INVESTIGATIONS ON THE FEASIBILITY OF USING PHYTOREMEDIATION FOR TREATMENT OF HYDROCARBON-CONTAMINATED SEDIMENTS AT HORSEA LAGOON

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October, 2012

A thesis submitted in partial fulfilment of the requirements for the award of the degree of Doctor of Philosophy of the University of Portsmouth

Whilst registered as a candidate for the above degree, I have not been registered for any other research award. The results and conclusions embodied in this thesis are the work of the named candidate and have not been submitted for any other award.

ABSTRACT

This project investigates phytoremediation at a disused fire training runoff lagoon at an ecologically sensitive area in Southern England called Horsea Island. The sediments in the semi-saline lagoon were highly contaminated with over 145,000 mg/kg Dry Weight (DW) Diesel Range Organics (DRO) and were classed as carcinogenic and unsafe for human exposure. Phytoremediation was attractive as an inexpensive and *in situ* remediation method. However there were limited field studies examining such extremely contaminated conditions.

Initial mesocosm studies indicated that both *Phragmites australis* and *Typha latifolia* were able to grow in this sediment without the need for additional nutrients and *P. australis* was subsequently used for test planting in the lagoon. *Ex situ* greenhouse microcosms as well as an *in situ* full scale trial was carried out and the response of DRO and microbial populations were investigated over a 15 month period. Using a novel colourmetric plate test developed during this study, planted sediments in both the microcosms and Horsea lagoon showed not only an increased diversity but also an increased number of hydrocarbon-degrading bacterial isolates when compared to unplanted sediments. However, although the overall DRO concentrations fell in both the *in situ* and *ex situ* systems in times of hot weather and low water levels, there were no significant differences between planted and unplanted sites. There was also no difference between microbial numbers in planted and unplanted sites in either the microcosms or Horsea lagoon however, the mesocosms that had been growing for three years showed significantly higher numbers of bacteria, hydrocarbon-tolerant bacteria and hydrocarbon-tolerant fungi as well as elevated numbers of fungi in the planted sites. This indicates that phytoremediation may require a longer period of time to enhance degradation in such conditions.

CONTENTS

PLATES

FIGURES

TABLES

ACKNOWLEDGEMENTS

First and foremost I would like to thank my three supervisors; Dr John Williams, Dr Catherine Mant and Dr Eric May for their constant support, encouragement and for always making time to see me. John, thank you for getting me through the mine field that was data analysis, Cath, thank you for accompanying me on my many Horsea digs through rain, wind and snow and Eric, thank you for making me realise that my English teacher has taught me very poorly and I really should not have got an A in GCSE!

My every day work was supported by a lovely group of scientists at the Institute of Naval Medicine and a huge thank you goes out to all the OES staff especially Mat Marshall and Dave Lewis for making sure I always had everything I needed.

During my time in the lab at the University of Portsmouth, I received a great deal of support (and cake) from the lovely technicians and post-docs. In particular, I would like to thank Vanessa Peters and Siobhan Watkins for letting me rant and giving me great feedback on my experiments and results. Thank you also goes to Joy Watts for the molecular analysis data and for all the advice.

To my Mum, Dad, Nan, brothers and sisters, thank you for the love and support you have given me over the years, sorry for never having any free time during the last 4 years!

This thesis is dedicated to Russell Vaughan for his constant support through my tears and tantrums and for bringing me endless cups of tea and snacks! I could not have done this without you!

ABBREVIATIONS

ASE	Accelerated Solvent Extractor
B	Sheeps Blood
BOD	Biochemical Oxygen Demand
BUG	Biolog Universal Agar
COD	Chemical Oxygen Demand
cm	Centimetre
DRO	Diesel Range Organics
DW	Dry Weight
EPA	Environmental Protection Agency
ESG	Environmental Science Group
FID	Flame Ionisation Detector
FTF	Fire Training Facility
g	Gram
GC	Gas Chromatography
GN	Gram Negative
GP	Gram Positive
HMDS	Hexamethyldisilazane
h	Hours
id	Internal Diameter
ID	Identification
kg	Kilogram
L	Litre
Log	Log_{10}
M	Molar
Ma	Maltose
m	Metre
mg	Milligram

min	Minutes
mL	Millilitre
mm	Millimetre
MOD	Ministry Of Defence
MPN	Most probable number
PAH	Polycyclic Aromatic Hydrocarbons
PBS	Phosphate Buffer Solution
PCR	Polymerase Chain Reaction
ppm	Parts Per Million
s	Seconds
SEM	Scanning Electron Microscopy
Spp.	Species
SPA	Special Protection Area
SSSI	Site of Special Scientific Interest
SS	Suspended Solids
T	Thioglycolate
TPH	Total Petroleum Hydrocarbons
TSA	Trypticase Soy Agar
TTC	2,3,5 TriphenylTetrazolium Chloride
UCM	Unresolved Complex Mixture

