Linking the degradation of model saturated and aromatic crude oil components to specific marine microbial taxa during biostimulation and bioaugmentation using RNA-stable isotope probing

Iman Fadlallah TALEB

A thesis in the fulfilment of the requirements for the degree of

Doctor of Philosophy

School of Biotechnology and Biomolecular Sciences
Faculty of Science
The University of New South Wales
Sydney Australia
March 2014

Abstract

In an effort to dissipate the notorious effect of the catastrophic oil spills that occur in the marine environment, several bioremediation techniques have been developed with variable outcomes depending on several environmental and nutritional factors. With the high level of sophistication in the development of molecular techniques, and their application in bioremediation, it has become possible to identify key hydrocarbon-degrading marine bacteria responsible for the degradation of specific hydrocarbon compounds. However, only recently, studies that linked the degradation of hydrocarbons to specific marine phyla have started to emerge.

The present study applied RNA-stable isotope probing in order to identify key marine hydrocarbonoclastic bacteria acquiring carbon from model oil hydrocarbons (hexadecane, benzene and naphthalene) under biostimulation and natural attenuation conditions in pristine as well as in oil pre-adapted seawater in laboratory microcosms. Pre-adaptation of seawater to oil induced the degradation of benzene, but slowed down the degradation of naphthalene and hexadecane. DGGE analysis showed high selection towards specific hydrocarbon degrading lineages that dominated the community profiles.

The effect of a new concept in bioremediation, autochthonous bioaugmentation, on the selected hydrocarbons in the presence or absence of nutrients was also investigated using hydrocarbon degrading isolates obtained from seawater. Bioaugmented cultures showed more rapid degradation of all compounds with variability in the extent of degradation per compound and nutritional conditions. Another highlight of the present study was the first application of RNA-SIP during autochthonous bioaugmentation in order to determine the fate of the labelled carbon isotopes. Profiling of the community showed that hexadecane was degraded mainly by the added *Rhodococcus qingshangii*, while benzene was not degraded by the added *Alteromonas addita*, but by members of the native community. Carbon from naphthalene, however, was consumed by the added *Pseudomonas alcaliphila* as well as by another indigenous taxon.

This thesis assigned the degradation of selected hydrocarbons to novel putative hydrocarbonoclastic bacteria and highlighted some of the microbial interactions during

bioremediation, which provided new insights for further developments of the promising autochthonous bioaugmentation strategy.

Dedication

I dedicate this thesis to:

***My mother*:** for the unconditional love and incomparable sacrifice and for teaching me how to stay strong and faithful despite all difficulties

***My father*:** for being my motive force, my role model and always my inspiration

***My uncle Khairallah*:** for being the second father, and for teaching me how to love and give unconditionally

***My husband Mohamad*:** for being my soul mate and the source of my comfort

***My little angels Ahmad and Omar*:** for making every single moment in my life meaningful… *Alhamdolilah* for this beautiful gift

Acknowledgments

سبحانك اللهم لا علم لي الا ما علمتني انك انت العليم الحكيم. يا ربي لك الحمد كما ينبغي لجلال وجهك وعظيم سلطانك, لك الحمد والفضل اولاً واخراً ودائماً وابداً

I am firstly grateful to my supervisor **Assoc. Prof. Mike Manefield** for making me a part of this amazing group of talented scientists. Thanks for your help at so many levels, for your guidance, patience and faith in me during my PhD journey. Thanks for making me discover myself and my capabilities, and most importantly thanks for teaching me how to achieve my work independently. It has been a great pleasure working under your supervision.

To the most amazing lady, scientist and friend, my co-supervisor **Dr. Maria-Luisa Gutierrez-Zamora,** thanks endlessly for the incomparable help and support, you are an essential reason I got to the end of it, and your friendship is one of the greatest outcomes of my PhD. Thanks for your kindness and for being *ALWAYS* there for me. Thanks for lifting me up every time I was about to fall down. You are one of a kind and I am very lucky to have met such a brilliant scientist who never ceased to inspire me. Thanks also for all the lab chats and for the great moments we had in the lab. Wade and Santiago (and the little "no-name-yet" baby boy) must be the luckiest people on the planet for having such a beautiful lady in their life. Much love and appreciation for you!!

Thanks also to a number of people who handed some of their expertise over to me throughout my candidature. Thanks to **Dr. Adrian Low** for patiently teaching me the art of Gas Chromatoraphy and especially for coming at very late, after hour times, to help me. I am also grateful to **Dr. Matt Lee** for helping me to use the different GC instruments and for the nice chats that definitely gave me insights to improve my work. My thanks also go to **Dr. Sania Wadud** for teaching me the basics of DGGE and showing me the nasty tricks of the technique.

I'm so grateful for Indo-Australia Biotechnology for funding this project and for the Centre for Marine BioInnovation at UNSW for the financial support. Special thanks to **Mr Adam Abdul** for all his support and help in organising my fee payments; I don't know what I would have done without you! I will always remember your help...Thank you!

I am glad to have had beautiful friends like Mona, Hazlin and Valentina**. Mona**, we had a lot in common literally, thanks for the long political talks. If politics were encoded in a gene, we both would have had it highly expressed☺! Thanks also for our morning coffee times with Valentina, they were unforgettable moments indeed! **Hazlin**, the pretty and calm lady, thanks for the chats, the laughs and the prayers together. ☺ I hope to see you a great mom as you've always wanted. **Valentina**, Thanks for being a great friend and for giving me a hand whenever I needed it, especially in the last weeks of lab work. Thanks also for saving my life by providing me with your own chemicals and reagents every time I needed. To my lab mates **Tammy** and **Ricardo**…Thanks for being so sweet and I will certainly miss all the lab chats we used to have.

I cannot forget my friend in France **Fidaa**. Despite distances, we still talk as we have never been away, this only happens with true friends. Thanks for being the great person you are, for sharing PhD stories that made me feel that I also can do it! You've always inspired me with your determination, faith and patience.

I must be the luckiest person on earth to be blessed with four loving families:

My Family in Lebanon, despite distances you were never far away from my heart and mind. **My mom**, you are an exceptional lady and teacher, my role model in forgiveness and sacrifice; without you, not only this thesis, but everything I achieved in my life would have never been possible. Thanks for your sincere prayers and your endless support and love for me and my family. My sisters **Olfat** and **Assala** and my brothers, **Ahmad**, **Bassel** & **Majd**, thanks for your love, support and respect for me the big sister. ☺ I love you!

My uncle's family, you are the reason I came to Australia, and the reason I kept up. I don't know if words can describe how much I love and appreciate what you all have done for me. **My uncle Khairallah**, what can I say, I hope I could finally make you proud. You, after Allah (SwT), are the reason I could achieve my goals. I always remember you and say you are a diamond; then I correlate it to science and remember that, graphite and diamond are both made of the same atom, "carbon", however, the first forms under standard conditions, while the latter needs extreme pressure and harsh conditions to form. You have been through a lot of extreme pain and sacrificed too much to help us being who we are, but this created a beautiful precious and unique

diamond that is you. People say you are one in a million, I rather say you are you and absolutely no one is ever like you…your sacrifice for us will be forever appreciated. **My Auntie Sabria**, Thanks for being the second mom and the loving relative and friend who took care of me and my family whenever I needed it, thanks for your love and support. My cousins: **Mona**, my friend, my lovely little sister, thanks for listening to me whenever I needed to vent, you are one in a million. **Rana**, my pretty, caring, loving cousin; how fortunate am I to have a person like you in my life. I will keep forever saying "I wish the world had so many clones of you". **Safa, Marwa (staph it :p ☺) Mariam, Asem and Tarek**, I love you all, ktir ktir ktir, thanks for being in my life. I thank Allah for blessing my beloved uncle with such an amazing family.

My family in law, especially **Houssein, Souad** and **Khaled** thanks so much for the nice chit-chats whenever I needed them, you are the best people ever and just great brothers to me. Special thanks for my mother in law. I could feel your prayers easing my pain in every single stressful moment I had during my PhD, thanks for thinking of me all the time.

My own little family, **my husband Mohamad**, how can I describe how important and vital you are to me?! You are my soul mate and the air I breathe, thanks for being with me all the time and for beautifying things whenever I felt they were getting gloomy. Thanks for making me believe in myself and for your care and tenderness. All those nice people made it possible, but you were certainly the "anonymous soldier" who always backed me up in all circumstances and replenished my heart with care and love. Thank you, Abou Ahmad ☺. My two little men **Ahmad** and **Omar**, having you made me not wanting to ask for more! My heart is very full and very warm because of the best feelings that you have given to me. You were definitely the reason I stayed strong. Thanks for making my life very rich; your smiles that beamed with innocence have always been powerful enough to make me achieve the hardest work and still feel happy and satisfied. I am proud of you my babies and I hope you will be proud of mommy in the future.

My dad, I wish you were still here to be with me in it. You are gone but never forgotten. I know the gap you left in my life is impossible to refill, but I am proud to be your daughter, and proud to have reached my goal and pleased your soul. I love you and miss you every moment!

Thanks to every single person who was there for me during my PhD in any way and whom I forgot to mention, I hope I remembered you all, otherwise, please forgive me.

Originality Statement

I hereby declare that this submission is my own work and to the best of my knowledge it contains no materials previously published or written by another person, or substantial proportions of material which have been accepted for the award of any other degree or diploma at UNSW or any other educational institution, except where due acknowledgement is made in the thesis. Any contribution made to the research by others, with whom I have worked at UNSW or elsewhere, is explicitly acknowledged in the thesis. I also declare that the intellectual content of this thesis is the product of my own work, except to the extent that assistance from others in the project's design and conception or in style, presentation and linguistic expression in acknowledged.

Signed: _____

Date: _____

Table of contents

List of figures

List of tables

List of abbreviations

µl	Microliter
µM	Micromolar
Altero	*Alteromonas addita*
APS	Ammonium persulphate
cDNA	Complementary DNA
CO_2	Carbon dioxide
CsTFA	Cesium trifluoroacetate
DCM	1,2-Dichloromehtane
DGGE	Denaturing gradient gel electrophoresis
DI	Deionised water
DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleotide phosphate
EPA	Environmental Protection Agency
FID	Flame ionasation detector
g	Gravity, in relation to relative centrifugal force
GC-FID	Gas chromatography- flame ionisation detector
h	Hour
i.d.	Internal diameter
K_2HPO_4	Dipotassium hydrogen phosphate
LB	Luria Bertani
mg/l	Milligram per litre
ml	Millilitre
mM	Millimolar
N	Nutrients
ng	Nanogram

NH_4Cl	Ammonium chloride
O	Crude oil
OD	Optical density
PAH	Polycyclic aromatic hydrocarbon
PLFA	Phospholipid-derived fatty acid
Pseudo	*Pseudomonas alcaliphila*
RDP	Ribosomal Database Project
Rhod	*Rhodococcus qingshangii*
RNA	Ribonucleic acid
rpm	Rounds per minute
RT	Reverse transcription
S	Seawater
SIP	Stable isotope probing
SSU	Small subunit (of rRNA)
TAE	Tris-Acetate-EDTA
TCD	Thermal conductivity detector
TE	Tris-EDTA
TEMED	Tetramethylethylenediamine
BTEX	Benzene, Toluene, Ethylbenzene, xylene

Chapter1

Introduction

1.1. Overview

Petroleum oil is an essential source of energy that the entire world heavily relies on in nearly all aspects of life. The consistent increase in the human population has led to a heavily increased exploitation of oil reservoirs and tanker operations all over the world in order to meet the increasing demand (from 85.62 million barrels in 2008 to 89.87 million barrels in 2013 (OPEC 2008 & 2013)). In addition to terrestrial sources, oil forms naturally from the decay of organic material in the deep-sea environment as a result of extreme temperature and pressure conditions. Thus, extraction and transport processes expose the ocean environment to oil components that exhibit toxicity to higher level organisms and disrupt natural ecosystem function. The sources of oil pollution vary and can be caused either by natural phenomena like earthquakes or movements of the earth's crust, or by human activity associated with oil transport and exploitation. Accidental leakage occurs due to the rupture of pipelines, operational discharge from ships and cargoes (Carpenter and Macgill 2001) and accidents in offshore oil platform or coastal refineries (Tolosa *et al.* 2005). The marine environment constitutes home for most of the transport of oil and petroleum products which, as indicated by Rodriguez (2012), hosted the shipping of 2.4 billion tons of petroleum by tankers, accounting for 62% of all petroleum produced in 2005.

Despite the strict environmental regulations and obligations imposed on industrial developers, oil spills happen all over the world every year at different magnitudes with the Exxon-Valdez (41.6 million litres) and the recent BP Deepwater Horizon (779 million litres) being the most catastrophic spills in the US (Atlas and Hazen 2011). The Deepwater Horizon oil spill was the largest oil spill in the US history (Graham *et al.* 2010) that costed 11 lives, perhaps it is also the world’s second largest spill after the Gulf War Oil Spill from Kuwait (Abbriano *et al.* 2011).

This error prone human activity constitutes a major risk to the marine environment and Earth more broadly given that oceans account for 70% of its surface. Oil pollution has negative impacts on the ocean's biological diversity. It can cause death to a large number of animals, it can create imbalance in the ocean's carbon cycle, and it can also impose a major risk to humans, as oil pollutants can be transferred via the food chain due to their tendency to bioaccumulate (Means *et al*. 1980, Morehead *et al.* 1986). Further, oil spreads rapidly on the sea surface enabling contact with shorelines and eventual contamination of terrestrial ecosystems.

1.2. Chemical composition of crude oil

Oil is a complex natural substrate. It consists of many thousands of individual compounds (Tissot and Welte 1984) that constitute a whole field of study on its own, hence the term petroleomics introduced by Marshall and Rodgers (2004). The more carbon atoms the molecules have, the less volatile, soluble and bioavailable they are, and the longer they persist in the environment. Oils mainly consist of hydrocarbons that are classified into 4 main groups (Fig.1.1): 1) saturated hydrocarbons (n- and branched-chain alkanes and cycloalkanes or cycloparaffins), 2) aromatic hydrocarbons (monoaromatic (BTEX i.e. benzene, toluene, ethylbenzene and xylene) and polycyclic aromatic hydrocarbons (PAHS)), 3) resins (nitrogen, sulfur and oxygen-containing molecules such as Quinolines, carbazoles, amides and pyridine) and 4) asphaltenes fatty acids, ketones, and porphyrins) (Colwell *et al.*1978).

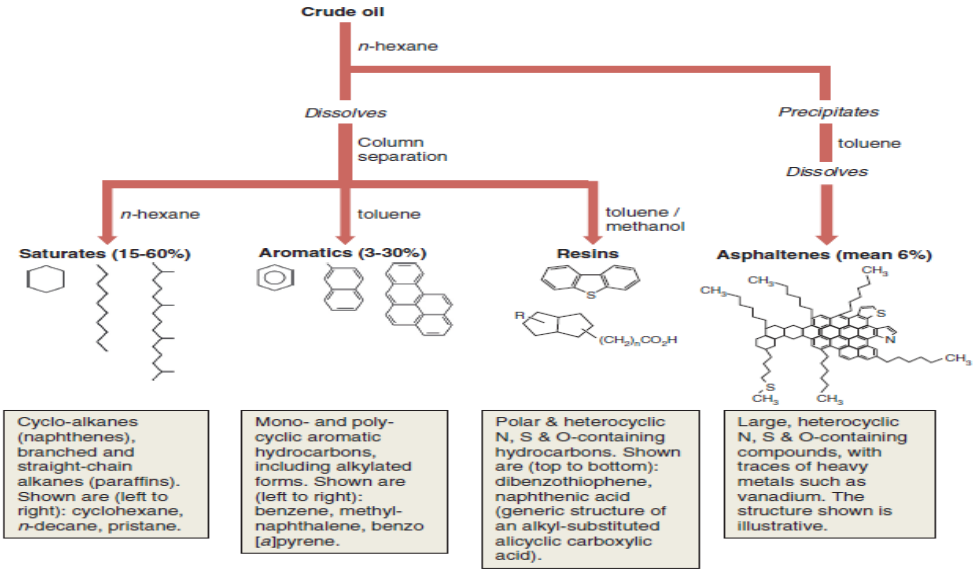


Figure1.1. Operational classification of the different classes of crude oil. Representative structures are provided for each class. Adapted from Mcgenity (2014).

Several factors such as age, source and geological history determine the relative proportion of each of these fractions in the oil (Fig.1.2) (Hyne 2001, Wang *et al.* 2003).

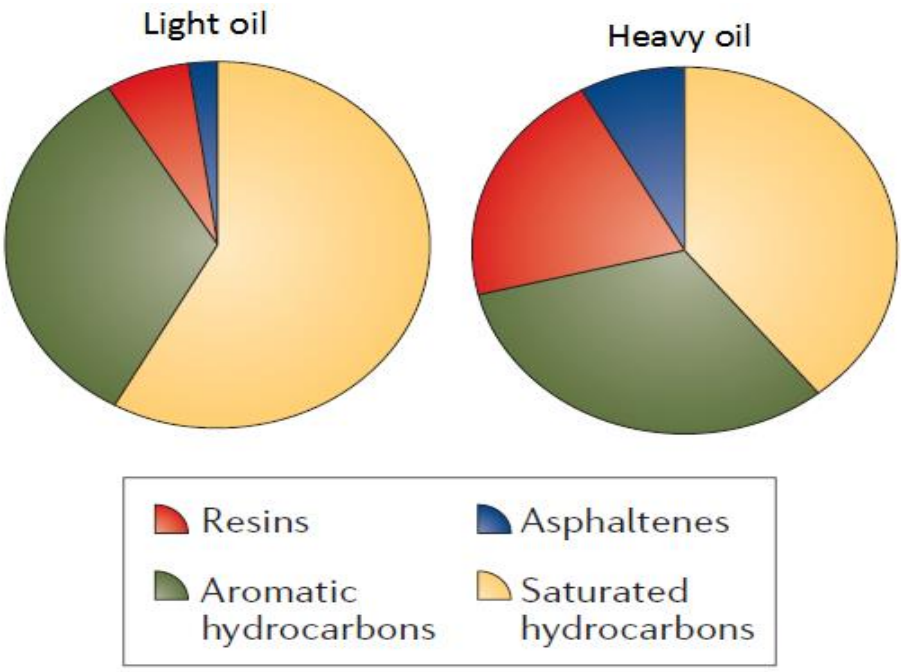


Figure 1.2. Composition of light and heavy crude oil. Light oils generally have high contents of saturated and aromatic hydrocarbon and a small proportion of resins and asphaltenes, while heavy oils are lower in saturated and aromatic hydrocarbons and have a higher proportion of resins and asphaltenes (Head *et al.* 2006).

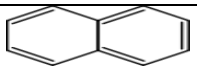
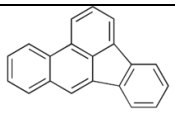
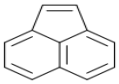
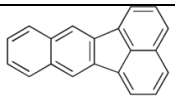
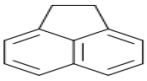
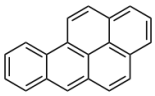
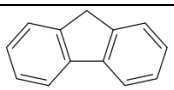
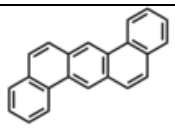
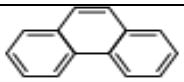
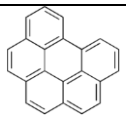
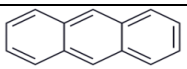
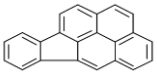
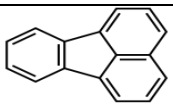
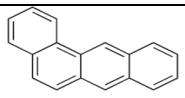
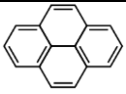
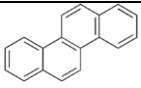
Saturated hydrocarbons include the linear and branched alkanes and the cycloalkanes. Alkanes constitute the major fraction of oils, and can account for 60-70% (Speight and Arjoon 2012) of crude oils (Fig. 1.2). The number of carbon atoms determines the physical and chemical properties of alkanes. Molecules with up to 4 carbon atoms are gaseous, 5 to 17 carbon atoms are liquid and alkanes with more than 18 carbon atoms are solid and known as waxes. Moreover chemical properties such as, boiling point, viscosity and density increase with increasing number of carbon atoms while solubility in water and toxicity decrease.

Aromatic hydrocarbons can be grouped into two main categories, the monoaromatics known as BTEX (benzene, toluene, ethylbenzene and xylene) and the polycyclic aromatic hydrocarbos (PAHs) that consist of two or more fused benzene rings. The

properties differ mainly in the volatility, aromatic ring number, molecular weight and water solubility (Kanaly and Harayama 2000, Harmsen 2004).

PAHs are considered widespread, ubiquitous environmental pollutants (Laflamme and Hites 1978, Johnson *et al.* 1985) that receive prominent attention in risk assessment due to their well-documented mutagenicity and carcinogenicity in humans (Phillips 1983, McAuliffe 1987, Cerniglia 1992). Sixteen PAHs have been listed as priority pollutants by the United States Environmental Protection Agency (USEPA) (Table 1.1.). Due to their toxicity and persistence, they are of greater long-term environmental significance (Head *et al.* 2006).

Table 1.1. List of the priority PAH pollutants and their molecular structures.

PAH	**Chemical structure**	**PAH**	**Chemical structure**
naphthalene		benzo[b]fluoranthene	
acenaphthylene		benzo[k]flouranthene	
acenaphthene		benzo[a]pyrene	
fluorene		dibenz(ah)anthracene	
phenanthrene		benzo[ghi]perylene	
anthracene		indeno(1,2,3-cd)pyrene	
fluoranthene		benzo[a]anthracene	
pyrene		chrysene	

Resins and asphaltenes are the most polar, structurally unresolved groups of crude oil components. They consist of aromatic structures with aliphatic substitutions and hetero atoms (oxygen, sulphur, or nitrogen). They are classified based on solubility in organic solvents (Akbarzadeh *et al.* 2007) rather than structure. Resins are smaller and more polar than asphaltenes, the latter have high density (up to 1.2 mg/L) and tend to precipitate (Goual and Firoozabadi 2004, Goual 2012). They are abundant in heavy oils and have limited bioavailability and toxicity. Asphaltenes have a complex structure and may contain trace amounts of heavy metals such as nickel and vanadium. Asphaltenes do not have a specific chemical formula and their exact molecular structure is hard to determine because their molecules tend to stick together in solution (McKenna et al., 2013).

1.3. Microbial degradation of hydrocarbons

Biodegradability of petroleum hydrocarbons has garnered the interest of microbiologists since 1895 when Miyoshi revealed that *Botrytis cinerea*, a common industrial fungus, attacks paraffins (Miyoshi, 1895). Extensive studies proceded then to demonstrate that bacteria, and to a lesser extent fungi, are able to use oil hydrocarbons as sole carbon and energy sources, or degrade them cometabolically, and are extremely diverse and ubiquitous in soil, marine and fresh water ecosystems (Sutiknowati 2007). Hydrocarbon biodegradation is an energetically favourable process (Speight and Arjoon 2012), the final products are carbon dioxide and water preceded by successive breakdown of the hydrocarbon molecule into a range of smaller products until achieving complete mineralisation.

Van Hamme *et al.* (2003) described the order in which hydrocarbons are biologically degraded based on their chemical properties as follows: *n*-alkanes > branched alkanes > monoaromatic hydrocarbons > cyclic alkanes > polynuclear aromatic hydrocarbons. Fig. 1.3 provides an overview of the processes involved in hydrocarbon biodegradation (Das & Chandran, 2011). Following passive influx into a cell, degradation is always initiated by an oxidative enzymatic attack with oxygenases and peroxidases that introduce oxygen atoms into the hydrocarbon molecules. Subsequently, these pre-intermediate compounds are further oxidised and introduced as intermediates into the TCA cycle where they can be used in vital metabolic processes in the bacterial cell such as energy harvesting (respiration) and synthesis of biomass.

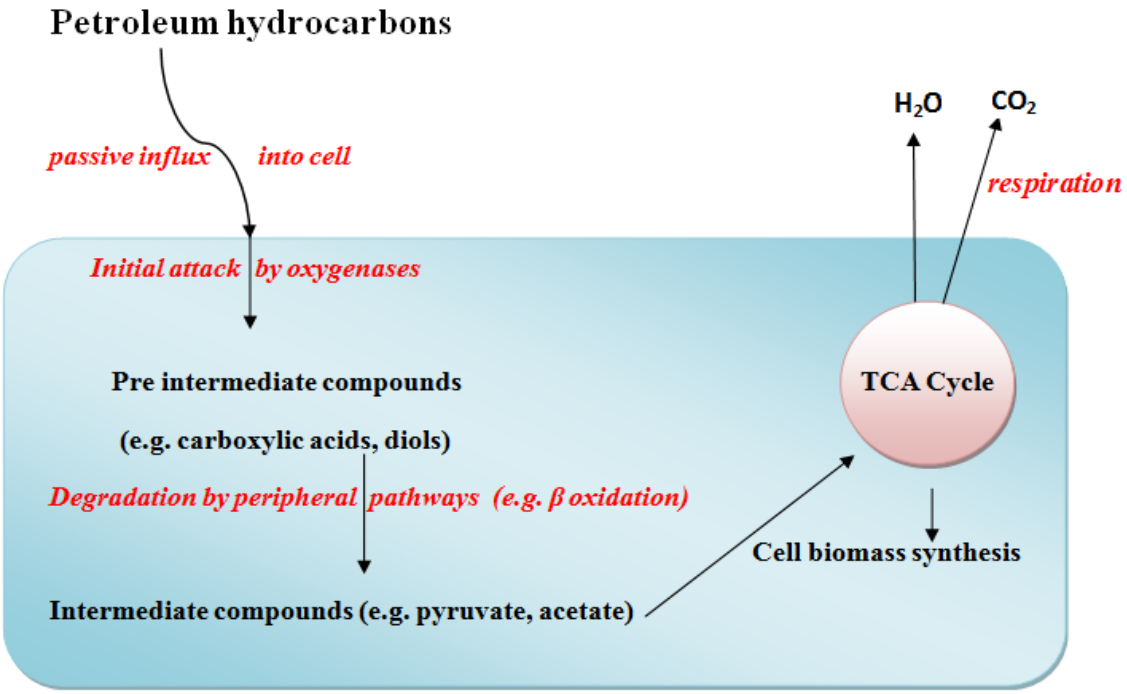


Figure 1.3. Overview of the main principle of aerobic hydrocarbon degradation by microorganisms (Das and Chandran 2011).

1.3.1. Metabolic pathways for degradation of saturated hydrocarbons

Due to the fact that they constitute the largest fraction of crude oil by mass, the biodegradation of saturated hydrocarbons is quantitatively the most important process in the removal of crude oil from the environment (Head *et al.* 2006). The mechanism by which microbes break down alkane molecules has been described in many bacterial species. Under aerobic conditions, the degradation always starts when oxygenases introducing one or more oxygen atoms into the alkane molecule. This attack can subsequently be followed by four different mechanisms (Fig. 1.4). The first and most commonly described pathway involves terminal oxidation (Van Beilen *et al.* 1994 & 2001, Li *et al.* 2008) where alkanes are attacked at their terminal methyl group to yield the corresponding primary alcohol. Alcohol dehydrogenase further oxidises alcohol to the corresponding aldehyde molecule and finally an aldehyde dehydrogenase catalyses the oxidation of that aldehyde into the corresponding carboxylic acid that further undergoes beta oxidation (Watkinson and Morgan 1990).

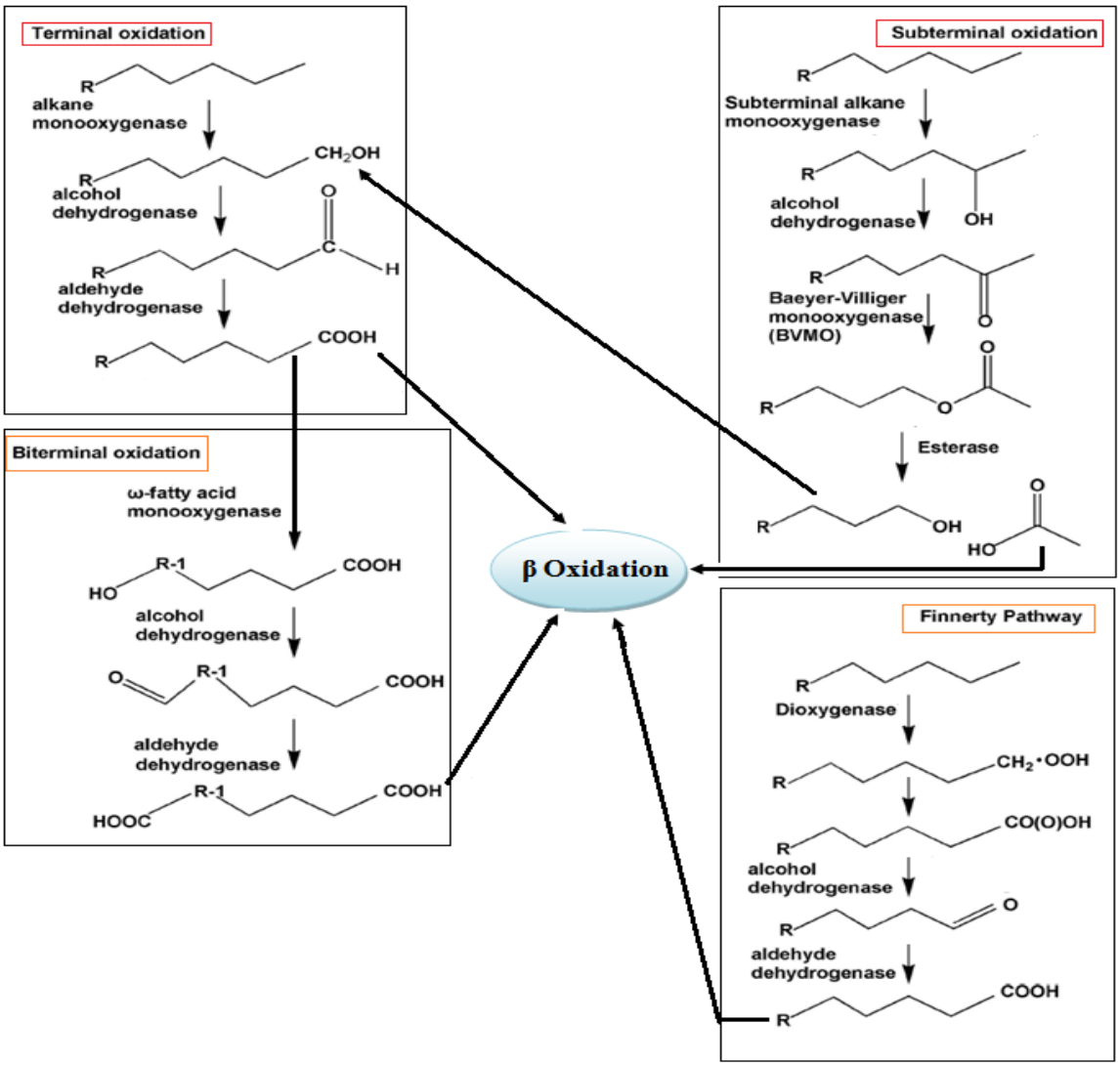


Figure 1.4. Degradation pathways of alkanes (van Beilen *et al.* 2003, Ji *et al.* 2013). Four mechanisms are illustrated all of which generate carboxylated alkanes for β-oxidation. The sub-terminal pathway generates a hydroxylated alkane which feeds into terminal oxidation pathway.

The fatty acid may also undergo a ω-hydroxylation at the ω position to generate a ω hydroxy fatty acid that proceeds to beta oxidation after having been converted to a dicarboxylic acid (Kester and Foster 1963, Coon 2005). This process is known as biterminal oxidation. The third pathway reported is subterminal oxidation when a primary alcohol and a secondary alcohol with the same length are formed by oxidation of the alkanes at their subterminal positions (Forney and Markovetz 1970, Kotani *et al.* 2003). Kotani *et al.* (2007) showed that the secondary alcohol is converted to the corresponding ketone, and then oxidized by a Baeyer–Villiger monooxygenase to form an ester that yields an alcohol and a fatty acid by hydroxylation with esterase.

In the Finnerty pathway the first step is oxidation of the n-alkane to n-alkyl hydroperoxide catalysed by a dioxygenase (Maeng *et al.* 1996). This is further oxidised to yield a peroxyacid, alkyl aldehyde and then a fatty acid (Finnerty 1988). This pathway is unique to *Acinetobacter* sp. (Finnerty 1988, Lin *et al.* 2010).

Cycloalkanes are most likely degraded by co-metabolism. Their degradation, otherwise, follows generally the terminal or peripheral pathway as demonstrated in Fig. 1.5.

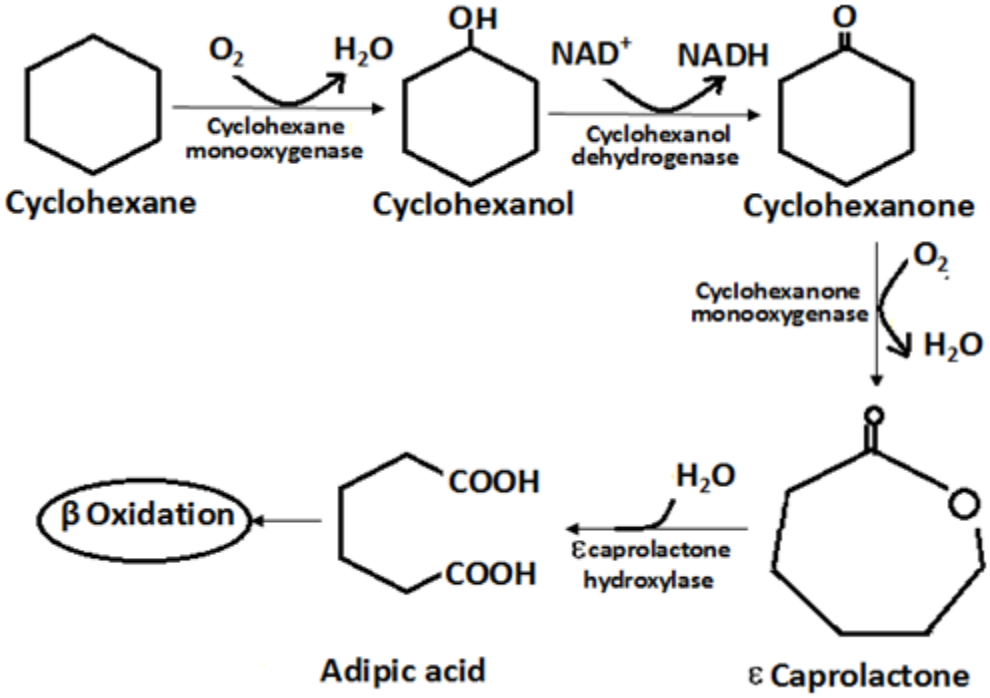


Figure 1.5. Metabolic pathway for cycloalkanes degradation represented by cyclohexane.

1.3.2. Metabolic pathways of degradation of aromatic hydrocarbons

Due to their notorious effects, the degradation of aromatic hydrocarbons has attracted the attention of scientists and several studies demonstrated the biodegradability of several compounds belonging to this class. Monoaromatics (BTEX) are easier to degrade compared to PAHs due to their less complex structure. PAHs with up to three benzene rings have been demonstrated to be prone to biodegradation, while PAHs with

four or more benzene rings are thought to be resistant to biodegradation. However, numerous studies demonstrated the biodegradability of high molecular weight PAHs (Bogan and Lamar, 1996, Silva *et al.* 2003) such as benzo [*a*] pyrene (Cerniglia and Gibson 1979) and phenanthrene (Hammel *et al.* 1992).

Bacteria belonging to the genus *Cycloclasticus* are demonstrated to be amongst the most significant players in aerobic aromatic hydrocarbon degradation in the marine environment (Head *et al.* 2006). A list of the main global aerobic hydrocarbon-degrading bacteria in the environment is provided in Head *et al.* (2006)

The general pathway for degradation of monoaromatic hydrocarbons results in the formation, by the action of dioxygenase, of either a diol, e.g., catechol, or a dicarboxylic acid e.g. 2-hydroxy-cis-cis-muconic acid by a process known as cis-hydroxylation (Fig. 1.6).

An ortho or meta cleavage results in the opening of the cathecol ring generating, in the first case, cis-muconic acid then beta-ketoadipic acid and finally the succinic acid plus acetatyl-CoA; and in the second case, 2-hydroxy-cis-cis-muconic semialdehyde, 2-keto-4-pentanoic acid and finally a pyruvic acid and acetaldehyde. When extensive, alkylation is known to inhibit the biodegradation of benzene, however, simple alkyl substitution facilitates its biodegradation. In addition, substituted aromatics are generally first metabolized by beta-oxidation of the side chain, and then the ring structure is cleaved.

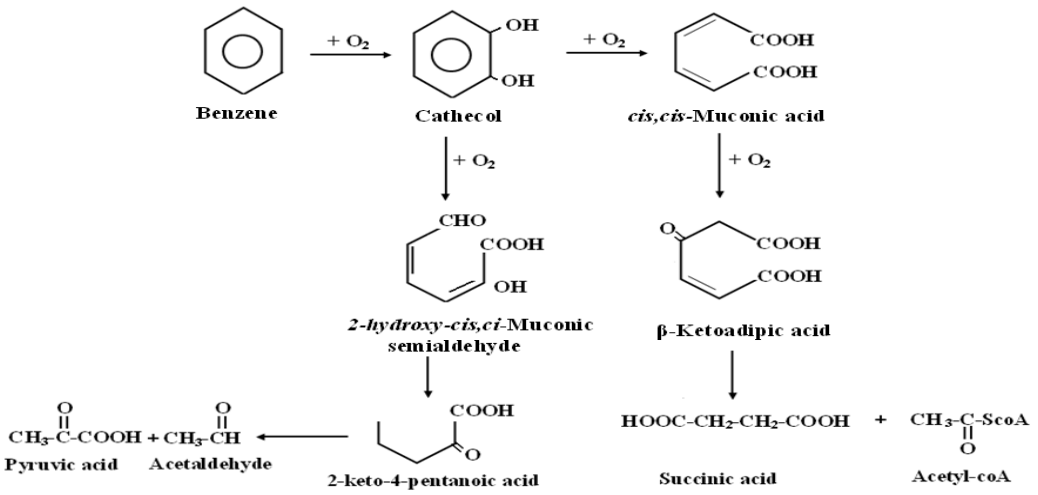


Figure 1.6. Microbial metabolism of the benzene aromatic ring (Atlas and Bartha 1998).

The metabolic pathway of PAH biodegradation has been well described for many bacterial and fungal strains (Cerniglia 1984 &1992, Menn *et al.* 1993, Kiyohara *et al.* 1994, Lin et al. 2010). Fig. 1.7 shows a general pathway for degradation of the simplest PAH naphthalene by *Bacilus fusiformis* as illustrated by Lin and co-workers (2010). The benzoic acid produced by this bacterium is further mineralised into carbon dioxide and water.

This pathway can have many variations and other possible pathways have been described in *Nocardia otitidiscaviarum* (Zeinali *et al.* 2008), *Pseudomonas putida* (Van Hamme *et al.* 2003) and *Bacillus thermoleovorans* (Annweiler *et al.* 2000).

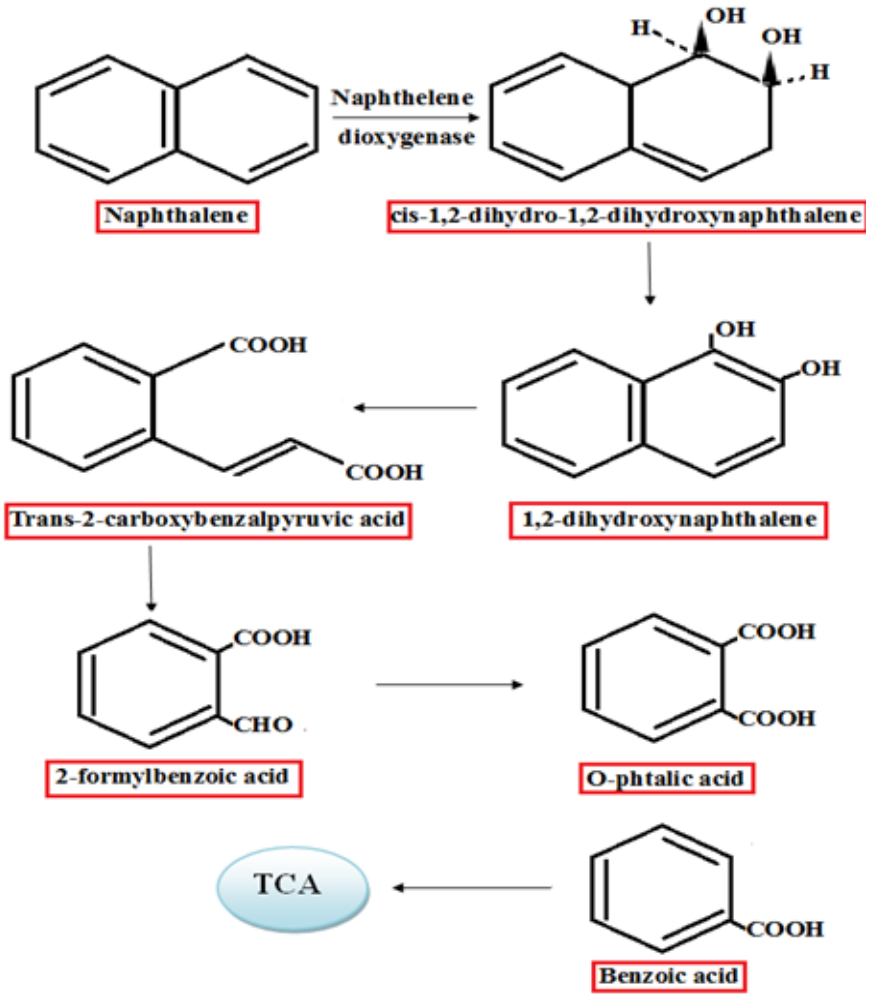


Figure1.7. Proposed metabolic pathway for the degradation of naphthalene by *Bacillus fusiformis* (BFN) (Lin *et al.* 2010). Dashed lines refer to probable but not confirmed metabolite.

Resins and asphaltenes are traditionally considered to be recalcitrant to microbial alteration. However, a few studies have demonstrated the possibility of their biodegradation (Connan 1984, Jenisch-Anton *et al*. 2000, Magot *et al.* 2000).

1.4. Environmental factors affecting hydrocarbon degradation in the ocean

The potential for hydrocarbon biodegradation in the natural environment can be hugely affected by conditions of the surrounding environment (Loehr and Webster 1996, Posada-Baquero and Ortega-Calvo 2011). Several studies have shown that the hydrocarbon biodegradation extent proved to be much lower in field studies than in the laboratory (Wild *et al.* 1990 & 1991, Cassidy *et al.* 1996, Balba *et al.* 1998, El Fantroussi and Agathos 2005, Gallego *et al.* 2010) due to the difficulty of controlling some environmental factors that dramatically affected the biodegradation process.

