University of Essex

Effect of Crude Oil on Light Attenuation and the Resulting Impacts on Marine Microbial Phototrophs and Heterotrophs

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Abstract

Oil spills spread over the surface of the seawater which cause the attenuation of light passing through it. Then, we showed that crude oil preferentially attenuates the shorter wavelengths of photosynthetically active radiation. Therefore, we proposed that this change in the spectrum and reduction in light intensity would affect microbial phototrophic community composition, which in turn would alter the heterotrophic community and potentially the rate of hydrocarbon degradation. Experiments were conducted using natural seawater with a crude-oil layer (but not in contact with), a no-oil control, oil spectrum and standard light-spectrum. Treatments included continuous light and light: dark regimes. The impact of oil in phototrophs (*Synechococcus* and *Thalassiosira weissflogii*) was measured and their alkane production to sustain hydrocarbonoclastic bacteria (HCB). Chlorophyll *a* and DNA concentrations were measured as a proxy for biomass. DGGE analysis of bacterial 16S rRNA and *psbA* genes was also performed. Miseq Illumina sequencing analysis was performed to measure the abundance of bacteria and phototrophs. A qPCR was also performed to quantify phototrophs and HCB and UPLC analysis was done to quantify phototroph`s pigments. With continuous light, cyanobacteria were the most abundant microorganism whereas with light: dark regime, diatoms were the most abundant. The bacterial and phototrophic community had a greater variability with light: dark regime. *Synechococcus spp.* and *T. weissflogii* produced different pigments in response to changes in light intensity and spectrum. *Synechococcus* was able to sustain the growth of *Marinobacter hydrocabronocasticus*. Thus, we have shown that, in addition to oil having a direct impact on the microbial community in seawater, it has an indirect effect by altering the spectrum and intensity of light.

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Abbreviation list

%- Percentage

×*g*- Relative centrifugal force

°C- Degree Celsius

µg- Micrograms

µL- Micro litre

µM- Micromolar

AAP- Aerobic anoxygenic phototroph

Chl *a*- Chlorophyll *a*

CTAB- Cetyltrimethylammonium Bromide

DGGE- Denaturing gradient gel electrophoresis

DNA- Deoxyribonucleic acid

dsDNA- Double-stranded DNA

EDTA-Ethylenediaminetetraacetic acid

EPS- extracellular polymeric substances

et al- Et alia (and others)

FRRf- Fast repetition rate flurometry

***Fv/Fm*-** Maximum photochemical efficiency of PSII

h- Hours

HPLC- High performance liquid chromatography

L- Continuous light (24 h)

L:D- Light and dark (12:12 h)

LED- Light-emitting diode

M - Molar

min- Minutes

mL- Millilitre

mM- Micromolar

mm- Millimetre

NaCl- Sodium chloride

NaH_2PO_4- Sodium dihydrogen phosphate

$NaNO_3$- Sodium nitrate

nm- Nanometers

PAH- Polycyclic aromatic hydrocarbon

PAR- Photosynthetically active radiation

PCR- Polymerase chain reaction

PEG- Polyethylene glycol

pH- Potential of hydrogen

***psbA*-** Gene that encodes the protein D1 of photosystem II.

PSII- Photosystem II

RFU- Raw fluorescence units

rpm- Revolutions per minute

rRNA- Ribosomal ribonucleic acid

s- seconds

SE- Standard error

T- Transmittance

TAE- Tris-acetate-EDTA

UK- United Kingdom

v/v- Volume/volume

WF-Weathered Forties crude oil

CHAPTER 1

Introduction

1.1 Crude Oil

Crude oils are a complex mixture of hydrocarbon and other compounds (Van Hamme *et al.*, 2003); which can be divided in four classes: the saturates (*n*-alkanes, branched alkanes, and cycloalkanes), the aromatics (monoaromatic with different degrees of branching and polycyclic aromatic hydrocarbons(PAH)), the asphaltenes (phenols, fatty acids, ketones, esters, and porphyrins) and the resins (pyridines, quinolines, carbazoles, sulfoxides, and amides) (Atlas, 1981; Leahy and Colwell, 1990). Crude oil is comprised almost entirely of the elements hydrogen and carbon in the ratio of about two hydrogen atoms to one carbon atom. It also contains elements such as nitrogen, sulphur and oxygen, all of which constitute less than 3% (v/v) (Okho, 2006).

Light oils are typically high in saturated and aromatic hydrocarbons, with a smaller proportion of resins and asphaltenes. Heavy oils have a lower content of saturated and aromatic hydrocarbons and a higher proportion of the more polar chemicals, the resins and asphaltenes (Head *et al.*, 2006).

The contamination of a marine environment with crude oil and/or its products through spillage or dumping causes sustained and serious problems to the natural environment (McGenity, 2014). Approximately 1.3 million tonnes (~1.3 billion litres) of petroleum is released to the marine environment each year, much of it from natural seeps (NRC, 2003). During the past

years, there has been many oil spills that had affected the marine environment, some of these major impact oil spills include the following.

In 1967, the SS Torrey Canyon grounded on the Seven stones reef off the western tip of Cornwall, releasing ~137 million of litres of Kuwait crude oil. What makes this oil spill distinctive from other oil spills is not the quantity of oil that arrived to the shore, but the enormous quantity of dispersants used to remove the oil which caused more environmental damaged (Southward and Southward, 1977).

Then, on 24 March 1989, the oil tanker Exxon Valdez went aground in Prince William Sound and spilled more than 30 million litres of Alaska North Slope crude oil, which constituted the largest oil spill ever to occur in an arctic environment (Piatt *et al*., 1990). Further, on 5 January 1993, the tanker MV *Braer* ran aground Garth's Ness on the southern Shetland Isles coast. Approximately 98 million of litres of Gullfaks crude oil was released into the sea. The *Braer* oil spill was unusual because the oil dispersed and scattered into the sea by the combinatuion of string winds and the light nature of the crude oil (Kingston *et al*., 1995).

Additionality, in 1996, the tanker Sea Empress ran aground on rocks at the entrance of Milford Haven harbour, releasing into the sea 83 million of litres of crude oil. The response included the response to this spillage was successfully spraying of 446 tonnes (~453,000 litres) of dispersants, preventing ~68 million of litres of emulsion to reach the ashore, despite this an approximation of 17 million of litres of emulsion reach the coastline around south-

west Wales which is a conservation interest to Wales (Lyons *et al*.,1999; Law and Kelly, 2004).

Also, in November 2002, the oil tanker Prestige sank about 240 km off the Finisterre Cape (NW coast of Spain) after releasing an estimated 54 billion litres of a Russian heavy fuel oil. The spill affected a large part of the Galician coast, coastal waters, and the Cantabrian coast (Jimenez *et al*., 2007). The clean-up procedure for the shore was a mechanical extraction. However, studies found that indigenous bacteria present in the Galician coast, can degrade crude oil (Medina-Bellver *et al*., 2005).

In addition to this, the Deepwater Horizon oil spill in 2010 is the largest offshore oil spill in history and released more than 636 million litres of oil into the Gulf of Mexico. (Camilli *et al*., 2010). The Deepwater Horizon spill was quite different due to several factors. Much of the oil was suspended or dissolved in a sub-sea plume of more than 35 kilometres in length and 1,100 meters in depth which was partly due to the first sub-sea addition of dispersants (Gros *et al*., 2017). Due to the influx of hydrocarbons from the plume, the indigenous microbial community structure was changed from a community dominated by *Oceanospirillales*, to *Colwellia* and *Cycloclasticus* and finally methylotrophic bacteria (Mason *et al*., 2012).

Lastly in 2017, the small tanker Agia Zoni II sank off Salamina Island, in which more than 2 million of litres of oil were released into the ocean, contaminating the coastlines of Salamina (Carpenter and Kostianoy, 2018). The majority of the oil was contained using an oil spill clean-up unit (Kostianoy and Carpenter, 2018).

1.1.1 Process following an oil spill

When petroleum is spilled in the sea, it spreads over the surface of the water and hydrocarbons rise to the surface and come into contact with air. Then, it is subjected to many modifications and the composition of the petroleum changes with time (Harayama *et al.*, 2004). This process is called weathering and depends on wind, waves, water currents, oil type, nutrient availability, temperature and light exposure (McGenity, 2014).

Natural attenuation has been focused on marine environments since the world's oceans are the largest and ultimate receptors of hydrocarbon pollutants (Atlas, 1981). Weathering allows oil/pollutants to be removed and degraded by natural means:

a) Evaporation of the low molecular weight fractions (volatile organic compounds into the atmosphere. This process may increase the density and viscosity of the remaining oil, and in some cases, make it more difficult to disperse. Volatile petroleum components, such as *n*-alkanes with a chain length shorter than C_{14} and monocyclic aromatic hydrocarbons (e.g. benzene and xylenes) are subjected to both evaporation and dissolution (Harayama *et al*., 2004). Evaporation can be enhanced by higher winds speeds and temperature.
b) Dissolution of the water-soluble components, such as light aromatic hydrocarbons compounds such as benzene and toluene. However, these compounds are also the first to be lost through evaporation, a process which is 10 -1000 times faster than dissolution. Most crude oils and all fuel oils contain relatively small proportions of

these compounds making dissolution one of the less significant processes (Dutta and Harayama, 2001; ITOPF, 2002).

c) Dispersion occurs when waves and turbulence cause a surface slick to break into fragments and oil droplets of different sizes, which mix and spread into the upper levels of the water column. These naturally dispersed droplets may float back to the surface where they recombine to form another slick. Some natural dispersion occurs with all oils, but it is especially common in light oils and in rough seas, light oils may even be completely dispersed by this process. It should be noted that dispersion (or oil-in-water emulsification) is generally beneficial to the degradation by increasing the surface area of hydrocarbons available for microbial degradation.

d) Emulsification is the process whereby sea water droplets become suspended in the oil to forma water-in-oil emulsion. The incorporation of water in the oil, ultimately leads to thickening and an increase in the total volume remaining. The emulsion sometimes is referring as chocolate mousse. At the same time, emulsification can reduce the other natural weathering processes and can complicate the response (Dutta and Harayama, 2001; ITOPF, 2002; Abu and Dike, 2008).

e) Sedimentation occurs when floating oil approaches the shore. When floating, semi submerged or dispersed oil comes into contact with suspended sediment, the sediment can bind to it. This particularly happens in shallow waters. Very few oils sink to the deep sediment in the marine environment (ITOPF, 2002). However, the Deepwater Horizon Spill resulted in some sinking when oil combined with marine oil snow, but the quantitate importance of this mechanism is highly debated (Brackstad et al., 2018).

f) Oxidation is the process by which hydrocarbons react chemically with oxygen either breaking down into soluble products or forming persistent compounds called tars. This process is promoted by sunlight, but is very slow and even in strong sunlight, thin films of oil break down at no more than 0.1% per day (ITOPF, 2002).

g) Microbial degradation is the process where naturally occurring bacteria, archaea and to a lesser extent fungus, consume hydrocarbons as an energy source and carbon source. The remaining hydrocarbons are metabolized relatively rapidly by microorganism's uptake of soluble hydrocarbons. These bacteria do not adhere to oil (Atlas, 1981).

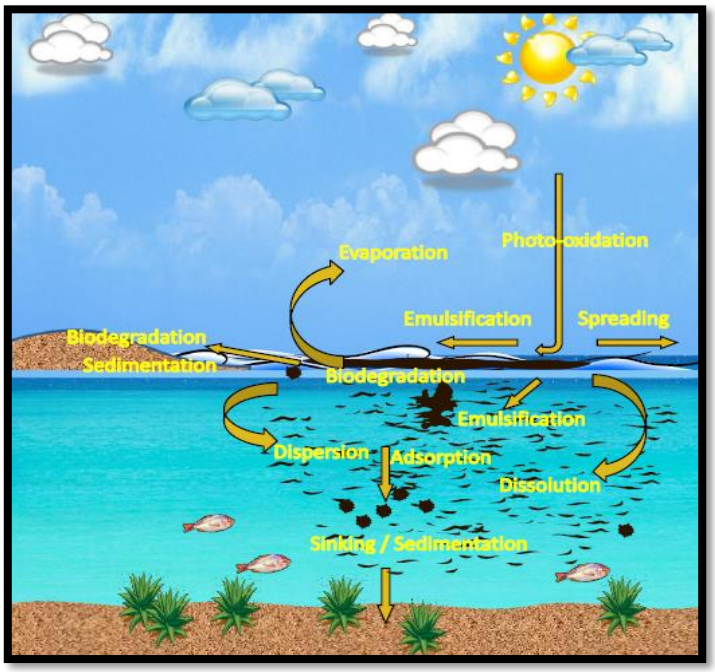

Figure 1.1 Process of weathering of crude oil spill on the ocean. McGenity *et al*. (2012).

1.1.2 Viscosity of oil

One of the main physical properties which affect the behaviour and the persistence of an oil spilled at sea is relative density. Most oils have relative density below 1 and are lighter than sea water which has a specific gravity scale about 1.025. Oils with a relative low density tend to contain a high proportion of volatile components and to be low viscosity (ITOPF, 2002).

All oils become more viscous as their temperature falls, some others depending on their composition (ITOPF, 2002). At low temperatures, the viscosity of the oil increases, the volatilization of toxic short chain alkanes is reduced, and their water solubility is increased, delaying biodegradation (Leahy and Colwell, 1990). The interaction between temperature, viscosity and bioavailability was shown recently in *Alcanivorax borkumensis*, where growth rate on individual n-alkanes fell sharply at the temperature that coincided with the liquid-wax phase transition for the specific n-alkanes (Lyu *et al*., 2018).

The different types of crude oil need to be classified for further treatment. Both a crude oil's viscosity and its API (American Petroleum Institute) gravity are important parameters for classification.

Table 1.1: Classification of oil according to the API gravity.

CLASIFICATION OF OIL	°API
Light	higher than 31.1
Medium	31.1 -22.3
Heavy	22.3-10
Extra-Heavy	below 10

1.1.3 Toxicity

Toxicity of crude oil depend on the solubility, the bioavailability and the concentrations of the hydrocarbons. Sometimes toxicity decreases during oil weathering in which less hydrophobic saturated and aromatic compounds disappear during the process of weathering. However, weathering can increase their hydrophobicity making the residues more bioavailable (Aeppli *et al*., 2012).

It is assumed that the water-soluble fraction is the most environmentally harmful fraction because it is available for uptake by microorganisms (Lampi *et al*., 2005). Although many polycyclic aromatic hydrocarbons (PAHs) are highly toxic, they have low water solubility and high affinity to accumulate in sediments, resulting in low accessibility to organisms and plants (Lampi *et al*., 2005).

PAHs are aromatic hydrocarbons with two or more benzene rings and/or pentacyclic molecules arranged in different structural configurations; when they have more than three aromatics rings, they are referred to as high-molecular-weight PAHs (HMW-PAHs) (Folwell *et al*., 2016). These compounds are the most persistent hydrocarbons and degrade very slowly under natural conditions (Haritash and Kaushik, 2009). Their persistence increases with the increase in molecular weight (HMW-PAHs) (Cerniglia, 1993). For the HMW-PAHs, biodegradation is greatly affected by the bioavailability, because they tend to adsorb to organic particles (Folwell *et al*., 2016). Also, some HMW-PAHs are mutagenic to bacteria and carcinogenic to mammals (Cerniglia and Sutherland, 2010).

The biodegradation of PAHs can be under aerobic or anaerobic conditions (Haritash and Kaushik, 2009). Many aerobic bacteria can use aromatic hydrocarbons as a source of carbon and energy in the presence of oxygen, most of these pathways have been elucidated (Meckenstock *et al*., 2004). On the contrary, the pathways of anaerobic degradation have not yet been elucidated. It is known, that the biodegradation of PAHs is catalysed by multicomponent dioxygenase which consists of a reductase, a ferredoxin and an iron-sulphur protein (Haritash and Kaushik, 2009).

When microorganisms use PAHs as the sole source of carbon and energy the microbial biomass can increase, on the other hand the microbial biomass can decrease if the pollutants become toxic in which the community composition may be affected (Johnston and Leff, 2014). Additionally, to reduce any harmful environmental impacts and control the rate of biodegradation it is important to have an adequate supply of nutrients like nitrates, phosphates and ferric iron when the quantity of hydrocarbons in the marine environment are critical (Atlas and Hazen 2011).

Finally, the toxicity of the oil could be due to other compounds other than PAHs, like the carboxylic acids, resins in crude oil, compounds containing hydroxyl, keto and nitro groups from contaminated sediments, oil sands or unknown oil weathering products (Aeppli *et al*., 2012).

1.2 Microbial degradation of crude oils

Microbes play a pivotal role in hydrocarbon degradation. The capacity to uptake and degrade hydrocarbons thereby lowering the concentration in the immediate vicinity of the cell and driving diffusive flux of poorly soluble compounds into the water column towards the cells is an important mechanism to enhance bioavailability of hydrocarbons (McGenity, 2014).

The microbial ability to increase the surface area of the bulk-phase hydrocarbon available to microbes by synthesis of emulsifying agents/biosurfactants (McGenity, 2014). Biosurfactants are polymers, totally or partially extra-cellular, with an amphipathic configuration, containing distinct polar and nonpolar moieties which allow them to form micelles that accumulate at interface between liquids of different polarities such as water and oil (Ilori *et al*., 2008). While bacteria can produce biosurfactants that stabilize emulsions, some microorganisms can emulsify hydrocarbons even in the absence of cell growth or uptake of hydrocarbons. The latter observation suggests that emulsification may be associated with the surface properties of the cells, as a result of attachment to the oil-water interface by general hydrophobic interactions rather than specific recognition of the substrate (Dorobantu *et al*., 2004).

For microbial degradation of crude oil to occur, it is necessary to have a population of oil degrading bacteria at the oil-water interface. Also, nutrients and an electron acceptor must be available to the degrading microorganisms (Venosa *et al*., 2010).

Generally, for hydrocarbons, the order of decreasing susceptibility to degradation is: *n*-alkanes>branched alkanes>low-molecular-weight aromatics>cyclo-alkanes>PAH>polar

compounds (Leahy *et al.*, 1990). Often the *n*-alkanes in the range C_{10} to C_{26} are viewed as the most readily degraded, but low-molecular-weight aromatics, such as benzene, toluene and xylene, which are among the toxic compounds found in crude oil, are also biodegraded by many marine microorganisms (Alvarez and Vogel, 1991).

Complex structures (those with branches and/or multiple condensed ring structures) are more resistant to biodegradation, meaning that fewer microorganisms can degrade those structures and the rates of biodegradation are lower than biodegradation rates of the simpler hydrocarbon structures found in crude oil (Atlas, 1995). It is therefore clear that hydrocarbons are not all biodegraded at similar rates, and not all hydrocarbons are degradable, what remain are principally the asphaltenes and resin compounds (Prince *et al*., 2003).

Biodegradation of hydrocarbons can be done by different microorganisms, as bacteria, archaea, fungi and yeast (Yakimov *et al.*, 2007). In the marine environment, bacteria are considered to represent the predominant hydrocarbon-degrading element of the microbial community (Atlas and Bragg, 2009).

Prior to 1900s it was thought that marine hydrocarbon degradation was primarily carried out by generalist species that also used carbon sources other than hydrocarbons (Van Hamme *et al*., 2003).

Degradation of alkanes and aromatic hydrocarbons have been investigated in different marine bacteria, including *Pseudomonas putida*, *Rhodococcus sp*., *Burkholderia cepacia* and *Sphingomonas* (Brakstad and Lodeng, 2005). Also, *P. fluorescens, P. aeruginosa, Bacillus subtilis, Bacillus* sp*., Alcaligenes* sp., *Acinetobacter lwoffi, Flavobacterium* sp*., Micrococcus roseus, and Corynebacterium* sp*.* were isolated from the polluted tropical stream which could degrade crude oil (Das and Chandran, 2011). Other genera that are typically found in petroleum-contaminated cold environments are *Acinetobacter*, *Arthrobacter*, *Mycobacterium*, *Pseudomonas*, *Rhodococcus* and *Sphingomonas* (Yakimov *et al*., 2003). Chronopoulou *et al*. (2015) using culture-dependent and culture-independent approaches detected only generealist hydrocarbon degrades, such as *Pseudoalteromonas*, beneath the oil layer in the North Sea. While in the cold deep-sea Gulf of Mexico, generalists like *Colwellia spp*. Were commonly detected (Redmond and Valentine, 2012).

From the 1990s onwards, based on isolation and also developments in investigating microbial communities by cultivation-independent methods, it became clear that specialist hydrocarbon-degrading microbes were very abundant in marine oil-polluted environments (Harayama *et al.,* 2014). These so-called obligate hydrocarbonoclastic bacteria (OHCB) include *Alcanivorax, Cycloclasticus, Oleispira*, *Oleibacter*, and *Thalassolituus* (McKew *et al*., 2007). Some species of Marinobacter primarily use hydrocarbons, such as *Marinobacter hydrocarbonoclasticus*, while others do not use hydrocarbons at all as a source of carbon and energy. *Marinobacter hydrocarbonoclasticus*, it is known to form biofilms in the interface between water and *n*-alkanes (C_8-C_{28}), biofilm formation is determined by the presence of nutrients in the interface and can also metabolize *n*-alcohols (C_{12}-C_{16}) (Mounier *et al*., 2014).

Alcanivorax strains grow on *n*-alkanes and branched alkanes but cannot use any sugars or amino acids as carbon sources (Harayama *et al*., 2004). Hara *et al*., (2003) found that *Alcanivorax borkumensis* strain ST-T1 isolated from the Sea of Japan in addition to using straight-chain alkanes, exhibited an ability to degrade branched alkanes (pristane and phytane), and that *Alcanivorax* formed the major population grown on squalane (2,6,10,15,19,23-hexamethyltetracosane), another branched long-chain alkane. It seems that this higher ability of *Alcanivorax* to degrade branched alkanes allowed this genus to predominate in oil-containing seawater. Also, it has been observed that *Alcanivorax* become the predominant microbial community established in oil-contaminated seawater when nitrogen and phosphorus nutrients are supplemented (Hara *et al*., 2003).

Species in the genus *Cycloclasticus* utilize aromatic hydrocarbons, including biphenyl, naphthalene, phenanthrene, toluene, and xylenes, as sole sources of carbon and energy. (Kasai *et al*., 2002). Geiselbrecht *et al*. (1998), isolated 23 strains of genus *Cycloclasticus* from the Gulf of Mexico, able to degrade naphthalene, phenanthrene, biphenyl, anthracene, acenaphthene, and fluorine. Kasai *et al*. (2002), isolated *Cycloclasticus* strains capable of degraded naphthalenes, dibenzothiophenes, phenanthrenes, and fluorenes. McKew *et al*. (2007) found *Cyclocasticus* to be the dominant community in a PAH mix (naphthalene, 1-methylnapthalene, 1,3-dimethylnaphtalene, fluorene, phenanthrene and pyrene) microcosm. More recently, certain lineages of *Cycloclasticus* have been shown to be able to growth on short-chain alkanes (Rubin-Blum *et al*., 2018)

Oleispira antarctica was isolated Antarctic coastal sea water (Yakimov *et al*., 2003) and has been found to be abundant in diverse cold, oil-polluted environments and alkane-containing microcosms (Coulon *et al*., 2007). *Thalassolituus* also can use a variety of branched and/or straight chain saturated hydrocarbons (Head *et al*., 2006). McKew *et al*., (2007) found that *Thalassolituus oleivorans* were the dominant *n*-alkane (C_{12}-C_{32}) degrader.

Marine phototrophic microalgae have also shown to partially degrade hydrocarbons. Cerniglia *et al*. (1980) demonstrated that the cyanobacterium *Oscillatoria* sp., can oxidized naphthalene to *cis*-1-2-dihydronaphthalene, 4-hydroxy-1-tetralone and 1-naphthol (major metabolite) in photoautotrophic conditions, and proposed three pathways for the metabolism of naphthalene (Figure 1.2). Also, Raughukumar *et al*. (2001), observed that three strains of cyanobacteria, *Oscillatoria salina*, *Plectonema terebrans* and *Aphanocapsa sp*. degraded 40% of Bombay High crude oil (50-60% of pure hexadecane, 20% of anthracene and 90% of phenantherene) in 10 days. Some diatoms also have the capacity of degrading hydrocarbons, e.g. *Cyclotella caspia*, have a high degradation ability for fluoranthene (Liu *et al*., 2006); *Anchanthes minutissima* can degrade *n*-alkanes (Antic *et al*., 2006); *Skeletonema costatum* and *Nitzchia sp*., can degrade phenanthrene and fluoranthene (Hong *et al*., 2008).

Although there are many reports of hydrocarbon degradation directly by phototrophs (microalgae and cyanobacteria), it is questionable whether these microorganisms would be competitive with the bacteria and some are probably involved only in partial oxidation (McGenity *et al*., 2012). Many studies did not use axenic cultures and it is probable that degradation of hydrocarbons was performed by associated heterotrophic degrading-

bacteria. Abed and Koster (2005) found that cyanobacteria *Oscillatoria* strain OSC**,** played an indirect role in biodegradation by supporting the activity of the heterotrophic bacteria degrades.

Fungi are not known to play a major role in hydrocarbon degradation within the marine environments (Harms *et al*., 2011), but in soils and potentially marine sediments, degradation by filamentous fungi may offer advantages over other microorganisms for several reasons: 1) movement of hyphae, which means more access to hydrocarbons and is not restricted by a hydrophobic environment (as it could be with bacteria); 2) similarly, fungal hyphae can penetrate hydrocarbon aggregates that may be anoxic internally; 3) many fungi are xerotolerant and osmotolerant; 4) some fungi possess extracellular enzymes which could help with the initial metabolism of hydrocarbons (April *et al*., 2000).

Also, some ligninolytic and non-ligninolytic fungi are known for their potential to degrade polycyclic aromatic hydrocarbons (PAHs) (Cerniglia and Sutherland, 2010). Exoenzymes produced by fungi have the advantage to diffuse into HMW-PAHs instead of entering into the cell as bacteria required (Folwell *et al*., 2016b).

For the yeast, there is even little information about their hydrocarbon-degradative potential (Margesin *et al*., 2003) it has been found that *Yarrowia lipolytica* degrade very efficiently hydrophobic substrates such as *n*-alkanes in soil samples (Fickers *et al*., 2005).

Also, some species of halophilic archaea are demonstrated to be able to degrade hydrocarbons in hypersaline environments like oil polluted salt marshes (Le Borgne *et al*., 2008). *Halobacterium sp*. has been shown able to degrade C_{10}-C_{30} *n*-alkanes in a medium contained 30% NaCl. Also hydrocarbon co-metabolization was shown for *H.salinarium*, *H. volcaniii* and *H.distributum* (Kulichevskaya *et al*., 1992). Strain EH4, was able to metabolize sutured hydrocarbons and PAHs while growing in a medium of 30 g/l of salts (Le Borgne *et al*., 2008).

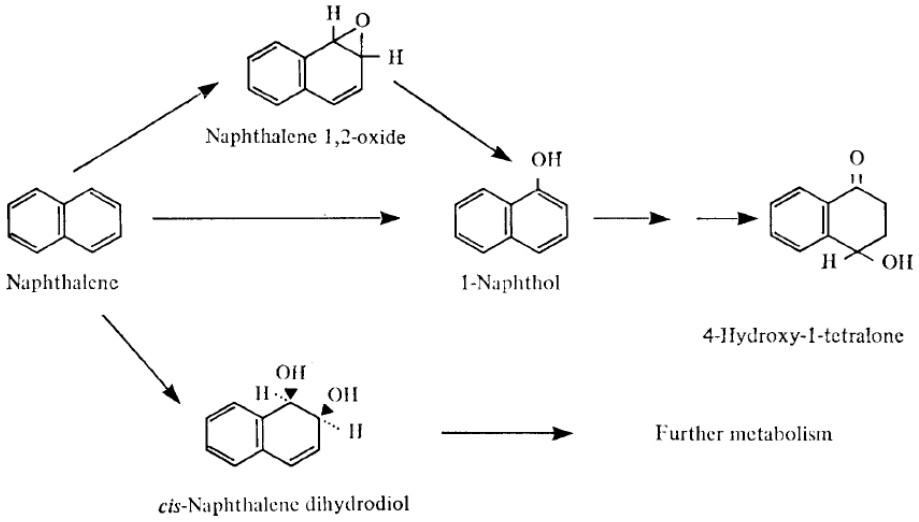

Figure 1.2 Pathways for metabolism of naphthalene by *Oscillatoria* sp. proposed by Cerniglia *et al*. (1980).

Crude oil biodegradation can occur under both oxic and anoxic conditions. In oxic conditions, primary attack on intact hydrocarbons always requires the action of oxygenases and, therefore, requires the presence of free oxygen as a reactant as well as a terminal electron acceptor (Okho, 2006). In the case of *n*-alkanes, aerobic degradation usually starts by oxygenation of a terminal methyl group to produce a primary alcohol which is further oxidized

to the corresponding aldehyde, and finally converted into fatty acid. Fatty acids are conjugated to CoA and further processed by β-oxidation to generate acetyl-CoA (Rojo, 2009) (Figure 1.3).

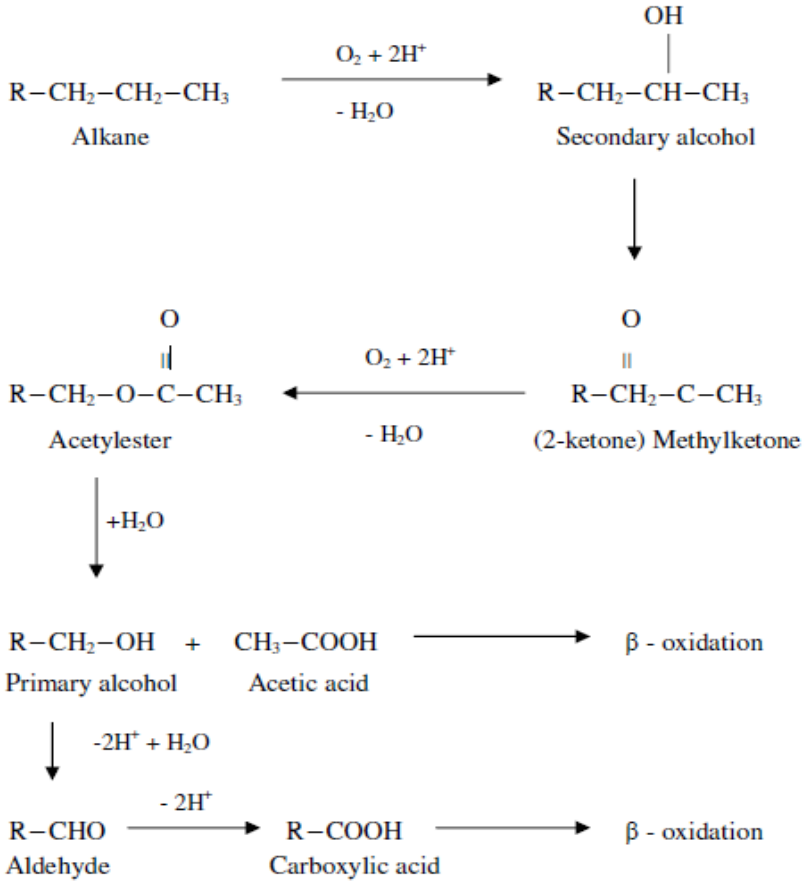

Figure 1.3 Aerobic alkane degraders use O_2 as a reactant for the activation of the alkane molecule (Rojo, 2009).

In the case of PAHs It is understood that the initial step in the aerobic catabolism by bacteria occurs via oxidation of the PAH to a intermediates may then be processed through either an *ortho* cleavage type of pathway or a *meta* cleavage of pathway, leading to central intermediates such as protocatechuates and catechols, which are further converted to tricarboxylic acid cycle intermediates (Kanaly and Harayama, 2000) (Figure 1.4)

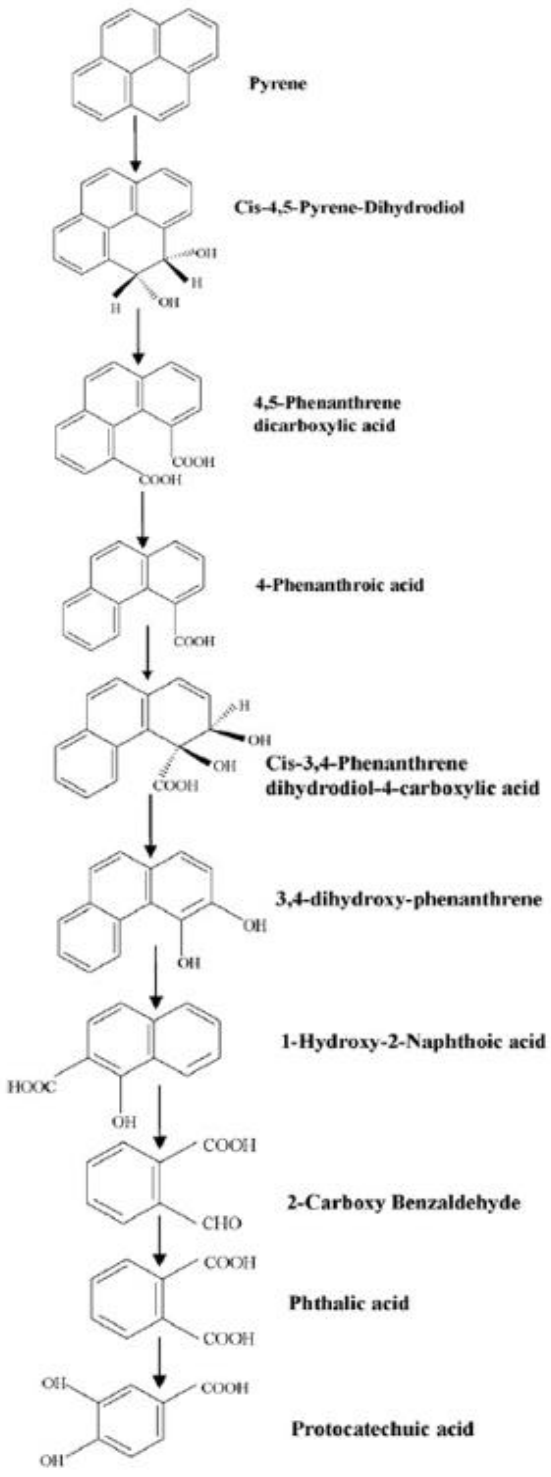

Figure 1.4 Aerobic pyrene degradation by *Mycobacterium sp*. (Haritash and Kaushik, 2009)

Several classes of petroleum hydrocarbons, including alkanes and mono and polycyclic aromatic compounds, can be degraded in the absence of oxygen with nitrate, ferric iron or sulphate as an electron acceptor, or under conditions of methanogenesis (Harayama *et al.*, 2004). Microorganisms able to utilize hydrocarbons anaerobically usually degrade only a very restricted range of them. Anaerobic degraders of hydrocarbons are bacteria, and the conversion of the hydrocarbon to CO_2 and reduction of the electron acceptor (nitrate, sulphate, ferric iron) may take place by a single species, but it is often carried out by syntrophic consortium. However, the anaerobic oxidation of methane is obviously carried out by consortia in a syntrophic mode of interaction in which archaeal cells oxidize methane and bacterial cells reduce sulfate with an intermediate scavenged from their partners. In the conversion of hydrocarbons to methane, the ‘‘tasks’’ are inverse: The bacteria activate and utilize the hydrocarbons whereas the archaea scavenge the intermediates (H_2 and acetate, or only H_2) for methanogenesis (Widdel *et al*., 2007).

Other terminal electron acceptors shown to be used during anaerobic hydrocarbon metabolism include manganese oxides, soil humic acids and the humic acid model compound anthraquinone-2,6- disulfonate, and fumarate in a fermentative oxidation process (Van Hamme *et al.*, 2003). An example of anaerobic degradation (Figure1.5): In the case of alkane initial activation occurs by addition of a fumarate molecule, which is later regenerated. Further degradation requires a C-skeleton rearrangement and the products are most likely processed by β-oxidation (Rojo, 2009). Other mechanisms of anaerobic degradation of hydrocarbons are discussed by Rojo (2009) and Widdle *et al*. (2010).

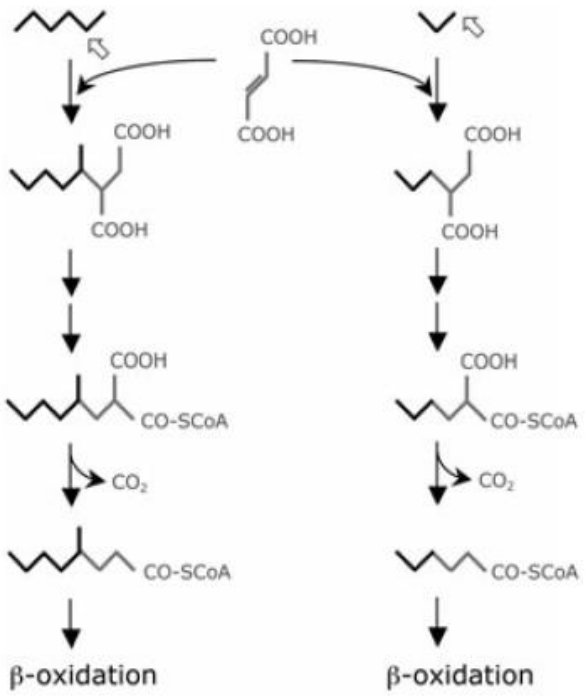

Figure 1.4 Anaerobic degradation of alkanes (Rojo, 2009).

1.3 Photooxidation

Light is a form of electro-magnetic radiation, and the property of this radiation that gives it characteristics, is its wavelength. The spectral colours are the basic components of white light (Hunt and Pointer, 2011).