1. INTRODUCTION

Although it is widely accepted that contamination of land and water has been occurring for millions of years due to volcanoes, forest fires and sedimentary rock erosion, many people regard anthropogenic pollution as a modern day problem (Baird & Cann, 2004). This is not the case as man-made pollution began occurring as soon as small communities were formed. However, in these early days of man the amount of waste was small and had virtually no effect on the environment (Dix, 1980). As the settlements increased in size, so did the amounts of pollution with cholera, typhus, typhoid and bubonic plague being associated with the lack of proper waste disposal methods in the thirteenth century. The increased use of coal during this time became such a nuisance that in 1273 Edward I made the first ever anti pollution law to prevent the use of coal for domestic heating. In 1800, the increased production of materials and chemicals in Europe at the start of the industrial revolution produced substantial pollution. However, the extent of contamination and the hazard from discarded materials expanded greatly in the last century particularly in the period after World War Two (Baird & Cann, 2004). With government policies prior to the 1970s not greatly helping to reduce pollution because of the low priority given to legislation and finance (Dix, 1980) many areas around the UK have been left highly polluted and in need of remediation.

1.1 Hydrocarbons in the environment

The term 'petroleum' comes from the Greek word 'petros' meaning stone or rock and 'oleum' meaning oil (Totten, 2004). Total petroleum hydrocarbons (TPH) such as those found in tar pits next to the Euphrates River have been used since 4000 BC to waterproof baths, roofs and boats. During the past century, the occurrence of the industrial revolution has increased the need for TPH and in turn increased the amount of TPH contamination and by products (Smith, 2011). Worldwide 10 million tonnes of oil and petroleum are used

per day and although major oil spills attract the attention of the media and the public, the majority (75%) of petroleum contamination in water occurs in port during routine ship operations such as loading, discharging and bunkering (Federation, 1987). Terrestrial TPH contamination comes mainly from pipelines (35%) and oil wells (24%) (Federation, 1987) although the movement of TPH from the oil fields to the consumer is also a large contributor to pollution. This movement of TPH involves as many as 10-15 transfers between many different modes of transportation. These include tankers, pipelines, rail cars and tank trucks. TPH is stored at transfer points and at terminals and refineries along the route and accidents can happen during any of these transportation steps or storage times (Fingas, 2001).

TPH in the environment are toxic to both animals and humans and can affect the liver, lungs, kidneys and nervous system, leading to cancer as well as having immunological and reproductive effects (Rushton *et al.*, 2007). They can also be problematic to plants growing in contaminated soil. Due to their physico-chemical properties, TPH can displace air in soil creating anaerobic conditions (Foth, 1984). They can also prevent water infiltration, reduce nutrient mobility (Kirk *et al.,* 2005) and are able to move through cell membranes easily into plants (Adam & Duncan, 2002; Overbeek & Blondeau, 1954) leading to inhibition of plant growth (Kaimi, 2007). TPHs are also able to form a layer around seeds preventing water or oxygen transfer thus delaying germination (Adam & Duncan, 2002).

1.2 Fate of hydrocarbons in the environment

TPHs are made up of 4 main structural classes of hydrocarbons namely alkanes, cyclo-alkanes, alkenes and aromatics (Marchal *et al.*, 2003).

Alkanes are saturated straight chain hydrocarbon compounds, meaning they consist only of hydrogen and carbon with each carbon having the maximum number of hydrogens bonded to it. Cycloalkanes are saturated hydrocarbon compounds in which the carbon atoms are

bonded to each other in a ring formation. The alkenes or unsaturated compounds are a group of compounds that contain fewer hydrogen atoms than the maximum possible. They also have at least one double carbon to carbon bond. The aromatic compounds include at least one benzene ring of six carbons and three double carbon to carbon bonds that float around the ring to add stability. Because of this stability benzene rings are very persistent and can have toxic effects on the environment. Polyaromatic hydrocarbons (PAH) are compounds consisting of at least two benzene rings (Fingas, 2001).

Diesel Range Organics (DRO) are a group of TPHs defined by the UK Environment Agency as being in the C_{10}-C_{25} range (Environment Agency, 2007) and are a complex mixture of 2000-4000 petroleum hydrocarbons. The DRO pollution limit in the UK for soil is currently 330 mg/kg therefore sites with higher levels will need to be remediated (Encia Geoenvironmental Investigations, 2011).