Several factors have been recognised to affect the degradation of hydrocarbons in the marine environment. Brusseau (1998) thoroughly discussed most of these factors. However, Atlas and Bartha (1972), Braddock *et al.* (1997) and Xia *et al.* (2006) determined that temperature and nutrient availability have the major effect. In addition, biodegradation is only one of several processes that take place immediately after an oil spill. These processes are known as "weathering" and the interaction between them determines the ultimate fate of oil in the ocean (Fig. 1.8).

The activity of enzymes involved in hydrocarbon degradation is highly temperature-dependant (Struvay and Feller 2012) which makes hydrocarbon degradation in any environments also temperature-dependant. Ambient temperature also affects the properties of spilled oil, hydrocarbon bioavailability, mass transfer and the overall growth rate and metabolism of microorganisms (Farrell and Rose 1967, Namkoong 2002, Feitkenhauer *et al.* 2003, Venosa and Zhu 2003, Sartoros *et al.* 2005). In addition, temperature also affects the composition of microbial communities (Atlas 1981). Hazen et al. (2010) also observed a surprisingly rapid hydrocarbon biodegradation rates in deep waters at 5°C during the Deepwater Horizon oil spill.

Nonetheless, hydrocarbon degradation has been found to occur at a wide range of temperatures (as low as below 0°C to as high as 70°C) (Cartaxana *et al.* 1999). Generally, at low temperatures, the oil viscosity increases and the rate of diffusion decreases which affects the biodegradation process negatively by reducing

bioavailability and volatilization of the short-chain alkanes that exhibit toxicity to the hydrocarbon degrading communities.

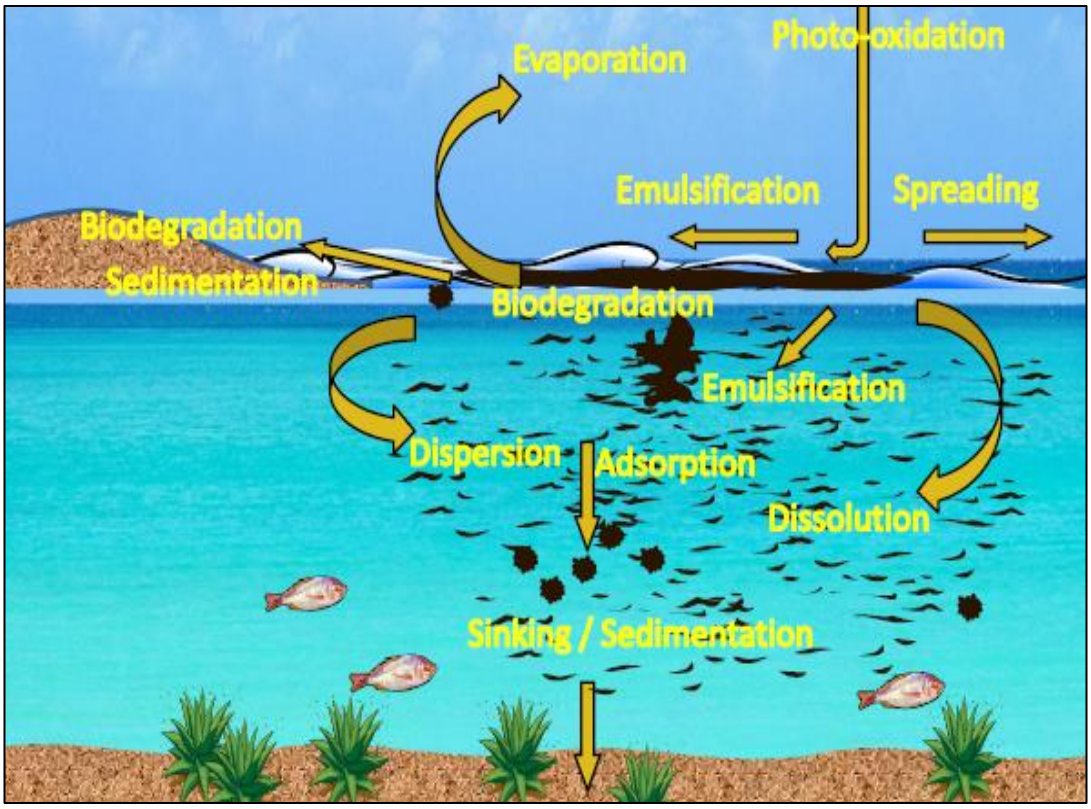


Figure 1.8. Schematic representation of the biological and chemical transformation of oil in seawater following an oil spill. **Spreading** of oil occurs at the sea surface as a single slick enhanced by wave and wind action (Reed et *al.* 1999). **Evaporation** of the light fractions (such as monoaromatics and short chain alkanes) takes place in the first 24 h (Sharma *et al.* 2002) which increases oil viscosity 1000 fold (Zhong and You 2011). **Dispersion** of oil droplets leads to solubilisation of water soluble fractions, which increases the bioavailability of oil and encourages the growth of hydrocarbonoclastic bacteria (Macnaughton *et al.* 1999, Venosa and Holder 2007) and promotes **biodegradation**, **dissolution** and **sedimentation**. Turbulence leads to the suspension of seawater droplets in the oil and the formation of water-in-oil emulsions (Fingas 1995) increasing the volume of pollutant (Scholz *et al.* 1999) and hindering dissipation of oil. Oil compounds can also be **photo-oxidised** (Dutta and Harayama 2000, Barron *et al.* 2003). Figure adapted from (McGenity *et al.* 2012).

Bossert and Bartha (1984) reported the highest biodegradation rates at 30-40°C in the soil, whereas in the marine environment, the highest biodegradation rates have been obtained at temperatures ranging from 15-20°C (Jordan and Payne 1980) and 20-30°C in freshwater ecosystems (Cooney 1984). Moreover, Westlake *et al.* (1974) detected a limited activity of microbial populations at 4°C compared to 30°C although they could metabolise hydrocarbons at both temperatures. However, several studies proved biodegradation efficiency at low temperatures. Colwell *et al.* (1978) showed that the

biodegradation of crude oil was best achieved at 3°C with a mixed culture in beach sand samples than at 22°C. Despite the wide range of temperatures at which the biodegradation may occur, the most common observation is that the lower the temperature the slower the biodegradation rate.

The other factor proved to play a crucial role in hydrocarbon degradation in marine water is the availability of nutrients. Following a major oil spill in the ocean, the carbon supply dramatically increases creating an environmental stress and an acute imbalance between the organic and mineral nutrients (Carbon:Nitrogen:Phosphorus) ratio needed for normal bacterial metabolism (Atlas 1985, Atlas 1995a, Röling *et al.* 2002). Nitrogen and phosphorus are the most important nutrients that become limiting. They are essential for amino acid and nucleic acid synthesis and are the most important mineral nutrients that affect hydrocarbon biodegradation as they quickly become depleted following the hydrocarbon overload. This problem seems to have a bigger impact in the marine environment as the levels of nitrogen and phosphorus are naturally low in seawater (Floodgate 1984). The outcome of the addition of nitrogen and phosphorus to polluted sites has been controversial and an adequate level of nutrients seems to be crucial (Boufadel *et al*. 1999). High levels may be detrimental as they may lead to eutrophication that enhances algal growth which ultimately reduces the dissolved oxygen concentration in water as the algal biomass is broken down aerobically (Nikolopoulou and Kalogerakis 2008). However, the positive effect of nutrient addition depends largely on the hydrocarbon molecule being degraded but also on the metabolism of the microorganisms that degrade it.

Other factors such as oxygen availability, salinity, pressure and pH have also been demonstrated to have an effect on the decontamination process in the ocean (Leahy and Colwell 1990, Das and Chandran 2011).

1.5. Ecology of hydrocarbon degradation in the environment

The hydrocarbon degradation process in the marine ecosystem is not a simple process involving a hydrocarbon degrader that attacks hydrocarbons by the action of enzymes. Hydrocarbons degraders are members of a network of interactions at many levels, with many other members of the community and with the environment. Cooperative interactions that enhance degradation, competition for limiting nutrients and resources,

predation by protozoa and bacterial lysis by phage constitute the main aspects of these interactions (Fig. 1.9) (Head *et al.* 2006).

Interactions between members of the microbial network can be of paramount importance in promoting the rate of hydrocarbon removal from the marine environment. Examples of these mutualistic interactions are illustrated by many studies. *Alcanivorax borkumensis* is known as an alkane degrading bacterium that does not degrade PAHs, however, when added to seawater microcosms containing crude oil, it promoted the degradation of PAHs (McKew *et al.* 2007).

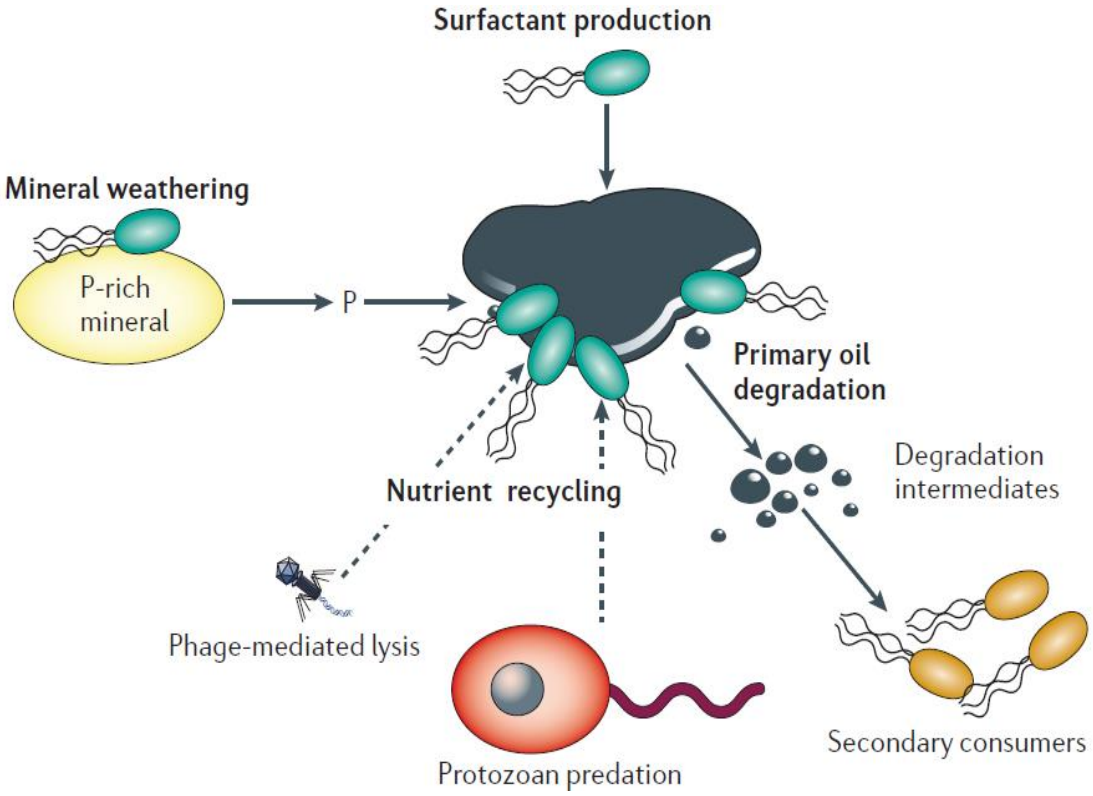


Figure 1.9. Schematic illustration of the interaction between hydrocarbon degraders and their environment (Head *et al.* 2006).

Schneiker and colleagues (2006) demonstrated that *Alcanivorax borkumensis* produces biosurfactants to enhance alkane solubility. These biosurfactants may have increased the availability of PAHs to enhance their attack by the PAH degraders that were used in the study of (McKew *et al.* 2007). On the other hand, some bacteria may secrete bioactive compounds to inhibit competitors and hence slow down the mineralisation process.

McKew and collaborators (2007) showed that *Alcanivorax* spp. could not grow or degrade alkanes in the presence of *Thalassolituus*. Another study by the same author demonstrated growth of *Alkanivorax* in all microcosms except the microcosm to which *Thalassolituus oleivorans* had been added previously. An enhancement in the degradation of a PAH and a change in the community composition marked by dominance of *Cycloclasticus* spp. were observed by Iwabuchi *et al.* (2002) due to the presence of extracellular polysaccahrides produced by *Rhodococcus rhodochrous* that resulted in the emulsification of oil. *Pseudonams* sp. grown on phenanthrene as a sole carbon and energy source produce an intermediate called salicylate that enhanced the degradation of high molecular weight PAH that the isolate could not use for growth by inducing the production of a PAH dioxygenase (Chen and Aitken 1999).

Jiang and Paul (1996) and Cochran *et al.* (1998) studied the occurrence of phage lysis in the marine environment, their studies demonstrated that phage-mediated lysis increased the turnover of biomass and resulted in lysis of a large proportion of the bacterial community which may be the reason for changes in community structure unrelated to degradation (Röling *et al.* 2002).

Protozoa have the ability to graze selectively and control the biomass of hydrocarbon degraders, therefore exhibit negative effects on oil degradation. Kota (1999) revealed that the numbers of hydrocarbon degrading bacteria were much higher in the absence of predators.

1.6. Bioremediation strategies

Following an oil spill, several physical (*e.g*. in situ burning, mechanical removal, and manual removal) and chemical (*e.g.* dispersants, emulsifiers) clean-up procedures can be applied immediately to lessen the extent of the pollution. However, these strategies can recover only 10-15% of the spilled oil according to the U.S. Congress (1991). Bioremediation is a cost-effective and environmentally friendly strategy that consists of using microorganisms that have the enzymatic machinery capable of breaking down the hydrocarbon pollutants into less harmful compounds. Bioremediation can substantially clean up oil contaminated environments and has become a widely adopted technique after its first successful application during the *Exxon-Vadez* oil spill in 1989 (Venosa and Zhu 2003) until present with the Deepwater Horizon spill. Three bioremediation

strategies have been developed in order to improve and accelerate the removal of pollutants from natural ecosystems namely natural attenuation, biostimulation and bioaugmentation (Iwamoto and Nasu 2001).

Natural attenuation is a natural process that utilises the intrinsic degradation capability of the indigenous microorganisms present in the environment to degrade contaminants under the natural environmental and nutritional conditions (Mills *et al.* 2003). This process is relatively slow, concentration-dependant, and complete removal of contaminant is either unlikely or takes a long time due to the low numbers of native hydrocarbon degraders in the environment (less than 1%) (Forsyth *et al.* 1995). It is reported that approximately 25% of all petroleum-contaminated land is being cleaned up using natural attenuation (Holden *et al.* 2002). Beazley *et al.* (2011) showed the efficacy of natural attenuation in removing hydrocarbons from a polluted salt marsh ecosystem that was impacted by the Deepwater horizon spill incident.

Biostimulation consists of promoting the growth of natural autochthonous hydrocarbon degrading communities in a given environment by addition of mineral nutrients that become deficient in a polluted ecosystem. This technique is most recommended as it uses autochthonous microbes already present and adapted to the conditions of life in a certain environment. This eliminates the risk of unexpected behaviour or loss of viability of allochthonous microbes that may be introduced. It is ideal for the removal of oil contaminants from ecosystems where hydrocarbon degraders are naturally present and mineral nutrients such as nitrogen and phosphorus are naturally low such as seawater (Burns *et al.* 1999).

Bioaugmentation consists of seeding the polluted environment with high numbers of highly specialised bacterial species or bacterial consortia in order to accelerate the decontamination process (Leahy and Colwell 1990, Gentry *et al.* 2004). Due to the low abundance of hydrocarbon degraders in some environments, the inoculation of these environments with microorganisms known to be metabolically capable of breaking down the contaminant has been regarded as a promising technology in speeding up the recovery of oil pollutants (Tyagi *et al.* 2011). However, it is still in the experimental stage due to the fact that survival of the introduced microbes and their biodegradation capability depend on environmental conditions (Vogel 1996, Gentry *et al.* 2004).

1.6.1. Application and comparison of bioremediation strategies

The choice of one of the bioremediation techniques requires a deep understanding of the environmental conditions at a specific site and the microbial communities that inhabit it, their metabolic capabilities and the factors that can affect their survival.

Successful and failed applications of these studies have been widely reported. Yu *et al.* (2005) showed best degradation of PAHs under natural attenuation conditions compared to biostimulation and bioaugmentation. Accordingly, Mills *et al.* (2003) reported up to 95% of PAH degradation under natural attenuation, while other studies reported ineffectiveness of natural attenuation in hydrocarbon removal (Holden *et al.* 2002). Johnson and Scow (1999) demonstrated an inhibitory effect of biostimulation on PAH degradation in soil, while many other studies showed that nutrient addition enhanced the hydrocarbon degradation rate (Bragget *et al.* 1994, Swannell *et al.* 1995, Venosa *et al.* 1996, Wright *et al.* 1997, Head and Swannell 1999, Nikolopoulou and Kalogerakis 2008, Prince 1997, Röling *et al.* 2002). In addition, bioaugmentation has been proved ineffective by several studies (Tagger *et al.* 1983, Venosa *et al.* 1996, MacNaughton *et al.* 1999), but enhanced the degradation rate of hydrocarbons in others (McKew *et al.* 2007). Yu *et al.* (2005) reported a slower biodegradation rate after bioaugmentation with a bacterial consortium and demonstrated the positive effect of biostimulation on the mineralisation rate of PAHs in mangrove sediment. The study illustrated that the autochthonous communities can not only interact with the added consortium but also compete for resources. In contrast, Mckew *et al.* (2007) enhanced alkane degradation in seawater microcosms after bioaugmentation with *Alcanivorax* compared with nutrient amendments.

The overall response of marine bacterial communities to oil pollution and bioremediation remains unpredictable (Head *et al.* 2006). The success of these strategies is inconsistent with dependency on many factors related to the ecosystem and the pollutant itself (Balba *et al.* 1998). To ensure a successful outcome, the choice of the bioremediation strategy to be applied must be delicately made based on a well-established investigation of the site, its natural inhabitants and the intrinsic and extrinsic factors that may promote or repress their activity. This is realistically hard to achieve given the hazardous nature of oil and the immediate mitigation action that needs to be taken which may explains the poor performance of certain bioremediation strategies.

1.6.2. Effect of bioremediation on microbial diversity and community dynamics following oil spills

The response of microbial populations in the environment to petroleum hydrocarbon pollution varies depending on several factors. Several studies focused on microbial dynamics in soil, freshwater and marine ecosystems following an oil spill and demonstrated the complexity and the peculiarity of the huge, poorly-understood microbial world.

Numerous studies reported a substantial reduction in microbial diversity after oil is spilled into the environment due to selection towards hydrocarbonoclastic bacteria (Kasai *et al.* 2002, Röling *et al.* 2002, Head *et al.* 2006, McKew *et al.* 2007). On the other hand, an increased diversity has been demonstrated to exist at a hydrocarbon-contaminated site (Juck *et al.* 2000). In addition, Atlas (1995a) reported an increase in the number of hydrocarbon degraders from 1% up to 10% of the microbial community upon exposure to oil pollutants. Röling *et al.* (2002 & 2004) demonstrated not only a marked change in the community composition within days, but also a complete disappearance of Achaea that could no longer be detected after exposure to oil.

Numerous studies detected changes in community structure in response to oil exposure and/or nutrient addition. For example, Röling *et al.* (2002) explained that the availability of nutrients dictates the structure of the hydrocarbon degrading community. Later studies (Röling *et al.* 2002 & 2004) showed that *Alkanivorax* sp. type 1 responded favourably to high concentrations of nutrients, while under lower nutrient concentration, another distinct genotype belonging to *Alkanivorax* sp. type 2 became more abundant. Accordingly, Head *et al.* (2006) revealed an obvious selection for microbial communities based on different nutrient-addition regimes in oil-contaminated sediments. Moreover, McKew *et al.* (2007) demonstrated that A*lkanivorax* was not detected in oil amended microcosms but became very abundant upon nutrient amendment.

Ultimately, microbial community composition after the oil spill in the ocean seems to shift according to oil pollution and bioremediation strategy used. Comparison between the community structure before and after the spill or the bioremediation is crucial to explain those dynamics and elucidate the way in which the native microbial populations are being affected.

1.7. Stable Isotope Probing (SIP) and potential application for improved bioremediation

With the increased demand on oil as an energy source, pollution with hydrocarbons happens daily in the marine environment. Microbes not only play a critical role in global biogeochemical cycling, but are also the main decontaminating agents in oil-polluted environments where they play an unparalleled role in restoring natural balance and dissipating environmental stress. The focus on the application of bioremediation *in situ* has been growing. As previously explained, an understanding of the community dynamics in response to the pollution and environmental factors at the contaminated site as well as identification of microorganisms and their metabolic capabilities is highly valuable to achieve successful cleanup. Amann *et al.* (1995) reported that most of the seawater bacteria detected microscopically cannot be cultivated in conventional media. Moreover, Torsvik and Øvreås (2002), Lozupone and Knight (2008) and Zhang and Xu (2008) have shown that only 1% of the microbes in the environment can be grown by cultivation-based techniques and their behaviour under realistic environmental conditions is hard to predict. Culture-independent techniques have provided insight into the phylogenetic diversity of microbial communities. However, linking a specific function to a specific microbial species remained a big challenge until the emergence of stable isotope probing.

Stable isotope probing (SIP) consists of pulsing heavy ^{13}C (Radajewski *et al.* 2003, Dumont and Murrell 2005, Neufeld *et al.* 2007, Uhlík *et al*. 2009, Chen and Murrell 2010); ^{15}N (Bell *et al.* 2011, Buckley *et al.* 2007a & 2007b), or rarely ^{18}O and ^{2}H (Aanderud and Lennon, 2011, Woods *et al.* 2011) isotope-labelled substrate into an environmental sample and tracking the incorporation of heavy isotopes into phylogenetically informative biomarkers including DNA (Radajewski *et al.* 2000, Whitby *et al.* 2001, Morris *et al.* 2002, Jeon *et al*. 2003, Padmanabhan *et al.* 2003, Ginige *et al.* 2004, Lueders *et al.*, 2004a & 2004b, Hutchens *et al.* 2004); RNA (Manefield *et al.* 2002a, Lueders *et al.* 2004a, 2004b & 2004c, Manefield *et al.* 2005) phospholipid lipid fatty acids (PLFA) (Boschker *et al.* 1998, Bull *et al.* 2000, Boschker *et al.* 2001, Johnsen *et al.* 2002, Crossman *et al.* 2004, Tillmann *et al.* 2005) or proteins (Jehmlich *et al.* 2008a, 2008b and 2009) that can eventually be recovered and analysed

allowing the identification of microbial populations with a defined metabolic function (Fig. 1.10).

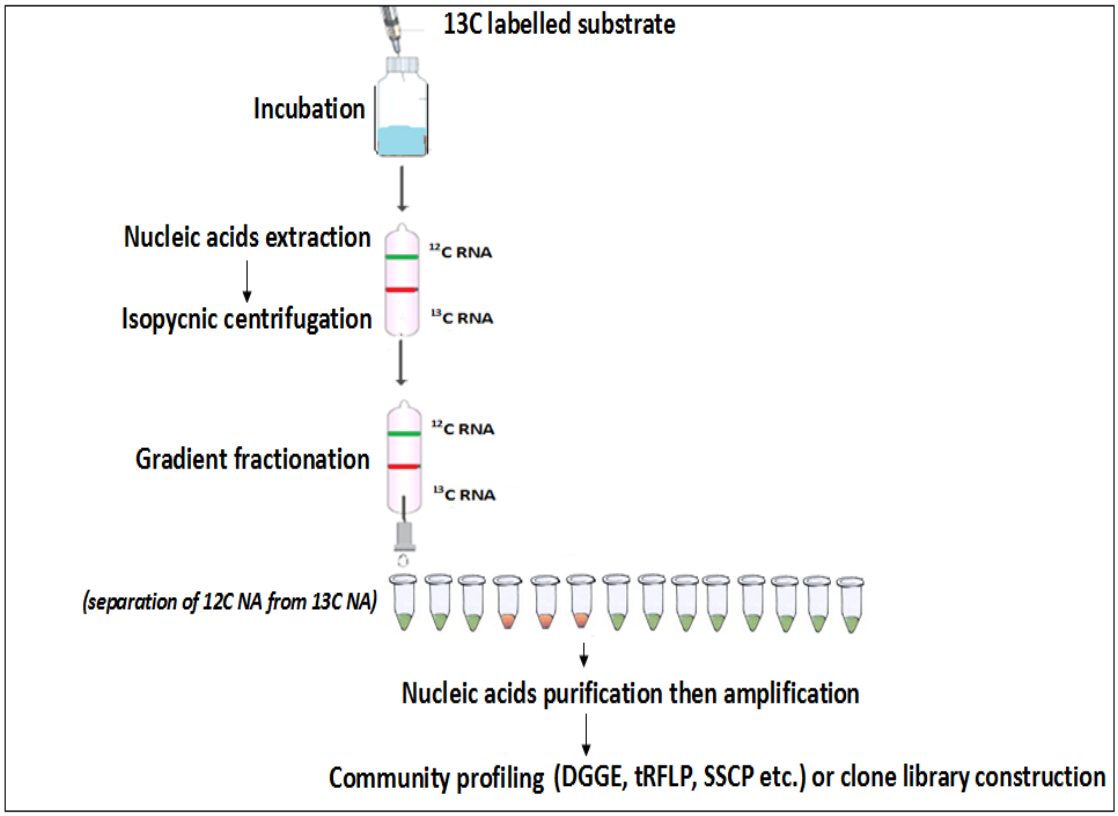


Figure 1.10. Principle of Nucleic acids stable isotope probing: Environmental sample is incubated with the ^{13}C labelled substrate. Native consumers incorporate the labelled isotopes into their biomarkers (e.g nucleic acids (NA)) that are then extracted and loaded on a heavy salt density gradient (Caesium chloride for DNA and Caesium trifluoroacetate for RNA). Isopycnic centrifugation separates the ^{13}C heavy NA from the light ^{12}C NA. Gradients are then fractionated and nucleic acids are precipitated and purified to be subsequently amplified and used for community profile analysis and identification of microbial taxa responsible for the consumption of the labelled substrate.

DNA-SIP is often used when the aim is to analyse functional or taxonomic genes coupled to specific metabolic process. However, it may require long incubation times for the cells to replicate and labelling to occur particularly for organisms with slow growth rates.

The use of ribosomal RNA in SIP studies is attractive because it allows the identification of the active members in a community due to its high production and rapid turnover rates (Manefield *et al.* 2002b), but also because it is directly related to

protein synthesis. However, functional genes encoding for the metabolic capability of the bacterial community cannot be accessed using rRNA analysis. Instead, mRNA can be used for this aim.

Huang *et al.* (2009) successfully detected naphthalene dioxygenase transcripts following SIP incubation with naphthalene. This can be hindered by the difficulty to isolate sufficient quantities of mRNA for SIP (Neufeld *et al.* 2007a). Dumont *et al.* (2011) demonstrated that mRNA was labelled at higher rates than their genes assigning more sensitivity to mRNA-SIP compared to DNA-SIP

PLFAs exhibit lower phylogenetic resolution compared to nucleic acids, which complicates the interpretation of complex communities. Proteins can also be used to obtain phylogenetic information, they can also indicate a specific microbial activity and hence, protein-SIP has recently been developed (Jehmlich *et al.* 2008a, 2008b and 2009).

A detailed discussion of the advantages and limitations of each of the above mentioned SIP methods is given by Gutierrez-Zamora and Manefield (2010).

The use of molecular techniques in monitoring dynamics in microbial communities during bioremediation is of great importance. Many studies have observed that following bioaugmentation, the growth of other hydrocarbon degraders is promoted, however, it is not clear whether these are responsible for the biodegradation results. Ueno *et al.* (2006 & 2007) used PCR and DGGE targeting 16S rRNA genes to monitor changes in soil communities during biostimulation and bioaugmentation treatments. The study demonstrated an increase in the degradation rate of diesel oil in the first week of bioaugmentation with *Pseudomonas aeruginosa* strain WatG paralleled with decreased band intensity for strain WatG. This coincided with a remarkable increase of the intensity of bands belonging to autochthonous bacteria that showed no diesel degrading activity. Thus, during biostimulation, the increase in the abundance of certain microbes in response to the addition of hydrocarbons is not necessarily always associated with their degradation. Some microbes may proliferate, competing for the added mineral nutrients but consuming carbon and energy from other compounds available in the same environment or simply, from by-products of degradation of these hydrocarbons. Studies that applied molecular techniques that link function to identity in an effort to study the

microbial dynamic during bioremediation are scarce and have just recently started to emerge (Sheppard *et al.* 2013).

In the light of this knowledge gap, a closer look at the carbon flow during bioremediation applications will assist in the identification of members of the community that derive carbon directly from the contaminant under investigation. In the case of bioaugmentation, SIP studies would help define whether biodegradation is achieved by the added strain or the latter is outcompeted by the indigenous microbes (Uhlik *et al.* 2012), which will serve as a powerful tool to achieve linkage between metabolic functions and corresponding phylogenetic identities in this context.

1.8. Research objectives and thesis structure

The aim of the present study is to compare the efficiency of the bioremediation strategies, biostimulation and bioaugmentation, in the degradation of model saturated and aromatic fractions of crude oil (*n*-hexadecane (long chain *n*-alkane), benzene (monoaromatic) and naphthalene (polyaromatic)) in cultures generated from pristine and oil-spiked seawater. Most importantly, this study aimed at monitoring the changes in community structure during the application of these strategies and at linking their degradation to specific microbial phyla in marine water using RNA stable isotope probing.

The following chapters describe the research work carried out in order to address the aim of this study. **Chapter two** describes the materials used and the methodology adopted throughout the whole study. **Chapter three** defines the preliminary experiments carried out in order to optimise the incubation and nutritional conditions required for the selection of active hydrocarbonoclastic bacteria in oil-spiked cultures and shows the effect of natural attenuation and biostimulation on the rate of oil mineralisation and on seawater community structure. **Chapter four** describes results of the biodegradability of saturated hydrocarbons represented by *n*-hexadecane, mono aromatic hydrocarbons (represented by benzene) and polycyclic aromatic hydrocarbons (PAHs) (represented by naphthalene) in pristine and oil-polluted seawater with and without nutrient amendments and shows the native *n*-hexadecane, benzene and naphthalene consumers in seawater that evolved under biostimulation conditions using RNA stable isotope probing. **Chapter five** includes results of experiments carried out to

study the effect of bioaugmentation in the presence or absence of fertilisation on the degradation rate of *n*-hexadecane, benzene and naphthalene and on the structure of the indigenous seawater bacterial community in response to bioaugmentation by using RNA-stable isotope probing. Finally, **Chapter six** provides a general discussion on the outcome of the study and perspectives for future research and applications.

Chapter 2

Materials and Methods

2.1. Cultures setup

2.1.1. Oil and nutrient-amended cultures

The oil used in the present study was a light crude oil obtained from Caltex Pty. Ltd. in Sydney. Unless otherwise indicated, the seawater used in the entire study was sampled from shallow marine waters (0-30cm depth) in Coogee Bay, NSW, Australia (33.921°S, 151.254°E) and transported directly to the University of NSW for immediate use in experimentation. Coogee sea water temperatures are always warm with a peak in the range 22 to 25° C reached in late January. Coldest temperatures are recorded towards the end of August in the range 16 to 19°C (According to the Australian Bureau of Meteorology).

A preliminary batch of enrichment cultures was prepared from seawater (Sampled in summer, February 2011) in order to optimise the incubation and nutritional conditions required to select for hydrocarbonoclastic bacteria and to determine the peak of their activity. Cultures consisted of four different enrichments and were named 1) **S**: seawater only; 2) **SN**: seawater amended with mineral nutrients (nitrogen and phosphorus); 3) **SO**: seawater spiked with crude oil and 4) **SON**: seawater spiked with crude oil and supplemented with nutrients. The final pH in the cultures ranged from 7.8 – 8. A description of these cultures is shown in Table 2.1.

Table 2.1. Composition of the oil and nutrient-amended enrichment cultures.

Oil and nutrient-amended cultures				
S	**SN**	**SO**	**SON**	**Sterile control**
1.4 L seawater	1.4 L seawater 40 mM NH_4Cl 2 mM K_2HPO_4	1.4 L seawater 14 ml crude oil	1.4 L seawater 14ml crude oil 40 mM NH_4Cl 2 mM K_2HPO_4	1.4 L heat-sterilised seawater

Based on the outcome of the preliminary cultures, the optimal conditions were then applied to set up two other batches of enrichment cultures for use in subsequent hexadecane (seawater sampled in autumn, March 2012) and aromatic hydrocarbon (seawater sampled in spring, early December 2012) biodegradation experiments. Crude oil was added at 1% (v/v) to the corresponding cultures (SO and SON). Nitrogen and phosphorus sources were NH_4Cl and K_2HPO_4 (Univar) respectively, and concentrations of these minerals were adjusted as recommended by Gibbs (1975) who demonstrated that 60 mg of N and 6 mg of P are needed to biodegrade 1 g of hydrocarbons. All cultures were prepared in 2 L glass bottles (Schott) closed with rubber stoppers. The volume of seawater dispensed into each bottle was 1400 ml. Cultures were incubated for two weeks on a rotary shaker (70 rpm) at room temperature (21°C). Pure (100%) molecular oxygen (50 ml) was injected with a syringe into the headspace on a daily basis to maintain aerobic conditions. Heat-sterilised seawater (1 h at 121° C) control (sterile control) was used to demonstrate that oil mineralisation in the oil-containing cultures (SO and SON) was solely achieved by microorganisms. Oil mineralisation in the enrichment cultures was measured by quantifying the carbon dioxide produced in the headspace. The concentration of mineral nutrients (nitrogen and phosphorus) was also measured using the colorimetric assays described below.

2.1.2. Oil and/or nutrient-adapted cultures

The oil and nutrient-adapted cultures generated were used to study the effect of preadaptation on the degradation of model hydrocarbons. Aliquots (100 ml) of each individual culture were added into 160 ml serum bottles in triplicate and spiked with 30 mg/l of ^{12}C or ^{13}C-fully labelled hexadecane ($^{13}C_{16}$, Sigma-Aldrich). Similarly, 100 ml of each individual culture coming from another equivalent set were dispensed into similar serum bottles and spiked with 30 mg/l of ^{12}C or ^{13}C-fully labelled benzene ($^{13}C_6$, Sigma-Aldrich) or10 mg/l of naphthalene ($^{13}C_{10}$, Sigma-Aldrich). ^{12}C and ^{13}C compounds-pulsed cultures were independent replicates. Sterile controls consisting of 100 ml of heat-sterilised seawater (1 h at 121° C) spiked with 30 mg/l of ^{12}C hexadecane or benzene or 10 mg/l of ^{12}C naphthalene respectively were used in triplicates to rule out the possibility of abiotic loss of substrates from cultures. An illustration of the setup of these cultures is provided in Fig. 2.1.

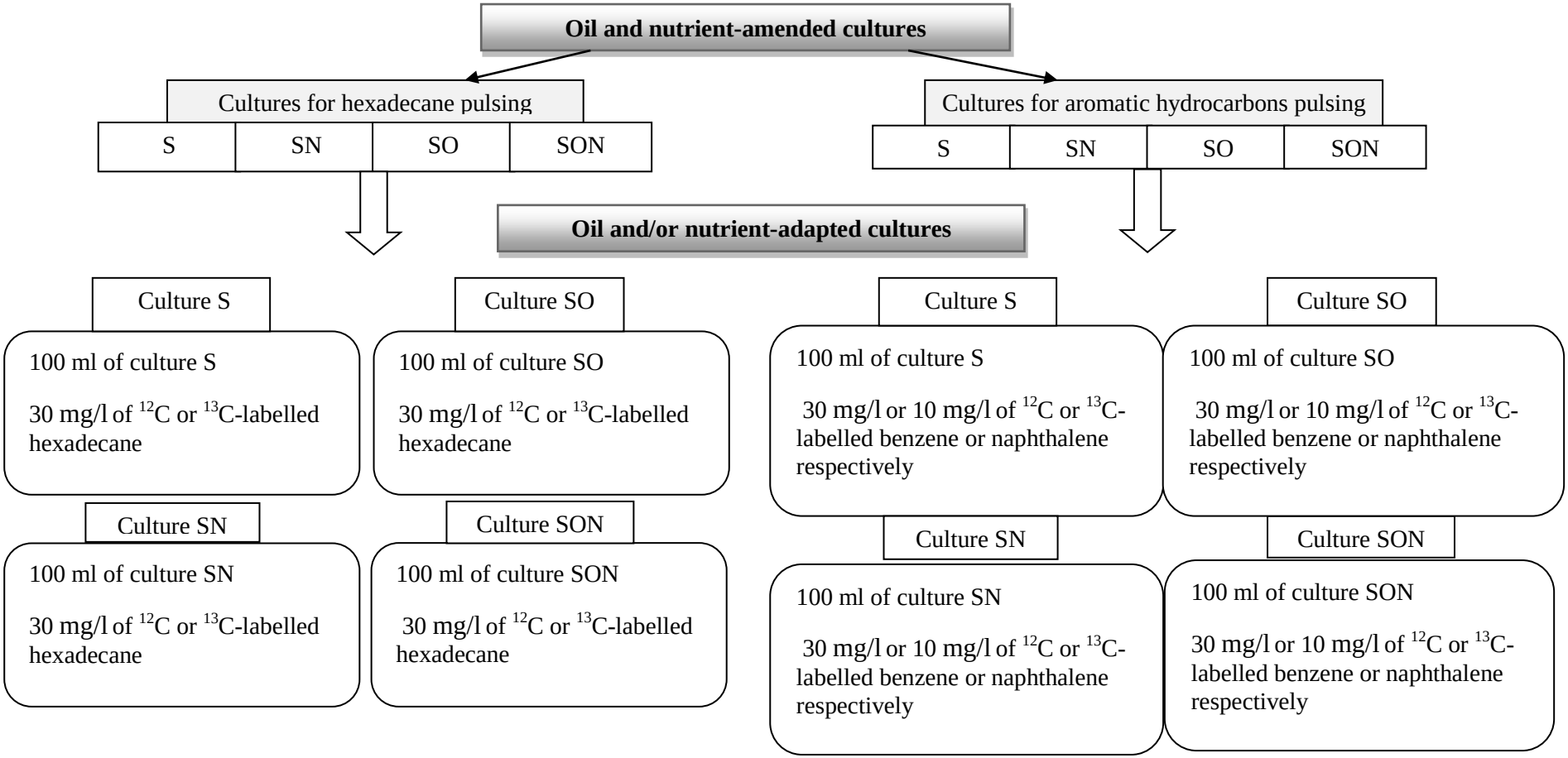


Figure 2.1. Flow chart illustrating the culture set up used in this work.

2.1.3. Bioaugmentation cultures

2.1.3.1. Isolation of bacterial species

The bacterial isolates used for hexadecane bioaugmentation were isolated from marine water sampled from Coogee beach. Bacterial species used in benzene and naphthalene bioaugmentation experiments were isolated from hydrocarbon-polluted seawater of Sydney Harbour (33.8583° S, 151.2333° E) that was exposed to chronic hydrocarbon pollution due to transportation activity (ferries and ships).

Bacterial isolation was performed by aseptically spreading 100 µl of Sydney Harbour or Coogee Bay seawater onto a seawater-agar medium (see appendix for composition). Hydrocarbons were supplemented as carbon sources as follows: 2 µl of benzene was introduced into a sterile wrapped piece of aluminium foil and placed onto the lid of the petri dish that was then incubated in an inverted position so benzene degraders could feed on the benzene vapour. The plate was well wrapped with parafilm to minimise loss of benzene by volatility. For hexadecane, 3 µl was spread onto the surface of the seawater-agar medium and mixed with the inoculum source. Naphthalene was dissolved in sterile seawater to a final concentration of 10 mg/l and 100 µl of this dilution was spread onto seawater-agar medium. Control plates consisting of seawater-agar inoculated with the same source of inoculum without addition of hydrocarbons were prepared. Plates were incubated at room temperature (21 ˚C) until colonies developed. Colonies that exhibited growth on the plates where hydrocarbons were added only were picked and subcultured into similar plates until pure cultures were obtained.

A further test was performed to confirm that the isolated microorganisms were able to use the provided hydrocarbon as a sole carbon and energy source. Each isolate was inoculated into a 160 ml serum bottle containing 100 ml of Bushnell-Haas broth (see appendix for composition) in triplicate and the hydrocarbon under consideration was added as a sole carbon source (30 mg/l of benzene or hexadecane or 10 mg/l of naphthalene). Bottles were sealed with Teflon-lined septa and crimped with aluminium caps. Gas chromatography analysis was carried out to monitor the disappearance of each hydrocarbon compound in the culture, as described below. Only the strains that showed degradation capability were kept for use in hexadecane, benzene and naphthalene bioaugmentation experiments. Pure cultures of the isolates that proved competent in biodegrading hexadecane, benzene or naphthalene were identified as

Rhodococcus qingshengii, *Alteromonas addita* and *Pseudomonas alcaliphila*, respectively.

2.1.3.2. Growth conditions

For bioaugmentation, biomass production was achieved by inoculating single colonies of the *Rhodococcus qingshengii, Alteromonas addita* or *Pseudomonas alcaliphila* strains isolated into 5 ml of a modified LB medium containing 3% NaCl (LB_{30} broth ,see appendix for composition). Cells were grown to the log phase then concentrated by centrifugation (5000 *g* for 10 min), washed three times with sterile seawater and resuspended in sterile seawater to obtain an OD_{600} of 3.2 (equivalent to approximately 10^9 cells/ml) using a SmartSpec Plus spectrophotometer (BioRad). A 1 ml aliquot of the cultures was then added into seawater incubations as described below. Cells numbers obtained by spectrophotometry were confirmed by performing a plate count technique using serial dilution of the cultures on LB_{30} medium.

2.1.3.3. Culture setup for bioaugmentation experiments

Hexadecane, benzene and naphthalene degradation by bioaugmentation was studied in the presence or absence of nutrients. For each individual compound, two bioaugmented and two corresponding control cultures lacking only the added bacterium were prepared as detailed in Table 2.2. Cultures were all prepared in 160 ml serum bottles sealed with Teflon-lined septa and crimped with aluminium seals. Oxygen was injected into the 60 ml headspace at a rate of 10 ml/day to provide aerobic conditions. Cultures were incubated at 21°C with shaking at 100 rpm and samples were taken from the headspace (benzene) and liquid phase (hexadecane and naphthalene) for GC analysis to determine the hydrocarbon concentration over time as described below.

Table 2.2. Detailed composition of the *R. qingshengii*, *A. addita* or *P. alcaliphila* augmented cultures. The name of each culture reflected its constituents: S=seawater; Rhod=Rhodococcus; Altero=Alteromonas; Pseudo=Pseudomonas; N=mineral nutrients (nitrogen and phosphorus). ^{12}C and ^{13}C cultures for each compound were independent replicates.