The behaviour of light is highly affected by the nature of the medium through which it is passing. Scattering and absorption processes within the atmosphere reduce the light intensity and change the spectrum of the direct solar beam. Light varies depending of the time of the day, solar radiation and the presence of dissolved organic matter and suspended particles in the water column (Kirk, 1994). Water strongly absorbs light in the red and infrared

wavelengths, and the absorption slightly increases towards UV wavelengths, which causes a progressive dominance of the blue-green (400–500 nm) spectral components with depth. Despite the strong attenuation of the red and infrared light from the sun, these components are still present in the sea, although at low intensities (Depauw *et al*., 2012).

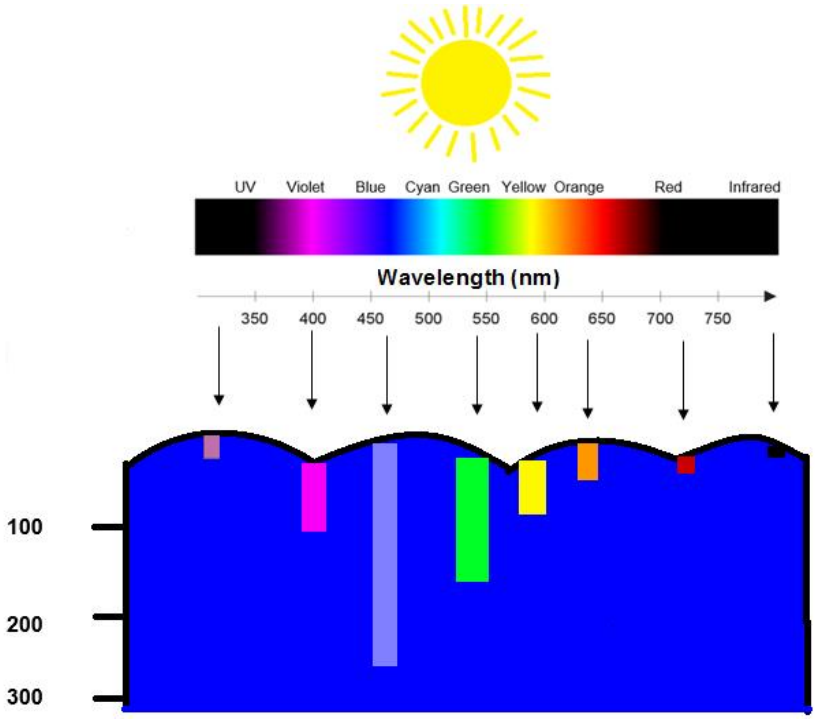

Figure 1.6 Light penetration in seawater at different wavelengths.

Among their many impacts, oil pollutants modify light fields (amount of light at every point in space travelling in every direction) in the water surface. These modifications are manifested by the attenuation of the light passing through an oiled water surface, by changes in light absorption in the seawater column due to the formation of an emulsion, and by the scattering of light by particles of such an emulsion (Krol *et al*., 2006).

The most important means of hydrocarbon degradation in the environment are driven by light and organisms. Whereas bacterial degradation results in total degradation of nearly all hydrocarbons, light is only able to make some photochemical modifications in the hydrocarbons (Prince *et al*., 2003).

Photooxidation is an important transformation pathway for crude oils spilles in the sea (Jacquot *et al.*, 1996). Under sunlight crude oil undergoes photochemical modifications, since aromatic compounds directly absorb sunlight, the aromatic fraction of the crude oil is decreased (Jacquot *et al.*, 1996; Harayama *et al.*, 2004; Garcia-Martinez *et al.*, 2006). Prince *et al*. (2003), determined that illumination has a significant effect on the composition of the oil, showing that the saturates are unaffected, but most of the aromatic hydrocarbons are converted to resins or polar molecules.

When PAHs are converted by photooxidation to more polar molecules it increase their water solubility and the bioavailability to microbial degradation (McConkey *et al.*, 1997). Photooxidation of Blend Arabian Light (BAL) crude oil asphaltenes showed that light exposure increased carbonyl, phenolic, sulfoxide and carboxylic groups (Fernandez-Varela *et al.*, 2006).

The portion of the solar spectrum responsible for photooxidation is primarily the ultraviolet region consisting of 5% UV-B (280-315 nm) and 95% UV-A (315-400 nm). Certain compounds of crude oil can also absorb and photooxidise in the visible region (400-700 nm) (Lee, 2003; Shankar *et al.*, 2015). These compounds can absorb light and initiate a chain of

photochemical reactions due to the presence of chromophores, an atom or group of atoms whose presence is responsible for the colour of the compound (D'Auria *et al.*, 2009).

It is important to notice that photooxidation of an oil spill is not homogenous and it depends on the climatic conditions (Fernandez-Varela *et al.*, 2006). The extent of photooxidation depends on the spectrum and irradiance of the incident light, the optical properties of surface water and the hydrocarbons themselves and on the presence of photosensitizers and photodegrades (Shankar *et al.*, 2015).

Attempts to quantify photochemical reactions are complicated by the diversity of possible chemical transformations and the mechanisms have been little studied. These reactions can be classified as: 1) direct photolysis, in which the molecule of interest absorbs light and is subsequently degraded; a mechanism that is largely responsible for degradation of higher molecular weight PAHs, since many of them absorb light in ultraviolet or visible wavelengths (Lee, 2003); 2) indirect photolysis (photosensitized), in which a different chemical (photosensitizer) present in the water absorbs light and then reacts to degrade the molecule of interest (Bertilsson and Windenfalk, 2002; Dutta and Harayama, 2001; Plata *et al.*, 2008); compounds such as common paraffin hydrocarbon and alkyl benzene cannot absorb light efficiently and must depend on sensitizers to obtain energy for initiating the photoreaction (Yang *et al.*, 2006).

Two major mechanisms by which photooxidation occurs are singlet oxygen and radical oxidation (Shankar *et al.*, 2015). The role of singlet oxygen is unknown, the existing

information is mostly indirect (Correa *et al.*, 2012). For example, Larson and Hunt in 1978, proposed that peroxides are initially formed by O_2 reactions and then photolyze to initiate radical chains. They demonstrated that β-carotene inhibits the photooxidation of fuel oil and suggested tentatively that this was due to quenching of singlet oxygen (Llchtenthalert, 1989). Further, the photooxidation of dimethylnaphthalenes sensitized by petroleum was found to involve singlet oxygen (Correa *et al.*, 2012).

In the singlet oxygen mechanism, the aromatic and polar fractions are the best singlet oxygen generators while asphaltene the least good, at wavelengths from 315 to 532 nm (Correa *et al.*, 2012). The molecule absorbing the light (aromatics and polar), transfers its energy from electronic excited states (usually triplet oxygen) to an oxygen molecule resulting in singlet oxygen which can then react with aromatic and heterocyclic sulphur compounds by addition. The singlet oxygen reacts with chemical compounds like PAH yielding peroxides that later convert into carbonyls and eventually hydroxyls (Shankar *et al.*, 2015). A phatway of photooxidation of naphthalene and anthracene using this mechanism is illustrated in Figure 1.7.

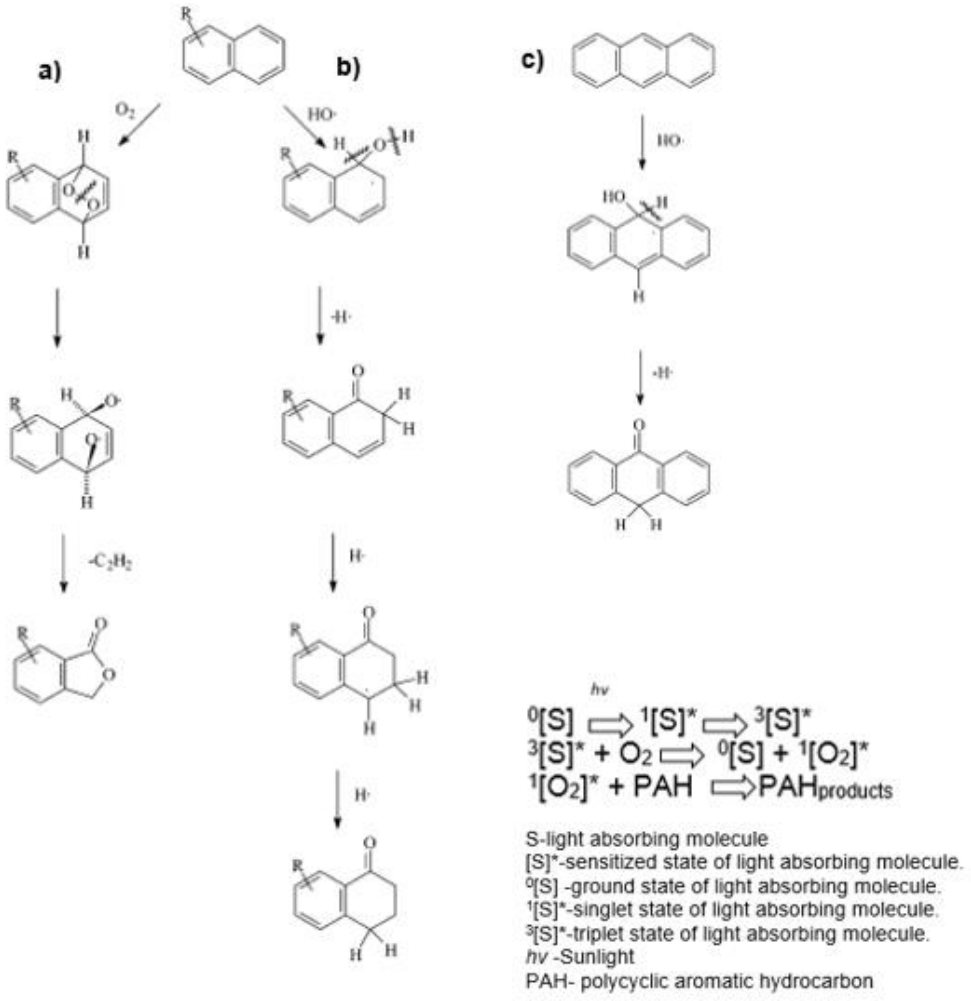

Figure 1.7. Pathways of photooxidation: Singlet oxygen mechanism: Naphthalene, products: a) 1(3H)-Isobenzofurane (phtalide) and b) 3, 4-dihydro-1(2H)-naphthalenone. Anthracene product: c) 9(1OH)-anthracenone (Garcia-Martinez *et al.*, 2006; Shankar *et al.*, 2015).

The free radical chain reaction mechanisms occur in the presence of hydrogens. For example, anthraquinone is sensitized initially by sunlight which reacts with alkyl benzene producing benzyl radical as a result of hydrogen abstraction. The benzyl radical then reacts with oxygen to form a peroxy radical which later stabilizes to form hydroperoxide. This hydroperoxide in the presence of sunlight splits to form benzyloxy radical and OH radical. The resulting benzyloxy radical may then proceed to react with oxygen resulting in

phenylalkanone and HO_2 radical (Shankar *et al.*, 2015). Mechanism of photooxidation of anthracene using this mechanism is illustrated in Figure 1.8.

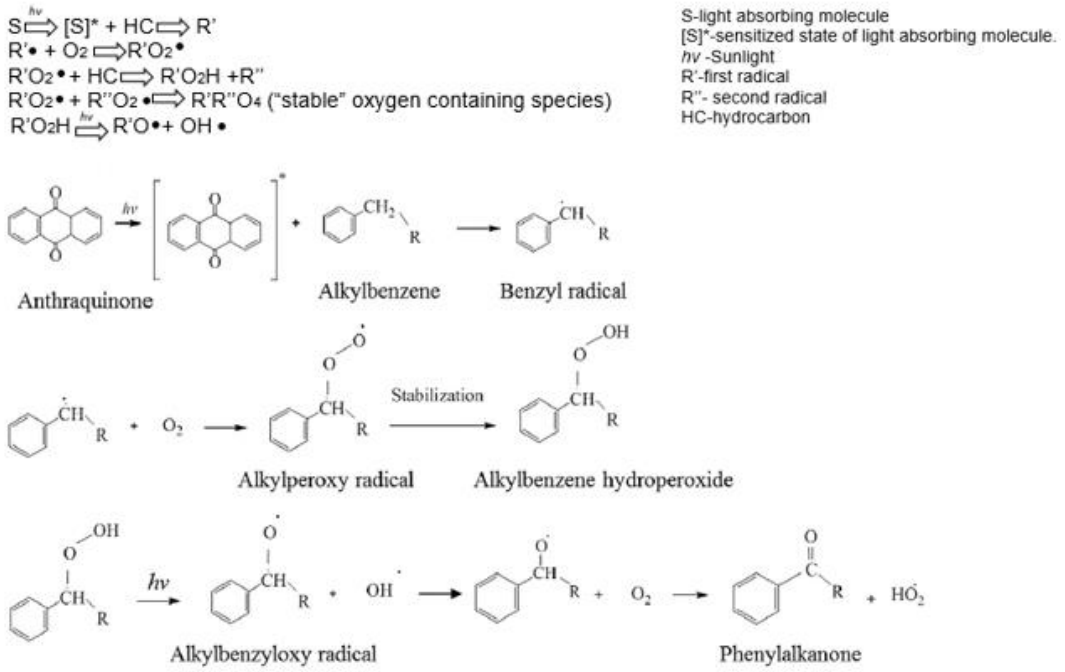

Figure 1.8. Pathways of photooxidation: Radical chain mechanism (Shankar *et al*, 2015).

Photooxidation could also have a negative effect, for example, it is known that photomodification of PAHs results in complex mixtures of photoproducts that are more toxic than the parent compounds (Duxbury *et al.*, 1997). The available evidence indicates that photoenhanced toxicity of oil occurs through a photosensitization mechanism, and the phototoxic components of oil are primarily 3 to 5 ring PAHs and heterocycles (PAHs containing an oxygen, sulphur, or nitrogen in place of a carbon) (Barron and Ka'aihue, 2001). It is probable that oxidized hydrocarbons are more toxic than sulphur compounds (Larson and Berenbaum, 1988), the difference may be correlated with content of free-radical and

singlet oxygen quenchers because the sulphur compounds have been shown to be readily photooxidized to sulfoxides and sulfones (Correa *et al.*, 2012). Aeppli *et al*. (2012) found that the accumulation of oxygen in oil residues which they called oxyhydrocarbons, are relatively persistent and have the potential to exhibit toxic effects. For example: a phenanthrene photoproduct, 9, 10-phenanthrenequinone (PHQ), has been shown to have greater toxicity that the parent compound in bacterial, plant and invertebrate assays (Lampi *et al.*, 2005) (Figure 1.9).

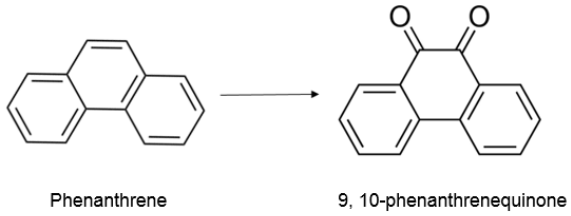

Figure 1.9. Structure of phenanthrene and structure of photomodified PAH product (oxy-PAH) (Lampi *et al.*, 2005).

Nevertheless, toxicity is often assumed to decrease during oil weathering. This is because bioavailability, which is a prerequisite for compounds to manifest toxic effect, usually negatively correlates with hydrophobicity, and because less hydrophobic saturated and aromatic compounds disappear during oil weathering (Aeppli *et al*., 2012).

1.4 Photosynthetic organisms

Another way that light can influence the microbiota, and ultimate the fate of oil, is via its impact on photosynthetic organisms. The oceans cover approximately 70% of the Earth's surface, and photosynthetic organisms living in the photic zone are responsible for half of the global primary productivity (Depauw *et al*., 2012). These organisms can convert light

energy into biologically usable (chemical) forms (Fuhrman *et al.*, 2015); via photosynthesis, representing the most important biological process on Earth (Madigan *et al.*, 2014). Phototrophic microorganisms are ubiquitously distributed throughout the planet in all types of lakes and ocean, where they can survive to different environmental stresses (e.g. heat, cold, salinity, photooxidation, UV exposure) (Amaro *et al*., 2011).

In the marine environments, the photic zone is composed by an oxic and anoxic part and these two parts are colonized by different groups of phototrophic microorganisms (Goericke, 2002); the oxic environment is dominated by eukaryotic microalgae, which together with cyanobacteria are collectively called phytoplankton (Depauw *et al*., 2012; Ramanan *et al*, 2015); and whose primary photosynthetic pigment is chlorophyll *a*. The discovery of phytoplankton in the late 1970s have a great impact because it could provide the missing link in the carbon supply/demand question in the oceans (Stockner, 1988). The anoxic environment is dominated by anoxygenic photosynthetic bacteria, whose primary photosynthetic pigment is bacteriochlorophylls *a*, *b*, *c*, *d*, *e* and *g* (Goericke, 2002). The anoxygenic photosynthetic bacteria are divided into: Green bacteria in the phylum Chlorobi, purple photosynthetic bacteria in the phylum Proteobacteria, green non sulfur bacteria in the phylum Chloroflexi, Heliobacteria in the phylum Firmicutes and Acidobacteria (Koblizek, 2015).

In addition to, there is another group of bacteria that mainly use bacteriochlorophyll *a* as a main light harvesting pigment but in contrast to anoxygenic photosynthetic bacteria, they can produce the pigment under oxic conditions, and they are called aerobic anoxygenic

phototrophic bacteria (Yurkov and Beatty, 1998). However, the concentration of bacteriochlorophyll *a* is significant lower compared to anoxygenic photosynthetic bacteria (Koblizek, 2015). Members of this group belong to the phylum Proteobacteria (Alphaproteobacteria, and Gammaproteobacteria) (Spring *et al*; 2013).

The oxygenic phototrophs use water as an electron donor whereas the anoxygenic phototrophs perform photosynthesis using reduced sulphur compounds, hydrogen or small simple organic compounds as electron donors and do not produce oxygen (Pfenning *et al*., 1981). The aerobic anoxygenic phototrophic bacteria utilize light as an additional source for their heterotrophic metabolism (Kloblizek, 2015) and because of that, these bacteria are classified as facultative photoheterotrophs, which can grow in the dark on organic carbon substrates but also can use light as a complement to their energy requirements (Goericke, 2002).

There is also another mechanism for light harvesting energy in which rhodopsins are utilized (Olson *et al*., 2018) these are integral membrane proteins that create a membrane-spanning proton gradient (Slamovits *et al*., 2011). Rhodopsins are present in bacteria, archaea and virus (Olson *et al*., 2018);and also can be found in some zooplankton, it seems that proteorhodopsins have been acquired by horizontal gene transfer from bacteria (Slamovits *et al*., 2011). The first rhodopsins were found in archaea (haloarchaea), and later were discovered in marine Proteobacteria (proteorhodopsin), including clades SAR-89 and SAR-11 that are the most abundant microbes in the ocean photic zone (Hohmann-Marriott and Blankenship, 2011). Light appears to be commonly utilized as a supplemental energy source

in heterotrophic bacterial carbon and energy cycling in the ocean (Olson *et al*., 2018) and some evidence shows that it does promote growth and survival in poor conditions (Slamovits *et al*., 2011). However, proteorhodopsins cannot achieve the coverage of the solar spectrum that chlorophyll-based pigments exhibit (Hohmann-Marriott and Blankenship, 2011).

The main pigments absorbing photosynthetically active radiation (PAR) include the chlorophylls, carotenoids and phycobilins (Porra *et al*., 1997). A light-harvesting pigment must absorb in a spectral region where radiant energy is available, and where quanta are energetic enough to move electrons from a high potential electron donor to a low potential electron acceptor molecule (Bjorn *et al.*, 2009). Chlorophyll traps light in blue and red part of the electromagnetic spectrum (Kuczynska *et al*., 2015). Carotenoids (carotenes and xanthophylls) have two major roles, they serve in light harvesting (Damjanovic *et al*., 1999) and protecting the photosynthetic apparatus against photo-oxidative damage (Fu *et al*., 2012). The peaks of absorption for some pigments given in table 1.2. The distributions of chlorophylls and accessory pigments can provide information on community and the abundance of primary producers (Airs *et al.*, 2001). The existence of different forms of chlorophyll or bacteriochlorophyll that absorbs light of different wavelengths allows phototrophs to make better use of the available energy in the electromagnetic spectrum (Bjorn *et al.*, 2009).

Cyanobacteria are considered algae by phycologists because of phenotypic similarities but the advent of molecular phylogeny and polyphasic studies proved their bacterial ancestry Most cyanobacteria in addition to some carotenoids, contain chlorophyll *a*, phycocyanin and

allophycocyanin, as pigments for photosynthesis, in addition, several cyanobacteria contain phycoerythrin (Govindjee and Shevela, 2011).

Two of the most abundant and environmentally significant cyanobacteria (Ahlgren and Rocap, 2012) are *Prochlorococcus* and *Synechococcus*, with an estimated global population sizes of $2.9 \pm 0.1 \times 10^{27}$ and $7.0 \pm 0.3 \times 10^{26}$ cells, respectively, and are together responsible for approximately a quarter of marine net primary production (Lea-Smith *et al*., 2015), having a fundamental biogeochemical role in the world's oceans (Tai and Palenik, 2009). *Prochlorococcus* dominates phytoplankton in the central oceanic gyres, while *Synechococcus* spp. can become very abundant in nutrient-rich tropical regions (Zubkov *et al.*, 2003).

Another important group of marine microorganisms are the diatoms, which are ubiquitous photosynthetic eukaryotes that are responsible for about 20% of photosynthesis on Earth They serve as the base of the marine food web when they are consumed by higher eukaryotes, and they can also serve as food for heterotrophic bacteria (Amin *et al.*, 2012).

Unlike the Rhodophyta and Phaeophyta, which are significant primary producers only along sea coasts, or the Haptophyta, significant only in the marine phytoplankton, diatoms are major constituents of benthic and planktonic algal communities worldwide, in terrestrial, freshwater and marine habitats (Mann and Droop, 1996). They are main players in the biogeochemical cycle of carbon (C), as they can account for 40% of the total primary production in the ocean and dominate export production, as well as in the biogeochemical cycles of the other macro-nutrients, nitrogen (N), phosphorus (P), and silicon (Si) (Sarthou *et al.*, 2005)

Table 1. 2 Photosynthetic organism and light harvesting pigments.

Pigments	Characteristic absorption maxima peak (nm)	Extraction Solvent	Organisms	Reference
Allophycocyanin	650, 671	Methanol	Cyanophyta and Rhodophyta	Govindjee and Shevela (2011)
Bacteriochlorophyll *a*	365, 605, 772	Methanol	Aerobic anoxygenic phototrophs, Purple non-sulphur, Chromatiacea, Ectothiorhodospiraceae, green sulphur bacteria and Heliobacteria	Madigan *et al.* (2014); Caumette (1993)
Bacteriochlorophyll *b*	368, 407, 582, 795	Acetone	Purple non-sulphur and Chromatiacea	Caumette (1993)
Bacteriochlorophyll c	435, 620, 670	Methanol	Chromatiacea, Chloroflexaceae,	Caumette (1993)
Bacteriochlorophyll *d*	427, 612, 659	Methanol	Chromatiacea, Chlorobiceae, Chloroflexaceae,	Caumette (1993)
Bacteriochlorophyll e	338, 456, 594, 649	Acetone	Chlorobiceae, Ectothiorhodospiraceae	Caumette (1993)
Bacteriochlorophyll *g*	365, 405, 566, 762	Acetone	Heliobacteria	Caumette (1993)
Chlorobium chlorophyll (Bacterioviridin)	750 or 760	Methanol	Green bacteria	Rabinowitch and Govindjee (1969)
Chlorophyll *a*	430, 668, 713	Acetone	All photosynthesizing oxygenic phototrophs and higher plants	Jeffrey *et al*., (1997)
Chlorophyll *b*	453, 652	Acetone	Higher plants, green algae and symbiotic prochlorophyte	Jeffrey *et al*., (1997)
Chlorophyll *c*	450, 633, 694	Acetone	Diatoms and brown seaweeds	Jeffrey *et al*., (1997)
Chlorophyll *d*	447, 688	Acetone	Some cyanophyta (e.g. *Acaryochloris sp.*)	Airs *et al.,* (2014)
Chlorophyll *f*	406, 706	Methanol	Some cyanophyta (e.g. *Chlorogloeopsis fritschii*)	Airs *et al.,* (2014)
Diadinoxanthin	426, 448, 478	Acetone	Xanthophyte, Bacillariophyte, Dinophyte	Takachi (2011), Jeffrey *et al*., (1997)
Diatoxanthin	427, 454, 482	Acetone	Xanthophyte	Takachi (2011), Jeffrey *et al*., (1997)
Fucoxanthin	446, 468	Acetone	Bacillariophyte, Haptophyte	Papagiannakis *et al.,* (2005)
Lutein	425, 448, 476	Acetone	Chlorophyta, Rhodophyta (Macrophyte type)	Takachi (2011), Jeffrey *et al*., (1997)
Phycocyanin	580, 625, 634	Methanol	Cyanophyta and Rhodophyta	Govindjee and Shevela (2011)
Phycoerythrin	496, 565	Methanol	Cyanophyta and Rhodophyta	Govindjee and Shevela (2011)
Proteorhodopsin	530	Dichloromethane	SAR 11 Expressed in *E.coli*	Giovannoni *et al*. (2005)
Zeaxanthin	428, 454, 481	Acetone	Cyanophyta	Takachi (2011), Jeffrey *et al*., (1997)
α-carotene	420, 440, 470	Hexane	Cryptophyta	Takachi (2011), Jeffrey *et al*., (1997)
β-carotene	440, 460, 495	Hexane	Cyanophyta, Heterokontophyte, Haptophyte, Chlorophyta	Takachi (2011), Jeffrey *et al*., (1997)

1.5 Interaction between phototrophic-heterotrophic microorganisms

The ubiquity and abundance of microbes (bacteria, archaea and protist) mean that they drive oceanic biogeochemical cycles, which in turn impact microbial biodiversity and community-level interactions (Amin *et al.*, 2012).

Microbial interactions are at the core of species success or failure. These interactions constitute an important component of microbial communities, where synergism or competition among species can drive the diversity of an ecosystem. The fact that no microbe exists alone in nature means that interactions with other species are required (Amin *et al.*, 2012). Phytoplankton blooms are often seasonal in nature and are thus transient events, the abundance and activity of heterotrophic bacteria varies accordingly. Indeed, secondary bacterial production typically correlates with the concentration of chlorophyll *a*, which is a proxy for phytoplankton abundance (Buchan *et al* 2014). Thus, marine microbial communities are in a constant state of flux and resilient, which means that their behaviours are widely predictable in terms of typical features of daily, seasonal and interannual variation in community composition (Fuhrman *et al*, 2015).

Bacteria can support the growth of phytoplankton via the recycling of nutrients, but at the same time, they also compete with phytoplankton for essential nutrients. Both healthy and dead (or dying) phytoplankton release organic compounds (e.g. excreted polysaccharides) that are consumed by heterotrophic bacteria, and the chemical nature and concentration of the released compounds varies with phytoplankton species and the physiological status of

the phytoplankton (Buchan *et al*, 2014). In addition to the exudation of organic components algal components released through cell lysis may be an important carbon source for bacteria (Arora *et al.*, 2012). On the other hand, the availability of inorganic nutrients (N, P and Si) is indirectly controlled by heterotrophic bacteria and Archaea that are responsible for remineralizing the organic matter produced by phytoplankton (Green *et al.*, 2015). Also, cyanobacteria can fix nitrogen and oxygen (via photosynthesis) to heterotrophic bacteria and thus maintain synergistic communities in, for instance, microbial mats (Rosenbaum *et al*, 2010). Bacterial action on components of the organic matter field near phytoplankton, influences carbon and nutrient fluxes in various pathways: microbial loop, sinking, grazing food chain, storage, and fixation (Grossart *et al.*, 1999). Bacteria also release exopolysaccharides (EPS) in response to the presence of phytoplankton, likely to initiate attachment (Amin *et al.*, 2012).

There is evidence that microbial communities dominated by phototrophic microorganism can be actively involved in the degradation of oil (Abed *et al.*, 2002). Also, Chronopoulou *et al.* (2013) observed that oil pollution in a mudflat sediment mesocosm led to an increase in benthic diatoms and a large increase in cyanobacteria. Cohen (2002) found that flasks with seawater and oil exposed to light were dominated by cyanobacteria, while those kept in the dark became brownish due to bacterial growth, and biodegradation of oil was slower in the dark than under light conditions.

Also, several works reported that cyanobacteria protect the associated bacteria and fungi from adverse ecological conditions like excessive light or dryness (Chaillan *et al.*, 2006).

Oxygen liberated by photosynthesis is likely to be very important in activating hydrocarbons and serving as an electron acceptor in aerobic respiration. In turn, the locally increased concentration of CO_2 produced by the heterotrophs, will generally allow enhanced photosynthesis (McGenity *et al.*, 2012).

Crude oil spills cause serious damaged to the natural environment and the fate of these spills will depend on the type of oil, the volume of the oil spilled and the weathering process. Two process are very important: Photooxidation in which light modifies the structure of hydrocarbons and could happen by two mechanisms: direct and indirect photolysis and microbial degradation. The principal hydrocarbon-degrading marine bacteria are *Alcanivorax*, *Cycloclasticus*, *Oleispira* and *Thalassolituus*; this group of specialists is called hydrocarbonoclastic bacteria (HCB). The interaction between HCB and phototrophic microorganisms, like cyanobacteria, diatoms and algae are very common in marine environments and they maintain synergistic communities in which the oxygen produced via photosynthesis by the phototrophs is an important electron acceptor in the aerobic respiration by HCBs and the CO_2 produced by HCBs enhances photosynthesis.

The overall aim of the thesis is to investigate the effect of an overlaying oil layer on both, quality and intensity of light transmission and the subsequent effects on the photosynthetic and bacterial community in seawater.

1.6 Objectives

1.6.1 To measure the photosynthetically active radiation (PAR) in seawater with and without and overlaying oil.

1.6.2 To determine the changes in the bacterial and photosynthetic community by an overlaying oil and by mimicking the effect of overlaying oil using artificial LED lights.

1.6.3 To determine the growth and production of pigments of two phototrophs, *Thalassiosira weissflogii* and *Synechococcus* under the overlaying oil, neutral density filter, control with no oil/filter and in contact with oil treatments.

1.6.4 To measure if *Thalassiosira weissflogii* and *Synechococcus* can sustain the growth of hydrocarbonoclastic bacteria (HBC) by potentially producing *n*-alkanes.

CHAPTER 2

Development of methods to measure the effects of crude oil overlaying (but no in contact with it) seawater on light attenuation and marine microbes, using a continuous-light regime.

2.1 Introduction

The oceans represent the largest biome on Earth. Bacteria and algae comprise the majority of organisms in the plankton of the ocean; Bacteria in marine systems are near to 10^6 cells ml^{-1}. The phytoplankton vary in abundance, generally between 10^2 and 10^5 cell.ml^{-1} (Cole, 1982).

Crude oils are complex mixtures of hydrocarbons and other organic compounds, with elemental compositions in the region of, carbon (83-87%), hydrogen (10-14%), nitrogen (0.1-2%), oxygen (0.1-1.5%) and sulphur (0.5-0.6%) (Van Hamme *et al.*, 2003). Hydrocarbon fractions can be oxidized either by sunlight radiation (both direct and indirect photooxidation reactions) or by marine microorganisms which that utilize them as a carbon and energy source (biodegradation) (Raghukumar *et al*., 2001).

Crude oil has a significant impact on light intensity and spectrum of PAR (photosynthetically active radiation) (Raghukumar *et al*., 2001). The presence of an oil layer can change the phototrophic community in seawater when they are exposed to continuous light. Also, the effects of natural or artificial light on microbial communities depends on organic matter, aquatic microorganisms, and length of exposure (Medina-Sanchez *et al*., 2002).

There are several studies about the degradation of oil by heterotrophs and their interaction with the phototrophic community. However, these studies have not taken into account the impact of oil on the intensity and spectrum of light received by phototrophs.

Chronopoulou *et al.* (2013), observed that oil pollution in an interdal mudflat sediment mesocosm led to 1.7 times more benthic diatoms and 7.2 times more cyanobacteria compared with the non-oiled control; and proposed that oil has a negative effect reducing grazing pressure and leading to a lower concentration of dissolved inorganic nitrogen, resulting in the increased photoautotrophic growth. However, oil-induced change in the light regime may also have an effect. For example, changes in the spectrum and intensity of light passing through and oil layer may favour cyanobacteria and microalgae which have different pigment able to absorb light at different wavelengths (430, 650, 680, 706 nm) (Rabinowitch and Govindjee, 1969).

Bacosa *et al*. (2015) demonstrated of the effect of light in the north of the Gulf of Mexico on microbial communities in the presence of Corexit, crude oil, separately and together. They found that in irradiated treatments the community structure was significantly affected, and the bacterial diversity was reduced.

The overall aim of this study is to investigate the effect that oil has on light, both its quality and intensity, and as a consequence of this, the effect on photosynthetic and the overall bacterial community in seawater under a continuous light regime.

2.1.1 Hypotheses

2.1.1.1 Crude oil will alter the spectrum and intensity of photosynthetically active radiation (PAR) in seawater.

2.1.1.2 The composition and abundance of photosynthetic and bacterial communities will change as a consequence of this change in PAR.

2.1.2 Objectives

2.1.1.3 To measure PAR spectrum and intensity in sea water with and without overlaying oil.

2.1.1.4 To measure the chlorophyll *a* to determine how changes in PAR affect the phototrophic biomass.

2.1.1.5 To determine changes causing by overlaying oil on the phototrophic and overall bacterial community composition in sea water by denaturing gradient gel electrophoresis (DGGE).

2.2 Methods

2.2.1 Design of experiments

2.2.1.1 Testing the effect of oil on transmitted light

Samples were prepared by dispensing an increasing amount of Gyda crude oil (1%, 2%, 3%, 4% and 5% v/v) into a series of sterile petri dishes (92 diameter x 16 mm depth) containing 20 ml of filter-sterilized (47 mm diameter, 0.2 µm pore-size polycarbonate filters, Whatman) natural sea water from oligotrophic waters of a cruise in 2011, and swirled in a slow circular motion to ensure even distribution of the oil. Controls were also established with 20 ml of filter-sterilized natural seawater but without the oil. They were exposed to artificial light using Helioespectra LED lamps. The light intensity from the LED source was 600 µmol photons m^{-2} s^{-1}. All the measurements were done in a range of 300 nm to 800 nm wavelength. PAR intensity and spectrum were measured with a *Macam* Spectroradiometer. For each sample, sensor readings from nine different positions under the petri dishes were taken in a predetermined order. All experiments were performed in triplicate or quadruplicate (Table 2.2).

The transmittance, i.e. the fraction of incident electromagnetic power that is transmitted through a sample curve, was obtained: $\% T = I / I_0 \times 100$, were I_0=Intensity of light passing through a control (seawater) and I=Intensity of light passing through the sample (seawater + oil).

2.2.1.2 Testing the effect of oil-induced light attenuation on the microbial community in sea water

Four experiments were carried out (as explained in Tables 2.2 and 2.3), using continuous light (L1, L2, L3, and L4). The design of the experiment was improved from L1 through L4. Samples were prepared with 160 mL of natural seawater plus nutrients (882 µM of $NaNO_3$ and 36.2 µM of NaH_2PO_4) in a cell-culture flask (Noc™ Easy Flask™ 75 cm^2, Nunclon™ Delta Surface, Thermo Scientific). Nutrients were added based on the concentration in f/2 Medium (Guillard and Ryther, 1962; Guillard, 1975) (Appendix A), which has been used extensively and is suitable for the growth of most algae. The sides of cell culture flask were covered with aluminium foil.

For all the continuous light (L1-L4) experiments (Figure 2.1), oil was evenly distributed in the bottom of four 150 mm diameter × 20 mm high petri dishes; as a control, four petri dishes without oil were used. Then, the petri dishes were placed on top of the cell culture flasks under the two LED lamps. The position of the cell culture flask was changed randomly every 8 h and the petri dishes with an oil layer were replaced every 24 h during the whole experiment. All samples were incubated at 20°C.

Seawater samples for DNA (30 mL), Chlorophyll *a* (45 mL), Fast repetition rate fluoremetry (FRRf) (2 mL) and microscopy (5 mL) analysis were collected in the middle and end of the experiment. For the details of each experiment see Tables 2.2 and 2.3.

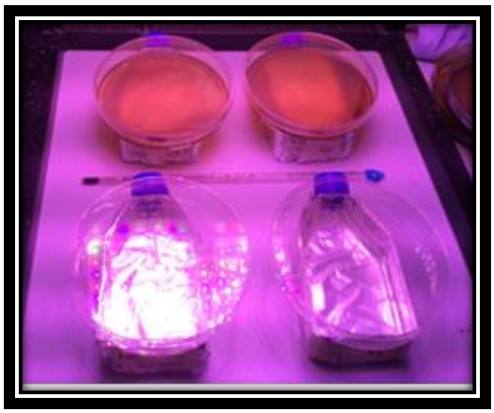

Figure 2.1 Design of experiments L1 to L4: cell culture flask containing seawater under the petri dish under an oil layer (top) and under petri dish under no-oil layer (below) using Heliospectra LED lights. All experiments were done separately, each with four cell culture flasks per experiment.