Theoretically all TPHs can be biodegraded (Bystrom & Hirtz, 2002) but the time it takes to degrade the substances differs considerably depending on their structure and molecular weight. There are seven stages of diesel biodegradation (Table 1), with short-chained hydrocarbons being broken down rapidly and as alkane chain length increases, so does its resistance to degradation (Potter & Simmons, 1998) with long-chained hydrocarbons being extremely persistent (Berset *et al*, 1999).

DROs released into the environment are subjected to chemical, physical and biological processes collectively referred to as weathering (Wang & Stout, 2007). These processes include evaporation, dissolution, dispersion, emulsification, adsorption on to suspended materials, microbial degradation or photooxidation. Weathering can alter the chemical fingerprint of spilled or discharged hydrocarbons so that it may no longer "match" the original as demonstrated by Serrano & Gallego, 2008 (Figure 1).

Table 1. The seven stages of diesel degradation adapted from Kaplan *et al.*, 1997

Stages of diesel biodegradation	Chemical composition
1	Abundant n-alkanes
2	Light-end n-alkanes removed
3	Middle range n-alkanes, olefins, benzene and toluene removed
4	More than 90% of n-alkanes removed
5	Alkylcyclohexanes and alkylbenzenes removed Isoprenoids an C_0-naphthalene reduced
6	Isoprenoids, C_1-naphthalenes, benzothiophene and alkylbenzothiophenes removed C_2-naphthalenes selectively reduced
7	Phenanthrenes, dibenzothiophenes and other polynuclear aromatic hydrocarbons reduced
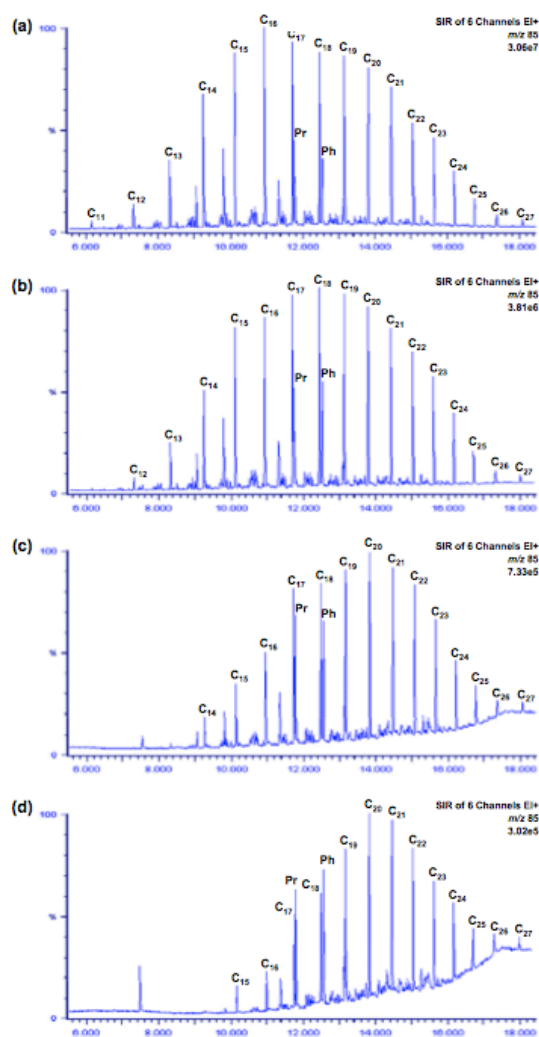
Figure 1. GC-FID chromatograms showing the affect of weathering on DRO after a) 3 hours b) 50 days c) 200 days and d) 400 days (Serrano & Gallego, 2008)

In the first few days after a petroleum spill the rate of non-biological degradation is very rapid (Alloway, 1997) with about 80% of photodecomposition occurring within this time (Fingas, 2001). This is due to the toxic properties of diesel fuel, which inhibit development

and metabolic activity in microorganisms at the early stages of a spill. However, after a so called "period of stress" where microorganisms gradually adapt to the pollutants, aliphatic hydrocarbons can be biodegraded and used as a source of carbon and energy with complete degradation brought about under aerobic conditions (Das & Chandran, 2011). Under aerobic conditions, the initial intracellular attack on alkane chains is to convert them into fatty acids. This is an oxidative process and the activation as well as incorporation of oxygen is the key enzymatic reaction, catalyzed by oxygenases and peroxidases. A monooxygenase introduces one atom of oxygen from molecular oxygen into the molecule thus forming a fatty alcohol. This alcohol is oxidized to an aldehyde and finally to a carboxylic acid which is channelled into central metabolism by β-oxidation. Alkenes with double and triple bonds are more chemically reactive and can undergo additional reactions such as epoxidation and hydration. The double bond of alkenes can be hydrated anaerobically to form an alcohol, which is then converted via the aerobic pathway (Bouwer & Zehnder, 1993). Incomplete degradation of hydrocarbons along with biogenic organic compounds (BOC) such as alkanes, sterols, sterones, fatty acids, fatty alcohols, waxes and wax esters cause a characteristic "hump" of Unresolved Complex Mixture (UCM) in the GC-FID chromatogram. The height and shape as well as position of the UCM can all give information about which hydrocarbons are present, how weathered they are and the age of the contamination.

