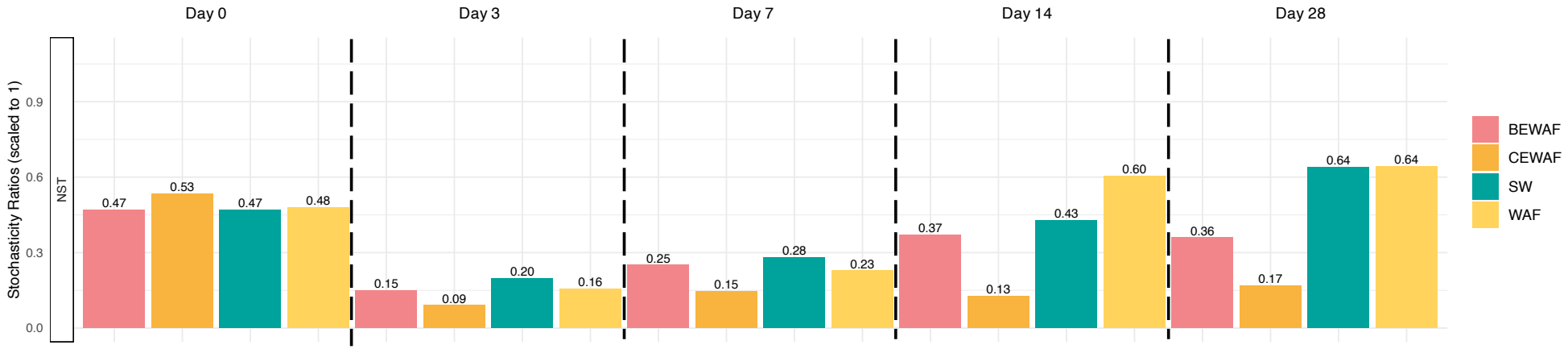


Figure 5-4. Temporal changes of the estimated NST for crude oil amended seawater (WAF), crude oil + dispersant-amended seawater (CEWAF), crude oil + rhamnolipid-amended seawater (BEWAF), and non-treated seawater (SW; control) treatments. NST was calculated based on abundance-based Ružička metrics using null model algorithm PF.

5.4.4 Tucker's beta null modelling

To quantify the relative contribution of niche and neutral processes in the assembly of the bacterial communities in seawater microcosms amended with crude oil (WAF) and dispersant (CEWAF) or rhamnolipid (BEWAF), Bray-Curtis and weighted UniFrac beta-diversity deviations from null expectation were computed. The Bray-Curtis (abundance only) and weighted UniFrac (abundance and phylogenetic relatedness) beta-null deviation results showed a dynamic pattern for the three treatments over time (Figure 5-5). The WAF treatment deviated from the null expectation towards a significant increase in the contribution of niche processes in the community assembly by the end of the incubation time period. In contrast, the relative contribution of niche processes in the BEWAF and CEWAF treatments varied more over time. The assembly of communities in both treatments was more prone to neutral processes during the first 3 days of incubations. Afterwards, the beta null deviation in the CEWAF treatment transitioned to more niche processes, before going back to neutral processes on day 14, and finally to an increase in niche processes' relative contribution. The analysis of the weighted UniFrac deviation from null model indicated that the relative contribution of neutral processes was more dominant in the BEWAF treatments assembly over the late phase of the studied period (day 14-28) in contrast to the Bray-Curtis null deviation (Figure 5-5). BEWAF's Bray-Curtis null deviation was not significantly different from that of the CEWAF on day 28, but it was statistically different when taking into account the phylogenetic relatedness (weighted UniFrac), indicating that phylogenetically distinct ASVs between the two treatments might play a role in shaping the bacterial community assembly towards more niche or more neutral processes.

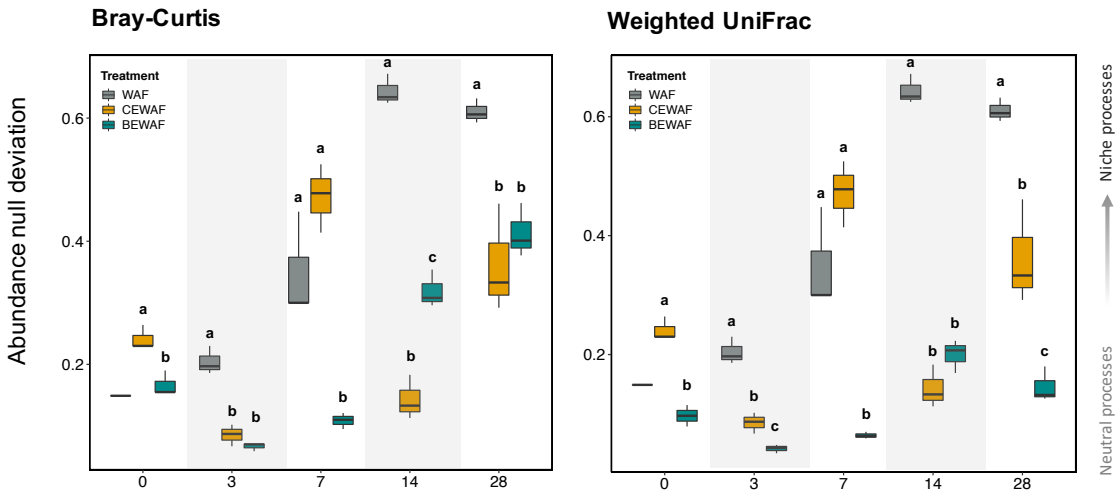


Figure 5-5. Boxplots of Bray-Curtis (left) and weighted UniFrac (right) abundance deviation from the null model of the microbial communities in treatments WAF (crude oil amended seawater), CEWAF (crude oil + dispersant-amended seawater) and BEWAF (crude oil + rhamnolipid-amended seawater) over time (days 0 to 28). Significance between group means for each time point were tested using Two-way ANOVA analysis and *post-hoc* Tukey's test. Groups that share different letters are significantly different from each other.

5.4.5 Quantitative process estimates (QPE)

The aim of the QPE was the estimate the relative importance of assembly processes. Considering the overall dynamics of these processes in the different treatments, there are some clear differences. For the *in-situ* FSC community, random processes or drift was the dominant assembly process (67%) followed by homogenising selection (33%) (Figure 5-6). On the other hand, the all the treatments, the dominant assembly processes were ecological drift, homogenising selection and dispersal limitation. The variation in the relative proportion of ecological drift among the treatments was greater than the variation in the rest of the assembly processes. Ecological drift was the least important in the CEWAF (29.5%) and the SWD (35.9%) treatments, while in the WAF and seawater control (SW) treatments the drift accounted for more than 50% of the assembly processes (Figure 5-6). Homogenising selection had similar relative importance in all of the treatments (except the *in-situ* FSC) ranging from 19.7% in the WAF to 27.6% in the CEWAF treatment. Dispersal limitation was also similarly important in the assembly of communities in all treatments (20.9-27.6%) except for the *in-situ* FSC community and the SWD treatment where the dispersal limitation was not important at all (0%) or in very

small proportion compared to other treatments (3.8%), respectively. Among all treatments, variable selection was relatively important in only two treatments – CEWAF (17%) and SWD (30.7%), while for the other treatments it was not found to be relatively important. The ecological process that had the lowest relative influence in the community assembly among all treatments was the homogenising dispersal. In fact, it had less than 1% of effect in the treatments BEWAF, CEWAF, SWBS and SW, just above 1% in the CEWAF and was highest in the SWD treatment (7.7%).