2.2.2 DNA extraction

DNA was extracted using the method described by Griffiths *et al*. (2000); 20 mL of seawater was centrifuged at 10,000 ×*g* for 20 min. The supernatant was discarded very carefully, leaving aproximately 1 mL of cells in seawater. The cell pellet was resuspendend in the seawater and added to a sterile microfuge tube, which was centrifuged at 11,300 ×*g* for 5 minutes and the supernatant was discarded. Then 0.6 mL of Cetyltrimethylammonium Bromide (CTAB) buffer (5% v/v CTAB in 120 mM sodium phosphate, pH 8) was added to the pellet, mixed and then added into a sterile bead beating tube containing 0.5 g of 0.1 mm zirconia/silica beads. Then, 0.5 ml of Phenol: Chloroform: Isoamyl-alcohol (25:24:1; pH 8) was added and the tube was mixed for 60s at 11,300 ×*g* in a Precellys Evolution homogenizer, Bertin Instruments. The tube was centrifuged at 11,300 ×*g* for 5 min and the upper aqueous layer was transferred to a sterile microfuge tube. Later, 1 ml of 30% PEG 6000 in 1.6M NaCl was added to the tube and inverted a few times. The tube was left to precipitate overnight at room temperature. The next day, the tubes were centrifuged (11,300

×*g* for 5 min) to obtain a DNA pellet. The supernatant was removed, and the pellet was washed in ice-cold 70% v/v ethanol, re-centrifuged and then ethanol was removed and the pellet air dried for 10-15 minutes. Finally, the pellet was resuspended in 50 µl of sterile MilliQ water and stored at -20°C.

2.2.3 Quantification of DNA

Double-stranded DNA (dsDNA) concentration was determined using the Quant-IT PicoGreen dsDNA Assay Kit (Thermo Fisher) and a NanoDrop 3300 Fluorospectrometer according to the manufacturer's protocol.

2.2.4 Composition of the bacterial community

Samples were analysed using PCR-DGGE (Denaturing gradient gel electrophoresis). After DNA extraction, the V3 region of the bacterial 16S rRNA gene and protein D1 of photosystem II reaction centre coded by *psbA* gene were amplified using primers shown in Table 2.1.

The amplification was carried out in a total volume of 25 µL containing 0.5 mM each primer, 1× REDtaq Ready Mix (Sigma-Aldrich, USA) and 1-2 µL of DNA template. The reactions were performed in a PCR Thermal Cycler (model 2720; Applied Biosystems). The PCR for the 16S rRNA gene was performed under the following conditions: initial denaturation at 95°C for 5 min, followed by 30 cycles of denaturation at 94°C for 30 s, annealing at 55°C for 30 s, and extension at 72°C for 30 s; a final extension at 72°C for 10 min (Muyzer *et al*., 1993). PCR conditions for *psbA* gene were: Initial denaturation at 92°C for 4 min, followed

by 35 cycles of denaturation at 92°C for 1 min, annealing at 55°C for 1 min and extension at 72°C for 1 min, a final extension at 72°C for 10 min (Zeidner *et al*., 2003).

Table 2.1 List of primers used for amplifying 16S rRNA and *psbA* genes.

Primer Name	Sequence (5'-3')	Specificity	Reference	Target Gene
341-F	CCTACGGGAGGCAGCAG	Bacteria	Muyzer *et al*. (1993).	16S rRNA
534-R	ATTACCGCGGCTGCTGG	Bacteria	Muyzer *et al*. (1993).	16S rRNA
58-VDIDGIREP-66-F	GTNGAYATHGAYGGNATHMGNGARCC	Eukaryotic algae and cyanobacteria	Zeidner *et al*. (2003).	*pbsA*
331-MHERNAHNFP-340-R	GGRAARTTRTGNGCRTTNCKYTCRTGCAT	Eukaryotic algae and cyanobacteria	Zeidner *et al*. (2003).	*psbA*

F (forward) and R (reverse) indicate the orientation of the primers. A GC-rich sequence (GC clamp) was attached to the 5' end of the primers 341F (5'-CGCCCGCCGCGCGCGGCGGGCGGGGCGGGGGCACGGGGGG-3') and 58-VDIDGIREP-66 (5'-CCGCCGCGCGGCGGGCGGGGCGGGGGCACGGGG-3').

DGGE was carried out using Bio-Rad DCode^{TM} universal mutation detection system, 8-12µL of PCR products of 16S rRNA gene was analyzed using 1× TAE (40 mM Tris base [pH 7.6], 20 mM acetic acid and 1 mM EDTA [pH 8]), in 8% acrylamide gel, with a denaturant gradient of 40% and 60% urea-formamide; the gel was run for 16h at 60°C and 60V (Muyzer *et al*., 1993). For PCR products of *psbA* gene DGGE was performed using 1× TAE in 6% acrylamide gel, with a denaturant gradient of 55%-45% urea-formamide; electrophoresis was performed at 60°C and 70V for 18h. Bands in both cases were visualized by silver nitrate stain.

The silver nitrate stain was carried out after the DGGE. Fixation was done by adding a solution of 100 mL of absolute ethanol + 5 mL of acetic acid dissolved in 1L of distilled water

for 30 min on a rotator at medium speed. After removing the fixation solution, the staining solution (0.2 g silver nitrate dissolved in 200 mL of distilled water) was added to the gel on a rotator at medium speed for 20 min. Then developing solution (3g of NaOH and 1.6 mL of formaldehyde dissolved in 200 mL of water) was added for 5-15 min until bands start appearing. Finally, the gel was washed by rocking in distilled water.

DGGE fingerprints were analysed based on the presence and absence of individual bands. The binary matrix was used to calculate a dissimilarity matrix using the Jaccard coefficient.

2.2.5 Microscopy analysis

A sample of 5 mL of seawater was taken at the end of the experiments and 0.2 mL of Lugo's iodine was added as a fixing agent. Lugol's iodine was prepared as follows: 100 g of KI and 50 g of iodine were dissolved in 900 mL of distilled water, then 100 mL of glacial acetic acid was added to the final solution and mixed again. The solution was stored in amber-glass bottle (Hasle, 1978). The samples were kept at 4°C in darkness until analysis. Later, photomicrographs of the seawater samples were taken using an incandescent tungsten-based light microscope.

2.2.6 Chlorophyll a extraction

Samples of 45 mL of seawater were centrifuged at 10,000 ×*g* for 40 min in a 50 mL centrifuged tube. The cells were collected on filters (47 mm diameter, 0.7 µm pore-size glass fibre filters (GF/F), Whatman) and stored in the freezer at -80°C until analysis. For chlorophyll

extraction, 3 ml of methanol (100%) was immediately added to the filters containing the cells and stored at -20°C for 24 h in darkness. Then methanol extracts were centrifuged at 10,000 ×*g* for 10 min and later the methanol was collected for fluorometric and pigment analysis (Ritchie, 2006).

2.2.7 Fluorometric chlorophyll analysis

After the chlorophyll extraction, 10 serially diluted standards were prepared using one of the sample extracts. Absorbance of the extract was measured at 632, 652, 696 and 750 nm in a spectrophotometer (Jenway 6300). Total chlorophyll *a* (µg/mL) was calculated using the following equation (Ritchie, 2006):

Chl *a* (µg/mL): $-2.0782\times A_{632-750} - 6.5079\times A_{652-750} + 16.2127\times A_{665-750} - 2.1372\times A_{696-750}$.

Each standard (2 mL) was measured in the Trilogy® Laboratory Fluorometer in raw fluorescence units (RFU) and a standard curve was produced. Then, the samples extract (2 mL) were measured in the fluorometer (RFU) and chlorophyll *a* concentration (µg/mL) was obtained using the standard curve and the linear equation: $y = mx + \mathrm{b}$.

2.2.8 Data analysis

Chlorophyll a, DNA concentrations and photochemical efficiency were analysed using two-tailed Student's t test in R studio 3.1.2. Significant difference: $P \leq 0.05$.

Table 2.2 Design of the continuous light experiments (L1-L4) for determining the effect of oil on light attenuation and on chlorophyll *a* concentration and microbial community composition.

EXPERIMENTAL OUTLINE				SEAWATER DETAILS			LIGHT DETAILS					OTHER EXPERIMENTAL VARIABLE	
Code	Date	Duration (days)	Number of samples	Collection Date	Source	Quantity used (mL)	Machine	Regime (hours)	Received Quantity (µmoles photons m^{-2} s^{-1})		Temp. (°C)	Oil type and %	Position of samples
									Oil	No-Oil			
L1[a]	9-04-15	4	3 + Oil 3 - Oil	8-04-15	Brightlingsea 51°48'17.9"N 1°01'04.5"E.	100	Heliospectra	24	c	600	20	WF 1%	Non-Random
L2[b]	10-06-15	5	3 + Oil 3 - Oil	8-06-15	Brightlingsea 51°48'17.9"N 1°01'04.5"E.	120	Heliospectra	24	c	500	20	Gyda 1%	Non-Random
L3[b]	24-08-15	6	4 + Oil 4 - Oil	21-08-15	Brightlingsea 51°48'17.9"N 1°01'04.5"E.	160	Heliospectra	24	40-50	600	20	Gyda 3%	Random every 8 h
L4[b]	24-10-15	6.5	4 + Oil 4 - Oil	22-10-15	St. Osyth 51°46'17.5"N 1°05'12.3"E.	160	IsoLight Technologica	24	40-50	600	20	Gyda 3%	Random every 8 h

[a] No nutrients added.

[b] Nutrients: 882 µM of $NaNO_3$ and 36.2 µM of NaH_2PO_4.

[c] No measurements of received light quantity were done.

* Experiment L4 was repeated three times with the same sweater sample.

Table 2.3 Details of the analyses performed on each continuous light experiment (L1-L4).

EXPERIMENTAL OUTLINE		ANALYSIS						
Code	Sampling day	DNA		PCR[ab]	DGGE	Chlorophyll *a*	Fast Repetition Rate	Microscopy
		Extraction	Quantification			Fluorescence	Fluorescence	
L1	4	✔	✘	✔[a]	✘	✘[c]	✘	✘
L2	5	✔	✘	✔[a]	✘	✘[c]	✘	✘
L3	3 and 6	✔	✔	✔[a]	✘	✔	✘	✘
L4	3 and 6.5	✔	✔	✔[ab]	✔	✔	✔	✔

[a] For the bacterial community, amplification of the variable V3 region of bacterial 16S rRNA gene was used.

[b] For the phototrophic community, amplification of photosystem II protein D1 coded by *psbA* gene was used.

[c] Absorbance instead of fluorescence was measured

2.3 Results

2.3.1 Effect of oil on transmitted light.

There is a change in both the quality (electromagnetic spectrum) and quantity of light after passing through oil. This was shown when the light source was natural sunlight (Appendix C) and with a different type of crude oil (Appendix D). Here, the focus is on using the Heliospectra LED lights L4a series (240 LEDs), which emits light in the visible spectrum with peaks at 400, 420, 450, 530, 630, 660 and 735 nm and Gyda crude oil. More light was attenuated by oil at short wavelengths (400-500 nm) and when the oil layer was thicker (0.07-0.08 mm). Also, there was negligible light attenuation by seawater without an oil layer (Figure 2.2), indicating that the seawater depth in these experiments has minimal effect on the attenuation of light. There is an inverse linear correlation between percent of transmittance and concentration of oil (Figures 2.3 and 2.4).

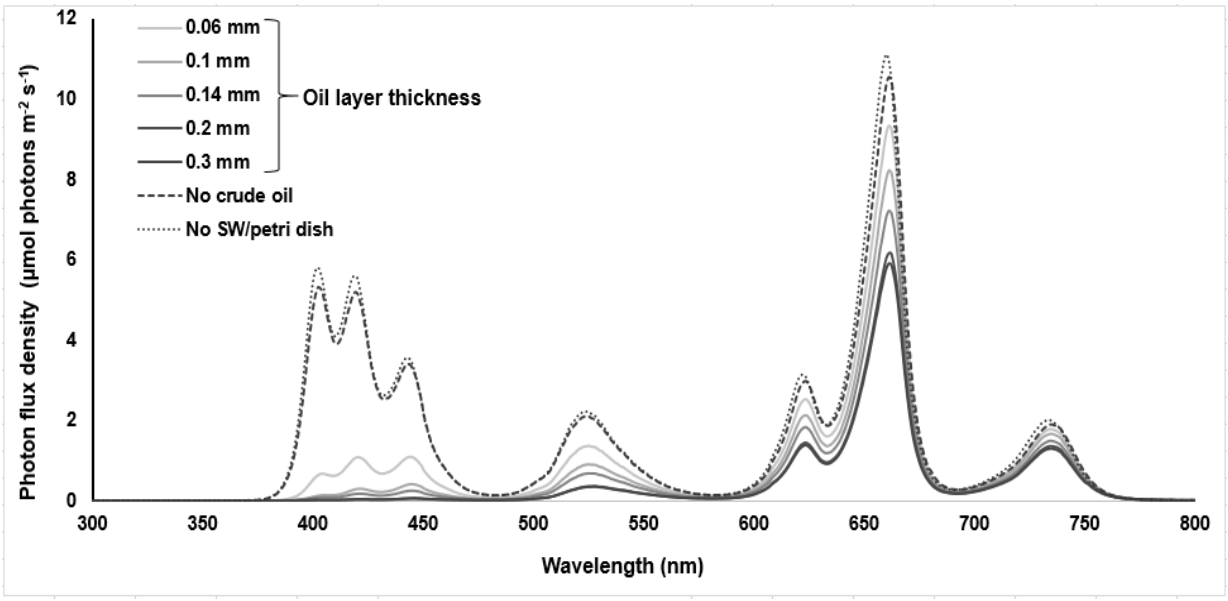

Figure 2.2: Spectrum of artificial LED lights (n=3), passing through samples containing natural filtered- seawater and different overlaying Gyda oil layer thicknesses (0.02, 0.03, 0.04, 0.07 and 0.08 mm). Control 1 (n=3), natural filtered seawater without an oil layer (dashed line). Control 2 (*n*=3), without seawater and without petri dish (dotted line).

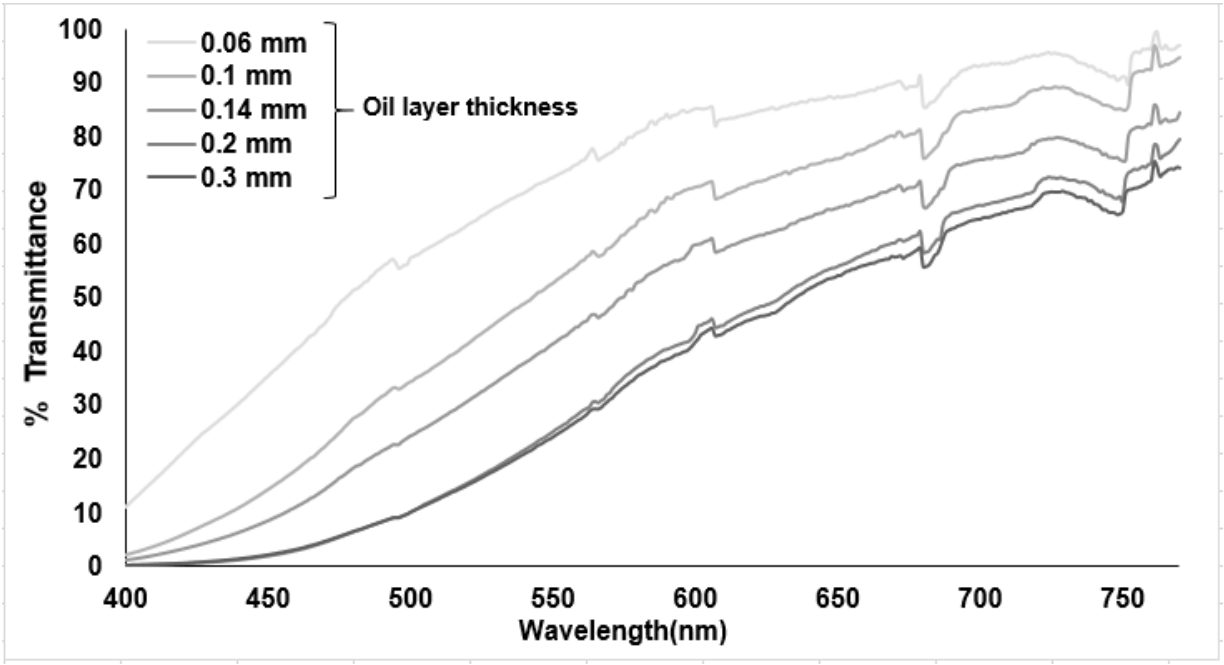

Figure 2.3: Percentage of light transmittance (mean; *n*=3) of samples: natural filtered seawater and different Gyda oil layer thicknesses (0.006, 0.1, 0.14, 0.2 and 0.3 mm).

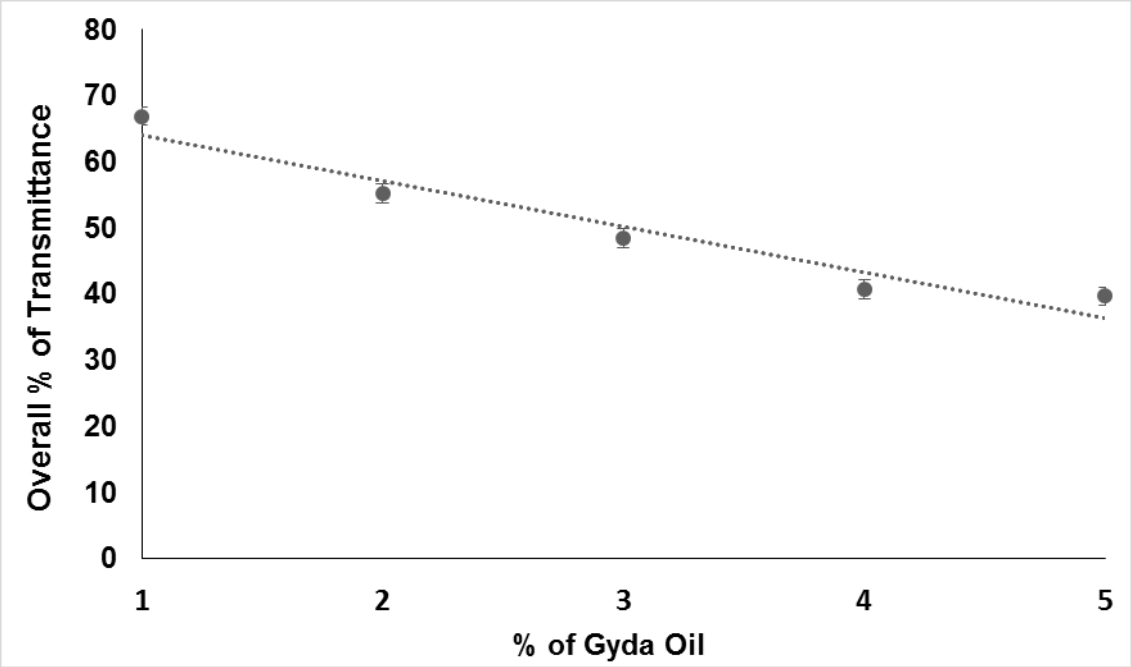

Figure 2.4: Overall percentage of light transmittance (mean ± SE; *n*=3), across the 400-750 nm spectrum for natural filtered seawater with 1% (0.06 mm), 2% (0.1 mm), 3% (0.14 mm), 4% (0.2 mm) and 5% (0.3 mm) of Gyda oil thicknesses. Regression: y=-6.88x +70.882. R^2=0.9363.

2.3.2 Effect of oil-induced light attenuation on the photosynthetic and bacterial community of seawater

Four experiments were performed (as explained in section 2.2.1.2). The differences between each experiment were a consequence of several factors: 1) availability of light sources (Heliospectra and Isolight Technologica); 2) ad-hoc observations (duration of experiment and quantity of light received); 3) improvement of the technique (with increased replication of samples and randomization of the positions under the artificial LED lights); 4) improvement in design of the experiments to test different treatments. The number and type of the analysis as well as the preservation of the samples for the subsequent analysis, also improved with each experiment. Brief experimental developments of L1 and L2 experiments are described below.

In experiment L1, the weathered Forties (WF) oil layer was floating on filtered-sterile seawater in a cell culture flask, but condensed water dropped on the oil and dispersed the oil monolayer. For this reason, the cell culture flask with seawater and oil was changed for 150 × 20 mm petri dishes, containing only oil that was spread evenly over the petri dish surface. Low levels of visual growth were observed for both treatments, seawater under an oil layer and seawater under no oil layer, consequently it was decided to add nutrients (882 µM of $NaNO_3$ and 36.2 µM of NaH_2PO_4) to the samples. Also, for the next experiments, it was decided to use Gyda crude oil because of the consistent results from the previous experiment (Section 2.3.1).

In experiment L2, now with added nutrients, it was observed that samples under an oil layer were coloured green and samples with no oil layer were coloured brown after five days of continuous light. The spectrophotometric method used first, was not able to detect the total concentration of chlorophyll *a*, therefore the quantity of methanol used for the extraction was changed from 10 mL to 3 mL and the spectrophotometric method (Ritchie, 2006) was replaced with fluorometric chlorophyll analysis, which has a lower detection limit.

Subsequent experiments, L3 to L4 were performed with 4 replicates and the position of the samples was changed randomly each day.

2.3.2.1 Microscopy

Selected illustrations of experiment L4 are shown in Figure 2.5. In this experiment there was a visible difference in the microbial growth. Treatments under an oil layer (Figure 2.5a) had round green colonies attached to the bottom of the cell culture flask while treatments without an oil layer (Figure 2.5b) had brown colonies floating in the cell culture flask. Microscopic observation showed a higher number and diversity of cells for treatments under an oil layer than those without an oil layer. The green colonies were formed of many cells (Figure 2.5c, d, g, h); and the dominant microorganism observed was a filamentous structure forming long branching chains of cells (Figure 2.5e, f). Also, different species of pennate diatoms were observed, as single cells (Figure 3j) or as part of green colonies (Figure 2.5h). On the other hand, samples without an oil layer only showed brown colonies (Figure 2.5k, l, m) and greater numbers of bacteria.

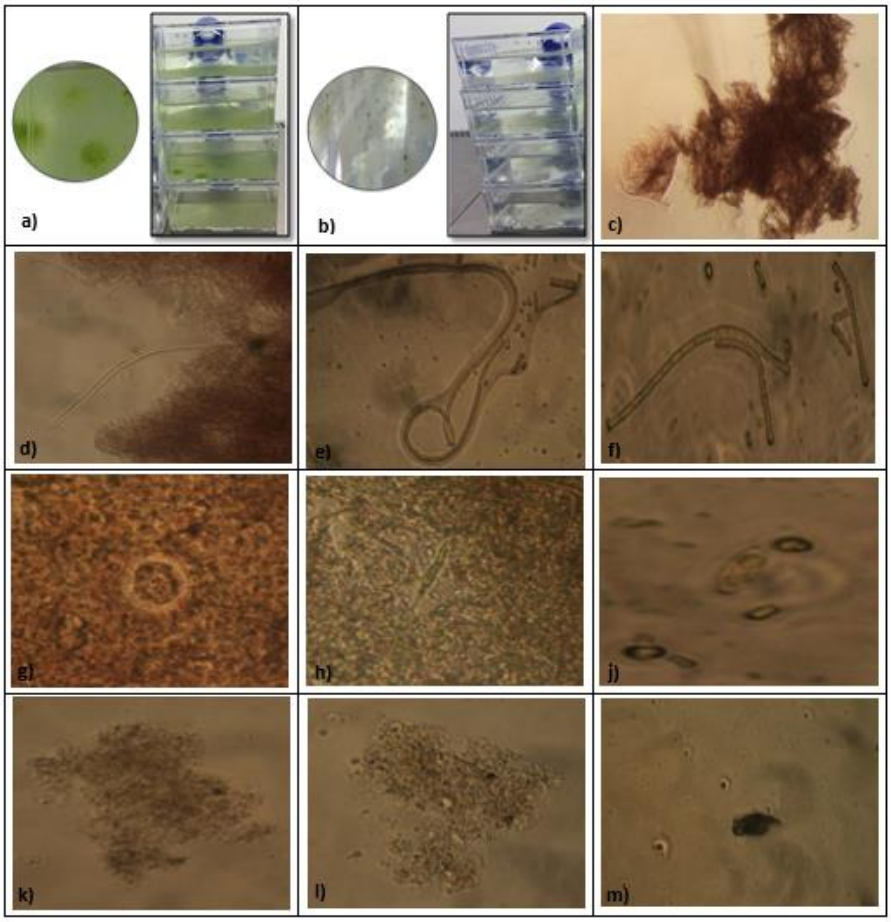

Figure 2.5. Photomicrographs of seawater samples from experiment L4 after 6.5 days of continuous light. Samples were fixed and stained using Lugol's iodine and observed using an incandescent tungsten-based light microscope. (a) Cell culture flasks with seawater after treatment under a Gyda oil layer (SW+OIL). (b) Cell culture flasks with seawater after treatment without an oil layer (SW). (c-j) photomicrographs from SW+OIL. (c) Green colony (×10 objective). (d) Side of the green colony (×40 objective). (d-f) Possible cyanobacteria (×100 objective). (g) Round shaped microorganism in the middle of a green colony (×100 objective). (h) Diatom in the middle of a green colony (×100 objective). (j) Diatom and Green fragments (×100 objective) (k-m) photomicrographs from seawater without an oil layer. (k-l) Brown colony (×40 objective). (m) Unknown microorganism (×100 objective).

2.3.2.2 Analysis of microbial populations

As explained in section 2.4.2, four experiments were performed, but DNA concentration and total chlorophyll *a* analysis were done only for experiments L3, L4.

In experiment L4, at day 3 there was no significant difference in the concentration of DNA (a measure of microbial biomass) between both treatments, under no-oil layer and under an oil layer. However, at day 6.5 the concentration of DNA was significantly and about seven-fold higher for samples under an oil layer (Figure 2.6 left). The concentration of chlorophyll *a* was also eight times higher in the samples under an oil layer at day 6.5 (Figure 2.6 right) and about three times higher at day 3.5. Thus, the change in light regime led to a large increase in phototroph biomass, especially after more time had elapsed.

For experiment L3 after 6 days of continuous light, only the concentration of chlorophyll *a* was measured; and it was observed that chlorophyll *a* was six times higher in treatments under an oil layer than under no-oil layer (Figure 2.6 right).

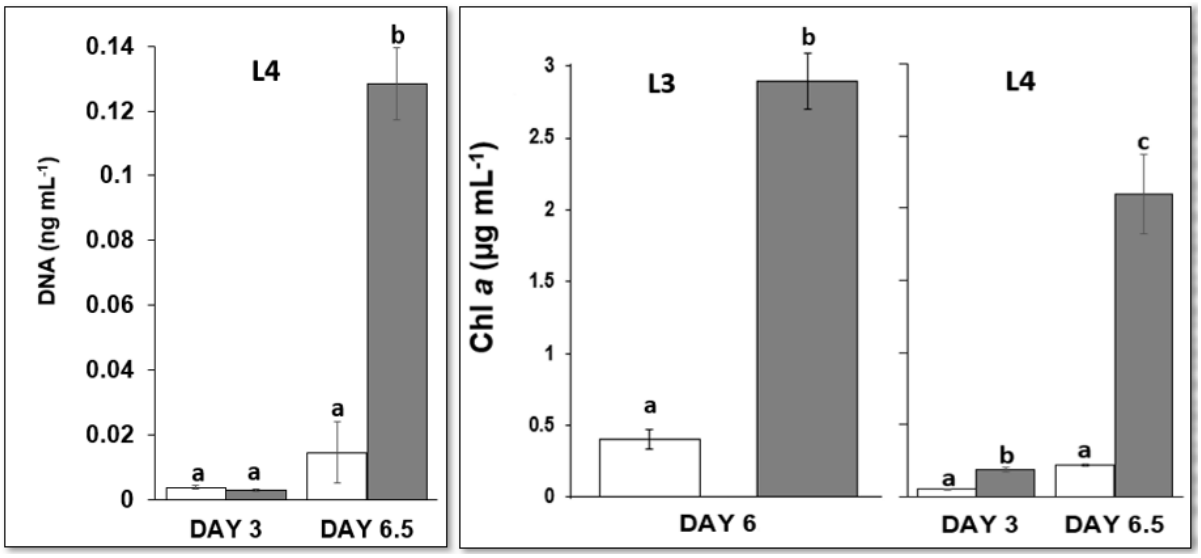

Figure 2.6. Left: DNA concentrations (ng / mL of seawater) on day 3 and 6.5 for L4 experiment. Right: Chlorophyll *a* concentration (µg mL^{-1}) for L3 and L4 experiments. All the experiments were done with continuous light (600 µm photons m^{-2} s^{-1}) between treatment under Gyda oil layer (grey bars) and under no-oil layer (white bars).

2.3.2.3 DGGE analysis

For experiment L4, hierarchical cluster analysis of 16S rRNA gene-based DGGE profile (Figure 2.7a) reveals that replicates of treatments were more similar to each other than between treatments. Bacterial communities were clearly influenced by both treatment and time, forming four distinct clusters, albeit deep, sub-clusters.

To obtain better results for the DGGE of *psbA gene*, different gradients (70-30%, 50-20%, 55-45%, 60-40%) and different percentage of polyacrylamide were used (6 and 8%), yet the results did not show many bands and the quality of the gel was not good.

The cluster analysis of the *psbA* gene (Figure 2.7b), reveals that samples under an oil layer and samples under no-oil are more similar during day 3 than during day 6.5, although two out of four samples under an oil layer at 6.5 day proved to be distinct.

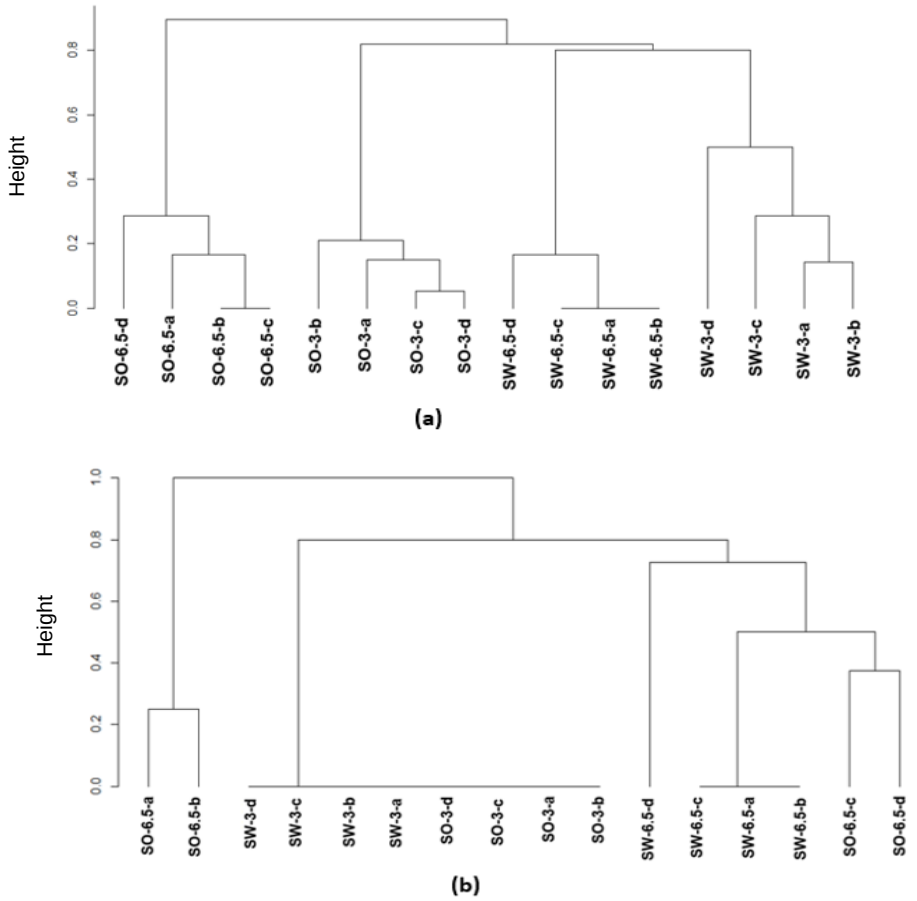

Figure 2.7. Hierarchical cluster analysis of DGGE profiles of (a) 16S rRNA and (b) *psbA* genes amplified from the continuous light experiment (L4). The analysis was based on a presence/absence matrix of bands and the Jaccard index using the complete linkage. Letters in the labels a, b, c and d indicate the replicates (n=4) of each sample, seawater without oil (SW) and seawater under an oil layer (SO). Numbers indicate the sampling day, either 3 or 6.5. The Y axis shows the distance between clusters.

2.4 Discussion

2.4.1 Effect of oil in light attenuation in seawater

Oil affected the spectral light quality (Figure 2.2 and 2.3), absorbing most of the violet and blue light, meaning that the attenuation of light is higher at shorter wavelengths (400-500 nm). Similarly, Otremba (1994), measured the reflection and transmission of light trough different oil layer thicknesses and also observed that light absorption in seawater, when an oil layer is present, depends on the wavelength, with more

absorption at short wavelengths. It is important to mention that for our experiment we do not know how much light was reflected and how much was actually absorbed. The optical absorption of the whole oil for the short wavelength range is largely due to the chromophores, a molecule that absorbs light at a specific wavelength (e.g. imine and nitro functional groups) (Downare and Mullins, 1995). All the oils possess chromophores but in very different concentrations (Ralston *et al*., 1996). For crude oils, the wavelength range of increasing absorption is much broader than the absorption bands of individual molecules and is due to the overlapping spectra of many molecular components. The spectral location of the absorption varies considerably and almost continuously for the different crude oils. The darkest crude oils, which are tar-like, show an absorption in the near-infrared; the lightest oil, barely absorbs any light in the visible region (Mullins, 2013). Additionally, polycyclic aromatic hydrocarbons can have an absorption spectrum from the range of 200-500 nm depending on the compounds present (Dabestani and Ivanov, 1999), which could explain the higher attenuation of light by an overlaying crude oil at short wavelengths (400-500 nm).

Two different crude oils were used, Forties that had been weathered in the laboratory by distillation at 230 °C by Coulon *et al*. (2007), thus losing its volatile compounds and Gyda, which was unweathered (Table 2.4). The exact composition of the Gyda oil was not avaible. The attenuation of light by Gyda (Figure 2.2) and weathered Forties crude oil (Appendix D) coincide with other types of oil, for example, Plata *et al*. (2008) studied the light-attenuating properties of oil extracted from rocks covered with oil films and observed that the amount of light in the oil film is significantly reduced (two orders of

magnitude) by No. 6 oil and that the light attenuation gradually decreases with increasing wavelength.

Table 2.4. Characteristics of the weathered Forties and Gyda crude oil.

Weathered Forties	Gyda
North Sea crude oil Light No volatile compounds (benzene, toluene, ethylbenzene, and xylene) Density: 0.84 g/mL API= 38.7°	North Sea Crude oil Light Unweathered Rich in asphaltenes Density: 0.88 g/mL API= 38.9°

Unsurprisingly, the attenuation of light depends on thickness of the oil layer, with higher concentrations of oil, more attenuation of light was observed; an observation that is consistent with the results found by Plata *et al*. (2008) and Otremba (1994).

2.4.2 Microbial communities are affected by oil-attenuation of light

The results in this study showed that photosynthetic and bacterial communities in seawater were affected by the oil layer after the exposure to continuous light (L3 and L4).

The DNA concentration was significantly higher in samples with an oil layer and the total concentration of chlorophyll *a* was also eight times higher for L4 experiment under an oil layer compared to the one under no-oil layer. The experiment L3 supports the high concentration of chlorophyll *a* data under an oil layer. This suggest that the high light intensity inhibited the cell growth and the production of chlorophyll. High light

intensity causes photoinhibition, in which the reaction centres of the photosystem II are damaged (Tyystjarvi, 2008). In response to a high irradiance, phytoplankton can change the quantity of its photosynthetic pigments to avoid photo-damaged and death of the cells (Fischer *et al*., 1996).

The results of microscopy, DNA and chlorophyll *a* concentration and the difference in communities between treatments, although partially supported by the DGGE of *psbA* gene, lead to speculation about the nature of microorganisms in the samples; and to a possible explanation about the differences between under an oil layer and under no-oil layer treatment. The major attenuation of light with an oil layer was at shorter wavelengths (400-550 nm), but not at longer wavelengths (640-680 nm) (Figure 2.2) and it is also known that the primary light harvesting pigment for most oxygenic photosynthetic microorganisms is chlorophyll *a*, which has a major peak of absorption at 430 nm, suggesting that in the presence of oil, the process of photosynthesis could be affected; however phototrophs can also trap light at different wavelengths depending on the phytoplankton species and the type and quantity of accessory pigments as well as their ratio to chlorophyll *a* (Fischer *et al*., 1996). The existence of different forms of chlorophyll and accessory pigments allows phototrophs to make better use of the available energy in PAR spectrum (Bjorn *et al.*, 2009). The absorption of light for some pigments it is mentioned in table 1.2. In the case of L4, the photosynthetic microorganisms found in the seawater under an oil layer could be using some of the accessory pigments, giving them an advantage over the ones which cannot absorbs light between 640-680 nm; for example, green algae also have a peak of light absorption for chlorophyll *b* at 650 nm (Jeffrey *et al*., 1997); and cyanobacteria have allophycocyanin with a peak of absorption at 650 nm (Govindjee and Sheveka,

2011) which could favour their growth under the samples with an oil layer over the ones which do not have these pigments.