1.3 Remediation of hydrocarbon contamination

Remediation of hydrocarbon-contaminated land can occur by removing the source of the pollution or by breaking the pathways to the receptors. Pollution can be removed physically by the removal of contaminated soils; installation of physical barriers; vapour extraction; soil flushing and thermal treatment (Ravikrishna *et al.,* 1998). These methods are expensive and normally require the addition of more chemicals, energy or the removal of large areas of polluted land, which can be damaging to the surrounding ecosystem.

Bioremediation involves engineering measures to intensify and enhance the natural degradation processes in the soil. This can be achieved by adding microbial seeds, mechanical aeration pumps to increase the oxygen levels and by the addition of fertilisers. Adding plants to contaminated soils can increase the microbial numbers, oxygen levels and nutrient levels increasing the rate of hydrocarbon degradation without the need for costly external engineering interventions making it an attractive option.

1.4 Phytoremediaton

Phytoremediation is the use of plants and their associated microorganisms for enhancing the remediation of polluted environments (Muratova, 2003). It is an attractive method for removing pollutants from soils for a variety of reasons, the vegetation is aesthetically pleasing (Flathman & Lanza, 1998); rhizosphere microbial communities are able to biodegrade a wide variety of organic contaminants (Shimp *et al*., 1993); it is a low cost (Gerhardt *et al*, 2000) and low energy operation (Anderson *et al.,* 1993; Ciria *et al.,* 2005; Kaimi, 2007; Shimp *et al*., 1993; Weis & Weis, 2004) and as the organic contaminants are mostly biodegraded there is no hazardous waste to be disposed.

Another additional benefit of phytoremediation is that organic nutrients and oxygen are added to soil via plant and microbial metabolic processes. This improves the overall quality and texture of soil at remediated sites. Plants also provide ground cover, and their roots help to stabilise soil, which mitigates erosion from both wind and water (Gerhardt *et al*., 2009; Schnoor, 1997).

The main mechanisms of phytoremediation are:

- phytostabilisation, where plants immobilise contaminants and store them below ground in roots and/or soils.

- phytovolatisation, where plants take up contaminants and release them as less toxic vapours into the atmosphere through the plants transpiration process.

- phytoextraction, where hyper accumulating plants accumulate the contaminants in shoots and above ground tissues (McDonald, 2006; Weis & Weis, 2004)

- phytodegradation, where plants and associated microorganisms degrade organic pollutants (Cunningham *et al.*, 1995)

- rhizofiltration, when plant roots absorb pollutants from a submerged environment (Salt *et al*., 1995).

1.5 The nature of phytoremediation

Soil microorganisms are considered to be the major or ultimate decomposers (Foth, 1984). The three major types of soil microorganisms in numerical terms are bacteria, fungi and the actinomycetes (Foth, 1984; Wild, 1993).

Bacteria are some of the smallest and most abundant microbes in the soil (Reid & Wong, 2005). In a single gram of soil, there are $1x10^6$ to $1x10^{10}$ individual cells (Atlas *et al.,* 1984; Foth*,* 1984). There are approximately 60,000 different bacteria species, and are present in all soils whether these are acid or alkaline, waterlogged or well drained and in regions that are hot or cold, wet or dry (Wild, 1993).

Bacteria play an important role in the decomposition of organic materials. Liste & Felgentreu (2006) carried out a 95 day greenhouse phytoremediation study on soil contaminated with 1,517 mg/kg TPH. They looked at the effect of annual ryegrass, summer vetch and white mustard on bacterial numbers and species type compared to unplanted and uncontaminated soil. After 15 days, contaminated soil contained up to 18-fold more bacteria than unpolluted soil due to the bacteria having an increased carbon source for growth. Bacteria were also particularly abundant in the root zones of flowering mustard and vetch reaching 10–30-fold the numbers of unplanted soil as bacteria live in colonies that can cover 4-10% of the root surface (Foth, 1984). The number of bacteria was similar in contaminated versus pristine soil but the species composition differed. TPH

degraders such as *Alcaligenes piechaudii*, *Pseudomonas putida*, and *Stenotrophomonas maltophilia, Flavobacterium johnsoniae, Pseudomonas fluorescens, Pseudomonas stutzeri,* and *Pseudomonas syringae* were particularly abundant in contaminated soil. Bacteria are especially important in the early stages of decomposition when moisture levels are high. In the later stages of decomposition, fungi tend to dominate.