Hexadecane		**Benzene**		**Naphthalene**	
Culture	**Composition**	**Culture**	**Composition**	**Culture**	**Composition**
S	100 ml seawater 30 mg/l of ^{12}C or ^{13}C-hexadecane	**S**	100 ml seawater 30 mg/l of ^{12}C or ^{13}C-benzene	**S**	100 ml seawater 10 mg/l of ^{12}C or ^{13}C-naphthalene
S+Rhod	100 ml seawater 10^7 cells/ml *R. qingshengii* 30 mg/l of ^{12}C or ^{13}C-hexadecane	**S+Altero**	100 ml seawater 10^7 cells/ml *A. addita* 30 mg/l of ^{12}C or ^{13}C-benzene	**S+Pseudo**	100 ml seawater 10^7 cells/ml *P. alcaliphila* 10 mg/l of ^{12}C or ^{13}C-naphthalene
S+N	100 ml seawater 1.3 mM NH_4Cl and 12 µM K_2HPO_4 30 mg/l of ^{12}C or ^{13}C-hexadecane	**S+N**	100 ml seawater 1.3 mM NH_4Cl and 12µM K_2HPO_4 30 mg/l of ^{12}C or ^{13}C-benzene	**S+N**	100 ml seawater 56 µM NH_4Cl and 6µM K_2HPO_4 10 mg/l of ^{12}C or ^{13}C-naphthalene
S+Rhod+N	100 ml seawater 1.3 mM NH_4Cl and 12 µM K_2HPO_4 10^7 cells/ml *R. qingshangii* 30 mg/l of ^{12}C or ^{13}C-hexadecane	**S+Altero+N**	100 ml seawater 1.3 mM NH_4Cl and 12µM K_2HPO_4 10^7 cells/ml *A. addita* 30 mg/l of ^{12}C or ^{13}C-benzene	**S+Pseudo+N**	100 ml seawater 56 µM NH_4Cl and 6µM K_2HPO_4 10^7 cells/ml *P. alcaliphila* 10 mg/l of ^{12}C or ^{13}C-naphthalene
Sterile control	100 ml autoclaved seawater (1h,121°C) 30 mg/l of ^{12}C or ^{13}C-hexadecane	**Sterile control**	100 ml Autoclaved seawater (1h,121°C) 30 mg/l of ^{12}C or ^{13}C-benzene	**Sterile control**	100 ml Autoclaved seawater (1h,121°C) 10mg/l of ^{12}C or ^{13}C-naphthalene

2.2. Analytical methods

2.2.1 Ammonium quantification assay

Ammonium concentration was measured colorimetrically. Polyvinyl alcohol dispersing agent (25 µl) (Hach) and mineral stabilizer (25 µl) (Hach) were added into 4 ml disposable cuvettes. Sample aliquots (200 µl) and 1800 µl of deionised water were then added, followed by 100 µl of Nessler's reagent (Hach). The mixture was covered with parafilm and shaken immediately. The contents were allowed to react for exactly 1 min. The absorbance was then read at 425 nm on a SmartSpec Plus spectrophotometer (BioRad). Standard NH_4^+ solutions (NH_4Cl) were prepared (concentrations ranged from 0 to 0.3 mM, with 0.05 mM increment) to generate a linear standard curve.

2.2.2 Phosphorus determination assay

Phosphorus was quantified as described by Rockstein and Herron (1951). To a 0.2 ml sample of standard phosphate (KH_2PO_4) solution (or unknown) in a 10 ml volumetric flask, 9 ml of acid molybdate and 0.8 ml of freshly prepared ferrous sulphate solution were added. A similar "blank" preparation was made, using 0.2 ml of distilled water instead of the unknown. The mixture was left for half an hour before reading absorbance at 720 nm on a SmartSpec Plus spectrophotometer (BioRad). Standard P solutions were prepared (concentrations ranged from 0 to 1.5 µg/ml, with 0.25 µg/ml increment) to generate a linear standard curve.

2.2.3. Gas chromatography methods

2.2.3.1. Carbon dioxide (CO_2) quantification

CO_2 production in enrichment cultures was quantified using a Shimadzu gas chromatograph equipped with a J&W PLOTQ column (30 m, 0.32 mm i.d, 20 µm film thickness). Headspace samples (100 µl) were drawn out using a lockable syringe and injected into a split inlet (with split ratio 44:1). The oven temperature was constant at 40°C for 3.5 min. The inlet was maintained at 200°C. Detector was Thermal conductivity detector TCD (25°C).

2.2.3.1. Hexadecane, naphthalene and benzene quantification

Naphthalene and hexadecane were extracted from 2 ml of the ^{12}C naphthalene or hexadecane-spiked cultures using an equal volume of dichloromethane (DCM) in screw-capped glass vials. After thoroughly mixing the samples with the solvent by

manual shaking, 1 ml of the organic phase was transferred into a 2 ml screw-cap GC vial and analysed using a Shimadzu gas chromatograph. The column used was a J&W DB-5 column (30 m, 0.32 mm i.d., 0.25µm film thickness) with helium as a carrier gas (1.95 mL/min) and a flame ionisation detector (FID) (320°C). Injection volume was 3 µl, with a splitless mode. The oven temperature program was: 70°C (1 min), increase to 250°C at 20°C/min, hold at 250°C.

Benzene was monitored using a Shimadzu GC fitted with J&W GS-GASPRO column (60 m, 0.32 mm i.d.) using flame ionisation detector (250°C). benzene was quantified by taking headspace samples (100 µl) using lockable syringe and injecting it into a split inlet (split ratio 10:1). The carrier gas was Helium (3.38 ml/min). The oven temperature program was: 200°C (1 min), increase to 250°C at 25°C /min. Benzene concentration in the aqueous phase was calculated using Henry's constants at 20°C (0.182, EPA, Athens), aqueous and gas phase volumes and volumes of benzene added.

2.3. Molecular methods

2.3.1. Total RNA extraction

For enrichment cultures, 100 ml of each culture at time zero and after 1 and 2 weeks incubation were filtered through a 0.22µm filters (Millipore) and total RNA was extracted using the RNeasy mini kit (Qiagen) according to the modified protocol described by Poretsky *et al.* (2009). Total RNA was extracted with the same method from the total volume of the SIP cultures and the bioaugmentation cultures (total volume) at the end of the experiment.

2.3.2. Stable isotope probing gradient setup

Five hundred nanograms (from hexadecane and naphthalene bioaugmentation experiments only) or one microgram (from all other experiments) of total RNA was subject to isopycnic centrifugation using CsTFA (GE Healthcare Life Sciences) density gradients as described by Whiteley *et al.* (2007) in 5.2 ml Optiseal tubes (Beckman). Ultracentrifugation was performed using a Himac CP 100WX ultracentrifuge (Hitachi) equipped with a P100VT rotor (at 150,000 g for >40 h at 20°C) (Whiteley *et al.* 2007). Gradients were fractionated into 16 fractions of 300 µl using a Beckman fraction recovery system connected to a syringe pump (Adelab scientific) set at a flow rate of 300µl/min. The density profiles of the gradients were determined by weighing 100 µl of

each fraction. RNA was precipitated with an equal volume of isopropanol and 20 µg/µl glycoblue (Ambion) from nine fractions with buoyant densities between 1.845 and 1.765 g/ml (average values), washed with 500 µl of isopropanol and resuspended in 30 µl of 1x Tris-EDTA (TE) buffer (Life Technologies), pH 7.5, in preparation for subsequent analysis.

2.3.3. Community analysis

2.3.3.1. Complementary DNA synthesis and polymerase chain reaction protocols

Construction of complementary DNA (cDNA) was achieved by subjecting 5 µl of total extracted RNA from oil and nutrient-amended cultures or 5 µl of gradient RNA for the rest of the cultures to reverse transcription with 1 µl of a 20 µM solution of the universal bacterial reverse primer (530R) (Table 2.3) into a 15 µl volume of molecular biology grade water (Thermo Fish). The mixture was incubated at 65°C for 5 minutes for denaturation and then 10 µl of a mixture containing 1 U of AMV reverse transcriptase enzyme (Promega), 2 µl of 100 mM dNTPs, 2.5 µl enzyme buffer (Promega) and 4.5 µl molecular biology grade water was added. Reverse transcription (RT) was achieved by incubating the mixture at 37°C for 1h. Amplification of cDNA was achieved by mixing 2 µl of RT product with 25 µl of 2X PCR master mix (Promega), 1 µl each of the universal bacterial DGGE primers 530R (10 µM) and GC-338F harbouring a 38 base pair GC clamp (Table 2.3) and 21 µl of molecular biology grade water. The reaction mixture was subject to 2 minutes at 94°C, 30 cycles of 1 minute at 94°C, 1 minute at 58°C, 2 minutes at 72°C, and a final extension at 72°C for 10 minutes. To sequence DGGE bands reamplified sequences were purified with a PCR purification kit (Zymo Research) amplified for sequencing with BigDye Terminator v3.1 cycle sequencing kit (Applied Biosystems) as per the manufacturer's instructions.

For bacterial isolates**,** selected colonies were subject to colony PCR in order to amplify almost full length 16S rRNA gene sequences for taxonomic identification. The PCR mix consisted of 12.5 µl of 2X PCR master mix (Promega), 0.5 µl of 27F (10 µM) and 1494R (10 µM) primers (Table 2.3) and 10.5 µl molecular biology grade water. The PCR mix was spiked with a few cells collected from a pure colony with a sterile pipette tip and subjected to the following PCR program: 30 cycles of 94°C for 3 min, 55°C for 1 min and 72°C for 1 min. The PCR purification kit (Zymo Research) was then used to

purify the PCR product before re-amplification with the BigDye Terminator v3.1 for sequencing. Composition of the Sanger sequencing reaction is provided in the appendix.

Table2.3. Primers used and their corresponding sequences

Primer	Sequence
27F	5'AGAGTTTGATCCTGGCTCAG3'
1494R	5'TACGGTTACCTTGTTACGAC3'
530R	5'GTATTACCGCGCCTGCTG3'
GC-338F	5'CGCCCGCGGCGCCCCCGCCCCGGCCCGCCGCCCCCGCACTCCTACGGGAGGCAC3'
28F	5'-GAGTTTGATYMTGGCTC3'
519R	5'-GWATTACCGCGGCKGCTG3'

2.3.3.2. Pyrosequencing analysis of oil and nutrient-amended cultures

The cDNA constructed from the different seawater samples used throughout the entire study and from the preliminary enrichment cultures were analysed using an FLX 454 sequencer (Roche). Amplicons were obtained with primers 28F and 519R (Table 2.3) targeting the variable V1-V3 regions of the 16S rRNA gene (Dowd et *al.* 2008). Downstream analysis including alignment of sequences with the Silva reference sequences, selection of quality sequences and a pre-clustering at 1% was performed using the Mothur software package (Schloss *et al*. 2009). Elimination of suspected chimeras was achieved using UCHIME (Edgar *et al.* 2011).

2.3.3.3. Denaturing gradient gel electrophoresis

DGGE was carried out using a DCode mutation detection system (Bio-Rad) for hexadecane bioaugmentation experiment and a Cipher Electrophoresis System (CBS-Scientific Company Inc.) for the rest of the experiments in 1xTAE buffer (40 mM Tris, 20 mM acetate, 10 mM EDTA), pH 7.5, at a constant temperature of 60 °C and 75 V for 16.5 h. PCR products (20 µl) were mixed with 6x loading dye (Fermentas) and loaded onto a 10% (v/v) acrylamide gel with a denaturing gradient ranging from 30-60% urea (Whiteley and Bailey 2000). Gels were stained with SYBR Gold solution (0.01% v/v) (Invitrogen) in 1xTAE buffer for 10 min and visualised with Bio-Rad gel documentation equipment (Gel doc XR). DGGE bands of interest were carefully cut out from gels with band cutting tips (Cleaver Scientific) and placed in 50 µl of molecular

biology grade water and left overnight at 4°C. DNA eluted from bands was re-amplified using 338F and 530R primers as described above (section 2.3.3.1) and purified using PCR purification kit (Zymo Research).

2.3.3.4. DNA Sequence analysis

BioEdit v.7.0.9.0 software (Hall 1999) was used for manual alignment of DNA sequences retrieved from DGGE-cut out bands or from colony PCRs. Sequences were then compared against the GenBank database using the Megablast algorithm of NCBI BlastN tool (Altschul *et al.* 1990) to obtain the taxonomic affiliation of bands.

Chapter3

Preparation of marine hydrocarbon-adapted cultures generated from pristine and oil-spiked seawater

3.1. Introduction

Marine oil pollution is unavoidable due to the increasing human demand on oil and petroleum components. The fact that the ocean is not covered with a layer of oil is due to active biodegradation by natural microbial communities (Atlas 1977). Marine hydrocarbon degrading bacteria (hydrocarbonoclastic bacteria) are naturally present at low or undetectable levels in pristine environments. However, successive blooms of otherwise relatively low abundant indigenous marine bacteria take place following addition of oil into seawater (Yakimov *et al.* 2007).

In the context of hydrocarbon degrading communities, adaptation is defined by the increase in the hydrocarbon-oxidising potential of a microbial community (Spain *et al.* 1980). This increase results from prior exposure of these communities to natural and/or anthropogenic sources of hydrocarbons. An understanding of this process is essential in determining how rapidly subsequent hydrocarbon inputs can be degraded (Leahy and Colwell 1990). Several studies demonstrated that oil hydrocarbons (Walker and Colwell 1975), mixed hydrocarbon substrates (Walker *et al.* 1976) as well as saturated (Caparello and LaRock 1975) and aromatic (Herbes and Schwall 1978) hydrocarbon compounds can be degraded to a greater extent and more rapidly in hydrocarbon-polluted environments compared to pristine environments.

Bacteria adapt to the presence of hydrocarbons in their environment using one of three interrelated mechanisms: 1) selective enrichment towards organisms that have the ability to transform the hydrocarbon molecules 2) expression/repression of specific enzymes and 3) development of new metabolic capabilities due to genetic changes (Spain *et al.* 1980, Spain and Van Veld 1983).

This chapter aimed to optimise the incubation and nutritional conditions required to obtain oil-adapted marine bacterial cultures with greater hydrocarbon-degrading potential. Analysis of the community structure using 454 pyrosequencing revealed changes that occurred in the marine microbial communities following addition of nutrient and/or crude oil to seawater microcosms. In subsequent chapters additional hydrocarbon-adapted cultures will be generated under the optimal conditions identified here and used to identify bacteria degrading model saturated and aromatic hydrocarbon compounds.

3.2. Results

3.2.1. Oil and nutrient amended seawater enrichment cultures

In order to generate oil-adapted cultures, a preliminary experiment was carried out. Large unreplicated incubations containing seawater (S) and crude oil (O) were prepared with and without addition of mineral nutrients (N). Four cultures namely S, SN, SO and SON were generated by incubating 1.4 L of fresh seawater aerobically in 2 L Schott bottles with crude oil and/or nutrients. For clarity, the thematic composition of these cultures are summarised in Table 3.1. (See Chapter 2, section 2.1. for experimental detail).

Table 3.1. Description of the preliminary cultures.

Culture	Content
S	Seawater only
SN	Seawater + nutrients
SO	Seawater + oil
SON	Seawater + oil+ nutrients

CO_2 production and RNA yield were monitored to detect the peak of microbial activity in response to oil addition. In addition, concentrations of nitrogen and phosphorus were measured over time using colorimetric assays (see chapter 2, sections 2.2.1 and 2.2.2) and re-amended where necessary to avoid nutrient limitation.

3.2.1.1. Oil mineralisation

Oil mineralisation was monitored by quantifying the CO_2 produced in the cultures containing crude oil. Fig. 3.1 shows the production of CO_2 over a period of four weeks in the unreplicated incubations.

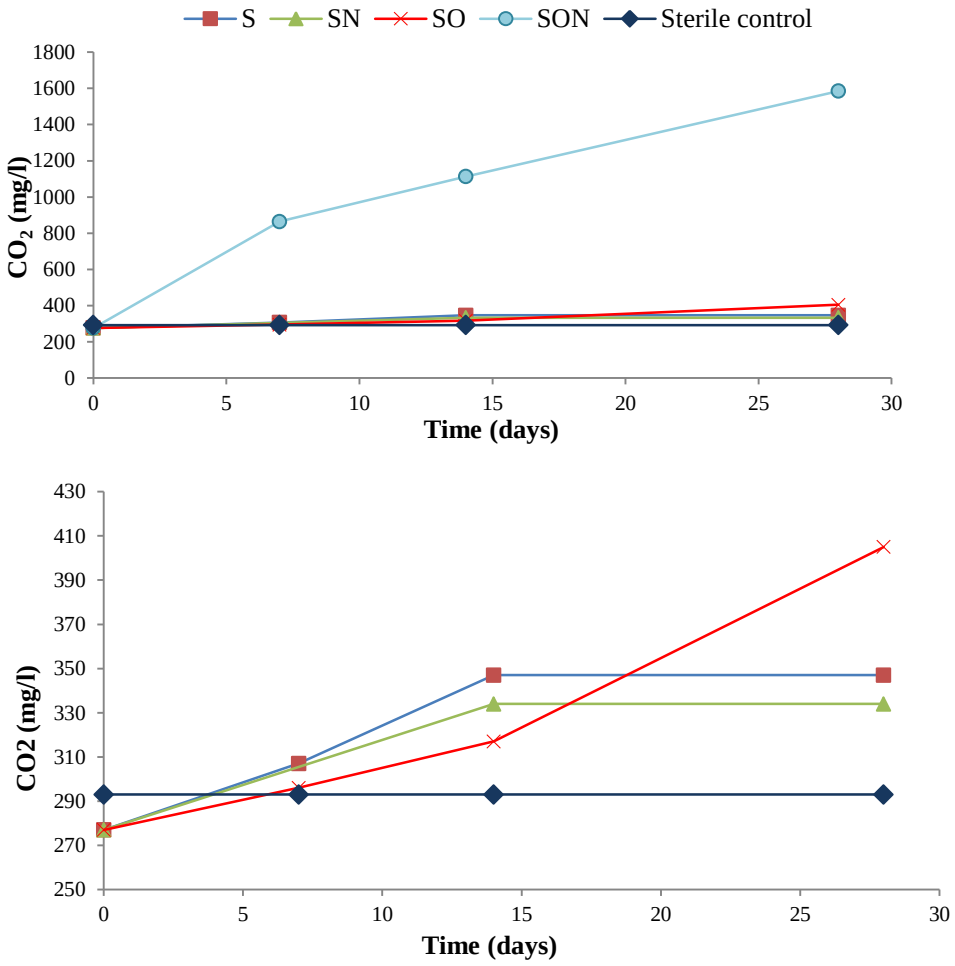


Figure 3.1. CO_2 production in the preliminary cultures over time (top panel). Bottom panel represents CO_2 from all the cultures except SON to show differences amongst these cultures (note the change in scale). Each line represents an individual culture. S = seawater only; SN = seawater and mineral nutrients nitrogen and phosphorus; SO = oil-spiked seawater; SON = oil-spiked seawater where nitrogen and phosphorus were added. Incubations were not replicated.

The nitrogen and phosphorus-amended culture containing oil (SON) (Fig 3.1.) showed a clear increase in CO_2 reaching 1585 mg/l CO_2 at week 4. Production of CO_2 over time in the culture spiked with oil only and the cultures lacking oil was relatively modest. No increase in CO_2 concentration was observed in the sterile control.

3.2.1.2. Nitrogen and Phosphorus uptake

The concentrations of nitrogen and phosphorus were monitored in the enrichments in order to ensure sufficient supply of mineral nutrients throughout the experiments. Fig. 3.2 shows the concentrations of NH_4^+ and total phosphorus in the enrichments cultures over four weeks. Concentrations of these nutrients in the cultures lacking nutrient amendments were not measured. The naturally available concentrations in seawater are 15.5 mg/l nitrogen and 0.088 mg/l total phosphorus according to Turekian (1968). A decrease in the concentration of NH_4^+ was observed over the first two weeks with approximately 50% of the nitrogen removed followed by a plateau (Fig. 3.2).

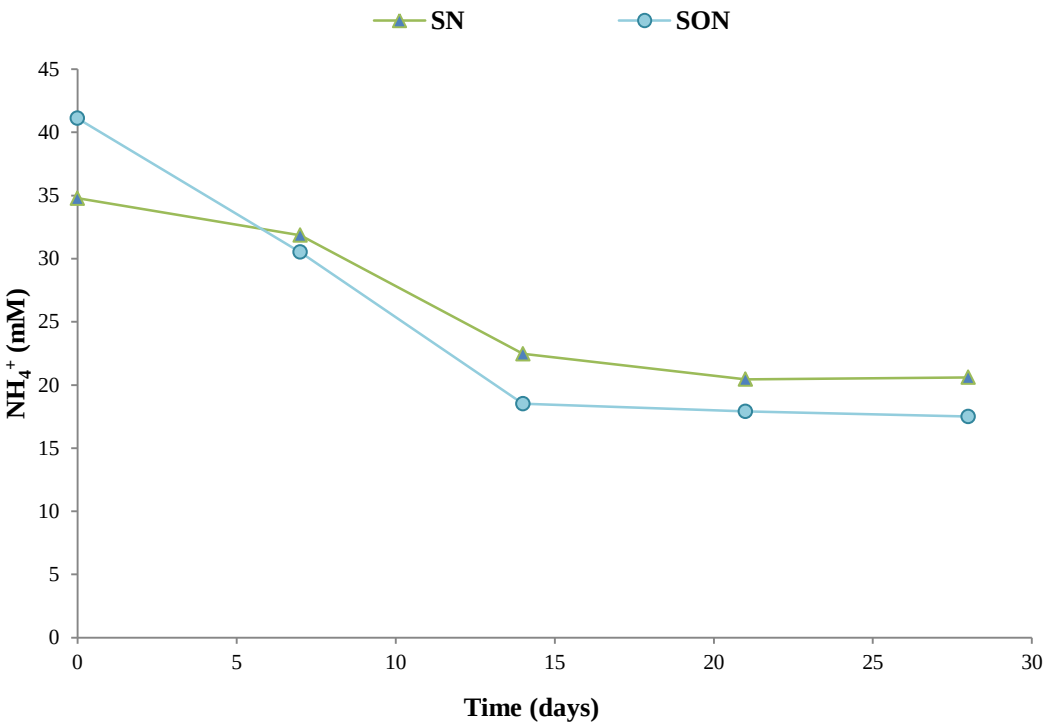


Figure 3.2. Concentrations of NH_4^+ nitrogen in nutrient-amended enrichments (SN and SON) of the preliminary cultures. Each line represents an individual culture.

The concentration of phosphorus showed a rise at week 1 followed by stabilisation at 0.03 mM and 0.04 mM in cultures SN and SON respectively (Fig. 3.3). The increase is likely due to the Phosphorus slowly solubilising over time. No decrease was observed.

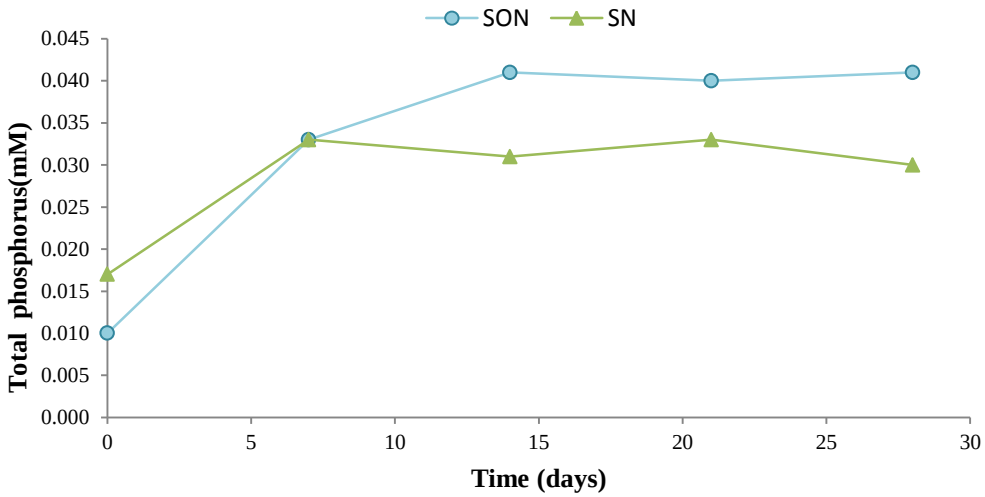


Figure 3.3. Concentrations of total phosphorus in nutrient-amended enrichments (SN and SON) of the preliminary cultures. Each line represents an individual culture.

3.2.1.3. RNA yield as a proxy of activity

Total RNA was extracted from cultures on a weekly basis in order to determine a peak time for maximum microbial metabolic activity (Fig. 3.4).

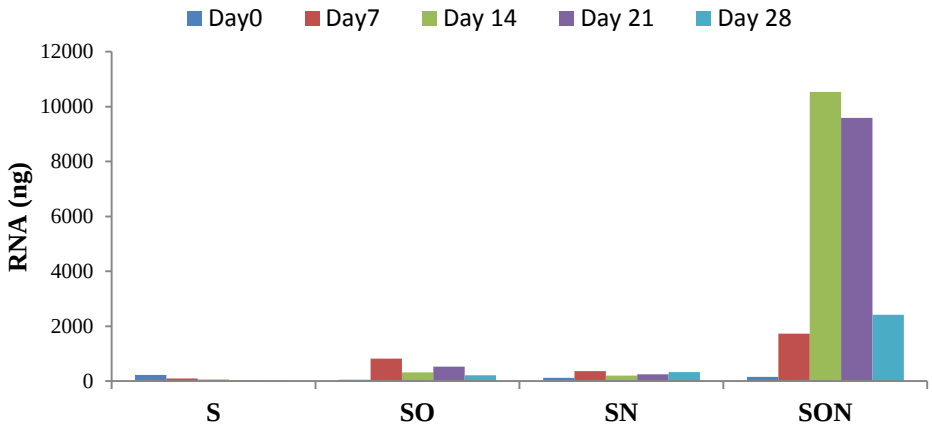


Figure 3.4. RNA yields from 100 ml of the preliminary cultures. S = seawater only; SN = seawater and mineral nutrients N and P; SO = oil-spiked seawater and SON = oil-spiked seawater where nitrogen and phosphorus sources were added. Bars represent individual cultures over time.

Culture SON showed the highest activity compared to the rest of the cultures which showed little or no difference in the RNA yield over time. An increase in the quantity of RNA obtained from SON was observed over two weeks (67 fold increase). RNA

concentrations then decreased over the ensuing two weeks. This suggests two weeks was an optimal enrichment incubation period for RNA based SIP analysis.

3.2.1.4. Community analysis by 454-pyrosequencing

SSU amplicons derived from complementary DNA generated from RNA extracted from cultures at the start of incubation were pyrosequenced to identify dominant marine bacteria in the seawater. Sequencing analysis was also conducted after 1 and 2 weeks incubation to describe the response of the bacterial community to oil and/or nutrient addition. Sequences (total number) that showed similarity above 97% with sequences in the SILVA 16S rRNA gene database were clustered into Operational Taxonomic Units (OTUs). OTUs were allocated to bacterial phyla, classes and genera, the latter being the lowest taxonomic rank identified (Fig. 3.5). The initial marine bacterial community showed relative dominance of Proteobacteria (63%). Planctomycetes represented 29% of the native marine communities. Within the Proteobacteria, Alphabroteobacteria were the most abundant (57% of Proteobacteria) followed by Deltaproteobacteria (22%), Gammaproteobacteria (19%) and Betaproteobacteria being the least abundant subgroup (2%). Addition of nitrogen and phosphorus led to a decrease in the abundance of Planctomycetes (3% and 1% at week 1 and 2 respectively). The Proteobacteria became even more prominent after nitrogen and phosphorus amendments (SN-week 1 and SN-week 2: 89% and 88% respectively in Fig 3.5). A modest increase in activity of Gammaproteobacteria was observed at week 1 (41% of total proteobacteria), which then dropped to 28% at week 2 in this culture. Alphaproteobacteria showed an increase at week 2 (72%). The activity of Deltaproteobacteria decreased after nutrient addition. It dropped down to represent only 4% of the active community after one week of N and P addition and then to show no activity at week 2. Betaproteobacteria seemed unaffected by mineral fertilisation at week 1 (culture SN) but their activity diminished substantially at week 2.

Addition of oil was highly selective for Proteobacteria that dominated the seawater communities in the cultures spiked with oil (SO and SON) from week 1 onwards (100%) (Fig.3.5). The activity of Deltaproteobacteria and Betaproteobacteria decreased

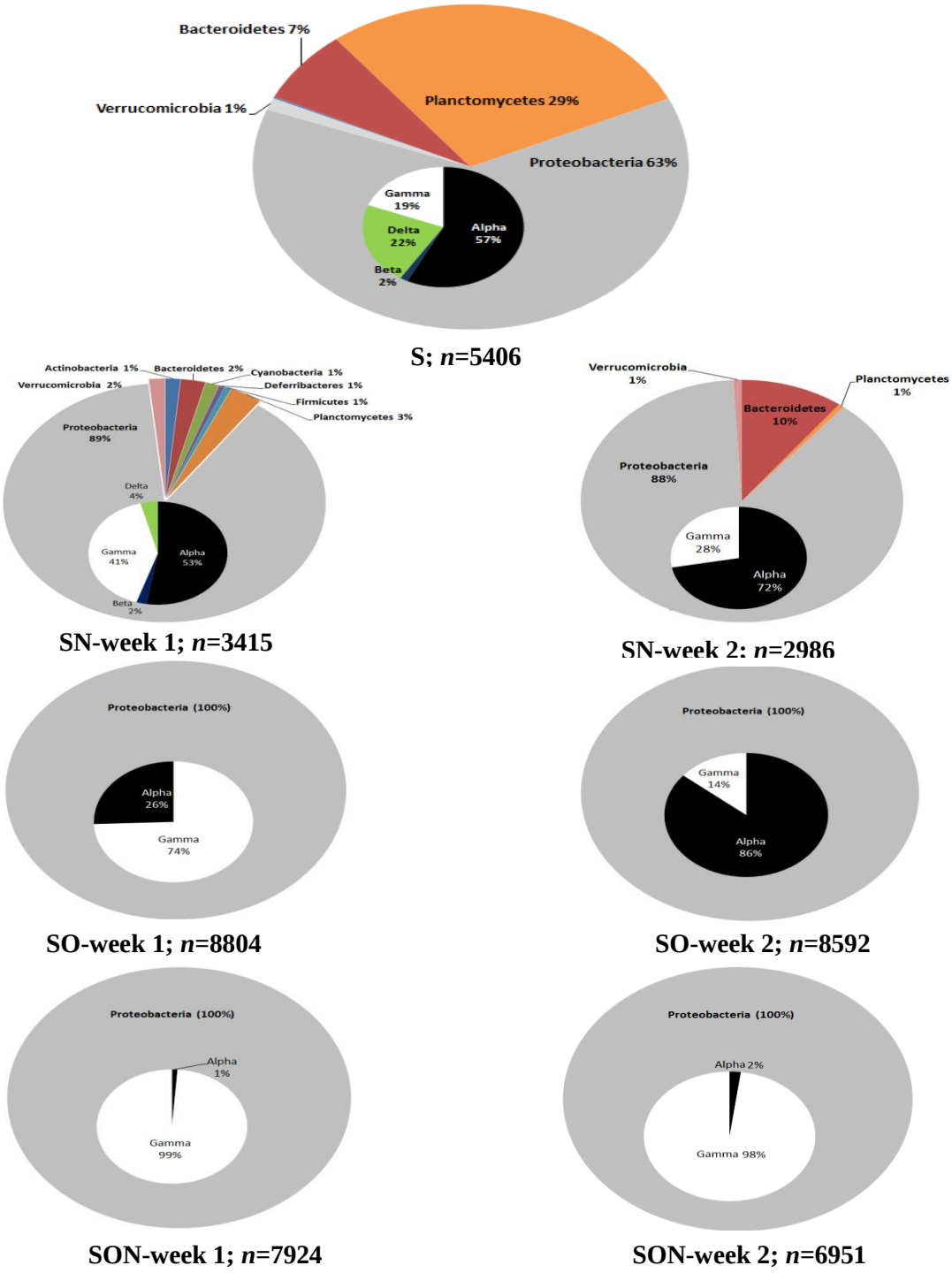


Figure 3.5. Composition and percentage of bacterial sequences of the preliminary cultures at the Phylum and Class level. S represents the initial seawater communities, SN-week 1 and SN-week 2 show the shifts in the communities after addition of nitrogen and phosphorus at week 1 and 2 respectively; SO-week1 and SO-week 2 illustrate the community composition after oil addition at week 1 and 2 respectively. SON-week 1 and SON-week2 shows the changes in bacterial populations in response to oil and mineral nutrients amendments at week 1 and 2 respectively. "*n*" refers to the total number of OTU sequences for each sample after computational processing. Phyla that accounted for less than 0.5% of the community are not shown. Only classes that showed changes over time in the different treatments are shown (Proteobacteria).

dramatically when oil was added to the seawater (cultures SO and SON). Alphaproteobacteria were outcompeted by Gammaproteobacteria at week 1, however flourished and started to take over at week 2 (86%). Conversely, Gammaproteobacteria responded more favourably to the presence of nutrients in oil-spiked cultures than Alphaproteobacteria (culture SON) showing an exceptional boost in activity at week 1 and 2 (99 and 98% respectively).

Fig. 3.6 represents the ten most abundant sequences in the cultures analysed at the genus level.

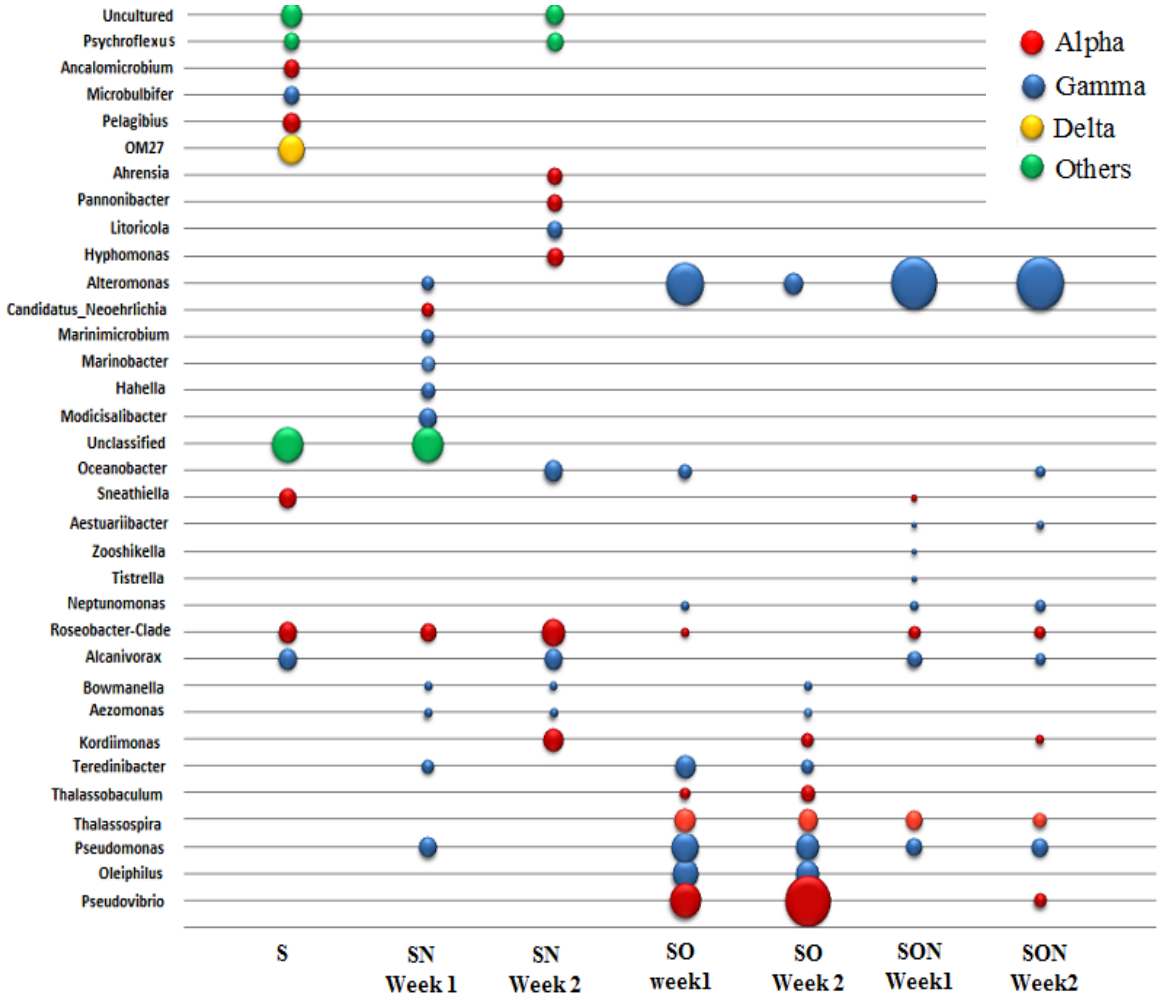


Figure3.6. Overview of the 10 most active OTUs in the enrichment cultures. "Others" in the legend refer to phyla other than Proteobacteria (e.g. *Psychroflexux* belongs to bacteroidetes); uncultured and unclassified OTUs belonging to different bacterial phyla (mainly Proteobacteria, followed by Bacteroidetes, Verrucomicrobia and Cyanobacteria) and classes were also grouped together as others in the legend. Numbers of sequences for each OTU were transformed to square roots to clarify data presentation. The size of the bubbles indicated the extent species activity.

Cultures SO and SON showed low diversity with the ten genera listed representing the only sequences retrieved from these cultures. Populations that thrived in the initial seawater showed similar relative abundance, with the OM27 clade being one of the most abundant bacteria. A similar profile was observed in culture SN at week 1 and 2 where all the OTUs showed similar abundance compared to each other.

Interestingly, some OTUs such as *Marinobacter*, *Marinimicrobium*, *Hahella*, as well as *uncultured* and *unclassified* bacteria belonging to different phyla (mainly proteobacteria, but also Cyanobacteria, Bacteroidetes and Verrucomicrobia) performed better in (no oil) nutrient-amended microcosms (SN). In addition, The *Roseobacter-Clade* was abundant in all the different treatments but exhibited highest abundance in SN at week 1. A remarkable change in the community was observed in SO and SON that exhibited an exceptional drop in diversity which supports previous class level observations. In culture SO there was an enormous enrichment for *Alteromonas*, followed by *Pseudovibrio*, *Thalassospira*, *Oleiphilus*, *Pseudomonas* and *Thalassobaculum* at week 1. *Pseudovibrio* flourished remarkably at week 2, followed by *Thalassospira*. This increase was paralleled by a notable decline in the activity/abundance of, most importantly, *Alteromonas*, and then *Pseudomonas* and *Teredinibacter*. Finally, an absolute dominance of *Alteromonas* in SON at week 1 and 2 was observed. All the other OTUs decreased in relative abundance.

In conclusion, results obtained from the preliminary cultures have demonstrated that two weeks incubation of seawater with crude oil and/or nutrients was enough to select for active hydrocarbon degrading communities. This finding came in accordance with the peak of activity observed at week 2 in SON (RNA yield) and supported by CO_2 concentrations. It has also demonstrated that the concentration of nitrogen and phosphorus was in excess but without exhibiting any inhibitory effect on the oil mineralisation.

3.3. Discussion

This chapter defined cultures generated from seawater and oil under natural attenuation and biostimulation conditions including a description of bacterial activity in the different treatments. This preliminary experiment was conducted to determine the

incubation conditions necessary to generate enrichment cultures to investigate the biodegradation of selected saturated and aromatic hydrocarbons.

CO_2 production was monitored in the preliminary experiment as an indication of metabolic activity and/or oil mineralisation (Atlas and Bartha 1972 & 1973). Incubations showed limited increase in microbial activity in seawater and nutrient-amended seawater over two weeks. The small increase in CO_2 production observed was likely due to consumption of available organic carbon endogenous to the seawater. Once this was depleted, CO_2 production ceased. Limited increases in CO_2 concentration were also observed in oil-amended incubations lacking nutrients; however, the concentration did not reach a plateau during the incubation period consistent with the organic carbon source not being depleted.

Addition of N and P into the microcosms spiked with oil substantially increased the rate of oil mineralisation as determined by CO_2 production. Atlas and Bartha (1972) demonstrated that when oil is added to marine water, a dramatic increase in the load of carbon paralleled by a limited availability of essential mineral nutrients, mainly nitrogen and phosphorus, takes place. Therefore, they concluded that an effective and economical means of enhancing the biodegradation of hydrocarbons was the addition of inorganic nitrogen and phosphorus. This has been supported over time by findings of numerous other studies that demonstrated a positive effect of fertilization with mineral nitrogen and phosphorus (Ruberto *et al.* 2003, Yu *et al.* 2005, Delille *et al.* 2009, Nikolopoulou *et al*. 2013). The results obtained here are consistent with the literature suggesting that the experimental system was appropriate for subsequent identification of functional phylotypes.

Bacterial metabolic activity was also evaluated by using total RNA as a proxy. The culture containing oil and nutrients yielded the highest amount of RNA at week 2 of 4 in comparison with the rest of the cultures. This was consistent with the CO_2 data suggesting this treatment resulted in the highest microbial activity. It also suggested that two weeks incubation was adequate to reach a maximum level of metabolic activity under these conditions.

RNA has been largely used to determine the activity of bacterial communities (Jones and Lennon 2010, Campbell *et al.* 2011). In addition, a recent study carried out by Hunt

et al. (2013) observed an uncoupling of abundance and specific activity of some marine bacteria. The study revealed highly active but less abundant marine genera (e.g. *Alteromonas*), and highly abundant but less active ones such as the heterotrophic SAR11. This finding highlights the importance of correlating RNA with activity rather than abundance in the marine environment. Hence, given that this study used RNA for community analysis, the phyla, classes and genera observed in the preliminary incubations described here and referred to as most abundant, were actually the most active ones.

Nitrogen is an essential element for bacterial metabolism, so it was provided to selected cultures at an excess amount to avoid N limitation. The concentration of supplemented nitrogen showed a decrease of approximately 50% after two weeks in seawater amended with oil and nutrients and to a slightly lesser extent in seawater amended with nutrients only. Given that nutrients enhanced CO_2 production in the oil-spiked culture, hydrocarbon degrading bacteria must have utilised the available nitrogen whilst mineralising hydrocarbons from oil. The decrease in activity after week 2 coincided with a drop in the uptake of nitrogen. In seawater amended with nutrients, nitrogen was possibly used by bacteria metabolising other organic carbon sources naturally available in the marine water. The decrease in nitrogen in the presence or the absence of oil, however, suggests that hydrocarbonoclastic bacteria were not the only consumers for nitrogen. Dortch *et al.* (1984) reported that phytoplankton in seawater accumulate nitrogen intracellularly and use it under starvation conditions. However, this cannot be the reason in the present study it contradicts with the mass balance (weight of ammonium consumed in culture SN ($2.5x10^{12}$pg/L) is higher than the total weight of cells ($63x10^{6}$pg/L)). Hence, the decrease in nitrogen concentration even in the absence of hydrocarbon inputs may be due to ammonium oxidation by marine bacteria (Martens-Habbena et al. 2009). This finding highlights the importance of taking this process into consideration when setting up bioremediation strategies as it may have direct implications due to the role played by the concentration of nitrogen added during bioremediation (Boufadel *et al.* 1999).