Samples from the experiment L4 were also analysed by Miseq Illumina sequencing described in Chapter 3. However, only a few replicates gave usable data, thus the results can only be considered as preliminary. Nevertheless, cyanobacteria constituted the 10-30 % of the oil-overlain community but less than 1% of the no-oil control community, while the diatoms under no-oil constituted about 15-24% and 3-13% under an oil layer. The data of the community analysis (albeit not properly replicated) support the microscopy findings. Kulk *et al*. (2011) found that eukaryotic species, such as diatoms performed better under high irradiance than prokaryotic species, such as cyanobacteria. And, they also observed that two different strains of *Synechococcus* sp. have a maximum growth rate during treatments with low light intensity (25 and 50 µmol m^{-2} s^{-1}). Ratchford and Fallowfield (2003), found that the onset of photoinhibition in *Synechococcus* occurred at irradiances >300 µmol photons m^{-2} s^{-1}; this could explain the abundance of cyanobacteria under an oil layer compared with no-oil control. On the other hand, one of the mechanisms that allows the diatoms to grow under a high irradiance is the non-photochemical quenching (NPQ) which dissipates the excess of energy and the use of photoprotective pigments with the xanthophyll cycle activity (Dimier *et al*., 2009).

2.5 Conclusion

Most studies, to my knowledge, on the impact of oil on light have focused on development of tools to detect oil spills at sea. This is the first study in which the changes to light caused by an oil layer have been investigated from the view of the impact on marine microbes, specifically keeping the oil separate from seawater to avoid the complicating effect that would be caused by hydrocarbons serving as a source of carbon and energy for some microbes and acting as a toxin for others.

The results demonstrated that in the presence of oil, both the intensity of transmitted light and its spectral light quality are affected. The oil absorbs most of the blue light (short wavelengths) but has almost no effect for the red light (longer wavelengths). As a consequence of these factors, the type and quantity of microorganisms found in the samples was also affected. Cyanobacteria was the most abundant microorganism under an oil layer and diatoms under no-oil control.

However, this chapter involved a lot of method development, and it could be argued that continuous light at the intensity used is not typical for most seas and certainly not for the temperate coastal waters used in this study. Continuous light does not provide an opportunity for the repair of light-induced damage to the photosystem II (Aro *et al*., 1993; Moore *et al*., 2006; Kulk *et al*., 2011). Therefore, the next chapter outlines the effects of oil-induced light attenuation on marine microbial communities using light and dark cycles and provides more detailed data on the effects on the phototrophic and overall bacterial communities.

CHAPTER 3

Effects of crude oil overlying seawater (but not in contact with) seawater on light attenuation and marine microbes, using a light-dark regime.

3.1 Introduction

The last chapter focussed on continuous light experiments and showed that there are changes in the intensity and spectrum of light when passing through an oil layer. Crude oil preferentially attenuates the shorter wavelengths of photosynthetically active radiation. This revealed that under an oil layer the quantity of microorganisms and chlorophyll *a* increased at the end of the experiment. Also, the most abundant microorganism found in samples under an oil layer was cyanobacteria (10-30%) while in the no-oil controls cyanobacteria account for less than 1%.

In this chapter, it was decided to use a light: dark (12:12 h) regime to be close to natural conditions. Having a light: dark cycle is important because physiological functions (e.g. photosynthesis and respiration) can be affected by circadian cycles (Jakobsen and Strom, 2004). Photoacclimation and photoregulation also allow microorganisms to increase photosynthesis (Dimier *et al*., 2009). In addition, it is known that photosynthesis is sensitive to light, leading to photoinhibition, thus continuous repair is necessary to keep it functional (Tyystjarvi, 2008). Moreover, light: dark cycles can stimulate different species, which in turn changes the microbial phototrophic community composition (Lichtman 2000) and as a consequence of this the heterotrophic community composition would be altered.

In order to distinguish the effects between the intensity and spectrum of light, artificial lights were used to mimic the spectrum of the oil while maintaining the same light intensity. Also, to have a photosynthetically active radiation (PAR) close to real conditions, in which the intensity of light fluctuates during the day, it was decided to use a ramped light. The amount of irradiance was determined from the study of Pankaew *et al*. (2014) on the variation of PAR through different seasons in the UK. Currently, there are no studies using the same intensity of light but a different spectrum and also using a ramped light.

Changes in the phototrophic community can alter the heterotrophic community in seawater. Phototroph-heterotroph interactions are very important for the degradation of oil. Many algae can produce hydrocarbons, which support hydrocarbon-degrading bacteria in the absence of an oil spill (McGenity *et al*., 2012; Leah-Smith *et al*., 2015). Some cyanobacteria can also fix nitrogen and along with eukaryote microalgae, supply organic matter (e.g. excreted polysaccharides), and oxygen to heterotrophic bacteria via photosynthesis and thus maintain synergistic communities as in microbial mats (Rosenbaum *et al.*, 2010). Oxygen liberated by photosynthesis is likely to be very important in both activating hydrocarbons (e.g. via oxygenase enzymes), as well as serving as an electron acceptor in aerobic respiration. In turn, the locally increased concentration of CO_2 produced by the heterotrophs, will generally allow enhanced photosynthesis (McGenity *et al.*, 2012). In the marine environment hydrocarbons degradation is carried out by obligate hydrocarbonoclastic bacteria (OHCB) (Yakimov *et al*., 2007).

An understanding of the impacts of oil on indigenous microbial communities and identification of oil degrading microbial groups are prerequisite for directing the management and clean-up of oil in contaminated environments (Kostka *et al*., 2011). A rise in marine microbial community studies over the past decade has led to a new information on the dynamics between microorganisms and their surrounding environments (Ul-Hasan *et al*., 2018). Also, the arrival of high throughput sequencing methods has allowed the investigation of the community composition in greater depth (Mukherjee *et al*., 2017).

The overall aim is to investigate the changes within bacterial community compositions in seawater when using a light: dark cycles with an overlaying crude oil and mimicking the effect of overlaying crude oil with artificial LED lights.

3.1.1 Hypotheses

3.1.1.1 Light: dark experiments will have a higher bacterial diversity than the continuous-light experiments, because the cycles are more similar to natural conditions and thus less stressful to the microbiota.

3.1.1.2 The light spectrum mimicking an oil layer will produce more changes in the bacterial community composition than a standard light spectrum.

3.1.2 Objectives

3.1.1.3 To determine changes in heterotrophic and phototrophic microbial composition in seawater by overlaying with crude oil or mimicking the effect of overlaying oil on the spectrum of light using Heliospectra artificial LED lights.

3.2 Methodology

3.2.1 Design of Experiments

3.2.1.1 Testing the effect of oil-induced light attenuation on the microbial community in sea water.

Light: dark (LD) experiments were performed, called L:D1 to L:D5 (Table 3.1). For each experiment four replicates were performed, and the position of the samples was changed randomly over time. All the experiments were prepared with 160 mL of natural seawater plus nutrients (882 µM of $NaNO_3$ and 36.2 µM of NaH_2PO_4) in a cell-culture flasks (Noc™ Easy Flask™ 75 cm^2, Nunclon™ Delta Surface, Thermo Scientific), except for L:D2 where no nutrients were added. The sides of cell culture flask were covered with aluminium foil.

Experiments L:D1 and L:D2 (Figure 3.1a) were prepared using an overlay of oil as in the continuous light experiments (see 2.3.1.2). For experiments L:D3, L:D4 and L:D5 (Figure 3.1 b, c), no oil was used, instead the spectrum of the oil was mimicked using the Heliospectra LED lights. The spectrum of the oil was obtained from previous experiments testing the effect of crude oil on transmitted light (Figure 2.2). For controls, a standard light spectrum was used; and the intensity of light was kept the same in both treatments, so the only variable was the spectrum of light. Finally for experiment L:D5 a ramped light intensity was used (Table 3.1).

Seawater samples for DNA (30 mL), Chlorophyll *a* analysis (45 mL), fast repetition rate fluorometry (FRRf) (2 mL) and microscopy (5 mL) analysis were collected at the

middle and end of the experiments. For details of each experiment see Tables 3.1 and 3.2.

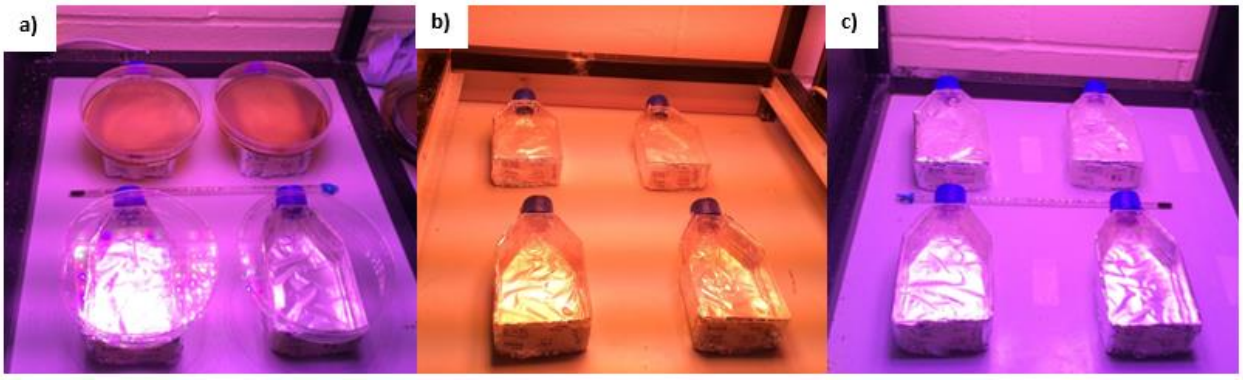

Figure 3.1. Design of experiments: (a) L:D1 and L:D2, cell-culture flask with seawater under the petri dish with an oil layer (top) and under a petri dish without an oil layer (below) using Heliospectra LED lights. (b) L:D3, L:D4 and L:D5, cell-culture flask with seawater under a light spectrum that mimics overlying oil using Heliospectra LED lights. (c) L:D3, L:D4, L:D5, cell-culture flask with seawater under a control standard-light spectrum using Heliospectra LED lights. All experiments were done separately, in quadruplicate.

3.2.2 DNA extraction (see section 2.2.2)

3.2.3 Quantification of DNA (see section 2.2.3)

3.2.4 Composition of the bacterial community (see section 2.2.4)

3.2.5 Microscopy analysis of L:D1 (see section 2.2.5)

3.2.6 Chlorophyll *a* extraction (see section 2.2.6)

3.2.7 Fluorometric chlorophyll analysis (see section 2.2.7)

3.2.8 Fast Repetition Rate (FRR) Fluorometry

The maximum photochemical efficiency (*Fv/Fm*) evaluates the efficiency by which absorbed light is utilized by photosynthesis in the Photosystem II complex (PSII) (Oxborough and Baker, 1997). A sample of 2 mL of seawater at 2.5 and 5 days was transferred to a glass 15mL centrifuge tube after 30 min of a dark-adaptation period in order to reach the maximum photochemical efficiency of photosynthetic microorganisms in the seawater. The tube was then introduced to the fluorometer where seawater was subjected to a series of controlled light pulses every four seconds, subsequently measuring the induced fluorescence. The analyses were carried out using FAST^{pro} v2.5.3 software and FAST^{act} fluorometer (Chelsea Technologies, UK) (Suggett et al., 2009).

3.2.9 Analysis of 16S rRNA gene using Miseq Illumina Sequencing

All the DNA extractions from the experiments L:D1, L:D2, L:D3 and L:D4 were used to amplify the variable V3 and V4 region of the 16SrRNA with the following primers:
Forward primer

5’TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCTACGGGNGGCWGCAG 3’

and reverse primer

5'GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGACTACHVGGGTATCTAATCC.

The PCR was done in a 96-well PCR plate. Each reaction of 25 µL contained 2.5 µL of DNA, 5 µL of each primer and 12.5 µL of REDtaq Ready Mix (Sigma-Aldrich, USA). The reactions were performed in Applied Biosystems® Veriti ® 96-well Thermal Cycler. The PCR was performed using the following conditions: 95°C for 3 min, 28

cycles of 95°C for 30 s, 55°C for 30 s, 72°C for 30 s and finally 72°C for 5 min. Then, the amplicons were cleaned using Agencourt® AMpure® XP (Beckman Coulter) magnetic bead-based purification. After that, the amplicons were visualized using a 1% agarose gel electrophoresis. The second PCR reaction, pooling and quantification were done by Benjamin H. Gregson and Boyd A. Mckew. The second PCR of 50µL was done using Nextera Xt Index Kit (Ilumina Nextera XT indexing primers, Illumina, Sweden). PCR reactions contained 5 µL of Nextera XT Index Primer 1 (N7xx) and 5µL of Nextera XT index Primer 2 (S5xx) and 25 µL of 2X KapaHiFi Hot start ready mix. The conditions of the PCR were: 95°C for 3 min, 8 cycles of 95°C for 30 seconds, 55°C for 30 seconds, 72°C for 30 seconds and 72°C for 5 minutes. PCR products were cleaned and again visualised using an electrophoresis gel and cleaned as described before. Samples were quantified using the Quant-IT PicoGreen dsDNA Assay Kit (Thermo Fisher). After, samples were sequenced following standard Illumina Miseq sequencing protocols.

3.2.10 Data analysis

Chlorophyll and DNA concentrations and photochemical efficiency were analysed using two-tailed student's t test in R studio 3.1.2. Significant difference: $P \leq 0.05$.

3.2.10.1 Bioinformatics

Bioinformatic analysis was done by Boyd A. Mckew using BioLinux operating system (Clark *et al.*, 2017; Dumbrell *et al.*, 2016). The forward and reverse sequences were pair-end aligned. The sequences were trimmed using Sickle version 1.33 (Joshi and Fass, 2011) at a threshold of Q20 and the ambiguous nucleotides were discarded.

Then, using the SPAdes version 3.10.1, with default parameters (Bankevich *et al*., 2012) the sequences were error corrected utilising the BayesHammer algorithm (Nikolenko *et al*., 2013). After, PANDAseq (Masella *et al*., 1993) was used to assemble paired-ends reads into single contigs. To cluster sequences into operational taxonomic units (OTUs) VSEARCH (Rognes *et al*., 2016) was used. First, sequences were de-replicated and singleton sequences discarded. Second, the sequences were clustered into OTUs at 97% sequences of similarity. Then, the chimeras were removed using UCHIME *de novo* (Edgar *et al*., 2011) and reference pipelines from VSEARCH. Lastly, taxonomy was assigned to OTUs using RDP classifier with a minimum confidence threshold of 0.7 (Wang *et al*., 2007).

The comparison of relative abundance of the taxa between treatments of metagenetic data was done with STAMP (Parks et al., 2014) bioinformatics software, using default parameters, two-sided Welch's t-test, except for filtering parameters when P value: P>0.05, the difference between proportions:<0.2 and/or difference between ratios: <1.5. All the data were sorted using the effect size.

Table 3.1 Design of the light: dark experiments (L:D1-L:D5) for determining the effect of oil on light attenuation and on chlorophyll *a* concentration and microbial community composition.

EXPERIMENTAL OUTLINE				SEAWATER DETAILS			LIGHT DETAILS					OTHER EXPERIMENTAL VARIABLE		
Code	Date	Duration (days)	Number of samples	Collection Date	Source	Quantity used (mL)	Machine	Regime (hours)	Received Quantity (µmoles photons m^{-2} s^{-1}) Oil	Received Quantity (µmoles photons m^{-2} s^{-1}) No-Oil	Temp. (°C)	Oil type and %	Position of samples
L:D1[a]	4-11-15	5	4 + Oil 4 - Oil	3-11-15	Brightlingsea 51°48'17.9"N 1°01'04.5"E.	160	IsoLight Technologica	12:12	40-50	600	Bench 21-24	Gyda 3%	Random every 12 h
L:D2[b]	12-04-16	5	4 + Oil 4 - Oil	12-04-16	Brightlingsea 51°48'17.9"N 1°01'04.5"E.	160	Heliospectra	12:12	40-50	600	20	Gyda 3%	Random every 12 h
L:D3[a]	13-04-16	5	4 Oil Spectrum 4 Natural Spectrum	12-04-16	Brightlingsea 51°48'17.9"N 1°01'04.5"E.	160	Heliospectra	12:12	600	600	20	-	Random every 12 h
L:D4[a]	26-04-16	5	4 Oil Spectrum 4 Natural Spectrum	26-04-16	Brightlingsea 51°48'17.9"N 1°01'04.5"E.	160	Heliospectra	12:12	400	400	20	-	Random every 12 h
L:D5[a]	27-04-16	5	4 Oil Spectrum 4 Natural Spectrum	26-04-16	Brightlingsea 51°48'17.9"N 1°01'04.5"E.	160	Heliospectra	12:12	7-10h: 400 10-12h: 500 12-14h: 850 14-16h: 600 16-19h:450 19-7h: 0	7-10h: 400 10-12h: 500 12-14h: 850 14-16h: 600 16-19h: 450 19-7h: 0	20 20 25 23 22 20	-	Random every 12 h

[a] Nutrients: 882 µM of $NaNO_3$ and 36.2 µM of NaH_2PO_4.
[b] No nutrients added.

Table 3.2 Details of the analyses performed on each light: dark experiments (L:D1-L:D5).

EXPERIMENTAL OUTLINE		ANALYSIS							
Code	Sampling day	DNA		PCR[ab]	DGGE	Chlorophyll *a* Fluorescence	Fast Repetition Rate Fluorescence	Microscopy	Illumina Miseq
		Extraction	Quantification						
L:D1	2.5 and 5	✔	✔	✔	✔	✔	✔	✔	✔[c]
L:D2	2.5 and 5	✔	✔	✔	✔	✔	✔	✘	✔[c]
L:D3	2.5 and 5	✔	✔	✔	✔	✔	✔	✘	✔
L:D4	2.5 and 5	✔	✔	✔	✔	✔	✔	✘	✔
L:D5	2.5 and 5	✔	✔	✔	✔	✔	✔	✘	✔

[a] For the bacterial community, amplification of the variable V3 region of bacterial 16S rRNA gene was used.
[b] For phototrophic community, amplification of photosystem II protein D1 coded by *psbA* gene was used.
[c] Results not shown.

3.3 Results

3.3.1 Effect of oil-induced light attenuation on the photosynthetic and overall bacterial community of seawater.

3.3.1.1 Microscopy

Selected illustrations of the effects of an oil-layer filtering light are shown in Figure 3.2. In experiment L:D1, treatment under an oil layer (Figure 3.2 b) and treatment without an oil layer (Figure 3.2 a), both have brown colonies floating in seawater; those with an oil layer, have a greater number of large cell aggregates (Figure 3.2 c, d, e) than those without an oil layer (Figure 3.2 k, l , m). Also, with an oil layer, pennate diatoms were observed, and they were mostly, single cells (Figure 3.2 f, g, h).

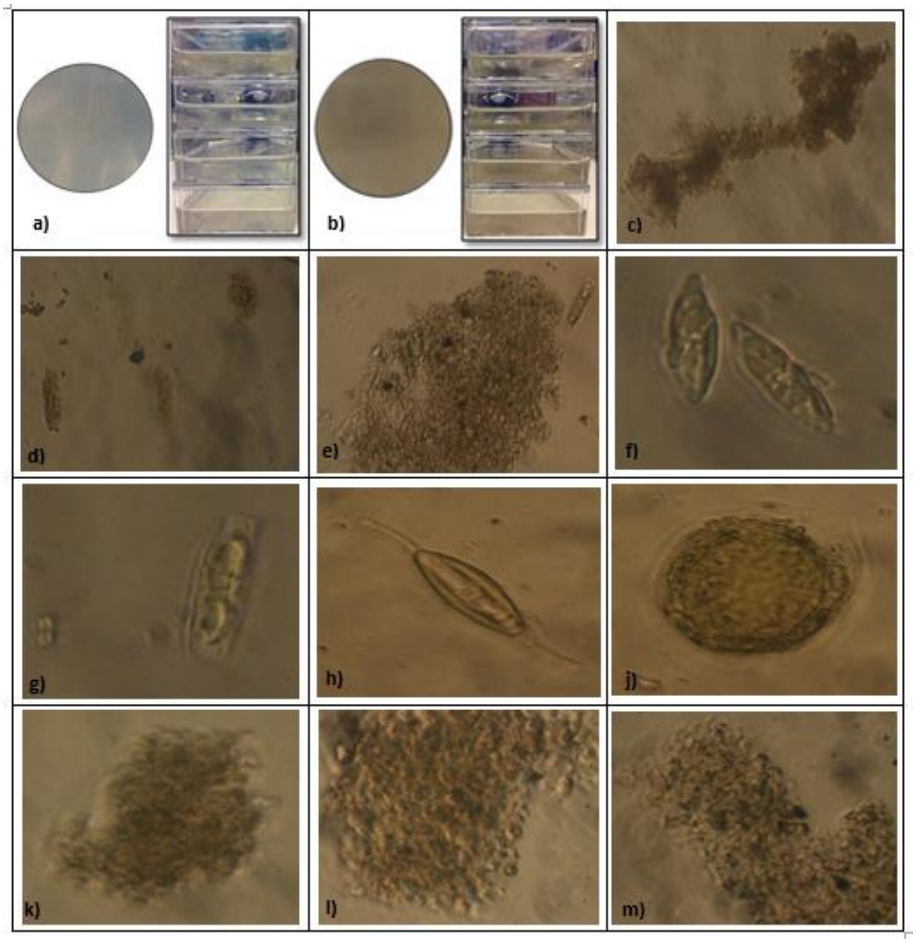

Figure 3.2 Photomicrographs of seawater samples from experiment L:D1 after 5 days of light: dark treatment. Samples were fixed and stained using Lugol's iodine and observed using an incandescent tungsten-based light microscope. (a) cell-culture flasks with seawater after treatment without an oil layer (SW). (b) cell-culture flasks with seawater after treatment under a Gyda oil layer (SW+OIL). (c-j) photomicrographs from SW+OIL. (c, d) brown colonies (×10 objective). (e) side of the brown colony and diatom (×40 objective). (f, g, h) Pennate diatoms (×100 objective). (j) cylindrical microorganism (×100 objective). (k, l, m) photomicrographs from seawater without an oil layer. (k) brown colony (×40 objective). (l, m) brown colonies (×100 objective).

3.3.1.2 Analysis of microbial populations.

In the four experiments DNA was measured as a proxy for microbial biomass, there was a notable increase in concentration with all the treatments from day 2.5 to day 5 (Figure 3.3). In all cases microbial biomass increased from day 2.5 to day 5 within the same

treatment. In contrast to the continuous-light experiment in Chapter 2 (Figure 2.6), where the oil layer resulted in higher microbial biomass at day 6. A very different trend was observed in the light: dark experiments, with either more biomass in the absence of an overlaying oil layer or with a standard-light spectrum (experiments L:D1 and L:D4), the same biomass (L:D2 and L:D3), or more biomass at 2.5 days and the same at 5 days (L:D5) (Figure 3.3).

In the experiments where the light intensity was constant between treatments, but the light spectrum differed (L:D3, L:D4 and L:D5), the concentration of chlorophyll *a* was the same between treatments at day 5 (Figure 3.4c, d), except for L:D3 in which a significant increment was seen at day 5. Similarly, in L:D2 there was a small but significantly higher chlorophyll *a* concentration with the oil spectrum treatment at both days 2.5 and 5 (Figure 3.4b). Experiment L:D1 was unusual in that the oil layer led to a decrease in chlorophyll *a* concentration at day 2.5 but a large increase in chlorophyll *a* at day 5 relative to the no-oil control (Figure 3.4a). Thus, overall, the more natural the light regime and the more similar the light intensity, the impact on chlorophyll *a* concentration was observed.

For the photochemical efficiency (*Fv/Fm*) there was a little difference between treatments at day 5. The only exception was day 5 for the L:D1 treatment where there was a 43.5% lower value in the absence compared with the presence of an oil layer. (Figure 3.4).

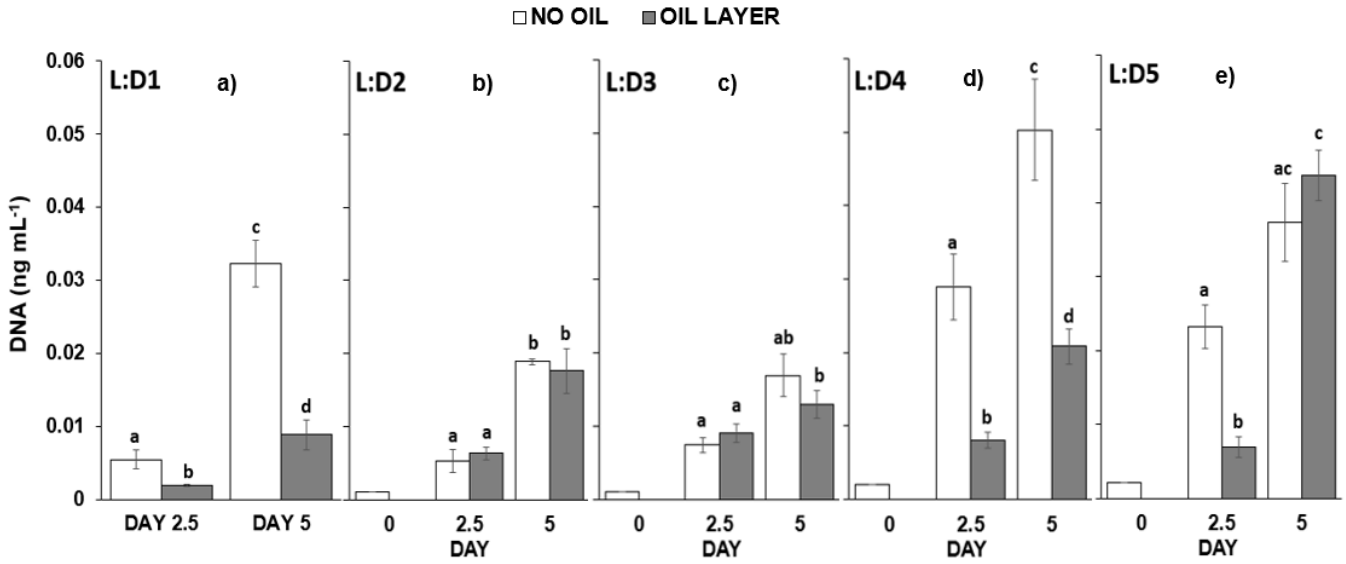

Figure 3.3 DNA concentrations (ng / mL of seawater) for light: dark experiments (L:D) on day zero, day 2.5 and day 5 between treatment under Gyda oil layer and under no-oil layer: (a) L:D1 and (b) L:D2 (without nutrients); between standard-light spectrum and oil spectrum: (c) L:D3, (d) L:D4 and (e) L:D5 (see table 3.1 for details of each experiment). Different letters (a, b, c and d) mark significant differences (Student's unpaired t-test, $p<0.05$) between treatments (under oil layer and no-oil layer) and between days (day 2.5 and day 5; Student's paired t-test, $p<0.05$), mean +/- SE (*n*=4).

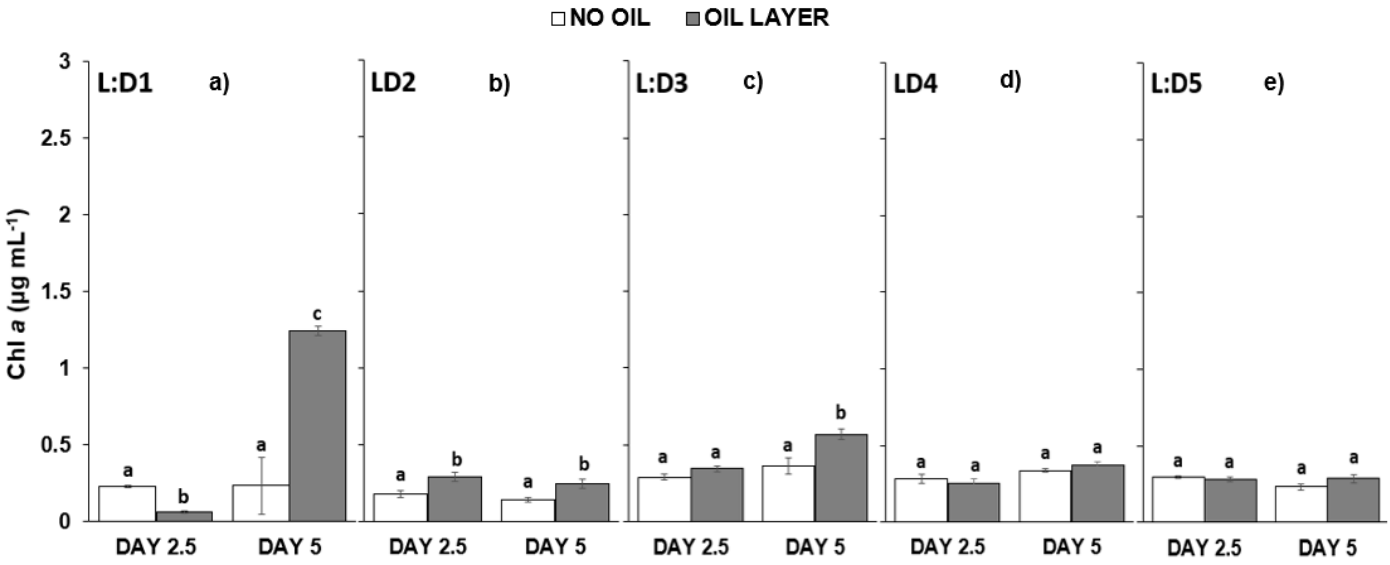

Figure 3.4 Chlorophyll *a* concentrations (µg mL^{-1}), for light: dark experiments (L:D) on day zero, day 2.5 and day 5 between treatment under Gyda oil layer and under no-oil layer: (a) L:D1 and (b) L:D2 (without nutrients); between standard-light spectrum and oil spectrum: (c) L:D3, (d) L:D4 and (e) L:D5 (see table 3.1 for details of each experiment). Different letters (a, b, c and d) mark significant differences (Student's unpaired t-test, $p<0.05$) between treatments (under oil layer and no-oil layer) and between days (day 2.5 and day 5; Student's paired t-test, $p<0.05$), mean +/- SE (*n*=4).

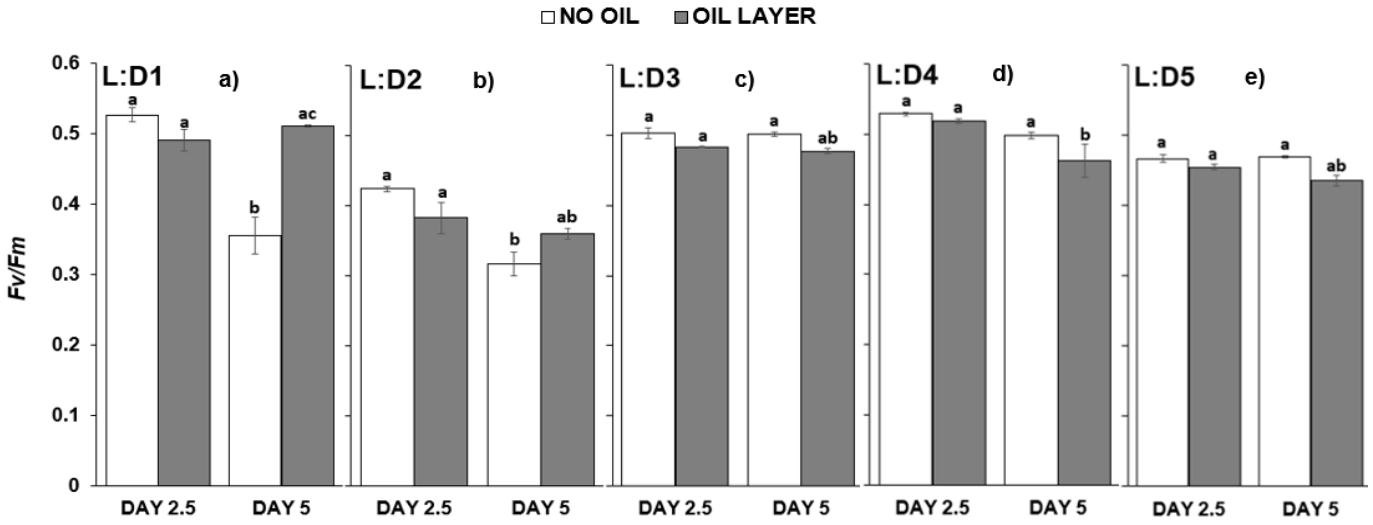

Figure 3.5 Yield values of PSII photochemical efficiency (*Fv/Fm*) on day 2.5 and day 5, after light: dark regime (L:D) between treatment under Gyda oil layer and under no-oil layer: (a) L:D1 and (b) L:D2 (without nutrients); between standard-light spectrum and oil spectrum: (c) L:D3, (d) L:D4 and (e) L:D5 (see table 3.1 for details of each experiment). Different letters (a, b, c and d) mark significant differences (Student's unpaired t-test, $p<0.05$) between treatments (under oil layer and no-oil layer) and between days (day 2.5 and day 5; Student's paired t-test, $p<0.05$), mean +/- SE (*n*=4).

3.3.1.3 DGGE analysis

Hierarchical cluster analysis of bacterial 16S rRNA gene-based DGGE profiles from experiment L:D1 (Figure 3.6a), showed very little difference in community composition between treatments at day 2.5. However, by day 5, there was a major difference in community composition, especially in the treatment without an oil layer, in which there was also a high degree of variability between replicates. Experiment L:D2 was almost identical to L:D1 except that the light source was Heliospectra rather than IsoLight Technologica, and the changes on the bacterial community were very similar for both experiments (Figure 3.6b) except that at 5 day in experiment L:D2 there was more variability in samples overlain by oil and less distinct difference between the two treatments. For the remaining experiments the light intensity was the same in all cases and the within-experiment treatment difference was that the spectrum of light differed (oil spectrum *versus* standard-light spectrum). As in the previous experiments the main distinction was at day 5 for experiments L:D4 and L:D5 (Figure 3.6d, e), but for experiment L:D3 there was more distinction at day 2.5 for three of the replicates (Figure 3.6c).

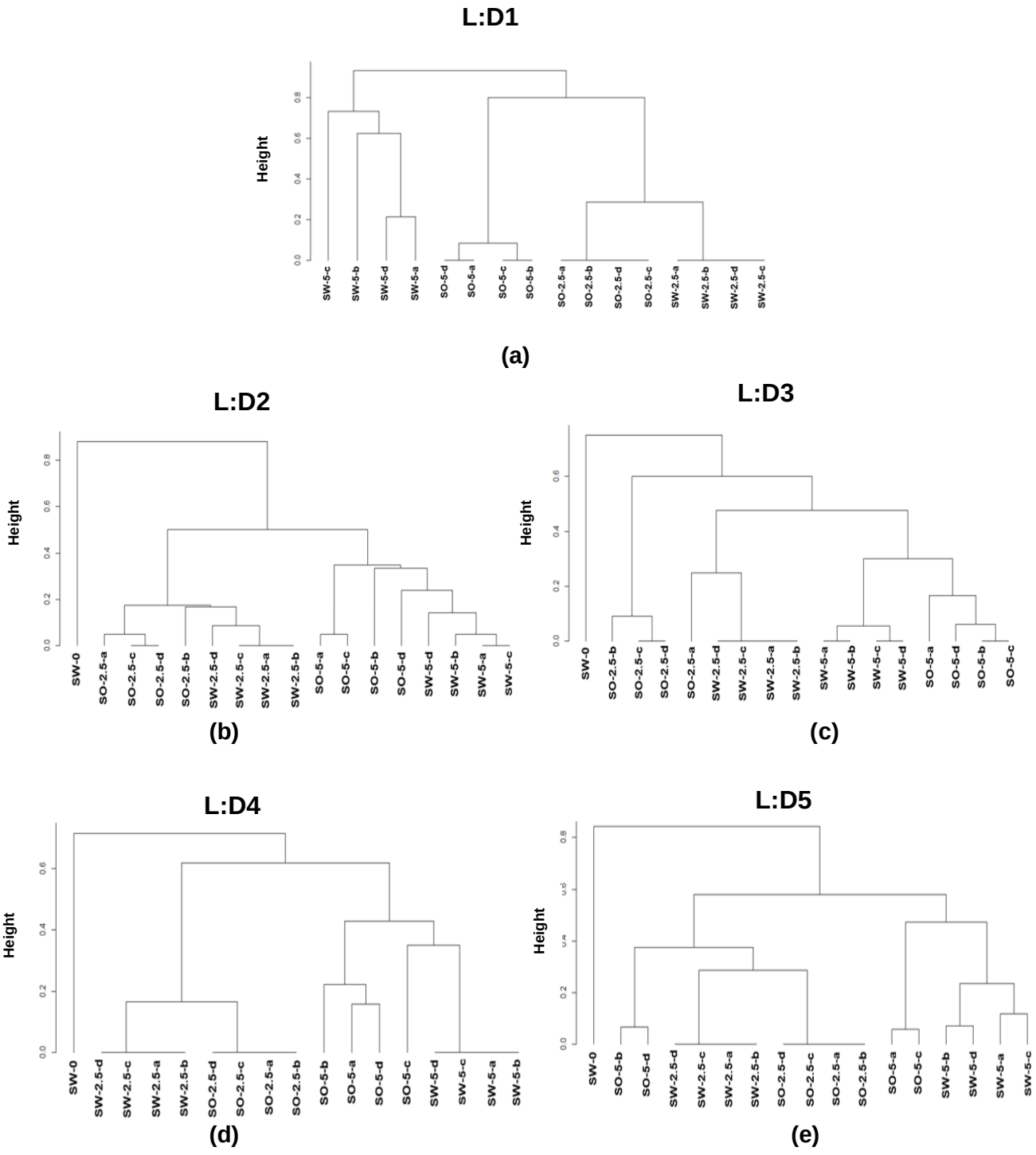

Figure 3.6 Hierarchical cluster analysis of DGGE profiles of 16S rRNA genes amplified from experiments: (a), L:D1 and (b) L:D2, after treatment without an oil layer (SW) and under Gyda oil layer (SO); (c) L:D3, (d) L:D4 and (e) L:D5 after treatment between standard-light spectrum (SW) and oil spectrum (SO). The analysis was based on presence/absence matrix of bands and Jaccard index using the complete linkage. Letters in the labels a, b, c and d indicate the replicates (n=4) of each sample (SW and SO). Numbers indicate the sampling day after 2.5 and 5. The Y axis shows the distance between clusters.