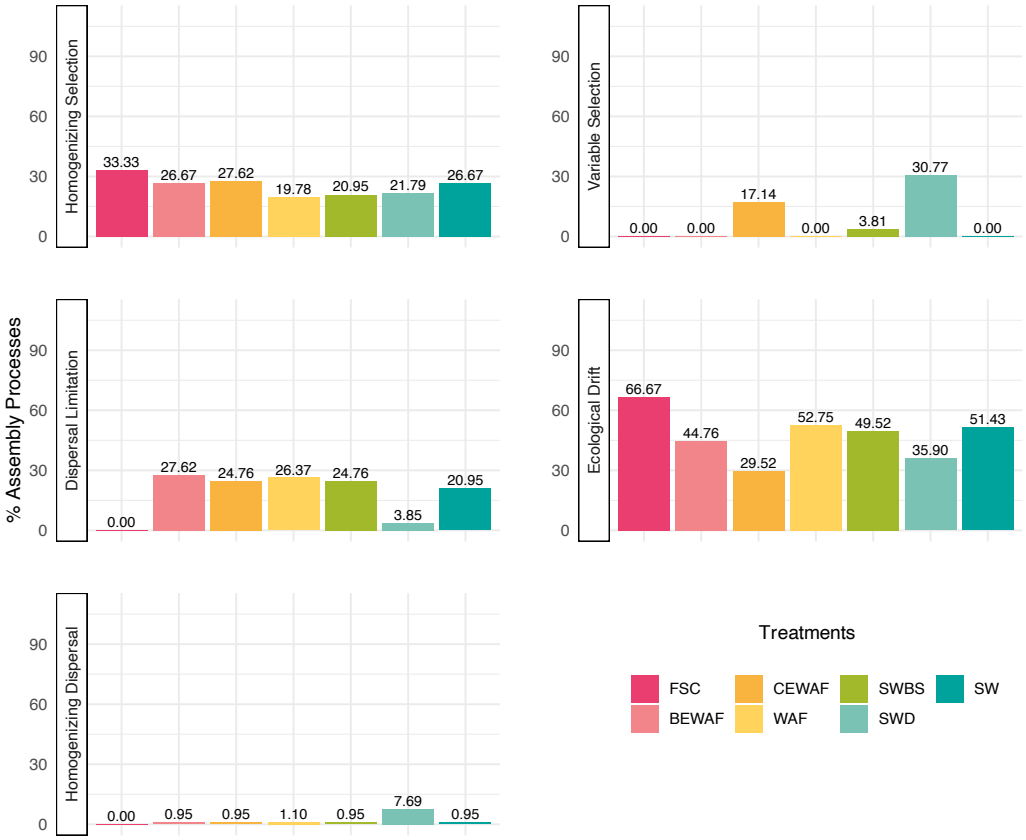


Figure 5-6. Overall dynamics of the relative importance of different community assembly processes expressed as the proportion of community pairs assembled either by species-sorting (variable or homogeneous selection), dispersal limitation or historical contingency, homogenising dispersal or ecological drift in the treatments: *in-situ* seawater (FSC), crude oil amended seawater (WAF), crude oil + dispersant amended seawater (CEWAF), crude oil + rhamnolipid amended seawater (BEWAF), seawater amended with Finasol (SWD) or rhamnolipid (SWBS), and non-treated seawater (SW; control) treatments.

5.4.6 Competitive lottery-controlled genera

The distribution of species across treatments and identity of lottery "winners" were characterised with the help of the lottery-based assembly model. The ASV distribution was expected to display two fundamental features. First, a single group member captures > 90% of the group's abundance (the "lottery winner") in each treatment and, second, different treatments should have different lottery winners. The winner prevalence (the fraction of samples in which one ASV was assigned > 90% of the genus abundance) and the winner diversity (the normalised diversity of lottery winners) for each genus in the WAF, CEWAF and BEWAF treatments were plotted in Figure 5-7. There were a number of genera with high winner prevalence. For example, *Pseudophaeobacter* was the lottery winner in the WAF treatment as it was present in 85% of samples and showed highest winner diversity (41%). Similarly, in one ASV of *Cycloclasticus* was in 71% of the WAF samples but it had a higher winner diversity than *Pseudophaeobacter.* The lottery winner in the BEWAF treatment was *Pseudohongiella* which was present in 80% of the samples. In the CEWAF treatment, the genus with the highest winner prevalence was a different *Cycloclasticus* ASV (from the one in the WAF treatment) but it had a winner diversity of 1, meaning that this particular *Cycloclasticus* ASV was not consistent with the competitive lottery schema. Groups that have low winner prevalence and have comparatively higher winner diversity likely reflected that the group abundances were more evenly distributed among the group ASVs (e.g., *Peredibacter*, *Hyphomonas*).

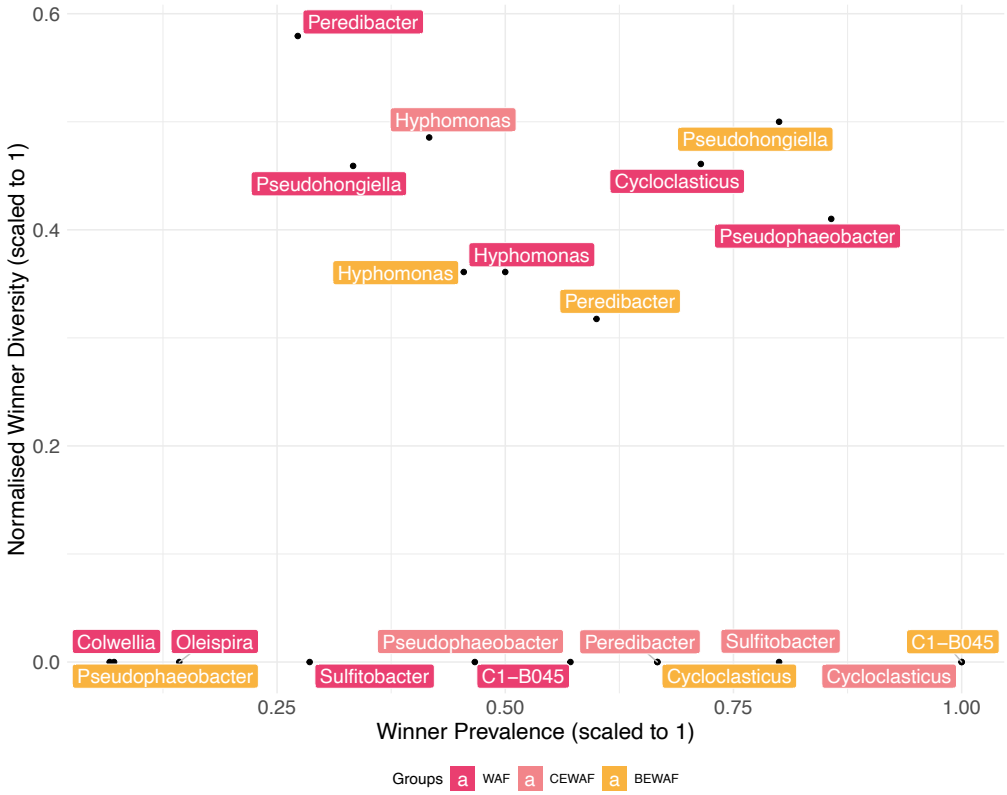


Figure 5-7. A scatter plot showing the winner prevalence and winner diversity for different genera in three crude oil-amended seawater treatments: WAF (oil + seawater), CEWAF (oil + seawater + Finasol), and BEWAF (oil + seawater + rhamnolipid).

