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Mpumalanga province mining operations expansion below old oil bunkers impacts on water quality: Petroleum oil bioremediation strategies.

By

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Mini Dissertation

Submitted in partial fulfilment

of the requirements for the degree

MASTER OF SCIENCE

in

WATER RESOURCES MANAGEMENT

at the

Centre of Environmental Studies

UNIVERSITY OF PRETORIA

SOUTH AFRICA

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June 2015

Declaration

I declare that the mini dissertation that I hereby submit for the degree of M.Sc. in Water Resources Management at the University of Pretoria in the Department of Microbiology and Plant pathology has not previously been submitted by me for degree purposes at any University.

Acknowledgements

To my ever-present God, Your unconditional love and grace gave me strength.
To my supervisor Wouter le Roux, I am most grateful for your support and unrelenting patience. This research would not be complete without your advice.

I would like to thank Liesl Hill for the training on water toxicity testing and advice with a smile.

To all my colleagues at the Centre of Environmental Studies at the University of Pretoria, for all your support and all the words of encouragement. I am thankful to Rodney Mashele for all the practical assistance and advices.

I would like to thank my family for always believing in me and supporting me throughout my academic career. Your patience, love and prayers have helped me tremendously. To My mother, Virginia M'Sarabuye your unconditional love and prayers gave me strength.

To the Landu's family for all you have done for me. You welcomed me in your family as I was your own. May God bless you abundantly.

To my friends Basa Mashamba, Bubbly Morgets, Clovis Mulala, Doreen Busane and her husband Amani, Eliud Mushina, Erikka Mokanya, Joseph Kandem, Jeremie Kipongo, Marie-Claude Palata, Maphuti Dibete, Pascal Zariki, Peter Meki, Raphael Mukanya, Rony Kabanga, Serges Fofou, and all my friends back home, thank you for always being there for me, for all your support and words of encouragement.

Last, but not certainly least, my deepest gratitude goes to my loving fiancée Patricia Mukash. You have always supported me throughout this project, thank you for always believing in me.

Abstract

Industrialization has brought to the modern society the benefit of a comfortable modern lifestyle: health-giving pharmaceuticals, labor-saving households appliances, automobiles and ships, paints and detergents, synthetic fibers and polythene packaging, personal computers and televisions, just to name a few from an endless list of manufactured goods. However, behind the luxury and convenience of modern living lies the real price of this industrial production: the generation of hundreds of millions of tons of hazardous waste every year, leading to air, soil and water pollution due to the use of vast quantities of fossil fuels such as petroleum, coal and natural gas as energy sources.

In Mpumalanga province, in South Africa, a coal mining company is currently conducting investigations to expand its mining operations near Ogies; the coal is situated below old oil bunkers where crude oil was historically stored. There are concerns that, since not all the crude oil has been removed from the underground storage bunkers, the proposed mining activities may pose a serious environmental threat to the underground and surface water resources in the event of an oil spill or seepage. Petroleum hydrocarbon contains hazardous chemicals such as benzene, toluene, xylenes and naphthalene that expose the local environment to great toxic dangers.

Bioremediation, involving nutrient addition, being an economical and eco-friendly approach, has emerged as the most advantageous water clean-up technique for contaminated sites containing toxic metals and organic pollutants. This study investigated the effectiveness of nutrient application treatment compared to natural attenuation on two crude oils from the mine site bunkers: the Alpha bunker and the North and South bunkers. Results of the Alpha bunker crude oil experiments showed that both treatments conducted lead to the degradation of almost 100% of the oil after eight weeks of incubation, and a gradually decreased toxicity level in the water. The results suggested that the native microbial population was able to detoxify hazardous components of the crude oil. Due to the fast degradation rate observed with nutrient addition treatment, we recommended that biostimulation be considered as the in situ oil spill remediation strategy. And for the North and South bunkers crude oil, which responded less to treatments, probably due to its heavy nature, compared to the Alpha bunker crude oil, we suggested a combined treatment technique involving biosurfactants and nutrient addition.

Abbreviations

Al	Aluminium
C: N: P	Carbon: nitrogen: phosphorus ratio
CAF	Central Analytical Facilities
CSIR	Council for Scientific and Industrial Research
DNA	Dioxyribonucleic acid
DO	Dissolved oxygen
EC	Electro-conductivity of water
Fe	Iron
GEF	Global Environment Facility
GM-MS	Gas chromatography-mass spectrometry
ID	Identity
MDEP	Massachusetts Department of Environmental Protection
Mn	Manganese
NAS	National Academy of Sciences
NCBI	National Centre for Biotechnology Information
NRC	National Research Council
NSO	Nitrogen, sulphur and oxygen containing compound
OPCSA	Oil Pollution Control South Africa
OTA	Office of Technology Assessment
PAH	Polycyclic aromatic hydrocarbon
SFF	Strategic Fuel Fund
TV	Television
UNEP	United Nations Environmental Program
US EPA	United States Environmental Protection Agency
WCCM	Witbank Consolidated Coal Mine
WMA	Water management area

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Chapter I: Introduction

1.1 General introduction

Ever since the industrial revolution took off in the 18th century, vast quantities of fossil fuels, such as petroleum, coal and natural gas, have been used as the ideal energy source to power the economy and thus, delivered to the society the benefits of a comfortable modern lifestyle: health-giving pharmaceuticals, labour-saving household appliances, automobiles and ships, paints and detergents, synthetic fibers and polythene packaging, personal computers and televisions (TVs), just to name a few out of an endless list of manufactured goods. However, behind the luxury and convenience of modern living lies the real price of this industrial production: the generation of hundreds of million of tons of hazardous waste every year, leading to air, soil and water pollution while also contributing to increasing concentrations of greenhouse gases in the atmosphere, exposing local communities to great dangers (UNEP, 2002).

Petroleum oil pollution has been recognized as one of the most serious problems for biotic life. Despite recent technological advances, accidental spills of crude oil and its refined products occur on a frequent basis during routine operations of extraction, transportation, storage, refining and distribution (US EPA, 2001). An estimated 1.3 tons of petroleum enter the world's water each year, of which more than 90% is directly related to human activities including deliberate waste disposal. Contrary to popular perception, only 10 per cent of the annual total amount of oils entering the marine environment is accountable to accidents involving oil tankers and offshore installations (NRC, 2003).

Most research on the fate and effects of oil entering the aquatic environment has focus on marine systems, as most of the large oil spills that have received much attention and evoked public outcry have occurred in marine environments. Parallel concern for the freshwater environment has lagged behind. However, oil spills do occur in freshwater and they are more frequent and are often more destructive as a consequence of the many oil-related activities in this environment (Green and Trett, 1989). Freshwater bodies are not just the main source of drinking water, they also serve as nesting grounds and food sources for various organisms. Oil spills can pose a serious threat to freshwater ecosystems as the freshwater organisms are very sensitive to the toxic effects of petroleum hydrocarbons. All types of freshwater organisms are susceptible to the deadly effects of spilled oil, including mammals, aquatic birds, fish, insects, microorganisms, and vegetation. In addition, the effects of spilled oil on freshwater microorganisms, invertebrates, and algae tend to move up the food chain and affect other species (Nomack, 2010).

When petroleum is spilled into a water body, it initially spreads in the water (primarily on the surface), depending on its relative density and composition. The oil slick formed remains cohesive, or may break up in the case of rough seas. Waves, water currents, and winds force the oil slick to drift over large areas, impacting the open water, coastal areas, and marine and terrestrial habitats in the path of the drift. As petroleum contains hazardous chemicals such as benzene, toluene, etylbenzene, xylenes, and naphthalene, its immediate toxic effects include mass mortality and contamination of fish and other food species, but long-term ecological effects may be worse (Water Encyclopedia, 2015).

Because oil is widely used and despite all the precautions, it is almost certain that oil spills and leakage will continue to occur. Thus it is essential that we have effective countermeasures to deal with the problem. A number of approaches and technologies have been developed for controlling oil spills in soil and water environments. These technologies include physical, chemical and biological processes. Although conventional methods, such as physical removal, are the first response option, they rarely achieve complete clean-up of oil spills (US EPA, 2001). According to the United States of America' Office of Technology Assessment (OTA, 1990), current mechanical methods typically recover no more than 10-15 percent of the oil after a major spill. Incineration is also a source of air pollution. Chemical treatment includes direct injection of chemical oxidants into contaminated soil and water, thereby altering native aquatic chemistry. Biological treatment or biodegradation, most commonly involves the breakdown of contamination into nontoxic forms using microbiological processes (Riser-Roberts, 1998).

Biodegradation as a natural process may proceed slowly depending on the type of oil (i.e., light crude oils degrade faster than heavier oils), the environment characteristics (temperature, pH and salinity, oxygen) and available nutrients sources (nitrogen and phosphorus) for microbial growth (US EPA, 2001). Bioremediation, defined as "the act of adding materials to contaminated environments to cause an acceleration of the natural biodegradation processes" (OTA, 1991) is considered to be one of the most promising treatment options for oil removal since its successful application after the 1989 Exxon Valdez spill (Bragg et al., 1994). The success of oil spill bioremediation depends on one's ability to optimize various physical, chemical, and biological conditions in the contaminated environment. If microorganisms with the appropriate metabolic capabilities are present, then optimal rate of growth and hydrocarbon biodegradation can be sustained by ensuring that adequate concentrations of nutrients and oxygen are present and that other environmental factors are suitable (Leahy and Colwell, 1990).

Bioremediation has several advantages over conventional technologies. First, the application of bioremediation is relatively inexpensive. For example, during the clean-up of the Exxon Valdez spill, the cost of bioremediating 120 km of shoreline was less than one day's costs for physical washing (Atlas, 1995). Secondly, bioremediation is also a more environmentally benign technology since it involves the eventual degradation of oil to mineral products (such as carbon dioxide and water), while physical and chemical methods typically transfer the contaminant from one environmental compartment to another. Since bioremediation is based on natural processes and is less intrusive and disruptive to the contaminated site, this "green technology" may also be more acceptable to the general public (Fingerman and Nagabhushanam, 2005).

South Africa is one the most industrialized countries in Africa. The country accounts for about 30% of the total primary energy consumed in all of Africa according to BP Statistical Review of World Energy 2014 (EIA, 2015). Energy production has been, and still is, one of the main contributing factors to the social and economic development of South Africa. It has lent prosperity and security to the country by providing heat and power for industry, transportation, and household use (Ogunlade et al., 2006). On the other hand, South Africa is a water-scarce country, with its freshwater systems heavily used. Eighty-two percent of South Africa's 120 river ecosystem types are threatened, and 44 percent are critically endangered (GEF, 2008). The availability of freshwater constitutes one of the most critical factors of development in the country, and freshwater is decreasing in quality because of an increase in pollution and the destruction of river catchments, caused by urbanization, deforestation, damming of rivers, destruction of wetlands, industry, mining, agriculture, energy use and accidental water pollution (Rand Water, 2015). An oil spill into the freshwater would put more pressure on the already very sensitive and critical resource.

In Mpumalanga province, a coal mine company is currently conducting investigations to expand its mining operations near Ogies; the coal is situated below an old oil bunker where crude oil was historically stored. There are concerns that, since not all the crude oil has been removed from the underground storage bunkers, the proposed mining activities may pose a serious environmental threat to the underground and surface water resources in the event of an oil spill or seepage. This work investigates the possible use of bioremediation strategies in the event of an oil spill, to mitigate the impact of oil toxicity in water and thus protect the life of biota depending on it.

1.2 Scope and aim

The bunker covers a surface area of 2 200 000 m^2 (CSIR, 2014; SRK, 2010) and forms part of the Ogies oil storage scheme which consists of four mined-out coal mines that have been converted to natural storage for crude oil (CSIR, 2014; Fraser et al., 2001). It is estimated that 2 604 337 m^3 of crude oil was initially stored in the bunker and that 125 515 m^3 still remains (CSIR, 2014; Arthur Partridge, OPCSA, personal communication, November 2013). For this purpose, the mining company appointed the CSIR, Natural Resources and the Environment (Water Ecosystem and Human Health research group) to establish a baseline study in support of the proposed expansion and to determine the ecological and human risk posed to water resources which are in close proximity of the proposed mining operations in the event of oil spill or seepage.

Ogies is a settlement in Nkngala District Municipality in the Mpumalanga province, it occupies an area of 1.95 km^2, with a population estimated at 1230 in 2011 (Frith, 2011). Ogies terminal is located approximately 100 km east of Johannesburg, in the Witbank coal mining belt, in a farming region where maize is the dominant crop (CSIR 2014; Crafford, 2007). It is situated in the B1 secondary catchment of the Olifants Water Management Area (WMA), in the Upper Olifants sub-catchment. The Olifants River constitutes one of the main river systems in South Africa but is also regarded as one of the most hard-working and polluted river in Southern Africa. Economic activities taking place in the WMA are highly diverse and consist principally of mining; the area has a rich reservoir of coal, metallurgic activities, commercial activities, agriculture (commercial, dry land and subsistence) and ecotourism. The surface water resources in the catchment, in particular the upper catchment, are largely stressed from numerous and extensive land use practices taking place in the catchment. This leaves the aquatic ecosystems in this area particularly vulnerable.

The study investigated potential bioremediation strategies in the event of unwanted oil pollution during mining activities. Since recent field studies have demonstrated that nutrient addition is the more effective bioremediation approach (Lee et al., 1997a), the aim of this study is to:

- Present the background information on the crude oil present in the bunkers as well as the quality conditions of the water resources in the surrounding of the mining activities that might be impacted by the oil spill. This study is one component in the overall current baseline study being conducted on the proposed mining operations expansions and their impacts on the local environment.

- Present the patterns of oil bioremediation in water that need to be taken account in the oil spill clean-up strategies.

- Investigate the nutrient doses, time frames and efficiencies associated with biostimulation, and to compare it to natural attenuation.

1.3 Background Study

The baseline study conducted by the CSIR, department of Natural Resources and the Environment, Water Ecosystem health and human research group, had the following objectives:

1) To monitor the resource quality of aquatic ecosystems (streams, rivers and wetlands) in the surrounding area of the oil bunkers before mining operations start, to establish a pre-mining baseline. The outcome of this baseline would serve as a control in the case of oil seepage and would further be used to inform the design of the long term monitoring programme to be implemented by the mine.
2) Set up a hydrological model to determine the direction of groundwater flow so that ecosystems that are at high risk, should spillage occur, can be identified;
3) Characterize and fingerprint crude oil in the bunkers, to distinguish traces of other types that may already be present in the surrounding aquatic system with the oil type in the bunkers.
4) Investigate clean-up options and develop an oil spill response action plan to minimize risk to freshwater resources in the study area
5) Design a long term monitoring programme to monitor and report on the status and trends of aquatic systems in the surrounding area of the oil bunkers during mining operations.

1.3.1 Water Quality conditions

Prior to any proposed land use activities, it is important to establish what the baseline water quality conditions are, in order to determine the exact impact of these activities on the surrounding water resources. This will serve as reference point from which to monitor any future change.