The hierarchical cluster analysis of DGGE profiles of *psbA* gene. For experiment L:D1 (Figure 3.7a), showed that the two treatments led to the formation of distinct phototrophic communities by day 2.5, which remained distinct by day 5. Differences were also observed for two samples under an oil layer by day 5 in which they are closer to those from day 2.5. Even though experiment L:D2 has the same conditions as L:D1 but different light source (Table 3.1), the samples without an oil layer were more similar to each other at both time points; in contrast two samples under an oil layer at 2.5 days were similar to the seawater without treatment at day zero, and the other two had a completely different community composition; and for samples at 5 days the community was more similar to samples without an oil layer at 2.5 days (Figure 3.7b).

For all the experiments with the oil spectrum and standard-light spectrum, the phototrophic communities were similar between treatments. But also, for L:D3 at day zero, the community was similar to those at day 5 (Figure 3.7c). For L:D4 two samples from the standard-light spectrum did not change between days and were different from the rest of the samples (Figure 3.7d). For experiment L:D5 (Figure 3.7e), two samples from standard-light spectrum and one from oil spectrum were different.

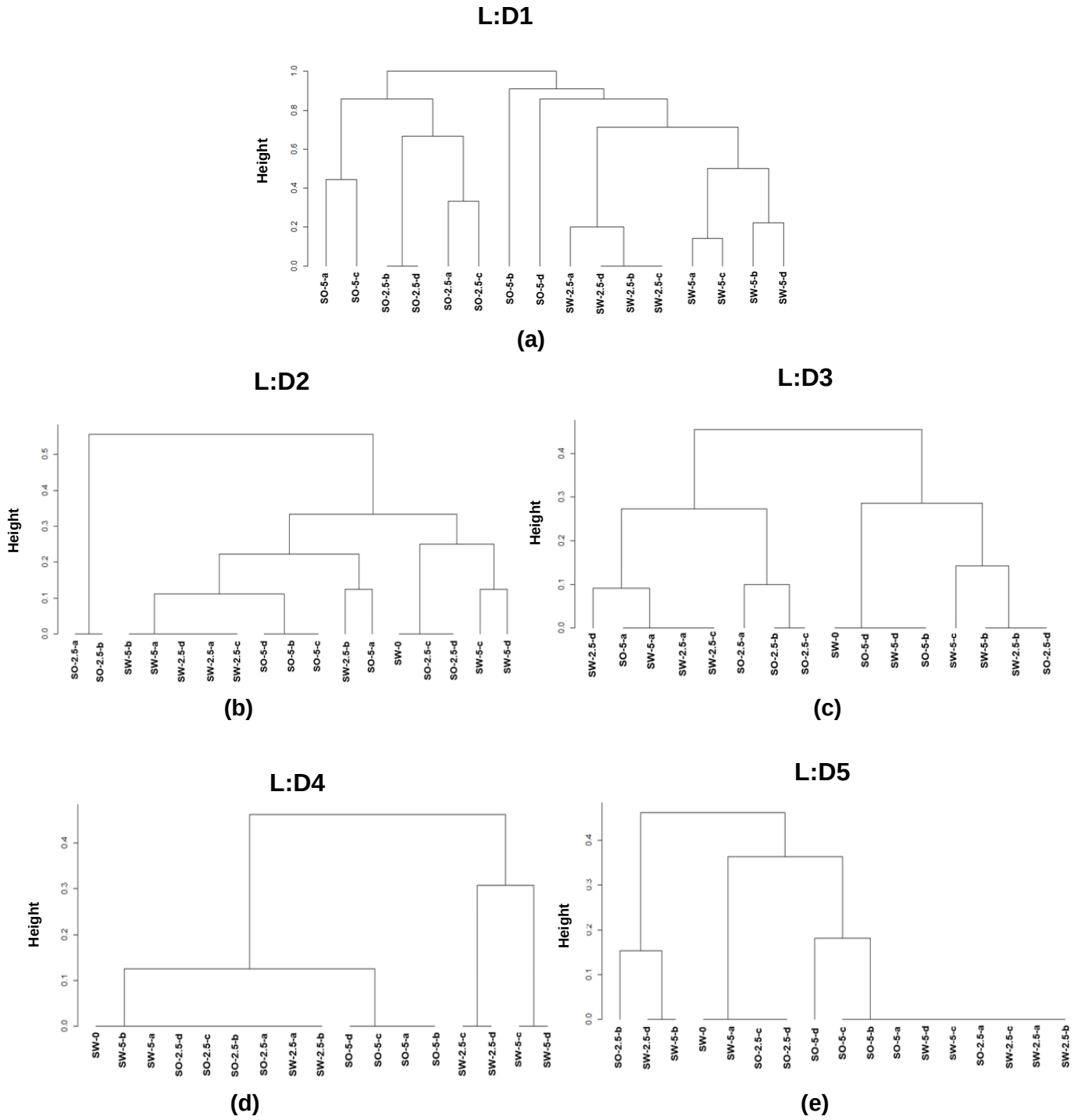

Figure 3.7 Hierarchical cluster analysis of DGGE profiles of *psbA* genes amplified from experiments: (a), L:D1 and (b) L:D2, after treatment without an oil layer (SW) and under Gyda oil layer (SO); (c) L:D3, (d) L:D4 and (e) L:D5 after treatment between standard-light spectrum (SW) and oil spectrum (SO). The analysis was based on presence/absence matrix of bands and Jaccard index using the complete linkage. Letters in the labels a, b, c and d indicate the replicates (n=4) of each sample (SW and SO). Numbers indicate the sampling day after 2.5 and 5. The Y axis shows the distance between clusters.

3.3.1.4 Analysis of 16S rRNA gene using Miseq Illumina Sequencing

DGGE analysis of 16S rRNA and *psbA* genes highlighted the differences in community composition between treatments under an oil layer and no-oil control (L:D1 and L:D2) and under an oil spectrum and standard-light spectrum (L:D3, L:D4 and L:D5). To understand better these differences, it was decided to study the community composition using Illumina Miseq sequencing. The DNA from all the experiments (L:D1-L:D5) were used to analyse the bacterial communities, however only the treatments with an oil spectrum and standard-light spectrum (L:D3, L:D4 and L:D5) produced enough replicas to statistically compare between treatments. Also, the data of the 2.5 and 5 day of each treatment (L:D3, L:D4 and L:D5) were combined to do the comparison of relative abundance because similar results were found for both days - 2.5 and 5.

The results showed that for all the light: dark experiments analyse by Miseq (L:D3, L:D4 and L:D5; oil spectrum *versus* standard-light spectrum), the most abundant sequences (45-55%) were from the Bacillariophyta (diatoms), identified via chloroplast 16S rRNA gene which supports the microscopy results (Figure 3.2) for light: dark experiment (L:D1) and contrasts with the relative abundance of cyanobacteria in the continuous light experiment (L4). Also, the relative abundance of Bacillariophyta was not significantly different between the oil spectrum and standard-light spectrum treatments (Figure 3.8 and 3.10) for L:D3 and L:D4; but for L:D5 a small but significant difference was observed whereby there was a higher relative abundance of diatoms in the standard-light spectrum (Figure 3.12).

The most abundant bacterial families detected in seawater in the three experiments (L:D2, L:D2 and L:D5) were Rhodobacteraceae, Alteromonadaceae, Flavobacteriaceae, Cyclobacteriaceae and Pseudoalteromonadaceae.

For experiment L:D3 the genera with the highest relative abundance under a standard-light spectrum (Figure 3.9) were *Glaciecola*, *Loktanella* and *Tenacibaculum*. *Glaciecola* and *Loktanella* were also found in higher relative abundance in experiments L:D4 (Figure 3.11) and L:D5 (Figure 3.13). For L:D5 also, under the standard-light spectrum, *Aestuariibacter*, *Winogradskyella* and *Roseovarius* had the highest relative abundance.

Several genera were found to have a higher relative abundance under an oil spectrum (Figure 3.9, 3.11 and 3.13): *Pseudoalteromonas*, *Alteromonas* and *Thalassomonas* in L:D3 (Figure 3.9), *Colwellia* and a large number of the other genera in L:D4 (Figure 3.11), and especially *Aliiglaciecola* in L:D5 (Figure 3.13). Some key physiological features of genera that were preferentially abundant under one treatment are shown in Table 3.3, and the possible reasons for this differential abundance will be discussed later.

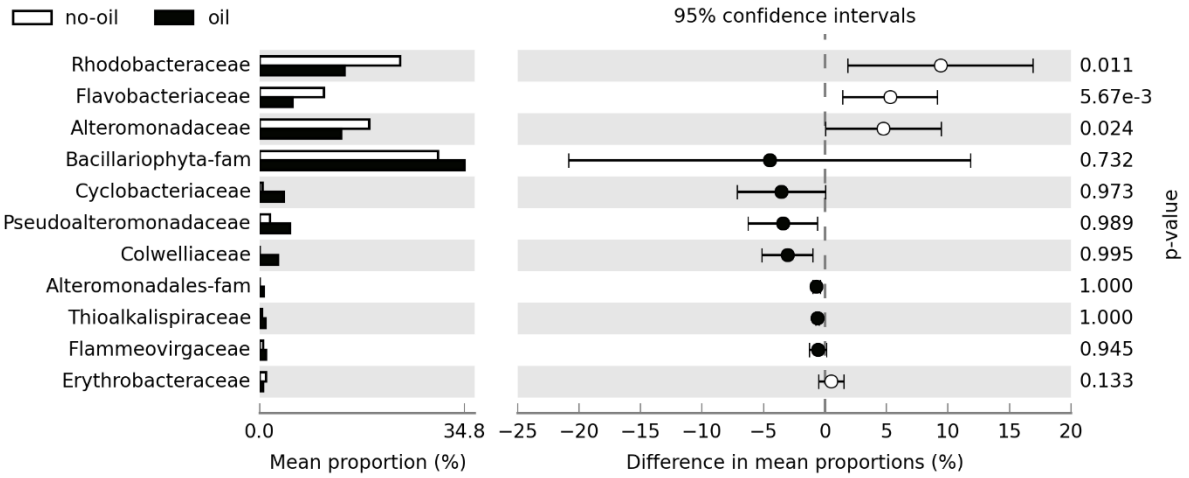

Figure 3.8 Abundance of microorganisms in experiment L:D3, between standard-light spectrum (no-oil, white) and oil spectrum (oil, black) using and incident light of 600 µm photons m^{-2} s^{-1}. The comparison was done using the hierarchy level 4.

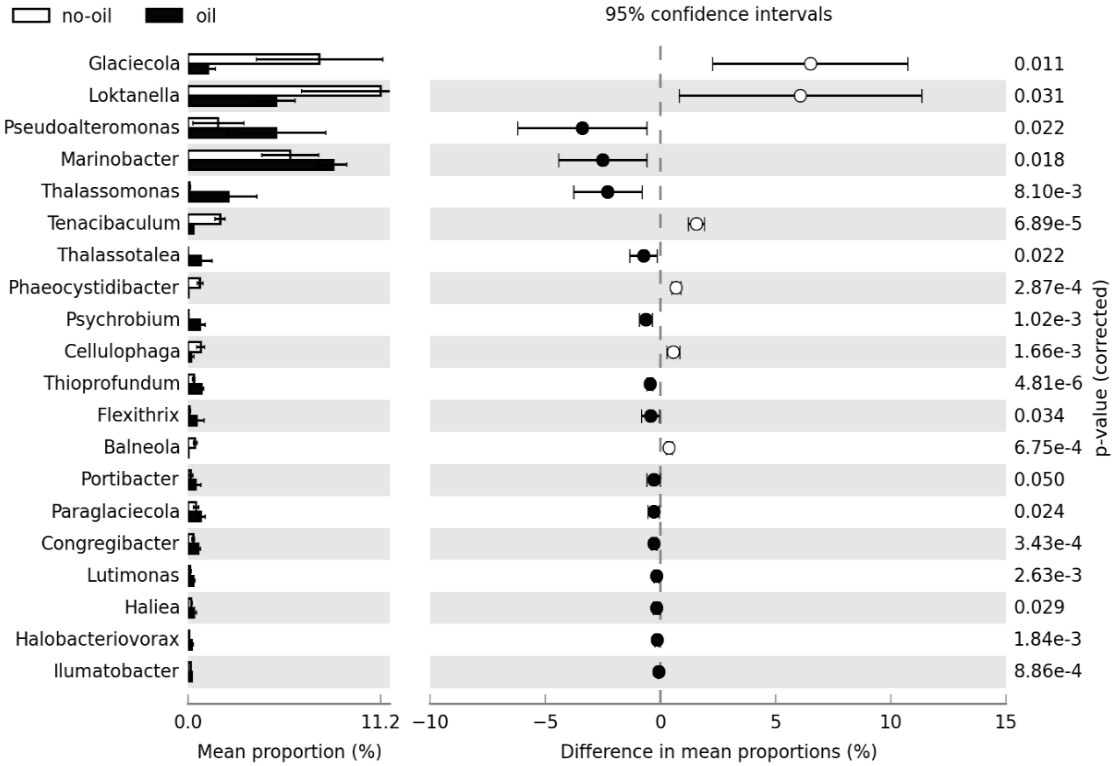

Figure 3.9 Relative abundance of microorganisms in experiment L:D3, between natural spectrum (no-oil) and oil spectrum (oil) using and incident light of 600 µm photons m^{-2} s^{-1}. The comparison was done using the hierarchy level 5.

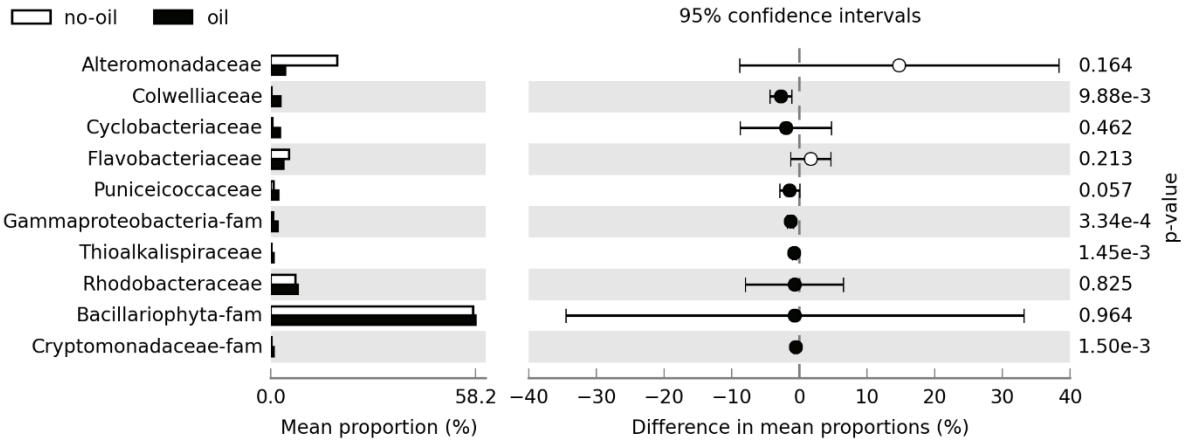

Figure 3.10 Relative abundance of microorganisms in experiment L:D4, between standard-light spectrum (no-oil, white) and oil spectrum (oil, black) using and incident light of 600 µm photons m^{-2} s^{-1}. The comparison was done using the hierarchy level 4.

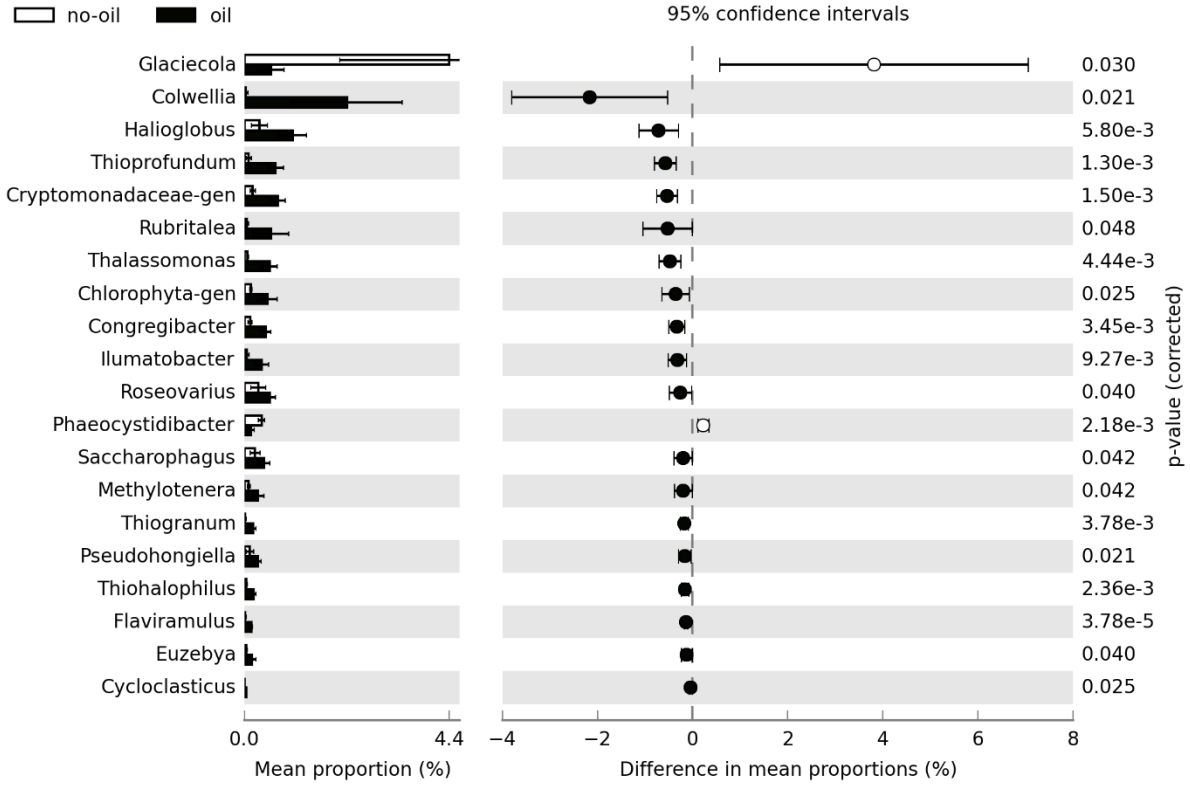

Figure 3.11 Relative abundance of microorganisms in experiment L:D4, between natural spectrum (no-oil) and oil spectrum (oil) using and incident light of 400 µm photons m^{-2} s^{-1}. The comparison was done using the hierarchy level 5.

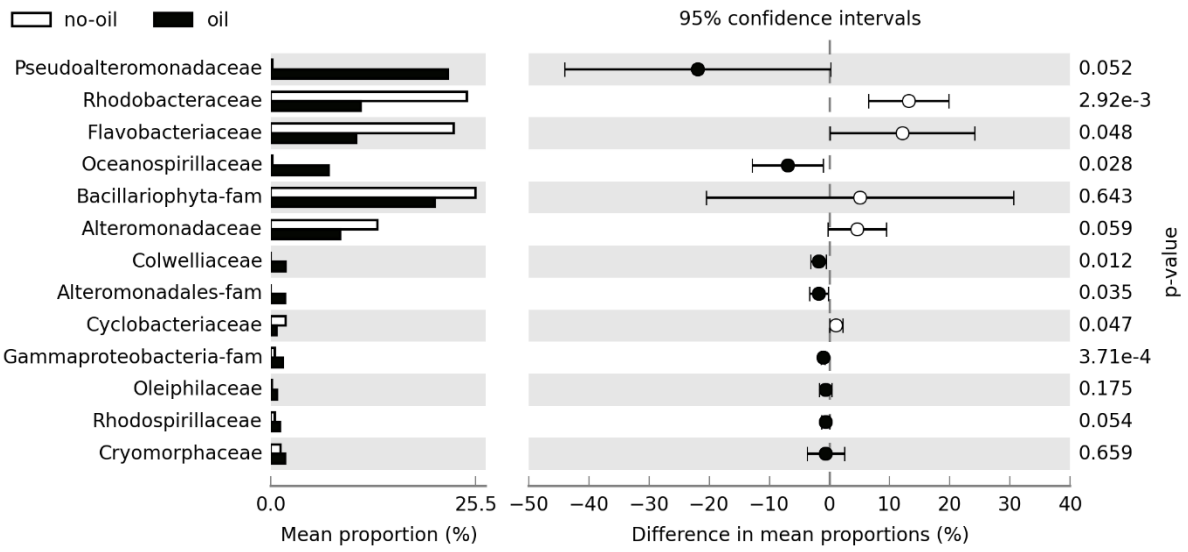

Figure 3.12 Abundance of microorganisms in experiment L:D5, between natural spectrum (no-oil) and oil spectrum (oil) .The comparison was done using the hierarchy level 4. For the different incident light used in this experiment see table 1.

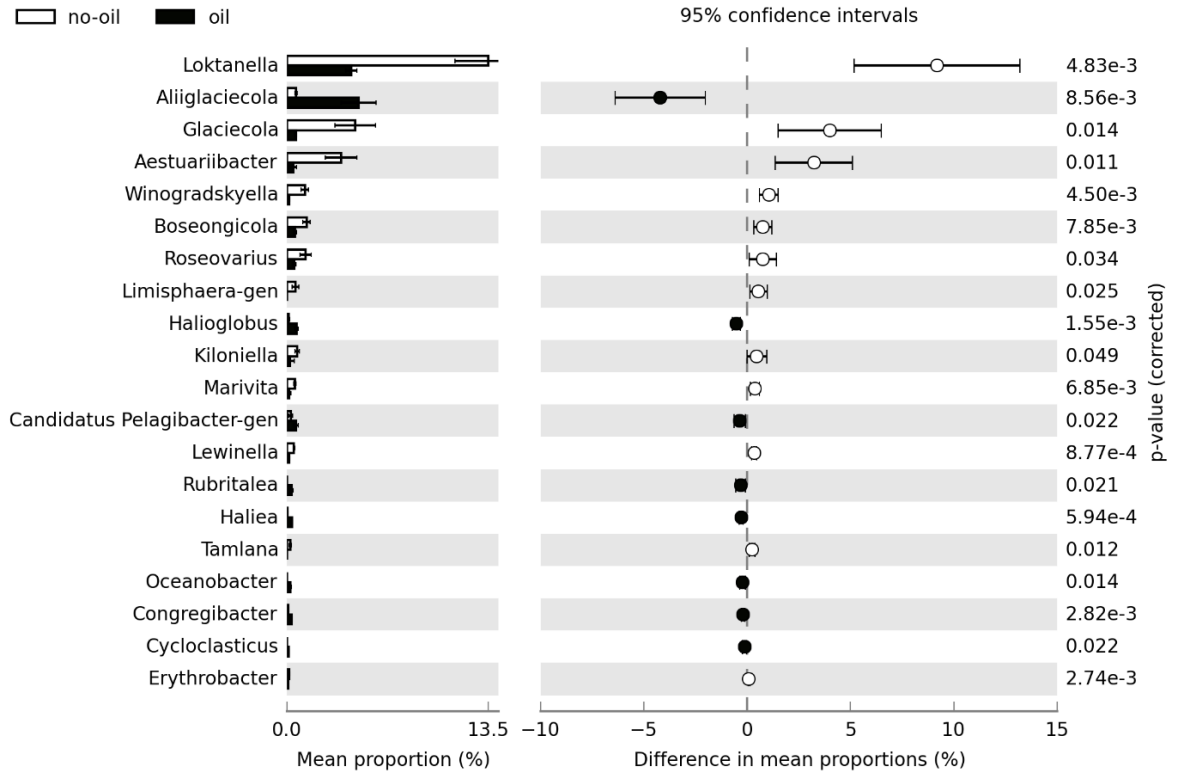

Figure 3.13 Relative abundance of microorganisms in experiment L:D5, between natural spectrum (no-oil) and oil spectrum (oil) .The comparison was done using the hierarchy level 5. For the different incident light used in this experiment see table 1.

Table 3.3 Characteristics of some of the genera that differ in relative abundance in light-standard spectrum compared with light that mimics an oil spectrum

Genus	Physiology	Presence in oil	Association with microalgae	Reference
Aestuariibacter	Heterotroph	Some species growth can growth with crude oil.	No	Wang *et al*. (2014)
Aliiglaciecola	Heterotroph	A. aliphaticivorans is an aliphatic hydrocarbon-degrader.	No	Jin *et al*. (2015)
Boseongicola	Heterotroph	No	No	
Candidatus Pelagibacter	Chemoorganoheterotrophs and has Proteorhodopsin	Inhibited by the presence of oil.	No	Stenindler et al. (2011); Chronopoulou *et al*. (2015); Bacosa *et al*. (2015)
Chlorophyta	Oxygenic phototroph	Some species grow in abundance in oil-contaminated environment.	It is a green alga	Lopez-Rodas (2009).
Colwellia	Heterotroph	Found in oil-contaminated seawater and able to degrade hydrocarbons.	No	Brakstad *et al*. (2008); Mason *et al*. (2014).
Congregibacter	AAP	Marine oil snow.	Associated to coccolithophorids e.g. *E. huxleyi* and *Coccolithus*.	Gutierrez *et al*. (2016); Green *et al*. (2015)
Cryptomonadacea	Oxygenic phototroph	No	-It is an alga, Cryptophyta	Lane and Archibald (2008)
Erythrobacter	AAP	Found in oil mousses. Some species contain cytochrome P450 alkane hydroxylases. May contributed to the aromatic hydrocarbon degradation in oil-contaminated beaches.	No	Liu & Liu (2013); Wang *et al*. (2010); Cappello *et al*. (2016)
Glaciecola	Heterotroph *Glaciecola punicea* has Proteorhodopsin	Oil degrader	Associated with phytoplankton blooms (diatoms).	Prabagaran *et al*., (2007); Brakstad *et al*. (2008); Chronopoulou *et al*. (2014). Scheibner *et al*. (2017) Qin *et al*. (2012)

Haliea	Some species are AAP	Sensitive to oil	No	Spring *et al*. (2013); Fragoroso-dos Santos et al. (2011)
Halioglobus	AAP	No	No	Spring *et al*. (2013)
Illumatobacter	Heterotroph	Found in the Deepwater Horizon spill site.	-Associated to coccolithophorids e.g. *E. huxleyi* and *Coccolithus pelagicus f. braarudii*	Olapade (2013); Green *et al*. (2015)
Kiloniella	Chemoorganotroph	No	Enzymes degrade macroalgae	Goecke *et al*. (2010)
Lewinella	Chemoorganotroph	Found in cyanobacterial-oil contaminated mats from a constructed wetland	Found in cyanobacterial-oil contaminated mats from a constructed wetland	Abed *et al*. (2014)
Limisphaera	Heterotroph	No	No	
Loktanella	Chemoheterotroph, but some species contain *pufM* gene (AAP)	Oil sediments, oil biofilms and oil seawater.	Present during algal blooms. Algicidal activity.	Trappen *et al*. (2014); Koblizek (2015); Sanni *et al*. (2015)
Marivita	Some species contain *pufM* and *pufL* gene in addition others produce Bchl *a*.	No	Associated with coccolithophorids e.g. *E. huxleyi* and *Coccolithus pelagicus f. braarudii* Associated with dinoflagellates	Hwang *et al*. (2019); Budinoff *et al*. (2011); Green *et al*. (2015)
Methylotenera	Methylotroph (use methanol or methylamine as a carbon source)	Found in oil contaminated soils; associated with organic contaminant degradation.	No	Liu *et al.* (2016); Wright *et al.* (2017)
Phaeocystidibacter	Chemoorganoheterotroph	No	No	Zhou *et al*. (2013)
Pseudoalteromonas	Chemoheterotroph	Oil contaminated seawater.	Present during algae blooms. Produce extracellular compounds than inhibit diatoms and algal spores.	Gutierrez *et al*. (2013); Chronopoulou *et al*. (2014); Holmstrom and Kjelleberg (1999)
Roseovarius	AAP	Some species can grow with crude oil, n-alkanes and PAH.	-Strain MCTG156(2b) was isolated from a phytoplankton net Trawl	Chronopoulou *et al*. (2014); Mckew *et al.* (2007); Vila *et al*. (2010); *Gutierrez et al*. (2017)
Rubritalea	Heterotroph	No	Some produce a carotenoid	Scheuermayer *et al*. (2006)
Saccharophagus	Heterotroph	Saccharophagus degradans was found in overlying water of an oil contaminated sediment.	Sometimes associated with macroalgae, contain enzymes that degrade algae.polysaccharides	Liu and Liu (2013) Minich *et al*. (2018).

Tenacibaculum	Heterotroph	Found in water contaminated with hydrocarbons.	Dominant bacteria in waters enriched with phytoplankton detritus. Grow on phytoplankton EPS.	Prabagaran *et al*. (2007); Dubinsky *et al*. (2013). Bohorquez *et al.* (2017)
Thalassomonas	Heterotroph	*Thalassomonas viridans* in oiled seawater after 7 days.	*Thalassomonas viridans* isolated from oyster is associated with microalgae culture.	Coulon *et al*. (2007); Nicolas *et al*. (2004)
Thalassotalea	Chemoheterotroph	No.	No	Deering *et al*. (2016)
Thiogranum	Chemilithoautotrophic Sulfur oxidizing	No	No	
Thioprofundum	Chemolithoautotroph Sulfur oxidizing	Found in high hydrocarbon concentration in salt marsh. Oil contaminated seawater and soil.	No	Engel *et al*. (2017); Dashti *et al*. (2018)
Winogradskyella	Heterotroph *Winogradskyella* PG-2 has Proteorhodopsin	Found as a core community in an oil-contaminated water	No	Bacosa *et al*. (2015); Newton *et al*. (2013); Kumagai et al. (2014)

3.4 Discussion

The change from continuous light (L4, Chapter 2) to a light: dark experiment (L:D1), showed differences in all the parameters measured. The concentration of DNA (an indicator of biomass) was lower than the L4 experiment (Figure 2.6), and contrary to L4, there was a higher concentration under the no-oil control, than under an oil layer (Figure 3.3). The concentration of chlorophyll *a* was also lower, with a decrease of 40% from L4 to L:D1 (Figure 3.4a). This was clearly visualized in the colour of the cultures, green for L4 and brownish for L:D1. Also, the composition of the community changed, while cyanobacteria dominated in L4 experiment under an oil layer, for all the light: dark experiments(L:D1-L:D5) the predominant type of phototroph was diatoms under both treatments, no-oil control and under an oil layer.

Light: dark cycles have a great influence on photosynthesis, therefore the productivity varies with the change in light: dark cycles (Ratchford and Fallowfield, 2003). The differences between continuous light and light: dark experiments could be due to several factors, one of them is photoacclimation. For example, Kulk *et al*. (2011) found as explained in chapter 2 (2.5.2) that eukaryotic phytoplankton, like diatoms *(Ostreococcus* sp., *Emiliania huxleyi* and *Thalassiosira oceanica*) can acclimate better to high and low irradiances even when there are irradiance fluctuations and with continuous light or light: dark period compared to some species of cyanobacteria (*Prochlorococcus marinus* and *Synechococcus* sp.), which in a high and constant irradiation the growth is diminished and growth better under low irradiance. This could explain the growth increase of diatoms under an oil layer for the dark and light experiments compared to continuous light experiments.

Also, some of the phototrophs could have experienced photoinhibition under the no-oil control. The photochemical efficiency (*Fv/Fm*) for L:D1 was higher under an oil layer at the end of the experiment (Figure 3.5 a). At high light-intensity levels there is less efficient use of absorbed light energy and also biochemical damage to photosynthetic machinery (photoinhibition), making light energy utilization even less efficient. Thus, the highest photosynthetic efficiency is realized at low-light intensity (Scott *et al*., 2010).

3.4.1 Effect on nutrient addition

To test whether the addition of nutrients (882 µM of $NaNO_3$ and 36.2 µM of NaH_2PO_4) has an influence on photosynthetic microorganisms' growth, experiment L:D2 had the same design as L:D1 but without nutrients, although, it is important to mention that the seawater for every experiment was taken at different seasons and a different set of lights were also used (Table 3.1).

The results for L:D2 (Figure 3.3b) showed that no nutrient addition had no influence on the total biomass (concentration of DNA),when comparing the two treatments, no-oil control and with oil layer and when comparing DNA concentrations with those from L:D1 (Figure 3.3a). Also, the DGGE of the 16S rRNA gene showed that the community composition for bacteria and phototrophs in the seawater with an oil layer were different between zero days, 2.5 days and 5 days; in contrast the community composition in seawater with no-oil control, were more similar between day 2.5 days and 5 days, but both different at zero day (Figure 3.6b and 3.7b).

Kocum *et al*. (2002) found that the seawater from the Brightlingsea, contains high concentrations of nutrients (N:P:Si) all year around, which could explain why the lack of added nutrients in L:D2 experiment did not significantly affect the total biomass of microorganisms. When nutrients were added there was a difference between treatments under an oil layer and under no-oil control, such that under the oil layer chlorophyll *a* was high (Figure 3.4 a), whereas under a standard-light regime total biomass was high (Figure 3.3 d, e). This pattern was not seen under the same light: dark regime when nutrients

where not added (Figure 3.4 b). Chlorophyll *a* concentration may vary due to differences in several factors including phototroph community composition, light, temperature, nutrient availability and grazing (Rosen and Lowe, 1984). Changes in the light are expected to affect phototroph pigment content but nutrient availability, along with light, can decrease the chlorophyll *a* content (Baulch *et al*, 2009). Even though chlorophyll *a* concentration decrease in L:D2 compared to L:D1, the photochemical efficiency for experiment L:D2 was not significantly affected.

Kocum *et al*. (2012) also found that throughout the Colne Estuary, algal photosynthesis was almost always light limited, and conditions in the Colne (high concentrations of nutrients and low availability of light) appear to favour the dominance of flagellates instead of diatoms; however, the results of light: dark cycles showed that in the presence of an oil layer, the dominant microorganism found in the seawater was the diatoms. The use of the cell-culture flasks in the experiment, may have encouraged growth of benthic phototrophs over planktonic phototrophs (both cyanobacteria and diatoms are planktonic and benthic phototrophs).

To have a better understanding of how the availably of nutrients can affect the production of algal biomass it would be necessary to measure the concentrations of the N and P, and the N: P ratios in the seawater before and after the treatments.

3.4.2 Use of LED lights to mimic the effect of oil on the spectrum of transmitted light

For the experiments L4, L:D1 and L:D2 the light intensity was different between treatments, with an oil layer (40-50 µm photons m^{-2} s^{-1}) and with no-oil control (600 µm photons m^{-2} s^{-1}). In order to know whether the changes in microbial community, were due to the changes light intensity or spectrum, the effect of the oil on the light spectrum was mimicked and the light intensity was kept the same for both treatments, oil spectra and standard-light spectra in experiments L:D3, L:D4 and L:D5.

The concentrations of chlorophyll *a* for L:D4 and L:D5 were not different between treatments, oil spectrum and standard-light spectrum, for both days 2.5 and 5, except for experiment L:D3 in which by the end (day-5) the chlorophyll *a* increased by about 37%. This could be due to the high intensity of the standard-light spectrum causing photoinhibition, so the quality of light received for samples with an oil spectrum favour somehow the growth of some phototrophs.

The photochemical efficiency (*Fv/Fm*) for L:D3, L:D4 and L:D5 (Figure 3.5 c, d, e) was slightly higher for samples under natural spectrum than with an oil spectrum or did not change between both treatments.

The community abundance for L:D3, L:D4 and L:D5 (Figure 3.6 and 3.7) showed that there are similarities between the treatments, with an standard-light spectrum and oil spectrum, for both days. There is more heterogeneity in communities where the oil spectrum was used in L:D3 and L:D4 experiments, this could mean that the oil, not only

favoured certain phototrophs but also favoured the growth of different bacteria. For experiment, L:D5 the community than change more was under standard-light spectrum. The changes in the bacterial and phototrophic community are due to a combination of direct and indirect effects of light and possible the sensory effects of an oil spectrum over the communities.

1) The direct effect of light over the communities can be observed in both treatments, under an oil spectrum and under standard-light spectrum, with the high abundance of species such as oxygenic phototrophs, aerobic anoxygenic phototrophs and species that have proteorhodopsin. For example, under the standard-light spectrum, it was found species that have proteorhodopsin, such as *Glaciecola* and *Winogradskyella* (Qin *et al*., 2012; Kumagai *et al*., 2014) and other species that are aerobic anoxygenic phototrophs such as *Roseovarius* and *Marivita*. Some species of *Loktanella* have the *pufM* gene (encode the M subunit of the reaction centre of aerobic anoxygenic photosynthesis) (Koblizek, 2015; Hwang *et al*., 2019).