5.4.7 Phylogenetic dispersion

The phylogenetic dispersion null model was used to estimate the extent of recruitment of new species into the microbial community of six natural seawater microcosms (SW) amended with crude oil (WAF), crude oil and dispersant Finasol (CEWAF), crude oil and rhamnolipid biosurfactant (BEWAF), Finasol only (SWD), or rhamnolipid only (SWBS) over time. Varying $D^{\wedge}$ changed the rate at which the phylodiversity was added to the resampled microbial communities over time and overall, the results showed that the D parameter successfully corresponded to over- and underdispersion relative to the neutral model (Appendix C). The CEWAF treatment had the highest D value ($D > 0$) compared to the other two oil-amended oils (WAF and BEWAF), indicating that the presence of dispersant changed the phylogenetic colonisation patterns to a preferential and faster recruitment of phylogenetically distant species in the community (i.e., overdispersion) (Figure 5-8). In contrast, the WAF and

BEWAF treatments had *D* value of less than 0 which suggests that there was underdispersion or phylogenetically similar new species were detected in the local community. In other words, the addition of dispersant caused the local community to become phylogenetically divergent as time progressed, whereas the addition of rhamnolipid caused the opposite trend – a phylogenetically constrained community. The oil by itself (WAF) also caused constrained community but less so compared to the rhamnolipid-amended oil treatment (BEWAF). All treatments had significantly different *D* values ($p < 0.001$) (Figure 5-8).

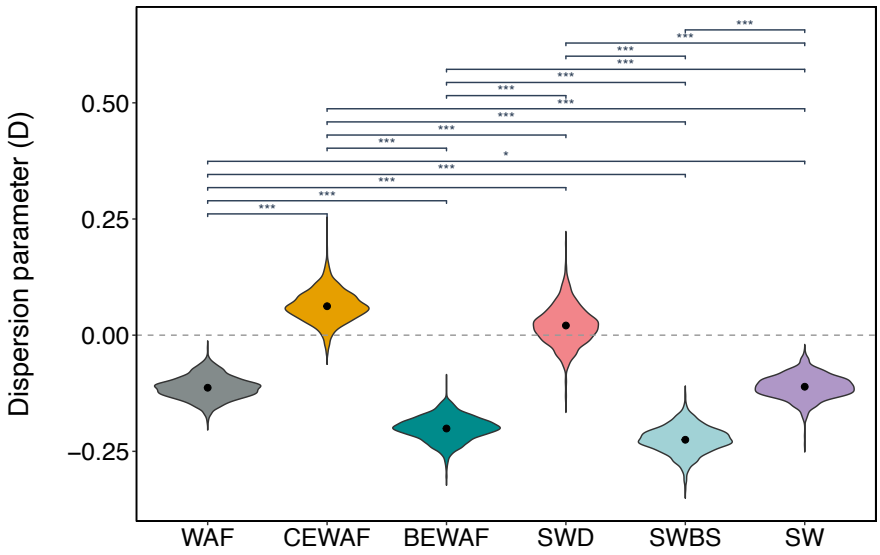


Figure 5-8. Violin plot of the distribution of dispersion parameter (*D*) estimates given by logistic error model bootstrap for six treatments (fill colour): *in-situ* seawater (FSC), crude oil amended seawater (WAF), crude oil + dispersant amended seawater (CEWAF), crude oil + rhamnolipid amended seawater (BEWAF), seawater amended with Finasol (SWD) or rhamnolipid (SWBS), and non-treated seawater (SW; control) treatments. Dots within each violin are means. Significantly different (t.test) treatments are share a bracket and the level of significance is shown with * ($p = 0.05$), ** ($p = 0.01$), or *** ($p = 0.001$).

5.5 Discussion

Seven different and recently developed null model frameworks were used to quantify the relative importance of ecological processes in the community assembly (Leibold and Mikkelson, 2002; Presley et al., 2010; Chase and Myers, 2011; Tucker et al., 2016; Lee et al., 2017; Verster and Borenstein, 2018; Ning et al., 2019; Darcy et al.,

2020). Elements of metacommunity structure (EMS), Incidence-based (Raup-Crick) beta-diversity, and normalised stochasticity ratio) were developed to detect patterns in binary incidence (i.e., presence/absence) matrices taking into account only taxonomic beta-diversity estimates. The next model used in this study was the beta-null framework developed by Tucker et al. (2016) to differentiate between neutral and niche communities and was based on quantitative abundance-based (Bray-Curtis) dissimilarity matrix. It was later complemented by Lee et al. (2017) to integrate phylogenetic relatedness information with the taxonomic abundance (weighted UniFrac). The QPE framework was also based on abundance and phylogenetic-based matrices to quantify the relative importance of ecological selection, drift, and dispersal. Although the lottery-based assembly model used in this study is abundance-based, it focuses more on the characterisation of the species distribution across treatments in relation to priority effects (Verster and Borenstein, 2018). The phylogenetic dispersion model provided a view into temporal dynamics in the recruitment of new species in the local community in relation to their phylogenetic similarity or dissimilarity to already existing members of the community which colonised it at a previous time point (Darcy et al., 2020).

5.5.1 Stochastic vs. deterministic assembly

It has been generally accepted that both deterministic and stochastic processes occur simultaneously in the assembly of local communities (Chase et al., 2011). According to incident-based beta-diversity ($\beta_{RC)}$ patterns, bacterial communities across all communities studied here were neither purely stochastic nor purely deterministic. However, there was a tendency toward more deterministically assembled communities ($\beta_{RC} \sim$ -0.5). EMS provided further evidence for deterministic assembly, and the NST ratio supported this finding. In particular, the NST showed that the oil-only control treatment (WAF) and untreated seawater control (SW) had more stochastically assembled communities, while the communities in the treatments with added dispersant Finasol (CEWAF and SWD) were more deterministic. It is likely that the presence of dispersant triggered a microbial response related to deterministic succession. Furthermore, NST also revealed that the relative importance of stochasticity over determinism varied substantially over time across treatments. Stochastic processes were more prevalent in the early and late phases of incubation, while deterministic processes are more important

in the middle phase in all treatments. The result in the middle phase seems fit to intuition that adding carbon source (e.g., crude oil and/or dispersant/biosurfactant) or altering the environmental conditions should drive selection and hence leads to a more deterministic outcome. This is a demonstration that drivers controlling biodiversity and community succession are dynamic rather than static in fluidic ecosystems (Zhou et al., 2014; Dini-Andreote et al., 2015).