Despite the addition of phosphorus as K_2HPO_4 at a final concentration of 2 mM no more than 0.043 mM phosphate was observed in the dissolved phase. This is due to the low solubility of K_2HPO_4 phosphate in seawater. The stability in concentration after two

weeks is likely due to consistent uptake and further solubilisation of K_2HPO_4 over time. Precipitate K_2HPO_4 was observed in the microcosms amended with this mineral source which supports the low solubility hypothesis. The low solubility of K_2HPO_4 in seawater has been previously demonstrated, as the presence of salts in seawater reduces the solubility of other solutes (Sverdrup *et al.* 1942).

Previous studies have demonstrated that bacteria are the main oil degraders in the ocean with Archaea no longer detected in experimental microcosms following exposure to oil (Röling *et al.* 2002, Röling *et al.* 2004). Hence, molecular microbial community analysis in this thesis focussed on the bacteria. The pyrosequencing data showed high abundance of rRNA sequences of Proteobacteria (63%) in the initial seawater community, followed by planctomycetes (29%) and then bacteroidetes (7%). Within the proteobacteria, Alphaproteobacteria were the most abundant (57%) followed by Deltaproteobacteria (22%) and Gammaproteobacteria (18%). Several studies that used pyrosequencing analysis for marine microbial communities demonstrated the predominance of Proteobacteria in the ocean (Teramoto *et al.* 2013, Kim *et al.* 2014).

Nutrient amendments exhibited an effect on the bacterial community structure in the seawater by favouring certain bacterial phyla showing no activity in the unamended seawater (e.g. Actinobacteria, Firmicutes, Cyanobacteria), and by promoting the growth of others (Proteobacteria) (Fig.3.5.). Interestingly, other phyla responded negatively to nitrogen and phosphorus fertilisation (Planctomycetes). Pollet *et al.* (2013) also observed a decrease in Planktomycetes after nutrient amendments. The same study concluded that Planktomycetes do not respond quickly to nutrient addition, which explains their low abundance in aquatic ecosystems.

Spiking the seawater with oil led to an apparent decline in diversity as determined by pyrosequencing. Proteobacteria were the only active phylum in oil amended incubations. In oil amended seawater, Gammaproteobacteria were active at week 1 (74%) but were outcompeted at week 2 by Alphaproteobacteria (86%). The oil and nutrient amended seawater incubations exhibited strong selection for Gammaproteobacteria that became highly dominant (99-98% of all sequences at week 1 and 2 respectively) and consisted mostly of *Alteromonas*. This outcome is consistent with the findings of Teramoto *et al.* (2013) who observed that Gammaproteobacteria flourish in hydrocarbon-rich environments with elevated nutrient concentrations. This

could also possibly explain the recession in the activity of this class at week 2 in oil amended seawater where nutrients were limiting. Teramato *et al*. (2103) also revealed that under high hydrocarbon and low nutrient regimes, Gammaproteobacteria were outcompeted by the Alpha class. In addition, the increase observed in the activity of Alphaproteobacteria at week 2 in oil amended seawater may also be relevant to the duration of exposure to hydrocarbons. It was previously demonstrated by Macnaughton *et al*. (1999) that Alphaproteobacteria become more abundant during the later stages of oil-spill bioremediation treatments. It should be noted that the apparent disappearance of certain sequences likely reflects the massive increase in relative abundance of the Proteobacteria, as opposed to actual extinction.

The drop in bacterial diversity and dramatic increase in activity of a few bacterial taxa following the addition of oil reflects selection towards hydrocarbonoclastic populations (Fig. 3.5 and Fig. 3.6.). *Alteromonas* constituted more than 95% of the active OTUs in oil and nutrient amended seawater. In addition, in seawater amended with oil only, *Alteromonas* was the most active OTU at week 1, but was outcompeted by *Pseudovibrio*, which became the main active OTU at week 2. Little is known about the role of *Pseudovibrio* in hydrocarbon degradation. However, several studies highlighted the hydrocarbon biodegradative capacity of *Alteromonas* (Jin *et al.* 2012, Math *et al.* 2012).

Members of the genus *Alteromonas*, proposed by Baumann *et al.* (1972), are amongst the most common heterotrophic bacteria found in the marine environment globally (Suzuki *et al.* 1997, García *et al.* 2005). They are r-strategist copiotrophs exhibiting sudden blooms in microcosms as soon as resources (organic carbon and nutrients) become available (Polz *et al.* 2006, Romera-Castillo *et al.* 2011, Tada *et al.* 2011, Hunt *et al.* 2013). *Alteromonas macleodii* is the most commonly studied species (Schäfer *et al.* 2000, López-lópez *et al.* 2005, Jin *et al.* 2012). Jin *et al.* (2012) noted that members of the genus *Alteromonas* have the potential to play important roles in metabolising crude oil contamination. They were shown to metabolise a variety of organic carbon sources. These studies are in accordance with the findings of the present study. As a result, the decrease of *Alteromonas* activity in SO at week 2 may be correlated with nutrient limitation in this nutrient-unamended microcosm after 2 weeks.

In conclusion, biostimulation with mineral nitrogen and phosphorus accelerated the rate of oil mineralisation and the presence of oil and nutrients led to an increase in microbial respiration and RNA production. The dominance of Gammaproteobacteria, specifically *Alteromonas* in the oil-spiked microcosms amended with nutrients demonstrates the utility of biostimulation in the bioremediation of possible hydrocarbon pollution in seawater. Most importantly, results shown in this chapter give evidence supporting selection of hydrocarbonoclastic bacteria after addition of oil into the seawater. Data obtained from this chapter served as a tool to subsequently generate other active hydrocarbon-degrading cultures in order to test the effect of pre-adaptation and/or biostimulation on the degradation of the selected saturated (*n*-hexadecane) and aromatic compounds (benzene and naphthalene) compared to non-hydrocarbon-adapted cultures. How oil-adapted cultures affect the biodegradation of the selected hydrocarbons and how community structure shifts in these cultures, is described in chapter 4.

Chapter 4

Linking the degradation of selected oil hydrocarbons to specific marine taxa from oil-adapted cultures using RNA-stable isotope probing

4.1 Introduction

Bioremediation of petroleum hydrocarbons in the ocean has been reported as a successful and cost effective strategy to clean up oil pollutants (Uhlik *et al.* 2012). The only large-scale successful application took place following the Exxon-Valdez oil spill (Atlas 1995a, Atlas 1995b, Bragg *et al.* 1994, Harayama *et al.* 1999). Due to the deleterious effect of those pollutants on marine biodiversity, it is of paramount importance that bioremediation strategies not only address the cleanup of the hydrocarbon pollutants, but also the speed with which they can be removed and the factors that speed up biodegradation. Two main phenomena have been demonstrated to boost the biodegradation rate. First, the addition of essential mineral nutrients (mainly nitrogen and phosphorus) has proven successful as it corrects the imbalance created by the carbon overload (Yu *et al.* 2005, McKew *et al.* 2007). In addition, hydrocarbonoclastic bacteria constitute a minor fraction of the microbial communities in the environment (Sivaraman *et al.* 2011). Therefore, it is essential to promote the growth of these bacteria over others in order to speed up the biodegradation of hydrocarbon inputs. Pre-adaptation has been demonstrated to accelerate the rate of hydrocarbon degradation by eliminating the lag phase needed for metabolic stimulation of hydrocarbonoclastic bacteria before initiating the attack on the hydrocarbon molecules (Leahy and Colwell 1990).

In this chapter, the effect of preadaptation and/or biostimulation on the degradation of three model hydrocarbons was investigated. Petroleum oil has a very complex chemical composition. It is comprised of several classes of compounds that differ enormously in their physical/chemical properties and in the mode of their degradation by bacteria. This has constrained most of the biodegradation studies to single hydrocarbon molecules that

represent classes of compounds sharing common properties, i.e. model compounds (Michaelsen *et al.* 1992, Annweiler *et al.* 2000, Piskonen *et al.* 2005, Parales *et al.* 2008). The model compounds selected for this study are benzene, naphthalene and *n*-hexadecane (representing BTEX, PAHs, and long-chain saturated hydrocarbons respectively). These are three of the most abundant components of petroleum spills (Smith *et al* 1998, Williams *et al.* 2005).

Bacteria are the predominant agents in hydrocarbon degradation in the environment (Leahy and Colwell 1990). With respect to oil degraders, more than 20 bacterial marine genera belonging to several groups have been described (Head *et al*. 2006,Yakimov *et al*. 2007). SSU or 16S rRNA gene-based studies have demonstrated a substantial reduction in the marine microbial diversity following exposure to hydrocarbons owing to strong selection for a limited number of hydrocarbonoclastic species (McKew *et al.* 2007, Röling *et al*. 2002) that belong mainly to the Gammaproteobacteria (Head *et al*. 2006). In addition, most of the degradation studies assigned the degradation of certain hydrocarbons to bacterial species based on their abundance following exposure to those hydrocarbons. However, a recent study by Hunt *et al.* (2013) demonstrated an uncoupling between abundance and activity for marine bacteria. As a result, the application of molecular methods that link metabolic functions to taxonomic identity has become the optimal strategy to correlate the degradation of hydrocarbon compounds to specific marine bacteria consuming these compounds. Stable isotope probing (SIP) constitutes a pivotal tool to link microbial assimilation of carbon sources and phylogeny. The application of SIP in microbial ecology, especially in the marine hydrocarbon degradation context is expanding and has enabled determination of key microbial taxa associated with ecological functions such as hydrocarbon biodegradation. A recent study conducted by Sheppard *et al.* (2013) demonstrated the advantage of applying RNA-SIP in better resolving the structure of the marine microbial communities compared to the standard molecular 16S rRNA-DGGE analysis.

The aims of this chapter were 1) to assess the effect of pre-adaptation and/or biostimulation on the degradation rate of the selected hydrocarbon compounds and 2) to identify marine microbial taxa deriving carbon these compounds and their dynamics in response to different nutritional regimes by applying RNA-SIP.

4.2 Results

4.2.1. Oil and nutrient-amended enrichment cultures

In order to identify hexadecane, benzene and naphthalene degrading bacteria in fresh or oil exposed seawater, two sets of enrichment cultures based on separate seawater sampling events were independently generated according to protocols developed in Chapter 3. One set of cultures, derived from seawater sampled in March 2012, was used for subsequent hexadecane biodegradation experiments whilst the second set, derived from seawater sampled in December 2012, was used for benzene and naphthalene degradation experiments. Briefly, 1.4 L of freshly sampled seawater in 2 L bottles was amended with oil and/or nutrients or left unamended and incubated aerobically for two weeks. A heat sterilised seawater control was also incubated. Carbon dioxide production was measured to monitor respiration and community profiles were generated using pyrosequencing on the seawater and denaturing gradient gel electrophoresis and band sequencing to monitor changes over time and in response to treatment.

Concerning CO_2 production, these enrichments were similar to each other and comparable to the preliminary trial presented in Chapter 3 suggesting the enrichment regime is reproducible. Respiration was more active in seawater amended with oil and nutrients than other treatments or the control (Fig. 4.1).

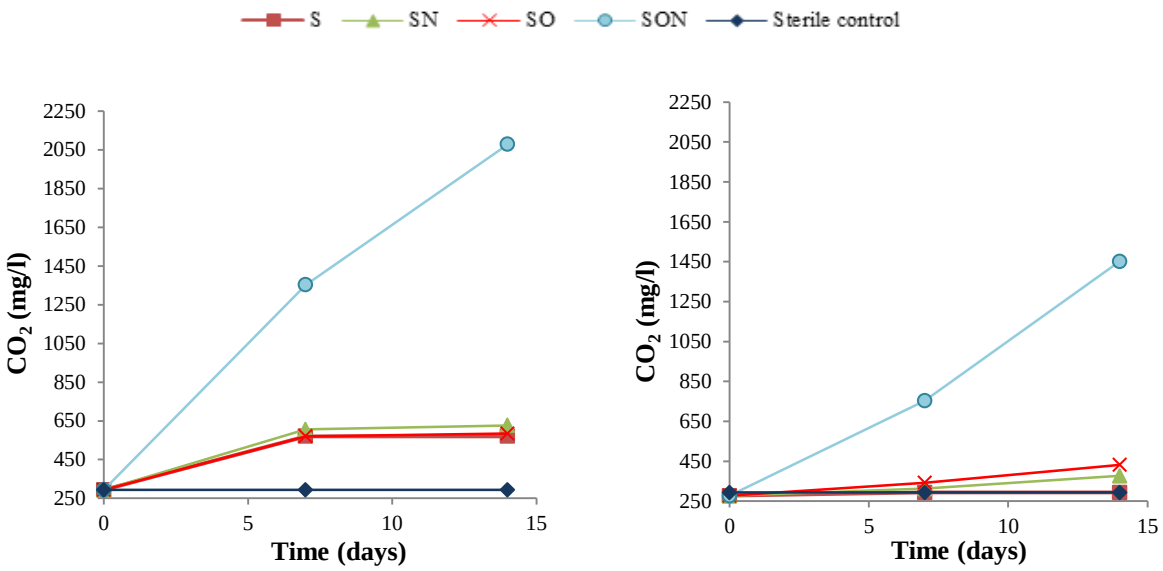


Figure 4.1. Carbon dioxide production in all the enrichment cultures used for hexadecane (left panel) and used for aromatic hydrocarbon degradation experiments (right panel). S= seawater only; SN= seawater and mineral nutrients (nitrogen and phosphorus), SO= oil-spiked seawater and SON= oil-spiked nutrient-amended seawater.

Pyrosequencing analysis on the seawater sampled on two distinct occasions for the two sets of enrichments at the start of incubation also generated results comparable to the preliminary assessment (Fig. 4.2). Proteobacteria were again observed to dominate the seawater community (67 - 89%). Proteobacteria were mainly represented by Alphaproteobacteria (24 - 63% of Proteobacteria) and Gammaproteobacteria (36% - 38% of Proteobacteria). Differences, however, were also observed. The Bacteroidetes were less consistent representing 4% of sequences in the set of enrichments subsequently amended with hexadecane and 31% in those subsequently amended with the aromatic compounds.

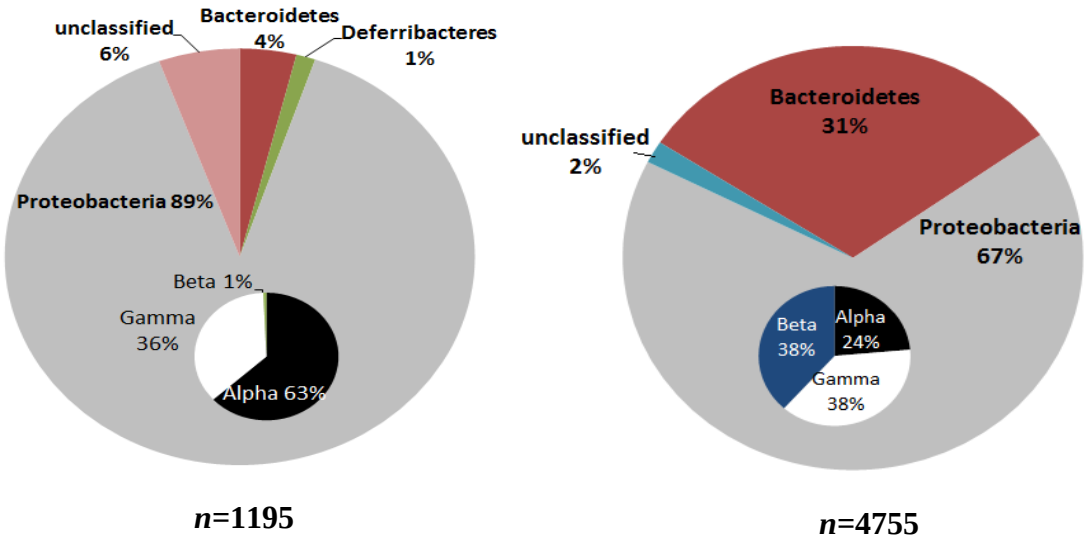


Figure 4.2. Composition and percentage of bacterial pyrosequences retrieved from the initial seawater used for hexadecane (left) and aromatic hydrocarbons pulsing (right) at the phylum and class level. "*n*" refers to the total number of OTU sequences for each sample after computational processing. Phyla accounting for less than 0.5% abundance were not represented.

Denaturing gradient gel electrophoresis (DGGE) was also performed using 16S rRNA RT-PCR products to visualise the community dynamics in the enrichments over the two weeks incubation (Fig. 4.3) and to identify putative hydrocarbon degrading community members. Sequencing of selected bands showed a prevalence of the *Alteromonas* genus in enrichments containing oil, which concurs with what was observed in the community profile of the preliminary enrichments, again supporting the reproducible nature of the enrichment process. Variation at finer phylogenetic resolution however revealed several differences in the distinct enrichments.

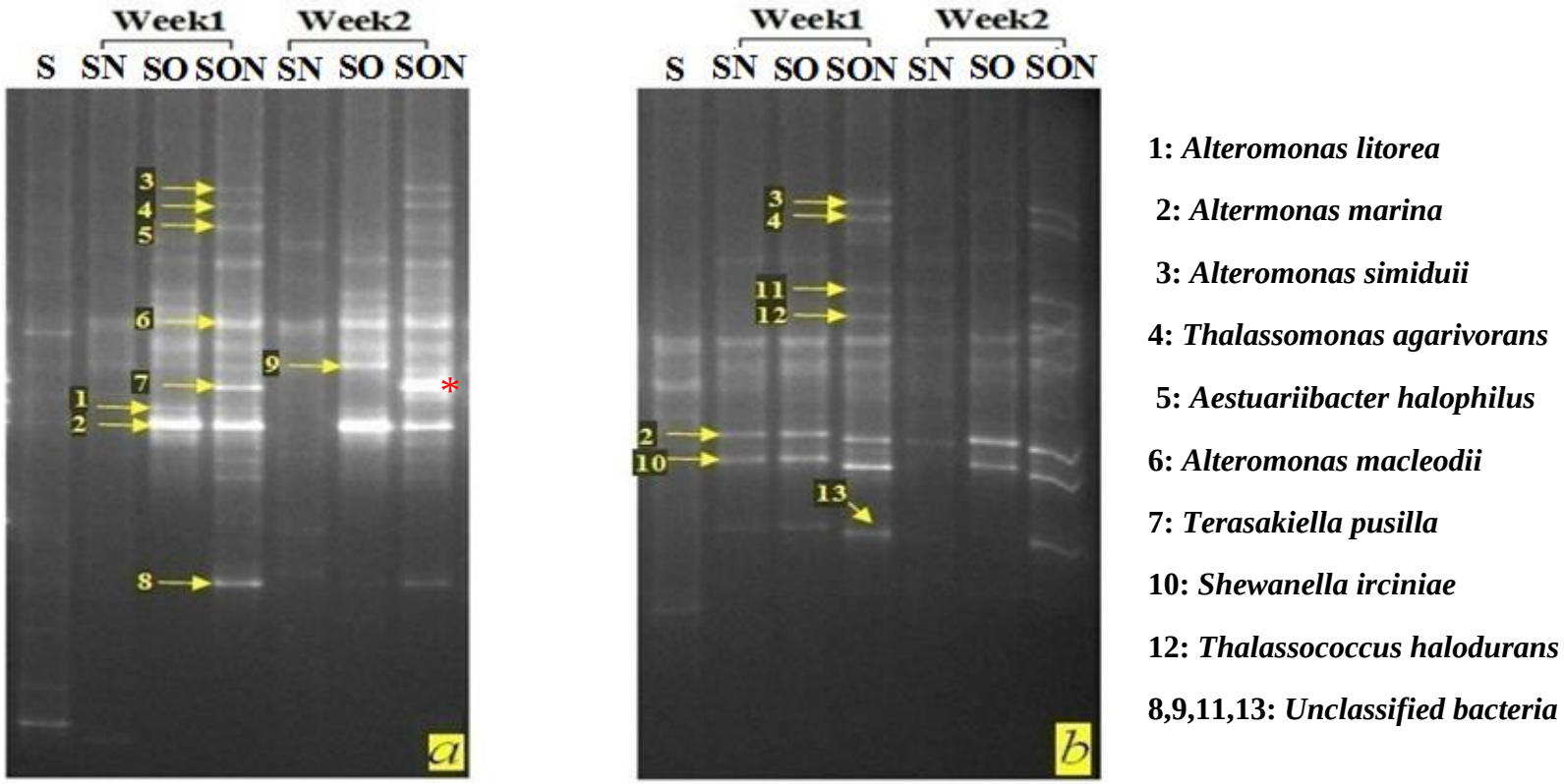


Figure 4.3. Denaturing gradient gel image showing the bacterial community composition in seawater (S), plus nutrients (SN), plus oil (SO) and plus both oil and nutrients (SON) of the two distinct enrichment culture sets for hexadecane (panel A) and aromatics (panel B) over time (after 1 and 2 weeks incubation).

Alteromonas simiduii (band 3) and *Thalassomonas agarivorans* (band 4), were active in oil and nutrient amended seawater used in the subsequent hexadecane pulse experiments (Fig.4.3-a) as well as in the corresponding culture for the subsequent aromatics pulses (Fig.4.3-b). *Aestuariibacter halophilus* (band 5), *Terasakiella pusilla* (band 7) and an *unclassified Proteobacterium* (band 8) were only seen in the oil-spiked nutrient-amended culture (SON, Fig.4.3a). In culture SON (gel a), *A. marina* (band2) was the most active bacterium at week 1 compared to *T. pussilla* (band 7). At week 2, *T. pussilla* took over and appeared like the most active member of the community (marked with an asterisk in Fig. 4.3-a). In culture SO, *A. marina* (band 2) was the most active community member at week 1 and 2 (Fig. 4.3 a) with increased band intensity at week 2 compared to week 1. Its activity was lower in the culture supplemented with nutrients (SON, Fig. 4.3 a) with decreased band intensity at week 2 compared to week 1. The unclassified bacterium represented by band 9 appared active at week 2 in cultures amended with oil only (SO, Fig. 4.3a).

With respect to the enrichment set for pulsing aromatics (Fig.4.3-b), *Altermonas marina* (band 2) and *Shewanella irciniae* (band 10) were the most active bacteria in the oil-spiked cultures with or without nutrients. Moreover, *A. simiduii* (band 3), *Thalassococcus halodurans* (band 4), *T. agarivorans* (band 12) and *unclassified bacteria* (bands 11 and 13) showed activity only in the presence of oil and nutrients. The marked differences observed between the two distinct enrichment sets may be due to the different seawater samples collected as inocula or heteroscedastic community processes.

4.2.2. Stable isotope probing with hexadecane

4.2.2.1. Biodegradability of hexadecane by the enrichments

The enrichments generated from oil-spiked seawater were used in order to assess the biodegradability of hexadecane in hydrocarbon pre-adapted cultures (SO and SON) compared to non-adapted control cultures (S and SN), and to link its degradation to specific marine bacterial taxa by applying RNA-stable isotope probing. Triplicate aliquots (100 ml) from the four enrichment types (S, SN, SO and SON) were dispensed into glass serum bottles (160 ml capacity) and spiked with 30 mg/l of ^{12}C or ^{13}C-labeled hexadecane under aerobic conditions. Gas chromatography analyses were carried out to monitor the degradation of hexadecane in the ^{12}C-hexadecane-spiked cultures. As

shown in Fig. 4.4, all cultures degraded hexadecane compared to the sterile control although at different rates. The concentration of hexadecane showed a decrease in nutrient amended cultures (SN) compared to the other enrichments. The decrease in this culture was initially rapid (4.6 mg/l/day) but slowed down significantly from day 3 onwards (1.1 mg/l/day) until hexadecane was 95% depleted at day 7.

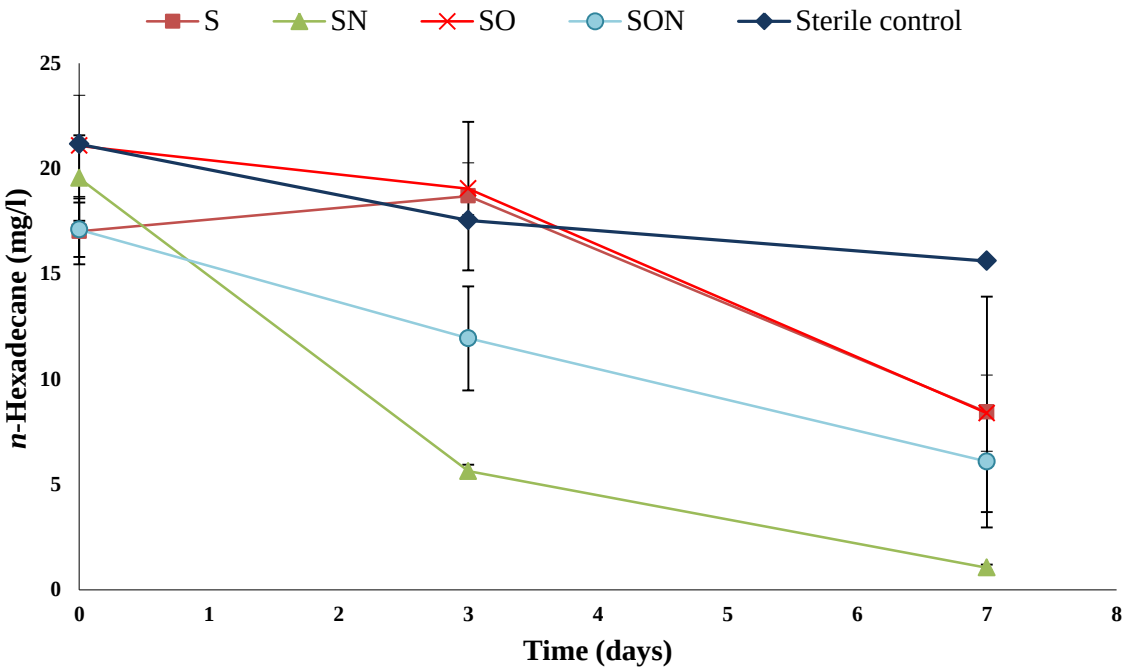


Figure 4.4. Biodegradation of *n*-hexadecane in the ^{12}C hexadecane-pulsed enrichment cultures. S = seawater only; SN = seawater and mineral nutrients (nitrogen and phosphorus), SO = oil-spiked seawater and SON = oil-spiked nutrient-amended seawater. Values are average of triplicates and error bars represent the standard deviation.

Oil plus nutrient amended cultures metabolised hexadecane at a relatively constant rate (1.6 mg/l/day) until day 7 where approximately 64% of hexadecane was consumed. Hexadecane degradation showed a similar trend in seawater and oil amended seawater enrichments where the uptake started at an initially slow rate that increased significantly over time. Culture SO exhibited a slow degradation rate (0.68 mg/l/day) until day 3 before increasing up to 2.6 mg/l/day until day 7 where 60% of hexadecane was consumed. The lowest degradation was observed in the unamended seawater (S) where only 50% of hexadecane was consumed in 7 days. Statistical analysis showed that the decrease in hexadecane concentration in all the cultures was statistically significant (P-value for t-test between replicates was greater than the significance level cut-off of 0.05).The increase observed in the concentration of hexadecane between the start and day 3 of the unamended seawater enrichments was statistically insignificant.

4.2.2.2 RNA-SIP of hexadecane amended pre-adapted cultures

After isopycnic centrifugation and fractionation of RNA extracted from the triplicate SIP pulses at day 7 , six fractions (fraction 2-7) containing the ^{13}C-labelled RNA were selected for community analysis based on buoyant density (average range: 1.85-1.80 g/ml) ((Lueders *et al.* 2004). In parallel, the same fractions were selected from the ^{12}C pulsed cultures and used as controls. Primers targeting the V3 region of the 16S rRNA gene were used to amplify the constructed cDNA. PCR products were then run on a 30-60 % denaturing gradient gel to identify bacterial species deriving carbon from hexadecane in the different enrichments. The community profiles for cultures SN and SON were generated (Fig 4.5 and 4.6 respectively). The low RNA yield obtained from seawater and oil-amended seawater enrichments following the hexadecane pulse was insufficient for stable isotope probing analysis.

Light and heavy RNA samples from gradients were characterized by DGGE fingerprinting. In nutrient amended seawater enrichments, the degradation of hexadecane seemed to be almost solely achieved by *Oceanobacter kriegii* (Fig.4.5, band 2), which dominated the assimilation of carbon from hexadecane in all the replicates. *Oceanobacter* sequences were not detected in the community profiles of the enrichments over time. In addition, *Alkcanivorax borkumensis* (Fig.4.5, band 1) appeared in the heavy fractions of replicate 1 only while another *Alcanivorax* species (*Alcanivorax jadensis*, band 3) was found in all the replicates suggesting that this bacterium also derived carbon from hexadecane.

The pre-addition of oil into the seawater generated a totally different SIP profile as shown in Fig.4.6. *Oceanobacter kriegii* that stood out as a hexadecane degrader in the nutrient amended seawater enrichments was not observed in the oil pre-adapted biostimulated culture. Bacteria dominating the acquisition of carbon from hexadecane in oil and nutrient amended seawater enrichments were *Marispirillum indicum* (Fig.4.6, band 5, all replicates), *Alcanivorax hongdengenesis* (Fig.4.6, band 2, replicates 1 and 3), *T. pusilla* (Fig.4.6, band 3, replicates 1 and 2) and *Kordiimonas gwangyangensis* (Fig.4.6, band 4, replicate 1 only). It was concluded that different preadaptation regimes affect that outcome of the SIP pulses with *Alcanivorax* and *Teraslakiella* dominating hexadecane degradation with enrichments preadapted with oil and nutrients and *Oceanobacter* dominating in nutrient amended seawater. Variation was also observed

between replicate SIP pulses highlighting the heteroscedastic nature of hexadecane consumption in seawater and possibly aspects of the methodology (RNA extraction, PCR, denaturing gradient fingerprinting).

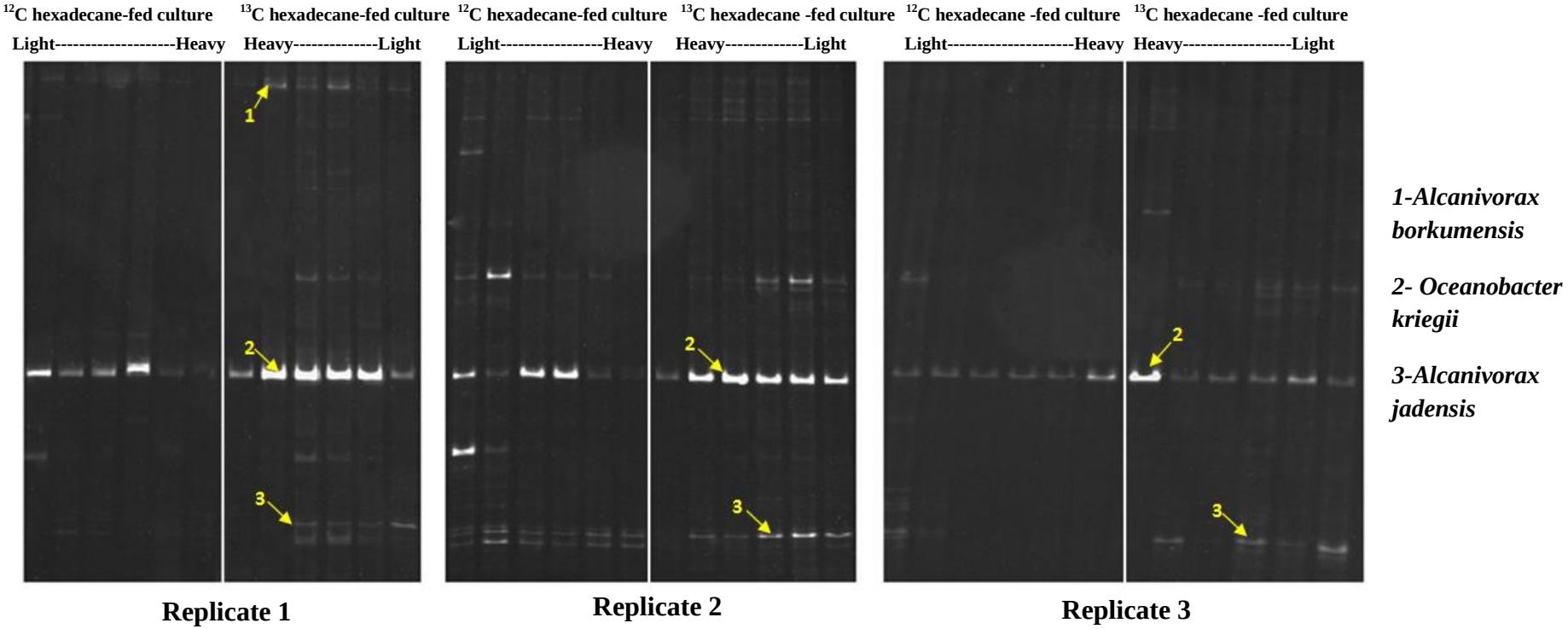


Figure 4.5. DGGE of RT-PCR-amplified 16S rRNA gene fragments from RNA (extracted at day 7) fractions retrieved from CsTFA density gradients of the ^{12}C and ^{13}C-hexadecane-fed nutrient amended seawater enrichments in triplicate. For each sample, 6 fractions with buoyant densities ranging from 1.852-1.80 g/ml the ^{12}C and ^{13}C hexadecane-pulsed cultures were selected for DGGE analysis. Yellow arrows refer to sequenced bands. Numbers define species (similar numbers denote the same species on replicate gels). All gel images were aligned with an internal marker run in parallel to samples.

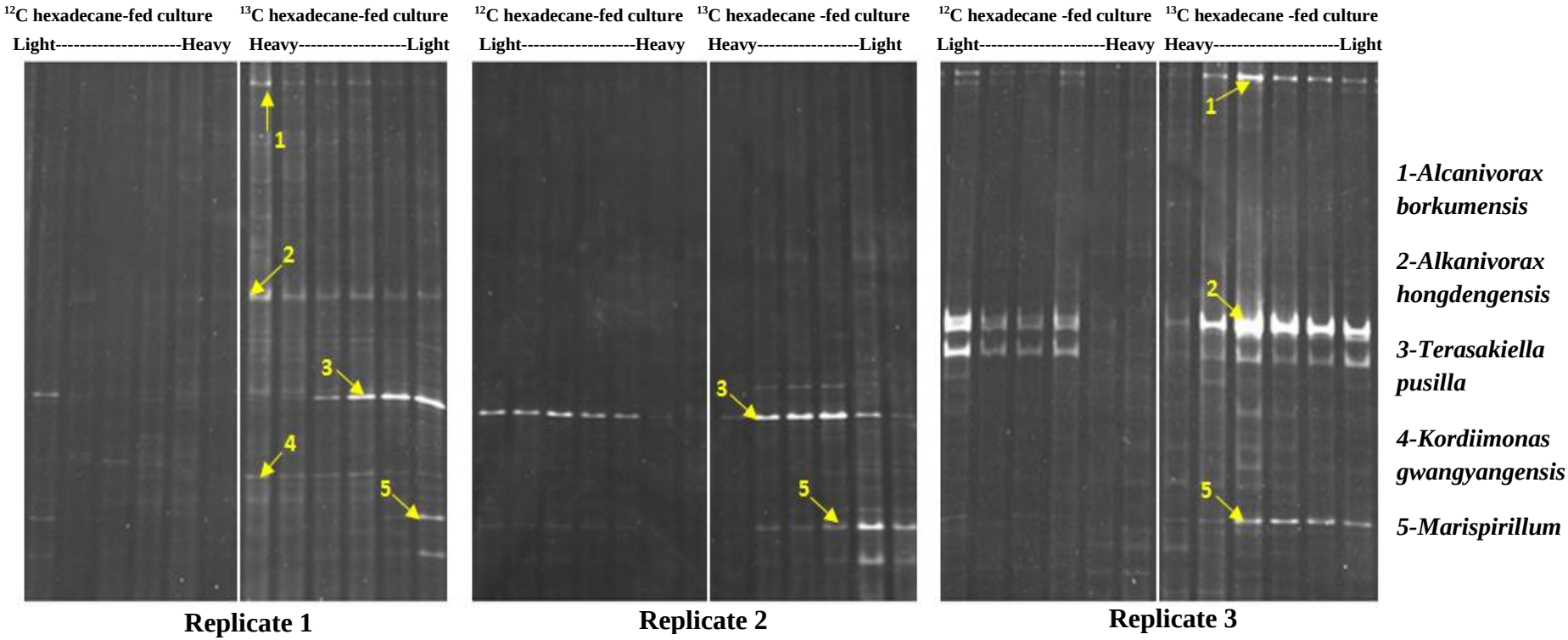


Figure 4.6. DGGE of RT-PCR-amplified 16S rRNA gene fragments from RNA (extracted at day 7) fractions retrieved from CsTFA density gradients from the ^{12}C and ^{13}C-hexadecane-fed culture SON in triplicate. For each sample, 6 fractions with buoyant densities ranging from 1.849-1.802 g/ml the ^{12}C and ^{13}C hexadecane-pulsed cultures were selected for DGGE analysis. Yellow arrows referred to sequenced bands. Numbers define species (similar numbers denote the same species on replicate gels). All gel images were aligned with an internal marker run in parallel to samples.

4.2.3. Stable isotope probing with aromatic hydrocarbons

4.2.3.1. Biodegradability of benzene and naphthalene by the enrichments

Biodegradability of benzene and naphthalene in the pre-adapted enrichment cultures was tested by dispensing 100 ml of each enrichment culture (S, SN, SO and SON) in triplicate into serum bottles (160 ml capacity). Cultures were then pulsed with 30 mg/l or 10 mg/l of ^{12}C or ^{13}C-labeled benzene or naphthalene under aerobic conditions. The degradation of benzene and naphthalene in the ^{12}C-benzene or ^{12}C-naphthalene-spiked cultures was monitored over time using gas chromatography.

Fig. 4.7 shows the biodegradability of benzene in the different enrichment cultures. As shown, SON was the only culture that exhibited benzene degradation ability. Benzene was metabolised rapidly in this culture (100% of benzene was degraded in 2 days). None of the other cultures showed an ability to metabolise benzene. The concentration of benzene was measured in the latter cultures for up to 40 days (data not shown) with no significant decrease in the concentration when compared to the sterile control.

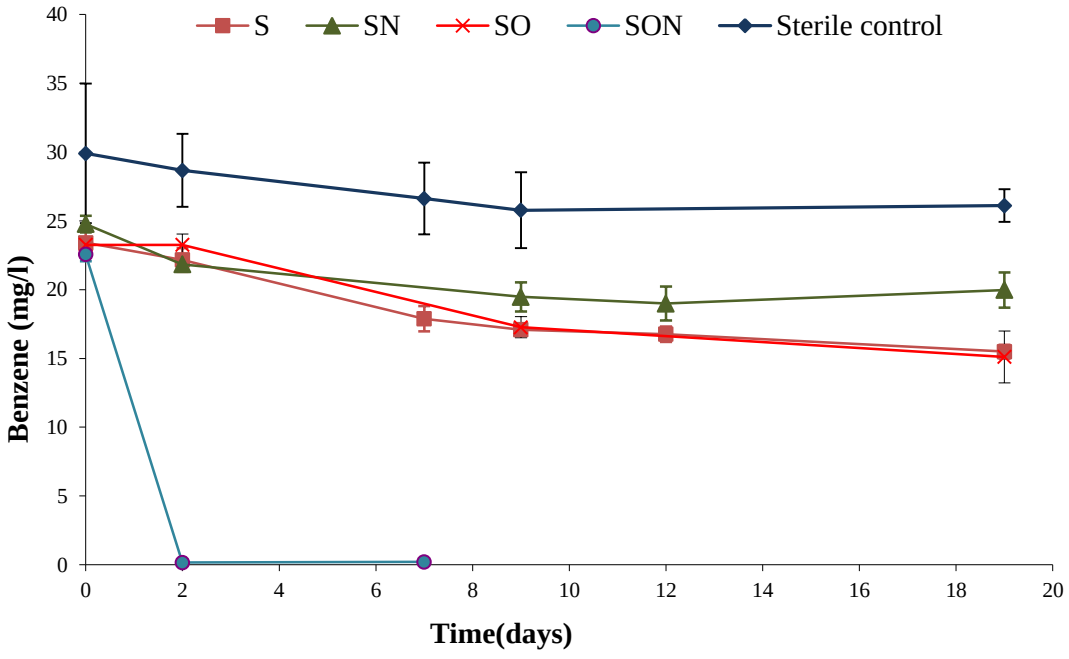


Figure 4.7. Biodegradation of benzene in the ^{12}C benzene pulsed cultures. S= seawater only; SN= seawater and mineral nutrients (nitrogen and phosphorus), SO= oil-spiked seawater and SON= oil-spiked nutrient-amended seawater. Values are average of triplicates and error bars represent the standard deviation.

To confirm the result obtained from benzene amended SON cultures, single smaller oil and nutrient-amended culture (300 ml of seawater spiked with 1% crude oil and amended with mineral nutrients) was prepared and incubated for 2 weeks before being split into 100 ml triplicates in serum bottles. Results were highly similar (data not shown) to the outcome obtained from SON, which supports the positive effect of oil-preadaptation combined with biostimulation on benzene degradation.

As for naphthalene amended cultures, those that contained oil showed no degradation of naphthalene (Fig.4.8) and it was degraded only by the oil-free cultures (S and SN). Interestingly, degradation occurred to a greater extent in seawater that had not received nutrients either. Naphthalene concentration was also checked for up to 30 days with no degradation observed compared to the sterile control. This result was in contrast to the previous observation that benzene degradation was greatly enhanced in oil and nutrient pre-adapted cultures.

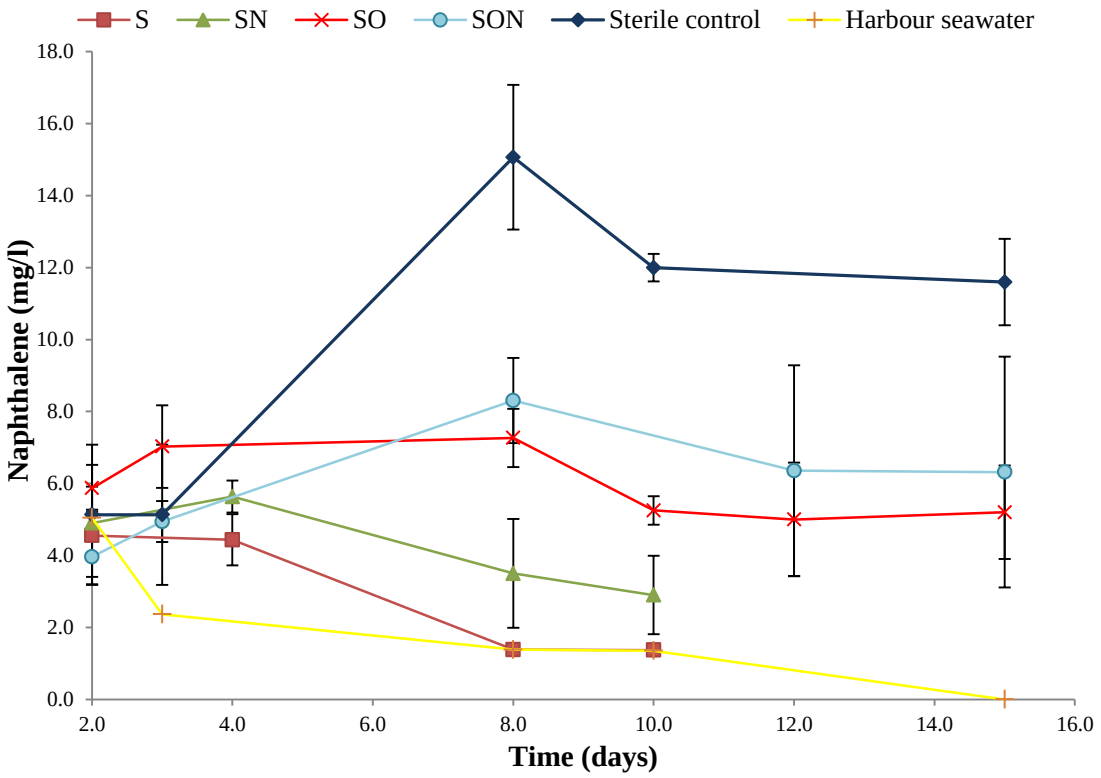


Figure 4.8. Biodegradation of naphthalene in the ^{12}C naphthalene pulsed cultures. S= seawater only; SN= seawater and mineral nutrients (nitrogen and phosphorus), SO= oil-spiked seawater and SON= oil-spiked nutrient-amended seawater. Values are average of triplicates and error bars represent the standard deviation.