Also, with the oil spectrum, microorganisms that have proteorhodopsin like *Candidatus pelagibacter* (Stenindler *et al*., 2011) in experiment L:D5 and microorganisms that are aerobic anoxygenic phototrophs such as *Haelioglobus* (experiments L:D4 and L:D5), *Haliea* (experiments L:D3 and L:D5), *Erythrobacter* (experiment, L:D5) and *Congregibacter* (experiments L:D3, L:D4 and L:D5) (Spring *et al*., 2013; Wand *et al*., 2010; Gutierrez *et al*., 2016) were observed. In addition to the bacteria, some oxygenic phototrophs species were relative by more abundant (experiment L:D4) under an oil

spectrum, such as *Cryptomonadacea and Chlorophyta* (Lane and Archibald, 2008; Lopez-Rodas, 2009).

2) The indirect effect of oil can be observed with the abundance and type of microorganisms that can be associated with different phototrophic communities, for example, live in close proximity to oxygenic or aerobic anoxygenic phototrophs and/or use their photosynthate as a source of carbon and energy. Under a light spectrum, *Glaciecola* is also known for being associated with phytoplankton blooms produced by diatoms (Scheibner *et al*., 2017). Some strains of *Roseovarius* has been found in phytoplankton net trawls (Gutierrez *et al*., 2017). *Kilonella* can produce enzymes that degrade macroalgae (Goecke *et al*., 2010), *Lewinella* was found to be associated with cyanobacterial mats (Abed *et al*., 2014) and *Tenacibaculum* has been found associated with phytoplankton detritus and can also grow on phytoplankton extracellular polymeric substances (EPS) (Dubinsky *et al*., 2013; Bohorquez *et al*., 2017).

Under an oil-spectrum, *Pseudoalteromonas* was found that is present during algae blooms and can produce extracellular compounds that inhibit diatoms and algal spores (Holmstrom and Kjelleberg, 1999). *Illumatobacter* that is associated with coccolithophorids (e.g. *E. huxleyi*) (Green *et al*., 2015), and *Saccharophagus* that sometimes is associated with microalgae and produce enzymes that degrade algae polysaccharides (Minich *et al*., 2018).

3) Specific sensory effects of an oil spectrum, this statement is highly speculative, but interesting. Some of these species that were found in high abundance under an oil spectrum, have representatives that can degrade hydrocarbons. For example, *Marinobacter* is known to have hydrocarbon degrading representatives such as *M. hydrocarbonoclasticus* (Mounier *et al*., 2014). Also, *Pseudoalteromonas* and *Thalassomonas* have been found in oil-contaminated seawater (Chronopoulou *et al*., 2014; Coulon *et al*., 2007). *Colwellia* species have shown to degrade hydrocarbons (Redmond and Valentine, 2012; Mason *et al*., 2014). *Thioprofundum* is a sulphur oxidizing bacterium (Engel *et al*., 2017) and has been found in hydrocarbon contaminated salt marshes (Dashti *et al*., 2018). *Aliiglaciecola*, have some species that degrade aliphatic hydrocarbons (Jin *et al*., 2015). Although most of them are metabolically versatile, *Cycloclasticus* can only degrade PAH (Staley, 2010) albeit found in low abundance.

3.5 CONCLUSION

The results showed that changing the light regime, from continuous light to light: dark (12:12) had an influence on the type and abundance of microorganisms found in the natural seawater. For the light: dark regimes, diatoms were the most abundant microorganism in all the treatments, under an oil layer and no-oil control (L:D1 and L:D2) and under an oil spectrum and standard-light spectrum (L:D3-L:D5). The changes in the bacterial and phototrophic community are due to a combination of direct and indirect effects of light and possible the sensory effects of an oil spectrum over the communities. In future work to be closer to the natural environment (changes in cloud cover, turbulence, waves, sediment resuspension in coastal zones) it will be interesting to have a fluctuating

light intensity instead of constant light of same overall intensity to study how this affects physiology of the microorganisms. Also, quantify the different types of bacteria and photosynthetic microorganisms, for example with a qPCR of *pufM*, that encode the M subunit of the bacterial reaction centre of aerobic anoxygenic phototrophs (Koblizek, 2015).

CHAPTER 4

Impact of crude oil on light attenuation and the growth of Synechococcus CY9201 and Thalassiosira weissflogii CCMP1051

4.1 Introduction

Crude oil has a significant impact on light intensity and the spectrum of transmitted light, specifically photosynthetically active radiation (PAR) (Raughukumar *et al*. 2001; Chapter 2.4.1). Thus, the presence of an oil layer on seawater can change the structure of the phototrophic community when they are exposed to continuous light (as shown in Chapter 2) or to a light and dark regime (as shown in 3). Also, the effects of light on bacterial production can depend on, differential susceptibility to solar radiation, the availability of organic matter, the presence of, and interaction with photosynthetic algae, and the length of exposure (Medina-Sanchez *et al*. 2002).

The oceans cover approximately 70% of the Earth's surface, and photosynthetic organisms living in the photic zone are responsible for half of the global primary productivity (Amin *et al.* 2012). The ubiquity and abundance of microorganisms, especially primary producers, mean they have an important role on biogeochemical cycles in the ocean (Falkowski *et al.* 2008).

Photosynthesis is the conversion of light energy to chemical energy and is the most important biological process on Earth (Madigan *et al.* 2014). Photosynthetic organisms can use chlorophylls (algae and cyanobacteria) and bacteriochlorophylls (anoxygenic

phototrophic bacteria and aerobic anoxygenic phototrophic bacteria) as light harvesting pigments (Bjorn *et al*., 2009; Kolber and Falkowski, 1993).

A light-harvesting pigment must absorb in a spectral region where radiant energy is available; on the present earth this means light of 300 to about 1200 nm wavelength (Bjorn *et al*., 2009). The absorption maxima of different phototrophic microorganisms are provided in Table 1.2 (Chapter 1).

Photosynthetic pigment levels usually change rapidly in response to environmental conditions because they are engaged in basic processes such as light harvesting, photosynthesis and photoprotection, which are essential for life. Pigment level is mostly regulated by light conditions, which drives their rapid conversion, usually without any changes in gene expression (Kuczynska et al. 2015). The concentrations and ratios of different chlorophylls and accessory pigments can provide information on both the community and the abundance of primary producers (Airs *et al.* 2001). The existence of different forms of chlorophyll or bacteriochlorophyll that absorb light of different wavelengths allows phototrophs to make better use of the available energy in the electromagnetic spectrum (Madigan *et al.* 2014).

For this experiment the selection of *Synechococcus* and *Thalassiosira weissflogii* was based on the previous results of the effect of Gyda crude oil on transmitted light where it was demonstrated that oil affects both the spectrum and the intensity of the received light and that the attenuation of light was greater at shorter wavelengths (see Chapter 2). Previous experiments also showed that the attenuation of light by crude oil, had

consequences for the process of photosynthesis within the phototrophic community (Figure 2.7 and 3.6). Specifically, seawater samples covered with an oil layer resulted in significantly higher concentrations of chlorophyll *a* after 6.5 days under continuous light (Figure 2.7) and after 5 days under a light and dark (12:12) regime (Figure 3.6). Microscopy, showed that mostly green coloured colonies were present when using continuous light (Figure 2.5), later confirmed by microbial community analysis (see Chapter 2) that cyanobacteria were one of the most abundant groups of microorganisms. In contrast, diatoms were more abundant under a light and dark regime (Figure 3.2); also confirmed by microbial community analysis (Chapter 3). The selection of the cyanobacterium *Synechococcus* CY9201 and the diatom *Thalassiosira weissflogii* CCMP1051 based upon these results and the analyses of different absorption spectra of several phototrophs (e.g. Figure 4.1) (Suggett D, unpublished), which indicated that *T. weissflogii* strains, may have a competitive advantage over *Synechococcus* in the presence of oil, due to having a higher proportion of their pigments that absorb longer wavelengths where the oil transmits relatively more light (Figure 2.3). In order to distinguish between the effect of the light intensity and compared with the light spectrum on the cultures, it was decided to use a neutral-density filter that resulted in an equal received light intensity to that in cultures with a 0.14 mm oil layer and also to test the susceptibility of the phototrophs when they were in direct contact with oil.

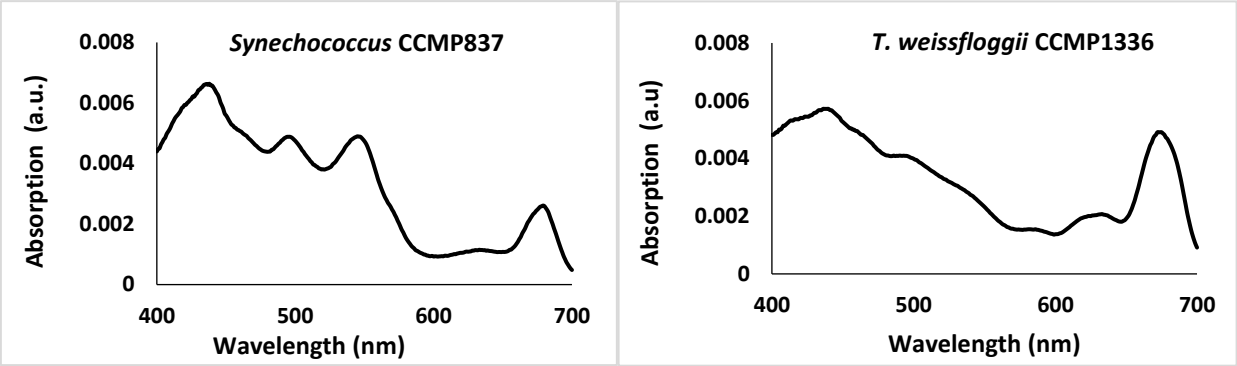

Figure 4.1. Absorption spectrum of *Synechococcus* CCMP837 and *Thalassiosira weissflogii* CCMP1336 (David Suggett, unpublish data)

The overall aim was to compare the effects of oil-induced PAR attenuation on the growth and physiology of *Synechococcus* CY9201 and *Thalassiosira weissflogii* CCMP1051 which have distinct pigment profiles, and to investigate whether the effect of PAR attenuation has a greater impact on the growth of the phototrophs than direct toxicity or indirect effects of crude oil itself.

4.1.1 Hypotheses

1. *Thalassiosira weissflogii* will grow better than *Synechococcus* with PAR attenuation due overlaying oil or a neutral density filter.
2. *Thalassiosira weissflogii* and *Synechococcus* will use their accessory light harvesting pigments for growth in the presence of an oil layer due to the attenuation of shorter wavelengths of light by oil, where chlorophyll *a* has the main absorption peak.

3. *Synechococcus* will growth better than *Thalassiosira weissflogii* when grown in direct contact with oil due to the ability of some species to grow in oil contaminated environments.

4.1.2 Objectives

1. To measure PAR spectrum and intensity in each treatment (oil layer, no oil/filter, neutral-density filter and oil in contact).
2. To determine growth of *Thalassiosira weissflogii* and *Synechococcus* with each treatment over 6 days.
3. To measure photosynthetic pigment abundance by UPLC for both strains in the four treatments.
4. To measure the photochemical efficiency of the cells by Fast Repetition Rate (FRR) Fluorometry.

4.2 Methodology

4.2.1 Design of the experiment

Axenic cultures of *Synechococcus* CCY9201 and *Thalassiosira weissflogii* CCMP1051 were pre-grown in cell-culture flasks (Noc™ Easy Flask™ 25 cm^2, Nunclon™ Delta Surface, Thermo Scientific) in marine BG11 for *Synechococcus* (Appendix B) and F/2 medium for *Thalassiosira weissflogii* (Appendix A) (Guillard and Ryther, 1962; Guillard, 1975), respectively, with a light intensity of 480-500 µmol photons m^{-2} s^{-1} in a light and dark regime (12:12 h) at 20 °C for 3 days. An aliquot of the pre-culture was inoculated into the same media in a in a cell-culture flask (Noc™ Easy Flask™ 25 cm^2, Nunclon™

Delta Surface, Thermo Scientific) and grown in a light chamber using the same conditions of light and temperature for 6 days. Four independent replicates of each culture were grown, with each growing on separate days under four different treatments as shown in Figure 4.2: cultures under (but not in contact with a Gyda crude oil layer (received light, 73-76 µmol photons m^{-2} s^{-1}), cultures without an oil layer or filter (received light, 540-601 µmol photons m^{-2} s^{-1}), cultures with a neutral-density filter on the top (Lee filters, 100 mm system stopper filter) (74-76 µmol photons m^{-2} s^{-1}) and Gyda oil in contact with the cultures (received light, 72-76 µmol photons m^{-2} s^{-1}). Light intensity was measured in triplicate with a light sensor positioned under the petri dishes for each treatment over the 6 days with two measurements per day (at the beginning and after 6 h of the light cycle).

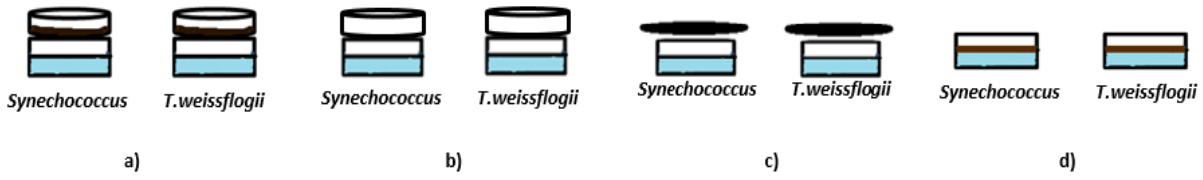

Figure 4.2. Experimental design for testing the influence of an oil layer, a neutral-density filter with equivalent light attenuation and oil in contact with two marine phototrophs: a) Gyda oil layer (73-76 µmol photons m^{-2} s^{-1}; b) no-oil/filter control: 540-601 µmol photons m^{-2} s^{-1} c) neutral-density filter: 74 µmol photons m^{-2} s^{-1} and d) oil in contact with the culture: 76 µmol photons m^{-2} s^{-1}).

4.2.2 Testing the effect of oil on transmitted light

Photosynthetically active radiation and the spectrum of light were measured for the oil layer, using a *Macam* Spectroradiometer over the range of 400 nm to 800 nm. The transmittance was obtained: % T= I / I_0 ×100, were I_0=Intensity of light passing through a control (seawater) and I=Intensity of light passing through the sample (seawater + oil).

4.2.3 Cell growth

Light microscopy with a Leica DME compound microscope (Meyer instruments, USA), was used for cell counts of *Thalassiosira weissflogii* CCMP1051 and *Synechococcus* CCY9201 (×10 and ×40 objective, respectively) using an improved Neubauer haemocytometer (Weber England). Cell numbers were measured every day to obtain a growth rate. At the end of the experiment the cells were collected to measure photosynthetic pigments (fluorometric chlorophyll *a* analysis and pigment analysis by UPLC) and measurement of photochemical efficiency using the Fast Repetition Rate (FRR) Fluorometry.

4.2.4 Chlorophyll a and pigments extraction (see 2.2.6)

4.2.5 Fluorometric Chlorophyll Analysis (see 2.2.7)

4.2.6 Fast Repetition Rate (FRR) Fluorometry (see 3.2.8)

4.2.7 Pigment analysis by UPLC

Pigment separation was performed using a method described by Zapata *et al.* (2000). The filtered methanol extracts were injected into an ACQUITY$_{TM}$ Waters System (MS, USA) ultra-performance liquid chromatograph. A C_8 column (50 mm × 2.1 mm, 1.7 µM particle size, 100 Å pore size: Waters; MS, USA) was used to separate the pigments. The mobile phases were HPLC-grade. Eluent A was a mixture of methanol:acetonitrile:aqueous pyridine (0.25 M, pH adjusted to 5.0 with pure acetic acid) (50:25:25 v/v); eluent B was prepared with acetonitrile: acetone (80:20 v/v). The

programmed flow rates and gradient are given in Table 4.1. Peak detection was determined using a diode array absorption detector (PDA Eλ Detector, Waters, USA). Pigments were identified by comparison with relative retention times given by Zapata *et al*. (2000) and reference absorbance spectra from Jeffrey *et al*. (1997). The detector was calibrated with a chlorophyll *a* sample from *Chlorella vulgaris*.

Table 4.1. Gradient elution of pigment analysis by UPCL

Time(min)	Flow (mL min^{-1})	%A	%B	Curve
0.00	0.2	100	0	-
1.00	0.2	100	0	1
2.57	0.2	80	20	2
6.50	0.2	0	100	8
9.00	0.2	100	0	6

%A: Eluent A (methanol:acetonitrile:aqueous pyridine) and %B: Eluent B (acetonitrile: acetone).

The concentration of the pigments was calculated using the area under the peaks with the integration parameters of the software equipment and each pigment concentration was normalized with the concentration of chlorophyll *a* calculated by fluorometric analysis (Figure 4.8) in each of the treatments to further calculate their concentration.

4.2.8 Statistical analysis

Differences among treatments were analysed by one-way analysis of variance (ANOVA). In the presence of significance differences ($P<0.05$), a post-hoc analysis was performed, using Tukey's test. Differences between days were analysed using two-tailed Student's test ($P<0.05$). Variations among pigments were done by Principal Component Analysis (PCA). All statistical analyses were performed using R studio 3.4.1.

4.3 Results

4.3.1 Effect of oil on transmitted light.

In order to quantify differences in light attenuation between the treatments (oil layer, no-oil/filter and neutral-density filter), the spectrum of light (400-800 nm) was measured. Figure 4.3 shows that light (Quartz halide lamp) was completely attenuated by oil at short wavelengths (400-530 nm) as found previously with Heliospectra LED lights (see Chapter 2.4.1). Also, greater light attenuation was observed at these wavelengths in the presence of neutral-density filter. However, at longer wavelengths (700-800 nm), the presence of the crude oil layer resulted in greater attenuation of light compared with the neutral-density filter (Figure 4.4).

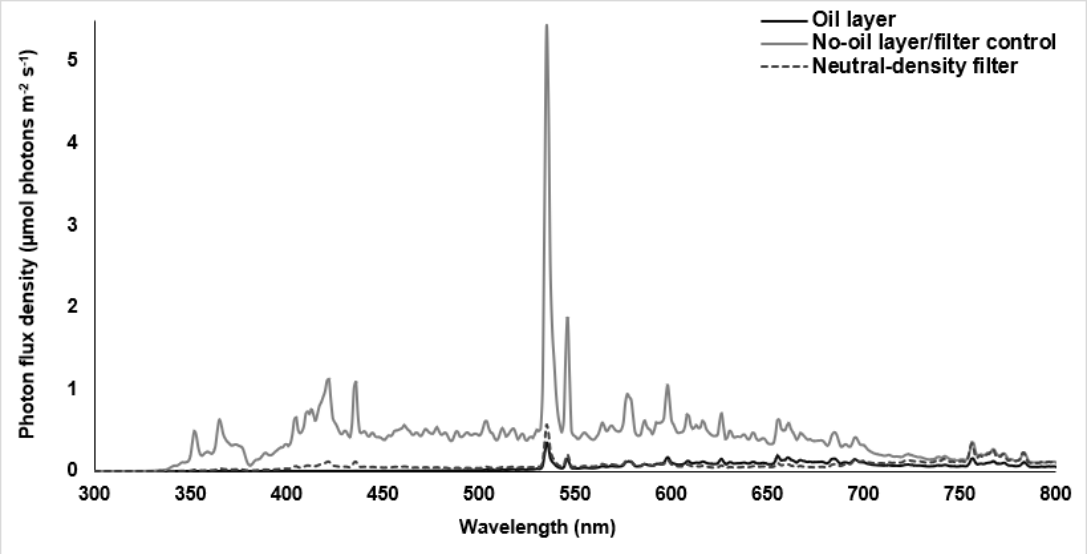

Figure 4.3: Spectrum of artificial lights (*n*=8) passing through: a) Gyda crude oil layer (73-76 µmol photons m^{-2} s^{-1}), b) No-oil/filter control (540-601 µmol photons m^{-2} s^{-1}) (74-75 µmol photons m^{-2} s^{-1}) and c) Neutral-density filter (74-76 µmol photons m^{-2} s^{-1}).

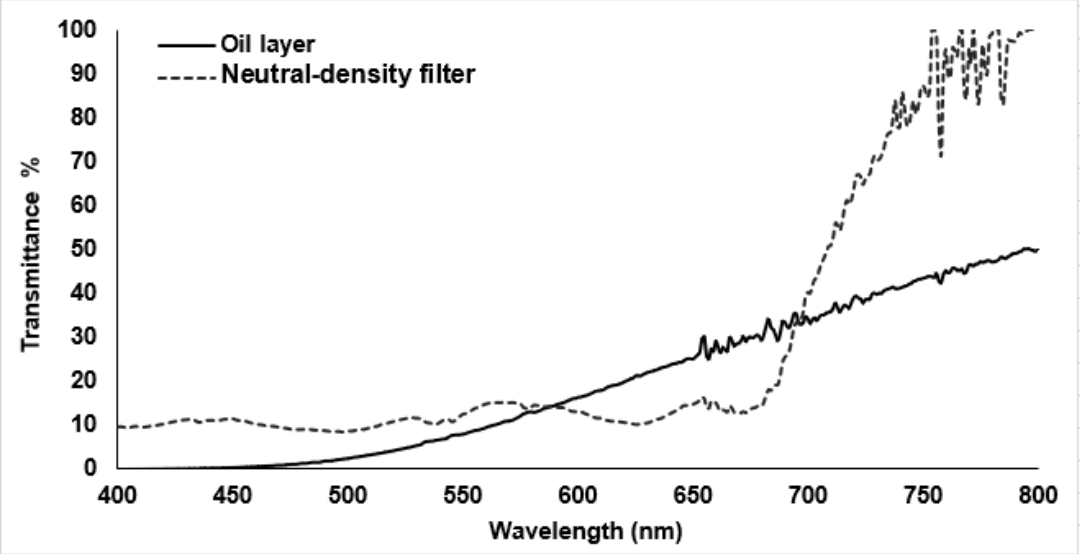

Figure 4.4: Percentage of light transmittance trough: **a) Gyda crude oil layer and b) neutral density-filter, both with the same light intensity (73-76 µmol photons m^{-2} s^{-1}), relative to the no-oil/filter control.**

4.3.2 Growth of *Synechococcus* and *Thalassiosira weissflogii*

Synechococcus displayed different responses to the four treatments (Figure 4.5). There was no difference in the growth rate of the cells grown under the neutral-density filter and the crude oil layer treatment, but these growth rates were higher than the very low growth rates observed in the no-oil/ filter control. Where the cells were in direct contact with oil no growth was observed. There were striking visual differences in the colour of the cultures grown under the different conditions. *Synechococcus* cells grown under an oil layer and under no-oil/ filter control were white, whilst when grown under the neutral-density filter the cells were coloured green (Figure 4.6).

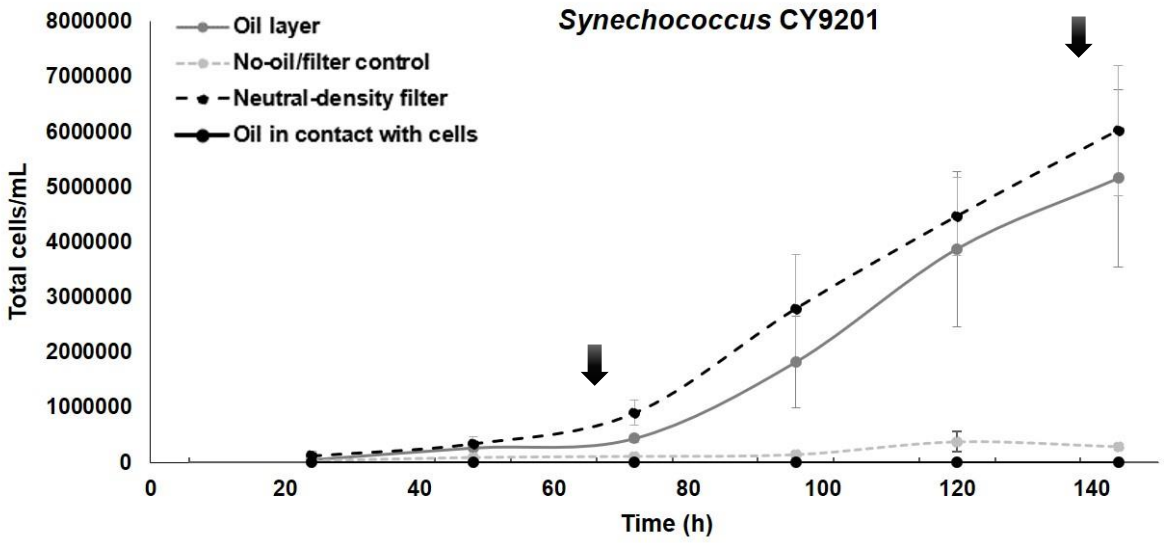

Figure 4.5 Growth of *Synechococcus* CY9201 over 6 days of light and dark (12:12 h) regime, growing under a crude oil layer (74 µmol photons m^{-2} s^{-1}), no-oil/filter control (540-601 µmol photons m^{-2} s^{-1}, neutral-density filter (74 µmol photons m^{-2} s^{-1}), or oil directly in contact with the culture (76 µmol photons m^{-2} s^{-1}). The arrows indicate when samples were taken for chlorophyll and pigment analysis. (Means +/- s.e *n*=4).

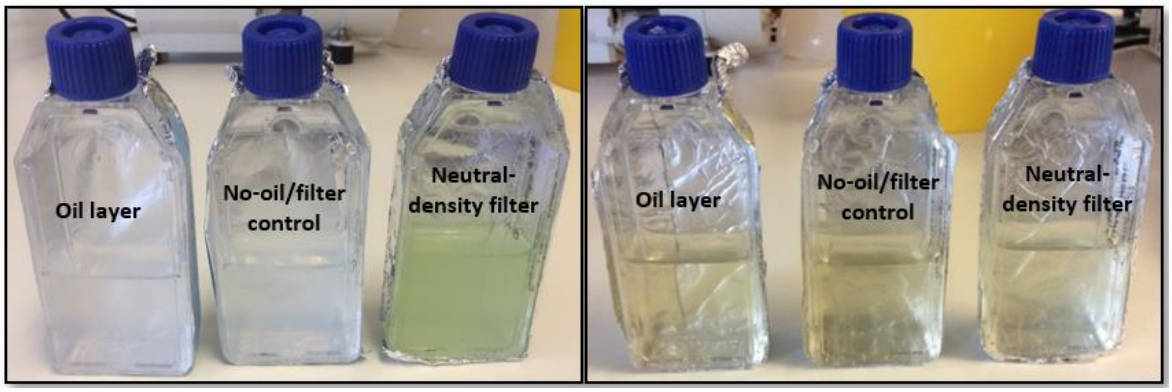

Figure 4.6. Cell-culture flasks with *Synechococcus* CCY9201 (left) and *Thalassiosira weissflogii* CCMP1051 (right) growth after 6 days of treatment: Oil layer (74 µmol photons m^{-2} s^{-1}); no-oil/filter control (540-601 µmol photons m^{-2} s^{-1}) Neutral-density filter (74 µmol photons m^{-2} s^{-1}) in a light and dark (12:12 h) regime.

T. weissflogii also displayed different growth rates between the treatments. The cells grew in all treatments, except when the oil was in direct contact with the culture (Figure 4.7). In the presence of the neutral-density filter and a crude oil layer, the cultures reached a similar cell density with a slower growth rate under the no-oil/filter treatment (Figure 4.7). Also, there were not visual changes in the colour of the cultures, which were all green-brown (Figure 4.6).

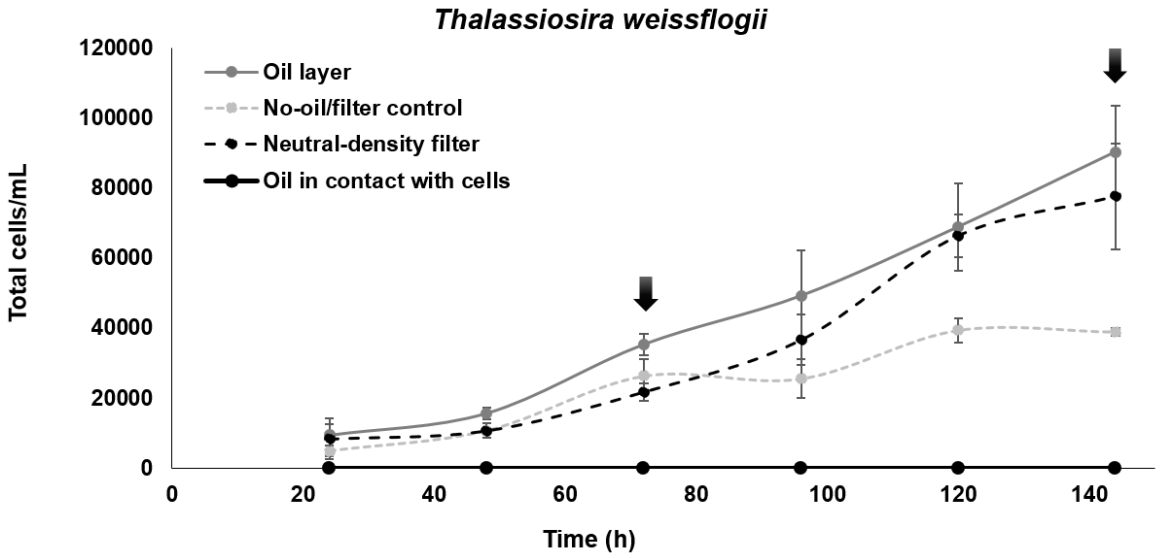

Figure 4.7. Growth of *Thalassiosira weissflogii* CCMP1051 over 6 days of light and dark (12:12 h) regime, growing under a crude oil layer (74 µmol photons m^{-2} s^{-1}), no-oil/filter control (540-601 µmol photons m^{-2} s^{-1}, neutral-density filter (74 µmol photons m^{-2} s^{-1}), or oil directly in contact with the culture (76 µmol photons m^{-2} s^{-1}). The arrows indicate when samples were taken for chlorophyll and pigment analysis. (Means +/- s.e *n*=4).

4.3.3 Fluorometric chlorophyll *a* (Chl *a*) analysis and PSII photochemical efficiency

The Chl *a* content in *Synechococcus* grown under an oil layer, showed no significant differences ($P>0.05$) at day 3 to the no-oil/layer control and neutral-density filter treatments. There was however a significant difference ($P<0.01$) for the cells grown under no-oil/filter control and neutral-density filter treatments. By day 6, the concentration of Chl *a* for the cells grown under the oil layer was significantly lower than those and neutral-density filter ($P<0.05$). But for the cells grown under an oil layer and no-oil/filter control, no significant difference was observed in the concentration of Chl *a* between day 3 and day 6, on the contrary the concentration of Chl *a* was significantly higher ($P<0.05$) at day 6 when using the neutral-density filter treatment (Figure 4.8).

For *Thalassiosira weissflogii* the concentration of Chl *a* (Figure 4.8) was higher at day 6 for the cells grown under a crude oil layer and neutral-density filter in comparison to the no-oil/filter control whereas between day 3 and day 6, no significant difference ($P<0.05$) was observed. In contrast, there was not a significant difference in the concentration of Chl *a* between day 3 and day 6 under the no-oil/filter control. For both days, day 3 and 6, the concentration of Chl *a*, in the control-no oil/filter showed significant differences ($P<0.05$) with the cells grown under an oil layer and neutral-density filter, on the other hand, for the oil and neutral-density filter, the cells showed no significant differences in the concentration of Chl *a* on both days.

There was no significant difference in the photochemical efficiency of photosystem II *(Fv/Fm)* of *T. weissflogii* when grown under a crude oil layer or neutral-density filter treatment (Figure 4.8) and the oil and no-oil/filter control. There was however, a significant

increase (P<0.01) in *Fv/Fm* when grown under the neutral-density filter and compared with the no-oil/filter control. For *Synechococcus*, there was no significant difference in the photochemical efficiency in any of the three treatments. The photochemical efficiency for *Synechococcus* grown in contact with the crude oil could not be measured due to the fact there was no growth observed (Figure 4.5).

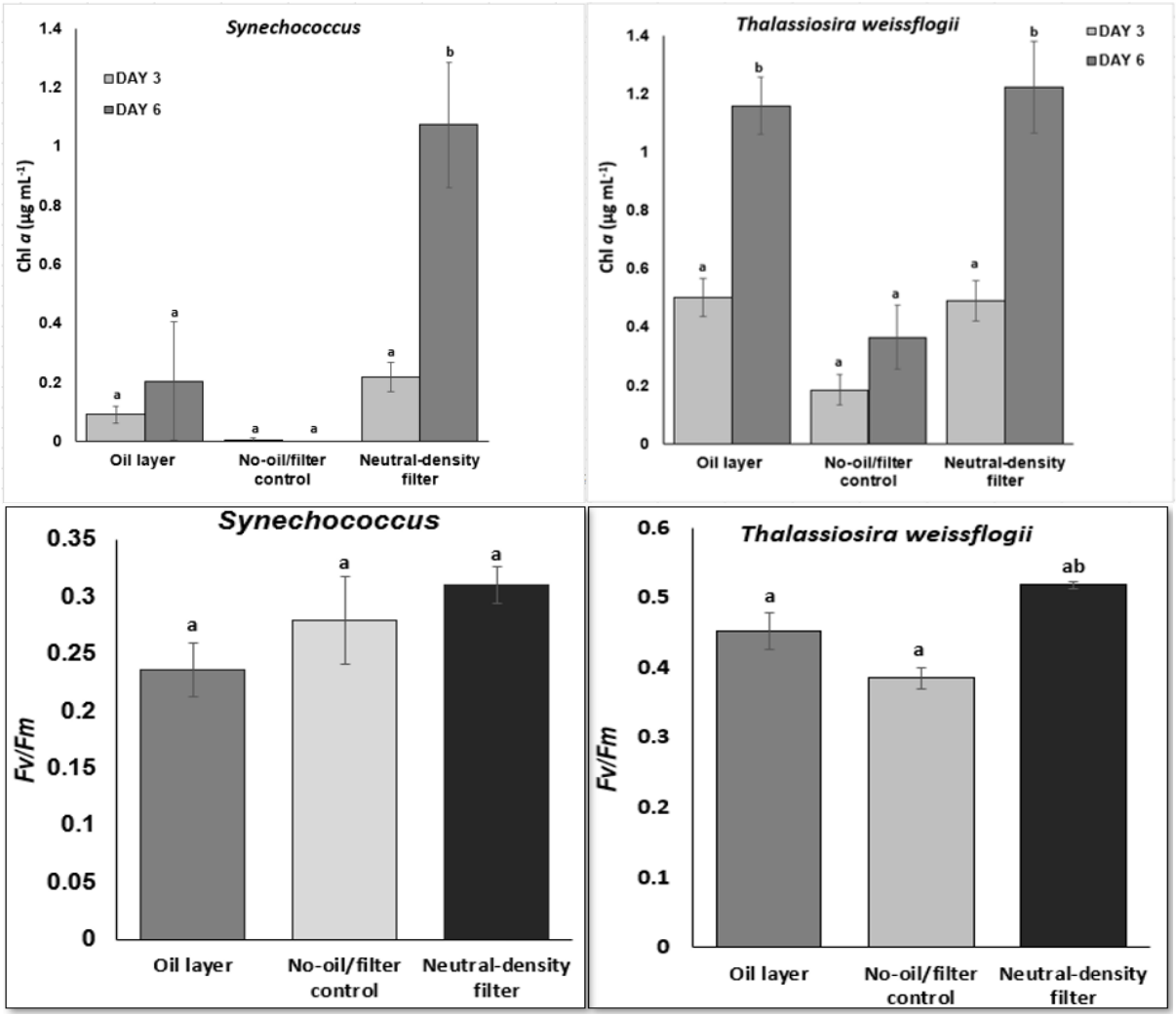

Figure 4.8. Chlorophyll *a* concentration (µg mL^{-1}) at 3 and 6 days, and yield values of PSII photochemical efficiency (*Fv/Fm*) after 6 days of treatment with a light and dark (12:12 h) regime. Oil layer (73-76 µmol photons m^{-2} s^{-1}), no-oil layer/filter control (540-601 µmol photons m^{-2} s^{-1}) and neutral-density filter (74-76 µmol photons m^{-2} s^{-1}). Letters indicate significance differences (one-way ANOVA (Tukey's test) p<0.05) between treatments (Oil layer, no-oil/filter control and neutral-density filter). Mean +/- standard error are shown (*n*=4).

4.3.4 Pigment analysis by UPLC

Photosynthetic pigments were identified by the retention times and the absorption spectra of each peak. In total 13 peaks were detected of which only seven were accurately identified (Table 4.2), the absorption spectra (λ max) of each pigment, the ratio of pigment to chlorophyll a and the pigment concentration per cell can be found in Table 4.3 for *Synechococcus* and Table 4.4 for *Thalassiosira weissflogii*.

For *Thalassiosira weissflogii* the major pigment Chl *a* was identified in all the treatments. Also, two xanthophyll pigments, fucoxanthin and diadinoxanthin were present in all the treatments. Diadinoxanthin was detected only at the end of the experiment (day 6) for oil-layer and neutral-density filter, on the other hand, for the no-oil/filter control it was detected in both days.

On the contrary, for *Synechococcus*, no pigments were identified in the no-oil/filter treatment; this result was expected since the cultures were coloured white (Figure 4.6) and the concentration of the Chl *a* (Figure 4.8) was almost undetectable. For the rest of the treatments, Chl *a* was the major pigment found. Allophycocyanin, another important pigment for cyanobacteria, was found to be present only at day 6 and the carotenoid zeaxanthin was only present during the treatment with the neutral-density filter.