5.5.2 Neutral vs. niche assembly

The Tucker's beta-null deviation model successfully differentiated patterns of niche and neutral processes in the three crude oil amended treatments (WAF, CEWAF, and BEWAF) in the presence of either dispersant or rhamnolipid over time, suggesting that the presence of dispersant, either synthetic (Finasol) or biogenic (rhamnolipid) did have an important role or even were selective factors in the community assembly processes. Neutral processes had a more prominent role in the assembly in the rhamnolipid-amended oil treatments than in the oil-only and Finasol-amended oil treatments. This finding suggests that the addition of rhamnolipid had not applied strong selection on the assembly of oiled seawater microcosms. The opposite was observed for the oil-only treatment (WAF) – clear niche community assembly pattern. A plausible explanation is that the oil selected for highly specialised species while the addition of rhamnolipid allowed more generalist taxa to thrive (see Chapter 4; Figure 4-3).

5.5.3 Importance of selection, dispersal and drift

The microbial succession patterns in response to either dispersed or non-dispersed crude oil in the marine environments have been well documented (Yakimov et al., 2007; Hazen et al., 2010; Hamdan and Fulmer, 2011; Gutierrez et al., 2013b; Kleindienst et al., 2015b; Liu et al., 2017; Miller et al., 2020; see also Chapter 4 in this thesis). In all cases, there was a distinct succession of obligate and generalist hydrocarbon-degrading taxa which rapidly increased in abundance to dominate the community structure composition. Furthermore, the microbial respond to chemically dispersed oil has been shown to be different to oil-only treatments, (Kleindienst et al., 2016; Techtmann et al., 2017). But which ecological process and to what extent do they govern such a response has not been

explored before, probably due to the many uncertainties around microbial ecology and/or the significant computational power required to process large 16S rRNA sequencing data.

The QPE framework showed that all treatments were dominated by drift, dispersal limitation, and homogenising selection. Homogenising dispersal was not found to be an important factor in the community assembly as expected for a marine ecosystem where there is a rapid dispersion and population movement (i.e., high connectivity) (Langenheder and Ragnarsson, 2007; Zhou et al., 2014). Drift was the most dominant ecological process detected in the QPE analysis in the majority of treatment. The importance of drift was notably lower in the both Finasol-amended treatments (CEWAF and SWD) compared to the rest of the treatments. The dispersal limitation was relatively higher than it would be expected for open ocean ecosystem likely because the microbial microcosms in this study (see Chapter 4) were enclosed in bottles where there was no water exchange, although constant rotation was applied to ensure adequate mixing and simulate the motion of seawater in the upper water column. The relative importance of dispersal limitation in the *in-situ* water sample (FSC), in comparison, was 0%, thus supporting that dispersal limitation in open ocean systems is not a decisive factor in community assembly. There is growing evidence, however, to support that the dispersal limitation can actually be a more important dominating factor in marine microbial communities than previously thought, especially when acting together with drift (Dumbrell et al., 2010; Stegen et al., 2015). Other microbial studies using quantitative process estimates have demonstrated the substantial proportion of the dispersal limitation or historical contingency (such as priority effects) in community assembly (Langenheder et al., 2017; Wu et al., 2018; Vass et al., 2020). It is reasonable to suggest that in a real-life oil spill, dispersant or biosurfactant application would not have a strong deterministic effect due to the higher dispersal rates observed in oceanic systems. The patterns of dispersal limitation and homogenising dispersal in the dispersant-only control treatment (SWD) particularly stand out from the rest of the treatments. It seems that the dispersant itself had high relative influence over the ecological processes. Indeed, the importance of variable selection was substantially higher only in the treatment containing Finasol dispersant (CEWAF and SWD). One explanation for this could be attributed to a smaller number of taxa that took over the community structure in CEWAF (see Chapter 4; Figure 4-3) that otherwise be selected against in communities without dispersant because of

spatial variation in the selective environment. In fact, the variable selection causes an increase in the spatial environmental heterogeneity as time (i.e., succession) progresses which leads to compositional differences across local communities (Dini-Andreote et al., 2015). Moreover, regression analysis confirmed that the dispersant caused a reduction in alpha diversity (see Chapter 4; Figure 4-12). Generally, selection has more detectable influence over microbial communities (Stegen et al., 2013; Zhou and Ning, 2017; Ning et al., 2019) and the same was observed in this study. Relatively strong homogenous selection was expected as selection is likely to be consistent across spatially homogenous systems (Zhou et al., 2014), such as the natural surface seawater ecosystem studied here. Stochasticity is influenced by an interaction between dispersal and selection, with stronger selection causing an increase in drift or priority effects (Evans et al., 2017). The importance of drift is considered higher when selection is weak and the local community is small. Pure drift is practically impossible to measure, and even more so for microbial communities, because no species in nature are exactly the same in a demographic sense. Distinct populations that share similar or the same ecological function (i.e., functional redundancy) are quite common in microbial communities. Functional redundancy tends to increase neutrality and sensitise functional redundant populations to ecological drift, which is unarguably stochastic. In conclusion, it was the direct or indirect interrelation of selection, dispersal, and drift with each other that assembled the microbial community structures in this study, as observed elsewhere (Evans et al., 2017).

5.5.4 Lottery winners

The findings of the lottery-based assembly model presented in this study confirmed that the lottery winner ASVs varied between treatments in accordance with the competitive lottery schema. Furthermore, the lottery winners in each treatment did not occur at the same frequency as assumed by the lottery schema (Verster and Borenstein, 2018). A winner diversity approaching 0 represents that same species is selected in all cohorts for a given clade. There were a number of genera with very high winner prevalence but very low winner diversity, including *Cycloclasticus* and *Sulfitobacter*, that did not involve complete competition-derived exclusion but rather strong coexistence with other species in the microcosms or even more complex assembly that combines exclusion and coexisting patterns (Verster and Borenstein, 2018). Taking into

consideration the relative abundance analysis of the top 25 most abundant ASVs from Chapter 4 (section 4.4.2 and Figures 4-3 and 4-4), it became apparent that the lottery winners in each treatment were not necessarily the most abundant species identified. For example, *Cycloclasticus* was identified as a potential lottery winner in the WAF treatment. Furthermore, *Cycloclasticus*, which is a PAH degrader, was found to dominate the WAF's community composition in the late stages of incubation, while early stages were previously dominated by *Colwellia* and *Oleispira*, which are aliphatic and low-molecular-weight PAH degraders. It is logical then to assume that microbial succession, driven by competition for resources, occurred in the WAF treatment - i.e., *Cycloclasticus* outcompeted *Colwellia* and *Oleispira*. The relative abundance analysis (see Chapter 4, section 4.4.2) showed that *Cycloclasticus* had a relative abundance of <1% across all CEWAF samples and it is highly likely that just one ASV was entirely responsible for the observed low abundance (see Chapter 4, Figure 4-4). According to the lottery-based assembly model, a single *Cycloclasticus* ASV (ASV_28) was present in 100% of all samples in the CEWAF treatments (Figure 5-7) but did not fit the assumptions of the competitive lottery schema. This suggests that this particular *Cycloclasticus* ASV was either outcompeted by other species (potentially more adept to the presence of dispersant or able to engage in resource partitioning) observed in the CEWAF treatment (e.g., by *Rhodobacteraceae*) to the point where its abundance never exceeded 1%, or the presence of the dispersant Finasol itself might have selected against it. Interestingly, generalist species *Pseudophaeobacter* and *Pseudohongiella* were projected the lottery winners in the WAF and BEWAF treatments, respectively, but both had less than 1 % abundance in the respective treatments (see Chapter 4, section 4.4.2). In fact, *Pseudophaeobacter* represented up to 40-fold higher abundance in the CEWAF treatment, while *Pseudohongiella* was most abundant in the WAF treatment. It is possible that there was other more complex assembly schema, which the lottery-based assembly method could not explain, that *Pseudophaeobacter* and *Pseudohongiella* conformed to in comparison to *Cycloclasticus*. For example, although they were lottery winners, both species could have facilitated subsequent species that join the ecosystem (i.e., respond to crude oil) to flourish. It is also possible that as generalists, *Pseudophaeobacter* and *Pseudohongiella* have more opportunities for niche diversification.