In addition, a positive control consisting of seawater sampled from the chronically hydrocarbon-exposed Sydney Harbour (Fortin *et al.* 2004) was included for comparison. Harbour seawater (100 ml) was dispensed into serum bottles in triplicates and spiked with unlabelled naphthalene (10 mg/l). Results showed degradation of naphthalene by the native microorganisms in the Harbour seawater (Fig. 4.7). It is possible that under extended incubation naphthalene might eventually get consumed in culture SON after complete depletion of the simpler bioavailable non-aromatic hydrocarbons carried over from the previous exposure of this culture to crude oil.

4.2.3.2. RNA-SIP of benzene and naphthalene amended pre-adapted cultures

Cultures that showed degradation of benzene or naphthalene were subject to stable isotope probing analysis to identify bacteria assimilating carbon from each of these hydrocarbons. After isopycnic centrifugation and purification of RNA (extracted at day 2 from the benzene-pulsed culture SON, and at day 10 from the naphthalene-pulsed culture SN) day obtained from gradient fractionation, DGGE profiling was carried out.

DGGE profiling of the benzene-degrading SON culture (Fig.4.9) showed one highly enriched band (band 4) that was more intense in the ^{13}C than in the ^{12}C benzene- spiked SON culture profiles and was present in all replicates (Fig.4.9, all replicates). Sequencing of this band revealed high similarity to *Marinobacter litoralis*. In addition, although to a lesser extent, *Thalassospira tepidiphila* (Fig.4.9, band 5) was also possibly directly implicated in benzene degradation as it showed more intense bands in the ^{13}C – pulsed SON culture's profile than in the corresponding ^{12}C one (less intense compared to band 4). In addition, *Marinobacter excellens* (Fig.4.9, band 1) and *T. pusilla* (Fig.4.9, band 2) showed higher activity in the ^{13}C-benzene SON culture than in the corresponding ^{12}C-benzene spiked one.

Stable isotope probing revealed the main naphthalene degraders in the SN culture replicates. Culture S could not be profiled due to the low RNA extracted. Bacteria that derived carbon from naphthalene in this culture included *M. aquaeolei* (Fig.4.10, band 1), *T. tepidiphila* (band 2 Fig. 4.10) and *Cycloclastic*us sp. (Fig. 4.10, band 3). These bands were more intense in the ^{13}C naphthalene-fed SN cultures than in the corresponding ^{12}C naphthalene-fed in all the three replicates. On the other hand, some species were observed in some replicates but not in others. *Phaeobacter gallaeciensis* (Fig. 4.10, band 4) was observed in the heavy fractions of the ^{13}C SN culture in replicate

1 and 3 and not in replicate 2. *Marinobacter hydrocarbonoclasticus* showed a clear enrichment only in replicate 1. Interestingly, *Alkanivorax jadensis* (Fig. 4.10, band 8) was observed in the ^{13}C naphthalene-fed culture profile (replicate 3), and in the ^{12}C profile in replicate 2. It was not at all seen in replicate 1. In addition, *A. burkomensis* was active in the ^{12}C naphthalene fed cultures in replicates 1 and 3 but not in the ^{13}C-naphthalene-spiked ones. This suggests that the last two species may have not derived carbon from naphthalene but from other available carbon sources or, perhaps from naphthalene degradation byproducts.

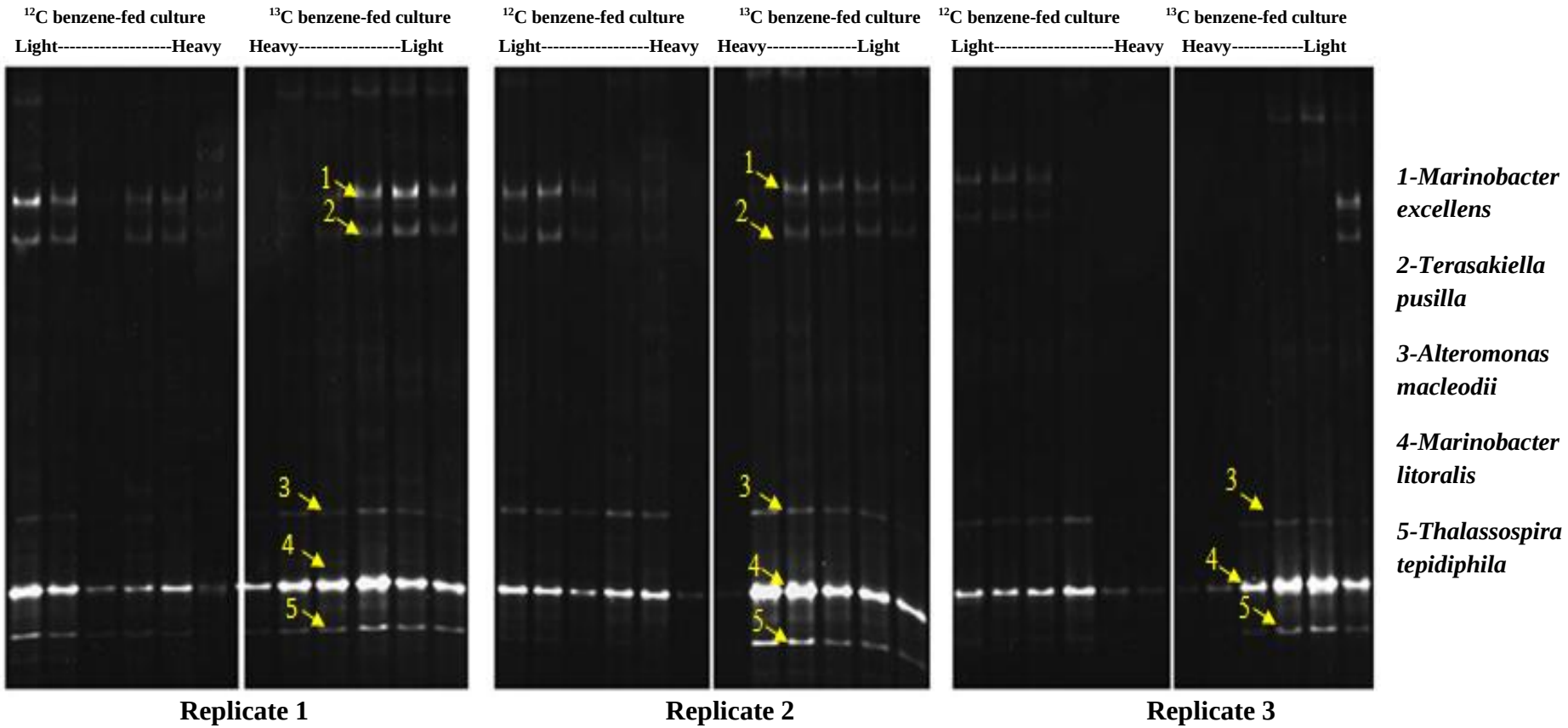


Figure 4.9. Denaturing gradient gels showing the bacterial community profiles of the ^{12}C and ^{13}C-benzene-fed culture SON in triplicates. RNA was extracted at day 2. For each sample, 6 fractions with buoyant densities ranging from 1.850-1.796 g/ml the ^{12}C and ^{13}C hexadecane-pulsed cultures were selected for DGGE analysis Species are represented by numbers on the gels. Species found in replicates are rep**r**esented by the same number. All gel images were aligned with an internal marker run in parallel to samples.

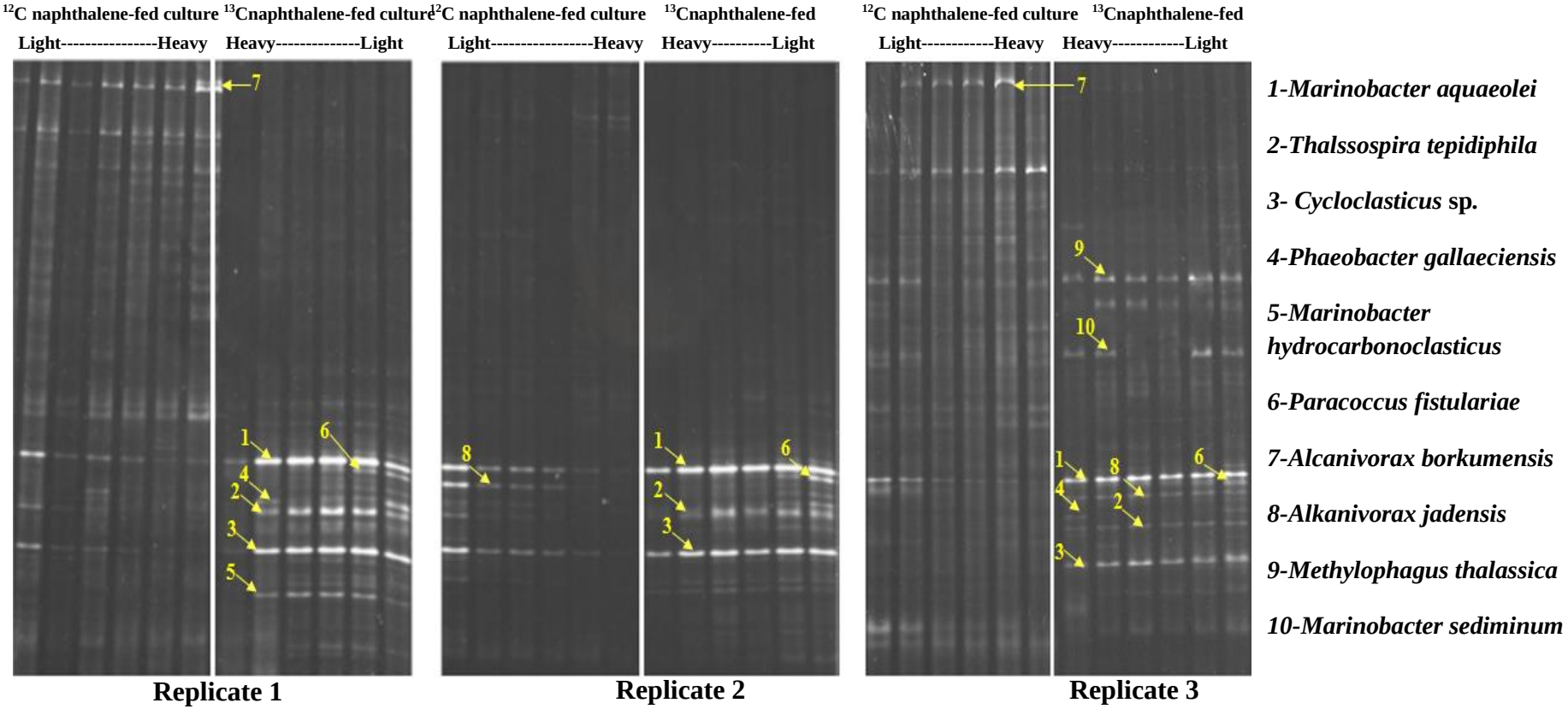


Figure 4.10. Denaturing gradient gels showing the bacterial community profiles of the ^{12}C and ^{13}C-naphthalene-fed culture SN in triplicates. RNA was extracted at day 10. For each sample, 6 fractions with buoyant densities ranging from 1.849-1.802 g/ml the ^{12}C and ^{13}C hexadecane-pulsed cultures were selected for DGGE analysis were selected for DGGE analysis. Sequenced bands are indicated by yellow arrows and numbers are assigned to different bands on the gels. Species found in more than one replicate are represented by the same number on replicate gels. All gel images were aligned with an internal marker run in parallel to samples.

4.3. Discussion

In order to investigate the effect of hydrocarbon preadaptation and/or biostimulation on the biodegradation rate and community dynamics of selected model saturated and aromatic oil hydrocarbons (hexadecane, benzene and naphthalene) in seawater, two sets of active hydrocarbon-adapted enrichment cultures were prepared. The enrichments were then spiked with labelled and unlabelled hydrocarbons in triplicate and subjected to stable isotope probing analysis. It should be recognised that triplicated analyses in SIP studies are rare. Microbial taxa deriving carbon directly from these compounds were identified by denaturing gradient gel electrophoresis followed by band sequencing.

It is worth noting that the oil-adapted cultures were spiked with single compounds rather than ^{13}C-compounds-spiked-oil because, with a subsequent oil load, the simpler hydrocarbons that had been consumed will become available again which may dramatically change the community structure by re-encouraging the simpler compounds degraders which would perhaps delay the degradation of more complex hydrocarbons like naphthalene. In addition, some bacteria have the capacity to degrade a range of compounds rather than one which may lead to dilution of the 13C isotopes or their even distribution between community members.

Pyrosequencing analysis of the initial seawater of both sets of oil and nutrient–amended enrichment cultures showed comparable results to the preliminary enrichment cultures, with high abundance of rRNA sequences of Proteobacteria in all of the initial seawater communities. However, some community differences between initial seawater samples were also observed. For example, Planctomycetes that were relatively active in the seawater used for the preliminary cultures were inactive in the seawater used to generate the enrichments used for SIP pulsing. Bacteroidetes represented 31% of amplicons in the enrichments for hexadecane pulsing but lower activity in the enrichments for pulsing aromatics (4%) and in the preliminary cultures (7%). Within Proteobacteria, Betaproteobacteria that exhibited modest activity in the seawater used for the preliminary enrichments and the seawater used to generate the enrichments for hexadecane pulsing, (2% and 1% respectively), showed a boost of activity in the seawater used to prepare enrichments for benzene and naphthalene pulsing (39%).

The structure of marine bacterial populations can be easily altered by numerous factors such as change in pH (Krause *et al.* 2012), bloom of diatoms (Riemann *et al.* 2000),

dust carried over by storms (Lekunberri *et al.* 2010) and, perhaps most prominently, seasonal variation (Delille 1993, Schauer *et al*. 2003). In addition, (Höppe 1978) demonstrated that only 10% of the marine communities are active in winter while 50% of the communities are active in summer. In this context, it is worth noting that the seawater used to generate the enrichments in Chapter three and in this chapter was sampled at different times of the year (February 2011, March 2012 and December 2012 respectively). Therefore, the reason behind the differences in community structure and activity between equivalent seawater samples may respond to any of these environmental variants. These results illustrate that changes in marine community structure should be expected even when samples from the same location are collected at different times.

To obtain community information at a higher phylogenetic resolution (species level), DGGE profiling and band sequencing of the enrichment cultures communities was carried out and showed predominance of *Alteromonas* genus (Fig 4.3). In particular, several *Alteromonas* species showed dominance in the cultures with oil and nutrients (SON) *(A. macleodii, A. marina* and *A. litorea).* This result agrees with the pyrosequencing data obtained from the preliminary cultures that showed dominance of *Alteromonas* in the oil-impacted cultures (See chapter 3, Fig.3.6).

To study the biodegradability of the long chain alkane *n*-hexadecane, triplicate aliquots (100 ml) of cultures S, SN, SO or SON derived from the prepared enrichment cultures were spiked with ^{12}C or ^{13}C-labelled hexadecane and incubated aerobically. Results showed that hexadecane was biodegraded in all the cultures with differences according to the different treatments (Fig.4.4). Oil pre-adaptation did not seem to exhibit any net positive effect on hexadecane biodegradation in the absence of mineral nutrients (cultures SO compared to cultures S). This was also reflected on the low RNA yields obtained from the SO cultures. On the other hand, the degradation rate of hexadecane was significantly higher in both the nutrient-treated enrichments (cultures SN and SON) which agrees with the findings of several studies (Atlas and Bartha 1972, Gibbs 1975, Graham *et al.* 1999, Van Hamme *et al.* 2003, Yu *et al.* 2005, Delille *et al.* 2009, McGenity *et al.* 2012).

In enrichments amended with nutrients only, hexadecane was the only carbon source provided with no potential competition or inhibition, hence the rapid utilisation.

Conversely, the slower rate of hexadecane biodegradation observed in culture SON compared to culture SN (not preadapted to oil) was possibly due to preferential degradation of other dissolved, simpler hydrocarbon molecules in this culture that were carried over from enrichments previously amended with crude oil.

Additionally, pre-treatment with oil may have favoured selection towards a broader range of hydrocarbonoclastic bacteria that can degrade a variety of hydrocarbon molecules rather than hexadecane only. Sequential utilisation of catabolites may have also been the reason behind this result. Bacteria that have the ability to metabolise multiple hydrocarbons generally prefer utilising simpler hydrocarbons (e.g. shorter chain alkanes) when several compounds are simultaneously available (Van Hamme *et al.* 2003). Furthermore, some dissolved hydrocarbons (especially PAHs) from the oil amendment might have exhibited either inhibition or toxicity on some hexadecane consumers (Samanta *et al.* 2002), leaving behind a smaller fraction of bacteria that can potentially degrade hexadecane.

RNA-SIP analysis revealed that the hexadecane degrading community in cultures SN and SON was dominated by the genus *Alcanivorax* (Fig 4.5 and Fig.4.6). *Alcanivorax* are widespread marine Gammaproteobacteria that are capable of utilising a broad range of *n*-alkanes as sole carbon and energy sources (Hara and Syutsubo 2003, Cappello *et al.* 2007) . This genus currently includes 5 species, 4 of them isolated from the marine environment, namely *A. borkumensis, A. jadensis , A. venustensis, A. dieselolei* (Wu *et al.* 2009).

Several studies demonstrated the predominance of *Alcanivorax* in oil-contaminated seawater during bioremediation especially after nutrient addition (Harayama *et al.* 1999, Röling *et al.* 2002, McKew *et al.* 2007, Teramoto *et al.* 2013). *A. borkumensis* is the best-studied species and consequently has several known adaptation mechanisms to alkanes (Naether *et al.* 2013). Sabirova *et al.* (2006) demonstrated the existence of three different alkane-oxidizing systems in *A. burkomensis* which explains its ability to degrade a wide range of alkanes. The same study also showed that when this bacterium is cultivated with hexadecane, the genes for 97 cytoplasmic membrane-bound proteins were upregulated. In the present study, *A. borkumensis* was observed in the heavy fractions of the ^{13}C hexadecane spiked SN and SON cultures.

Sei et al. (2003) studied the dynamics of alkane-degrading bacteria followed by quantifying three groups of alkane- hydroxylase genes. This study showed three groups of bacteria: those that carry group I alkane hydroxylase gene (degrade short alkanes) detected in the first 3 days of the experiment, those that carry group II alkane hydroxylase gene (degrade higher molecular weight alkanes) which were not detected until day 10 of the experiment and others that carry group III alkane hydroxylase gene that were initially undetectable but appeared after 3 days, however, they were less abundant than group I and more abundant than group II. This finding indicates that Group III degrades the high molecular weight alkanes all throughout the period of alkane degradation which rules out the possibility of sequential utilisation (low then higher molecular weight alkanes). The apparent sequential utilisation is rather created by the slow rate of degradation of higher molecular weight alkanes rather than the sequential utilisation.

O. kriegii was also prominent in the ^{13}C-hexadecane pulsed culture SN. The *Oceanobacter* lineage, and more specifically *O. kriegii*, has recently been identified as a key marine *n*-alkane degrader possessing a metabolic capacity to degrade linear chain alkanes comparable to the capacity of *A. borkumensis* to degrade branched-chain alkanes (Teramoto *et al.* 2009). The same study demonstrated that several *O. kriegii* strains are hydrocarbonoclastic marine bacteria and showed their abundance in nutrient-amended and unamended seawater microcosms suggesting their natural abundance in oil-impacted marine environments and persistence during bioremediation. This supports the findings of the present study; however, until now, *O. kriegii* has not yet been specifically implicated in hexadecane degradation.

Of the bacterial lineages besides *Alcanivorax* implicated in hexadecane degradation in oil and nutrient adapted seawater, *K. gwangyangensis* was recently identified as an alkane degrading species, although not with hexadecane specifically (Giebler *et al.* 2013). *M. indicum* (Lai *et al.* 2009) and *T. pusilla (*aka *Oceanospirillum pisillum)* (Satomi *et al.* 2002) are known marine bacteria that have not yet been linked to hydrocarbon degradation. This study demonstrated the ability of these bacteria to derive carbon specifically from hexadecane. Interestingly, none of the *Alteromonas* members that dominated the hexadecane-pulsed starting community derived carbon from hexadecane suggesting those taxa actively consumed hydrocarbons from oil other than

hexadecane. On the other hand, *T. pusilla* that was highly active in the starting community (culture SON) (Fig.4.3, gel a, band 7) acquired the labelled isotopes from hexadecane. This highlights the ability of SIP in linking the degradation of hexadecane to specific taxa among many other active members within the same community even in presence of a complex carbon source like crude oil.

Benzene degradation was only observed in seawater enrichments amended jointly with oil and nutrients. Leahy and Colwell (1990) highlighted the importance of oil pre-adaptation in accelerating the degradation of eventual hydrocarbon inputs, the same study and many others also highlighted the importance of nutrient amendments on promoting the mineralisation of hydrocarbons (Yu *et al.* 2005, Jiménez *et al.* 2007). This study demonstrated that benzene-degrading bacteria thrived and mineralised benzene only after being exposed to oil only in the presence of nutrients.

As for naphthalene, the addition of nutrients did not affect the rate of naphthalene degradation in pristine seawater. This is in conflict with the findings of Mckew *et al*. (2007) who observed a promotion in the degradation of naphthalene in seawater after biostimulation. However, it concurs with other studies that reported no positive effect of nutrient addition on naphthalene biodegradation (Bauer and Capone 1985, William *et al.* 1980). In addition, naphthalene was not degraded in seawater enrichments containing oil, with or without nutrients which may be due to carbon catabolite repression. This phenomenon occurs in several microorganisms that do not degrade complex carbon sources (such as naphthalene) unless the simple carbon source is completely depleted (biphasic or diauxic growth) suggesting a sequential rather than simultaneous utilisation of metabolites (Collier *et al.* 1996). In culture SON, catabolite repression could have been exerted by the available hydrocarbons themselves or by their degradation by-products (Doughty *et al.* 2006). This hypothesis is supported by a lab observation; some oil was carried over during aliquoting from the oil-adapted enrichment cultures which left a thin layer of oil floating on the surface in cultures SO and SON. Quantification of $^{12}CO_2$ in the 12C naphthalene fed cultures vs $^{13}CO_2$ in the ^{13}C naphthalene spiked cultures would have been of paramount importance to support this hypothesis further.

Interestingly, the potential diauxic growth suggested in culture SON after naphthalene addition was not observed in the case of benzene. Benzene is easier than naphthalene to

biodegrade due to its simpler structure (one *vs.* two benzene rings), which makes it more prone to microbial attack than naphthalene in the context of sequential utilisation (Van Hamme *et al.* 2003). In addition, Bauer and Capone (1985) observed an optimal acclimation to polyaromatic hydrocarbons (PAH) (through pre-exposure to the compounds) at the highest acclimation concentration. Benzene is more abundant than naphthalene in crude oil (0.16 and 0.069 % w/w, respectively) (Williams *et al.* 2005). The findings of these studies may explain a faster adaptation of the indigenous bacteria in culture SON to benzene than to naphthalene, hence the rapid benzene depletion. However, these benzene pre-adapted bacteria could only degrade benzene when nutrients (nitrogen and phosphorus) were amended.

Stable isotope probing analysis of the 16S rRNA gene sequences recovered from incubations with ^{12}C and ^{13}C-labeled benzene and naphthalene revealed a number of frequently encountered hydrocarbonoclastic organisms with limited phylogenetic diversity that derived carbon from these compounds (Fig.4.9 and Fig.4.10). The profile of the ^{13}C labelled benzene-pulsed SON showed two more intense bands in the heavy fractions than in the ^{12}C control cultures (Fig.4.9). The most intense band belonged to *M. litoralis. Marinobacter* are marine hydrocarbon degrading Gammaproteobacteria. The first species identified, *M. hydrocarbonoclasticus*, was discovered by Gauthier *et al.* (1992). *M. litoralis* was first identified by Yoon (2003). *T. tepidiphila*, also involved in benzene consumption (Fig 4.9) was recently characterised by Kodama *et al.* (2008). All these species were isolated in the corresponding studies from hydrocarbon-contaminated seawater and were associated with the degradation of hydrocarbons; however, none of them were specifically associated with benzene degradation, as observed here. Interestingly, none of the hydrocarbonoclastic bacteria that were enriched at the beginning of the pulse degraded benzene. For example, members of the genus *Alteromonas* that were active at the start of the pulse did not derive carbon from benzene. *A. macleodii*, however, showed only a modest activity compared to *M. litoralis*. Despite the fact that members of the *Alteromonas* genus are key aromatic hydrocarbon degraders in coastal sediments (Jin *et al*. 2012), their disappearance following benzene addition may suggets their inability to derive carbon specifically from benzene. Moreover, *M. litoralis* and *T. tepidiphila* may have outcompeted *Alteromonas* via interactions that remain unrevealed here. Studying those interactions and the mechanisms that favoured *M. litoralis* over other community members would be

of interest. The DGGE profile of the naphthalene-spiked culture SN, showed 4 bands prominently more enriched in the heavy fractions of the ^{13}C than the ^{12}C naphthalene-fed culture, corresponding to *M. hydrocarbonoclasticus, M. aquaeolei*, *Cycloclasticus* sp. and *M. tepidiphila* (Huu *et al.* 1996). *Cycloclasticus* and *Thalassospira* genera that derived carbon from naphthalene (Fig 4.10) are defined as key marine PAH degraders (Head *et al.* 2006, Mckew *et al.* 2007). *T. tepidiphila* was linked to both benzene and naphthalene degradation in the present study explained by its ability to metabolise both compounds. The metabolic capability of some bacteria to degrade several aromatic hydrocarbons was previously demonstrated by Bauer and Capone (1988). *Alcanivorax* species have already been shown to only degrade alkanes and not aromatic hydrocarbons (Mckew *et al*. 2007). *A. jadensis* is a putative marine alkane degrader not known to degrade PAHs. It was observed in the heavy fractions of the ^{13}C naphthalene spiked SN culture (Fig. 4.10, replicate 3) which suggests this bacterium rather derived carbon from naphthalene degradation by-products or cellular debris. In addition, members of the genus *Alcanivorax* are surfactant producers (yakimov *et al.* 1997). This suggests that *A. jadensis* was possibly releasing a biosurfactant that solubilised naphthalene and increased its availability. This could explain the presence of multiple lineages deriving carbon from naphthalene.

In conclusion, the effect of oil pre-adaptation on the degradation of hydrocarbons in this study was compound specific. In fact, it exhibited three different effects: acceleration (benzene), deceleration (hexadecane) and inhibition (naphthalene). In the case of benzene, pre-adaptation was only beneficial when combined with biostimulation. Whereas biostimulation showed no effect on naphthalene degradation, it boosted the degradation of hexadecane. The community profiles of the hexadecane cultures were also affected by preadaptation of seawater to oil. This study also revealed that the major consumers of hydrocarbons in the seawater sampled are likely to be *Kordiimonas gwangyangensis*, *Marispirillum indicum, Terasakiella pusilla*, *Oceanobacter kriegii* and *Alcanivorax borkumensis* for alkanes and *Marinobacter hydrocarbonoclasticus, Marinobacter aquaeolei, Marinobacter litoralis Cycloclasticus* sp. and *Thalassospita tepidiphila* for aromatics. Most of the bacteria identified here (*Marinobacter*, *Cycloclasticus*, *Alcanivorax* etc.) were also identified as hydrocarbon degraders in the first application of SIP during the Deepwater Horizon spill (Gutierrez 2011).

Chapter 5

RNA-stable isotope probing elucidates the outcome of competition between indigenous bacteria and bioaugmentation strains

5.1. Introduction

The biodegradation of complex hydrocarbon molecules in the environment has been demonstrated to start with a lag period that depends on the complexity of hydrocarbons and the mechanism of attack (Bauer and Capone 1985). During the lag phase, evaporation of the low molecular weight hydrocarbons (e.g. BTEX, low molecular weight alkanes etc.) takes place immediately exposing humans and other living organisms to potential risk. Immediate transformation of organic pollutants into harmless compounds is therefore important to minimise human and environmental hazard. In the preceding chapter, it was demonstrated that preadaptation of native marine microbial communities was compound-specific, triggering the degradation of benzene but delaying hexadecane and naphthalene degradation. Therefore, in pristine marine environments where hydrocarbonoclastic bacteria may constitute a small fraction of the total microbial population (Sivaraman *et al.* 2011) bioaugmentation can be adopted.

Bioaugmentation is a controversial bioremediation strategy for the mineralisation of oil hydrocarbons, with outcomes ranging from advantageous to unsuccessful (McKew *et al.* 2007, Hassanshahian *et al.* 2013). Controversy emerges from the high dependency of the microorganisms added on prevailing environmental conditions and their viability in a given environment. In addition, following the Exxon Valdez oil spill, several studies applied bioaugmentation in field studies with no reported success (Venosa *et al.* 1992 & 2006, Lee and Levy 1987). Atlas (1995b) suggested that the application of microbial seeding was unsuccessful due to disappearance of the added bacterium shortly after being released into an ecosystem that is totally different to its natural habitat. The latter author explained that the foreign environmental factors are what compromised the survival of the added bacteria and their poor performance was not due to metabolic

incapacity. As a result, autochthonous bioaugmentation (El Fantroussi and Agathos 2005, Ueno et al. 2006), involving microbes exclusively indigenous to the contaminated environment was regarded promising in overcoming these limitations. However, Venosa et al (2006) in a randomised block experiment, seeded with an indigenous consortium, could not achieve successful acceleration of biodegradation which disagrees with the previous suggestion; therefore, bioaugmentation was deemed unsuccessful and was abandoned for a long time. With the development in biotechnology, recent studies renewed the focus on seeding with bacterial species or tailored consortia native to the environment (Nikolopoulou et al. 2013a, Nikolopoulou et al. 2013b, Hassanshahian et al. 2013).

Bioaugmentation studies predominantly focus on the rate of hydrocarbon degradation, with relatively little attention given to changes in the indigenous community in response to bioaugmentation. For example, to date there has been no explicit demonstration of the fate with respect to consumers of hydrocarbons in bioaugmentation experiments. It is not clear therefore if bacteria added to affect hydrocarbon removal are deriving carbon from the contaminants or alternatively stimulating the native microflora. Uhlik *et al.* (2012) recommended the use of stable isotope probing in order to determine the ultimate fate of labelled isotopes and identify hydrocarbon degraders during bioaugmentation.

This chapter examined the effect of autochthonous bioaugmentation using indigenous bacterial species on the degradation rate of hexadecane, benzene and naphthalene in seawater microcosms. RNA stable isotope probing was also applied in order to link the degradation of these model hydrocarbons to their consumers. The results are discussed in the context of the interactions that potentially take place between members of the bacterial community and the added microbes during bioaugmentation.

5.2. Results

5.2.1. Identification of bacterial isolates and growth conditions

For bioaugmentation, three bacterial species were isolated from pristine (Coogee) or hydrocarbon-exposed (Sydney Harbour) seawater and grown on selective media containing hexadecane, benzene or naphthalene as sole carbon sources. Isolates were then subcultured until pure cultures were obtained and tested for the ability to use the

selected hydrocarbon compounds as sole carbon and energy sources. Sequencing of 16S rRNA gene amplicons revealed the identity of these bacterial species (Table 5.1)

Table 5.1. Identified bacterial isolates used for bioaugmentation.

Bacterial species (similarity%)	**Compound degraded**	**Marine source**
Rhodococcus qingshangii (99%)	*n*-Hexadecane	Coogee
Alteromonas addita (99%)	Benzene	Sydney Harbour
Pseudomonas alcaliphila (99%)	Naphthalene	Sydney Harbour

Unlike *R. qingshangii* that has not yet been associated with hydrocarbon degradation, *A. addita* and *P. alcaliphila* are putative marine hydrocarbonclastic bacteria. The ability of all the three bacteria to grow in LB_{30} as well as to maintain their hydrocarbon degradation capability when reintroduced into the seawater was tested in preliminary experiments (data not shown). Subsequently, the strains were used to bioaugment seawater microcosms.

5.2.2. Biodegradation of the model hydrocarbons in bioaugmented microcosms

The effect of bioaugmentation on the degradation of hexadecane, benzene and naphthalene and on the marine community dynamic was tested in 100 ml seawater in 160 ml bottles in the presence and/or absence of mineral nutrient amendments (added simultaneously with the hydrocarbons) in triplicate. Sterile controls (heat-sterilised) were included to account for abiotic losses (Table 5.2). Stable isotope probing was applied by spiking all cultures with 30 mg/l of ^{12}C or ^{13}C-hexadecane, 30 mg/l of ^{12}C or ^{13}C-benzene or 10 mg/l of ^{12}C or ^{13}C-naphthalene and incubated aerobically. Gas chromatography analysis was performed to quantify the hydrocarbon compounds in the head space (benzene) or aqueous phase (hexadecane and naphthalene) of cultures, while CsTFA density gradient centrifugation followed by DGGE profiling allowed the identification of hexadecane, benzene and naphthalene degraders during bioaugmentation.

Table 5.2. Description of the *R. qingshengii*, *A. addita* or *P. alcaliphila* augmented cultures.

Hexadecane		**Benzene**		**Naphthalene**	
Culture	**Composition**	**Culture**	**Composition**	**Culture**	**Composition**
S	seawater only	**S**	seawater only	**S**	seawater only
S+Rhod	seawater + *R. qingshengii*	**S+Altero**	seawater + *A. addita*	**S+Pseudo**	seawater + *P. alcaliphila*
S+N	seawater + mineral nutrients (nitrogen and phosphorus)	**S+N**	seawater + mineral nutrients (nitrogen and phosphorus)	**S+N**	seawater + mineral nutrients (nitrogen and phosphorus)
S+Rhod+N	seawater+ *R. qingshengii* +mineral nutrients	**S+Altero+N**	seawater+ *A. addita* + mineral nutrients	**S+Pseudo+N**	seawater+ *P. alcaliphila* +mineral nutrients
Sterile control	Heat-sterilised seawater	**Sterile control**	Heat-sterilised seawater	**Sterile control**	Heat-sterilised seawater

5.2.2.1. Saturated hydrocarbons

The concentration of hexadecane decreased faster in the presence of *R. qingshengii* (S+Rhod+N and S+Rhod) compared to the un-bioaugmented controls (culture S+N and S respectively) (Fig.5.1).

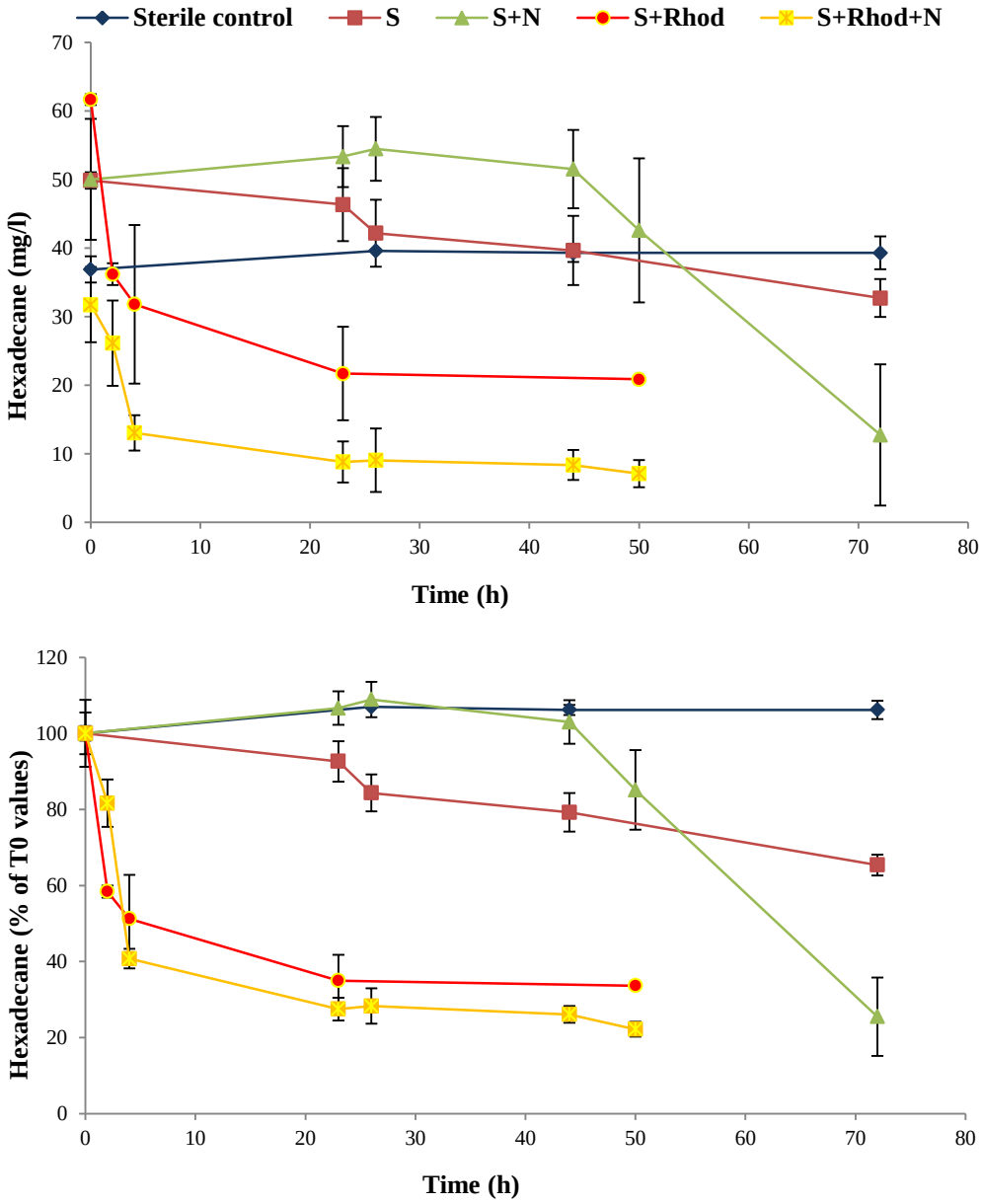


Figure 5.1. Biodegradation of hexadecane in all the ^{12}C-hexadecane-spiked microcosms (top graph). S: seawater only; S+N: nutrient-amended seawater; S+Rhod: seawater bioaugmented with *R. qingshengii*; S+Rhod+N: nutrient-amended seawater bioaugmented with *R. qingshengii*. Values are represented in percentage of T0 values (bottom graph) to clarify the decrease in concentration at the start. Values are average of triplicates and error bars represent the standard deviation

Bioaugmentation with *R. qingshengii* boosted the degradation of hexadecane irrespective of the nutritional conditions with 68% and 60% of hexadecane consumed during the first 4 h of incubation in culture S+Rhod+N and S+Rhod respectively. The degradation slowed down in these cultures after 4 h, with 10 mg/l and 4 mg/l of hexadecane consumed in the subsequent 19 h. In addition, the rate of degradation was faster in culture S+Rhod than S+Rhod+N in the first 4 h (7.4 mg/l/h and 4.7 mg/l/h respectively). This may be explained by the difference in the initial concentration of hexadecane in these cultures possibly due to a manual error during the pulse (62 and 37 mg/l respectively), that affected the degradation rate. In the non-bioaugmented control cultures, the addition of nutrients (culture S+N) resulted in a lag phase of 44 h after which 75% of the hexadecane was consumed (between 44 h and 72 h) while the degradation proceeded slowly in the seawater-only treatment compared to all the other cultures all throughout the experiment where only 35% of hexadecane was consumed in 72 h (the same amount of hexadecane was degraded in only 2 h when *R. qingshengii* was added (culture S+Rhod)).

5.2.2.2. Aromatic hydrocarbons

Benzene degradation was only observed in the *A. addita* augmented microcosms (culture S+Altero and S+Altero+N) (Fig.5.2). In these cultures, the addition of nutrients promoted the degradation of benzene right from the start of the incubation (culture S+Altero+N). A quick and complete consumption of benzene was observed in the latter culture within four days (Fig.5.2). On the other hand, the lack of nutrient amendments (culture S+Altero) resulted in a 4 day lag phase after which, benzene was consumed at a rate of 6 mg/l/day. It is likely that the native microorganisms in the seawater were unable to degrade benzene on their own regardless of the nutritional conditions (culture S and S+N). Cultures S and S+N were monitored for 25 days (not shown on the graph) and no decrease in benzene concentration was observed when compared to the sterile control.

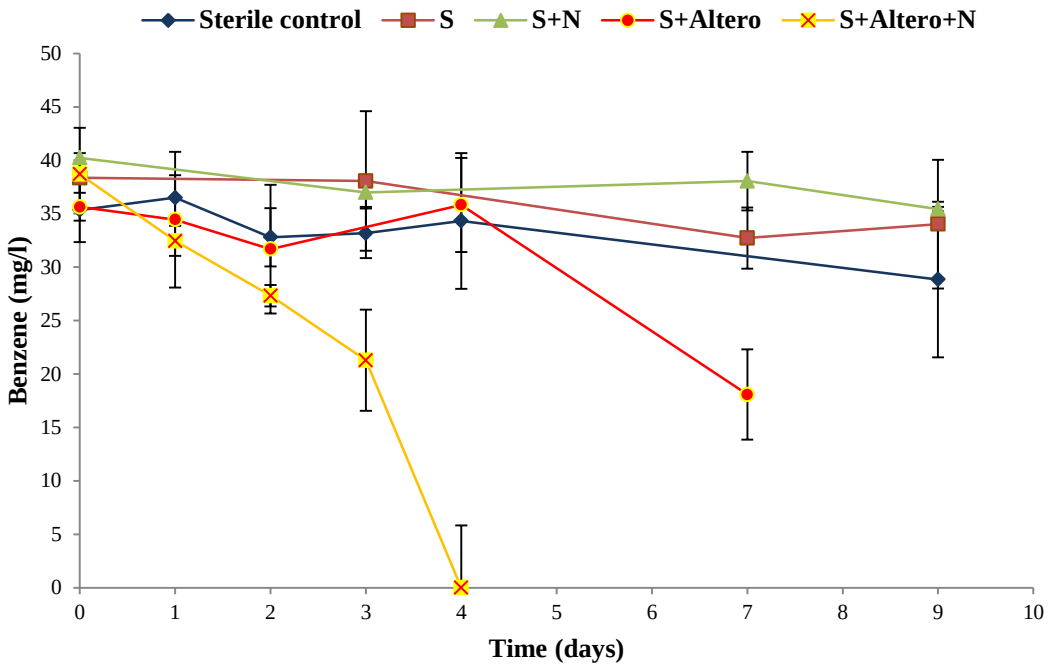


Figure 5.2. Biodegradation of benzene in all the ^{12}C benzene-spiked microcosms. S: seawater only; S+N: nutrient-amended seawater; S+Altero: seawater bioaugmented with *A. addita*; S+Altero+N: nutrient-amended seawater bioaugmented with *A. addita*. Values are average of triplicates and error bars represent the standard deviation.