Table 4.2. Concentration of pigments (µg mL^{-1}) of *Synechococcus sp.* and *Thalassiosira weissflogii* during the treatments: OL (Oil layer), CN (no-oil/filter control) and F (neutral-density filter). Number 3 and 6 indicate the day. Unidentified pigments are indicated by ?. Zero (0) indicates pigments were absent or below the limit of detection. *n*=4

Microorganism			*Synechococcus sp.*						*Thalassiosira weissflogii*					
			Concentration µg mL^{-1}											
Peak	Pigment	Retention time	OL3	OL6	CN3	CN6	F3	F6	OL3	OL6	CN3	CN6	F3	F6
1	Fucoxanthin	4.18	0	0	0	0	0	0	0.157	0.534	0.160	0.324	0.119	0.418
2	?	4.32	0	0.001	0	0	0	0	0.018	0	0	0	0	0
3	?	4.52	0	0	0	0	0	0	0.026	0.040	0	0	0.015	0.034
4	Diadinoxanthin	4.97	0	0	0	0	0	0	0	0.092	0.023	0.109	0	0.058
5	?	5.2	0.072	0.061	0	0	0	0	0	0	0.025	0.037	0	0
6	Zeaxanthin	5.48	0	0	0	0	0.207	0.467	0	0	0	0	0	0
7	?	6.13	0.072	0.014	0	0	0	0	0	0	0	0	0	0
8	?	6.34	0	0	0	0	0.015	0.043	0	0	0	0	0	0
9	Allophycocyanin ?	6.7	0	0.013	0	0	0	0.019	0	0	0	0	0	0
10	Chl *a* -like pigment	7.75	0	0	0	0	0	0	0.085	0.095	0	0.018	0.060	0.107
11	Chlorophyll *a*	7.84	0.091	0.205	0	0	0.218	1.072	0.502	1.160	0.183	0.364	0.490	1.224
12	?	8.35	0.013	0.010	0	0	0.027	0.034	0	0	0	0	0.009	0.012
13	Solvent	14.56	0.453	0.057	0	0	0.197	0.114	0.410	0.190	0.351	0.356	0.254	0.190

Table 4.3. Concentration of pigments per cell (µg/cell), ratio of pigment to chlorophyll a and absorption spectra (λ max) of each pigment (nm) of *Synechococcus sp.* during the treatments: OL (Oil layer) and F (neutral-density filter). Number 3 and 6 indicate the day. Unidentified pigments are indicated by ?. Zero (0) indicates pigments were no concentration because there were absent of below the limit of detention. No pigments were detected under CN (no-oil/filter control). *n*=4

Treatment	OL3			OL6			F3			F6		
Pigment	Concentration Pigment per cell	Ratio	λ Max (nm)	Concentration Pigment per cell	Ratio	λ Max (nm)	Concentration Pigment per cell	Ratio	λ Max (nm)	Concentration Pigment per cell	Ratio	λ Max (nm)
Fucoxanthin	0	1:0	-	0	1:0	-	0	1:0	-	0	1:0	
?	0	1:0	-	5.7×10^{-11}	20000:7	636, 646	0	1:0	-	0	1:0	-
?	0	1:0		0	1:0	-	0	1:0	-	0	1:0	-
Diadinoxanthin	0	1:0	-	0	1:0	-	0	1:0	-	0	1:0	-
?	9.7×10^{-08}	9:7	450, 486, 635	5.2×10^{-09}	10:3	458, 636	0	1:0	-	0	1:0	-
Zeaxanthin	0	1:0		0	1:0		1.5×10^{-07}	21:20	455	3.2×10^{-08}	107:46	453
?	9.7×10^{-08}	9:7	636	1.2×10^{-09}	20:1	637	0	1:0		0	1:0	
?	0	1:0	-	0	1:0	-	1.1×10^{-08}	21:1	632	2.9×10^{-09}	107:4	640
Allophycocyanin?	0	1:0	-	1.1×10^{-09}	20:1	643	0	1:0	-	1.3×10^{-09}	107:1	633
Chl *a* like pigment	0	1:0	-	0	1:0	-	0	1:0	-	0	1:0	-
Chlorophyll *a*	1.2×10^{-07}	1:1	434, 642	1.8×10^{-08}	1:1	430, 642	1.6×10^{-07}	1:1	434, 642	7.3×10^{-08}	1:1	431, 662
?	1.7×10^{-08}	9:1	486, 642	8.7×10^{-10}	20:1	524, 642	1.9×10^{-08}	21:2	642	2.4×10^{-09}	107:3	642
Solvent	0	1:5	640	0	4:1	640	0	21:19	640	0	107:11	640

Table 4.4. Concentration of pigments per cell (µg/cell), ratio of pigment to chlorophyll a and absorption spectra (λ max) of each pigment (nm) of *Thalassiosira weissflogii* during the treatments: OL (Oil layer), CN (no-oil/filter control) and F (neutral-density filter). Number 3 and 6 indicate the day. Unidentified pigments are indicated by ?. Zero (0) indicates pigments were no concentration because there were absent of below the limit of detention. *n*=4

Treatment	OL3			OL6			CN3			CN6			F3			F6		
Pigment	Conc. Pigment per cell	Ratio	λ Max (nm)	Conc. Pigment per cell	Ratio	λ Max (nm)	Conc. Pigment per cell	Ratio	λ Max (nm)	Conc. Pigment per cell	Ratio	λ Max (nm)	Conc. Pigment per cell	Ratio	λ Max (nm)	Conc. Pigment per cell	Ratio	λ Max (nm)
			448,			448,			450,						446,		122:4	446,
Fucoxanthin	2.62×10^{-06}	10:3	479	1.99×10^{-06}	116:53	477	3.84×10^{-06}	9:8	476	2.23×10^{-06}	9:8	446,476	2.94×10^{-06}	49:11	476	1.89×10^{-06}	1	475
?	3.05×10^{-07}	50:1	633	0	1:0		0	1:0	-	0	1:0	-	0	1:0		0	1:0	
?	4.28×10^{-07}	25:1	635	1.49×10^{-07}	29:1	651	0	1:0	-	0	1:0	-	3.71×10^{-07}	49:1	652	1.57×10^{-07}	122:3	635
									423,			448,						445,
Diadinoxanthin	0	1:0	-	3.43×10^{-07}	116:9	453,486	5.62×10^{-07}	9:1	485	7.52×10^{-07}	18:5	479	0	1:0	-	2.63×10^{-07}	122:5	487
												445,						
?	0	1:0	-	0	1:0	-	6.01×10^{-07}	9:1	632	2.57×10^{-07}	12:1	642	0	1:0	-	0	1:0	
Zeaxanthin	0	1:0	-	0	1:0	-	0	1:0	-	0	1:0	-	0	1:0	-	0	1:0	
?	0	1:0	-	0	1:0	-	0	1:0	-	0	1:0	-	0	1:0	-	0	1:0	
?	0	1:0	-	0	1:0	-	0	1:0	-	0	1:0	-	0	1:0	-	0	1:0	
Allophycocyanin?	0	1:0	-	0	1:0	-	0	1:0	-	0	1:0	-	0	1:0	-	0	1:0	
Chl *a* like															434,			428,
pigment	1.42×10^{-06}	25:4	646	3.54×10^{-07}	116:9	642	0	1:0		1.25×10^{-07}	36:1	642	1.48×10^{-06}	49:6	642	4.87×10^{-07}	61:5	646
			432,			430,			430,			433,			431,			430,
Chlorophyll *a*	8.32×10^{-06}	1:1	643	4.32×10^{-06}	1:1	662	4.4×10^{-06}	1:1	642	2.5×10^{-06}	1:1	642	1.21×10^{-05}	1:1	662	5.54×10^{-06}	1:1	662
?	0	1:0		0	1:0		0	1:0		0	1:0		2.44×10^{-07}	49:9	642	5.6×10^{-08}	122:1	642
																	122:1	
Solvent	0	50:41	642	0	116:19	642	0	18:35	642	0	36:35	642	0	49:25	642	0	9	642

The profiles of pigments produced by *Synechococcus* and *Thalassiosira weissflogii* were different in all the treatments (Figure 4.9 and 4.10). The major differences observed were at day 6 in cultures grown under the oil layer (OL) and neutral-density filter (F) for *Thalassiosira weissflogii* (t) and neutral-density filter (F) for *Synechococcus* (s). Early at day 3, there are less differences in the pigment profiles between the treatments.

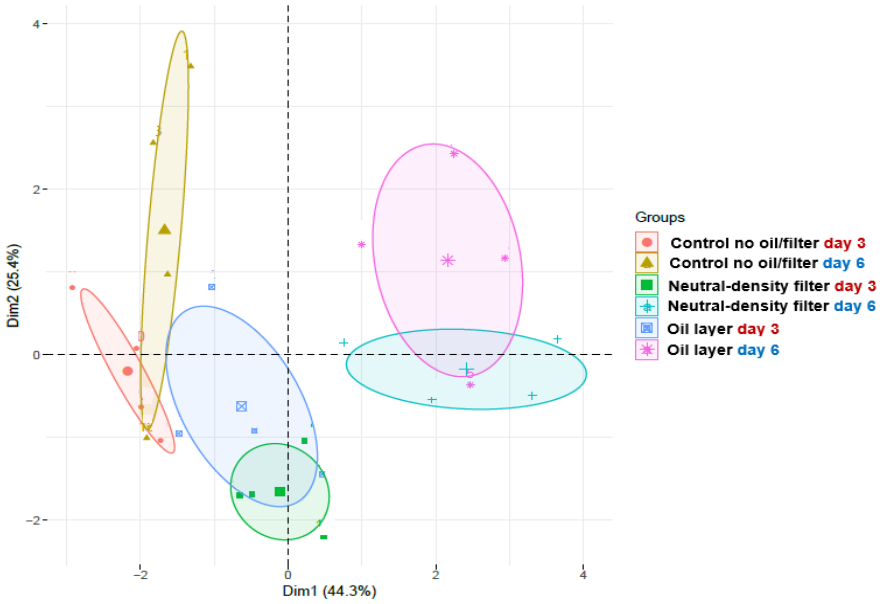

Figure 4.9 Principal component analysis (PCA) of the pigment profiles *Thalassiosira weissflogii* CCMP1051 in all the treatments at day 3 and 6. (OL) Oil layer (73-76 µmol photons m^{-2} s^{-1}), (CN) no-oil layer/filter control (540-601 µmol photons m^{-2} s^{-1}) and (F) neutral-density filter (74-76 µmol photons m^{-2} s^{-1}) in light and dark conditions (12:12 h).

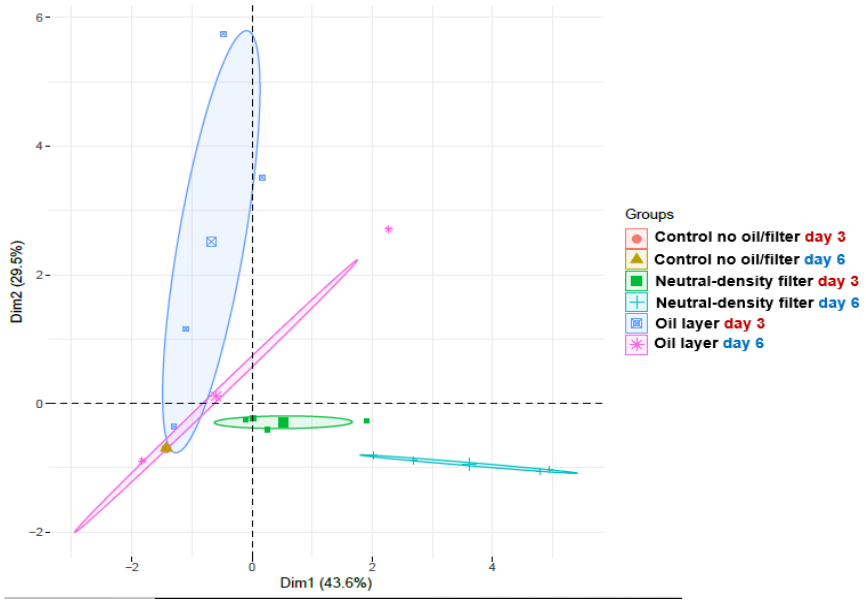

Figure 4.10 Principal component analysis (PCA) of the pigment profiles of *Synechococcus* CY9201 in all the treatments at day 3 and 6. (OL) Oil layer (73-76 µmol photons m^{-2} s^{-1}), (CN) no-oil layer/filter control (540-601 µmol photons m^{-2} s^{-1}) and (F) neutral-density filter (74-76 µmol photons m^{-2} s^{-1}) in light and dark conditions (12:12 h).

4.4 Discussion

The effect of crude oil on the transmitted light (Figure 4.3 and 4.4) was similar to the effect observed using Heliospectra artificial lights (Figure 2.2 and 2.3) in which the attenuation of light by oil is higher in the shorter wavelengths (400-530 nm). The neutral-density filter and oil layer attenuated similar amounts of light, although the neutral-density filter allows the transmission of significantly more light in the longer wavelengths than the oil (700-800 nm). The neutral-density filters absorbed light evenly across the visible spectrum (350-700 nm) and because they are made up of yellow, magenta and cyan colours, the absorption declines at 700 nm, and more red light is transmitted (Thomas-Davis, 2018).

The growth rate for *Thalassiosira weissflogii*, under a crude oil layer or neutral-density filter was equally fast and with no difference in the production of Chl *a*, but in the no-oil/filter control treatment, the growth rate was significantly lower and coupled with a significantly lower concentration of Chl *a*. This could indicate that light intensity is affecting the growth of *Thalassiosira weissflogii*, Goericke and Welschmeyer (1992) found that 75 µmol photons m^{-2} s^{-1} of irradiance is the growth rate saturation for *Thalassiosira weissflogii*, also it is known in general that the concentration of Chl *a* declines before light saturation of growth rate (Myers, 1970) with increasing irradiance (Thompson, 1999) indicating photoacclimation of the cells (McIntyre *et al*., 2002). The response to the pigments to changing irradiance is different depending on the phototrophic species (Moore *et al*., 2006). These results showed that the presence of oil protects the cell from high amounts of irradiance, but if the oil is significantly reducing the transmittance of shorter wavelengths (400-530 nm) *Thalassiosira weissflogii* may need to produce

accessory pigments to allow it to survive this condition. The only two accessory pigments, identified were fucoxanthin and diadinoxanthin. Fucoxanthin in diatoms is part of the protein fucoxanthin-chlorophyll (FCP) complex responsible for light-harvesting (Gelzinis *et la*., 2015). Fucoxanthin may play an important role in absorption of light for all the treatments, especially under an oil layer, as a 3.5-fold increase in the concentration of this pigment was observed at day 6. Fucoxanthin pigments absorb light better than the Chl *a* in the region known as the green gap (Gelzinis *et la*., 2015) which is between 400-500 nm (Gundlach *et al*., 2009). Despite the observed differences in the growth rate and concentration of Chl *a*, the photochemical efficiency showed no difference among the three treatments, which implies that the cells in the no-oil/filter control treatment also are using others pigments to absorb light and utilized it for photosynthesis. One of these pigments is the carotenoid diadinoxanthin that plays an important role in the photoprotection of diatoms (Kuczynska *et al*., 2015) and which was only present at both days with the no-oil/filter treatment, which had a relatively high light intensity. Diadinoxanthin is part of the xanthophyll cycle and its content depends of the amount of light (Dimier *et al*., 2009). This is a strategy to minimize the damage cause by the excess of energy at the high intensity levels (Lavaud *et al*., 2002).

In the case of *Synechococcus*, the growth rate based on cell counts between cultures grown under neutral-density filters or crude oil layer showed no significant differences (Figure 4.5). However, at day 6, the concentration of Chl *a* was 4.5 times higher with the neutral-density filter than with the oil layer (Figure 4.8). Due to the fact that these two treatments received the same amount of light intensity, the changes in the chlorophyll *a*

concentration may more likely be related to the received light spectrum. The cells grown under the oil layer were white and lacked the characteristic colour green of the *Synechoccocus* strain CCY9201 (Stomp *et al*., 2008). The green colour of this strain is from the dominant pigment phycocyanin along with chlorophyll *a* (Stomp *et al*., 2008; Simis *et al*., 2012); although phycocyanin was not identified in any of the treatments. Nevertheless, allophycocyanin (phycobiliprotein) was detected at day 6 with both treatments, oil layer and neutral-density filter. Allophycocyanin along with phycocyanin, phycoerythrin (some cyanobacteria) and Chl *a*, is part of the multiprotein complex called phycobilisome, responsible of the harvesting light energy in cyanobacteria (Govindjee and Shavela, 2011). The content and composition of the phycobiliprotein changes in relation of the quality and quantity of light (Masamoto and Furukawa, 1997). Phycocyanin was not detected in any of the treatments, possible because the absorption band of this protein is at 580 and 625 nm, and allophycocyanin have an absorption band at 625 and 650 nm (Govindjee and Shevela, 2011).

Wang *et al*. (2007) found that the largest specific growth rate occurred when using red LED light, and Govindjee and Shevela (2011) mention that “long-wave” (red light) photosystem in cyanobacteria is run by Chl *a* (670-680 nm). Also, it has been reported that *Synechococcus* strain CCY9201 absorbs more efficiently red light (Stomp *et al*., 2008). These explanations are inconsistent with our findings, the neutral-density filter transmitted more light at longer wavelengths (Figure 4.4) than the oil layer, however, *Synechococcus* produce a higher concentration of chlorophyll *a* under the neutral-density filter. Possibly, the answer is in the carotenoids but the only one detected was zeaxanthin,

which is one of the two major carotenoids present in *Synechococcus* and is essential for photoprotection (Sarnaik *et al*.,2017) which mean that *Synechococcus* depends more on the light intensity; however, zeaxanthin was only found under the treatment with a neutral-density filter.

The results also showed that the growth rate of *Synechococcus* was extremely slow with no-oil/filter control than with the other two treatments, indicating a physiological response of the cells to high light intensity, which leads to a decline of photosynthetic capacity, and oxygen production and CO_2 fixation, known as photoinhibition (Kulkarni and Golden, 1994). Exposure of microalgae to bright light can result in a photoinhibitory reduction in the number of functional PSII reaction centres and a consequential decrease in the maximum quantum yield of photosynthesis (Behrenfeld *et al*. 1998); however, *Synechococcus* showed an equally low photochemical efficiency with all the treatments.

There are some limitations in the study for both phototrophs, which are important to consider: 1) when cells were pre- grown for *Synechococcus* and *T. weissflogii*, the cell density of the inoculum was not measured properly, which is an important factor to standardize the growth of microorganisms. This could affect the capacity of the microorganisms to growth within the different treatments. 2) Lack of proper controls, as media only control without any of the phototrophs.

Also, for both microorganisms, the presence of other pigments not identified could play a part in the way they react to the treatments, although most of these pigments have a very low concentration and this also contributed on difficulty of the identification.

4.5 Conclusion

The overall aim was to compare the effect of oil attenuation on the growth and physiology of *Synechococcus* CY9201 and *Thalassiosira weissflogii* CCMP1051. Both phototrophs were subject to four treatments, under a Gyda crude oil layer, under a neutral-density filter, under no-oil/filter control and oil in direct contact with the cultures. The results showed that for *T.weissflogii*, the growth and concentration of chlorophyll *a*, was not significantly different between the crude oil and neutral-density filter. For *Synechococcus* the cell-growth was no significant different between crude oil and neutral-density filter but the concentration of chlorophyll *a* was higher with the neutral-density filter. With the no-oil/filter control for both phototrophs, the growth and concentration of chlorophyll *a* was lower than the other two treatments. Also, it was observed that in response to the changes on light intensity and spectrum with the four treatments *T. weissflogii* and *Synechococcus* produced different pigments besides chlorophyll *a*. *T. weissflogii* produced fucoxanthin, a component of the light-harvesting complex for photosynthesis in diatoms and diadinoxanthin, used for photoprotection. *Synechococcus* produced allophycocyanin, responsible of the harvesting light energy in cyanobacteria and zeaxanthin, used for photoprotection.

CHAPTER 5

Do *Synechococcus* CCY9201 and *Thalassiosira weissflogii* CCMP1051 support the growth of marine hydrocarbon-degrading bacteria by the production of alkanes?

5.1 Introduction

The oceans represent the largest biome on Earth. Bacteria, archaea and protists comprise the majority of biomass in the ocean (Amin *et al.*, 2012). Phytoplankton mass in the ocean only amounts ~1-2 % of the total global plant carbon (Falkowski, 1994). Despite this relative low biomass, marine phytoplankton contribute roughly one half of the global carbon fixation and producing almost half of the oxygen in the atmosphere playing a key role in regulating global biogeochemical cycles (Field *et al.*, 1998), especially through the process of photosynthesis (Helbling *et al*., 2011).

Among the cyanobacteria, two groups, *Synechococcus* and *Prochlorococcus* were reported to be the predominant component of the phytoplankton (Liu *et al*., 1995). *Phrochlorococcus* is estimated to account for ~15% of the ocean primary production (Valentine and Reddy, 2015). Also, diatoms, which can be found worldwide in oceans and freshwater, play an important role in the global carbon cycle as they are thought they may contribute ~20% to global primary production (Malviya *et al*., 2016).

Phytoplankton release considerable amounts of organic components into the extracellular medium, most of which are utilised by bacteria (Arora *et al.*, 2012). Carbohydrates, especially polysaccharides, can comprise 80-90% of the total extracellular release

(Myklestad, 1995). Planktonic algae can also produce few hydrocarbons of simple structure compared to those of ancient sediments and fossil fuels (Blumer *et al.*, 1971). Cyanobacteria synthesize C_{15}, C_{17} and C_{19} hydrocarbons via two pathways, via acyl-ACP reductase and aldehyde deformylating oxygenase enzymes that produce alkanes, and via a polyketide synthase enzyme that generates alkenes (Lea-Smith *et al*., 2015). Based on global population sizes Leah-Smith *et al*. (2015) estimated that *Synechococcus* and *Prochlorococcus* have the capacity to produce 2–540 pg alkanes per ml per day, which translates into a global ocean yield of ~308–771 million tons of hydrocarbons annually; and they demonstrated that facultative and obligate hydrocarbonoclastic bacteria (OHCB) can consume alkanes produced by cyanobacteria thus preventing the accumulation of these hydrocarbons in the environment (Figure 5.1).

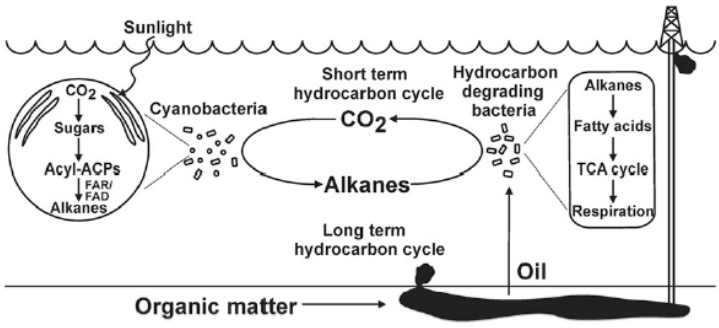

Figure 5.1. Hydrocarbon cycles: short term cycle (days) that use alkanes from cyanobacterial photosynthesis and long-term hydrocarbon (thousand to millions of years) where alkanes are used by hydrocarbon-degrading bacteria (Leah-Smith *et al*., 2015).

In the marine environment, bacteria are generally considered to represent the predominant hydrocarbon-degrading element of the microbial community (Atlas and Bragg, 2009). Many OHCB also have been isolated from marine areas: *Alcanivorax,*

Cycloclasticus, *Marinobacter* and *Thalassolituus* (Harayama *et al*., 2004). Hara *et al*. (2003) found that *Alcanivorax borkumensis* exhibited higher ability to degrade branched alkanes; species of *Cycloclasticus* utilize aromatic hydrocarbons; including biphenyl, naphthalene, phenanthrene, toluene, and xylenes, as sole sources of carbon and energy (Kasai *et al*., 2002). *Thalassolituus oleivorans* were the dominant n-alkane (C_{12}-C_{32}) degraders (Mckew *et al*., 2007) and can use a variety of branched and/or straight chain saturated hydrocarbons (Head *et al*., 2006). *Marinobacter hydrocarbonoclasticus*, form biofilms between water and n-alkanes (C_8-C_{28}), the biofilm formation is determined by the presence of nutrients in the interface and can also metabolize n-alcohols (C_{12}-C_{16}) (Mounier *et al*., 2014).

The overall aim is to investigate if hydrocarbonoclastic bacteria can grow on hydrocarbons produced by photosynthetic microalgae and the effect of hydrocarbon-degrading bacteria on microalgae growth.

With this information the next questions were formulated:

- How much and which types of hydrocarbon are produced by *Synechococcus* and *Thalassiosira weissflogii*?
- Can photosynthetic microalgae sustain the growth of hydrocarbonoclastic bacteria by producing hydrocarbons?
- What is the effect of hydrocarbon-degrading bacteria on microalgal growth?

5.1.1 Hypothesis

- *Synechococcus* and *Thalassiosira weissflogii* will be able to produce n-alkanes which will sustain the growth of HCB.

5.2 Methodology

5.2.1 Experiment design

Two microalgae, *Synechococcus* CCY9201, *Thalassiosira weissflogii* CCMP1051, and three hydrocarbonclastic bacteria (HCB), *Alcanivorax borkumensis* SK2, *Marinobacter hydrocarbonoclasticus* SP17 and *Thalassolituus oleivorans* MIL-1 were used in this experiment.

The cultures were pre-grown in a cell-culture flask (Noc^{TM} Easy Flask^{TM} 25 cm^2, Nunclon^{TM} Delta Surface, Thermo Scientific) for 7 days in marine BG11 (Appendix E) for *Synechococcus* and f/2 medium (Appendix A) for *T.weissflogii*. For the HCB, tetradecane (0.1 % v/v) was added as a carbon source. All the microorganisms were grown in a light:dark regime (12:12 h) with a light intensity of 250-280 µm photons m^{-2} s^{-1} at 20°C. The HCB were harvested by centrifugation (15,000 ×*g*, for 10 min), rinsed three times with sterile BG11 or f/2 medium, resuspended and diluted in the same media. These cells, were used to inoculate the experimental media to achieve a final bacterial cell density of ~ 1× 10^5. *Synechococcus* and *T. weissflogii* were harvested in exponential phase and the heterotrophs and phototrophs were combined in a ratio of ~5: 1 HCB: *Synechococcus* ratio and ~50: 1 HCB: *T. weissflogii*.

To determine the cell density (number of cells mL^{-1}) of the inoculum, cells counts were made along with optical density in a 10-fold diluted culture. Light microscopy examination, Leica DME compound microscope (Meyer instruments, USA), was used for cell counts of *Thalassiosira weissflogii* (×10 objective), *Synechococcus* and HCB (×40 objective) using an improved Neubauer haemocytometer (Weber England). Optical density was determined by measuring absorbance at 600 nm with a spectrophotometer (Jenway 6300).

Cell culture flasks with 40 mL of BG11 were prepared as follows in duplicates at two different times (i.e. in quadruplicate overall):

1- *Synechococcus* (S)
2- *Synechococcus* + *Alcanivorax borkumensis* (S + A)
3- *Synechococcus* +*Marinobacter hydrocarbonoclasticus* (S + M)
4- *Synechococcus* + *Thalassolituus oleivorans* (S + T)
5- *Alcanivorax borkumensis* + tetradecane (0.01% v/v) (As)t
6- *Marinobacter hydrocarbonoclasticus* + tetradecane (0.01% v/v) (Ms)t
7- *Thalassolituus oleivorans* + tetradecane (0.01% v/v) (Ts)t
8- Tetradecane (0.01% v/v)
9- No alkane/inoculum only media

Cell culture flasks with 40 mL of f/2 medium were also prepared as follows in duplicates, at two different times (i.e. in quadruplicate overall):

1- *Thalassiosira weissflogii* (Tw)

2- *T. weissflogii* + *Alcanivorax borkumensis* (Tw + A)

3- *T. weissflogii* + *Marinobacter hydrocarbonoclasticus* (Tw + M)

4- *T. weissflogii* + *Thalassolituus oleivorans* (Tw + T)

5- *Alcanivorax borkumensis* + tetradecane (0.01% v/v) (Atw)t

6- *Marinobacter hydrocarbonoclasticus* + tetradecane (0.01% v/v) (Mtw)t

7- *Thalassolituus oleivorans* + tetradecane (0.01% v/v) (Ttw)t

8- Tetradecane (0.01% v/v)

9- No alkane/inoculum only media

For experiments with both phototrophs growth consisted of a light:dark regime (12:12 h) with a light intensity of 250-280 µm photons m^{-2} s^{-1} at 20°C for 10 days.

5.2.2 Chlorophyll a extraction (see section 2.2.6)

5.2.3 Fluorometric Chlorophyll Analysis (see section 2.2.7)

5.2.4 DNA extraction (see section 2.2.2)

5.2.5 Quantification of heterotrophs and phototrophs by qPCR

In order to prepare standard curves for qPCR, cultures (*Synechococcus*, *T.weissflogii*, and HCB) were grown in the same conditions as the experiments, except for HCB which were grown with 1 % (v/v) of tetradecane. After 7 days, 2 mL was taken from each culture and genomic DNA was extracted (section 2.3.2) to prepare the standards. The amplification was carried out in a total volume of 50 µL containing 0.5 mM of each primer, 25 µL of 2× AppTaq RedMix and 1µL of DNA template. The reactions were performed in

Applied Biosystems™ SimpliAmp™ thermal cycler. The PCR was performed under the following conditions: initial denaturation at 95°C for 3 min, followed by 30 cycles of denaturation at 95°C for 15 s, annealing at 55°C for 15 s, extension at 72°C for 30 s; and final extension at 72°C for 7 min.

Primers were designed to amplify the alkane 1-monooxygenase (*alkB*) gene in HCB, the phycocyanin subunit A (*cpcA*) gene in *Synechococcus* and the ribulose-1,5-biphosphate carboxylase/oxygenase small subunit (*rcbS*) gene of *T. weissflogii*. The sequence of the primers are shown Table 1. All the primers were designed to be specific for the HCB and phototrophs strains.

Table 5.1. List of primers used for amplifying *alkB*, c*pcA* and *rcbS* genes. F (forward) and R (reverse) indicate the orientation of the primers.

Primer Name	Sequence (5'-3')	Insert Size	Microorganism Target	Target Gene
A*alkB*-F	CGCCGTGTGAATGACAAGGG	132	*Alcanivorax borkumensis* SK2*	*alkB*
A*alkB*-R	CGACGCTTGGCGTAAGCATG			
T*alkB*-F	GACGTCGCCACACCTGCC	217	*Thalassolituus oleivorans** MIL-1	*alkB*
T*alkB*-R	GGGCCATACAGAGCAAGCAA			
M*alkB*-F	CTGGGTCCTTTCTGTGCTGT	193	*Marinobacter hydrocarbonoclasticus* SP17	*alkB*
M*alkB*-R	ACGATCCTGTTCAAGCCGAG			
S*CpcA*-F	TCTCCAACACCGAGCTCAAC	103	*Synechococcus* CCY9201	c*pcA*
S*CpcA*-R	TTCACCAGCTCATCGGCTTT			
TW*rcbS*-F	TGCAGCTGGTTACATCCGT	92	*Thalassiosira weissflogii* CCMP1051	*rcbS*
TW*rcbS*-R	CGTTAGCTGGACGGTTTGTG			

*Designed by Benjamin H. Gregson

The primer sets were designed using primer3 and BLAST. Sequence of the genes *alkB*, *cpcA* and *rcbS*, were obtained from UniProt for each specific microorganism, then were

compared against their whole or partial genome using GenBank database and aligned. The expected size of the PCR products was confirmed by gel electrophoresis. Each primer was chosen to amplify a product of a size between 90-200 bp, with an content between 50-55% of GC and a primer melting temperature (Tm) of 60°C; the annealing temperature was optimised with a using a gradient PCR with three different annealing temperatures (55, 59, 60, 65). The PCR was done with the same DNA and conditions as previously described.

Each amplicon was cleaned with QIAquick PCR purification kit as follows: to bind the DNA 5 volumes of binding buffer (PBI) were added to 1 volume of the amplicon, mixed and placed in a QIAquick spin column in a 2 mL collection tube and centrifuged at 11,300 ×*g* for 30 s. After, the flow-through was dicarded and the QIAquick column was returned to the same tube and 750 µL of wash buffer (PE) with ethanol was added to the QIAquick column and centrifuged at 11,300 ×*g* for 30 s. The flow-through was dicarded and the QIAquick column was placed into the same tube and centrifuged again for an additional 1 min. To elute the DNA, the QIAquick column was placed in a 1.5 mL microfuge tube and 100 µL MilliQ water was added left to stand for 1 min and centrifuged at 11,300 ×*g* again for 1 min.

The concentration of each cleaned double-stranded DNA (dsDNA) was determined using the Quant-IT PicoGreen dsDNA Assay Kit (Thermo Fisher) and a NanoDrop 3300

Fluorospectrometer according to the manufacture's protocol. The number of copies of each gene was determined using the following equation:

$$\text{Number of gene copies (molecules): } \frac{X\text{ng} * 6.0221\times10^{23}\ \text{molecules/mole}}{(N * 660\ \text{g/mole}) * 1\times10^{9}\ \text{ng/g}}$$

Where X = amount of amplicon (ng)
N = Number of base pairs (bp) of dsDNA amplicon
660 g/mole = average mass of 1 bp dsDNA

A standard curve for each primer set was created using ten-fold serial dilutions of their target dsDNA ranging from 10^1 to 10^7. The qPCR amplification and analysis were performed using the real time PCR thermal cycler (CFX96 Bio-Rad). The PCR mixture was prepared using: 5 µL of mastermix SensiFAST™ SYBR® green (Bioline), 0.4 µL of each primer (10 µM), 3.2 µL of PCR-grade water and 1 µL of DNA template. A 96 well white optical plate and optical film (Bio-Rad) for sealing were used. The thermal cycling protocol was as follows: initial denaturation at 95 °C for 3 min, followed by 40 cycles of denaturation at 95°C for 10 s, annealing at 60°C for 30 s, at the end of the cycles, a melting curve from 65°C to 95°C. Standards and no-template control (NTC) reactions were done in triplicate, unknowns were performed with technical duplicates (in addition to the experimental quadruplicates described above). Gene copies mL^{-1} were estimated using Bio-Rad CFX Manager Software 3.1. To calculate from gene copies to cells/mL, it was assumed that genes (*alkB*, *cpcA* and *rcbS*) are present as a single copy per genome, except for *Alcanivorax borkumensis* which has two copies of *alkB* genes (*alkB*1 and *alkB*2).

5.2.6 Statistical analysis

Differences among treatments were analysed by one-way analysis of variance (ANOVA) and a post-hoc analysis, Tukey's test. Statistical analyses were performed using R studio 3.4.1.

5.3 Results

5.3.1 Quantification of hydrocarbon degraders and phototrophs by qPCR

A set of primers was designed for each species targeting specific genes. All primers were tested by end-point PCR before being used for qPCR, and the product size was confirmed by agarose gel electrophoresis. Also, in the qPCR, all the primer sets produced a single curve which confirm the specificity of the primer annealing, except for *Thalassolituus oleivorans* (Appendix E) which produced two more peaks that could indicate some non-specific annealing or/and primer-dimer formation.

The positive controls, HCB growing on tetradecane, demonstrated that the HCB were actively growing on tetradecane as their abundance was consistently higher by 50-60% for *A. borkumensis*, 95% for *M. hydrocarbonoclasticus* and 63-83% for *T. oleivorans* comparing with the starting abundance. Growth on this relatively high concentration of tetradecane was also (as expected) higher than in the presence of phototrophs as a source of alkanes.

Compared to the inoculum there was a reduction in abundance of all three HCB when grown in the presence of the two phototrophs (Figure 5.3 and 5.4), with the notable exception of *M. hydrocarbonoclasticus* in the presence of *Synechococcus* (Figure 5.2). On the other hand, *M. hydrocarbonoclasticus* and *T. oleivorans* were both more significantly abundant ($P<0.01$) when grown with *Synechococcus* compared to grown with *T. weissflogii*, whereas *A. borkumensis* ($P>0.05$) was not significantly different when grown with both phototrophs.

Synechococcus was significantly abundant ($P>0.05$) when grown alone than in the presence of HCB, but there was no difference in the grown of *Synechococcus* with the three HCB (Figure 5.5); whereas the abundance of *T. weissflogii* was the same when grown alone as in the presence of HCB (Figure 5.6).

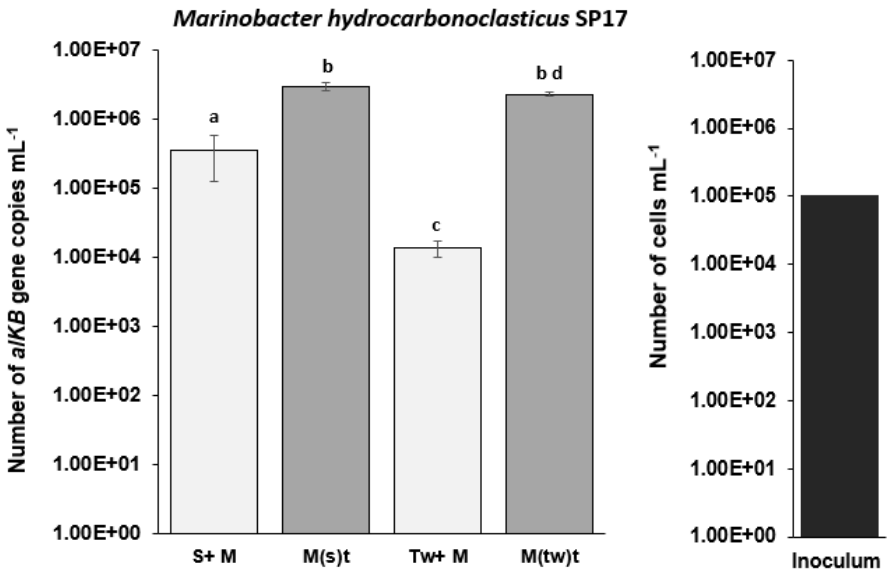

Figure 5.2. Left: Number of *alkB* genes mL^{-1} after 10 days in a 12:12 light and dark regime, 20°C and light intensity of 250-280 µm photons m^{-2} s^{-1}. *Synechococcus* + *Marinobacter hydrocarbonoclasticus* (S + M), *M. hydrocarbonoclasticus* with tetradecane (0.01% v/v) in marine BG11 media (M(s)t); *T.weissflogii* + *M. hydrocarbonoclasticus* (Tw + M), *M. hydrocarbonoclasticus* with tetradecane (0.01% v/v) in f/2 media (M(tw)t). Mean +/- standard error are shown (n=4). Right: Total of cells counts of *M. hydrocarbonoclasticus* by optical density at 600 nm. Letters a, b, c and d indicate significance differences (one-way ANOVA (Tukey's test) p<0.05) between phototrophs + HCB and HCB with tetradecane.