5.5.5 Phylogenetic dispersion

The phylogenetic dispersion model is the most recently developed null model and hence, it has not been tested in other environmental microbial studies. Nevertheless, the model provides a valuable insight into the largely unknown area microbial ecology of how and when microbial communities are colonised by phylogenetically similar or dissimilar relatives (Darcy et al., 2020). Microcosms treatments with added dispersant Finasol (CEWAF and control SWD) were the only two treatments that had *D* values > 0 indicating that the microbial community in these treatments become more phylogenetically divergent as time progresses and there is little preference of which species are recruited first (i.e., random colonisation). The rest of the treatments (WAF, BEWAF and the controls SWBS and SW) had *D* values < 0 and followed a "nepotistic" pattern of new species recruitment. This "nepotistic" pattern was described as a recruitment pattern in which new species that are closely related to the already existing member of the community are more likely to be recruited than distantly related species (D'Andrea et al., 2019; Darcy et al., 2020). Traditionally, community ecology assumes that competition among closely related species would be strongest but also allows similar species to coexist, especially when dispersion is high as is in ocean ecosystems (D'Andrea et al., 2019). One explanation for the overdispersion observed in the CEWAF treatment is that the dispersant caused the formation of multiple environmental gradients in the community which provided an opportunity for different species to colonise. This was further supported by the findings of the QPE model which showed that variable selection was only observed in the CEWAF and SWD treatments. Conversely, variable selection in BEWAF and WAF treatments was not detected, and hence, explain the observed phylogenetic underdispersion which posits that recruitment of new species is slow, i.e., there is a single environmental gradient available to colonise. This is somewhat opposite to the Tuckey's beta null model which showed that the community in the BEWAF treatment was assembled more by neutral processes than the CEWAF treatment.

CHAPTER 6

FINAL CONCLUSIONS AND FUTURE RECOMMENDATIONS

6.1 Overview

The purpose of this chapter is to summarise the main conclusions from each chapter and provide future recommendations for research development.

6.2 Final conclusions

6.2.1 Conclusions from Chapter 1

Chapter 1 provided a review of the types of surface-active compounds produced by marine bacteria, growing conditions, and their application in different industries including the oil and gas industry. The main conclusions from this chapter were:

- There are two main classes of surface-active molecules produced by bacteria – low-molecular-weight (LMW) biosurfactants and high-molecular-weight (HMW) bioemulsifiers (also known as EPS). The main characteristics of biosurfactants is that they reduce the ST and IFT between two immiscible phases such as water/oil. Bioemulsifiers, on the other hand, enable the formation of oil-in-water or water-in-oil emulsions.

- The main types of LMW biosurfactants are glycolipids (e.g., rhamnolipid, sophorolipids, trehalose lipids, and MELs), lipopeptides (e.g., surfactin, lichensysin, viscosin, iturin, and fengycin), lipoproteins, and phospholipids. HMW bioemulsifiers have more complex chemical structure consisting of mixtures of heteropolysaccharides, lipopolysaccharides, lipoproteins, and proteins (e.g., emulsan, alasan, liposan, sphingan, and xanthan gum).

- Known marine biosurfactant and EPS-producing strains have been identified to belong to several genera that are also able to degrade hydrocarbons – *Pseudomonas, Bacillus, Acinetobacter, Antarctobacter, Rhodococcus, Halomonas, Alcanivorax, Pseudoalteromonas, Marinobacter,* and a growing number of other less known species. The apparent high diversity of marine biosurfactant producing bacteria provide a reliable source of novel, and potentially non-pathogenic, strains that produce a wide range of molecules that can be used for different industrial applications.

- Biosurfactant-producing bacteria are found in a wide range of habitats including in extreme environments, highlighting their versatile use and application. Furthermore, temperature, dissolved oxygen, carbon and nitrogen sources are the main factors limiting the growth and production of biosurfactants.

- There is growing support for the use of cheaper and renewable growth substrates, especially industrial waste products from the agriculture and food industries, that can substantially reduce the production costs of biosurfactants from biosurfactants.

- Biosurfactants are already being used in the food (e.g., xanthan gum and sphingans) and cosmetics (e.g., rhamnolipids, sophorolipids, EPS) industries. Promising antimicrobial, antifungal, anticancer, anti-adhesive, and visco-elastic properties have also been reported for some biosurfactants, highlighting their use in biomedical and pharmaceutical industries.

In the oil and gas industry, biosurfactants have been shown to be effective in soil and marine biodegradation, as well as enhanced oil recovery.

6.2.2 Conclusions from Chapter 2

In Chapter 2, a bioemulsifier was extracted from a marine hydrocarbon degrading species of *Halomonas* sp. strain TGOS-10. The TGOS-10 bioemulsifier was tested for its ability to emulsify different hydrocarbon compounds and its chemical structure was analysed. The data from the analyses conducted in this chapter showed:

- *Halomonas* sp. strain TGOS-10 was able to grow well on glucose and produce stable emulsions of *n*-hexadecane (~60%). The yield of extracted freeze-dried polymer was 1.23 g/L, which is comparable to other EPS yields reported in literature.

- The extracted and freeze-dried bioemulsifier polymer retained its emulsification activity after one-year storage. Overall, the TGOS-10 polymer effectively emulsified (> 60%) all of the three tested hydrocarbons (*n*-hexadecane, motor oil and diesel). Furthermore, emulsions produced with

diesel and motor oil remained stable for at least two weeks at room temperature.

- The chemical structure analysis revealed that the TGOS-10 polymer was composed of 46% protein and 1.5% carbohydrate. In fact, NMR analysis confirmed that the polymer is a glycosylated glycoprotein composed of galactose and mannose and had a low uronic acid content. The strong emulsification properties of the TGOS-10 polymer could be attributed to the high protein content. However, further work will be needed to better resolve the role of protein in the functionality of this polymer.

6.2.3 Conclusions from Chapter 3

The aim of Chapter 3 was to investigate the biosurfactant production by *Halomonas* sp. strain TGOS-10 and a non-pathogenic marine *Pseudomonas* sp. strain MCTG214(3b1), and the effectiveness of the two biosurfactants to disperse crude oil. The key findings from this work were:

- Both strains grew well on sunflower oil while reduced the ST to 31-32 mN/m, indicating biosurfactant production. *Halomonas* spp. are usually EPS producers and biosurfactant production by this genus (especially marine) is rarely reported.

- The surface activity of the two types of biosurfactants was relatively stable under extreme conditions (i.e., temperature, NaCl concentration, and pH), highlighting their possible versatility for use in different industrial applications. However, the production yield from both biosurfactants was relatively low and further decreased after purification.