The process involved the evaluation of chemical parameters in water resources and the assessment of pollutants toxicity on aquatic organisms.

1.3.1.1 Water chemistry monitoring

The concentration of various constituents in water column and in sediment was determined, water quality indicators were calculated, and water quality diagrams were constructed and compared to standards. In addition, hydrocarbon presence was also tested in water bodies to assess if it was due to a possible spill of oil from the bunkers or from another external source. The chemistry monitoring involved the following water bodies:

-Three wetlands/pans

- Outflowing streams from these pans,

- To the north, the Zaalklip river, and

- To the west, the Wilge river

Results from the analyses showed that:

● In the water column: some water bodies presented an increased alkalinity level, along with sulfates, chloride, sodium and calcium. The alkaline conditions also resulted in elevated metal concentrations, of which Al, Fe and Mn all exceeded the Target Water Quality Guidelines for water used for drinking and irrigation purposes (DWAF, 1996b; DWAF, 1996c).

The outflowing streams presented lower pH, higher sulfates and lower alkalinity, and were likely impacted by acid mine drainage. The sampling sites along the Zaalklip and Wilge River had relatively good water quality, yet had been impacted by anthropogenic activity to some extent.

All the sites presented similar hydrocarbons contamination, limited to volatile and short chain hydrocarbons suggesting no evident contamination of surface water by the oil in the bunkers.

● In the sediment: there was evidence of contamination by several metals at one station. Polycyclic aromatic hydrocarbons (PAH) were found to be relatively widespread contaminants at concentrations high enough at certain points to suggest a toxicological risk to sediment-dwelling organisms. However, it was unknown whether the chemical concentrations were in a bioavailable form due to a significant limitation of the use of sediment quality guidelines (McDonald et al., 2000) in interpreting the toxicological significance of chemical concentrations in sediment. Toxicity testing and the analysis of sediment-dwelling organism communities were performed to shed more light on the bioavailability and toxicity effect of chemicals.

1.3.1.2 Toxicity of pollutants

Different aquatic organisms were tested to assess the toxicity of pollutants in water bodies. These tests consisted of:

1) Aquatic macro-invertebrates as indicator of water quality impacts: Aquatic macro-invertebrates are the commonly used aquatic organisms for biomonitoring (CSIR, 2014; Haase and Nolte, 2008). They act as indicators of water quality disturbance because of their adaptation to specific habitats, subtract types and specific physico-chemical parameters. These disturbances result in the change of the types and abundances of aquatic macro-invertebrates (Rinne, 1990; Hillman and Quinn, 2002). From the selected monitored sites, one site presented the lower macro-invertebrate diversity, with disappearance of sensitive macro-invertebrate taxa, and the dominance of tolerant taxa. This indicated that high levels of pollutants were present at this site.

2) Vegetation condition of macrophytes using field spectroscopy: Most of the vegetation species when exposed to contaminants (mining impacted water for example) will show signs of toxicity, with impacts that include increased mortality rates, modifications to biochemical and physiological responses, and changes to metabolic processes (CSIR, 2014; Goetz et al., 1983; Mcfarlane et al., 2003). Vegetation may therefore provide valuable information to determine the status and condition of wetlands receiving mining waste water. Results of the field spectroscopy showed that two of the three sampling sites showed poor vegetation condition and thus constituted the most impacted sites.

3) Toxicity screening bioassays: Ecotoxicity is an approach that aims to identify the effects that chemical pollutants, alone or in combination with other stressors, have on biota in the

environment (Ester and Hermens, 2004). *Daphnia magna* is a widely used invertebrate species that is commonly utilized during toxicity testing. Algae may also be employed, and the unicellular green alga *Selenastrum caprivornutum* is more sensitive than some other standard test organisms to many common compounds and is used as a screening test for phytotoxicity. In this study, daphnids and algae were exposed to water samples collected from two pans on the mine property as well as three riverine sites named. Results of short term exposures (48 hours) for *Daphnia* showed no or a slight acute hazard. The alga test however, which is mostly more sensitive than *Daphnia*, pointed to the eutrophication potential at three of the sampling sites (one from the pans and two from the riverine sites).

1.3.2 Crude oil analysis

1.3.2.1 Location and composition

The bunker under which the mining operations will occur, named Alpha bunker, forms part of the Ogies oil storage scheme which consists of four mined-out coal mines that have been converted to natural storage containers for crude oil (Fraser et al., 2001)

At the moment, these underground oil storage bunkers are managed by Oil Pollution Control South Africa (OPCSA) on behalf of the Strategic Fuel Fund (SFF, SRK, 2010). The proposed mining expansion will occur north and south-east of the Alpha bunker (Figure 1). The Alpha bunker covers a surface area of 2 200 000 m^2 (SRK, 2010). Estimates suggest that 2 604 337 m^3 of crude oil was initially stored in the Alpha bunker and that 125 515 m^3 still remain (Arthur Partridge, OPCSA, personal communication, 8 November 2013). The under-recovery may be a result of the evaporation of volatiles, oil remaining behind (stuck on side walls and in pools), accounting inaccuracies, and migration of oil out of the container (Fraser et al., 2001).

Current operations at the Alpha bunker involve the periodic abstraction of water, via several dewatering boreholes, from the oil storage bunker. The abstraction of water creates a cone of drawdown (shallow Karoo aquifer), which ensures that there is no outflow of oil or degraded contaminants from the bunker, i.e. it induces groundwater flow towards the bunker. According to the CSIR (2008), approximately 80 000 m^3 a-1 (220 m^3 day^{-1}) is abstracted to maintain the water level close to the container floor. The abstracted water is passed through an oil separation process where oil is skimmed from the water and the remaining water is diverted to an evaporation dam.

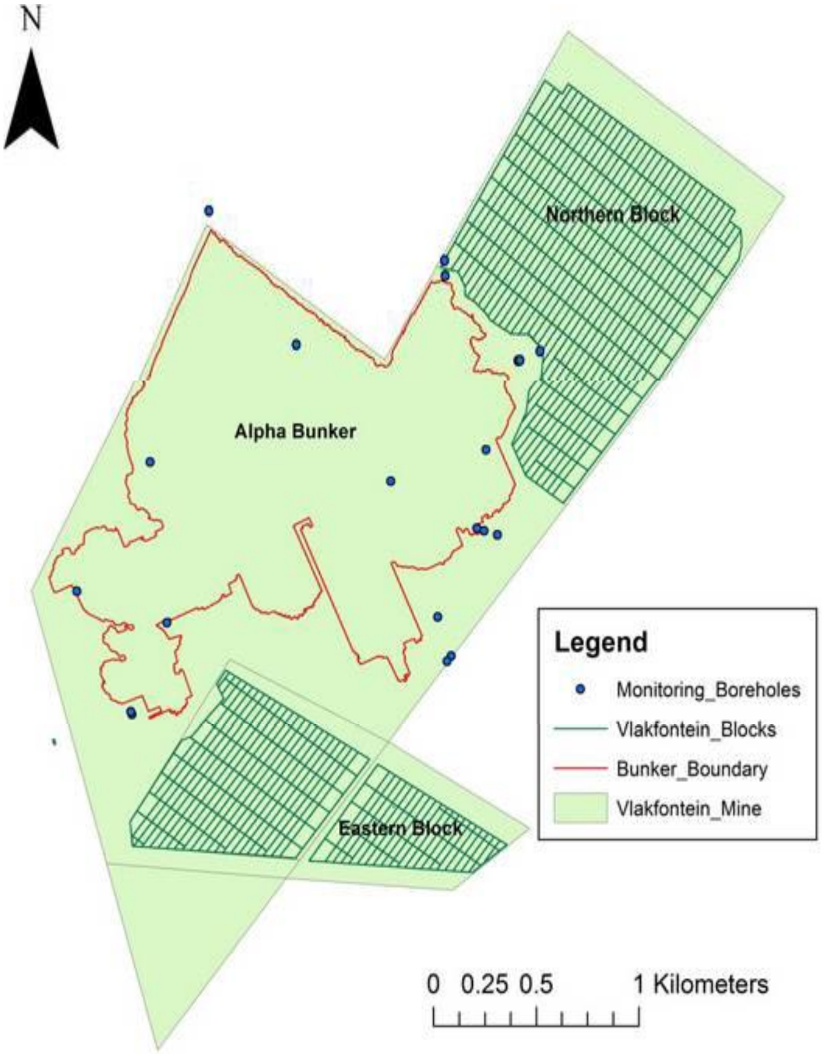


Figure 1: The Alpha Bunker position on the site.

The proposed mining operations will be deeper than the Alpha bunker, located in the N^o.4 seam, as both the N^{o}2 and N^{o}4 coal seams will be mined. A cone of groundwater drawdown will therefore develop around these new operations. If the lateral extent reaches the Alpha bunker, it will affect the groundwater gradient within the vicinity of the bunker which may lead to the migration of oil and degraded contaminants out of the bunker.

Chemical analysis of the North and South bunkers crude oil (which also served as fingerprinting of the Alpha bunker crude oil), through gas chromatography-mass spectrometry (GC-MS) at the CAF laboratory at Stellenbosch University (South Africa) revealed the following characteristics: The major PAH distribution for Naphthalenes C0-C4, Phenanthrenes C0-C3, Dibenzothiophene C0-C3 and Fluorene C0-C2 was determined from the normalized peak areas for the specific target ions in the GC-MS chromatogram. Herein, it was found that naphthalenes were the major PAH and that no chrysene was present. The preliminary results corresponded well to chromatograms for bunkers and heavy crude oils reported in the literature (Stout et al., 2002; Papazova and Pavlona, 1999; Wang et al., 2006).

1.3.2.2 Toxicity assessments

Crude oil constitutes a global environmental concern, due to components such as PAH which may induce non-lethal health impairment, such as disrupted endocrine signaling, immunodeficiency, metabolic disorders and developmental abnormalities (e.g. teratogenicity) (Irigaray et al., 2006; Ball and Truskewycz, 2013).

Aquatic vertebrates such as amphibians, form an important component of natural ecosystems and can function as indicator species for pollution due to trans-trophic bioaccumulation. Results of vertebrates exposed to contaminated water with oil from the Alpha bunker pointed to low toxicity in the water sample, suggesting limited impacts in terms of lethal toxicity in aquatic vertebrates during development. However these findings should be considered with caution due to possible prevalence of sub-organismal health impairment and developmental abnormalities.

1.3.3 Groundwater flow screening

The area groundwater flow was studied in order to understand the current groundwater dynamics within the vicinity of the Alpha bunker before the proposed mining operations take place. Knowing the direction of groundwater flow will facilitate the identification of ecosystems which may be at risk due to migration of oil and/or degraded contaminants out of the container; and to identify strategic long term groundwater monitoring points which will identify any groundwater contamination or significant deviations of the hydraulic gradient at the site. In terms of groundwater flow, results indicated that the regional direction of groundwater flow was towards the north/north-east. In the vicinity of the Alpha bunker (constant pressure boundary), the flow was distorted due to the induced hydraulic gradient, and thus flow is towards the bunker.

In this study, bioremediation strategies will be investigated (as a first step) under laboratory conditions (ex situ). Further research will therefore be required to provide field validated bioremediation strategies.

Chapter II: Literature review: Bioremediation of petroleum hydrocarbons

2.1 Introduction

Petroleum-based products are the major source of energy for industry and daily life. Despite recent technological advances, accidental spills of crude oil and its refined products occur on a frequent basis during routine operations of extraction, transportation, storage, refining and distribution (US EPA, 2001). Release of hydrocarbons into the environment whether accidentally or due to human activities is a main cause of water and soil pollution (Holliger, 1997). Soil and water contamination with hydrocarbons causes extensive damage of local ecosystems since accumulation of pollutants in animals and plant tissue may cause death or mutations (Alvarez and Vogel, 1991).

Because oil is widely used and despite all the precautions, it is almost certain that oil spills and leakage will continue to occur. Thus, it is essential that we have effective countermeasures to deal with the problem. A number of approaches and technologies have been developed for controlling oil spills in soil and water environments. These include mechanical, burying, evaporation, dispersion and washing methods. However, they are expensive and can lead to incomplete decomposition of contaminants (US EPA, 2001).

Bioremediation is considered to be one of the most promising treatment options for oil removal since its successful application after the 1989 Exxon Valdez spill (Bragg et al., 1994). In nature some microorganisms are capable of using petroleum oil as a source of carbon and energy. The process of bioremediation, consisting of the use of microbes to clean up contaminated soil and water, is based on the premise that a large percentage of oil components are readily biodegradable in nature (Atlas 1981). In addition, bioremediation is believed to be non-invasive and relatively cost-effective (April et al., 2000).

However, bioremediation like other technologies has its limitations. The success of oil bioremediation depends on many factors that include, amongst others, the behavior of oil in the environment, the availability in the polluted environment of the appropriate microorganisms under suitable environmental conditions, and the chemical composition of the oil (US EPA, 2001).

2.2 Factors affecting natural oil biodegradation and bioremediation success

Oil bioremediation is a complex process involving interactions of oil and microorganisms under the conditions of the prevailing environment. To understand the scope and strategies of oil bioremediation, it is essential to first understand the properties of oil, the environment of concern, the mechanisms of oil biodegradation and the factors that control its rate (EPA, 2001).

2.2.1 Chemical composition of petroleum hydrocarbon

Before considering the degradation of petroleum oil it is first essential to focus on its composition. Petroleum is defined as a mixture of natural gas, petroleum condensate, and crude oil. Crude oil is a heterogeneous liquid, comprised of hydrocarbon compounds (accounting for 50-98% of the total composition) consisting almost entirely of the elements hydrogen and carbon in the ratio of about two hydrogen atoms to one carbon atom, and non-hydrocarbon compounds containing sulphur, nitrogen, oxygen, and various trace metals, all of which constitute less than 3% of the total composition (Atlas, 1981).

Petroleum components may be classified into four major groups based on their differential solubility in organic solvents (Leahy and Colwell, 1990):

1. Saturated hydrocarbons: Include normal and branched alkanes with structures of C_nH_{2n+2} (aliphatics) and cyclic alkanes with structures of C_nH_{2n} (alicyclics), which range in chain length from one to over 40 carbons. Saturates usually are the most abundant constituents in crude oils.

2. Aromatic hydrocarbons: Include monocyclic aromatics (e.g., benzene, toluene, and xylenes) and polycyclic aromatic hydrocarbons (PAHs) (e.g., naphthalene, anthracene, and phenanthrene), which have two or more fused aromatic rings. Polycyclic aromatic hydrocarbons are of particular environmental concern because they are potential carcinogens or may be transformed into carcinogens by microbial metabolism.

3. Resins: Includes polar compounds containing nitrogen, sulfur, and oxygen, they are often referred to as NSO compounds (e.g., pyridines and thiophenes).

4. Asphaltenes: Consist of poorly characterized high molecular weight compounds that include both high molecular weight and poorly characterized hydrocarbons and NSOs. Metals such as nickel, vanadium, and iron are also associated with asphaltenes.