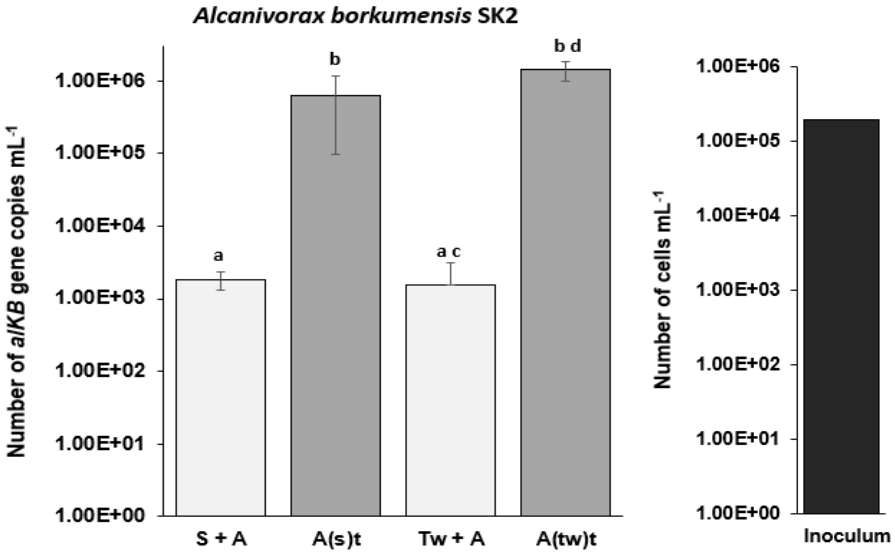

Figure 5.3. Left: Number of *alkB* genes mL^{-1} after 10 days in a 12:12 light and dark regime, 20°C and light intensity of 250-280 µm photons m^{-2} s^{-1}. *Synechococcus* + *Alcanivorax borkumensis* (S + A), *A. borkumensis* with tetradecane (0.01% v/v) in marine BG11 media (A(s)t), *T.weissflogii* + *A. borkumensis* (Tw + A), *A. borkumensis* with tetradecane (0.01% v/v) in f/2 media (A(tw)t). Mean +/- standard error are shown (n=4). Right: Total of cells counts of *A. borkumensis* by optical density at 600 nm. Letters a, b, c and d indicate significance differences (one-way ANOVA (Tukey's test) p<0.05) between phototrophs + HCB and HCB with tetradecane.

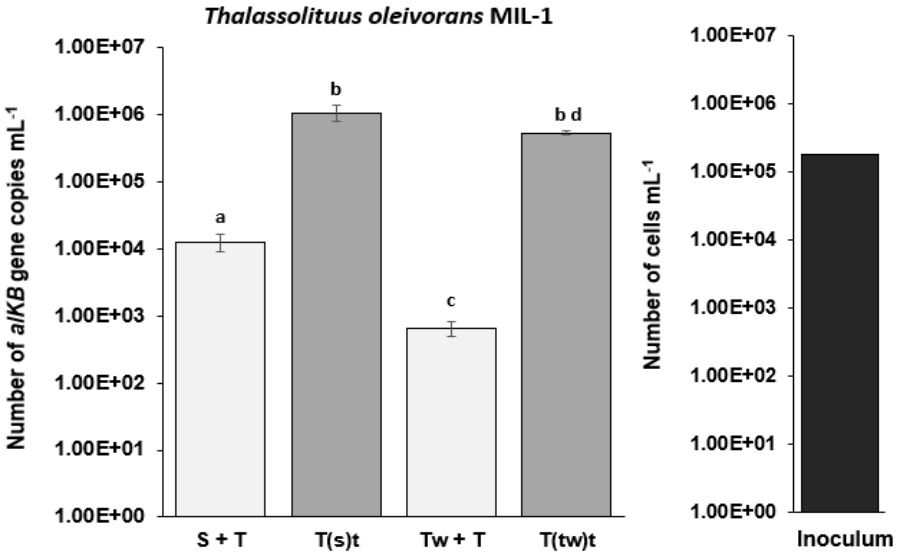

Figure 5.4. Left: Number of *alkB* genes mL^{-1} after 10 days in a 12:12 light and dark regime, 20°C and light intensity of 250-280 µm photons m^{-2} s^{-1}. *Synechococcus* + *Thalassolituus oleivorans* (S + T), *T. oleivorans* with tetradecane (0.01% v/v) in marine BG11 media (T(s)t), *T.weissflogii* + *T. oleivorans* (Tw + T), *T. oleivorans* with tetradecane (0.01% v/v) in f/2 media (T(tw)t). Mean +/- standard error are shown (n=4). Right: Total of cells counts of *T. oleivorans* by optical density at 600 nm. Letters a, b, c and d indicate significance differences (one-way ANOVA (Tukey's test) p<0.05) between phototrophs + HCB and HCB with tetradecane.

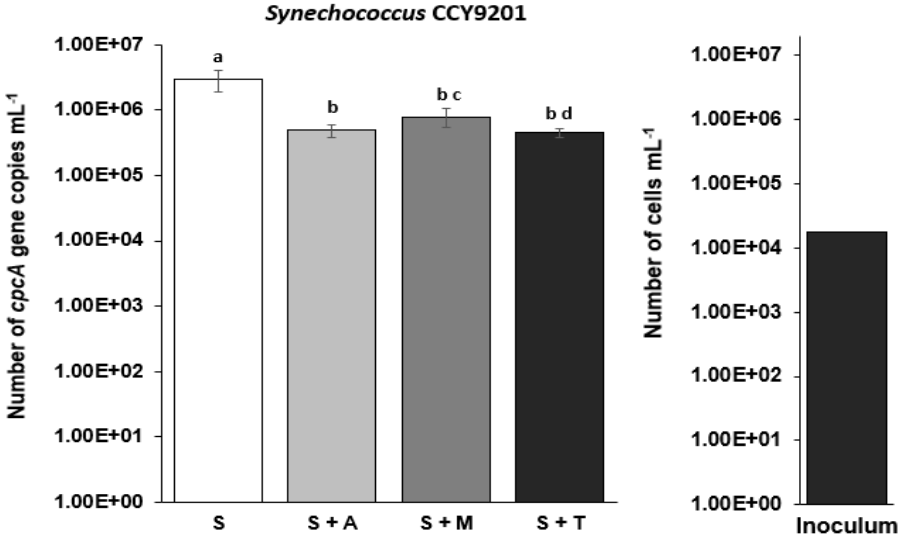

Figure 5.5. Left: Number of *cpcA* genes mL^{-1} after 10 days in a 12:12 light and dark regime, 20°C and light intensity of 250-280 µm photons m^{-2} s^{-1}. *Synechococcus* (S); *Synechococcus* + *Alcanivorax borkumensis* (S + A); *Synechococcus* +*Marinobacter hydrocarbonoclasticus* (S + M) and *Synechococcus* + *Thalassolituus oleivorans* (S + T). Right: Total of cells counts of *Synechococcus* by optical density at 600 nm. Letters a, b, c and d indicate significance differences (one-way ANOVA (Tukey's test) p<0.05) between phototrophs + HCB and HCB with tetradecane.

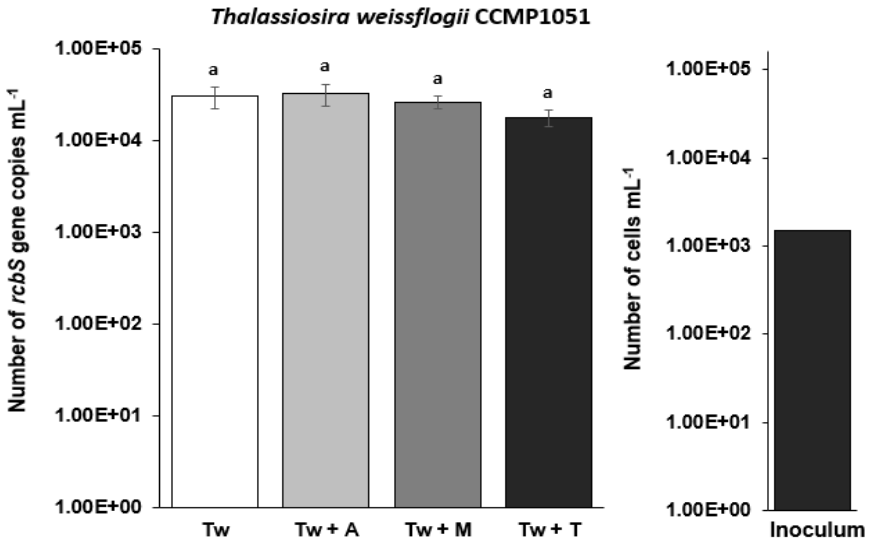

Figure 5.6. Left: Number of *rcbS* genes mL^{-1} after 10 days in a 12:12 light and dark regime, 20°C and light intensity of 250-280 µm photons m^{-2} s^{-1}. *Thalassiosira weissflogii* (Tw); *T.weissflogii* + *Alcanivorax borkumensis* (Tw + A); *T.weissflogii* + *Marinobacter hydrocarbonoclasticus* (Tw + M); and *T.weissflogii* + *Thalassolituus oleivorans* (Tw + T). Mean +/- standard error are shown (n=4). Right: Total of cells counts of *T.weissflogii* by optical density at 600 nm. Letters a, b, c and d indicate significance differences (one-way ANOVA (Tukey's test) p<0.05) between phototrophs + HCB and HCB with tetradecane.

5.3.1 Chlorophyll *a* Analysis

For all the experiments, the concentration of chlorophyll *a*, showed no significant differences when the phototrophs were grown alone or in the presence of any of the hydrocarbonoclastic bacteria (Figure 5.7).

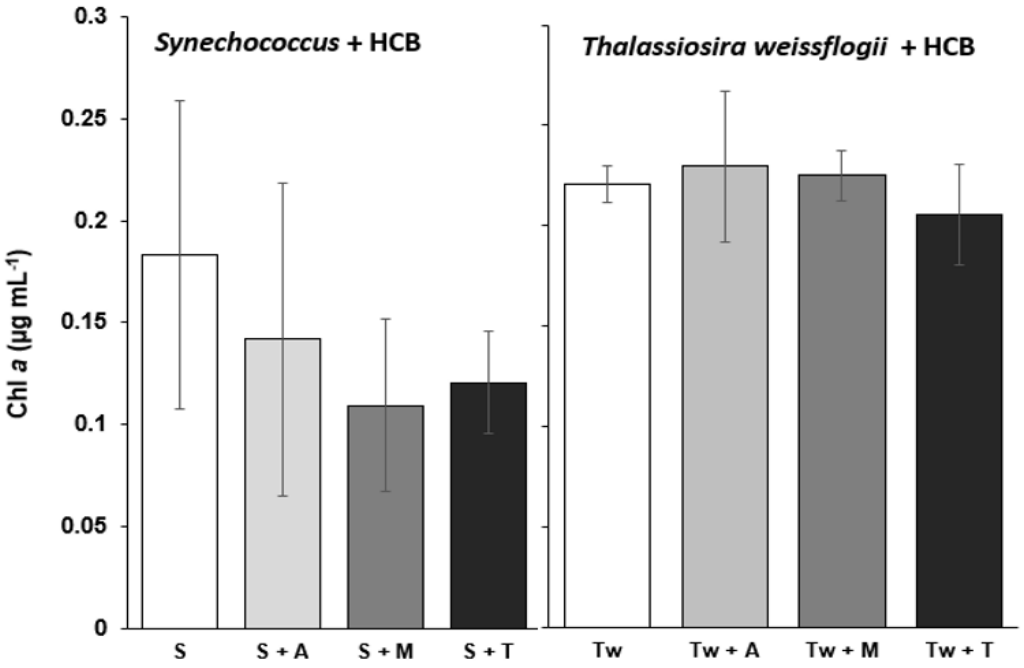

Figure 5.8. Chlorophyll *a* concentration (µg mL^{-1}) of *Synechococcus* +/- HCB and *Thalassiosira weissflogii* +/- HCB after 10 days in a 12:12 light and dark regime, 20°C and light intensity of 250-280 µm photons m^{-2} s^{-1}. Left panel: *Synechococcus* (S); *Synechococcus* + *Alcanivorax borkumensis* (S + A); *Synechococcus* +*Marinobacter hydrocarbonoclasticus* (S + M) and *Synechococcus* + *Thalassolituus oleivorans* (S + T). Right panel: *Thalassiosira weissflogii* (Tw); *T.weissflogii* + *Alcanivorax borkumensis* (Tw + A); *T.weissflogii* + *Marinobacter hydrocarbonoclasticus* (Tw + M); and *T.weissflogii* + *Thalassolituus oleivorans* (Tw + T). Mean +/- standard error are shown (*n*=4)

5.4 Discussion

There is evidence that diatoms can produce alkanes, for example, Blumer (1971) found that centric diatoms contained traces of alkanes in the C_{15}-C_{25} range, and pristane occurs at similarly low concentrations; McKay *et al*. (1996) they found that diatoms can produce short-chain hydrocarbons, ethane and propane. However, in my study the three hydrocarbonoclastic bacteria (HCB) showed no growth in the presence of *T. weissflogii*, and the phototroph was not affected by the presence of the HCB, confirmed by the quantification of the *rcbS* gene and the production of Chl *a*; which could mean that the diatom is not producing any or enough hydrocarbons to support the growth of the HCB.

Although, for *A. borkumensis* and *T. oleivorans* no growth was observed in the presence of *Synechococcus*; *M. hydrocarbonoclasticus* showed a small but significant increase when grown with the phototroph. Cyanobacteria are one of only few types of organisms that are known to directly produce hydrocarbons (Coates *et al*, 2014). Leah-Smith *et al*. (2015) found that two species of cyanobacteria, *Synechococcus* and *Prochlorococcus* can synthesize alkanes in the range of C_{15}-C_{19}. Also, it is known that *M. hydrocarbonoclasticus* SP17 is found to be able to form biofilms at the interface between water and n-alkanes (C_8-C_{28}), and this bacterium only forms biofilms in substrates that it can metabolize (Mounier *et al*., 2014). This may imply that *Synechococcus* CCY9201 was able to produce one or several alkanes and *M. hydrocarbonoclasticus* used them to grow.

It is important to mention that even if the quantification of the *alkB* gene mL^{-1} in *M. hydrocarbonoclasticus* was higher compared with the cells mL^{-1} of the initial inoculum, it

is not clear if this represent an actual growth of the HCB because two different methods were used, to quantify the number of genes and cells in the experiments. For this reason, it essential to add another control in the experiment, the HCB without any carbon source, tetradecane or a phototroph.

Another point to be made is that the availability of hydrocarbons produced by cyanobacteria depend heavily on their physical state (Valentine and Reddy, 2015). Leah-Smith et al. (2016) found that hydrocarbons were shown to accumulate in thylakoid and cytoplasmic membranes suggesting that these compounds aggregate in the centre of the lipid bilayer, potentially promoting membrane and flexibility. Also, the production of hydrocarbons by diatoms is most likely during the autolysis of the cells (McKay *et al*., 1996; Ramachandra *et al*., 2009). If hydrocarbons are released from the membrane by the both phototrophs, it will be interesting to add another experiment where HCB are grown with dead cells of the phototroph.

The HCB could also be growing on other compounds produced by the phototrophs. For example, it has been observed that isoprene and hexane appear during phytoplankton growth and are most likely produced directly by the phytoplankton with the photolysis of polyunsaturated lipids or through the oxidation of organic matter (Ramachandra *et al*., 2009). Acuña Alvarez *et al*. (2009) found that isoprene can be degraded by heterotrophic bacteria, such as *A. borkumensis* but suggested that the levels of isoprene in the oceans cannot be enough to allow a rapid growth of HCB.

On the other hand, the experiments also showed that the abundance of *Synechococcus* decreased with the presence of HCB compared when grown alone, but the production of Chl *a* was not significant affected by the presence of HCB. It is known that microalgae and heterotrophs, have mutualistic interactions (McGenity *et al*., 2012) in which cyanobacteria can fix nitrogen and along with microalgae, supply organic matter (e.g. excreted polysaccharides), and oxygen to heterotrophic bacteria via photosynthesis (Rosenbaum *et al.*, 2010); antagonistic interactions may occur (McGenity *et al*., 2012).

This study requires GC-MS analysis of the cultures to quantify the hydrocarbons produced by the phototrophs and degraded by HCB. Albeit the hydrocarbons of *Synechococcus* were extracted and the GC-MS analysis was done, the results produced were unusable and the experiment could not be repeated because of the lack of time and material.

5.5 Conclusion

In this study the principal objective was to know if photosynthetic microalgae, in this case, *Synechococcus* and *Thalassiosira weissflogii*, could sustain the growth of hydrocarbonoclastic bacteria by producing n-alkanes. The results showed that *T. weissflogii* was not able to produce alkanes and therefore no growth of HCB was observed, however, the growth and activity of the *T. weissflogii* was not affected by the presence of HCB. On the other hand, it seems that *Synechococcus* was able to produce alkanes but could not sustain the growth of *Alcanivorax borkumensis* and *Thalassolituus oleivorans*. *Marinobacter hydrocarbonoclasticus* was the only HCB to slightly growth with *Synechococcus*. Moreover, the growth of the HCB could be due to the realised of other compounds by the phototroph. To confirm these statements, it is important to do more analysis including the quantification of hydrocarbons by GC-MS.

CHAPTER 6

DISCUSSION

During the past years, the contamination by oil to the marine environment has become a large problem - environmental, economic and social factors had an enormous impact within countries that have been affected by oil spills.

When the oil is spilled into the ocean it suffers some changes through the weathering process, some of them are evaporation, photooxidation, emulsification and microbial degradation (McGenity *et al*., 2012). Most of the studies in recent years regarding oil has been focused on the improvement and design of the necessary tools utilised to both detect and clean-up the oils spills; leading to a more efficient procedure of clean-up when an oil spills does occur. In this investigation the overall aim is about the impact of the oil has on the attenuation of light and as consequence the impact on the bacterial and photosynthetic community.

In chapter 2.3, it was demonstrated that an overlaying oil preferentially attenuates shorter wavelengths (400-500 nm) of photosynthetically active radiation and it was also observed that the attenuation of the light depends on the thicknesses of oil layer. Although the exact composition of the oil was not measured, it was known that the oil used in this study, Gyda crude oil, was not weathered; and the attenuation of light on short wavelengths can be explained due to the presence of polycyclic aromatic compounds which absorb light in the range of 200-500 nm (Dabestani and Ivanov, 1999). These findings are similar with

the experiments found it by Plata *et al*. (2008) and Otembra *et al*. (1994) where it was observed that the attenuation of light decreases with the increasing wavelength.

After, with the results of the attenuation of light by oil in short wavelengths it was decided to investigate how this would this would affect the microbial community composition in seawater. Experiments were conducted using natural seawater from the Brightlingsea with an overlaying oil and with no oil layer while being irradiated with a continuous light, in which there was a clear increase phototroph biomass (DNA and chlorophyll *a* concentration) within the samples where there was an oil layer at the end of the experiment. It was suggested that the oil layer reduced the quantity of light received by the phototrophs, allowing them to grow in comparison with samples with no oil layer. Also, the phototrophs could have an advantage while using different pigments accessories that can trap light at longer wavelengths (Fischer *et al*., 1996; Bjorn *et al*., 2009). It is important to highlight the fact that the quantity of light reflected was not measured and it was assumed that all light was absorbed.

Continuous irradiance does not effectively repair of the light-induce damaged to the photosystem II of the photosynthetic microorganisms (Aro *et al*.,1993; Moore *et al*., 2006; Kulk *et al*., 2011). Therefore, a light and dark (12:12 h) regime (L:D1-L:D2) was used in the next Chapter in which photoacclimation and photoregulation can be carried out by the phototrophs (Lichtman, 2000; Tyystjarvi, 2008; Dimier *et al.* 2009). In order to distinguish between the intensity of light and the spectrum of light, artificial LEDs lights were tuned to mimic the effect of the oil layer (L:D3-L:D5). Finally, to have a more realistic simulation

to the natural environment, it was decided to use a ramped light in which the intensity of light fluctuates throughout day (L:D5).

The results show that the community composition was changed, while the cyanobacteria were the dominant phototroph under an oil layer sample with a continuous light (L4), the diatoms were the dominant phototroph in all the treatments for the light and dark regime (L:D1-L:D5).

It was observed that nutrient limitation (L:D2) had no significant influence within the concentration of DNA, but chlorophyll *a* concentration was lower compared to the experiment with added nutrients (L:D1). Kocum *et al*., 2000, found that the seawater from the Brightlingsea, have higher concentrations of nutrients through the whole year (N:P:Si) which explained what it did not affect the total biomass. However, it is necessary to measure the concentrations of N and P, and the N:P ratio before and after the experiment to get a better understanding of how the nutrients affect both the concentration of DNA and Chl *a*.

Further, through the analysis of the microbial community, it was concluded that the changes within microbial community were due to a combination of direct effect, with high abundance of oxygenic phototrophs, aerobic anoxygenic phototrophs and species that have proteorhodopsin; an indirect effect, with the abundance of microorganisms that are closely associated to oxygenic and anoxygenic phototrophs that use their photosynthate as a source of carbon an energy; and lastly, specific sensory effects to the oil spectrum

by the presence of microorganisms able to degrade hydrocarbons like *Marinobacter hydrocarbonoclasticus* and *Cycloclasticus*.

The last point is highly speculative but very interesting, leading to more questions about the physiology of bacteria capable of degrading hydrocarbons and their interactions with the photosynthetic community. For this reason, it is suggested that in further work, different types of bacteria and photosynthetic microorganisms, anoxygenic phototrophs and the ones with proteorhodopsin; these are quantify using specific genes.

Also is important to take in account the changes in the environment as cloud cover, turbulence, waves, sediment resuspension in coastal zones. Additionally, all experiments are required to have a fluctuating light intensity factor to observe how this would affect community composition.

Due to the changes observed in the photosynthetic community in previous experiments, in which cyanobacteria was the most abundant microorganism when using a continuous light regime and diatoms were dominant when using a light and dark regime, it was decided to use two phototrophs, *Synechococcus* sp. and *Thalassiosira weissflogii* CCMP1051.

The results show, that for *Thalassiosira weissflogii* the growth rate and chlorophyll *a* concentration was not significantly affected by the presence of an oil layer or neutral-

density filter but under control with no oil/filter, the growth and the production of chlorophyll was significantly lower.

This suggest that, the diatom, was affected by high the light intensity (540-601 µmol photons m^{-2} s^{-1}), Goericke and Welschmeyer (1992) found that for *T. weissflogii*, the growth rate saturation is at 75 µmol photons m^{-2} s^{-1} of irradiance. The ability of *T. weissflogii* to grow under an oil layer and under a neutral-density filter could be explained by the produced accessory pigments detected, like Fucoxanthin, which absorb light better than chlorophyll *a*. The growth under the cells with no oil/filter can be explained by the presence of the carotenoid, diadinoxanthin, via the xanthophyll cycle and non-photochemical quenching (NPQ) which minimise the damage caused by the excess of light in diatoms (Lavaud *et al*., 2002; Dimier *et al*; 2009). The capacity of the diatoms to grow with an excess of light could also explain the findings in chapter 3, where the diatoms were the most abundant microorganism in all the light and dark treatments.

On the other hand, it was observed that any changes that occurred varied greatly in for *Synechococcus*, despite the fact that the growth rate for the oil layer and the neutral-density filter showed no significant differences, the amount of chlorophyll *a* and therefore the colour of the cyanobacteria was different -green for the neutral-density filter and white for the oil layer treatment. For this specific strain, the phycocyanin is the main pigment produced (Stomp *et al*., 2008) but, in this study, only allophycocyanin was detected. Also, a photoprotector pigment was detected, zeaxanthin, but only under the neutral-density filter. Again, this behaviour was under high light intensity as observed in chapter 2 where

cyanobacteria were affected by the high light intensity (600 µmol photons m^{-2} s^{-1}) under control treatments.

Other pigments were also detected, but unable to identified, which could help to explain in more depth the differences between treatments and the behaviour of both microorganisms.

Additionally, both phototrophs, *Thalassiosira weissflogii* and *Synechococcus* did not grow when they were directly in contact with the oil, proving that the oil was toxic for them, which could affect the presence of these phototrophs during oil spill.

For future work, the cell density of the pre-inoculum must be standardized, and more controls should be added like the medium without microorganisms. Also, after extracting the pigments from the cells, it is important to immediately do the pigment analysis to avoid any the degradation of the pigments. In addition to this, different concentrations of oil must be tested with both diatoms and cyanobacteria.

Knowing that the diatom *T. weissflogii* and cyanobacteria, *Synechococcus* are one of the most abundant components of phytoplankton (Liu *et al*., 1995), it was decided to investigate if these strains can produce hydrocarbons (*n*-alkanes) that can support the growth of hydrocarbonoclastic bacteria (HCB) like *Alcanivorax borkumensis*, *Marinobacter hydrocarbonoclasticus* and *Thalassolituus oleivorans*; based on the findings by *Lea-Smith* et al., 2015, in which they estimated that *Synechococcus* and *Prochlorococcus* have the capacity to produce 2-540 pg. alkanes per day.

The results show that *T. weissflogii* was not able to produce any *n*-alkanes since no HCB was able to grow in its presence. For *Synechococcus*, only *Marinobacter hydrocarbonoclasticus* grow slightly compared to the controls. However, it is important to notice, different methods were used to quantify the number of cells and the number of genes. For future work it is better to add more controls, like the HCB without any carbon source or a phototroph.

Continuing for the future work, it is important to consider is that the hydrocarbons are likely accumulate in thylakoid and cytoplasmic membranes suggesting that the autolysis of the cells will help to realise the hydrocarbons from the cell (McKay *et al*., 1996; Ramachandra *et al*., 2009; Leah-Smith *et al*., 2016). In this case, it is important to add another experiment in which the HCB grows alongside the dead cells of phototrophs.

Another point to consider it is that some HCB can grow using another compound besides *n*-alkanes, like isoprene and hexane (Ramachandra *et al*., 2009; Acuña Alvarez *et al*., 2009). Which are important to measure in future studies through GC-MS alongside the other *n*-alkanes.

This investigation was able to demonstrate that in the presence of an overlaying oil, both the intensity and spectrum of the light is affected, and because of this the bacterial and the photosynthetic community composition of the seawater changed. These findings improve our understanding of how bacterial and photosynthetic microorganisms interact

in the marine environment when an oil spill occurs. This will aid us with understanding of how the oil degrades in the presence of certain microbial communities and how to implement new bioremediation techniques.

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Appendices

Appendix A

f/2

(Guillard and Ryther 1962, Guillard 1975)

To prepare, autoclave each stock separately then add aseptically to artificial seawater which had been autoclaved. Vitamin solution have to be Filter sterilize and store in refrigerator or freezer covered from light.

Component	**Conc stock g/L**	**mL stock/ L**	
$NaNO_3$	75	1	
NaH_2PO_4 H_2O	5	1	
Na_2SiO_3 9 H_2O	30	1	
Trace metal solution		1	
Vitamin solution		05	

Trace metal solution

Component	**Conc stock g/L**	**mL stock/ L**	
$FeCl_3$ $6H_2O$	---	3.15 g	
Na_2EDTA $2H_2O$	---	4.36 g	
$CuSO_4$ $5H_2O$	9.8	1	
$NaMoO_4$ $2H_2O$	6.3	1	
$ZnSO_4$ $7H_2O$	22	1	
$CoCl_2$ $6H_2O$	10	1	
$MnCl_2$ $4H_2O$	0.18	1	

Vitamin solution

Component	**Conc stock g/L**	**mL stock/ L**	
Thiamine HCl (vit. B1)	---	0.2 g	
Biotin (vit. H)	0.1	10	
Cyanocobalamin (vit.B12)	1	1	

Appendix B:

Marine BG11 for Baltic Sea strains

Minerals	[stock g/L]	[media g/L]	Molar/ Stock	Molar/ Media
NaCl	250	33.3	4.2808219	1.43E-01
$MgCl_2$. $6H_2O$	200	3.3	0.9837547	3.25E-03
KCl	50	3.3	0.6706791	2.21E-03
$MgSO_4.7H_2O$	350	3.3	1.4200246	4.69E-03
$CaCl_2.2H_2O$	50	3.3	0.3401023	1.12E-03
Na_3-citrate	0.6	8.3	0.0023250	1.93E-05
Na_2-EDTA	0.1	8.3	0.0002686	2.23E-06
$NaNO_3$	150	8.3	1.7648159	1.46E-02
Trace metal mix		1	-	-

Adjust to 900 mL with distilled water. In the case of solid medium only 400 mL.

Minerals	[stock g/L]	[media g/L]	Molar/ Stock	Molar/ Media
$K_2HPO_4.3H_2O$	4	8.3	0.0175285	1.45E-04
Na_2CO_3	20	2.3	0.1886992	4.34E-04

Adjust to 100 mL with distilled water

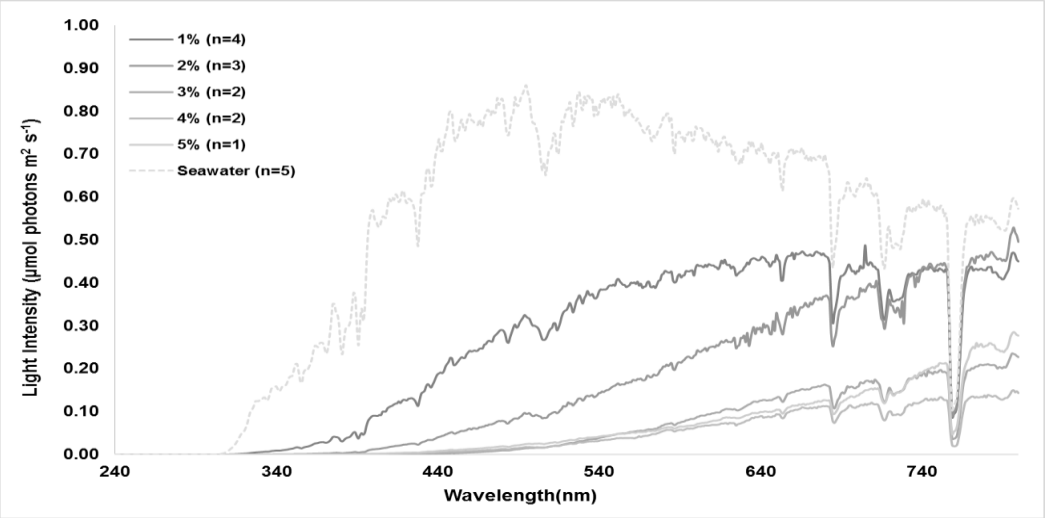
Minerals	[stock g/L]	[media g/L]	Molar/ Stock	Molar/ Media
Fe-NH_4-citrate	6	0.8	0.0225557	1.80E-05
Vitamin B12	0.002	1	0.0000147	1.47E-08

Add after autoclaving when medium is cold

- Sterile the phosphate and carbonate separately from the mineral medium and complete the medium after cooling.
- Fe-NH_4-citrate stock solution is already autoclaved. Vitamin B12 solution sterile filtrated. Add with a sterile filter (0.22 µ) after cooling.

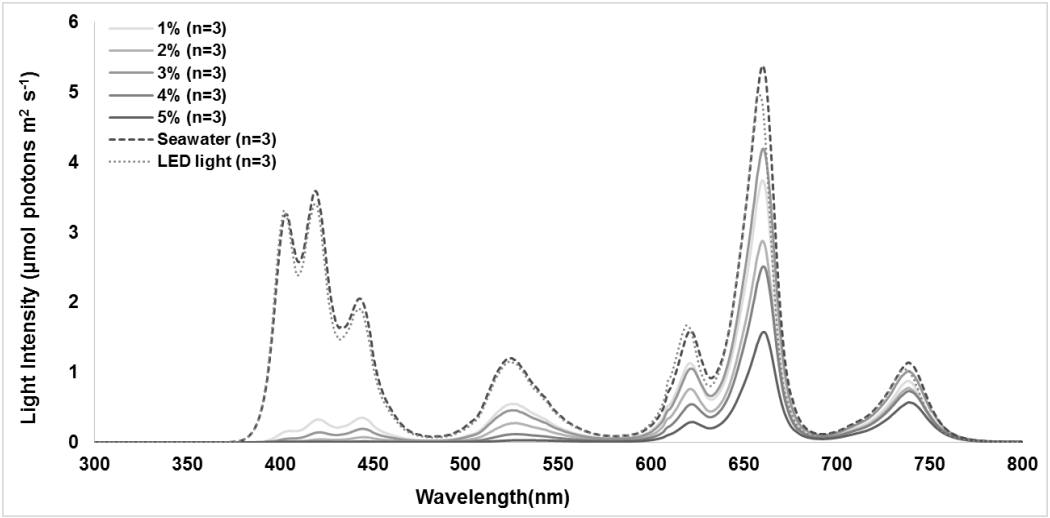
Trace metal sln.	[stock g/L]	[media g/L]	Molar/ Stock	Molar/ Media
H_3BO_3	2.86	1	0.0462558	4.63E-05
$MnCl_2$.$4H_2O$	1.81	1	0.0091457	9.15E-06
$ZnSO_4$.$7H_2O$	0.222	1	0.0007720	7.72E-07
Na_2MoO_4 .$2H_2O$	0.39	1	0.0016118	1.61E-06
$CuSO_4$.$5H_2O$	0.079	1	0.0003163	3.16E-07
$Co(NO_3)_2$.$6H_2O$	0.0494	1	0.0001697	1.70E-07

Appendix C:



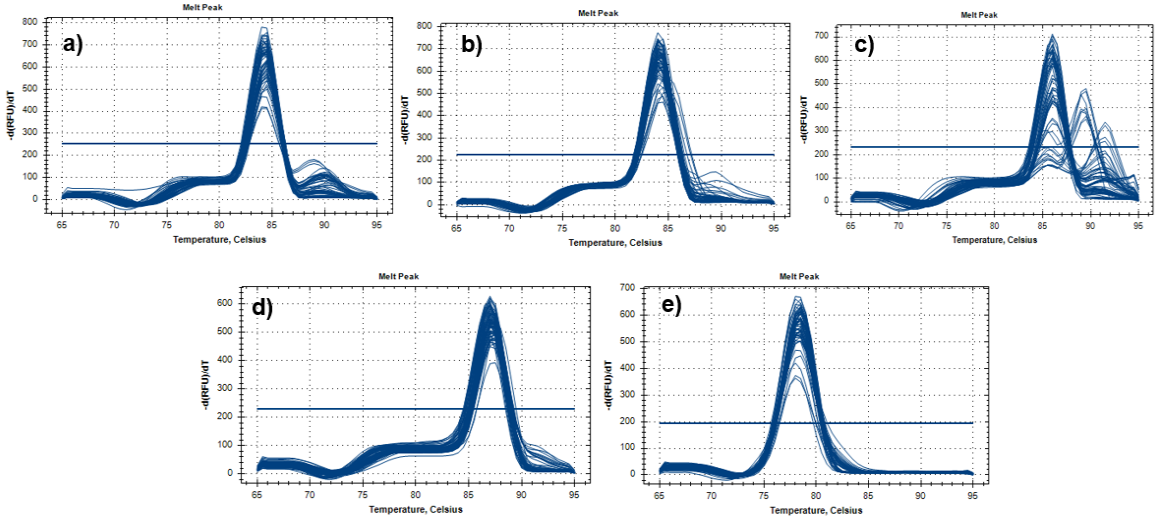
Spectrum of sunlight, passing through samples containing natural filtered- seawater and a weathered Forties oil layer at different concentrations (1, 2, 3, 4 and 5% v/v). Control sample (n=5), natural filtered-seawater without oil (dashed line).

Appendix D:



Spectrum of artificial lights LED lights (n=3), passing through samples containing natural filtered- seawater and a weathered Forties oil layer at different concentrations (1, 2, 3, 4 and 5% v/v). Control sample (n=3), natural filtered-seawater without oil (dashed line). Measurement of LED lights (n=3), without control or samples (r dotted line).

Appendix E:



Melting curve analysis of the designed primers: a) *Marinobacter hydrocarbonoclasticus* (M*alkB*-F and M*alkB*-R), b) *Alcanivorax borkumensis* (A*alkB*-F and A*alkB*-R), c) *Thalassolituus oleivorans* (T*alkB*-F and T*alkB*-R), d) *Synechococcus* sp. (S*CpcA*-F and S*CpcA*-R), e) *Thalassiosira weissflogii* (TW*rcbS*-F and TW*rcbS*-R).