Fungi are aerobic, eukaryotic heterotrophs that generally grow from spores and form thread-like hyphae on the exterior of roots. These external hyphae function as an extension of roots for water and nutrient absorption and are called mycorrhiza (Foth, 1984). Whereas bacteria grow on a localised site, the fungal mycelium grows out into the surrounding environment. Mycorrhizal fungi are found naturally in all soils with one gram of soil containing 10-100 m of mycelial threads (Shimp *et al*., 1993). Fungi have an important relationship with plants; ectomycorrhizae*,* grow on the surface layers of the roots and are commonly associated with trees and endomycorrhizae that grow within the root cells and are commonly associated with grasses, row crops, vegetables, and shrubs. Mycorrhizal fungi can help solubilise phosphorus and bring soil nutrients (phosphorus, nitrogen, micronutrients and perhaps water) to the plant (Ingham, 2000b). They can also bind the soil particles together to create water-stable aggregates which in turn create the pore spaces in the soil that enhance water retention and drainage. In periods of water deficit, fungi can survive by living in dead plant roots and/or as spores or fragments of hyphae (Jenkins, 2005) or by bridging gaps between pockets of moisture (Ingham, 2000b).

Actinomycetes are a group of prokaryotic soil bacteria that are similar in structure to fungi. They are single-celled but form filaments resembling mycelia and often produce spores (Shimp *et al*., 1993). Actinomycetes numbers typically vary from 1×10^6 to 4×10^7 per gram of soil. Although actinomycetes are abundant in aerobic soils (Wild, 1993), it is generally noted they that are not as important as bacteria and fungi as decomposers but they are able to degrade compounds resistant to decomposition by other bacterial and fungal species

(Ingham, 2000a). This class of microbe may be able to degrade many complex chemicals often found in contaminated soil (Foth, 1984).

Plant-microorganism interactions have been known and studied for more than a century (Shimp *et al*., 1993). Both fungi and actinomycetes have been shown to increase with the addition of plants. Juwarkar & Jambhulkar (2008) carried out a phytoremediation field study in an old coal mine dump in India. Thirty six months after planting 12 assorted, native plants, the number of fungi and actinomycetes increased from $9x10^1$ to $8.3x10^4$ CFU/g and from $6x10^1$ to $9x10^4$ respectively. This brought the numbers of fungi and actinomycetes close to those found in a good productive soil leading to the previously barren dump turning into a fertile and productive green area.

The term rhizosphere was first used by Lorenz Hiltner in 1904 (Anderson *et al*., 1993) and is used to describe the area of soil closely surrounding the plant roots. It is colonised by a predominately Gram-negative microbial community, specifically *Pseudomonas, Flavobacterium* and *Alcaligenes* (Euliss *et al*, 2008). The rhizosphere supports microbial populations up to 100 times larger than the surrounding bulk soil (Erickson, Davis, & Narayanan, 1995; Shimp *et al*., 1993; Wild, 1993). This is because the rhizosphere has increased oxygen levels, plant exudates and an increased surface area.

The most rapid and complete degradation of the majority of pollutants is brought about under aerobic conditions (Riser-Roberts, 1998a). There is increased oxygenation in the rhizosphere because of the continuous dying of root hairs producing tiny channels and pores in the soil, facilitating the diffusion of oxygen from the top surface to the lower soil depths (Shimp *et al*., 1993). Some plants also have several internal gas transport pathways raising the oxygen concentration in the proximity of the roots close to that of air (Shimp *et al*., 1993). Many wetland plants are also able to absorb oxygen through their leaves, lenticels of twigs, stems, bark and even the unflooded roots and then release oxygen into the rhizosphere by mechanisms such as thermo-diffusion and a venturi and humidity-

induced convection of air (Armstrong & Armstrong, 1990; Armstrong *et al.,* 1992; Callaway & King, 1996).

The length and surface area of the root system is another factor affecting the increased number of microbes found in the rhizosphere (Shimp *et al*., 1993). Plant systems with higher root densities and larger root surface areas may create a more effective environment for soil microorganisms due to the greater concentrations of root exudates supporting growth (Keller *et al.,* 2008).

Plants sustain an increased microbial population in the rhizosphere by secreting substances such as carbohydrates and amino acids through root cell walls and by sloughing root epidermal cells (Anderson *et al*., 1993; Erickson *et al.*, 1995; Foth, 1984). More than 25% of the photosynthate produced by plants may be lost from the roots to the soil (Foth, 1984). The rates of exudation and composition of exudates vary with the age of the plant (Shimp *et al*., 1993). This continuous supply of root exudates is necessary to maintain the $1x10^6$-$1x10^{10}$ vegetative microbes per gram of soil in the rhizosphere in a metabolically active state (Erickson *et al.*, 1995). Root exudates also increase the diversity and numbers of microbial species and can stimulate aerobic microbial degradation of pollutants (Anderson *et al*., 1993; Erickson *et al.*, 1995; Wild, 1993).