Hydrocarbons differ in their susceptibility to microbial attack and, in the past, have generally been ranked in the following order of decreasing susceptibility: n-alkanes > branched alkanes > low-molecular-weight aromatics > cyclic alkanes (Perry, 1984). Biodegradation rates have been shown to be highest for the saturates, followed by the light aromatics, with high-molecular-weight aromatics and polar compounds exhibiting extremely low rates of degradation (Fusey and Oudot, 1984). This pattern is not universal, however, as Cooney et al. (1985) reported greater degradation losses of naphthalene than of hexadecane in water-sediment mixtures from a freshwater lake and Jones et al. (1983) observed extensive biodegradation of alkylaromatics in marine sediments prior to detectable changes in the n-alkane profile of the crude oil tested. Fedorak and Westlake (1981) also reported a more rapid attack of aromatic hydrocarbons during the degradation of crude oil by marine microbial populations from a pristine site and a commercial harbor.

Horowitz and Atlas (1977), using an in situ continuous flow system in a study of biodegradation in Arctic coastal waters, and Bertrand et al. (1983), using a continuous-culture fermentor and a mixed culture of marine bacteria, observed degradation of all fractions of crude oil at similar rates, in marked contrast to the results of most other studies. In the latter investigation, experimental conditions were optimized and extensive degradation of resins (52%) and asphaltenes (74%) were observed. The microbial degradation of these fractions, which have previously been considered relatively recalcitrant to biodegradation (Rontani et al., 1985), can be ascribed to cooxidation, in which hydrocarbons that do not support growth are oxidized in the presence of hydrocarbons which can serve as growth substrates (Perry, 1984). Evidence for cooxidation of asphaltenes was provided by Rontani et al. (1985), who reported degradation of asphaltenic compounds in mixed bacterial cultures to be dependent upon the presence of n-alkanes 12 to 18 carbon atoms in length.

Compositional heterogeneity among different crude oils and refined products influences the overall rate of biodegradation both of the oil and of its component fractions. Walker et al. (1976) compared the degradation of two crude and two fuel oils by a mixed culture of estuarine bacteria. Low-sulfur, high-saturate South Louisiana crude oil was the most susceptible to microbial degradation, and high-sulfur, higharomatic Bunker C fuel oil was the least susceptible. Percent losses of saturated, aromatic, resinous, and asphaltenic hydrocarbons were highly variable among the four oils.

2.2.2 Physical state of petroleum hydrocarbons

Oil spilled in water tends to spread across the water surface and form a slick (Berridge et al., 1968). As a result of wind and wave action, oil-in-water or water-in-oil emulsions may form (Cooney, 1984). The mixing of oil with seawater occurs in several forms. Dispersion of the oil droplets into a water column is induced by the action of waves, while water-in-oil emulsification occurs when the petroleum contains polar components that act as emulsifiers. A water-in-oil emulsion containing more than 70% of seawater becomes quite viscous; it is called chocolate mousse from its appearance (Harayama et al., 1999). Dispersion of hydrocarbons in the water column in the form of oil-in-water emulsions increases the surface area of the oil and thus its availability for microbial attack. However, large masses (or plates) of oil establish unfavourably low surface to volume ratios, inhibiting biodegradation (Fedorak and Westlake, 1981). Tarballs, which are large aggregates of weathered and undegraded oil, also restrict access by microorganisms because of their limited surface area (Colwell et al., 1978).

2.2.3 Hydrocarbon-degrading microorganisms

Hydrocarbons and their derivatives, including solid, liquid and gaseous fossil carbon deposits, compounds of biological origin such as lipids and fatty acids from plants, animals and microbes and the products of their conversion in anoxic zones, are ubiquitous in the biosphere. Given the high carbon content available for biomass production, and the high energy content of such highly reduced compounds, it is hardly surprising that many microbes have evolved or acquired the ability to utilize hydrocarbons as sources of carbon and energy (Prince et al., 2003). Almost a century has passed since the first hydrocarbon-degrading bacteria were isolated and described, and the most recent list includes almost 200 bacterial, cyanobacterial, algal and fungal genera, representing more than 500 species and strains (Head et al., 2006). In the marine environment, bacteria are considered to be the predominant hydrocarbon-degraders with a distribution range that even covers extreme cold Antarctic and Arctic environments (Floodgate, 1984; Jordan and Payne, 1980). In the freshwater environment, yeast and fungi may also play a significant role in degrading petroleum hydrocarbons (Cooney, 1984). Table 1 lists of some of the most important hydrocarbon-degrading microorganisms known in both marine and freshwater environments.

Table 1: Microorganisms capable of degrading petroleum hydrocarbons (EPA, 2001, Based on Atlas, 1984; Jordan and Payne, 1980; Leahy and Colwell, 1990).

Bacteria	Yeast and Fungi
Achromobacter	*Aspergillus*
Acinetobacter	*Candida*
Alcaligenes	*Cladosporium*
Arthrobacter	*Penicillium*
Bacillus	*Rhodotorula*
Brevibacterium	*Sporobolomyces*
Cornybacterium	*Trichoderma*
Flavobacterium	
Nocardia	
Pseudimonas	
Vibrio	

2.2.4 Environmental factors affecting oil biodegradation

Abiotic factors influence the weathering of petroleum hydrocarbons in the environment, and impact on the biodegradation of the oil. Factors which influence rates of microbial growth and enzymatic activities affect the rates of petroleum hydrocarbon biodegradation. These factors include weathering processes, temperature, availability and concentration of nutrients, availability and concentration of oxygen, and pH (Atlas, 1981).

2.2.4.1 Temperature

Temperature influences petroleum biodegradation by its effect on the physical nature and chemical composition of the oil, rate of hydrocarbon metabolism by microorganisms, and composition of the microbial community (Atlas, 1981). At low temperatures, the viscosity of the oil increases, the volatilization of toxic short-chain alkanes is reduced, and their water solubility is increased, delaying the onset of biodegradation (Atlas and Bartha, 1973). Rates of degradation are generally observed to decrease with decreasing temperature; this is believed to be a result primarily of decreased rates of enzymatic activity, or the "Qlo" effect (Atlas and Bartha, 1973). Higher degradation rates generally occur in the range of 30 to 40°C in soil environments, 20 to

30°C in some freshwater environments, and 15 to 20°C in marine environments (Bossert and Bartha, 1984).

2.2.4.2 Oxygen

The initial steps in the catabolism of aliphatic (Singer and Finnerty, 1984), cyclic, and aromatic (Cerniglia, 1984) hydrocarbons by bacteria and fungi involve the oxidation of the substrate by oxygenases, for which molecular oxygen is required. Aerobic conditions are therefore necessary for this route of microbial oxidation of hydrocarbons in the environment. Conditions of oxygen limitation normally do not exist in the upper levels of the water column in marine and freshwater environments. Aquatic sediments, however, are generally anoxic except for a thin layer at the surface of the sediment (Cooney, 1984). The availability of oxygen is dependent on rates of microbial oxygen consumption, the type of soil, whether the soil is waterlogged, and the presence of utilizable substrates which can lead to oxygen depletion, the wave and water flow and the physical state of the oil (EPA, 2001; Bossert and Bartha, 1984). The concentration of oxygen has been identified as the rate-limiting variable in the biodegradation of petroleum in soil and of gasoline in groundwater (Jamison et al., 1975).

2.2.4.3 Nutrients

In theory, approximately 150 mg of nitrogen and 30 mg of phosphorus are utilized in the conversion of 1 g of hydrocarbon to cell materials (Rosenberg and Ron, 1996). The release of hydrocarbons into aquatic environments which contain low concentrations of inorganic nutrients often produces excessively high carbon: nitrogen or carbon: phosphorus ratios, or both, which are unfavorable for microbial growth (Atlas, 1981). It is well established that the availability of nitrogen and phosphorus limits the microbial degradation of hydrocarbons in estuarine water and sediment, seawater, marine sediment, freshwater lakes, Arctic ponds, freshwater sediments (Cooney et al., 1985) and groundwater (Jamison et al., 1975)

Adjustment of carbon:nitrogen:phosphorus ratios by the addition of nitrogen and phosphorus in the form of oleophilic fertilizers, including paraffinized urea, octylphosphate, ferric octoate, paraffin-supported $MgNH_4P0_4$, and 2-ethylhexyldipolyethylene oxide phosphate, stimulates the biodegradation of crude oil and individual hydrocarbons in seawater and in Arctic ponds and lakes (Atlas and Bartha, 1973). Inorganic salts of nitrogen and phosphorus are effective in enclosed systems but tend to wash out in simulated field experiments (Atlas and Bartha, 1973).

2.2.4.4 Salinity and pH

Other important factors affecting biodegradation of petroleum hydrocarbons include pH and salinity. The pH of seawater is generally stable and slightly alkaline (Bossert and Bartha, 1984). In contrast, the pH of freshwater and soil environments can vary widely. Organic soils in wetlands are often acidic, while mineral soils have more neutral and alkaline conditions. Most heterotrophic bacteria and fungi favor a neutral pH, with fungi being more tolerant of acidic conditions. Studies have shown that degradation of oil increases with increasing pH, and that maximum degradation occurs under slightly alkaline conditions (Dibble and Bartha, 1979).

There are few published studies which deal with effects of salinity on the microbial degradation of hydrocarbons. Changes in salinity may affect oil biodegradation through alteration of the microbial population. Dramatic variation in salinity may occur in estuarine environments where marine organisms mingle with freshwater forms. Many freshwater organisms can survive for long periods in seawater although few can reproduce. In contrast, most marine species have an optimum salinity range of 2.5 to 3.5% and grow poorly or not at all at salinity lower than 1.5 to 2% (Zobell, 1973).

2.2.5 Behavior of oil in the environment

2.2.5.1 Weathering process

When petroleum is spilled into the sea, it spreads over the surface of the water and immediately goes through a variety of modifications which change its composition. This process is called weathering, and is mainly due to evaporation of the low-molecular-weight fractions, dissolution of the water-soluble components, mixing of the oil droplets with seawater, photochemical oxidation, and biodegradation (Harayama et al., 1999).

2.2.5.1.1 Evaporation

Soon after spreading of oil on water, petroleum components with low-molecular-weight, with a boiling point below 250°C are subject to evaporation (Harayama et al., 1999). In terms of environmental impact, evaporation is the most important weathering process during the early stages of an oil spill in that fact that it can be responsible for the removal of a large fraction of the oil including the more toxic, lower molecular weight components. Evaporation removes virtually all the normal alkanes smaller than C_{15} within 1 to 10 days (Harayama et al., 1999). Volatile aromatic compounds such as benzene and toluene can also be rapidly removed from an oil spill slick through evaporation. However these oil components may be more persistent when oil is stranded in sediments. The volatile components make up 20-50% of most crude oils, about 75% of fuel oil, and about 100% of gasoline and kerosene. As a result, the physical properties of the remaining slick change significantly (e.g., increased density and velocity). Major factors influencing the rate of evaporation include composition and physical properties of the oil, wave action, wind velocity, and water temperature (US EPA, 2001; Jordan and Payne, 1980).

2.2.5.1.2 Dissolution

Although dissolution is less important from the view point of mass loss during an oil spill, dissolved hydrocarbon concentrations in water are particularly important due to their potential influence on the success of bioremediation and the effect of toxicity on biological systems. The extent of dissolution depends on the solubility of the spilled oil, weather conditions, and the characteristics of the spill site. The low molecular weight aromatics are the most soluble oil components, and they are also the most toxic components in crude and refined oils. Although many of them may be removed through evaporation, their impact on the environment is much greater than simple mass balance considerations would imply (US EPA, 2001; NAS, 1985). Dissolution rates are also influenced by photochemical and biological processes.

2.2.5.1.3 Photooxidation

Under sunlight, petroleum discharged at sea is subjected to photochemical modification. In the presence of oxygen, natural sunlight has sufficient energy to transform many complex petroleum compounds such as high molecular weight aromatics and polar compounds into simpler compounds through a series of free-radical chain reactions. This process may increase the solubility of oil in water, due to formation of polar compounds such as hydroperoxides, aldehydes, ketones, phenols, and carboxylic acids. Detrimental effects may be associated with this increase in the solubility of oil in water (i.e., bioavailability) and the formation of toxic compounds mediated by photooxidation. On the other hand, the formation of polar compounds may increase the rate of biodegradation of petroleum, particularly at lower concentrations where acute toxicity effects are limited (Nicodem et al. 1997).

2.2.5.1.4 Dispersion

Dispersion, or formation of oil-in-water emulsions, involves incorporating small droplets of oil into the water column, resulting in an increase in surface area of the oil. In general, oil-in-water emulsions are not stable. However, they can be maintained by continuous agitation, interaction with suspended particulates, and the addition of chemical dispersants. Dispersion may influence oil biodegradation rates by increasing the contact between oil and microorganisms and/or by increasing the dissolution rates of the more soluble oil components (US EPA, 2001).

2.2.5.1.5 Emulsification

The process of emulsification of oils involves a change of state from an oil-on-water slick or an oil-in-water dispersion to a water-in-oil emulsion, with the eventual possible formation of a thick, sticky mixture that may contain up to 80% water, commonly called “chocolate mousse”. The formation and stability of emulsions are primarily related to the chemical composition of the oils and are enhanced by wax and asphaltic materials. Surface-active materials (surfactants) generated through photochemical and biological processes are also involved in formation of the emulsions. The formation of emulsions makes oil clean-up operations more difficult by decreasing the effectiveness of physical oil spill recovery procedures and suppressing the natural rates of oil biodegradation (US EPA, 2001). Biosurfactants consist of amphiphilic compounds that reduce surface and interfacial tensions by accumulating at the interface of immiscible fluids or of a fluid and a solid and increase the surface area of insoluble compounds leading to increased mobility, bioavailability and subsequently biodegradation (Banat et al., 2000).

2.2.5.1.6 Biodegradation

Biodegradation of oil is one of the most important processes involved in weathering and the eventual removal of petroleum from the environment, particularly for the non-volatile components of petroleum (US EPA, 2001).

Microorganisms capable of degrading petroleum hydrocarbons and related compounds are ubiquitous in marine, freshwater, and soil habitats. Bacteria and fungi, and to a lesser extent, heterotrophic phytoplankton, utilize hydrocarbons as a carbon source to produce energy, while subsequently degrading the long-chained molecules in a metabolic process called oxidative phosphorylation, or respiration (Prince, 2002). Moreover, there are different types of microorganisms that use other metabolic pathways such as nitrate reduction and sulphate reduction to degrade hydrocarbons into carbon dioxide and water. However, a consortium of bacterial strains usually uses multiple metabolic pathways in order to degrade complex hydrocarbons such as branched alkanes and multicyclic compounds (polycyclic aromatic and aliphatic hydrocarbons) (Sugiura et al., 1997).

2.3 Petroleum oil spill remediation strategies

Strategies for cleaning up an oil spill are greatly affected by a variety of factors, such as the type of soil, the characteristics of the spill site, and occasionally political considerations. A number of approaches and technologies have been developed for controlling oil spills in marine shorelines and freshwater environments. These methods are briefly described in the following table.

Table 2: Conventional Oil spill clean-up options (US EPA, 2001).