- HPLC analysis revealed that the biosurfactants possibly contained low-molecular-weight rhamnolipid congeners. However, there were other abundant compounds in the respective NMR spectra that were not identified by the HPLC analysis. NMR analysis was attempted to further determine the chemical structure of the biosurfactants, but the produced NMR spectra had very low quality and interpretation of results proved difficult.

- Biosurfactants TGOS-10 and MCTG214(3b1) effectively dispersed light (API 32) and medium crude (API 22) oil as demonstrated by the baffle flask test and consequent oil droplet size measurements. Both biosurfactants were more effective in dispersing the light crude oil. Overall, however, the oil droplet sizes were inconsistent in the biosurfactant-facilitated dispersions suggesting that they would not be stable and some of the oil droplet may return to the surface slick soon after agitation stops.

6.2.4 Conclusions from Chapter 4

This chapter aimed to advance the understanding of how rhamnolipid biosurfactant affect marine microbial communities and their ability to degrade oil in oil-amended seawater microcosms compared to synthetic dispersant Finasol OSR52. The main conclusions from the work conducted in this chapter were:

- Microbial communities exposed to crude oil (WAF) amended with either synthetic chemical dispersant (CEWAF) or biosurfactant (BEWAF) were initially similar in composition. Over time, the microbial succession patterns dramatically changed and different treatments became more dissimilar with time. Finasol suppressed *Cycloclasticus*, but favoured *Vibrio* and *Pseudophaeobacter*. In contrast, the rhamnolipid sustained *Oleispira* and *Micavibrionaceae* abundances.

- There was a significant difference in the alpha diversity between the BEWAF and CEWAF treatments, with species richness and Shannon values being lower in the CEWAF and SWD treatments.

- Generally, WAF, BEWAF and CEWAF treatments were mostly closely clustered to each other as indicated by PCoA plot of weighted UniFrac beta-diversity. However, with time CEWAF and SWD treatments became more dissimilar to the rest of the treatments.

- Differential abundance analysis identified the key taxa that represent the major shift in the communities. Oil-degrading taxa *Marinobacter*, *Oleispira* and *Pseudomonas* were enriched in CEWAF, BEWAF and WAF. However,

Vibrio, *Oleiphilus*, and *Glaciecola* were enriched exclusively in CEWAF, while *Alteromonas*, *Moritella* and *Paraglaciecola* having become enriched exclusively in BEWAF.

- Subset analysis revealed that only two taxa, namely unclassified member of the *Altermonadaceae* family and *Amylibacter* (from the *Rhodobacteraceae* family) cause the significant shift in beta diversity dynamics in the CEWAF treatment, whereas *Amylibacter*, *Sedimentitalea*, *Pseudophaeobacter*, *Colwellia*, *Oleispira,* and *Micavibrionaceae* were the taxa responsible for the community shift in the BEWAF treatment, highlighting the different taxa responding to dispersant and rhamnolipid.

- Nearest-relative-index (NRI) indicated that deterministic environmental filtering was responsible for the changes in the microbial communities in each treatment over time. Moreover, the nearest-taxon index (NTI) indicated co-occurrence of more closely related species in all treatments.

- Regression analysis revealed that Finasol-amended treatments (CEWAF and SWD) negatively affected the variation in alpha diversity (i.e., the presence of dispersant decreased alpha diversity) but positively affected the variation in local contribution to beta diversity (i.e. the presence of dispersant increase the dissimilarity of the community composition compared to the rest of the treatments). In contrast, the rhamnolipid-amended treatment (BEWAF) mostly had non-significant effect on the alpha and beta diversity measures.

- The functional abundance and diversity analysis (PICRUSt2) predicted that the number of KEGG orthologs was significantly lower in the CEWAF and SWD treatments than in the BEWAF treatment. Genes involved the short-chain and medium-chain length alkanes degradation and biosurfactant production were detected in the three oil-amended treatments (i.e., WAF, CEWAF, and WAF).

- Overall*, n*-alkane degradation was faster in the CEWAF treatment than the BEWAF treatment, while the biodegradation of the aromatic fraction remained mostly unchanged in both treatments.

6.2.5 Conclusions from Chapter 5

The computational work in this chapter aimed to quantify and better understand the ecological processes that drive the microbial community assembly in treatments amended with crude oil (WAF) and either chemical dispersant Finasol (CEWAF) or a rhamnolipid biosurfactant (BEWAF).

- The seven different null models that were used to quantify the ecological processes that assembled the microbial communities generally agreed about the dominant community assembly processes.

- According to the EMS model, the microbial communities in all treatments were shaped by patterns of species distribution caused by deterministic competitive exclusion (i.e., species loss).

- The incidence-based (Raup-Crick) beta diversity model indicated that the community assembly in all treatments was slightly more deterministic than stochastic.

- The NST model revealed that the relative importance of deterministic processes was more dominating in the CEWAF and SWD microbial communities' assembly. Over time, stochastic processes played more important role in controlling community succession in the early and late phases of the incubation period, while deterministic processes were more important in the middle phase.

- The Tucker's beta-null model also showed a dynamic pattern in the beta diversity null deviation. The WAF treatment consistently deviated towards a significant increase in the contribution of niche processes over time. On the other hand, the CEWAF and BEWAF communities were more likely to be neutral.

- According to the QPE model, the dominant assembly processes in all treatments were ecological drift, homogenising selection and dispersal limitation. In addition, variable selection was found to be a major factor in the community assembly only in the CEWAF and SWD treatments.

- The Lottery-based model identified *Pseudophaeobacter* to be the lottery-winner species in the BEWAF and *Pseudohongiella* in the WAF treatment. There was no clear lottery winner in the CEWAF treatment. Furthermore, the model further revealed that the lottery-winners were not necessarily the most abundant species in the microbial communities in the respective treatments.

- The phylogenetic dispersion model demonstrated that CEWAF and SWD were the only two treatments whose microbial communities became more phylogenetically divergent as time progresses and that there was little preference of which species are recruited first (i.e., random colonisation). The rest of the treatments (WAF, BEWAF and the controls SWBS and SW) followed a "nepotistic" pattern of new species recruitment.

6.3 Future recommendations and perspectives

This thesis provides new insights into the biotechnological potential of marine biosurfactant-producing bacterial strains that are often found to dominate crude oil contaminated marine environments. As such, they have been "targeted" as suitable candidates for biosurfactant production which can be used in oil spill response either as environmentally friendly substitutes for some of the synthetically derived chemical dispersants currently used by the oil industry or as stand-alone biogenic dispersants. The effect of rhamnolipid biosurfactants on a North Atlantic marine community was then assessed in *ex-situ* microcosms compared to that of synthetic dispersant. And lastly, the community assembly in each treatment was investigated with promising results. A number of natural progressions to this work are outlined below.

- The process optimisation of biosurfactant production from *Halomonas* sp. strain TGOS-10 and *Pseudomonas* sp. strain MCTG214(31b) have been investigated by another non-related multinational project, MARISURF, coordinated by Heriot-Watt University, and hence, production optimisation was not a goal of this thesis. However, as discussed in Chapter 1, low yield of product is one of the main reasons for the currently high production cost which translates into economically inefficient production on an industrial scale compared to cheaper synthetically derived products. A potential progression from this work would be to investigate

the possibility for bioengineering of the strains so that they can overproduce biosurfactants for a shorter time and in smaller bioreactors and/or improve purification processes.