As for naphthalene (Fig. 5.3), the increase observed in the initial concentration of naphthalene was due to its slow solubilisation in seawater. This suggests the difference in the maximal initial concentration between different treatments was a function of simultaneous biodegradation and solubilisation rates. As shown, all cultures exhibited a metabolic capacity for actively degrading this hydrocarbon. However, the addition of *P. alcaliphila* reduced the time needed for naphthalene degradation by approximately 50% (cultures S+Pseudo and S+Pseudo+N) when compared to the non-bioaugmented controls (cultures S and S+N respectively). The culture bioaugmented with *P. alcaliphila* in the absence of nutrient amendments showed a rapid degradation where 100% of the naphthalene was consumed in 7 days in contrast to the control (culture S) where naphthalene started to get consumed by day 7 following a lag period. Nutrient addition on its own exhibited no net positive effect on the degradation of naphthalene (culture S+N) except for the later stage (day 11-14) where the degradation rate increased slightly when compared to the nutrient–unamended culture (consumed naphthalene: 2.6 mg/l/day and 1 mg/l/day respectively) but this decrease was statistically insignificant. However, the simultaneous addition of nutrients and *P. alcaliphila* led to complete degradation of naphthalene in 8 days. Interestingly, the

addition of *P. alcaliphila* eliminated the lag phase observed in the non-bioaugmented cultures (S and S+N) in the absence of nutrients where naphthalene was simultaneously solubilised and biodegraded (maximum soluble concentration detected was 6 mg/l). This effect seemed to have been repressed by the addition of nutrients (culture S+Pseudo+N).

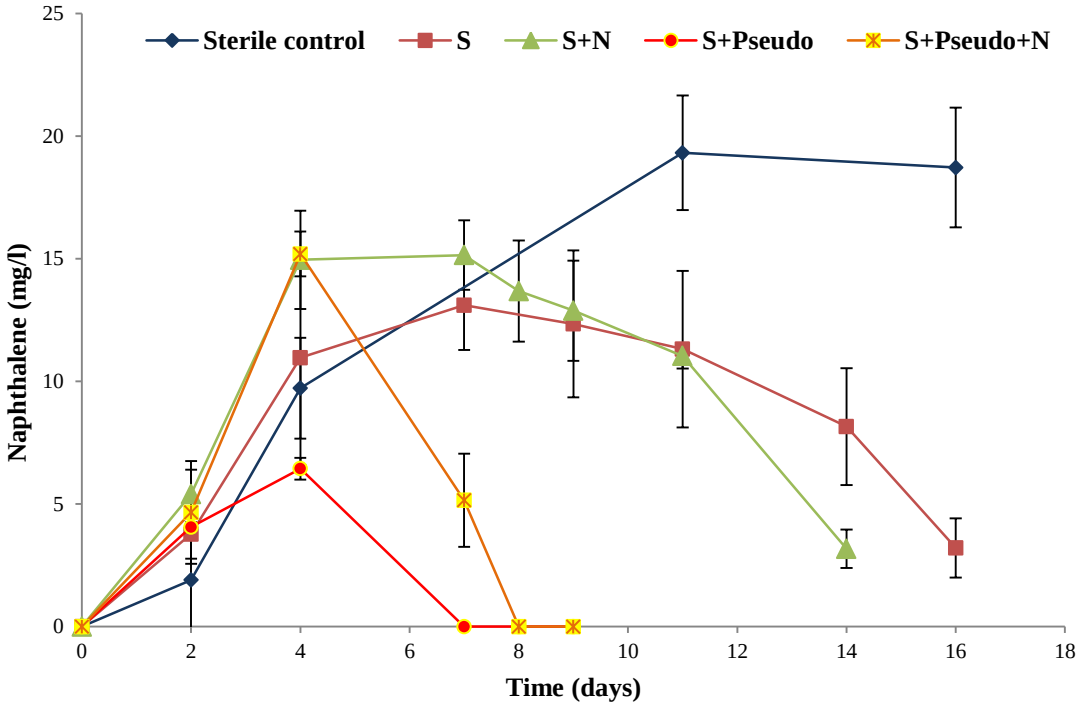


Figure 5.3. Biodegradation of naphthalene in all the ^{12}C naphthalene-spiked microcosms. S: seawater only; S+N: nutrient-amended seawater; S+Pseudo: seawater bioaugmented with *P. alcaliphila*; S+Pseudo+N: nutrient-amended seawater bioaugmented with *P. alcaliphila*. Values are average of triplicates and error bars represent the standard deviation.

5.2.3. RNA-stable isotope analysis and community profiling by DGGE

In order to determine the effect of bioaugmentation on the marine community structure and determine the possible interactions that resulted in the acceleration of the degradation rate of hexadecane, benzene and naphthalene, RNA stable isotope probing was applied. Total RNA was extracted from the cultures at the end of the incubation (marked by the last time point shown on the graphs in Fig. 5.1, Fig. 5.2 and Fig. 5.3 respectively) and subject to stable isotope probing analysis. RNA loaded into a CsTFA gradient followed by isopycnic centrifugation and gradient fractionation. RNA was then

precipitated and purified. Out of the 16 fractions obtained, five heavy fractions of each ^{13}C hexadecane, benzene or naphthalene-fed cultures containing the ^{13}C RNA (based on buoyant density values described by Lueders *et al.* (2004a)) as well as their parallel fractions of the ^{12}C hexadecane, benzene or naphthalene-fed control cultures were subject to RT-PCR amplification and then fingerprinting analysis by DGGE.

DGGE profiling showed that the heavy isotopes from ^{13}C-hexadecane were mainly assimilated by the bioaugmented bacterium *R. qingshengii* in the absence or presence of nutrients (Fig.5.4 and Fig.5.5 respectively). In culture S+Rhod, *R. qingshengii* apparently outcompeted three bacterial species namely: *A. hondengensis* (band 1), *O. beijerinckii* (band 4) and *N. caesariensis* (band 5) that derived carbon from hexadecane in the non-bioaugmented control culture (culture S). On the other hand, *S. degradans* (band 3) exhibited a boost of activity after addition of *R. qingshengii* and exhibited a higher activity in replicate 3 where *R. qingshengii* was modestly observed. On the other hand, the simultaneous addition of nutrients and *R. qingshengii* (culture S+Rhod+N) promoted the growth of the latter over *S. degradans*, represented by a slightly enriched band in the ^{13}C hexadecane fed culture profile when compared to the nutrient unamended culture (S+Rhod) and to the non-bioaugmented control (culture S+N).

In the case of benzene, the degradation occurred only in the bioaugmented microcosms (culture S+Altero and S+Altero+N) but did not occur in the non bioaugmented controls (culture S and S+N respectively) which suggested that the degradation was solely achieved by *A. addita*. Surprisingly, community profiling showed that ^{13}C from benzene was assimilated by an indigenous species *R. opacus* (band 2) (Fig.5.6). Another native species, *A. halophilus*, was also represented by a ^{13}C enriched band (band 1) although at a much lesser extent, and more prominently in the absence of nutrients (all replicates) than in the nutrient-amended culture (only in replicate 3). These two native marine bacteria outcompeted *A. addita* for carbon from benzene but exhibited no benzene degradation ability in its absence (cultures S and S+N) regardless of the nutritional regime.

The community profile of the naphthalene-degrading cultures showed that the acceleration of naphthalene biodegradation was solely achieved by *P. alcaliphila* in culture S+Pseudo with enrichment in the heavy fractions of the ^{13}C naphthalene-fed culture (Fig.5.7). In addition, *Cycloclasticus* sp. (band 2) that was deriving carbon from

naphthalene in the control non-bioaugmented culture (S) simultaneously with *P. alcaliphila,* was outcompeted at higher numbers of the latter (i.e. culture S+Pseudo). Interestingly, in the presence of nutrients, the heavy carbon isotopes from naphthalene were shared between *P. alcaliphila* and the indigenous *Ketogulonigenium vulgare* not only in the bioaugmented culture (S+Pseudo+N), but also in the non bioaugmented control (culture S+N) although the latter was only observed in one replicate in the control (replicate 2). Nutrient addition slowed down the activity of *P alcaliphila* possibly due to stimulation of other indigenous naphthalene degrading bacteria (e.g. *K. vulgare*) competing for the same carbon source.

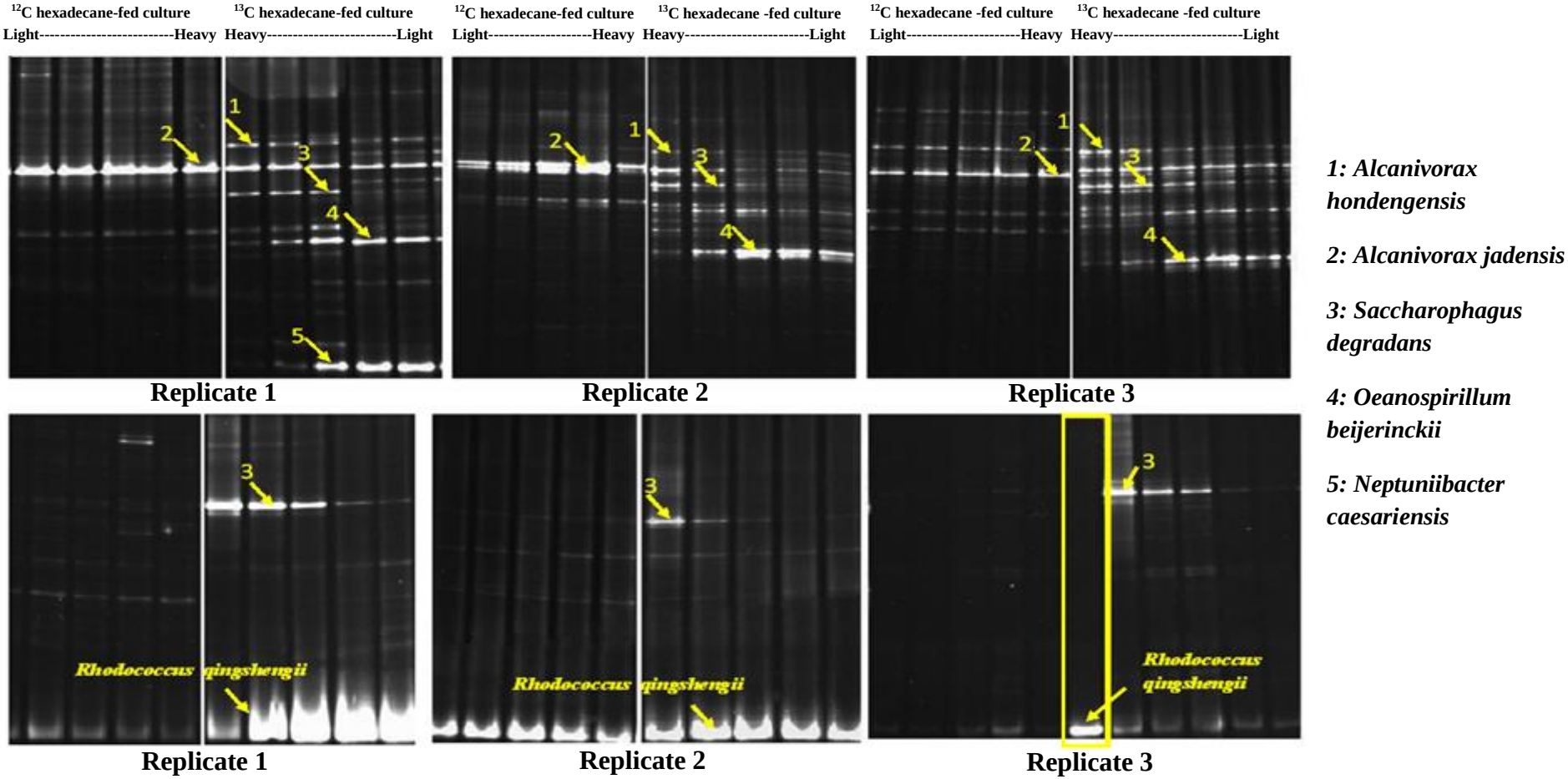
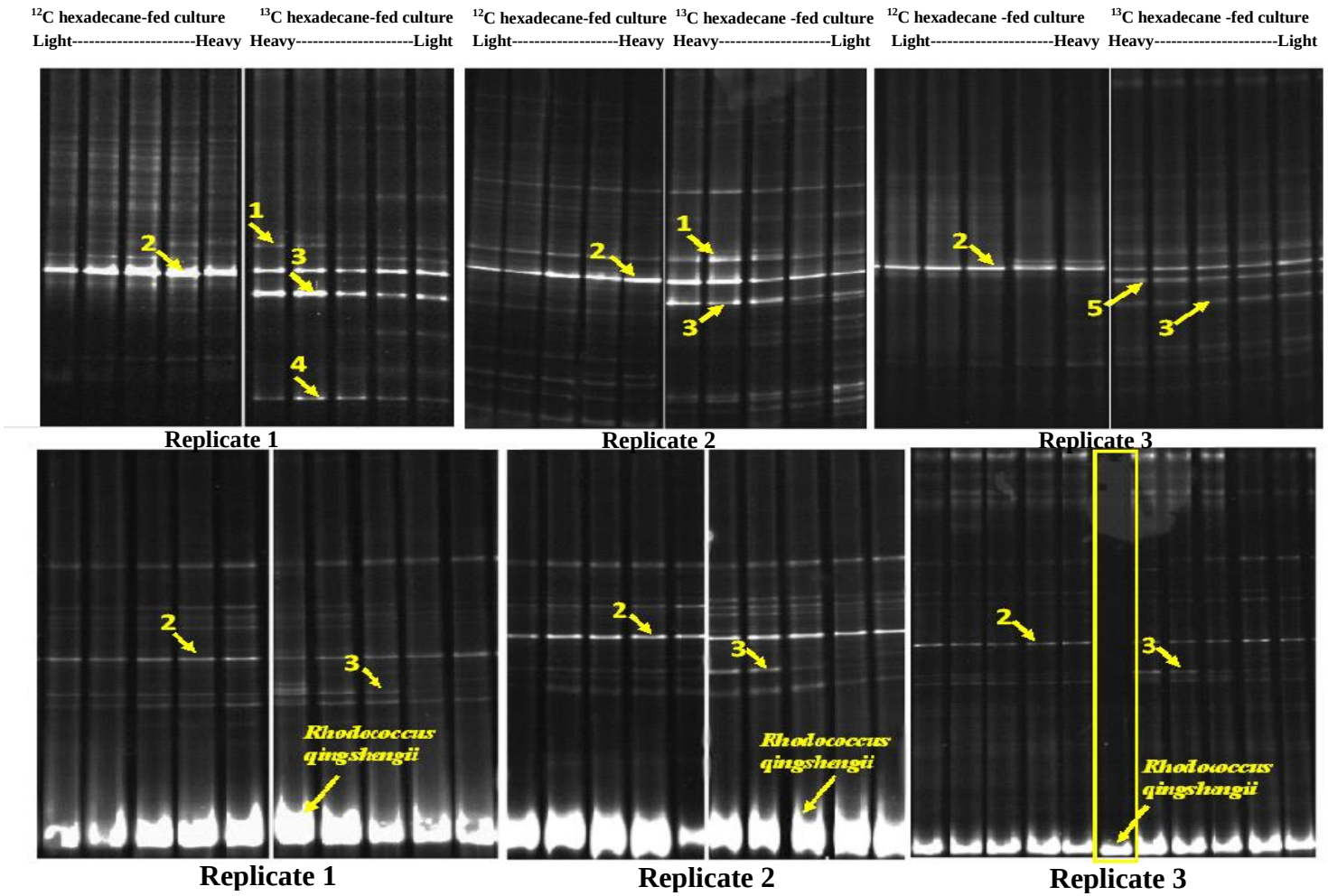


Figure 5.4. DGGE gels showing the community profiles in (top gels) the non-augmented control cultures (S) and (bottom gels) the nutrient unamended, *R. qingshengii*-augmented cultures (S+Rhod) in triplicates. Five heavy fractions (fractions 2-6) of average buoyant densities ranging from 1.832 to 1.789 g/mL from the ^{13}C hexadecane-pulsed cultures and five parallel fractions of the ^{12}C hexadecane-pulsed control cultures were selected for DGGE analysis. Yellow numbered arrows refer to sequenced bands. Numbers define species and similar numbers on replicate gels denote same species. Colony PCR products from a pure *R. qingshengii* culture are shown in one replicate gel (replicate 3, bottom gel) denoted with a yellow frame. All gel images were aligned with an internal marker run in parallel to samples



1: Alcanivorax hondengensis

2: Alcanivorax jadensis

3: Saccharophagus degradans

4: Thalassospira tepidiphila

5: Teredinibacter turnerae

Figure 5.5. DGGE gels showing the community profiles in the nutrient amended, non-augmented control (top gels) cultures (S+N) and the nutrient amended, *R. qingshengii*-augmented (S+Rhod+N) (bottom gels) in triplicates. Five heavy fractions of average buoyant densities ranging from 1.826 to 1.795 g/mL from the ^{13}C hexadecane-pulsed cultures and five parallel fractions of the ^{12}C hexadecane-pulsed control cultures were selected for DGGE analysis. Yellow numbered arrows refer to sequenced bands. Numbers define species and similar numbers on replicate gels denote the same species. Colony PCR products from a pure *R. qingshengii* culture were shown on one gel replicate (replicate 3, bottom gel) denoted with a yellow frame. All gel images were aligned with an internal marker run in parallel to samples.

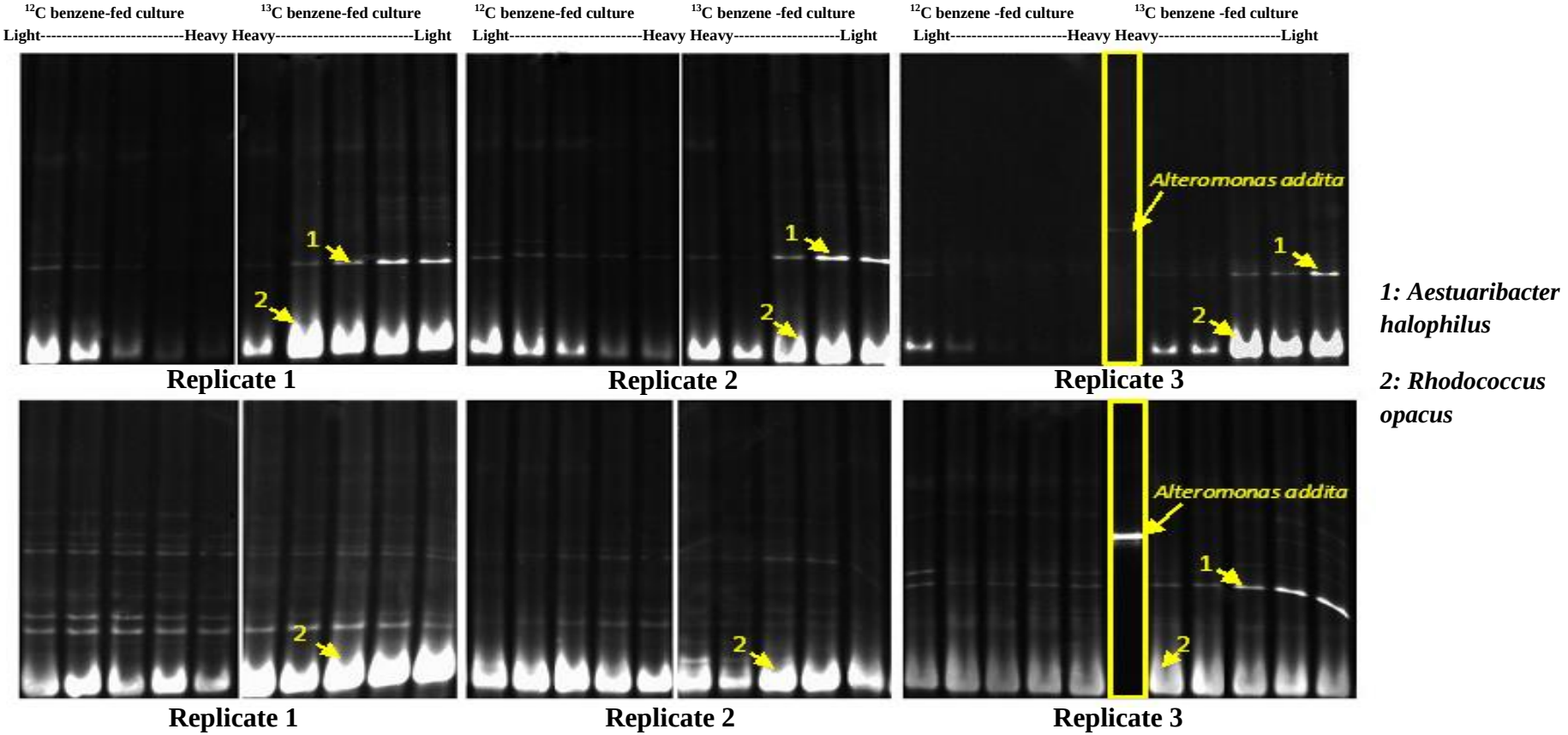
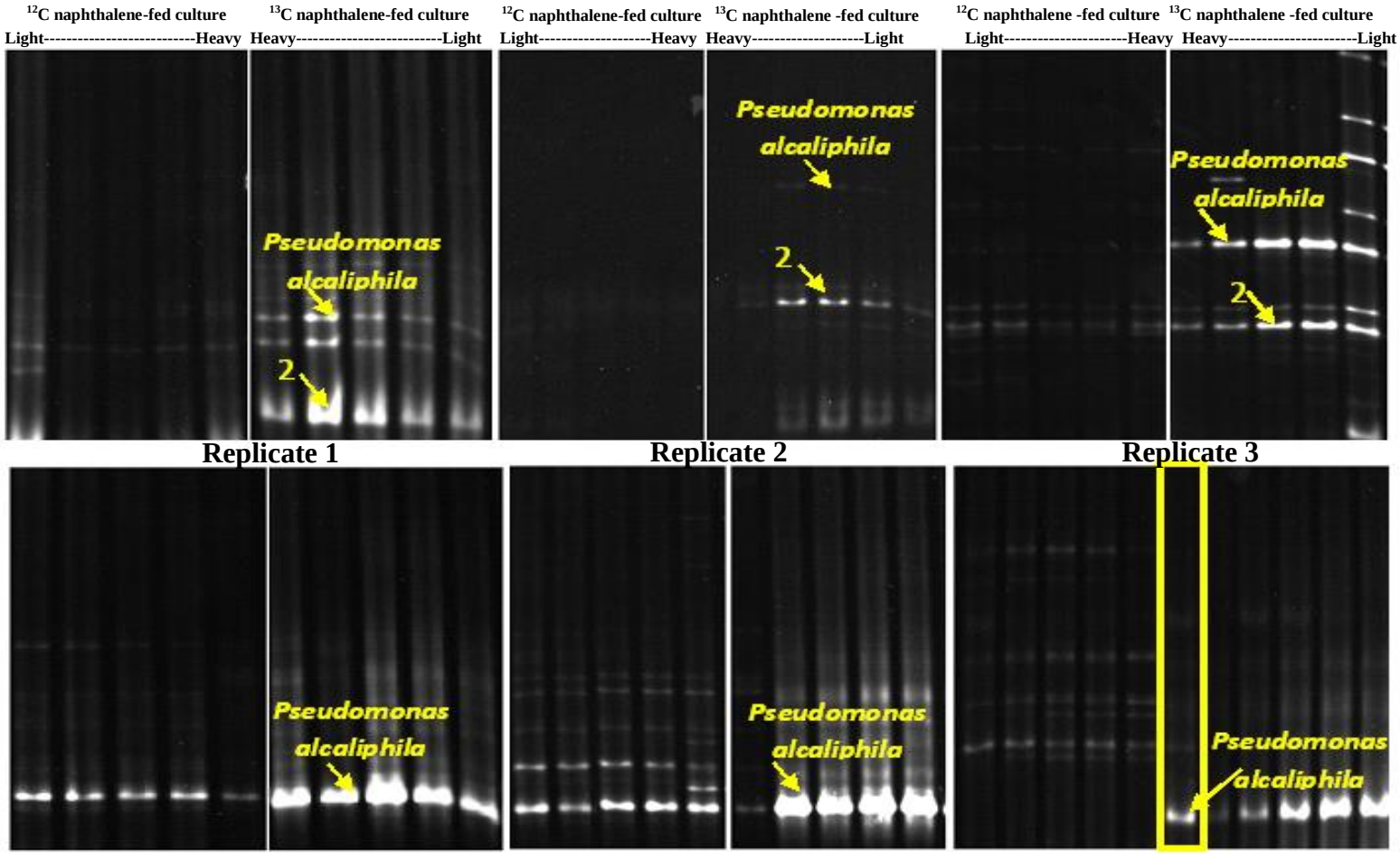
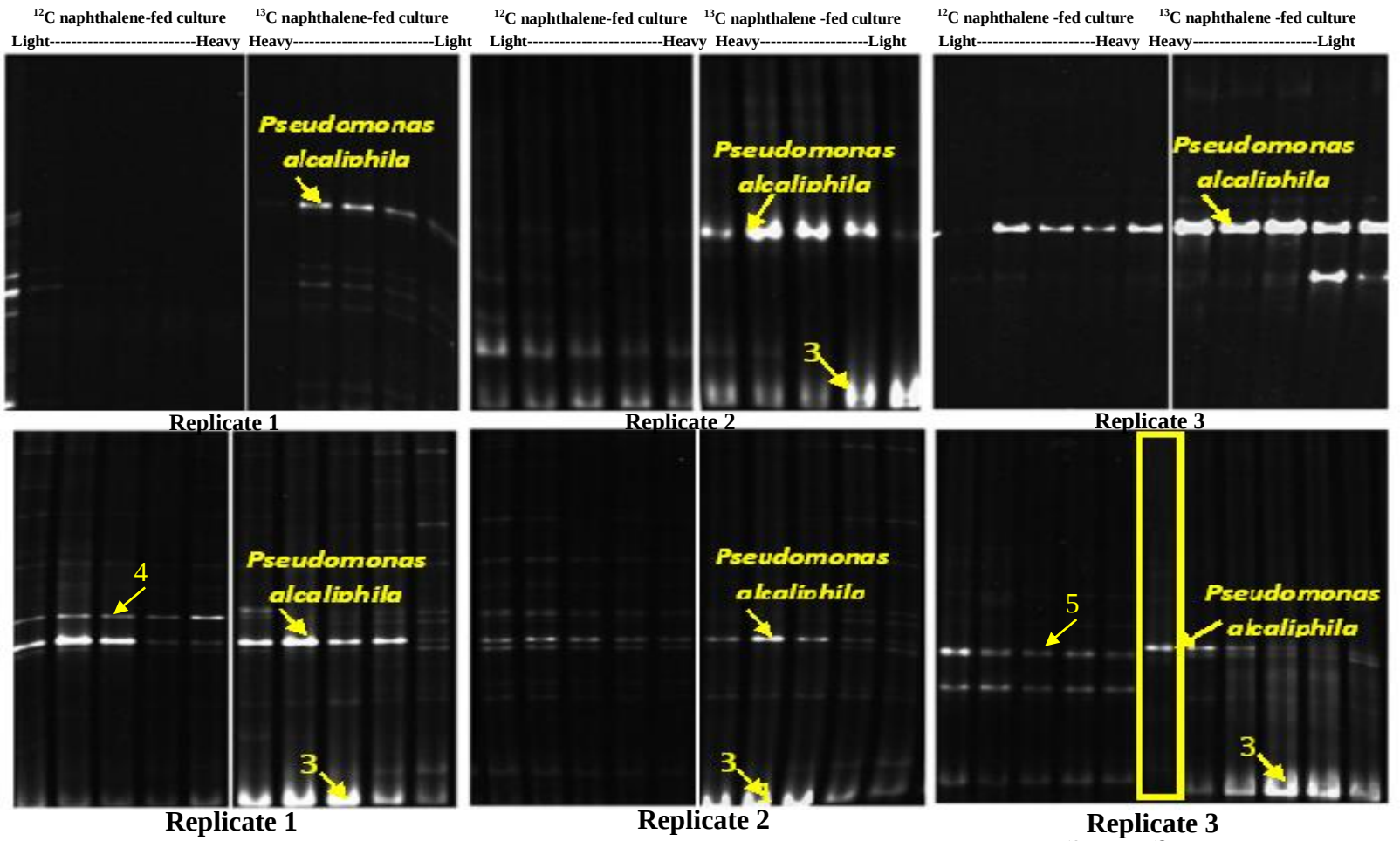


Figure 5.6. DGGE of RT-PCR-amplified 16S rRNA gene fragments from RNA fractions retrieved from CsTFA density gradients from the ^{12}C and ^{13}C-benzene-fed, *A. addita*–bioaugmented cultures S+Altero (top gels) and S+Altero+N (bottom gels) in triplicates. For each sample, 5 heavy fractions of average buoyant densities ranging from 1.840 to 1.793 g/mL from the ^{13}C benzene-pulsed cultures and five parallel fractions of the ^{12}C benzene-pulsed control cultures were selected for DGGE analysis. Yellow numbered arrows refer to sequenced bands. Numbers define species and similar numbers on replicate gels denote the same species. Colony PCR products from a pure *A. addita* culture were used as a control and are shown on one gel replicate per culture (replicate 3, top and bottom gels) and are denoted with a yellow frame. All gel images were aligned with an internal marker run in parallel to samples.



***2: Cycloclasticus* sp.**

Figure 5.7. DGGE gels showing the amplified 16S rRNA gene fragments from RNA fractions of the ^{12}C and ^{13}C-naphthalene-fed, non-bioaugmented control culture (S) (top gels) and the *P. alcaliphila*-bioaugmented cultures (S+Pseudo) (bottom gels) in triplicates. Five heavy fractions with average buoyant densities ranging from 1.830 to 1.785 g/mL from the ^{13}C naphthalene-pulsed cultures and five parallel fractions of the ^{12}C naphthalene -pulsed control cultures were selected for DGGE analysis. Yellow numbered arrows refer to sequenced bands. Numbers define species and similar numbers on replicate gels denote same species. Colony PCR products from a pure *P. alcaliphila* culture were used as a control and are shown on one gel replicate (replicate 3, bottom gel) and denoted with a yellow frame. All gel images were aligned with an internal marker run in parallel to samples.



3: Ketogulonigenium vulgare

4:Marinomonas potica

5:Hyphomonas oceanitis

Figure 5.8. DGGE gels showing the amplified 16S rRNA gene fragments from RNA fractions of the ^{12}C and ^{13}C-naphthalene-fed, non-bioaugmented nutrient-amended control culture (S+N) (top gels) and the *P. alcaliphila*–bioaugmented nutrient-amended cultures (S+Pseudo+N) (bottom gels) in triplicates. Five heavy fractions with average buoyant densities ranging from 1.835 to 1.790 g/mL from the ^{13}C naphthalene-pulsed cultures and five parallel fractions of the ^{12}C naphthalene -pulsed control cultures were selected for DGGE analysis. Yellow numbered arrows refer to sequenced bands. Numbers define species and similar numbers on replicate gels denote the same species. Colony PCR products from a pure *P. alcaliphila* culture were used as a control and are shown on one gel replicate (replicate 3, bottom gel). All gel images were aligned with an internal marker run in parallel to samples.

5.3. Discussion

The effect of bioaugmentation with *R. qingshengii, A. addita* and *P alcaliphila* on the biodegradation of hexadecane, benzene and naphthalene respectively in the presence and/or absence of nutrients was investigated. Stable isotope probing was also applied in order to monitor changes in community composition and potential interactions resulting from the addition of competent hexadecane, benzene or naphthalene degraders and to identify the bacterial species (indigenous or added) that have incorporated the heavy isotopes from these compounds into their RNA.

Results varied widely depending on the different treatments, on the added bacteria and on the hydrocarbon compounds themselves, indicating that the study was well justified. Bioaugmentation of the hexadecane or naphthalene spiked cultures with *R. qingshengii* or *P. alcaliphila* respectively dramatically increased the rate of degradation of these compounds, while addition of *A. addita* rather triggered the degradation of benzene in the benzene-spiked cultures. Autochthonous bioaugmentation is thought to overcome the limitations imposed by the environmental conditions on allochthonous bacteria. *R. qingshengii, A. addita* and *P. alcaliphila* used in this study were indigenous marine bacteria, possibly explaining their ability to increase the degradation rates of the studied hydrocarbons.

The joint application of biostimulation and bioaugmentation had little or no effect on the degradation of hexadecane or naphthalene but promoted the degradation of benzene compared to bioaugmentation alone. Nikolopoulou *et al*. (2013) observed an increase in the biodegradation rate of a range of oil hydrocarbons by the combined application of autochthonous bioaugmentation and biostimulation compared to bioaugmentation alone. In fact, Nikolopoulou *et al*. (2013) used a pre-adapted consortium rather than a single species and oil as a carbon source rather than single hydrocarbons compounds as in this study. The addition of a consortium and of a complex hydrocarbon source like oil causes much more potential interactions making comparisons between these studies difficult. In addition, biostimulation exhibited a positive effect on the degradation of hexadecane but had little effect on the degradation of naphthalene and failed to stimulate the degradation of benzene in the present study. The differences observed in the biodegradation rates of the different compounds used according to the different

bioremediation strategies applied confirm that the success of any bioremediation strategy depends on the hydrocarbon compound itself.

The application of stable isotope probing enabled identification of bacteria consuming carbon from the model substrates and characterised the nature of some of the bacterial interactions with bioaugmented strains that took place during biodegradation. Studies that focus only on the outcome of bioaugmentation linked the acceleration in biodegradation rate of hydrocarbons to the added bacteria or bacterial consortia without revealing the identity of bacteria that are deriving carbon from those hydrocarbons. The present study followed the flow of heavy carbon isotopes from hexadecane, benzene and naphthalene and demonstrated that degradation is not always achieved by the added bacteria.

For hexadecane, the acceleration in biodegradation rate during bioaugmentation with *R. qingshangii* was achieved by the added bacterium. DGGE analysis showed that *R. qingshangii* was the main consumer of the heavy isotopes from hexadecane regardless of the nutritional regime. However, the activity of *S. degradans*, a native of the bacterial marine community that was also deriving carbon from hexadecane in the control non-bioaugmented culture, was remarkably increased in the bioaugmented culture in the absence of nutrients, and decreased in the nutrient-amended bioaugmented culture when compared to the corresponding controls.

R. qingshengii is a species that was isolated from a carbendazim (broad-spectrum benzimidazole fungicide)-contaminated soil in China (Xu *et al.* 2007). It has not previously been demonstrated to use hexadecane as a sole carbon and energy source. The indigenous bacterium *S. degradans* that also acquired carbon from hexadecane is a versatile marine bacterium belonging to the *Saccharophagus* genus, identified by Ekborg *et al.* (2005). It has the ability to utilise a range of complex polysaccharides. Its ability to degrade hydrocarbons and more specifically hexadecane has not previously been reported. It is also possible that *R. qingshengii* is producing a polysaccharide that is being consumed by *S. degradans*. No studies have yet demonstrated its ability of *R. qingshangii* to produce exopolysaccharides, but another *Rhodococcus* species *(R. rhodochrus* S-2*)* was reported to produce an exopolysaccharide that promoted the degradation of aromatic hydrocarbons of crude oil by indigenous marine bacteria (Iwabuchi *et al.* 2002). This suggests that the increase in activity of *S. degradans* can be

partially due to consumption of potentially released exopolysaccharides but does not rule out its hexadecane degrading potential because it also consumed the heavy carbon isotopes from hexadecane in the non-bioaugmented controls where the indigenous *R. qingshagii* did not show activity. The decrease in the activity of *S. degradans* due to the simultaneous addition of nutrients and *R. qingshangii* suggests either an inhibitory effect of nutrients on *S. degradans* or an enhancement in the activity of *R. qingshangii* that dominated the community in the nutrient-amended culture, or a combination of both.

The *A. addita* used for bioaugmentation of the benzene-spiked microcosms triggered the degradation of benzene, which did not happen in the non-bioaugmented controls. Interestingly, after pulsing with labelled benzene, it was not enriched in heavy density gradients fractions suggesting it did not utilise benzene as a carbon source. The physiology of these bacteria helps understand the nature of the observed result. *A. addita* is a recently characterised marine bacterium that releases *O*-specific polysaccharides (Ivanova *et al.* 2005). It is possible that *R. opacus* and *A. halophilus*, which did take up label from the benzene pulse, benefitted from the *O*-specific exopolysaccharide released by *A. addita.*

R. opacus produces substantial amounts of triacylglycerol from different carbon sources including hydrocarbons. This is then stored in intracytoplasmic lipid inclusions (especially when a nitrogen source is abundant) and uses them as a carbon source when carbon and nitrogen are limiting (Alvarez *et al*. 1996 and 2000). It is possible that this behavior had an effect directly or indirectly on *A. addita,* but certainly not through consumption of the triacylglycerol, otherwise A. Addita would have been seen in the heavy fractions of the 13C benzene pulsed culture.

Moreover, there is a possibility that *A. Addita* had consumed benzene rather very quickly and lysed, which means that R.opacus and *A. halophilus* may have been cross-feeding on labelled *A. Addita* cell components rather than deriving carbon directly from benzene. This would be hard to determine as the RNA was extracted at the later stages of benzene degradation. In this context, it is important to mention that *A. Addita* required five days to completely consume the same amount of benzene (30mg/l) in preliminary experiments (data not shown). However, RNA extraction at the early stages of benzene degradation would have been very important in order to rule out other

possible unknown interactions that would have led to a quicker degradation ability of *A. Addita* followed by lysis and then cross feeding by *R. opacus* and *A. halophilus.*

As for naphthalene, the bioaugmented strain *P. alcaliphila* shared the carbon source with an indigenous *P. alcaliphila. P. alcaliphila* was labelled in all the microcosms including the non-bioaugmented controls. DGGE profiles showed intense bands belonging to *P. alcaliphila* in the heavy fractions of all the ^{13}C naphthalene-spiked cultures. Despite the competition, the bioaugmentation strain *P. alcaliphila* still acquired carbon from naphthalene regardless of the nutritional conditions.

Naphthalene degradation in the non-bioaugmented nutrient-unamended control was a shared metabolic activity between *P. alcaliphila* and *Cycloclasticus* sp. that was outcompeted when bioaugmented with *P. alcaliphila*. Members of the genus *cycloclasticus* are amongst the main marine aromatic hydrocarbon degrading bacteria that used PAHs as carbon and energy sources almost exclusively (Head *et al.* 2006). They have been isolated from different marine ecosystems including the Gulf of Mexico (Geiselbrech *et al.* 1998) and Puget Sound (Washington) (Dyksterhouse *et al.* 1995) and were demonstrated to use a variety of PAHs including naphthalene, biphenyl, phenanthrene, toluene and xylenes (Kasai *et al.* 2002). *Cycloclasticus* sp. was also previously demonstrated to derive carbon from naphthalene in the present study (chapter 4, Fig.4.10). The rapid biodegradation rate observed was translated by DGGE into a *P. alcaliphila* band that dominated the profile. It was concluded that it was degrading naphthalene almost exclusively. However, its activity was slowed down by the addition of nutrients. This drop in the degradation rate could possibly be correlated with the emergence of *K. vulgare* that exhibited a high naphthalene degrading activity. This nutrient dependent outcome could be simply due to competition for the carbon source or to other possible unfavourable interactions that are not revealed in this study. *K. vulgare* is an Alphaproteobacterium used industrially to produce 2-keto-l-gulonic acid (2-KLG), a key intermediate in the chemical synthesis of vitamin C (Urbance *et al.* 2001). Further research is recommended to determine the potential effect of this molecule on *P. alcaliphila* activity and replication during naphthalene biodegradation. The naphthalene degradation capability of this bacterium has not yet been reported, nor has it been identified as native to the marine environment. It is worth noting that *K. vulgare* belongs to the Rhodobacteraceae family, within which several genera consume

a wide spectrum of hydrocarbons and are commonly isolated from different environments (Sorkhoh *et al.* 1990, Di Gennaro *et al.* 2001, Xu *et al.* 2007). Finally, some bacterial species that showed activity in the ^{12}C naphthalene-fed cultures but not in the ^{13}C naphthalene-fed ones (*Marinomonas potica* and *Hyphomonas oceanitis)*, suggest that these bacteria rather derived carbon from other available carbon sources. It is also possible that these bacteria derived carbon from naphthalene, however, their absence in the ^{13}C naphthalene pulsed cultures could be due to sampling heterogeneity as the ^{13}C naphthalene-pulsed cultures and their control ^{12}C fed-replicates were independent.

In conclusion, this chapter demonstrated acceleration in the biodegradation rate of hexadecane, benzene and naphthalene in seawater microcosms bioaugmented with autochthonous strains, irrespective of the nutritional conditions. Through replicated application of RNA SIP, it was revealed that two of the bioaugmented strains dominated carbon acquisition from their respective target pollutants (hexadecane and naphthalene), whilst the third stimulated biodegradation without acquiring carbon from the pollutant (benzene). Indigenous flora, some of which have not previously been affiliated with biodegradation of the model pollutants used, were also identified. This study achieved the goal of revealing the actual biodegrading bacteria of the model hydrocarbons during bioaugmentation and highlighted potential interactions that led to observed result. The overall consistency observed between replicates lends credibility to the results and highlights the suitability of the approach to address the stated aims.

Chapter 6

General discussion

The marine environment constitutes a massive reservoir of biological diversity including different trophic levels of biota interacting to achieve vital global environmental functions. Disturbance of these interactions may negatively affect the biological diversity in the ocean and ultimately perturb the total environment. The unavoidable human need for petroleum products and the hard-to-control oil spills have created a need for the development of immediate as well as long-term clean-up strategies following oil spill. Bioremediation strategies have been applied worldwide and stories of success and failure abound for each strategy (Tyagi *et al.* 2011). However, complete recovery of the altered ecosystem is difficult to achieve promptly due to the complex biological and abiotic interactions that happen following oil spills and that are still poorly investigated. Understanding the mechanisms behind those interactions is key in reaching successful bioremediation outcomes.