Category of response option	Example technology
Natural method	Natural attenuation
Physical method	Booming Skimming Manual removal (Wiping) Mechanical removal Washing Sediment relocation/Surf-washing Tilling *In-situ* burning
Chemical method	Dispersants, Demulsifies, Solidifiers Surface film chemicals
Bioremediation	Biostimulation, Bioaugmentation

2.3.1 Natural methods

Natural attenuation or natural recovery is basically a no-action option that allows oil to be removed and degraded by natural means. For some spills, it is probably more cost-effective and ecologically sound to leave an oil-contaminated site to recover naturally than to attempt to intervene (US EPA, 2001). Examples of such cases are spills at remote or inaccessible locations when natural removal rates are fast, or spills at sensitive sites where clean-up actions may cause more harm than good. It should also be noted that when natural attenuation is used as a clean-up method, a monitoring program is still required to assess the performance of natural attenuation. Major natural processes that result in the removal of oils include:

- Evaporation: Evaporation is the most important natural cleaning process during the early stages of an oil spill, and it results in the removal of lighter-weight components in oil (US EPA, 2001).

- Photooxidation: Photooxidation leads to the breakdown of more complex compounds into simpler compounds that tend to be lighter in weight and more soluble in water, allowing them to be removed further through other processes (US EPA, 2001).

- Biodegradation: Biodegradation is a particularly important mechanism for removing the non-volatile components of oil from the environment. This is a relatively slow process and may require months to years for microorganisms to degrade a significant fraction of an oil stranded within the sediments of marine and/or freshwater environments (US EPA, 2001).

2.3.2 Physical methods

Commonly used physical methods include:

- Booming and skimming: Use of booms to contain and control the movement of floating oil and use of skimmers to recover it. The environmental impact of this method is minimal if traffic of the clean-up work force is controlled (US EPA, 2001).

- Wiping with absorbent materials: Use of hydrophobic materials to wipe up oil from the contaminated surface. While the disposal of contaminated waste is an issue, the environmental effect of this method is also limited if traffic of clean-up crew and waste generation is controlled (US EPA, 2001).

- Mechanical removal: Collection and removal of oiled surface sediments by using mechanical equipment. This method should be used only when limited amounts of oiled materials have to be removed. It should not be considered for clean-up of sensitive habitats or where beach erosion may result (US EPA, 2001).

- Washing: Washing of the oil adhering along the shorelines to the water's edge for collection. Washing strategies range from low-pressure cold water flushing to high-pressure hot water flushing. This method, especially using high-pressure or hot water, should be avoided for wetlands or other sensitive habitats (US EPA, 2001).

- Sediment relocation and tilling: Movement of oiled sediment from one section of the beach to another or tilling and mixing the contaminated sediment to enhance natural cleansing processes by facilitating the dispersion of oil into the water column and promoting the interaction between oil and mineral fines. Tilling may cause oil penetration deep into the shoreline sediments. The potential environmental impacts from the release of oil and oiled sediment into adjacent water bodies should also be considered (US EPA, 2001).

- In situ burning: Oil on the shoreline is burned usually when it is on a combustible substrate such as vegetation, logs, and other debris. This method may cause significant air pollution and destruction of plants and animals (US EPA, 2001).

2.3.3 Chemical methods

Chemical methods, particularly dispersants, have been routinely used in many countries as a response option. For some countries, such as the United Kingdom, where rough coastal conditions may make mechanical response problematic, dispersants are the primary choice (Lessard and Demarco, 2000). However, chemical methods have not been extensively used in the United States due to the disagreement about their effectiveness and the concerns of their toxicity and long-term environmental effects (US EPA, 1999b). Major existing chemical agents include:

- Dispersants: Dispersing agents, which contain surfactants, are used to remove floating oil from the water surface to disperse it into the water column before the oil reaches and contaminates the shoreline. This is done to reduce toxicity effects by dilution to benign concentrations and accelerate oil biodegradation rates by increasing its effective surface area (US EPA, 2001).

- Demulsifiers: Used to break oil-in-water emulsions and to enhance natural dispersion.

- Solidifiers: Chemicals that enhance the polymerization of oil can be used to stabilize the oil, to minimize spreading, and to increase the effectiveness of physical recovery operations (US EPA, 2001).

- Surface film chemicals: Film-forming agents can be used to prevent oil from adhering to shoreline substrates and to enhance the removal of oil adhering to surfaces in pressure washing operations (US EPA, 2001).

2.3.4 Bioremediation

Although conventional methods, such as physical removal, are the first response option, they rarely achieve complete clean-up of oil spills. According to the United States of America's Office of Technology Assessment (OTA, 1990), current mechanical methods typically recover no more than 10-15% of the oil after a major spill. Bioremediation has emerged as one of the most promising secondary treatment options for oil removal since its successful application after the 1989 Exxon Valdez spill (Bragg et al., 1994; Prince et. al., 1994).

Bioremediation has several advantages over conventional technologies. First, the application of bioremediation is relatively inexpensive. For example, during the clean-up of the Exxon Valdez spill, the cost of bioremediating 120 km of shoreline was less than one day's costs for physical washing (Atlas, 1995). Bioremediation is also a more environmentally benign technology since it involves the eventual degradation of oil to mineral products (such as carbon dioxide and water), while physical and chemical methods typically transfer the contaminant from one environmental compartment to another. Since it is based on natural processes and is less intrusive and disruptive to the contaminated site, this "green technology" may also be more acceptable to the general public.

Bioremediation has been defined as "the act of adding materials to contaminated environments to cause an acceleration of the natural biodegradation processes" (OTA, 1991). Biodegradation as a natural process may proceed slowly, depending on the type of oil (i.e., light crude oils degrade faster than heavier oils). Bioremediation strategies are based on the application of various methodologies to increase the rate or extent of the biodegradation process. The success of oil spill bioremediation depends on the ability to optimize various physical, chemical, and biological conditions in the contaminated environment. If the microorganisms with the appropriate metabolic capabilities are present, then optimal rate of growth and hydrocarbon biodegradation can be sustained by ensuring that adequate concentrations of nutrients and oxygen are present and that other environmental factors are suitable. There are two main approaches to oil spill bioremediation:

- Bioaugmentation, in which known oil-degrading bacteria are added to supplement the existing microbial population, and

- Biostimulation, in which the growth of indigenous oil degraders is stimulated by the addition of nutrients or other growth-limiting cosubstrates, and/or by alterations in environmental conditions (e.g. surf-washing, oxygen addition by plant growth, etc.).

Both laboratory studies and field tests have shown that bioremediation, with biostimulation in particular, can enhance oil biodegradation on contaminated shorelines (Swannell, et al., 1996). Field studies have also demonstrated that biostimulation is a more effective approach because the addition of hydrocarbon-degrading microorganisms often does not enhance oil degradation more than simple nutrient addition (Lee et al., 1997a). Indigenous microorganisms isolated from the contaminated site are certainly adapted to the prevailing climatic, physicochemical and nutrient conditions. Bioremediation by these microorganisms is expected accelerate after nutrient addition and/or seeding with enriched microorganism cultures. According to Atlas et al. (1978) bioremediation using introduced microorganisms pose issues of (1) lack of controlled experiments demonstrating superior performance of introduced microorganisms compare to indigenous ones, (2) time lag between microorganism application and hydrocarbon break-down, (3) lack of information on microorganism pathogenicity to humans, genetic stability and toxicity of metabolic by-products, (4) large quantities of microorganisms required for frequent application to the contaminated site, (5) logistics of culture preparation and mixing just prior to application on site, and (6) fate of these microorganisms once they have completed their role in bioremediation.

However, as petroleum hydrocarbons exist as a complex mixture of hydrocarbons, and despite their huge potential to degrade organic compounds under favorable conditions, no single species of microorganism can degrade all the components of a given oil. These microbial communities may not include the full range of species or enzymes required for effective oil biodegradation.

Commercially available preparations of oil-biodegrading microorganisms usually include many species and have an increased potential to degrade various oil components effectively. Suppliers claim that these mixtures can be custom-made for the specific oil or environmental conditions and can also be easily produced for emergency situations (US EPA, 2001).

In final, bioremediation is not a panacea against organic contamination. The spectacular results of laboratory experiments cannot always be transferred directly to the field. Many compounds that are easily metabolized in vitro are often not broken down efficiently in contaminated soils and aquifers. This is probably due to reduced contaminant bioavailability caused by adsorption on soil particles or solution in no aqueous-phase liquids (US EPA, 2001).

2.4. Petroleum hydrocarbon toxicity and the environment

2.4.1 Petroleum hydrocarbon toxicity

Assessing toxicity of petroleum hydrocarbon is not an easy task. The main reason is that petroleum hydrocarbon is a mixture of thousands of components. The toxicity of petroleum hydrocarbons depends on the solubility and the bioavailability of the hydrocarbons. Only the bioavailable forms of chemicals are toxic to aquatic organisms (Neff, 2002).

A chemical is said to be bioavailable if it is in a form that can move through or bind to the surface membranes of an organism (e.g., skin, gill epithelium, gut lining, cell membrane). Thus, the exposure concentration of hydrocarbons to aquatic and sediment-dwelling organisms is the fraction of the total hydrocarbon in the ambient medium (including the gut) that is in a bioavailable form (dissolved in the water and in contact with a permeable membrane) (MDEP, 2007).

In the past it was assumed that the water soluble fractions of the aromatics and polyaromatics were the most harmful and thus these compounds were the molecules for considering in toxicological studies. They are assumed to be mutagenic and carcinogenic (Keith and Telliard, 1979). Polyaromatic hydrocarbons with 4 or 5 rings are known carcinogens (Van der Heul, 2009; Cerniglia, 1992). The non-aromatic substances in the petroleum were not considered very harmful. This is in fact not true, and alkanes and cycloalkanes are now also taken into account (Van der Heul, 2009; Peterson, 1994). Hydrophobic hydrocarbons are toxic for microorganisms by accumulation in the membrane, which causes the loss of membrane integrity (Van der Heul, 2009; Sikkema et al., 1995). As toxicology depends on concentration, biodegradation is an important topic in petroleum toxicology because it changes both the nature and concentration of the chemical compounds (US EPA, 2001).

2.4.2 Oil spill impact on the environment

Spilled oil poses serious threats to aquatic environments. It affects surface resources and a wide range of subsurface organisms that are linked in a complex food chain that includes human food resources. Spilled oil can harm the environment in several ways, including physical damage that directly impacts wildlife and habitats (such as coating birds or mammals with a layer of oil), and the toxicity of the oil itself, which can poison exposed organisms. The severity of an oil spill's impact depends on a variety of factors, including the physical properties of the oil, whether oils are petroleum-based or non-petroleum-based, and the ultimate fate of the spilled oil (Nomack, 2010).

The various freshwater and marine habitats have different sensitivities to the harmful effects of oil contamination, as well as different abilities to recuperate. Although some organisms may be seriously injured or killed very soon after contact with oil, other effects are more subtle and often longer lasting. For example, freshwater organisms are at risk of being smothered by oil that is carried by the current, or of being slowly poisoned by long-term exposure to oil trapped in shallow water or stream beds. In addition, oil can potentially have catastrophic effects on birds and mammals (Nomack, 2010).

2.4.2.1 Sensitivity of freshwater habitats

Oil spills occurring in freshwater bodies are given less attention than spills into the ocean, even though freshwater oil spills are more frequent and often more destructive to the environment. Freshwater bodies are highly sensitive to oil spills and are important to human health and the environment. They are often used for drinking water and frequently serve as nesting grounds and food sources for various freshwater organisms. All types of freshwater organisms are susceptible to the deadly effects of spilled oil, including mammals, aquatic birds, fish, insects, microorganisms, and vegetation. In addition, the effects of spilled oil on freshwater microorganisms, invertebrates, and algae tend to move up the food chain and affect other species (Nomack, 2010).

Freshwater can be divided in to two types: Standing water (lakes, marshes, and swamps) and flowing water (rivers and streams). The effects of an oil spill on freshwater habitats vary according to the rate of water flow and the habitat's specific characteristics (Nomack, 2010).

Standing water such as marshes or swamps with little water movement are likely to incur more severe impacts than flowing water because spilled oil tends to "pool" in the water and can remain there for long periods of time. In calm water conditions, the affected habitat may take years to restore. The variety of life in and around lakes has different sensitivities to oil spills (Nomack, 2010).

2.4.2.2 Sensitivity of marine habitats

The marine environment is made up of complex interrelations between plant and animal species and their physical environment. Harm to the physical environment will often lead to harm for one or more species in a food chain, which may lead to damage for other species further up the chain. Where an organism spends most of its time (in open water, near coastal areas, or on the shoreline) will determine the effects an oil spill is likely to have on that organism.

In open water, marine organisms such as fish and whales have the ability to swim away from a spill by going deeper in the water or further out to sea, reducing the likelihood that they will be harmed by even a major spill. Marine animals that generally live closer to shore, such as turtles, seals, and dolphins, risk contamination by oil that washes onto beaches or by consuming oil-contaminated prey. In shallow waters, oil may harm sea grasses and kelp beds that are used for food, shelter, and nesting sites by many different species (Nomack, 2010).

2.4.2.3 Sensitivity of birds and mammals

An oil spill can harm birds and mammals by direct physical contact, toxic contamination, and destruction of food resources. One of the more difficult aspects of oil spill response is the rescue of oiled birds and mammals (Nomack, 2010). Sensitivity of birds and mammals to oil spill include:

- Physical contact: When fur or feathers come into contact with oil, they get matted down. This matting causes fur and feathers to lose their insulating properties, placing animals at risk of freezing to death. As the complex structure of the feathers that allows birds to float becomes damaged, the risk of drowning increases for birds (Nomack, 2010).

- Toxic contamination: Some species are susceptible to the toxic effects of inhaled oil. Oil vapours can cause damage to an animal's central nervous system, liver, and lungs. Animals are also at risk from ingesting oil, which can reduce the animal's ability to eat or digest its food by damaging cells in the intestinal tract. Some studies show that there can be long-term reproductive problems in animals that have been exposed to oil (Nomack, 2010).

- Destruction of food resources**:** Even species that are not directly in contact with oil can be harmed by a spill. Predators that consume contaminated prey can be exposed to oil through ingestion. Because oil contamination gives fish and other animals unpleasant tastes and smells, predators will sometimes refuse to eat their prey and may begin to starve. Sometimes, a local population of prey organisms is destroyed, leaving no food resources for predators (Nomack, 2010).

2.4.3 Petroleum oil toxicity testing

The toxicity level of hydrocarbons in marine environment can be assessed by estimating the hydrocarbon concentration in the sediment porewater and then compare the estimated concentration to water quality criteria for the hydrocarbon, as described in USEPA guidance (Hansen et al., 2003). The hydrocarbon compounds that are taken into account in toxicity testing are given in **Table 3**.