- Blending of the two biosurfactants at different concentrations could be performed to determine whether the dispersion effectiveness can be improved and therefore be used either as substitutes for synthetic surfactants in commercial dispersant formulations or on their own. Different solvents, including biosolvents, can be tested in combination with the biosurfactants to determine the most effective formulation.

- Crude biosurfactant samples from the two studied strains along with three types of crude oil (API° light, medium, and heavy) were sent last year to the US National Oil Testing Facility Ohmsett in New Jersey for a small-scale herding test. Unfortunately, due to the development of the COVID-19 global pandemic and consequent lockdown for months in the US, the testing was repeatedly postponed and, in the end, not performed by the time of submission of this thesis. The results of the herding test, however, can give further confirmations of the effectiveness of the biosurfactants produced by *Halomonas* sp. strain TGOS-10 and *Pseudomonas* sp. strain MCTG214(31b) to be used as an effective tool in oil spill response.

- Both biosurfactants studied in this work showed very good surface activity under challenging environmental conditions. Therefore, the two biosurfactants should be investigated for their potential to be effective in MEOR. For this, core flooding experiments can be done in rock cores to test the ability of biosurfactants to reduce the capillary forces that keep the oil within the pores of reservoir rocks, alter the wettability of rock formation, and change the flow of fluids.

- In this thesis, *in-silico* approach (PICRUSt2) was used to predict the microbial functional diversity and abundance of the oil-amended microbial microcosms. Transcriptomic analysis can be done to accurately quantify the expression of genes responsible for biosurfactant production and/or hydrocarbon degradation in the presence of synthetic dispersants and rhamnolipids or other biogenic oil spill

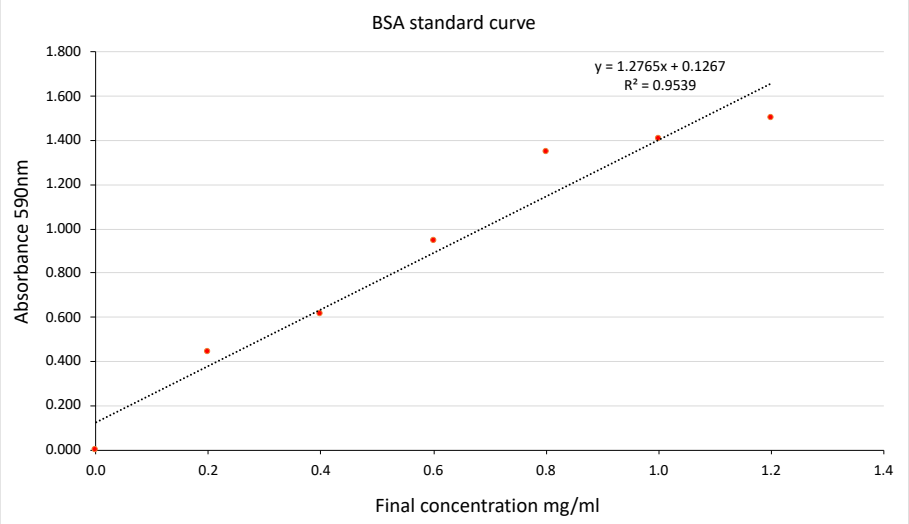
treating agents. To avoid the effect of genetic "noise" in environmental samples, a consortium of bacterial species that are typically enriched in the presence of crude oil could be used. The best-known genes for rhamnolipid *(rhl*AB), surfactin (*sfp*), and EPS (*eps*C) can be targeted as well as alkane (e.g., *alk*B) and PAH degrading genes. After suitable genes are selected, the temporal response of each of these genes after exposure should provide invaluable insights into how quickly (or not) biosurfactant genes respond to crude oil with dispersants/biosurfactants.

- Null models worked well with the 16S rRNA sequence data but the NST model highlighted the importance of having more biological replicates and fewer treatments in order to have more reliable results. Furthermore, collection of multiple microbial samples from *in-situ* oil spills would be recommended to obtain more meaningful estimation of the ecological processes responsible for the assembly of communities in response to crude oil with or without dispersant addition (either synthetic or biogenic).

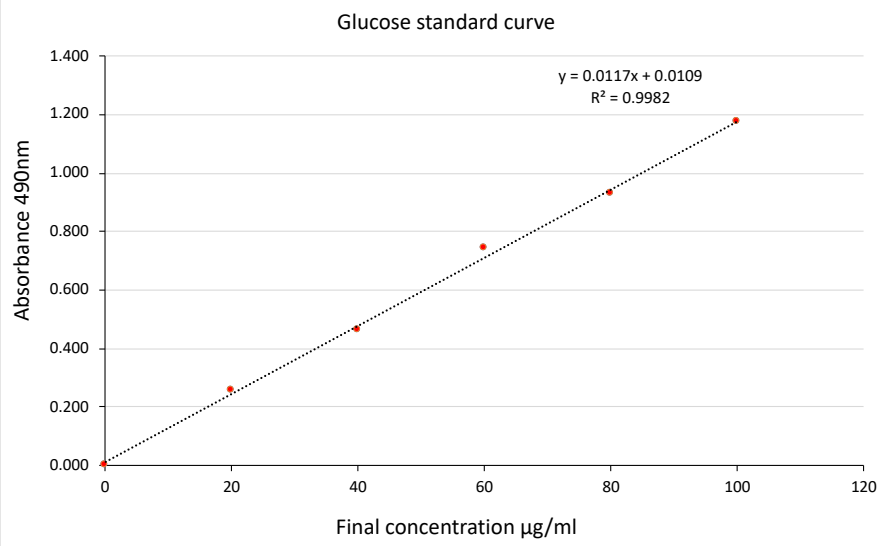
Overall, this research project resulted in a few key findings that can potentially find invaluable biotechnological applications. The results from this work contribute to the ever-growing research effort to tap in the huge phylogenetic and metabolic diversity of marine microorganisms and their potential for industrial applications. Particularly interesting was the finding that *Halomonas* sp. strain TGOS-10 produced effective biosurfactant and bioemulsifier by utilising different growth substrates. Therefore, this strain can potentially have a unique economic advantage over other microorganisms that can produce only one type of surface-active compounds. Next, this project revealed that the two biosurfactants (by *Halomonas* sp. strain TGOS-10 and *Pseudomonas* sp. strain MCTG214(3b1)) were able to disperse three types of crude oil and therefore, become suitable candidates for further research in their use for treating oil spills.