Laboratory studies have helped dissecting some of these interactions and have revealed identities of microbes playing a key role in hydrocarbon degradation in the marine environment (Head *et al.* 2006). Field-based studies remain more realistic as they take into consideration the ecological biotic and abiotic interactions between the microbial communities in their actual context (Cravo-Laureau and Duran 2014). The use of experimental systems that mimic the environmental conditions offers the possibility of replication which allows robust statistical analysis as well as the advantage of extrapolation to the natural environment (Prosser 2010).

Hydrocarbonoclastic bacteria occur in low abundance in pristine marine ecosystems. They have developed the metabolic capacity to utilise oil hydrocarbons as carbon and

energy sources due to the natural occurrence of these molecules in the ocean (Mcgenity 2014). Several lineages have been identified as key degraders of *n*-alkanes (*Oceanobacter* genus), branched alkanes (*Alcanivorax* genus), and aromatic hydrocarbons (*Thalassospira*, *Cycloclasticus* and *Alteromonas* genera) (Head *et al*. 2006, Teramoto *et al.* 2009, Jin *et al.* 2012, McGenity *et al.* 2012). Hydrocarbon biodegradation is garnering more and more attention nowadays owing to negligent release of petroleum hydrocarbons worldwide, stemming from growing demand and increased transport of this resource over the oceans.

In this study, an oil spill in seawater was simulated in laboratory microcosms under biostimulation and natural attenuation regimes with the aims of studying the effect of pre-adaptation on the degradation rate of the model hydrocarbons hexadecane, benzene and naphthalene, and to identify members of the hydrocarbonoclastic bacterial community deriving carbon from these compounds. Additionally, the effect of autochthonous bioaugmentation on the degradation rate of these model hydrocarbons was investigated to identify bacterial lineages playing a role in their degradation and to examine possible interactions that resulted in the acceleration of the biodegradation rates.

6.1 Selection *vs* functional redundancy during hydrocarbon degradation

Pyrosequencing analysis of the initial seawater communities exposed to crude oil showed variation in the taxonomic composition of native communities. Although Proteobacteria were always the dominant phylum, Bacteroidetes for example were more varied across different sampling occasions. Seawater communities are very dynamic responding to environmental perturbation (discussed in chapter 3); hence it is difficult to determine the reason behind the change in community composition of the different seawater samples used. Despite the change in community composition, the mineralisation of oil followed a similar pattern in all the oil-spiked enrichments. There are two possible reasons for this continuity in function despite compositional change in the community. Firstly, the phenomenon of functional redundancy (Bowen *et al.* 2011), whereby the community as a whole maintained a specific common metabolic function (i.e. capacity for hydrocarbon breakdown) despite the community structure perturbation exerted by the oil hydrocarbons. Secondly, a strong selection towards specific hydrocarbonoclastic bacteria that are more efficient in utilising hydrocarbons as growth

substrate allowing them to flourish following the addition of oil. It is also possible that a combination of both phenomena occurred.

The DGGE and pyrosequencing analyses demonstrated dominance of the genus *Alteromonas* in all the oil-spiked enrichments, which supports the selection hypothesis. However, the presence of other active genera in the oil-spiked cultures of some enrichments but not all (e.g. *Aestuariibacter halophilus* or *Shewanella irciniae*), support the hypothesis of functional redundancy. At a higher taxonomic level, however, marine hydrocarbon biodegradation seems to be conserved in one phylum, the Proteobacteria, as the vast majority of the marine hydrocarbonoclastic bacteria identified to date (including those identified in the present study), belong to this phylum (Head *et al.* 2006, McGenity *et al.* 2012). This may also explains the reason why none of the lineages identified by SIP belonged to bacteroidetes despite their higher abundance in the seawater used for aromatic pulsing. Understanding the role of functional redundancy in oil degradation and the mechanisms that maintain the metabolic activity of microbes despite potential perturbations in the community composition may also help plan bioremediation strategies (Cravo-Laureau and Duran, 2014). More detailed functional redundancy analysis can be carried out in future experiments using a metagenomic approach in combination with bioremediation strategies.

The generation of enrichment cultures under optimised conditions (chapter 4) aimed at stimulating the growth of potential hydrocarbonoclastic bacteria in order to eliminate the lag phase that normally precedes the degradation of the majority of hydrocarbon compounds. Pre-exposure to oil, however, gave mixed results. It delayed the biodegradation of hexadecane and degradation of naphthalene, but triggered the degradation of benzene, which could not be degraded by the pristine seawater communities regardless of the nutritional conditions. The time needed for hexadecane biodegradation in biostimulated cultures in chapter 4 was approximately double the time needed for its degradation in the biostimulated cultures in chapter 5. Two possible experimental reasons may explain this difference. Firstly, the incubation conditions differed where the bacterial community of the former culture was pre-exposed to nutrients for 2 weeks before hexadecane addition, while for the latter; hexadecane was simultaneously added with mineral nutrients. Preadaptation to nutrients may have selected for bacterial lineages that thrived in nutrient-rich conditions and outcompeted

those that preferred low-nutrient environments. Secondly, the seawater used for both experiments was sampled at two different occasions, which again suggests a change in the community composition depending on environmental factors or seasonal variations. This difference was also reflected in the community composition where the hexadecane degrading community in the nutrient pre-adapted culture was less diverse (almost only *Oceanobacter kriegii*). Results obtained from this chapter may to some extent explain some of the interactions that take place in the marine environment following an oil spill.

To understand the community dynamics behind these results RNA stable isotope probing was used to identify the key hydrocarbon degrading community members deriving carbon from these model compounds. Community analysis showed that some bacteria degraded hexadecane solely after nutrient-preadaption (*Oceanobacter kriegii*), while others derived carbon from hexadecane only after pre-exposure to oil (*Terasakiella pusilla*). In addition, other hydrocarbonoclastic bacteria exhibited a capacity to acquire carbon specifically from benzene (*Marinobacter litoralis*) and/or naphthalene (*Thalassospira tepidiphila / Marinobacter aquaeolei* respectively), which was observed in this study for the first time. Some bacteria did not derive carbon from the amended labelled substrates despite showing high activity in the nutrient and oil amended enrichment cultures at the end of incubation (*Alteromonas* members). This suggests first, limited metabolic capacity of these bacteria to utilise specific hydrocarbons other than the ones provided here, and second they were outcompeted by the ones that dominated the acquisition of the labelled isotopes from the tested hydrocarbons (e.g. *Marinobacter litoralis* in the case of benzene).

The sequential utilisation of hydrocarbons correlated with sequential growth of specific marine taxa (Head *et al.* 2006) suggests an effect of the duration of pre-exposure to oil. The oil-adapted cultures generated here were incubated for 2 weeks. Perhaps only a limited number of bacteria that degraded the oil hydrocarbons available during this period of time were identified by SIP. These same bacteria may have also inhibited the growth of others degrading other hydrocarbons. This suggests that if seawater enrichments were incubated with oil for longer, application of SIP may have revealed totally different community profiles. Incubation of larger volumes of seawater with oil with application of SIP on cultures subsampled at different times throughout the incubation may be of paramount importance. It may identify key marine

microorganisms responsible for the degradation of specific, perhaps more toxic hydrocarbons and the time of their appearance during an oil spill. It can also highlight potential microbial interactions that potentially help in designing more effective bioremediation techniques.

6.2. On the potential success of bioaugmentation using SIP

Biostimulation of the native hydrocarbonoclastic community has shown many successful applications (Röling *et al*. 2002, Nikolopoulou and Kalogerakis 2008). However, when time is of the essence, waiting for the native hydrocarbonoclastic bacteria to establish themselves and synthesise the necessary enzymes is not ideal. Firstly, because the adaptation time needed may be quite long and secondly, because techniques that can accelerate this process are available.

Bioaugmentation studies focused on beached oil as this oil becomes trapped into the sediment and persists for a long time. However, oil spills primarily impact marine water, and degradation of the oil at sea is preferable to degradation once it has reached the shore, becomes unsightly and affects coastal life. It is hence a prevention vs a cure scenario. Simons *et al.* (2013) used carriers for delivery of bacterial inocula into oil-impacted seawater. Those carriers additionally sorb oil increasing the contact between oil and hydrocarbonoclastic bacteria and have recently shown great promise in treating oil spills at sea. In addition, and the spilled oil affects the biological life in the sea with potential transfer to humans via food webs. Furthermore, bioaugmentation is generally acknowledged to accelerate the degradation of hydrocarbons if the conditions of its success were carefully considered. Therefore bioaugmentation was applied to seawater in order to test the isolated microorganisms for potential acceleration of the degradation of selected hydrocarbons in order to optimise the removal of the light hydrocarbon fractions that dissolve rapidly into the marine water (due to the action of waves) which poses a risk on zooplankton and phytoplankton due to the toxicity of those compounds. The application of bioaugmentation to seawater has been growing recently (Nikolopoulou *et al.* (2013a), Nikolopoulou *et al*. (2013b), Hassanshahian *et al.* (2013)).

No successful applications of bioaugmentation in the marine environment have been reported. It has been disregarded due to poor performance related to disappearance of the added bacterium in the early stages of spill remediation (Tagger *et al.* 1983).

However, autochthonous bioaugmentation has recently received renewed interest (Hassanshahian *et al.* 2013, Nikolopoulou *et al*. 2013a, Nikolopoulou *et al.* 2013b) as a promising technology that can accelerate the biodegradation of oil hydrocarbons and potentially reduce the amounts of oil that reach the shore and contaminate coasts. Several questions are yet to be answered in order to better plan a suitable and promising bioremediation strategy (Cravo-Laureau and Duran 2014). One of these questions is whether, during bioaugmentation, the added microbes consume the substrate or instead encourage potential native hydrocarbonoclastic bacteria to do so through complex stimulatory interactions. Uhlik *et al.* (2012) highlighted the importance of the application of stable isotope probing in linking the degradation to the actual performers during bioaugmentation. The present study successfully addressed this issue (chapter 5).

Autochthonous bioaugmentation showed acceleration in the biodegradation rate of all the three studied hydrocarbons. Profiling of the community showed that the labelled isotopes were either consumed solely by the added bacterium (hexadecane), or shared between the added and native bacteria (naphthalene), or, interestingly, consumed by native bacteria only in the presence of the added strain (benzene). This result emphasises the need to further investigate the nature of these interactions to better understand this process and be able to predict outcomes of this technique *in situ.* Furthermore, the importance of other biological interactions between photosynthetic autotrophs and hydrocarbonoclastic bacteria has been recently highlighted for hydrocarbon biodegradation. The study of Chronopoulou *et al.* (2013) demonstrated that cyanobacteria and diatoms bloomed in a tidal mesocosm consisting of intact sediment cores where an oil spill was simulated.

This study investigated the degradation of single hydrocarbon molecules, but for a complex mixture like oil comprising more than 10,000 hydrocarbons (McGenity 2014) alternative strategies have to be investigated. The existence of bacterial species that have the metabolic capacity to degrade more than one hydrocarbon compound makes tailoring a consortium containing a handful of these microbes for bioaugmentation a promising strategy for bioremediation. In this context, the use of a bacterial consortium rather than single bacterial species has shown various results. McGenity *et al.* (2012) emphasised the importance of using a microbial consortium with complementary catabolic capacities in order to degrade a complex mixture like petroleum oil rather than

one species with a limited metabolic capability. However, Hassanshahian *et al.* (2013) who bioaugmented seawater with a consortium composed of *Alcanivorax borkumensis* and *Thalassolituus oleivorans,* known to degrade aliphatic and aromatic hydrocarbons respectively, demonstrated a negative effect exerted by the addition of this consortium on oil degradation when compared to bioaugmentation with *Alcanivorax borkumensis* on its own. The authors suggested an unfavourable interaction between the two members of the consortium, but the explanation of these interactions was rather complicated. On the other hand, Abdel-Megeed *et al.* (2010) observed a higher hexadecane degradation potential in a microcosm bioaugmented with a consortium of three soil bacteria (*Pseudomonas putida, Rhodococcus erythroplolis* and *Bacillus thermoleovorans*) than in microcosms bioaugmented with the members of the consortium individually. These studies demonstrate that our understanding of relevant microbial interactions is still "*in its infancy*" (Head *et al.* 2006) and confirm the need for more intensive research to improve our knowledge in this context. The failed application of bioaugmentation is a consequence of our lack of knowledge; therefore, study of the potential interactions between members of the consortium and the native communities is crucial for the success of bioremediation.

Moreover, in addition to the bacteria-bacteria interaction suggested as possible reasons for the outcome of this study, interactions with other phylogenetic taxa such as protozoan predators that selectively graze and control the bacterial biomass may also affect indirectly the pollutant biodegradation (Head *et al.* 2006). Sherr and Sherr (2002) reported that bacterial biomass is highly controlled by protozoan grazers. In addition, Moreno *et al.* (2010) tracked the carbon flow into higher trophic levels in activated sludge using RNA-SIP and demonstrated the flow of carbon from bacteria consuming the provided labelled carbon source into protozoa. This suggests the importance of studying those interactions in future investigations and their effect on the active hydrocarbonoclastic bacteria which will help better explain the outcome of bioremediation strategies.

6.3. Significance of the findings in the light of SIP limitations

RNA-SIP was used to shed some light on the potential interactions of hydrocarbonoclastic bacteria in this study. However, it is important to mention that as a molecular method, RNA-SIP has got some limitations. Cross feeding is an issue that

needs to be addressed whenever SIP is the technique of choice. This problem can be avoided by sampling at different stages of the course of compound degradation to detect the bacterial species that are active at the early stages of the degradation process (Manefield *et al.* 2002). However, in the present study, though incubations were not protracted, RNA was extracted at the end of the incubation when most or all of the added compounds were consumed. Sampling at different time points of the incubation was not possible due to technical difficulties associated with the volatility of the studied compounds (especially benzene). Teflon-lined septa (commercially available only for the 160 mL serum bottles used throughout this study) to seal microcosm bottles were necessary for accurate analytical chemistry, but limited the removal of sufficient culture volume for RNA extraction.

6.4. Future perspectives

Future developments should consider the microbial interactions more deeply and put more efforts into optimising the biotic and abiotic factors that lead to favourable interactions in order to promote the hydrocarbon biodegradation. Application of real time PCR using strain specific primers would be important in determining the increases in the activity of specific bacteria during the degradation process. This would add more confirmation to the present SIP finding. Conducting metabolomic studies that enable chemical analysis of compounds released by the native members of the community and potentially by the added bacteria could be pivotal for the success of autochthonous bioaugmentation. It could help correlate changes in the bacterial community to changes in the concentration of specific compounds released. The hydrocarbons studies here were representative of classes of oil hydrocarbons. However, more research is needed on more compounds belonging to the same classes in order to demonstrate the potential of comparable outcomes. In addition, studying the expression of specific genes in correlation with increased hydrocarbon degradation rates could be very valuable in revealing regulation mechanisms of these genes and ultimately help promoting their expression during bioremediation. Finally, application of shotgun sequencing on fractions containing ^{13}C labelled RNA recovered from SIP fractions would also be beneficial especially during bioaugmentation.

6.5. Concluding remarks

The present study demonstrated that bioremediation strategies such as biostimulation can be compound specific. It was ineffective in accelerating naphthalene or benzene degradation but accelerated hexadecane degradation. Preadaptation to hydrocrabons and nutrients triggered benzene degradation but slowed down the degradation of naphthalene and hexadecane. Bioaugmentation with indigenous marine bacteria boosted the degradation rate of all the selected compounds irrespective of the nutritional regimes.

This study also defined new marine hydrocarbonoclastic taxa deriving carbon from specific hydrocarbon molecules, and linked the degradation of these hydrocarbons as a new function to putative marine hydrocarbon-degrading lineages. It also correlated changes in the hydrocarbon degradation rates to microbial community changes during bioaugmentation.

Findings of this study provide key knowledge to the growing and promising bioremediation strategy of autochthonous bioaugmentation. Stable isotope probing has recently been applied to isolate hydrocarbon degrading bacteria from different environments. The present study, however, pioneered the application of RNA-stable isotope probing during bioaugmentation. It showed that the performance of the added microorganisms varied depending on the organism and on the hydrocarbon compound providing valuable insights into developing new concepts and conduct new investigations towards a better and more successful bioremediation.

References

Aanderud, Z. T., & Lennon, J. T. (2011). Validation of heavy-water stable isotope probing for the characterization of rapidly responding soil bacteria. *Applied and Environmental Microbiology*, *77*(13), 4589–96.

Abbriano, R.M., Carranza, M.M., Hogle, S.L., Levin, R.A., Netburn, A.N., Seto, K.L., Snyder, S.M., Franks, P.J.S. (2011). Deepwater Horizon Oil Spill: A Review of the Planktonic Response. *Oceanography 24*(3),294-301.

Abdel-Megeed, A., Al-Harbi, N., & Al-Deyab, S. (2010). Hexadecane degradation by bacterial strains isolated from contaminated soils. *African Journal of Biotechnology*, *9*(44), 7487–7494.

Akbarzadeh, K., Hammami, A., Kharrat, A., Zhang, D., Allenson, S., Creek, J., Kabir, S., Jamaluddin, A., Marshall, A. G., Rodgers, R. P., Mullins, O. C., & Solbakken, T. (2007). Asphaltenes - Problematic but rich in potential. *Oilfield Review*, *19*(2), 22–43.

Altschul, S., Gish, W., Miler, W. & Lipman, D. (1990). Basic local alignment search tool. *Journal of Molecular Biology*, *215*, 403–410.

Alvarez, H. M., Mayer, F., Fabritius, D., & Steinbüchel, A. (1996). Formation of intracytoplasmic lipid inclusions by Rhodococcus opacus strain PD630. *Archives of microbiology*, *165*(6), 377–86.

Alvarez, H. M., Kalscheuer, R., & Steinbüchel, A. (2000). Accumulation and mobilization of storage lipids by *Rhodococcus opacus* PD630 and *Rhodococcus ruber* NCIMB 40126. *Applied Microbiology and Biotechnology*, *54*(2), 218–223.

Amann, R. I., Ludwig, W., Schleifer, K. H., Amann, R. I., & Ludwig, W. (1995). Phylogenetic identification and in situ detection of individual microbial cells without cultivation. *Microbiological Reviews*, *59*(1) 143-69.

Annweiler, E., Richnow, H. H., Antranikian, G., Hebenbrock, S., Garms, C., Franke, S.,Frank, W., & Michaelis, W. (2000). Naphthalene degradation and incorporation of naphthalene-derived carbon into biomass by the thermophile *Bacillus thermoleovorans*. *Applied and Environmental Microbiology*, *66*(2), 518–23.

Atlas, R.M. (1981). Microbial degradation of petroleum hydrocarbons: an environmental perspective. *Microbiological Reviews*, *45*(1), 180–209.

Atlas, R. M., & Bartha, R. (1972). Degradation and mineralization of petroleum in sea water: limitation by nitrogen and phosphorous. *Biotechnology and Bioengineering*, *14*(3), 309–18.

Atlas, R.M. (1985). Effects of hydrocarbons on micro-organisms and biodegradation in Arctic ecosystems. In F. R. Engelhardt (Ed.), *Petroleum Effects in the Arctic Environment* (pp. 63–99). London, UK: Elsivier.

Atlas, R.M. (1977). Stimulated petroleum biodegradation, *Critical Reviews in Microbiology*, *5*, 371–386.

Atlas, R.M., & Hazen, T. C. (2011). Oil biodegradation and bioremediation: a tale of the two worst spills in U.S. history. *Environmental Science & Technology*, *45*(16), 6709–15.

Atlas, R.M. (1995a). Petroleum biodegradation and oil spill bioremediation. *Marine Pollution Bulletin*, *31*(4-12), 178–182.

Atlas, R. M. (1995b). Bioremediation of Petroleum Pollutants. *International Biodeterioration and Biodegradation*, 311–321.

Atlas, R.M. & Bartha, R. (1972). Degradation and mineralization of petroleum in seawater: limitation by nitrogen and phosphorus. *Biotechnology and Bioengineering*, *14*, 309–318.

Atlas, R. & Bartha, R. (1973). Stimulated biodegradationof oil slicks using oleophilic fertilizers. *Environmental Science & Technology*, *7*(6), 538–541.

Atlas, R.M. & Bartha, R. (1998). *Microbial ecology; Fundamentals and applications* (4th Ed., pp. 570–573). California: Benjamin Cummings Science Publishing.

Balba, M. , Al-Awadhi, N., & Al-Daher, R. (1998). Bioremediation of oil-contaminated soil: microbiological methods for feasibility assessment and field evaluation. *Journal of Microbiological Methods*, *32*(2), 155–164.

Barron, M. G., Carls, M. G., Short, J. W., & Rice, S. D. (2003). Photoenhanced toxicity of aqueous phase and chemically dispersed weathered Alaska North Slope crude oil to Pacific herring eggs and larvae. *Environmental Toxicology and Chemistry / SETAC*, *22*(3), 650–60.

Bauer, J. E., & Capone, D. G. (1985). Degradation and mineralization of the polycyclic aromatic hydrocarbons anthracene and naphthalene in intertidal marine sediments. *Applied and Environmental Microbiology*, *50*(1), 81–90.

Bauer, J. E., & Capone, D. G. (1988). Effects of co-occurring aromatic hydrocarbons on degradation of individual polycyclic aromatic hydrocarbons in marine sediment slurries. *Applied and Environmental Microbiology*, *54*(7), 1649–55.

Baumann, L., Baumann, P., Mandel, M., & Allen, R. D. (1972). Taxonomy of aerobic marine eubacteria. *Journal of Bacteriology*, *110*(1), 402–29.

Beazley, M.J., Martinez, R.J., Rajan, S., Powell, J., Piceno, Y.M., Tom, L.M., Andersen, G.L., Hazen, T.C., Van Nostrand, J.D., Zhou, J. et al. (2012). Microbial Community Analysis of a Coastal Salt Marsh Affected by the Deepwater Horizon Oil Spill. *PLoS ONE 7*(7):e41305

Beilen, J. B., Wubbolts, M. G., & Witholt, B. (1994). Genetics of alkane oxidation by *Pseudomonas oleovorans*. *Biodegradation*, *5*(3-4), 161–174.

Bell, T. H., Yergeau, E., Martineau, C., Juck, D., Whyte, L. G., & Greer, C. W. (2011). Identification of nitrogen-incorporating bacteria in petroleum-contaminated arctic soils by using [^{15}N]DNA-based stable isotope probing and pyrosequencing. *Applied and Environmental Microbiology*, *77*(12), 4163–71.

Bogan, B. W., & Lamar, R. T. (1996). Polycyclic aromatic hydrocarbon-degrading capabilities of *Phanerochaete laevis* HHB-1625 and its extracellular ligninolytic enzymes. *Applied and Environmental Microbiology*, *62*(5), 1597–603.

Boschker, H. T.S., de Graaf, W., Köster, M., Meyer-Reil, L.-A., & Cappenberg, T. E. (2001). Bacterial populations and processes involved in acetate and propionate consumption in anoxic brackish sediment. *FEMS Microbiology Ecology*, *35*(1), 97–103.

Boschker, H. T. S., Nold, S. C., Wellsbury, P., Bos, D., de Graaf, W., Pel, R., Parkes, J. & Cappenberg, T. E. (1998). Direct linking of microbial populations to specific biogeochemical processes by ^{13}C-labelling of biomarkers. *Nature*, *392*(6678), 801–805.

Bossert, I., & Bartha, R. (1984). The treatment and disposal of petroleum wastes. In R. M. Atlas (Ed.), *Petroleum Microbiology* (pp. 553–578). New York, NY, USA.: Macmillan.

Boufadel, M. C., Reeser, P., Suidan, M. T., Wrenn, B. A., Cheng, J., Du, X., Tiehung, L., & Venosa, A. D. (1999). Optimal nitrate concentration for the biodegradation of n-heptadecane in a variably-saturated sand column. *Environmental Technology*, *20*(2), 191–199.

Bowen, J. L., Ward, B. B., Morrison, H. G., Hobbie, J. E., Valiela, I., Deegan, L. A., & Sogin, M. L. (2011). Microbial community composition in sediments resists perturbation by nutrient enrichment. *The ISME journal*, *5*(9), 1540–8.

Braddock, J. F., Ruth, M. L., Catterall, P. H., Walworth, J. L., & McCarthy, K. A. (1997). Enhancement and inhibition of microbial activity in hydrocarbon-contaminated arctic soils: implications for nutrient-amended bioremediation. *Environmental Science & Technology*, *31*(7), 2078–2084.

Bragg, J. R., Prince, R. C., Harner, E. J., & Atlas, R. M. (1994). Effectiveness of bioremediation for the Exxon Valdez oil spill. *Nature*, *368*(6470), 413–418.

Buckley, D. H., Huangyutitham, V., Hsu, S.-F., & Nelson, T. A. (2007a). Stable isotope probing with ^{15}N achieved by disentangling the effects of genome G+C

content and isotope enrichment on DNA density. *Applied and Environmental Microbiology*, *73*(10), 3189–95.

Buckley, D. H., Huangyutitham, V., Hsu, S.-F., & Nelson, T. A. (2007b). Stable isotope probing with $^{15}N_2$ reveals novel noncultivated diazotrophs in soil. *Applied and Environmental Microbiology*, *73*(10), 3196–204.

Bull, I. D., Parekh, N. R., Hall, G. H., Ineson, P., & Evershed, R. P. (2000). Detection and classification of atmospheric methane oxidizing bacteria in soil. *Nature*, *405*(6783), 175–8.

Campbell, B. J., Yu, L., Heidelberg, J. F., & Kirchman, D. L. (2011). Activity of abundant and rare bacteria in a coastal ocean. *Proceedings of the National Academy of Sciences of the United States of America*, *108*(31), 12776–81.

Caparello, D.M., & LaRock, P. A. (1975). A radioisotope assay for the quantification of hydrocarbon biodegradation potential in environmental samples. *Microbial Ecology*, *2*, 28–42.

Cappello, S., Denaro, R., Genovese, M., Giuliano, L., & Yakimov, M. M. (2007). Predominant growth of Alcanivorax during experiments on "oil spill bioremediation" in mesocosms. *Microbiological Research*, *162*(2), 185–90.

Carpenter, A., & Macgill, S. (2001). Charging for port reception facilities in North Sea ports: putting theory into practice. *Marine Pollution Bulletin*, *42*(4), 257–66.

Cartaxana, P., Caçador, I., Vale, C., Falcão, M., & Catarino, F. (1999). Seasonal variation of inorganic nitrogen and net mineralization in a salt marsh ecosystem. *Mangroves and Salt Marshes*, *3*(2), 127–134.

Cassidy, M. B., Lee, H., & Trevors, J. T. (1996). Environmental applications of immobilized microbial cells: A review. *Journal of Industrial Microbiology*, *16*(2), 79–101.

Cerniglia, C E, & Gibson, D. T. (1979). Oxidation of benzo[a]pyrene by the filamentous fungus Cunninghamella elegans. *The Journal of Biological Chemistry*, *254*(23), 12174–80.

Cerniglia, C. E. (1984). Microbial metabolism of polycyclic aromatic hydrocarbons. In R.M.Atlas (Ed.), *Petroleum Microbiology* (pp. 99–152). Macmillan, New York.

Cerniglia, C. E. (1992). Biodegradation of polycyclic aromatic hydrocarbons. *Biodegradation*, *3*(2-3), 351–368.

Chen, S.-H., & Aitken, M. D. (1999). Salicylate Stimulates the degradation of high-molecular weight polycyclic aromatic hydrocarbons by *Pseudomonas saccharophila* P15. *Environmental Science & Technology*, *33*(3), 435–439.

Chen, Y., & Murrell, J. C. (2010). When metagenomics meets stable-isotope probing : progress and perspectives. *Trends in Microbiology*, *18*(4), 157–163.

Chronopoulou, P.-M., Fahy, A., Coulon, F., Païssé, S., Goñi-Urriza, M., Peperzak, L., Acuna Alvarez, L., McKew, B.A., Lawson, T., Timmis, K.N., Duran, R., Underwood, G.J., & McGenity, T. J. (2013). Impact of a simulated oil spill on benthic phototrophs and nitrogen-fixing bacteria in mudflat mesocosms. *Environmental Microbiology*, *15*(1), 242–52.

Cochran, P. K., Kellogg, C. A., & Paul, J. H. (1998). Prophage induction of indigenous marine lysogenic bacteria by environmental pollutants. *Marine Ecology Progess Series*, *164*, 125–133.

Collier, D. N., Hager, P. W., & Phibbs, P. V. (1996). Catabolite repression control in the Pseudomonads. *Research in Microbiology*, *147*(6-7), 551–561.

Colwell, R. R., Mills, A. L., Walker, J. D., Garcia-Tello, P., & Campos-P., V. (1978). Microbial ecology studies of the Metula spill in the Straits of Magellan. *Journal of the Fisheries Research Board of Canada*, *35*(5), 573–580.

Connan, J. (1984). Biodegradation of crude oils in reservoirs. In D. H. Brooks, J., Welte (Ed.), *Advances in Petroleum Geochemistry I* (pp. 298–335). London, UK: Academic Press.

Coon, M. J. (2005). Omega oxygenases: nonheme-iron enzymes and P450 cytochromes. *Biochemical and Biophysical Research Communications*, *338*(1), 378–85.

Cooney, J. J. (1984). The fate of petroleum pollutants in fresh water ecosystems. In R.M. Atlas (Ed.), *Petroleum Microbiology* (pp. 399–434). New York, NY, USA.: Macmillan.

Cravo-Laureau, C., & Duran, R. (2014). Marine coastal sediments microbial hydrocarbon degradation processes: contribution of experimental ecology in the omics'era. *Frontiers in Microbiology*, *5*, 1–8.

Crossman, Z. M., Abraham, F., & Evershed, R. P. (2004). Stable isotope pulse-chasing and compound specific stable carbon isotope analysis of phospholipid fatty acids to assess methane oxidizing bacterial populations in landfill cover soils. *Environmental Science & Technology*, *38*(5), 1359–1367.

Das, N., & Chandran, P. (2011). Microbial degradation of petroleum hydrocarbon contaminants: an overview. *Biotechnology Research International*, *2011*, 941810.

Delille, D. (1993). Seasonal changes in the abundance and composition of marine heterotrophic bacterial communities in an Antarctic coastal area. *Polar Biology*, *13*(7).

Delille, D., Pelletier, E., Rodriguez-Blanco, A., & Ghiglione, J.-F. (2009). Effects of nutrient and temperature on degradation of petroleum hydrocarbons in sub-Antarctic coastal seawater. *Polar Biology*, *32*(10), 1521–1528.

Di Gennaro, P., Rescalli, E., Galli, E., Sello, G., & Bestetti, G. (2001). Characterization of *Rhodococcus opacus* R7, a strain able to degrade naphthalene and o-xylene isolated from a polycyclic aromatic hydrocarbon-contaminated soil. *Research in Microbiology*, *152*(7), 641–51.

Dortch, Q., Clayton, J. R., Thoresen, S. S., & Ahmed, S. I. (1984). Species differences in accumulation of nitrogen pools in phytoplankton. *Marine Biology*, *81*(3), 237–250.

Doughty, D. M., Sayavedra-Soto, L. A., Arp, D. J., & Bottomley, P. J. (2006). Product repression of alkane monooxygenase expression in *Pseudomonas butanovora*. *Journal of Bacteriology*, *188*(7), 2586–92.

Dowd, S. E., Callaway, T. R., Wolcott, R. D., Sun, Y., McKeehan, T., Hagevoort, R. G., & Edrington, T. S. (2008). Evaluation of the bacterial diversity in the feces of cattle using 16S rDNA bacterial tag-encoded FLX amplicon pyrosequencing (bTEFAP). *BMC Microbiology*, *8*(1), 125.

Dumont, M. G., & Murrell, J. C. (2005). Stable isotope probing - linking microbial identity to function. *Nature Reviews. Microbiology*, *3*(6), 499–504.

Dumont, M. G., Pommerenke, B., Casper, P., & Conrad, R. (2011). DNA-, rRNA- and mRNA-based stable isotope probing of aerobic methanotrophs in lake sediment. *Environmental Microbiology*, *13*(5), 1153–67.

Dutta, T. K., & Harayama, S. (2000). Fate of crude oil by the combination of photooxidation and biodegradation. *Environmental Science & Technology*, *34*(8), 1500–1505.

Dyksterhouse, S. E., Gray, J. P., Herwig, R. P., Lara, J. C., & Staley, J. T. (1995). *Cycloclasticus pugetii* gen. nov., sp. nov., an aromatic hydrocarbon-degrading bacterium from marine sediments. *International Journal of Systematic Bacteriology*, *45*(1), 116–23.

Edgar, R. C., Haas, B. J., Clemente, J. C., Quince, C., & Knight, R. (2011). UCHIME improves sensitivity and speed of chimera detection. *Bioinformatics (Oxford, England)*, *27*(16), 2194–200.

Ekborg, N. A., Gonzalez, J. M., Howard, M. B., Taylor, L. E., Hutcheson, S. W., & Weiner, R. M. (2005). *Saccharophagus degradans* gen. nov., sp. nov., a versatile marine degrader of complex polysaccharides. *International Journal of Systematic and Evolutionary Microbiology*, *55*(4), 1545–9.

El Fantroussi, S., & Agathos, S. N. (2005). Is bioaugmentation a feasible strategy for pollutant removal and site remediation? *Current Opinion in Microbiology*, *8*(3), 268–275.

Farrell, J., & Rose, A. (1967). Temperature effects on microorganisms. *Annual Review of Microbiology*, *21*, 101–20.

Feitkenhauer, H., Müller, R., & Märkl, H. (2003). Degradation of polycyclic aromatic hydrocarbons and long chain alkanes at 60-70 degrees C by *Thermus* and *Bacillus* spp. *Biodegradation*, *14*(6), 367–72.

Fingas, M. (1995). Water-in-oil emulsion formation: A review of physics and mathematical modelling. *Spill Science & Technology Bulletin*, *2*(1), 55–59.

Finnerty, W. (1988). Lipids of *Acinetobacter*. In T. H. A.- White (Ed.), *Proceedings of the World Conference on Biotechnology for the Fats and Oil Industry* (pp. 184–188). Urbana:Amer Oil Chemists Society.

Forney, F. W., & Markovetz, A. J. (1970). Subterminal oxidation of aliphatic hydrocarbons. *Journal of Bacteriology*, *102*(1), 281–2.

Forsyth, J.V., Tsao, Y.M., & Bleam, R. D. (1995). Bioremediation: when is augmentation needed. In B. C. Hinchee, R.E., Fredrickson, J., Alleman (Ed.), *Bioaugmentation for Site Remediation* (pp. 1–14). Columbus: Battelle Press.

Fortin, N., Beaumier, D., Lee, K., & Greer, C. W. (2004). Soil washing improves the recovery of total community DNA from polluted and high organic content sediments. *Journal of Microbiological Methods*, *56*(2), 181–91.

Gallego, J. L. R., Sierra, C., Permanyer, A., Peláez, A. I., Menéndez-Vega, D., & Sánchez, J. (2010). Full-Scale Remediation of a Jet Fuel-Contaminated Soil: Assessment of Biodegradation, Volatilization, and Bioavailability. *Water, Air, & Soil Pollution*, *217*(1-4), 197–211.

García, M. T., Ventosa, A., & Mellado, E. (2005). Catabolic versatility of aromatic compound-degrading halophilic bacteria. *FEMS Microbiology Ecology*, *54*(1), 97–109.

Gauthier, M., & Lafay, B. (1992). *Marinobacter hydrocarbonoclasticus* gen. nov., sp. nov., a new, extremely halotolerant, hydrocarbon-degrading marine bacterium. *International Journal of Systematic Bacteriology*, *42*(4), 568–576.

Geiselbrecht, A. D., Hedlund, B. P., Tichi, M. A., & Staley, J. T. (1998). Isolation of marine polycyclic aromatic hydrocarbon (PAH)-degrading *Cycloclasticus* strains from the Gulf of Mexico and comparison of their PAH degradation ability with that of puget sound *Cycloclasticus* strains. *Applied and Environmental Microbiology*, *64*(12), 4703–10.

Gentry, T., Rensing, C., & Pepper, I. (2004). New Approaches for Bioaugmentation as a Remediation Technology. *Critical Reviews in Environmental Science and Technology*, *34*(5), 447–494.

Gibbs, C. F. (1975). Quantitative Studies on Marine Biodegradation of Oil. I. Nutrient Limitation at 14 degrees C. *Proceedings of the Royal Society B: Biological Sciences*, *188*(1090), 61–82.

Giebler, J., Wick, L. Y., Schloter, M., Harms, H., & Chatzinotas, A. (2013). Evaluating the assignment of alkB Terminal Restriction Fragments and sequence types to distinct Bacterial Taxa. *Applied and Environmental Microbiology*, *79*(9), 3129–3132.

Ginige, M. P., Hugenholtz, P., Daims, H., Wagner, M., Keller, J., & Blackall, L. L. (2004). Use of stable-isotope probing, full-cycle rRNA analysis, and Fluorescence In Situ Hybridization-Microautoradiography to study a methanol-fed denitrifying microbial community. *Applied and Environmental Microbiology*, *70*(1), 588–596.

Goual, L. (2012). Petroleum asphaltenes. In *Crude oil emulsions-Composition stability and Characterization* (Abdel-Raouf., pp. 27–42). InTach, Rijeka.

Goual, L. & Firoozabadi, A. (2004). Effect of resins and DBSA on asphaltene precipitation from petroleum fluids. *AIChE Journal*, *50*(2), 470–479.

Graham, D. W., Smith, V. H., Cleland, D. L., & Law, K. P. (1999). Effects of nitrogen and phosphorus supply on hexadecane biodegradation in soil systems. *Water, air and soil pollution*, 111(1-4), 1–18.

Graham, W.M., R.H. Condon, R.H. Carmichael, I. D'Ambra, H.K. Patterson, L.J. Linn, and F.J. Hernandez Jr. (2010). Oil carbon entered the coastal planktonic food web during the Deepwater Horizon oil spill. *Environmental Research Letters5*, 045301, http://dx.doi.org/10.1088/1748-9326/5/4/045301.

Gutierrez-Zamora, M.-L., & Manefield, M. (2010). An appraisal of methods for linking environmental processes to specific microbial taxa. *Reviews in Environmental Science and Bioechnology*, *9*(2), 153–185.

Gutierrez, T. (2011). Identifying polycyclic aromatic hydrocarbon-degrading bacteria in oil-contaminated surface waters at Deepwater Horizon by cultivation, stable isotope probing and pyrosequencing. *Reviews in Environmental Science and Biotechnology, 10*(4), 301-305

Hall, T. A. (1999). BioEdit: a user-friendly biological sequence alignment editor and analysis program for windows 95/98/NT. *Nucleic Acids Symposium Series*, *41*, 95–98.

Hammel, K. E., Gai, W. Z., Green, B., & Moen, M. A. (1992). Oxidative degradation of phenanthrene by the ligninolytic fungus *Phanerochaete chrysosporium. Applied and Environmental Microbiology*, *58*(6), 1832–8.

Hara, A., & Syutsubo, K. (2003). *Alcanivorax* which prevails in oil-contaminated seawater exhibits broad substrate specificity for alkane degradation, *5*, 746–753.

Harayama, S., Kishira, H., Kasai, Y., & Shutsubo, K. (1999). Petroleum biodegradation in marine environments. *Journal of Molecular Microbiology and Biotechnology*, *1*(1), 63–70.

Harmsen, J. (2004). Landfarming of Polycyclic aromatic Hydrocarbons and Mineral Oil Contaminated *sediments*. Washington: The Netherlands: Alterra Scientific contribution 14.

Hassanshahian, M., Emtiazi, G., Caruso, G., & Cappello, S. (2013). Bioremediation (bioaugmentation/biostimulation) trials of oil polluted seawater: A mesocosm simulation study. Marine Environmental Research, 1–11.

Hazen, T.C., E.A. Dubinsky, T.Z. DeSantis, G.L. Andersen, Y.M. Piceno, N. Singh, J.K. Jansson, A. Probst, S.E. Borglin, J.L. Fortney, and others (2010). Sea oil plume enriches indigenous oil-degrading bacteria. *Science, 330,* 204–207,http://dx.doi.org/10.1126/science.1195979.

Head, I. M. & Swannell, R. P. (1999). Bioremediation of petroleum hydrocarbon contaminants in marine habitats. *Current Opinion in Biotechnology*, *10*(3), 234–9.

Head, I. M., Jones, D. M., & Röling, W. F. M. (2006). Marine microorganisms make a meal of oil. *Nature reviews, Microbiology*, *4*(3), 173–82.

Herbes, S.E. and Schwall, L. R. (1978). Effect of estuarine sediment pH and oxidation-reduction potential on microbial hydrocarbon degradation. *Applied and Environmental Microbiology*, *35*, 306–316.

Holden, P. A., LaMontagne, M. G., Bruce, A. K., Miller, W. G., & Lindow, S. E. (2002). Assessing the role of *Pseudomonas aeruginosa* surface-active gene expression in hexadecane biodegradation in sand. *Applied and Environmental Microbiology*, *68*(5), 2509–18.

Hoppe, H.-G. (1978). Relations between active bacteria and heterotrophic potential in the sea. *Netherlands Journal of Sea Research*, *12*(1), 78–98.

Huang, W. E., Ferguson, A., Singer, A. C., Lawson, K., Thompson, I. P., Kalin, R. M.,Larkin, M.J., Bailey, M.J., & Whiteley, A. S. (2009). Resolving genetic functions within microbial populations: in situ analyses using rRNA and mRNA stable isotope probing coupled with single-cell raman-fluorescence in situ hybridization. *Applied and Environmental Microbiology*, *75*(1), 234–41.

Hunt, D. E., Lin, Y., Church, M. J., Karl, D. M., Tringe, S. G., Izzo, L. K., & Johnson, Z. I. (2013). Relationship between abundance and specific activity of bacterioplankton in open ocean surface waters. *Applied and Environmental Microbiology*, *79*(1), 177–84.

Hutchens, E., Radajewski, S., Dumont, M. G., McDonald, I. R., & Murrell, J. C. (2004). Analysis of methanotrophic bacteria in Movile Cave by stable isotope probing. *Environmental Microbiology*, *6*(2), 111–20.

Huu, N., Denner, E. B. M., Ha, D. T. C., Wanner, G., & Stan-lotter, H. (1996). *Marinobacter aquaeolei* bacterium isolated from a Vietnamese oil- producing well. *International Journal of Systematic Bacteriology*, *49* (2), 367–375.

Hyne, N. J. (2001). *Nontechnical Guide to Petroleum Geology, Exploration, Drilling and Production* (2nd ed., pp. 1–4). Oklahoma: PennWell Corporation.

Ivanova, E. P., Bowman, J. P., Lysenko, A. M., Zhukova, N. V, Gorshkova, N. M., Sergeev, A. F., & Mikhailov, V. V. (2005). *Alteromonas addita* sp. nov. *International Journal of Systematic and Evolutionary Microbiology*, *55*(Pt 3), 1065–8.

I**wabuchi, N., Sunairi, M., Urai, M., Itoh, C., Anzai, H., Nakajima, M., & Harayama, S. (2002).** Extracellular polysaccharides of *Rhodococcus rhodochrous* S-2 stimulate the degradation of aromatic components in crude oil by indigenous marine bacteria. *Applied and Environmental Microbiology*, *68*(5), 2337–43.