Table 3: Hydrocarbon compounds considered in eco toxicity (MDEP, 2007)

Aliphatic hydrocarbons	Aromatic hydrocarbons
n-Pentane	Benzene
2,2-Dimethylbutane	Toluene
Cyclopentane	Ethylbenzene
2,3-Dimethylbutane	p-Xylene
2-Methylpentane	o-Xylene
3-Methylpentane	Isopropylbenzene
n-Hexane	n-Propylbenzene
2,2-Dimethylpentane	1-Methyl-4-ethylbenzene
Methylcyclopentane	1,3,5-Trimethylbenzene
2,4-Dimethylpentane	Isobutylbenzene
2,2,3-Trimethylbutane	Sec-Butylbenzene
3,3-Dimethylpentane	1-Methyl-4-isopropylbenzene
Cyclohexane	n-Butylbenzene
2-Methylhexane	1,2,4,5-Tetramethylbenzene
2,3-Dimethylpentane	Naphthalene
3-Methylhexane	2-Methylnaphthalene
2,2,4-Trimethylpentane	1-Methylnaphthalene
Heptane	2-Ethylnaphthalene
n-Propylcyclopentane	Biphenyl
Methylcyclohexane	2,6-Dimethylnaphthalene
1,1,3-Trimethylcyclopentane	2,3-Dimethylnaphthalene
2.3.4-Trimethylpentane	Acenaphthene
2,3-Dimethylhexane	Fluorene
2-Methylheptane	Phenanthrene
3-Methylheptane	Anthracene
1,4-Dimethylcyclohexane	1-Methylphenanthrene
2,2,5-Trimethylhexane	Pyrene
1,2-Dimethylcyclohexane	Fluoranthene
Octane	Benzo(a)fluorine
4-Methyloctane	Benz(a)anthracene
3-Methyloctane	Chrysene
Nonane	Benzo(a)pyrene
Decane	Coronene
Undecane	Benzo(ghi)perylene
Dodecane	Dibenz(a,h)anthracene
Tetradecane	
Pentadecane	
Hexadecane	
Heptadecane	
Octadecane	
Nonadecane	
Eicosane	
Tetracosane	

Another approach is bioassay tests which involve the use of a biological organism to test for chemical toxicity. Bioassays provide a more accurate picture of ecosystem health at a contaminated site than chemical analyses, because their result is an integration of the interaction that occurs between the contaminant and environmental variables. Bioassay endpoints are quantitative measures of toxicity (USEPA, 2001). Organisms used in bioassays include:

- Invertebrates: Chronic toxicity test using macro invertebrates have been extensively used in aquatic risks assessment studies. The parameters measured are mortality or reproduction. One of the most common invertebrate toxicity tests uses *Daphnia* and *Ceriodaphnia*, both freshwater species pertaining to *Cladocera*. Tests are carried out by exposing the test organisms to toxic substances under control conditions (US EPA, 2002).

- Algae and plants: Test species, such as marine unicellular algae *Selenastrum capricornutum* or *Dunaliella tertiolecta* are used as indicator species. Inhibition of algal growth is used as the indicator of toxicity. The main disadvantages of algal methods are a lack of reproducibility between consecutive assays (US EPA, 2002).

- Fish: Due to their economic, recreational, and aesthetic value, fish have been historically selected as a primary bioassay organism. Difficulties in using fish as biomonitors of sediment contamination arise from their preference for particular sediments or habitats and their residence time in or over contaminated areas. Furthermore, their absence in a water body may more directly reflect water quality (USEPA, 2002).

The response of the test organisms to the toxicant or test sediment is often affected by its life stage. Larval or juvenile life stages are generally more sensitive than adults.

Chapter III: Material and methods

3.1 Study area

Ogies, which is close to where the mine expansions are taking place, is a settlement in Nkngala District Municipality in the Mpumalanga province of South Africa. Ogies terminal is located 100 km east of Johannesburg, it is a coal mining town 29 km south-west of Witbank and 70 km north-east of Springs. It was laid out in 1928 on the farm Oogiesfontein, 'fountain with many "eyes" or springs'. The name is derived from that of the farm (Raper, 2010).

Ogies occupies an area of 1.95 km^2, with a population estimated at 1230 in 2011 (Frith, 2011). It is situated in the B1 secondary catchment of the Olifants Water Management Area (WMA), in the Upper Olifants sub-catchment. The Olifants River constitutes one of the main river systems in South Africa and is one of the most hard-working and polluted rivers in Southern Africa due to numerous and extensive land use practices taking place, particularly in the upper catchment of the surface water resources (CSIR, 2014).

Economic activities taking place in the WMA are highly diverse and consist principally of mining; the area has a rich reservoir of coal, metallurgic activities, commercial activities, agriculture (commercial, dry land and subsistence) with maize as the dominant crop, and eco-tourism, placing the Olifants WMA as one of the most economically important in South Africa (CSIR, 2014; DWA, 2011). The rich mineral deposits present in the Olifants River catchment are a key economic driver in the area. Mining within the upper Olifants sub-catchment consists almost entirely of thermal coal mining for the power stations in the WMA (CSIR, 2014).

3.2. Sampling site

For the bioremediation experiment, water samples were collected from an impacted pan, which was named PAN 1 (Figure 2) and is closest to the mining activities (**Figure 3**).

Figure 2: The sampling site

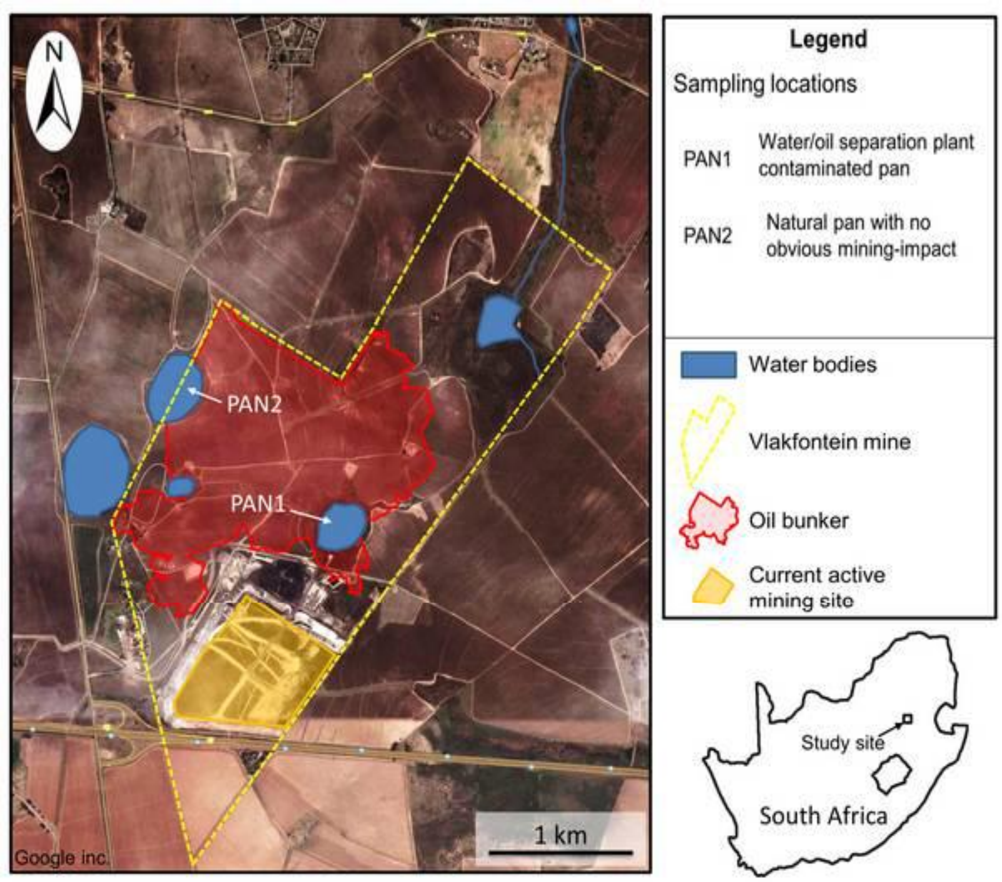


Figure 3: Location of the sampling site

3.3 Microorganism sampling

The bacterial consortium used in the bioremediation experiments was obtained by passing water samples from PAN 1 through 0.45 µm nitrite cellulose filters. This pan is impacted both by coal dust (as it is situated close to the coal loading zone of the mine) and hydrocarbon pollution (water from an oil/water separator is pumped into this pan). This site was chosen due to the likelihood that microbial populations present here were already adapted/selected for hydrocarbon metabolism.

The microbial diversity present at PAN 1 was assessed by means of pyrosequencing, described briefly below.

3.4 Microbial pyrosequencing

The pyrosequencing process consisted of:

- DNA extraction: A volume of 2 L of water was sampled from the site and filtered through 0.45 µm cellulose nitrate filters (Sartorius Stedium). The remaining cell debris was gently scraped from the filter and resuspended in 2 mL of 1 × phosphate buffered saline (PBS) (137 mM NaCl [Merck Chemicals, Germany, Grade AR]; 2.7 mM KCl [Merck Chemicals, Germany, Grade AR]; 10 mM Na_2HPO4 [Merck Chemicals, Germany, Grade AR] 1.8 mM K_2HPO4 [BDH Laboratories, England, Grade GPR]) with a of pH 7.4.. The suspension in 1 × PBS was centrifuged at 13 000 rpm to pellet the cells and the supernatant was removed. DNA was extracted using the DNeasy blood and tissue kit (Qiagen) and the manufacturer’s protocol for extraction of Gram-positive bacteria was followed.

- Pyrosequencing reaction: Pyrosequencing is a next-generation DNA sequencing technique, wherein 16S rDNA fragments from the bacterial community can be sequenced and used as identity tags. Based on the presence and abundance of specific 16S rDNA sequences information can be gained on the bacterial consortium present within a specific niche. Amplification and pyrosequencing was carried out by Inqaba Biotec (Pretoria, South Africa). The universal 16S rRNA primers 27F (5'-AGAGTTTGATCCTGGCTCAG-3') (Weisburg et al., 1991) and 518R (5'-ATTACCGCGGCTGCTGG-3') (Muyzer et al., 1993) were used to amplify the V1, V2 and V3 hypervariable regions of the gene. Each amplicon was gel purified in equimolar amounts for sequencing. Sequencing was carried out on a 454 GS FLX Titanium sequencing platform (Roche 454 Life Sciences).

3.5 Crude oil Sampling

Crude oil used in the bioremediation experiment originated from two bunkers, the Alpha bunker and the North and South bunkers. Chemical analysis, of the North and South bunkers crude oil sample (which also served as oil fingerprinting for the Alpha bunker crude oil), was done using gas chromatography-mass spectrometry (GC-MS), by the CAF laboratory at Stellenbosch University, South Africa. The instrument used for the analysis was an Agilent 6890N GC with CTC CombiPAL Autosampler and Agilent 5975B MS. The column was a ZB 274305 used for Semi Volatiles (30 m, 0.25 mm ID, 0.25 µm film thickness)

3.6 Bioremediation experiments

Two treatment options were investigated on oil samples from the Alpha and North and South bunkers, to evaluate the efficiency of crude oil bioremediation in contaminated water. The treatments were: a) natural attenuation (microorganism natural ability to degrade the contaminant) which also served as control, and b) biostimulation (adding nutrients to improve the natural biodegradation rate).

The water soluble nutrient products used consisted of the chemical compounds ammonium nitrate NH_4NO_3 (molecular weight: 80.046 mg) and monosodium phosphate NaH_2PO_4 (molecular weight: 119.98 mg), at a C:N:P ratio of 100:15:3 following the assumption that 150 mg of nitrogen and 30 mg of phosphorus are consumed in the conversion of 1g of carbon to cell material by the microorganisms (Rosenberg and Ron, 1996); and a C:N:P ratio of 100:1.5:0.3.

Distilled water (10 l) was placed in each of eight pre-sterilized glass tanks prepared for the experiment. A 300 ml volume of gravel (sterilized by autoclaving at 121 °C for 30 minutes) was then added to the tanks as a bottom layer. Next, microbial concentrates obtained by filtrating 1.25 l of sampled water (from PAN 1, see section 3.2) through 0.45 µm nitrite cellulose filters were added to each of the treatments. The tanks were divided in two groups: two tanks received 30 ml of the Alpha bunker oil (C1 and T2), with C1 representing natural attenuation (microbial concentrates but no nutrient addition) and T2 representing biostimulation (microbial concentrates and nutrients addition), and a group of six tanks that received the North and South bunkers crude oil was also divided in two groups: three tanks received 100 ml of oil with one tank serving as control C2 (no nutrients and no microbial consortia added) and two tanks (T4 and T5) were used to investigate natural attenuation and biostimulation, the last three tanks containing 10 ml of oil each had a control tank (C3) (no nutrient and no microbial consortiums added) and two test tanks (T7 and T8 respectively serving for natural attenuation and biostimulation). Tank T8 received nutrients at the C: N: P ratio of 100:1.5:0.3, while the T2 and T5 received a C: N: P ratio of 100:15:3. In all the tanks the nutrients were applied at weekly intervals (until five weeks) to test the effect of nutrient dosage on the bioremediation process, and to avoid excessively high pH and high concentrations of nitrogen that might be toxic to water microbes (Prince and McMillen, 2002). **Table 4** presents the design of the experiment:

Table 4: Summary of the experiment layout

Crude oil source	Tank	Oil Sample		Treatment	Microbe addition in1.2 L	Nutrient application		
		Volume (ml)	Mass (g)			Frequency	N(mg)	P(mg)
Alpha bunker	C1	30	17.5	Natural	Yes	-	0	0
	T2	30	17.5	BS	Yes	Weekly	7.9	2.14
North and South bunkers	C2	100	93.39	Control	No	-	0	0
	T4	100	93.39	Natural	Yes	-	0	0
	T5	100	93.39	BS	Yes	Weekly	7.9	2.14
	C3	10	9.339	Control	No	-	0	0
	T7	10	9.339	Natural	Yes	-	0	0
	T8	10	9.339	BS	Yes	Weekly	0.79	0.214

Natural: Natural attenuation, BS: Biostimulation, Control: Zero treatment

During the eight week experiment, the tanks were placed under constant light, and at laboratory ambient temperature (22°C) (Figure 4). Aeration (*ca.* 500 cc/min) was supplied with aquarium air pumps (Dophin, South Africa).