1.6 Phytoremediation of hydrocarbons

The use of plants has been shown to be effective in many TPH phytoremediation studies. The process by which organic contaminants in the soil are broken down by microbial communities on plant roots is called rhizodegradation (Anderson *et al*., 1993). The carbon in the TPH is a good energy source, or substrate, for many microbes and causes their growth to be greatly stimulated (Adam & Duncan, 2002; Foth, 1984). Although many microorganisms can metabolise TPHs, no single microorganism possesses the microbial capacity to degrade all, or even most of the compounds in a petroleum mixture. More rapid

rates of degradation occur when there is a mixed microbial community than can be accomplished by a single species (Atlas, 1984). The diversity and high degrading activity of microorganisms in the rhizosphere may account for this efficacy of plants in stimulating the removal of hydrocarbons from the soil (Nichols *et al*, 1997).

Kirk *et al.,* (2005) conducted a laboratory study into the numbers of bacteria and the number of hydrocarbon-degrading bacteria found in planted and unplanted soils contaminated with 31,000 ppm TPH. They found that after 7 weeks perennial ryegrass altered the rhizosphere by hosting 233 times more bacteria and 37 times more hydrocarbon-degrading bacteria compared to the unplanted soil. Alfalfa also increased the number of both bacteria and hydrocarbon-degrading bacteria, although the effect for alfalfa was not as substantial as perennial ryegrass, probably due to the different root morphologies.

Heterotrophic microorganisms require inorganic nutrients to degrade organic contaminants (Walworth *et al.*, 1997). The most important macro nutrients for plant growth and function are nitrogen, phosphorus and potassium. Nitrogen increases the plant biomass which has been shown to increase the rate of TPH degradation and so is therefore required in the highest concentration for effective phytoremediation (White *et al.,* 2003). The addition of nutrients during phytoremediation trials has had mixed results; Karthikeyan *et al.,* (2000) found there was a 73% removal in TPH concentration in planted sites with added fertiliser during a 2 year phytoremediation study whereas Ji *et al.*, (2004) found a 96% removal in TPH in planted sites with no added fertiliser during a two year phytoremediation trial.

The most common microbial genera responsible for TPH degradation include: *Nocardia, Pseudomonas, Acinetobacter, Flavobacterium, Micrococcus, Arthrobacter, Corynebacterium, Achromobacter, Bacillus, Aspergillus, Mucor, Fusarium. Penicillum, Rhodotorula, Candida* and *Sporobolomyces* (Balba *et al.,* 1998).

Plants shown to phytoremediate hydrocarbon contaminated soils include: fescue; Sudan grass (*Sorghum sudanese*); switch grass (*Panicum virgatum*) (Keller *et al.*, 2008); ryegrass (*Lolium perene*) (Kaimi *et al.,* 2006; Kirk *et al.*, 2005); maiden cane (*Panicum hemitomon);* bulltongue arrowhead *(Sagittaria lancifolia)* (Dowty *et al., 2001*); saltmarsh arrowhead (Spartina *alterniflora)* (Lin *et al*., 2002) and cattail (*Typha latifolia*) (Gessner *et al.,* 2005; Groudeva *et al.,* 2001).

Phytoremediation has been studied extensively *ex situ* in laboratory and microcosm experiments and the findings are very encouraging (Williams, 2002). Gunther *et al* (1996) conducted a laboratory scale experiment using agricultural top soil spiked with 4,330 mg/kg PAH. After 22 weeks, the levels of PAH had decreased by 97% in the column planted with ryegrass compared to only 82% removal in the unplanted control column. Liste & Felgentreu (2006) also found similar findings in a greenhouse study using soil contaminated with 1,517 mg/kg TPH from a former coal gasification site. After 95 days, the TPH levels had decreased by 84% (white mustard) and 81% (vetch) and 69% (unplanted control). Only a few full scale *in situ* applications have been tested (EPA, 2001) meaning the next phase in advancing acceptance of phytoremediation as a regulatory alternative must be to demonstrate sustained contaminant removal in real life, *in situ* sites (Wang *et al.,* 2010). Although soil microcosm experiments can be a useful tool to access the biodegradation potential of hydrocarbon contamination (Balba *et al.*, 1998), evaluation of phytoremediation in the field is difficult because of the inherent spatial heterogeneity and the hydraulic and chemical properties of the soil (Nedunuri *et al.,* 2000). In greenhouse and laboratory experiments soils are generally well mixed to achieve a uniform matrix. The special heterogeneity of contaminant levels can result in variation of concentrations between locations, which may make it difficult to statistically identify significant treatment effects in field trials (Gerhardt, 2009).