Appendix A

Bradford Protein Assay standard curve



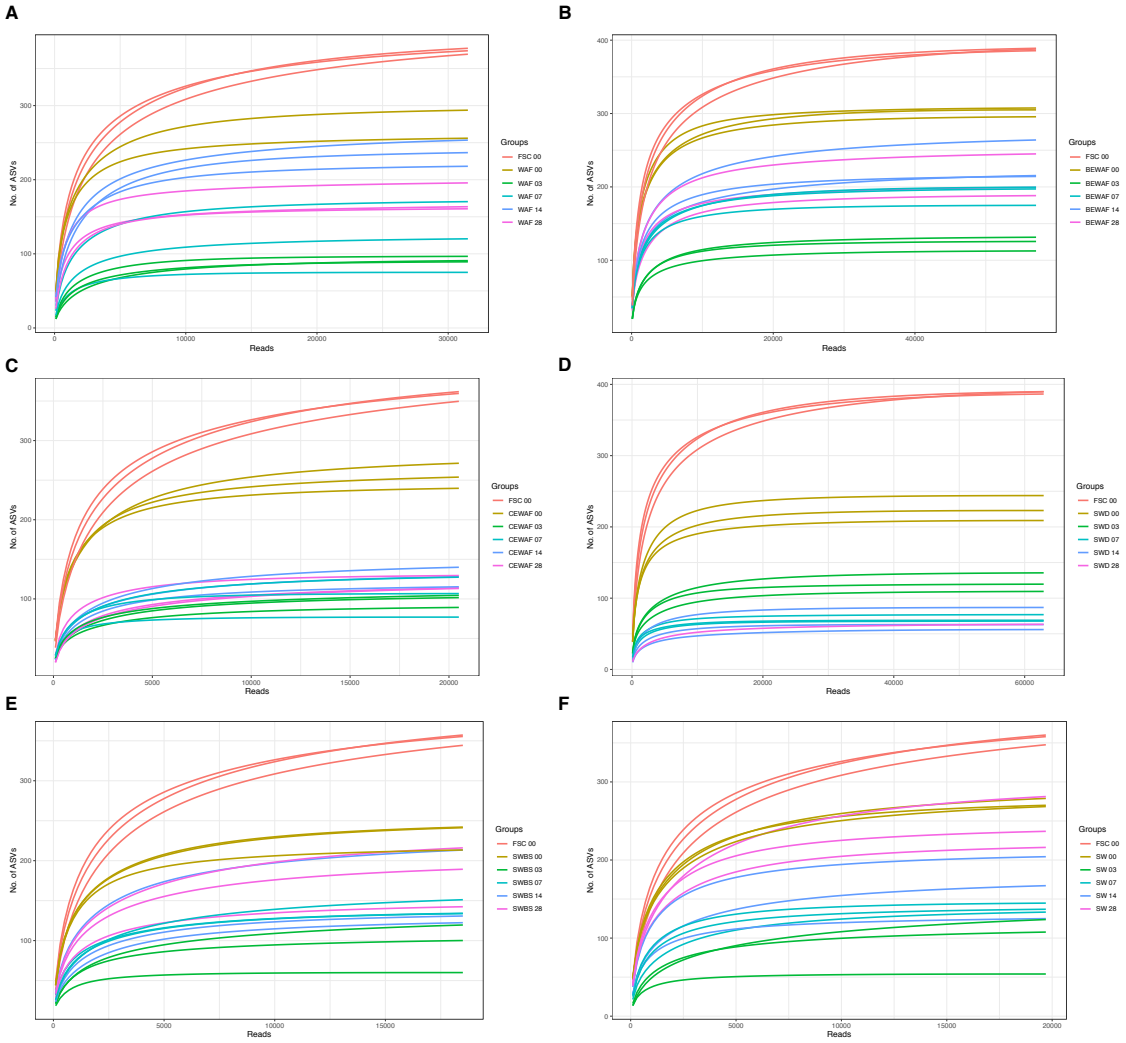
Dubois carbohydrate assay standard curve



Appendix B

Rarefaction curves

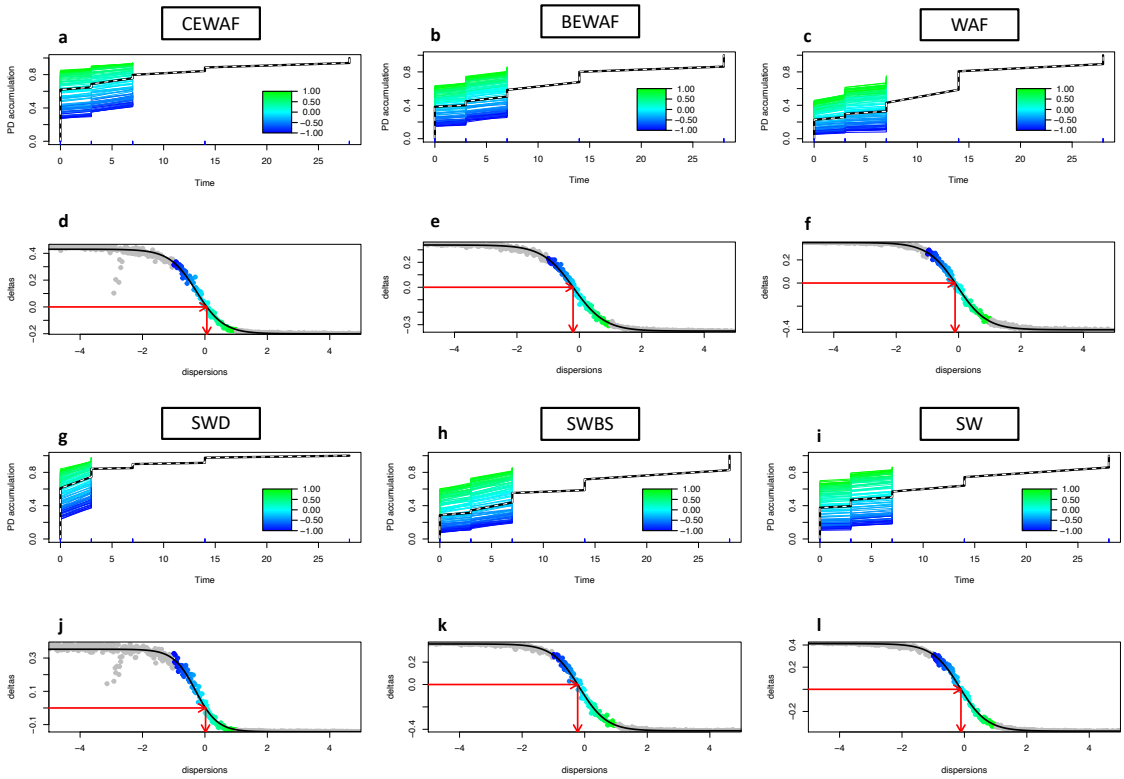
Rarefaction curves of treatments over 28-day incubation period compared to *in-situ* FSC seawater. (**A**) WAF, (**B**) BEWAF, (**C**) CEWAF, (**D**) SWD, (**E**) SWBS, and (**F**) SW. Colours represent incubation days: red – baseline microbial community at the time of seawater sampling, olive green – day 0, green – day 3, teal – day 7, blue – day 14, and pink – day 28



Appendix C

Phylodispersity accumulation curves

Plots **a-c** and **g-i** show the empirical (dashed) and surrogate polydispersity accumulation curves coloured according to $D^{\wedge}$ value. Blue colour corresponds to new species that have a previously detected close relative contribute little polydiversity and cause slow phylodiversity accumulation. Green colour corresponds to new species, that do not have a close relative, contribute more phylodiversity and cause faster accumulation. Teal colour corresponds to the neutral model which is above the empirical model (dashed line), signifying underdispersion in the order of first-time species detections. The time of sampling points (days) are shown on the x-axis. Plots **d-f** and **j-l** show how empirical and surrogate data compare with generation of D estimates. The y-axis represents the difference between empirical and surrogate data at time m and $D^{\wedge}$ value used to generate the surrogate datasets is shown on the x-axis. Colour-coded points correspond to surrogate datasets, grey-coloured points are extreme values of $D^{\wedge}$ (which help to fit the logistic error model – black line), and the red arrow show the process of error minimisation, producing a D estimate.



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