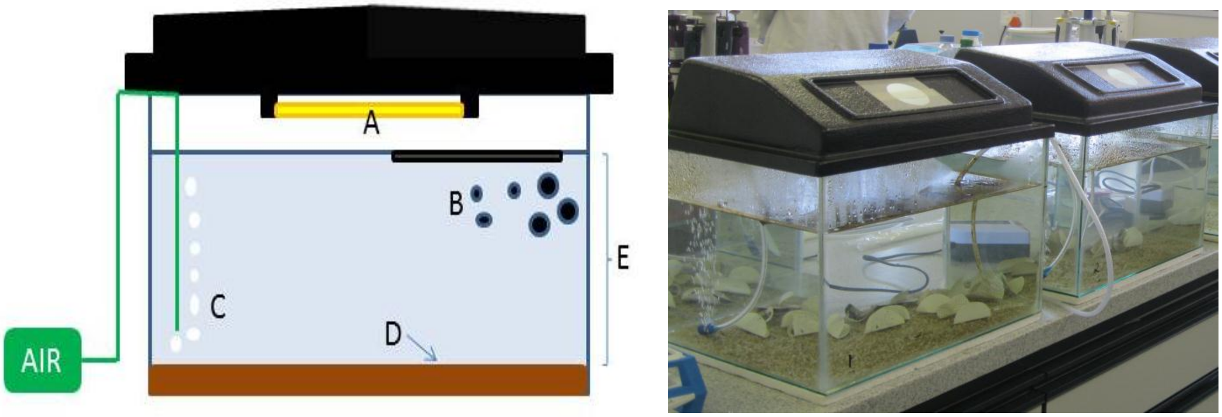


Figure 4: The basic set-up of the microcosm, which depicts A) Light source B) Crude oil (floating or emulsified) C) Aeration D) Sediment layer (synthetic) E) Water.

3.7 Treatment evaluation

To assess the efficiency of the remediation experiments the following variables were monitored weekly:

1) Visual observation of the reduction in the concentrations of oil. In order to demonstrate that biodegradation is taking place in the field, the chemistry and physical state of the oil must be shown to change in ways that would be predicted if bioremediation were occurring (NRC, 1993). For this purpose, a set of photos was taken every week to illustrate oil degradation.

2) Environmental effects: In addition to demonstrating oil concentration diminution, it is also necessary to demonstrate that bioremediation products have low toxicity and do not produce any undesired environmental and ecological effects. The toxicity level in the tanks was assessed weekly by conducting bioassay tests using *Daphnia magna*. *Daphnia* constitute a major component of the freshwater zooplankton throughout the world, plus they are highly sensitive to certain metals.

The *Daphnia* organism bioassay relies on the measurement of the biological response of the test organism to a mixture of contaminants present in a water sample, in a standardized test, usually conducted in the laboratory. The observed toxic impact is generally the result of the bioavailability of the complex mixture of pollutants that may be present in the sample, but also dependent on the physic-chemical parameters of the water (Allan et al., 2006)

Acute, 48 hours *Daphnia magna* bioassays were conducted every week to assess the degradation of toxic components of oil into less toxic particles. Toxicity assays were performed in accordance with the USEPA's Methods for Measuring the Acute Toxicity of Effluents and Receiving Waters to Freshwater and Marine Organisms (USEPA, 2002). The *Daphnia magna* bioassays were performed with neonates less than 24 hours old. Four replicates with five daphnids per test vessel were exposed to the test samples (collected on the same day every week), under standard conditions (USEPA, 2002) (Table 5). The mortality of test organisms was recorded after 24 hours and 48 hours. Survival of test organisms that is at least 10% lower than the mean test organism response in the negative control sample (provided that control lethality is ≤10%), indicates the toxicity potential of a sample (Thursby et al., 1997).

Table 5: Summary of test conditions and test acceptability criteria for Daphnia magna acute toxicity tests with effluents and receiving waters (USEPA, 2002).

Summary of Toxicity test	
Test system	*Daphnia* test
Test species	*Daphnia magna*
Age of test organisms	Less than 24h old
Trophic level	Grazer
Toxicity level	Acute toxicity
Test procedure	USEPA, 2002
Summary of test conditions for the *Daphnia magna* acute toxicity test	
Test type	Static-renewal
Water temperature	20°C to 25 °C
Light quality	Ambient laboratory illumination
Photoperiod	8 hours dark: 16 hours light
Feeding regime	Fed algae and commercial fish flakes while in holding prior to test
Aeration	None
Size of test chamber	50 ml
Volume of test sample	25 ml
Number of test organisms per chamber	5

Control and dilution water	Moderately hard, reconstituted water
Test duration	48 hours
Effect measured	Percentage lethality (no movement on gentle prodding), calculated in relation to control
Test acceptability	90% greater survival in control
Interpretation	Lethality >10% indicates toxicity, provided that control lethality is ≤10%

Chapter IV: Results and Discussion

4.1 Results

The effectiveness of bioremediation, defined as a process that enhance the rate at which microbes biodegrade organic chemical and detoxify organic contaminated areas by transforming undesirable and harmful substances into non-toxic compounds, was assessed through the disappearance of oil in biostimulated tanks compared to unstimulated tanks. The reduction of toxicity levels in the tanks was also measured during the experimental period.

4.1.1 Hydrocarbon degraders and bacterial community composition

Pyrosequencing generates sequence reads, one read for each 16S rDNA template present in the reaction (under ideal conditions). The generated read sequences act as identity tags or barcodes from which the identity of the micro-organisms can be deduced, and provide an indication of the abundance of a specific entity. A total of 539 sequence reads was obtained for the PAN 1 water sample (used as the microbial consortium within the microcosms in this study). The most abundant bacterial entities are given in **Table 6**:

Table 6: The most abundant entities as detected by pyrosequencing

NCBI Blast Hit ID	Number if Reads
No hits	185
Uncultured beta bacteria	112
Uncultured bacterium	87
Uncultured *Actinobacterium*	35
Gram-negative bacterium	28
Uncultured gamma	19
Uncultured soil bacteria	16
Rhodobacter sp.	12
Comamonadaceae bacterium	11
Uncultured *Betaproteobacterium*	11

A large portion of the sequence reads could not be identified to genius or species level, the reason for this was: a) there are still a huge number of largely uncharacterised micro-organisms in nature, b) the pysosequencing reaction may not have progressed optimally due to inhibitors and contaminants. The dominant (or most abundant) microorganisms could not be described identified due to the fact that no such organism had been described (sequence characterised) to such an extent that it could be included in the NCBI's sequence data base.

The identified sequence reads indicated the presence of many bacterial strains such as *Actinobacterium*, gamma bacteria, *Betaproteobacterium*, *Rhodobacter* sp. and *Comamonadaceae bacterium* which are known to be able of degrading petroleum oil.

4.1.2 Crude oil composition

Representative crude oil samples originating from the Alpha bunker and the North and South bunkers were selected for the bioremediation experiment.

Chemical analysis of the North and South bunkers crude oil (which also served as fingerprinting of the Alpha bunker crude oil), through GC-MS revealed:

- The distribution of n-alkanes in the oil sample was similar to the bunker diesel distribution, which is representative of heavy residual fuels such as crude oils.

- C2-alkylated naphthalene (C_2N) was the major PAH, followed by C_3N. No chrysene was present in the oil sample. The results corresponded well to reported chromatograms for bunker and heavy crude oils reported in the literature.

The Alpha bunker crude oil was lighter and less viscous than the North and South bunkers crude oil. A 100 mL aliquot of the Alpha bunker crude oil sample weighed 58.33 g whereas the North and South crude oil sample was 93.39 g.

4.1.3 Bioremediation process

The results of the eight weeks Alpha bunker bioremediation process are given in **Figure 5-1** and **5-2**, representing pictures of the continual disappearance of oil in the tanks, proof of oil degradation.

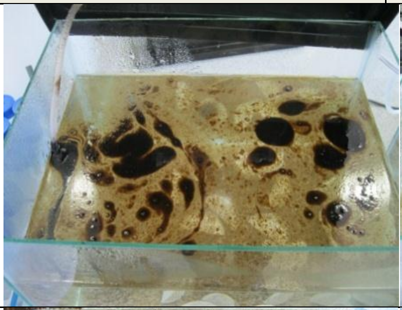
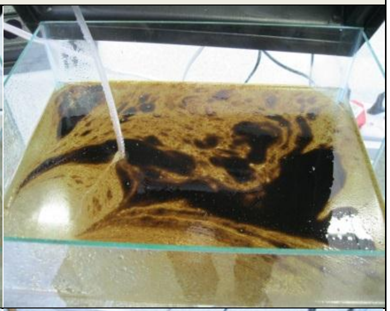
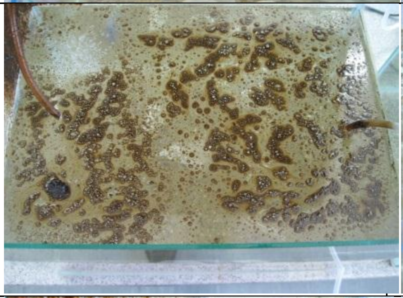
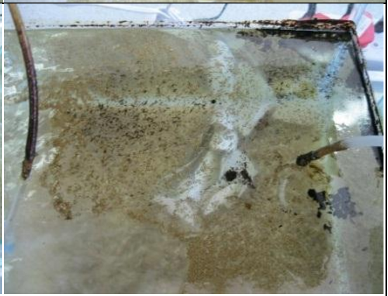
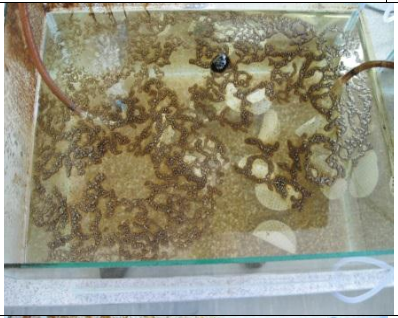
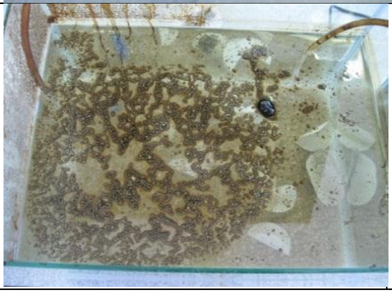
Week	C1 (natural attenuation)	T2 (biostimulation)
Week 1		
Week 2		
Week 3		
Week 4		

Figure 5-1: First 4 Weeks of the Alpha bunker oil bioremediation experiment

Week	C1 (natural attenuation)	T2 (biostimulation)
Week 5		
Week 6		
Week 7		
Week 8		

Figure 5-2: Second Four weeks of the Alpha bunker oil bioremediation experiment

The white papers at the bottom of the tanks are the filters used to concentrate the microbial consortium.

Both natural attenuation and nutrient addition treatments degraded almost 100% of the Alpha bunker crude oil after eight weeks of experiment. The highest rate of degradation was observed in the first 4 weeks of the biostimulation treatment. During that period the majority of hydrocarbons were degraded (as judged visually), with a small and continual decrease in degradation until the end of the experiment. In the beginning, petroleum oil microorganisms were stimulated by nutrient addition (N and P) and more readily degraded hydrocarbon components (probably linear and open-chain hydrocarbons). It is likely that as these easily degradable forms decreased, microbial populations had to use the more recalcitrant hydrocarbons (probably aromatic hydrocarbons with higher molecular weight), and do so less efficiently. Also, diminution of the oil surface ratio induced a restriction on the oil degraders' growth and therefore decreased biodegradation (Atlas and Bartha, 1992), in the later weeks of experiment.

The natural attenuation process of the Alpha bunker crude oil showed a slow (as compared to the biostimulated test tank) but continual degradation rate during the whole process until almost complete oil degradation at the end of the experiment. In both treatments, experimental conditions being similar (microbial population, water, pH, oxygen supply, temperature, light), the slow biodegradation rate in natural attenuation might have been due to the absence of nutrient supplementation (visually almost half of the oil was not degraded four weeks into the experiment).

Results of the North and South bunker oil bioremediation treatments are presented in **Figure 6-1**, consisting of 100ml of oil treated in tank T5 with a C:N:P nutrient ratio of 100:15:3; And in **Figure 6-2**, which consists of 10ml of oil treated in tank T8 with a C:N:P nutrient ratio of 100:1.5:0.3; And where Tanks T4 and T7 served respectively as control for tank T5 and T8, with water, oil, microbial consortium but no additional nutrient. Tanks C2 and C3, with no microbial consortium, serving as controls, respectively for tank T4 and tank T7, to check the impact of the microbial consortium on the bioremediation process.

Unused filter papers were added to tanks that were not enriched with microbial consortia as the filter paper itself may (or may not) act as an energy source for microbes, and every effort was made to ensure consistency between all tanks in this regard.

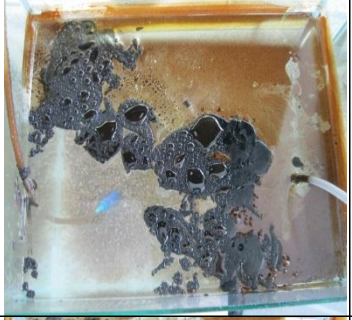
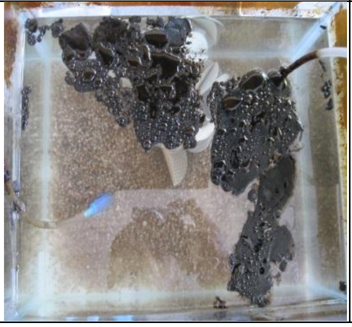
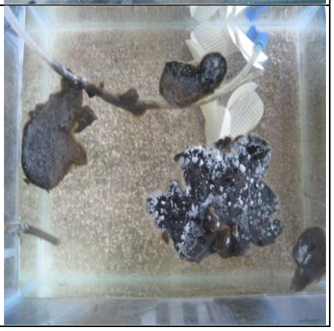
Week	C2 (control)	T4 (natural attenuation)	T5 (biostimulation)
Week 1			
Week 5			
Week 7			
Week 8			

Figure 6-1: 100 mL North and South bunker oil biodegradation in eight weeks of the experiment

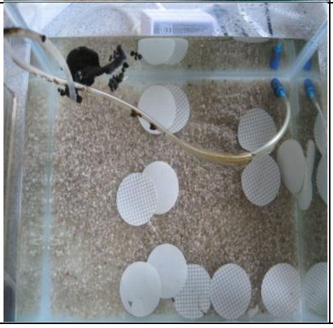
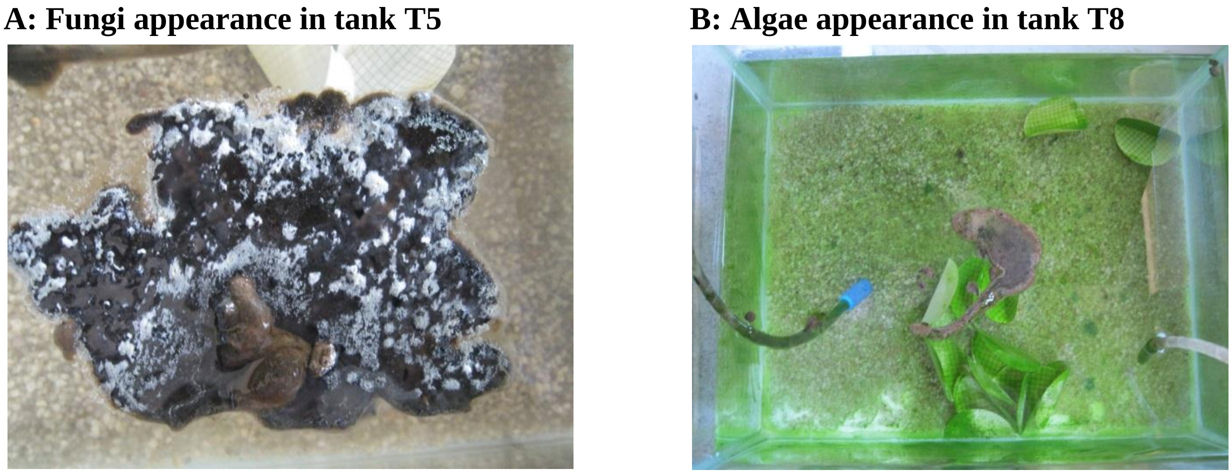
Week	C3 (control)	T7 (natural attenuation)	T8 (biostimulation)
Week 1			
Week 5			
Week 7			
Week 8			

Figure 6-2: 10 mL North and South bunker oil biodegradation in eight weeks of experiment

In comparison to the Alpha bunker crude oil bioremediation experiment, little degradation was observed in the North and South bunker crude oil experiments after eight weeks, neither in the experiments with 100 mL oil or those with 10 mL of oil, or any significant difference between the natural attenuation or nutrient addition treatments. The almost complete degradation of the

Alpha bunker crude oil in both tanks after 8 weeks of incubation could possibly be due to it being light oil, which makes it easily biodegraded by microorganisms and demonstrates that the native PAN 1 water microbes were capable of degrading hydrocarbons to a large extent. DNA extraction results of the microbial consortium used in the experiment indicated the presence of many bacterial strains such as *Actinobacterium*, gamma bacteria, *Betaproteobacterium*, *Rhodobacter* sp. and *Comamonadaceae bacterium* (**Table 6**) are known to be able of degrading petroleum oil. The two bacterial groups *Comamonadaceae bacterium* and *Rhodobacter* sp. clearly identified by the pyrosequencing contain members known to degrade heterocyclic aromatics (Watanabe et al., 2012), naphthalene, benzene and toluene (Aburto et al., 2009). Although a portion of the hydrocarbon reduction might have been due to volatilisation, abiotic loss of hydrocarbon oil has been reported to be generally below 10% at 25°C in the first 30 days (Margesin and Schinner, 1997).