Challenges faced by plants during *in situ* trials include, temperature variation; nutrient variation; precipitation; herbivory (insects or animals); predation; plant pathogens and competition by weed species that are better adapted to the site (Nedunuri *et al.,* 2000).

1.7 Phytoremediation of hydrocarbons using wetland plants

The selection of an appropriate set of plants that are adapted to the site conditions and most capable of increasing the contaminant-degradation potential of the soil microbial community is crucial for the successful application of phytoremediation. The plant species selected should possess characteristics that enable them to grow on contaminated sites. Simultaneously, they should be able to establish microbial associations that facilitate the degradation of contaminants (Wang & Oyaizu, 2009).

Two wetland plants commonly used in phytoremediation studies are *Phragmites australis* (common reed) and *Typha latifolia* (common cattail). These plants are found in shallow water in marshes, bogs and the edges of shallow lakes, salt marshes and estuaries throughout the world. *P. australis* have round, hollow stems which grow up to 1.5 to 6 m in height and 8 to 32 mm in diameter (Clayton *et al.,* 2002; Massacci, 2001). In the UK, they flower in June/July with seed germination in late autumn (Massacci, 2001) after which the stems harden and the leaves die. These stems remain standing for 2-3 years before breaking near the surface. The underground rhizomes for the following year are then formed before winter (Haslam, 1972; Rodwell, 1995). *T. latifolia* can grow up to 3 m in height with spongy strap like leaves (Rook, 2002).

P. australis and *T. latifolia* are useful for the phytoremediation of eutrophic lakes and waste waters, as well as contaminated soils (McNaughton *et al*., 1974; Taylor & Crowder, 1984) and possess the traits for an ideal phytoremediation crop: rapid growth; high biomass and a high tolerance for contaminated soils (McDonald, 2006).

Massacci (2001) calculated that a typical plant of *P. australis* has an average above ground biomass dry weight of 50 g that includes about 13 g of leaves. As the number of plants per square metre can be up to 300, an incredibly high above ground biomass per square metre can be then accumulated in healthy and dense stands of *P. australis*. *T. latifolia* also produces a large biomass from a stand (Wang *et al.,* 1997) in similar amounts to *P. australis* (Solano *et al.,* 2004). This large biomass is advantageous for phytoremediation of eutrophic systems because a large amount of nitrogen, phosphorus and other elements must be invested in the shoots for their structure and function (Massacci *et al.*, 2001).

Both *P. australis* (Muratova, 2003) and *T. latifolia* (Ciria *et al.*, 2005) have demonstrated an increased degradation rate of hydrocarbons. *P. australis* has been shown to decrease the levels of bitumen tar in a 16 week laboratory study by 43% more than unplanted soil (Tischer, 2002). *T. latifolia* was shown to remove 90% of hydrocarbons from a starting concentration of 60 ppm in a wetland experiment over two years (Salmon *et al*., 1998) as well as removing 100% TPH from a 14 day constructed wetland contaminated with 15 to 20 mg/L TPH (Moore *et al.,* 1999).

Wetland soils are usually anaerobic due to water filling the air spaces of soil. Studies have shown that aerated soils enhance hydrocarbon degradation (Dowty *et al*., 2001). Both *T. latifolia* (Bendix *et al.,* 1994; Brix *et al.,* 1992) and *P. australis* (Brix *et al.,* 1996) have effective aeration systems based on internal convective through-flow of gases which give them outstanding capacities to vent their underground tissues. Both plants have highly developed gas aerenchymatous tissues (Armstrong, 1964; Callaway & King, 1996). During the growing season (Armstrong *et al.,* 1992), oxygen from the atmosphere can move downwards through these tissues (Brix *et al*., 1996; Moorhead & Reddy, 1998) and biogenic gas from the sediments can move upwards and into the atmosphere (Armstrong & Armstrong, 1991). Plant roots can then transport oxygen to the surrounding rhizosphere

(Armstrong et al., 1992; Boon & Sorrell, 1991; Brix et al., 1996, Jespersen *et al.,* 1998; Wießner *et al.,* 2002; Wright & Otte, 1999).

A second type of pressurised gas flow in *P. australis* is venturi-induced convection. This process can operate both during the growing season and in winter (Armstrong *et al.,* 1992) and involves oxygen being transported from the atmosphere to the rhizosphere when the plants are senesced by a 'venturi' mechanism through the open ended (cut or broken) culms (Ciria *et al*., 2005).