Iwamoto, T., & Nasu, M. (2001). Current bioremediation practice and perspective. *Journal of Bioscience and Bioengineering*, *92*(1), 1–8.

Jehmlich, N., Schmidt, F., Hartwich, M., von Bergen, M., Richnow, H.-H., & Vogt, C. (2008a). Incorporation of carbon and nitrogen atoms into proteins measured by protein-based stable isotope probing (Protein-SIP). *Rapid Communications in Mass Spectrometry : RCM*, *22*(18), 2889–97.

Jehmlich, N., Schmidt, F., von Bergen, M., Richnow, H.-H., & Vogt, C. (2008b). Protein-based stable isotope probing (Protein-SIP) reveals active species within anoxic mixed cultures. *The ISME Journal*, *2*(11), 1122–33.

Jehmlich, N., Schmidt, F., Taubert, M., Seifert, J., von Bergen, M., Richnow, H.-H., & Vogt, C. (2009). Comparison of methods for simultaneous identification of bacterial species and determination of metabolic activity by protein-based stable isotope probing (Protein-SIP) experiments. *Rapid Communications in Mass Spectrometry : RCM*, *23*(12), 1871–8.

Jenisch-Anton, A., Adam, P., Michaelis, W., Connan, J., Herrmann, D., Rohmer, M., & Albrecht, P. (2000). Molecular evidence for biodegradation of geomacromolecules. *Geochimica et Cosmochimica Acta*, *64*(20), 3525–3537.

Jeon, C. O., Park, W., Padmanabhan, P., DeRito, C., Snape, J. R., & Madsen, E. L. (2003). Discovery of a bacterium, with distinctive dioxygenase, that is responsible for in situ biodegradation in contaminated sediment. *Proceedings of the National Academy of Sciences of the United States of America*, *100*(23), 13591–13596.

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

Jiang, S. C. & Paul, J. H. (1996). Occurrence of lysogenic bacteria in marine microbial communities as determined by prophage induction. *Marine Ecology Progress Series*, *142*, 27–38.

Jiménez, N., Viñas, M., Bayona, J. M., Albaiges, J., & Solanas, A. M. (2007). The Prestige oil spill: bacterial community dynamics during a field biostimulation assay. *Applied Microbiology and Biotechnology*, *77*(4), 935–45.

Jin, H. M., Kim, J. M., Lee, H. J., Madsen, E. L., & Jeon, C. O. (2012). *Alteromonas* as a key agent of polycyclic aromatic hydrocarbon biodegradation in crude oil-contaminated coastal sediment. *Environmental Science & Technology*, *46*(14), 7731–40.

Johnsen, A. R., Winding, A., Karlson, U., & Roslev, P. (2002). Linking of microorganisms to phenanthrene metabolism in soil by analysis of ^{13}c-labeled Cell lipids. *Applied and Environmental Microbiology*, *68*(12), 6106–6113.

Johnson, A. C., Larsen, P. F., Gadbois, D. F., & Humason, A. W. (1985). The distribution of polycyclic aromatic hydrocarbons in the surficial sediments of Penobscot Bay (Maine, USA) in relation to possible sources and to other sites worldwide. *Marine Environmental Research*, *15*(1), 1–16.

Johnson, C. R., & Scow, K. M. (1999). Effect of nitrogen and phosphorus addition on phenanthrene biodegradation in four soils. *Biodegradation*, *10*(1), 43–50.

Jones, S. E., & Lennon, J. T. (2010). Dormancy contributes to the maintenance of microbial diversity. *Proceedings of the National Academy of Sciences of the United States of America*, *107*(13), 5881–6.

Jordan, R.E. & Payne, J. R. (1980). *Fate and Weathering of Petroleum Spills in the Marine Environment*. Ann Arbor, MI: Ann Arbor Science Publishers Inc.

Juck, D., Charles, T., Whyte, L., & Greer, C. (2000). Polyphasic microbial community analysis of petroleum hydrocarbon-contaminated soils from two northern Canadian communities. *FEMS Microbiology Ecology*, *33*(3), 241–249.

Kanaly, R. A., & Harayama, S. (2000). Biodegradation of high-molecular-weight polycyclic aromatic hydrocarbons by bacteria. *Journal of Bacteriology*, *182*(8), 2059–2067.

Kasai, Y., Kishira, H., & Harayama, S. (2002). Bacteria belonging to the genus *Cycloclasticus* play a primary role in the degradation of aromatic hydrocarbons released in a marine environment. *Applied and Environmental Microbiology*, *68*(11), 5625-33.

Kester, A. S., & Foster, J. W. (1963). Diterminal oxidation of long-chain alkanes by bacteria. *Journal of Bacteriology*, *85*, 859–69.

Kim, I. S., Lee, J., Kim, S.-J., Yu, H.-W., & Jang, A. (2014). Comparative pyrosequencing analysis of bacterial community change in biofilm formed on seawater reverse osmosis membrane. *Environmental Technology*, *35*(2), 125–136.

Kiyohara, H., Torigoe, S., Kaida, N., Asaki, T., Iida, T., Hayashi, H., & Takizawa, N. (1994). Cloning and characterization of a chromosomal gene cluster, pah, that encodes the upper pathway for phenanthrene and naphthalene utilization by *Pseudomonas putida* OUS82. *Journal of Bacteriology*, *176*(8), 2439–43.

Kodama, Y., Stiknowati, L. I., Ueki, A., Ueki, K., & Watanabe, K. (2008). *Thalassospira tepidiphila* sp. nov., a polycyclic aromatic hydrocarbon-degrading bacterium isolated from seawater. *International Journal of Systematic and Evolutionary Microbiology*, *58*(3), 711–5.

Kota, S. (1999). Influence of protozoan grazing on contaminant biodegradation. *FEMS Microbiology Ecology*, *29*(2), 179–189.

Kotani, T., Yamamoto, T., Yurimoto, H., Sakai, Y., & Kato, N. (2003). Propane monooxygenase and NAD^+-dependent secondary alcohol dehydrogenase in propane metabolism by *Gordonia* sp. strain TY-5. *Journal of Bacteriology*, *185*(24), 7120–8.

Kotani, T., Yurimoto, H., Kato, N., & Sakai, Y. (2007). Novel acetone metabolism in a propane-utilizing bacterium, *Gordonia* sp. strain TY-5. *Journal of Bacteriology*, 189(3), 886–93.

Krause, E., Wichels, A., Giménez, L., Lunau, M., Schilhabel, M. B., & Gerdts, G. (2012). Small changes in pH have direct effects on marine bacterial community composition: a microcosm approach. *PloS one*, *7*(10), e47035.

Laflamme, R. E., & Hites, R. A. (1978). The global distribution of polycyclic aromatic hydrocarbons in recent sediments. *Geochimica et Cosmochimica Acta*, *42*(3), 289–303.

Lai, Q., Yuan, J., Gu, L., & Shao, Z. (2009). *Marispirillum indicum* gen. nov., sp. nov., isolated from a deep-sea environment. *International Journal of Systematic and Evolutionary Microbiology*, *59*(6), 1278–1281.

Lane, D. J. (1991). 16S/23S rRNA sequencing. In E. S. & M.Goodfellow (Ed.), *Nucleic Acid Techniques in Bacterial Systematics* (pp. 115–175). New York, N.Y.: John Wiley & Sons.

Leahy, J. G., & Colwell, R. R. (1990). Microbial degradation of hydrocarbons in the environment. *Microbiological Reviews*, *54*(3), 305–315.

Lee, K., and Levy, E.M. (1987). Enhanced biodegradation of a light crude oil in sandy beaches.Proceedings of 1987 Oil Spill Conference. American Petroleum Institute, Washington, DC, pp411-416.

Lekunberri, I., Lefort, T., Romero, E., Vazquez-Dominguez, E., Romera-Castillo, C., Marrase, C., Peters, F., Weinbauer, M., & Gasol, J. M. (2010). Effects of a dust deposition event on coastal marine microbial abundance and activity, bacterial community structure and ecosystem function. *Journal of Plankton Research*, *32*(4), 381–396.

Li, L., Liu, X., Yang, W., Xu, F., Wang, W., Feng, L., Bartlam, M., Wang, L., & Rao, Z. (2008). Crystal structure of long-chain alkane monooxygenase (LadA) in complex with coenzyme FMN: unveiling the long-chain alkane hydroxylase. *Journal of Molecular Biology*, *376*(2), 453–65.

Lin, C., Gan, L., & Chen, Z.-L. (2010). Biodegradation of naphthalene by strain *Bacillus fusiformis* (BFN). *Journal of Hazardous Materials*, *182*(1-3), 771–7.

Loehr, R. C., & Webster, M. T. (1996). Behavior of fresh vs. aged chemicals in soil. *Soil and Sediment Contamination*, *5*(4), 361–383.

López-lópez, A., Bartual, S. G., Stal, L., Onyshchenko, O., & Rodríguez-valera, F. (2005). Genetic analysis of housekeeping genes reveals a deep-sea ecotype of *Alteromonas macleodii* in the Mediterranean Sea. *Environmental Microbiology*, *7*, 649–659.

Lozupone, C. A., & Knight, R. (2008). Species divergence and the measurement of microbial diversity. *FEMS Microbiology Reviews*, *32*(4), 557–78.

Lueders, T., Manefield, M., & Friedrich, M. W. (2004a). Enhanced sensitivity of DNA- and rRNA-based stable isotope probing by fractionation and quantitative analysis of isopycnic centrifugation gradients. *Environmental Microbiology*, *6*(1), 73–78.

Lueders, T., Pommerenke, B., & Friedrich, M. W. (2004b). Stable-isotope probing of microorganisms thriving at thermodynamic limits: syntrophic propionate oxidation in flooded soil. *Applied and Environmental Microbiology*, *70*(10), 5778–86.

Lueders, T., Wagner, B., Claus, P., & Friedrich, M. W. (2004c). Stable isotope probing of rRNA and DNA reveals a dynamic methylotroph community and trophic interactions with fungi and protozoa in oxic rice field soil. *Environmental Microbiology*, *6*(1), 60–72.

Brusseau, M.L. (1998). The impact of physical, chemical and biological factors on biodegradation. In R. Serra (Ed.), *International Conference on Biotechnology for Soil Remediation: Scientific Bases and Practical Applications* (pp. 81–98). Milan, Italy: C.I.P.A S.R.L.

MacNaughton, S. J., Stephen, J. R., Venosa, A. D., Davis, G. A., Chang, Y. J., & White, D. C. (1999). Microbial population changes during bioremediation of an experimental oil spill. *Applied and Environmental Microbiology*, *65*(8), 3566–74.

Macnaughton, S. J., Stephen, J. R., Venosa, A. D., Davis, G. A., Chang, Y., & White, D. C. (1999). Microbial Population Changes during Bioremediation of an Experimental Oil Spill . *Applied and Environmental Microbiology*, *65*(8),3566-3574.

Maeng, J. H., Sakai, Y., Tani, Y., & Kato, N. (1996). Isolation and characterization of a novel oxygenase that catalyzes the first step of n-alkane oxidation in *Acinetobacter* sp. strain M-1. *Journal of Bacteriology*, *178*(13), 3695–700.

Magot, M., Ollivier, B., & Patel, B. K. (2000). Microbiology of petroleum reservoirs. *Antonie van Leeuwenhoek*, *77*(2), 103–16.

Manefield, M., Griffiths, R. I., Leigh, M. B., Fisher, R., & Whiteley, A. S. (2005). Functional and compositional comparison of two activated sludge communities remediating coking effluent. *Environmental Microbiology*, *7*(5), 715–22.

Manefield, M., Whiteley, A. S., Griffiths, R. I., & Bailey, M. J. (2002a). RNA Stable Isotope Probing , a Novel Means of Linking Microbial Community Function to Phylogeny RNA. Appied and Environmental Microbiology, *68*(11),5367-5373.

Manefield, M., Whiteley, A. S., Ostle, N., Ineson, P., & Bailey, M. J. (2002b). Technical considerations for RNA-based stable isotope probing: an approach to associating microbial diversity with microbial community function. *Rapid Communications in Mass Spectrometry : RCM*, *16*(23), 2179–83.

Marshall, A. G., & Rodgers, R. P. (2004). Petroleomics: the next grand challenge for chemical analysis. *Accounts of Chemical Research*, *37*(1), 53–9.

Martens-Habbena, W., Berube, P., de la Torre, J.R. and Stahl, D. A. (2009). Ammonia oxidation kinetics determine niche separation of nitrifying Archaea and bacteria. *Nature*, *461*, 976–979.

Math, R. K., Jin, H. M., Kim, J. M., Hahn, Y., Park, W., Madsen, E. L., & Jeon, C. O. (2012). Comparative genomics reveals adaptation by *Alteromonas* sp. SN2 to marine tidal-flat conditions: cold tolerance and aromatic hydrocarbon metabolism. *PloS one*, *7*(4), e35784.

McAuliffe, C. D. (1987). Organism exposure to volatile/soluble hydrocarbons from crude oil spills-a field and laboratory comparison. In *Proceedings of the 1987 Oil Spill Conference* (pp. 275–288). Baltimore, Maryland.

McGenity, T. J. (2014). Hydrocarbon biodegradation in intertidal wetland sediments. *Frontiers in Microbiology*, *27*, 46–54.

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

McKenna, A. M., Donald, L. J., Fitzsimmons, J. E., Juyal, P., Spicer, V., Standing, K. G.,Marshall, A.G., & Rodgers, R. P. (2013). Heavy petroleum composition. 3. Asphaltene Aggregation. *Energy & Fuels*, *27*(3), 1246–1256.

Mckew, B. A., Coulon, F., Yakimov, M. M., Denaro, R., Genovese, M., Smith, C. J., Osborn, A.M, Timmis, K.N., & Mcgenity, T. J. (2007). Efficacy of intervention strategies for bioremediation of crude oil in marine systems and effects on indigenous hydrocarbonoclastic bacteria. *Environmental Microbiology*, *9*(6), 1562–1571.

Means, J. C., Wood, S. G., Hassett, J. J., & Banwart, W. L. (1980). Sorption of polynuclear aromatic hydrocarbons by sediments and soils. *Environmental Science & Technology*, *14*(12), 1524–8.

Menn, F. M., Applegate, B. M., & Sayler, G. S. (1993). NAH plasmid-mediated catabolism of anthracene and phenanthrene to naphthoic acids. *Applied and Environmental Microbiology*, *59*(6), 1938–42.

Michaelsen, M., Hulsch, R., Höpner, T., & Berthe-Corti, L. (1992). Hexadecane mineralization in oxygen-controlled sediment-seawater cultivations with autochthonous microorganisms. *Applied and Environmental Microbiology*, *58*(9), 3072–3077.

Mills, M. A., Bonner, J. S., McDonald, T. J., Page, C. A., & Autenrieth, R. L. (2003). Intrinsic bioremediation of a petroleum-impacted wetland. *Marine Pollution Bulletin*, *46*(7), 887–99.

Miyoshi, M. (1895). Die durchbohrung von membranen durch pilzfaden. Jahrbücher für wissenschaftliche. *Botanik*, *28*, 269–289.

Morehead, N. R., Eadie, B. J., Lake, B., Landrum, P. F., & Berner, D. (1986). The sorption of PAH onto dissolved organic matter in Lake Michigan waters. *Chemosphere*, *15*(4), 403–412.

Moreno, A. M., Matz, C., Kjelleberg, S., & Manefield, M. (2010). Identification of ciliate grazers of autotrophic bacteria in ammonia-oxidizing activated sludge by RNA stable isotope probing. *Applied and Environmental Microbiology*, *76*(7), 2203–2211.

Morris, S. A., Radajewski, S., Willison, T. W., & Murrell, J. C. (2002). Identification of the functionally active methanotroph population in a peat soil microcosm by stable-isotope probing. *Applied and Environmental Microbiology*, *68*(3), 1446–53.

Naether, D. J., Slawtschew, S., Stasik, S., Engel, M., Olzog, M., Wick, L. Y., … Heipieper, H. J. (2013). Adaptation of the hydrocarbonoclastic bacterium *Alcanivorax borkumensis* SK2 to alkanes and toxic organic compounds: a physiological and transcriptomic approach. *Applied and Environmental Microbiology*, *79*(14), 4282–93.

Namkoong, W. (2002). Bioremediation of diesel-contaminated soil with composting. *Environmental Pollution*, *119*(1), 23–31.

Neufeld, J. D., Dumont, M. G., Vohra, J., & Murrell, J. C. (2007). Methodological considerations for the use of stable isotope probing in microbial ecology. *Microbial Ecology*, *53*(3), 435–42.

Neufeld, J. D., Vohra, J., Dumont, M. G., Lueders, T., Manefield, M., Friedrich, M. W., & Murrell, J. C. (2007b). DNA stable-isotope probing. *Nature Protocols*, *2*(4), 860–6.

Nikolopoulou, M., & Kalogerakis, N. (2008). Enhanced bioremediation of crude oil utilizing lipophilic fertilizers combined with biosurfactants and molasses. *Marine Pollution Bulletin*, *56*(11), 1855–61.

Nikolopoulou, M., Pasadakis, N., & Kalogerakis, N. (2013a). Evaluation of autochthonous bioaugmentation and biostimulation during microcosm-simulated oil spills. *Marine Pollution Bulletin*, *72*(1), 165–73.

Nikolopoulou, M., Eickenbusch, P., Pasadakis, N., Venieri, D., & Kalogerakis, N. (2013b). Microcosm evaluation of autochthonous bioaugmentation to combat marine oil spills. *New Biotechnology*, *30*(6), 734–42.

OPEC, Organisation of Petroleum Exporting Countries (2013). Global oil inventory article. Helferstorferstrasse 17, A-1010 Vienna, Austria. Retrieved from http://www.opec.org/opec_web/static_files_project/media/downloads/publications/MOMR_November_2013.pdf.

OPEC, Organisation of Petroleum Exporting Countries. (2008). In *Ibrahim OK (ed) Annual Report 2008*. Retrieved from http://www.opec.org/opec_web/static_files_project/media/downloads

Padmanabhan, P., Padmanabhan, S., DeRito, C., Gray, A., Gannon, D., Snape, J. R., Tsai, C.S., Park, W., Jeon, C., & Madsen, E. L. (2003). Respiration of ^{13}C-labeled substrates added to soil in the field and subsequent 16S rRNA gene analysis of ^{13}C-labeled soil DNA. *Applied and Environmental Microbiology*, *69*(3), 1614–22.

Parales, R. E., Parales, J. V, Pelletier, D. A., & Ditty, J. L. (2008). Diversity of microbial toluene degradation pathways. *Advances in Applied Microbiology*, *64*(08), 1–73.

Phillips, D. H. (1983). Fifty years of benzo(a)pyrene. *Nature*, *303*(5917), 468–472.

Piskonen, R., Nyyssönen, M., Rajamäki, T., & Itävaara, M. (2005). Monitoring of accelerated naphthalene biodegradation in a bioaugmented soil slurry. *Biodegradation*, *16*(2), 127–134.

Pollet, T., Humbert, J.-F., & Tadonléké, R. D. (2013). Planctomycetes in lakes: poor or strong competitors for phosphorus? *Applied and Environmental Microbiology*, *80* (3), 819-828.

Polz, M. F., Hunt, D. E., Preheim, S. P., & Weinreich, D. M. (2006). Patterns and mechanisms of genetic and phenotypic differentiation in marine microbes. *Philosophical transactions of the Royal Society of London. Series B, Biological Sciences*, *361*(1475), 2009–21.

Poretsky, R. S., Gifford, S., Rinta-Kanto, J., Vila-Costa, M., & Moran, M. A. (2009). Analyzing gene expression from marine microbial communities using environmental transcriptomics. *Journal of Visualized Experiments : JoVE*, (24), 1–6.

Posada-Baquero, R., & Ortega-Calvo, J.-J. (2011). Recalcitrance of polycyclic aromatic hydrocarbons in soil contributes to background pollution. *Environmental Pollution (Barking, Essex : 1987)*, *159*(12), 3692–9.

Prosser, J. I. (2010). Replicate or lie. *Environmental Microbiology*, *12*(7), 1806–10.

Prince, R.C. (1997). Bioremediation of marine oil spills. *Trends in Biotechnology*, *15*, 158–160.

Radajewski, S, Ineson, P., Parekh, N. R., & Murrell, J. C. (2000). Stable-isotope probing as a tool in microbial ecology. *Nature*, *403*(6770), 646–9.

Radajewski, Stefan, McDonald, I. R., & Murrell, J. C. (2003). Stable-isotope probing of nucleic acids: a window to the function of uncultured microorganisms. *Current Opinion in Biotechnology*, *14*(3), 296–302.

Reed, M., Brandvik, P. E. R. J., Daling, P. E. R., Lewis, A., Fiocco, R., Mackay, D. O. N., & Prentki, R. (1999). Oil Spill Modeling towards the Close of the 20th Century : Overview of the State of the Art. *Spill Science & Technology Bulletin*, *5*(1), 3–16.

Riemann, L., Steward, G. F., & Azam, F. (2000). Dynamics of Bacterial Community Composition and Activity during a Mesocosm Diatom Bloom. *Applied and Environmental Microbiology*, *66*(2), 578–587.

Rockstein, M., & Herron, P. W. (1951). Colorimetric Oetermination of Inorganic Phosphate in Microgram Quantities. *Analytical Chemistry*, *23*(10), 1500–1501.

Rodriguez, J.-P. (2012). The Geography of Transport Systems. Retrieved from http://people.hofstra.edu/geotrans

Röling, W. F. M., Milner, M. G., Jones, D. M., Lee, K., Daniel, F., Swannell, R. J. P., & Head, I. M. (2002). Robust hydrocarbon degradation and dynamics of bacterial communities during nutrient-enhanced oil spill bioremediation. *Applied and Environmental Microbiology*, *68*(11), 5537–48.

Röling, W. F. M., Couto de Brito, I. R., Swannell, R. P. J., & Head, I. M. (2004). Response of Archaeal Communities in Beach Sediments to Spilled Oil and Bioremediation. *Applied and Environmental Microbiology*, *70*(5), 2614–2620.

Romera-Castillo, C., Sarmento, H., Alvarez-Salgado, X. A., Gasol, J. M., & Marrasé, C. (2011). Net production and consumption of fluorescent colored dissolved organic matter by natural bacterial assemblages growing on marine phytoplankton exudates. *Applied and Environmental Microbiology*, *77*(21), 7490–8.

Ruberto, L., Vazquez, S. C., & Mac Cormack, W. P. (2003). Effectiveness of the natural bacterial flora, biostimulation and bioaugmentation on the bioremediation of a hydrocarbon contaminated Antarctic soil. *International Biodeterioration & Biodegradation*, *52*(2), 115–125.

Sabirova, J. S., Ferrer, M., Regenhardt, D., Timmis, K. N., & Golyshin, P. N. (2006). Proteomic insights into metabolic adaptations in *Alcanivorax borkumensis* induced by alkane utilization. *Journal of Bacteriology*, *188*(11), 3763–73.

Samanta, S. K., Singh, O. V, & Jain, R. K. (2002). Polycyclic aromatic hydrocarbons: environmental pollution and bioremediation. *Trends in Biotechnology*, *20*(6), 243–248.

Sartoros, C., Yerushalmi, L., Béron, P., & Guiot, S. R. (2005). Effects of surfactant and temperature on biotransformation kinetics of anthracene and pyrene. *Chemosphere*, *61*(7), 1042–50.

Satomi, M., Hamada, T., Harayama, S., & Fujii, T. (2002). Phylogenetic study of the genus *Oceanospirillum* based on 16S rRNA and gyrB genes : emended description of the genus *Oceanospirillum* , description of *Pseudospirillum* gen . nov ., *Oceanobacter* gen . nov . and *Terasakiella* gen . nov . and transfer of *Oceanos Oceanospirillum jannaschii* and *Pseudomonas stanieri* to *Marinobacterium* as *Marinobacterium jannaschii* comb. nov. and *Marinobacterium stanieri* comb. no. *International Journal of Systemtic and Revoutionary Microbiology*,*52*, 739–747.

Schäfer, H., Servais, P., & Muyzer, G. (2000). Successional changes in the genetic diversity of a marine bacterial assemblage during confinement. *Archives of Microbiology*, *173*(2), 138–45.

Schauer, M., Balagué, V., Pedrós-Alió, C., & Massana, R. (2003). Seasonal changes in the taxonomic composition of bacterioplankton in a coastal oligotrophic system. *Aquatic Microbial Ecology*, *31*, 163–174.

Schloss, P. D., Westcott, S. L., Ryabin, T., Hall, J. R., Hartmann, M., Hollister, E. B., Lesniewski, R.A., Oakley, B.B., Parks, D.H., Robinson, C.J., Sahl, J.,W., Stres, B., Thallinger, G.G., Van Horn, D.J., & Weber, C. F. (2009). Introducing mothur: open-source, platform-independent, community-supported software for

describing and comparing microbial communities. *Applied and Environmental Microbiology*, *75*(23), 7537–41.

Schneiker, S., Martins dos Santos, V. A. P., Bartels, D., Bekel, T., Brecht, M., Buhrmester, J., & Golyshin, P. N. (2006). Genome sequence of the ubiquitous hydrocarbon-degrading marine bacterium *Alcanivorax borkumensis*. *Nature Biotechnology*, *24*(8), 997–1004.

Scholz D. K., Kucklick, J. H., Pond, R., Walker, A. H.., Bostrom, A., & Fischbeck, P. (1999). Fate of spilled oil in marine water. *Health and Environment Science Department, API, 4691,* 1–57.

Sei, K., Sugimoto, Y., Mori, K., Maki, H. and Kohno, T. (2003). Monitoring of alkane- degrading bacteria in a seawater microcosm during crude oil degradation by polymerase chain reaction based on alkane-catabolic genes. *Environmental. Microbiology 5,* 517–522.

Sharma, V. K., Hicks, S. D., Rivera, W., & Vazquez, F. G. (2002). Characterization and Degradation of Petroleum Hydrocarbons Following an Oil Spill into a Coastal Environment of South Texas, U.S.A. *Water, Air, and Soil Pollution*, *134*(1-4), 111–127.

Sheppard, P. J., Adetutu, E.M., Young, A., Manefield, M., Morrison, P.D., & Ball, A.S. (2013). Stable Isotope Probing - A Tool for Assessing the Potential Activity and Stability of Hydrocarbonoclastic Communities in Contaminated Seawater. *Journal of Bioremediation & Biodegradation*, *4*, 5.

Sherr, E. B., & Sherr, B. F. (2002). Significance of predation by protists in aquatic microbial food webs. *Antonie van Leeuwenhoek*, *81*(1-4), 293–308.

Silva, M. , Cerniglia, C. E., Pothuluri, J. V., Canhos, V. P., & Esposito, E. (2003). Screening filamentous fungi isolated from estuarine sediments for the ability to oxidize polycyclic aromatic hydrocarbons. *World Journal of Microbiology and Biotechnology*, *19*(4), 399–405.

Simons, K.L., Sheppard, P.J., Adetutu, E.M., Kaladi, K., Jushaz, A.L., Manefield, M., Sarma, P.M., Lal, B. & Ball, A.S. (2013). Carrier mounted bacterial consortium facilitates oil remediation in the marine environment. *Bioresource Technology*, *134,* 107-116.

Sivaraman, C., Ganguly, a., Nikolausz, M., & Mutnuri, S. (2011). Isolation of hydrocarbonoclastic bacteria from bilge oil contaminated water. *International Journal of Environmental Science & Technology*, *8*(3), 461–470.

Smith, V. H., Graham, D. W., & Cleland, D. D. (1998). Application of resource-ratio theory to hydrocarbon biodegradation. *Environmental Science & Technology*, *32*(21), 3386–3395.

Sorkhoh, N. A., Ghannoum, M. A., Ibrahim, A. S., Stretton, R. J., & Radwan, S. S. (1990). Crude oil and hydrocarbon-degrading strains of *Rhodococcus rhodochrous*

isolated from soil and marine environments in Kuwait. *Environmental Pollution*, *65*(1), 1–17.

Spain, J.C., & van Veld, P. a. (1983). Adaptation of the natural microbial communities to degradation of xenobiotic compounds: effects of concentration, exposure time, inoculum and chemical structure. *Applied and Environmental Microbiology*, *45*, 428–435.

Spain, J.C., Pritchard, P.H., & Bourquin, A. W. (1980). Effects of adaptation on biodegradation rates in sediment/water cores from estuarine and freshwater environments. *Enviromental Microbiology*, *40*, 726–734.

Speight, J. G., & Arjoon, K. K. (2012). *Bioremediation of Petroleum and Petroleum Products*. Beverly, Massachusetts: Scrivener Publishing.

Struvay, C., & Feller, G. (2012). Optimization to low temperature activity in psychrophilic enzymes. *International Journal of Molecular Sciences*, *13*(9), 11643–65.

Sutiknowati, L. I. (2007). Hydrocarbon degrading bacteria :isolation and identification. *Makara Sains*, *11*(2), 98–103.

Suzuki, M. T., Rappé, M. S., Haimberger, Z. W., Winfield, H., Adair, N., Ströbel, J., & Giovannoni, S. J. (1997). Bacterial diversity among small-subunit rRNA gene clones and cellular isolates from the same seawater sample. *Applied and Environmental Microbiology*, *63*(3), 983–9.

Sverdrup, H.U, Johnson, M.W., & Feming, R. H. (1942). *The Oceans Their Physics, Chemistry, and General Biology*. New York: Prentice-Hall, Inc.

Swannell, R. P. J., Croft, B. C., Grant, A. L., & Lee, K. (1995). Evaluation of bioremediation agents in beach microcosms. *Spill Science & Technology Bulletin*, *2*(2-3), 151–159.

Tada, Y., Taniguchi, A., Nagao, I., Miki, T., Uematsu, M., Tsuda, A., & Hamasaki, K. (2011). Differing growth responses of major phylogenetic groups of marine bacteria to natural phytoplankton blooms in the western North Pacific Ocean. *Applied and Evironmental Microbiology*, *77*(12), 4055–65.

Tagger, S., Bianchi, A., Julliard, M., Petit, J., & Roux, B. (1983). Effect of microbial seeding of crude oil in seawater in a model system. *Marine Biology*, *78*(1), 13–20.

Teramoto, M., Suzuki, M., Okazaki, F., Hatmanti, A., & Harayama, S. (2009). *Oceanobacter*-related bacteria are important for the degradation of petroleum aliphatic hydrocarbons in the tropical marine environment. *Microbiology*, *155*(10), 3362–3370.

Teramoto, Maki, Queck, S. Y., & Ohnishi, K. (2013). Specialized hydrocarbonoclastic bacteria prevailing in seawater around a port in the Strait of Malacca. *PloS one*, *8*(6), e66594.

Tillmann, S., Strömpl, C., Timmis, K. N., & Abraham, W.-R. (2005). Stable isotope probing reveals the dominant role of *Burkholderia* species in aerobic degradation of PCBs. *FEMS Microbiology Ecology*, *52*(2), 207–17.

Tissot, B P., & Welte, D. H. (1984). *Petroleum Formation and Occurrence*. Berlin: Springer verlag.

Tolosa, I., de Mora, S. J., Fowler, S. W., Villeneuve, J.-P., Bartocci, J., & Cattini, C. (2005). Aliphatic and aromatic hydrocarbons in marine biota and coastal sediments from the Gulf and the Gulf of Oman. *Marine Pollution Bulletin*, *50*(12), 1619–33.

Torsvik, V., & Øvreås, L. (2002). Microbial diversity and function in soil: from genes to ecosystems. *Current Opinion in Microbiology*, *5*(3), 240–5.

Turekian, K. (1968). *Oceans*. Prentice-Hall: Englewood, Cliffs, N.J.

Tyagi, M., da Fonseca, M. M. R., & de Carvalho, C. C. C. R. (2011). Bioaugmentation and biostimulation strategies to improve the effectiveness of bioremediation processes. *Biodegradation*, *22*(2), 231–241.

U.S. Congress (1991). Bioremediation for marine oil spills background paper. *OTA-BP-O-70*.

Ueno, A., Hasanuzzaman, M., Yumoto, I., & Okuyama, H. (2006). Verification of degradation of n-alkanes in diesel oil by *Pseudomonas aeruginosa* strain WatG in soil microcosms. *Current Microbiology*, *52*(3), 182–5.

Ueno, A., Ito, Y., Yumoto, I., & Okuyama, H. (2007). Isolation and characterization of bacteria from soil contaminated with diesel oil and the possible use of these in autochthonous bioaugmentation. *World Journal of Microbiology and Biotechnology*, *23*(12), 1739–1745.

Uhlík, O., Jecná, K., Leigh, M. B., Macková, M., & Macek, T. (2009). DNA-based stable isotope probing: a link between community structure and function. *The Science of the total Environment*, *407*(12), 3611–9.

Uhlik, O., Leewis, M.-C., Strejcek, M., Musilova, L., Mackova, M., Leigh, M. B., & Macek, T. (2012). Stable isotope probing in the metagenomics era: a bridge towards improved bioremediation. *Biotechnology Advances*, *31*(2), 154–65.

Urbance, J. W., Bratina, B. J., Stoddard, S. F., & Schmidt, T. M. (2001). Taxonomic characterization of *Ketogulonigenium vulgare* gen. nov., sp. nov. and *Ketogulonigenium robustum* sp. nov., which oxidize L-sorbose to 2-keto-L-gulonic acid. *International Journal of Systematic and Evolutionary Microbiology*, *51*(Pt 3), 1059–70.

Van Beilen, J. B., Panke, S., Lucchini, S., Franchini, A. G., Röthlisberger, M., & Witholt, B. (2001). Analysis of Pseudomonas putida alkane-degradation gene

clusters and flanking insertion sequences: evolution and regulation of the alk genes. *Microbiology*, *147*, 1621–1630.

Van Beilen, J.B., Li, Z., Duetz, W. A., Smits, T.H.M., and Witholt, B. (2003). Diversity of alkane hydroxylase systems in the environment. *Oil Gas Science and Technology*, *58*, 427–440.

Van Hamme, J. D., Singh, A., & Ward, O. P. (2003). Recent Advances in Petroleum microbiology. *Microbiology and Molecular Biology Reviews, 67*(4), 503-549.

Venosa, A. D., & Holder, E. L. (2007). Biodegradability of dispersed crude oil at two different temperatures. *Marine Pollution Bulletin*, *54*(5), 545–53.

Venosa,A. D., Albert D., & Zhu, X. (2003). Biodegradation of Crude Oil Contaminating Marine Shorelines and Freshwater Wetlands. *Spill Science & Technology Bulletin*, *8*(2), 163–178.

Venosa, Albert D., Suidan, M. T., Wrenn, B. A., Strohmeier, K. L., Haines, J. R., Eberhart, B. L., & Holder, E. (1996). Bioremediation of an Experimental Oil Spill on the Shoreline of Delaware Bay. *Environmental Science & Technology*, *30*(5), 1764–1775.

Venosa, A.D., Haines, J.R., and Allen, D.M. (1992). Efficacy of commercial inocula in enhancing biodegradation of crude oil contaminating a Prince William Sound beach. *Journal of industrial Microbiology*, *10*, 1-11

Vogel, T. M. (1996). Bioaugmentation as a soil bioremediation approach. *Current Opinion in Biotechnology*, *7*(3), 311–6.

Walker, J.D., & Colwell, R. R. (1975). Some effect of petroleum on estuarine and marine microorganisms. *Canadian Journal Of Microbiology*, *21*, 305–313.

Walker, J.D. , Colwell, R.R., & Petrakis, L. (1976). Biodegradation of petroleum by Cheasapeake Bay sediment bacteria. *Canadian Journal Of Microbiology*, *22*, 423–428.

Wang, Z., Hollebone, B. P., Fingas, M., Fieldhouse, B., Sigouin, L, Landriault, M, Smith, P., Noonan J., & Thouin, G. (2003). Characteristics of spilled oils, fuels, and petroleum products: 1. Composition and properties of selected oils. North Carolina: Durham.

Watkinson, R. J., & Morgan, P. (1990). Physiology of aliphatic hydrocarbon-degrading microorganisms. *Biodegradation*, *1*(2-3), 79–92.

Westlake, D. W. S., Jobson, A., Phillippe, R., & Cook, F. D. (1974). Biodegradability and crude oil composition. *Canadian Journal of Microbiology*, *20*(7), 915–928.

Whitby, C. B., Hall, G., Pickup, R., Saunders, J. R., Ineson, P., Parekh, N. R., & McCarthy, A. (2001). (^{13})C incorporation into DNA as a means of identifying the

active components of ammonia-oxidizer populations. *Letters in Applied Microbiology*, *32*(6), 398–401.

Whiteley, A. S., & Bailey, M. J. (2000). Bacterial Community Structure and Physiological State within an Industrial Phenol Bioremediation System. *Applied and Environmental Microbiology*, *66*(6), 2400–2407.

Whiteley,A.S., Andrew S., Thomson, B., Lueders, T., & Manefield, M. (2007). RNA stable-isotope probing. *Nature Protocols*, *2*(4), 838–44.

Wild, S.R., Simon R., Waterhouse, K. S., McGrath, S. P., & Jones, K. C. (1990). Organic contaminants in an agricultural soil with a known history of sewage sludge amendments: polynuclear aromatic hydrocarbons. *Environmental Science & Technology*, *24*(11), 1706–1711.

Wild, S.R., Obbard, J. P., Munn, C. I., Berrow, M. L., & Jones, K. C. (1991). The long-term persistence of polynuclear aromatic hydrocarbons (PAHs) in an agricultural soil amended with metal-contaminated sewage sludges. *The Science of The Total Environment*, *101*(3), 235–253.

William, D., Westlake, S., & Cook, F. (1980). *Petroleum biodegradation potential of northern Puget Sound and Strait of Juan de Fuca environments: Volume 1* (Volume 1.). United states: Office of Energy, Minerals, and Industry, Office of Research and Development, U.S. Environmental Protection Agency.

Williams, S.D., Ladd, D.E., & Farmer, J. J. (2005). Fate and Transport of Petroleum Hydrocarbons in Soil and Ground Water at Big South Fork National River and Recreation Area , Tennessee and Kentucky , 2002-2003. *U.S Department of the Interior, U.S. Geological Survey*: Scientific Investigations Report 2005-5104.

Woods, A., Watwood, M., & Schwartz, E. (2011). Identification of a toluene-degrading bacterium from a soil sample through H(^{2})(^{18})O DNA stable isotope probing. *Applied and Environmental Microbiology*, *77*(17), 5995–9.

Wright, A. L., Weaver, R. W., & Webb, J. W. (1997). Oil bioremediation in salt march mesocosms as influenced by N and P fertilization, flooding, and season. *Water, Air, & Soil Pollution*, *95*(1-4), 179–191.

Wu, Y., Lai, Q., Zhou, Z., Qiao, N., Liu, C., & Shao, Z. (2009). *Alcanivorax hongdengensis* sp. nov., an alkane-degrading bacterium isolated from surface seawater of the straits of Malacca and Singapore, producing a lipopeptide as its biosurfactant. *International Journal of Systematic and Evolutionary Microbiology*, *59*(6), 1474–1479.

Xia, W. X., Li, J. C., Zheng, X. L., Bi, X. J., & Shao, J. L. (2006). Enhanced biodegradation of diesel oil in seawater supplemented with nutrients. *Engineering in Life Sciences*, *6*(1), 80–85.

Xu, J.-L., He, J., Wang, Z.-C., Wang, K., Li, W.-J., Tang, S.-K., & Li, S.-P. (2007). *Rhodococcus qingshengii* sp. nov., a carbendazim-degrading bacterium. *International Journal of Systematic and Evolutionary Microbiology*, *57*(12), 2754–7.

Yakimov, M. M. Golyshin, P. N., Lang, S., Moore, E. R., Abraham, W. R., Lünsdorf, H., & Timmis, K. N. (1998). *Alcanivorax borkumensis* gen. nov., sp. nov., a new, hydrocarbon-degrading and surfactant-producing marine bacterium. *International Journal of Systematic Bacteriology*, *48*, 339–48.

Yakimov, M. M., Timmis, K. N., & Golyshin, P. N. (2007). Obligate oil-degrading marine bacteria. *Current Opinion in Biotechnology*, *18*(3), 257–66.

Yoon, J.-H. (2003). *Marinobacter litoralis* sp. nov., a moderately halophilic bacterium isolated from sea water from the East Sea in Korea. *International Journal of Systematic and Evolutionary Microbiology*, *53*(2), 563–568.

Yu, K. S. H., Wong, a H. Y., Yau, K. W. Y., Wong, Y. S., & Tam, N. F. Y. (2005). Natural attenuation, biostimulation and bioaugmentation on biodegradation of polycyclic aromatic hydrocarbons (PAHs) in mangrove sediments. *Marine Pollution Bulletin*, *51*(8-12), 1071–7.

Zeinali, M., Vossoughi, M., & Ardestani, S. K. (2008). Naphthalene metabolism in *Nocardia otitidiscaviarum* strain TSH1, a moderately thermophilic microorganism. *Chemosphere*, *72*(6), 905–9.

Zhang, L., & Xu, Z. (2008). Assessing bacterial diversity in soil. *Journal of Soils and Sediments*, *8*(6), 379–388.

Zhong, Z., & You, F. (2011). Oil spill response planning with consideration of physicochemical evolution of the oil slick: A multiobjective optimization approach. *Computers & Chemical Engineering*, *35*(8), 1614–1630.

Appendix

1-Media composition

1.1.Luria Bertani broth (LB_{30}): In 1L of milliQ water:

-10.0 g tryptone

-5.0 g yeast extract

-30.0 g NaCl.

1.2. Seawater-agar medium:

15g Agar/L of natural seawater

1.3. Bushnal-Haas medium: In 1L of milliQ water:

-0.2g Magnesium sulphate ($MgSO_4$)

-0.02g calcium chloride ($CaCl_2$)

-1.0g monopotassium phosphate ($KH2PO_4$)

-1.0g diammonium hydrogen phosphate ($(NH_4)2HPO_4$)

-1.0g potassium nitrate (KNO_3)

-0.05g ferric chloride ($FeCl_3$)

-15g agar (for the solid medium)

3-DGGE gel solution formulation

	30% denaturant	60% denaturant
40% Bis Acrylamide (1:37.5)	62.5ml	62.5ml

50xTAE	5ml	5ml
Deionised formmide	30ml	60ml
Urea	31.5g	63g
MilliQ water	Up to 250 ml	Up to 250 ml

For polymerisation, Ammonium persulfate (APS) and Tetramethylethylenediamine (TEMED) are used in the following proportions: to 12.5 ml of high and low-denaturing solutions , 62 µl of APS solution (1g of APS in 1ml of milliQ water) and 6.2 µl of TEMED are added.

4-Conposition of the Sanger sequencing reaction

Component	Volume
20 - 50 ng Band PCR Product	2 µl
BigDye® Terminator v3.1 (Life Technologies)	1 µl
338F Bacterial 16S rRNA forward primer (IDT)	0.32 µl (10 µM stock)
5X Buffer (Life Technologies)	3.5 µL
Nuclease-, RNase- free Molecular Grade Water (Thermo Scientific)	13.18 µl