North and South bunkers crude oil consists in heavy residual fuels, containing C2-alkylated naphthalene (C_2N) as the major PAH (CSIR, 2014) and which are considered to be refractory to degradation (Leahy and Colwell, 1990). Observations made from the 5th week onwards indicate the appearance of fungal and algal growth (**Figure 7**) on the oil and in the water, in tank T5 and tank T8, due to the nutrient supplementation, probably favoured by the inefficient use of nutrients by the oil degraders



A: Fungi appearance in tank T5

B: Algae appearance in tank T8

Figure 7: Algae and fungi growth appearance in the water body and on the surface of the oil.

Algae identification by means of direct microscopic examination was performed at the end of the experiment on water samples from all the tanks and a sample of algal growth that formed on the surface of the oil. Only tanks T5 and T8 contained measurable algae. The samples contained two algal taxa (Cyanobacteria and Chlorophyta divisions) in T5 and seven taxa, all belonging to the Chlorophyta in T8, with *Pseudococcomyxa simplex* being the most dominant species (**Table 7**).

Table 7: Algae identification from the tanks

Tank	T5 (nutrient addition)	T8 (biostimulation)
Algae Species	*Synechocystis* sp. (Cyanobacteria) Maybe *Pseudococcomyxa simplex* (Chlorophyta)	Possibly *Pseudococcomyxa simplex* (Chlorophyta) Possibly some *Chlorella* species (Chlorophyta) *Chlamydomonas* sp. (Chlorophyta) *Chlorella* (Possibly *minutissima*) (Chlorophyta) *Chlorococcum* sp. (Chlorophyta) Possibly *Pseudococcomyxa simplex* (Chlorophyta) *Scenedesmus* sp. (Chlorophyta) *Coelastrum* sp. (Chlorophyta) Maybe *Pseudococcomyxa simplex* (Chlorophyta) *Euglena* sp (Chlorophyta)

4.1.4 Toxicity analysis

Weekly bioassay tests using *Daphnia magna* provided acute toxicity data, expressed as a percentage of *Daphnia* deaths per sample (**Tables 8-1 to 8-4**).

Table 8-1: 24 hours Alpha bunker oil treatment toxicity results

Treatment	Mortality (%)							
	Week 1	Week 2	week 3	Week 4	Week 5	Week 6	Week 7	Week 8
C1 (natural attenuation)	85	50	15	15	0	5	0	15
T2 (biostimulation)	100	100	100	90	80	75	75	65

Table 8-2: Results of the Alpha bunker oil experiment *Daphnia magna* screening assay expressed as the mortality at 48 hours.

Treatment	Mortality (%)							
	Week 1	Week 2	Week 3	Week 4	Week 5	Week 6	Week 7	Week 8
C1 (natural attenuation)	100	90	65	55	55	50	50	55
T2 (biostimulation)	100	100	100	100	100	90	85	75

Table 8-3: Results of the North and South bunkers crude oil experiments *Daphnia magna* screening assay expressed as the percentage mortality at 24 hours.

Treatment	% mortality							
	Week 1	Week 2	week 3	Week 4	Week 5	Week 6	Week 7	Week 8
C2 (control)	25	40	15	45	25	10	30	20
T4 (natural attenuation)	100	25	40	40	30	40	35	35
T5 (nutrient addition)	100	100	100	100	100	100	100	100
C3 (control)	55	50	40	30	30	35	40	35
T7 (natural attenuation)	20	15	5	0	0	5	0	5
T8 (nutrient addition)	30	20	70	100	70	80	100	100

Table 8-4: Results of the North and South bunkers crude oil experiments *Daphnia magna* screening assay expressed as the percentage mortality at 48 hours.

Treatment	% mortality							
	Week 1	Week 2	Week 3	Week 4	Week 5	Week 6	Week 7	Week 8
C2 (control)	100	100	95	80	70	80	80	90
T4 (natural attenuation)	95	70	65	70	50	60	50	50
T5 (biostimulation)	100	100	100	100	100	100	100	100
C3 (control)	50	65	70	50	45	45	50	35
T7 (natural attenuation)	45	55	25	20	20	25	40	15
T8 (biostimulation)	90	90	100	100	100	100	100	100

Forty eight hours toxicity results of Alpha bunker crude oil contaminated water showed 100% *Daphnia magna* mortality during the first five weeks for tanks with nutrient addition. This was followed by a slight decrease in mortality from week 6 to the end of experiment (**Figure 8**), a period when nutrient addition was ceased, suggesting that ammonia (as source of nitrogen) toxicity and low pH (**Table 9**) probably caused by acid production associated with ammonia metabolism (US EPA, 2001) might have caused the *Daphnia magna* mortality. The toxic effect seen with *Daphnia* did not, however, inhibit the microbial biodegradation. Nutrient toxicity effect on microbial population may gradually decrease possibly because of an increase in the rate of nitrification with increased tillage and aeration; NO_3-N is typically not toxic to microbes (Zhou and Crawford, 1995). Also increased rates of degradation may have led to increased toxicity as the toxic by-products may be produced during this time. A necessary evil before final and complete non-toxicity is reached.

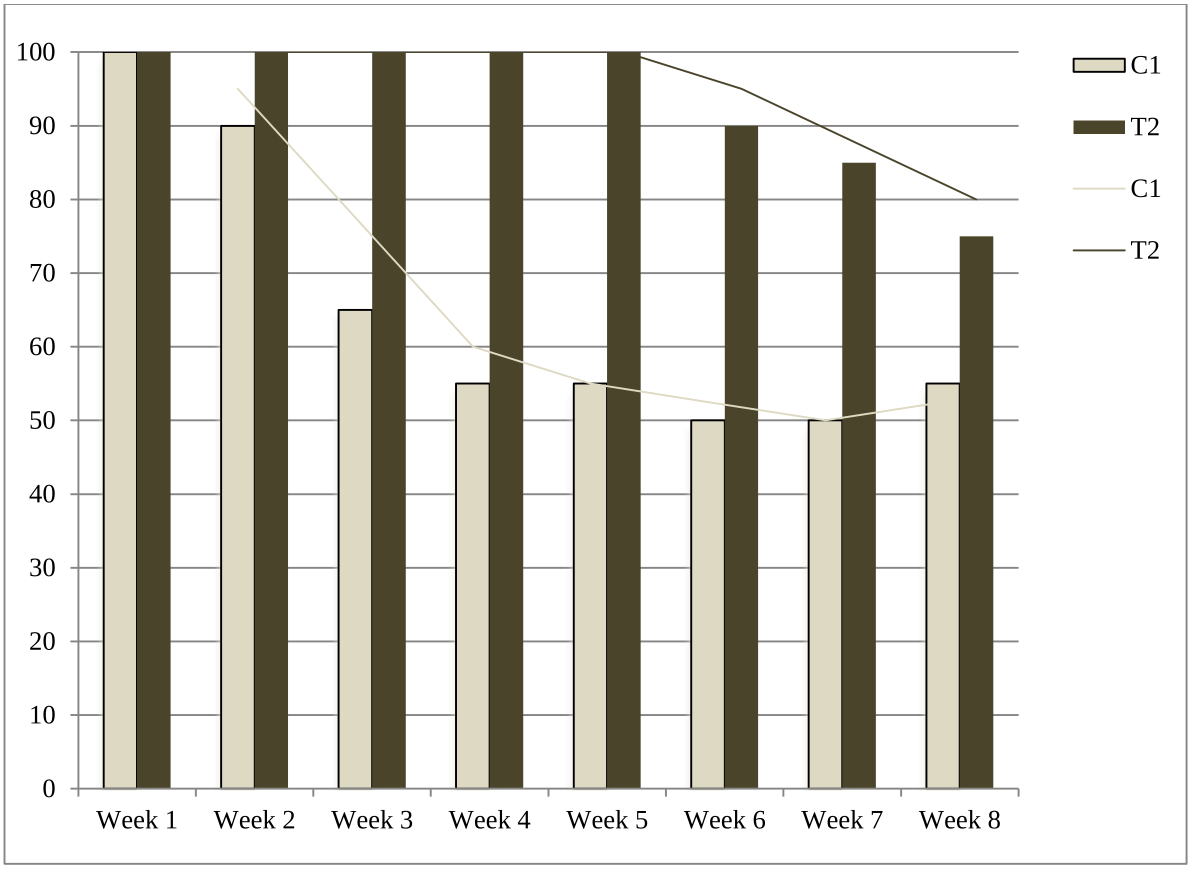


Figure 8: Decreased *Daphnia magna* mortality in Alpha bunker crude oil bioremediation experiment

The highest Daphnia magna mortality rates (100% mortality) were often in 100 mL and 10 mL North and South bunkers crude oil contaminated water treated with nutrient addition (T5 and T8) (**Table 8-4, Figure 9 and 10**), caused possibly by ammonia toxicity and low pH (Table 10 and Table 11). The low mortality rates observed in 10 mL North and South bunkers crude oil contaminated water treatments compared to the 100 mL North and South bunkers crude oil treatments suggest an impact of the spill concentration in toxicity. The more petroleum hydrocarbons that are spilled in a water body, the larger the impact it will have on water ecosystems.

A continual slight decrease in Daphnia mortality was observed in both 100 mL and 10 mL North and South crude oil natural attenuation treated tanks (T4 and T7) (**Figure 9 and 10**), with the higher decrease recorded in 10 mL North and South crude oil experiment, suggesting that the microbial consortium used in the experiment were able to convert the bioavailable toxic substances contained in the crude oil into non-toxic or non-toxic forms.

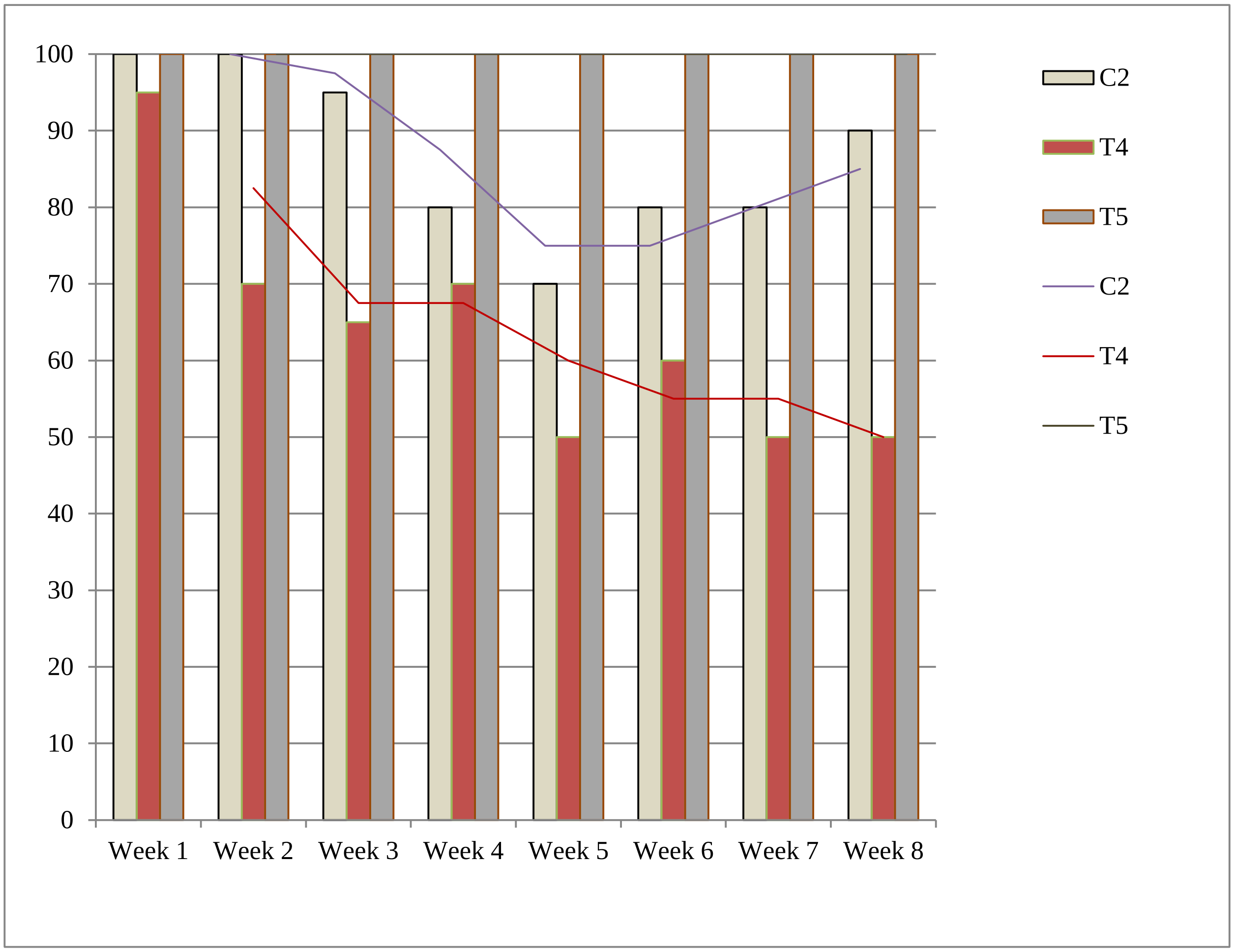


Figure 9: Decreased mortality in 100 mL North and South bunker oil natural attenuation treated tanks.

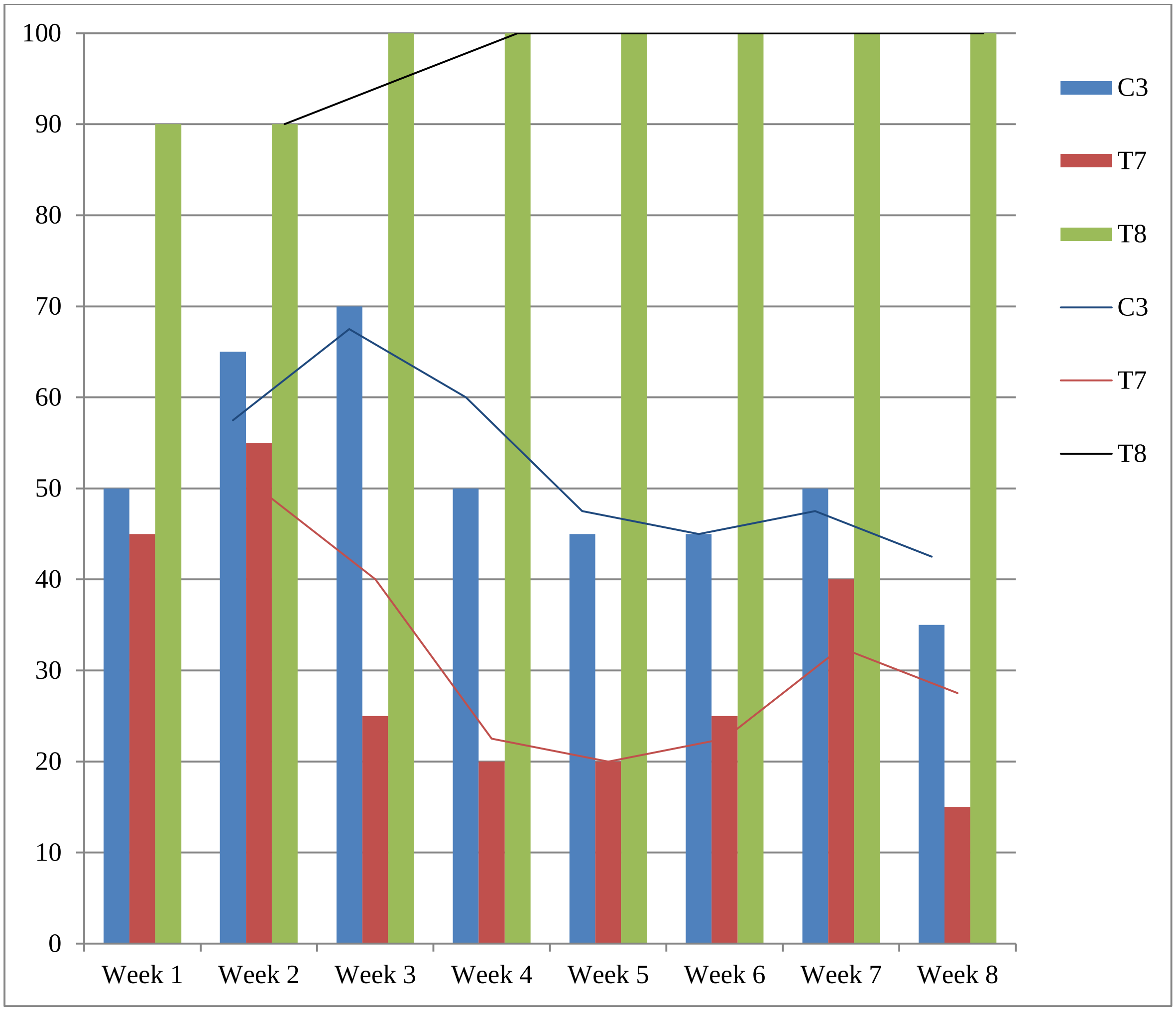


Figure 10: Decreased mortality in 10 mL North and South bunker oil natural attenuation treated tanks.

Physicochemical characteristics of water samples taken during *Daphnia magna* toxicity tests at different weeks indicate decreased dissolved oxygen (DO) in 10 mL and 100 mL of the North and South bunkers crude oil biostimulation experiments (T5 and T8) from week 5 (**Tables 10 and 11**), and low pH values (< to 6, which is the standard in *Daphnia magna* ecotoxicity test) for the Alpha bunker crude oil biostimulation experiment (T2) during the first 5 weeks of experiment (**Table 9**) and low pH values during the 8 weeks of experiment for the North and South bunkers crude oil biostimulation experiments (T5 and T8) (Table 10 and table 11). However, temperature and the electro-conductivity (EC) have not changed significantly.

Table 9: Physicochemical properties of water samples from Alpha bunker crude oil bioremediation experiments taken during weekly *Daphnia magna* toxicity tests.

Treatment	Week	Temperature (^{o}C)	pH	EC (µS/cm)	Do (mg/L)	DO (%)
C1(natural attenuation)	1	20	7.97	18.92	7.1	91.3
	2	20.3	8	36.2	7.02	91.6
	3	20.3	6.21	27.3	7.59	97.9
	4	20.5	6.85	19.35	7.63	97.2
	5	20.3	8.32	22.7	7.8	99.4
	6	20.3	7.8	26.6	6.77	87.7
	7	20.8	8.4	24.7	6.61	85.4
	8	20.7	8.62	26.4	7.19	91.5
T2 (nutrient addition)	1	20	5.18	1139	7.05	91
	2	20.4	5.1	1891	7.12	92.6
	3	20.9	5.1	3.25	7.42	95.9
	4	20.5	5.09	4.5	7.65	97.4
	5	20.1	5.25	575	7.8	99.2
	6	20.3	6.8	26.6	6.77	87.7
	7	20.6	7.31	5.69	6.8	87.7
	8	20.7	6.16	5.9	7.21	91.8

Table 10: Physicochemical properties of water samples from 100 mL North and South bunkers oil bioremediation experiments taken during weekly *Daphnia magna* toxicity tests.

Treatment	Week	Temperature (°C)	pH	EC (µS/cm)	Do (mg/L)	DO (%)
C2 (Control)	1	20.1	7.2	8.81	7.01	90.5
	2	20.4	6.58	12.51	6.95	90.8
	3	20.3	6.8	15.3	7.4	95.3
	4	20.5	6.12	17.7	7.54	95.2
	5	20.1	6.67	13.27	7.67	97.5
	6	20.3	6.57	15.53	6.52	84.4
	7	20.6	6.81	14.76	6.49	83.6
	8	20.7	6.26	20.91	6.42	81.7
T4 (natural attenuation)	1	20	7.48	22.2	7.06	91.2
	2	20.8	6.4	37.3	6.95	90.6
	3	20	6.33	23.8	7.45	96.3
	4	20.6	6.29	24	7.57	96.6
	5	20.1	7.17	22.6	7.73	98.5
	6	20.1	6.84	24.1	6.56	84.8
	7	20.7	6.79	23.2	6.32	81.4
	8	20.7	6.58	21.86	6.65	84.7
T5 (nutrient addition)	1	20.1	5.54	1140	7	90.4
	2	20.5	5.45	2186	7.05	92
	3	20	4.42	3.34	7.44	96
	4	20.5	4.62	4.74	7.58	96.7
	5	20.8	4.98	6.15	7.72	97.7
	6	20.3	5.53	6.04	0.12	15
	7	20.6	4.9	6.24	3.66	47.2
	8	20.7	4.94	6.38	4.67	59.5

Table 11: Physicochemical properties of water samples from 10 mL North and South bunkers oil bioremediation experiments taken during weekly Daphnia magna toxicity tests.

Treatment	Week	Temperature (°C)	pH	EC (µS/cm)	Do (mg/L)	DO (%)
C3 (Control)	1	20.1	7.23	10.04	6.82	88.2
	2	20.4	6.54	25.4	6.86	89.2
	3	20.1	6.24	10.04	7.38	95.5
	4	20.6	6.42	13.41	7.55	96.2
	5	20.1	6.62	11.4	7.65	97.3
	6	20.1	6.45	19.57	6.43	83
	7	20.6	6.06	14.38	6.42	82.7
	8	20.7	6.11	15.32	7.09	90.4
T7 (natural attenuation)	1	20.2	7.47	22.6	6.85	88.7
	2	20.6	6.59	32.7	6.95	90.6
	3	20.3	66.02	21.73	7.39	95.8
	4	20.6	6.1	22.1	7.49	95.6
	5	20	7.05	23.9	7.65	98.5
	6	20.1	6.79	21.7	6.6	85.2
	7	20.5	6.59	24.5	6.8	87.6
	8	20.7	5.8	23.5	7.17	91.3
T8 (nutrient addition)	1	20.3	6.75	132	6.88	89.3
	2	20.7	5.39	242	7.01	91.3
	3	20.3	5.48	384	7.48	97.2
	4	20.8	5.67	569	7.65	97.8
	5	20.6	5.13	560	7.77	99.7
	6	20.2	6.38	543	3.18	41.2
	7	20.7	6.24	574	4.93	63.6
	8	20.6	6.08	586	4.91	62.5

The oxygen and pH rate drop observed in the North and South bunkers crude oil biostimulated tanks (T5 and T8) from weeks 6 to 8 (Table 10 and table 11), may also be due to the increased algal growth. This could have caused increased *Daphnia* mortality during these weeks. In fact, algal blooms have dramatic effects on water chemistry, most notably pH and DO. When algae remove carbon dioxide during photosynthesis they raise the pH and increase the level of hydroxide. The opposite reaction occurs during respiration when carbon is produced lowering hydroxide and lowering the pH (Detlef, 2004). While oxygen limitation may cause mortality, pH of surface water is an important factor to aquatic life, because it affects the normal physiological functions of aquatic organisms, including the exchange of ions with the water and respiration (Mahassen, 2011). Physiological processes operate normally in most aquatic biota, including *Daphnia magna* under a relatively wide pH range, (typically at a pH of 6-9) (US EPA, 2002).

4.2 Discussion

One problem encountered in this experiment was that the Alpha bunker crude oil sample used in the experiment was in quantity not enough to perform multiple experiments as could be done for the North and South bunkers crude oil (a control test with no bacteria and no nutrient addition, and a different nutrient rate addition).

Although conventional methods, such as physical removal, are the first response option, they rarely achieve complete clean-up of oil spills. Current mechanical methods, typically recover no more than 10-15% of the oil after a major spill (OTA, 1990). Bioremediation, is considered to be one of the best and cost-effective approaches of restoring contaminants of soil and water, and rely on the ability of microorganisms present naturally in the environment to use petroleum oil as a source of carbon and energy and thus to detoxify or remove pollutants. Biostimulation, supplying additional nutrients enhance the rate of biodegradation in stimulating microbial growth.

Previous studies showed that biostimulation with addition of inorganic nutrients to oil-contaminated water enhanced their biodegradation rates (Lee and Levy, 1991; Mills et al., 2004). A study carried out by Chorom et al (2010) investigated the efficacy of inorganic fertilizer (NPK) in enhancing microbial degradation of petroleum hydrocarbons. Gas chromatography results showed that normal paraffin and isoperoid (Phitane and Pristane) decreased in the range 40-60% in less than ten weeks. Agarry et al (2012) using kerosene as source of TPH and inorganic NPK (4.30g) as source of nutrients, obtained total petroleum hydrocarbon degradation of 75.06%. Results of the present study showed that both, biostimulation using nitrogen and phosphorus as nutrients, and natural attenuation (natural biodegradation), conducted to the degradation of almost 100% of the Alpha bunker crude oil (consisting of a light crude oil), within eight weeks. However, the biostimulation treatment had the fastest degradation rate, with more than 80% of the crude oil being degraded during the first four weeks of experiment. Adjustment of carbon:nitrogen:phosphorus ratios by the addition of nitrogen and phosphorus in the form of ammonium nitrate (NH_4NO_3) and monosodium phosphate (NaH_2PO_4), at a C:N:P ratio of 100:15:3 stimulates the biodegradation of crude oil and individual hydrocarbons in water. This based on the assumption that 150 mg of nitrogen and 30 mg of phosphorus are consumed in the conversion of 1g of carbon to cell material by the microorganisms (Rosenberg and Ron, 1996).

However, when applied to the North and South bunker crude oil (which consists of heavy residual fuels containing C2-alkylated naphthalene (C_2N) as the major PAH (CSIR, 2014) and which is considered to be refractory to degradation (Leahy and Colwell, 1990)) the biostimulation treatment did not show any major improvements. Even though it was applied at different crude oil concentrations as well as different nutrients ratios. After eight weeks, no major visible sign of oil degradation was observed. Similar effects had been reported by previous researchers. Walker et al. (1976) compared the degradation of two crude oils, one lighter and another heavier, the lighter oil was the most susceptible to microbial degradation. Furthermore, Agarry et al (2012) using kerosene as source of TPH and inorganic NPK (4.30g) as source of nutrients, obtained total petroleum hydrocarbon degradation of 75.06%. The better performance of NPK in reducing TPH in kerosene contaminated soil when compared to spent engine oil contaminated soil was probably due to the presence of lighter chains of hydrocarbons in the latter as revealed by chromatographic results. This confirmed earlier studies (Venosa et al., 2002) reporting that microorganisms more readily degraded light end hydrocarbons than heavy end hydrocarbons. Compositional heterogeneity among different crude oils and refined products influences the overall rate of biodegradation both of the oil and of its component fractions.

Chapter V: Conclusions

This investigation compared the effectiveness of natural attenuation and biostimulation on two different crude oils from old oil storage bunkers, the Alpha bunker and the North and South bunkers.

Results revealed that both treatments (natural attenuation and biostimulation) degraded almost 100% of the Alpha bunker crude oil within eight weeks, however the fastest degradation rate was observed with the nutrient addition treatment. Decreased toxicity suggested that native PAN 1 site water microbes are capable of detoxifying components of the crude oil to a large extent. However, when applied to the North and South bunkers crude oil, neither nutrient addition nor natural attenuation showed a significant improvement in term of oil biodegradation after eight weeks, probably due to its heavy nature compared to the Alpha bunker crude oil. Biostimulated North and South bunkers crude oil showed a change in texture, and the edge of the heavy oil clumps degraded over time, suggesting that the biodegradation was taking place even though it was not as clear cut with the Alpha bunker crude oil experiment. Hence, we suggest nutrient addition as a potential efficient response to an Alpha bunker crude oil spill. An extended observation period would inform better about the time required to decrease the toxicity level in treated water to the background level, and whether longer time frame extended might lead to complete degradation of the heavier oil from the North and South bunkers. In situ application will be required to determine the background nutrient concentration, and therefore the level of additional nutrients needed during biostimulation. This in order to avoid the addition of excess nutrients that could lead to the inhibition of the biodegradation.

The North and South bunkers crude oil may require combined biostimulation treatment and use of biosurfactants, due to its recalcitrant nature.

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