Plants with dense roots and/or greater specific surface area increase populations of soil microorganisms due to greater concentrations of root exudates supporting their growth (Kaimi *et al*., 2006; Keller *et al*., 2008). *T. latifolia* has rhizomes up to 3 cm in diameter and 69 cm in length growing up to 10 cm below the surface (Rook, 2002). *P. australis* has rhizomes up to 10 m long (Duke, 1979) which makes it an ideal plant to use for remediation of contaminated soil, the long rhizomes have a large surface area per unit volume of soil and fibrous root systems with extensive surface area for microbial colonisation (Adam & Duncan, 2002; Kaimi, 2007).

The actual composition of the microbial community in the rhizosphere is dependent on root type, plant species, plant age and soil type, as well as other factors such as exposure history of the plant roots to xenobiotics (Anderson *et al.*, 1993). Muratova (2003) showed that when *P. australis* was grown in hydrocarbon contaminated soil, it stimulated the development of heterotrophic microorganisms, significantly increasing the population of bacterial degraders by 40 times the levels found in unplanted soil.

P. australis can thrive in shallow and almost stagnant water, where few others species can survive, because its dense and vertically extended rhizome mat takes up nutrients and oxygen (Massacci *et al*., 2001). It can also survive in water up to 2 m above its substratum although optimal performance occurs at between 50 cm above substratum and 20 cm

below (Armstrong, 1979). It can survive flooding for several months of the year due to it having a low metabolic requirement of oxygen, but one third of its leaves need to be above the water (Tyler-Walters, 2003). *T. latifolia* can only tolerate deep water up to 95 cm (Callaway & King, 1996; Grace, 1988, 1989) and in drier areas of marsh *P. australis* may be out-competed by *T. latifolia* (Tyler-Walters, 2003). *T. latifolia* has been shown to allocate more of its biomass to leaves with increasing water depth and have decreasing shoot density and flowering. This is because when all other factors are constant, the dominant effect of increasing depth is light limitation. Since light penetration through water has a negative exponential function, plants in deeper water will have less total biomass capable of photosynthetic activity (less roots and flowers) and a larger proportion of their biomass would contribute to the respiratory burden of the plant (leaf and stem number and stem height) (Grace, 1989).

P. australis can tolerate a wide range of pH (4.8 to 8.2) and also a wide range of temperatures with an optimum range of 6.6 to 26.6^{o}C (Duke, 1979) which is similar to the 6 to 28°C reported for *T. latifolia* (Rook, 2002).

Although nitrogen is abundant in the atmosphere, combined nitrogen is generally a limiting factor in plant growth (Shimp *et al*., 1993). Nitrogen is a critical component in the mineralisation of hydrocarbon-contaminated soil (Adam & Duncan, 2002; Atlas, 1981). The addition of N-P-K fertilisers has been found to accelerate hydrocarbon degradation probably due to microbial enhancement (Leahy & Colwell, 1990). Many reports even state that healthy *P. australis* plants tolerate the over-loading of nitrogen and phosphorus in the aqueous environment (Comin *et al.,* 1997) and exhibit enhanced growth of shoots and rhizomes (Massacci *et al*., 2001).

1.8 Site description

As discussed in section 1.1, TPH contamination originates from many sources. This study is concerned with TPH contamination from a disused fire-training facility (FTF). Live fire training is obligatory to meet the Health and Safety training needs of the Fire Service (personal communication G. Coley, 2011). In England alone there are 46 Fire and Rescue Services each of which have a FTF or contracted access to a neighbouring facility. FTFs are also found at UK airports, military bases, ports, and police public order units in addition to commercial training providers offering basic fire extinguisher training (Justice, 2011). As well as burning a wide variety of hydrocarbons many FTFs have historically burnt solvents, wood, paints and many other combustibles. Combined with the fire fighting foams used to extinguish these fires, there are many compounds in the fire debris sediment that have arisen by pyrolysis, combustion and distillation of these substrates. These FTFs have left a legacy of soil contamination at sites operated before environmental control measures were legally required.

1.8.1 Site location

The study site is an old Royal Navy FTF and the contaminated lagoon is called Horsea lagoon. It is a 25 m wide by 75 m long shallow lagoon, located on Horsea Island to the immediate North East corner of Portsmouth Harbour, Hampshire, UK (Plate 1), its National Grid Reference is SU 635 044. Portsmouth Harbour is a designated Site of Special Scientific Interest (SSSI), Special Protection Area (SPA) and Ramsar site. This is due to the unique tidal basin, mudflats and the wide range of flora and fauna. The site is also in close proximity to a residential development and approximately 200 m from a large shopping and leisure complex named Port Solent.
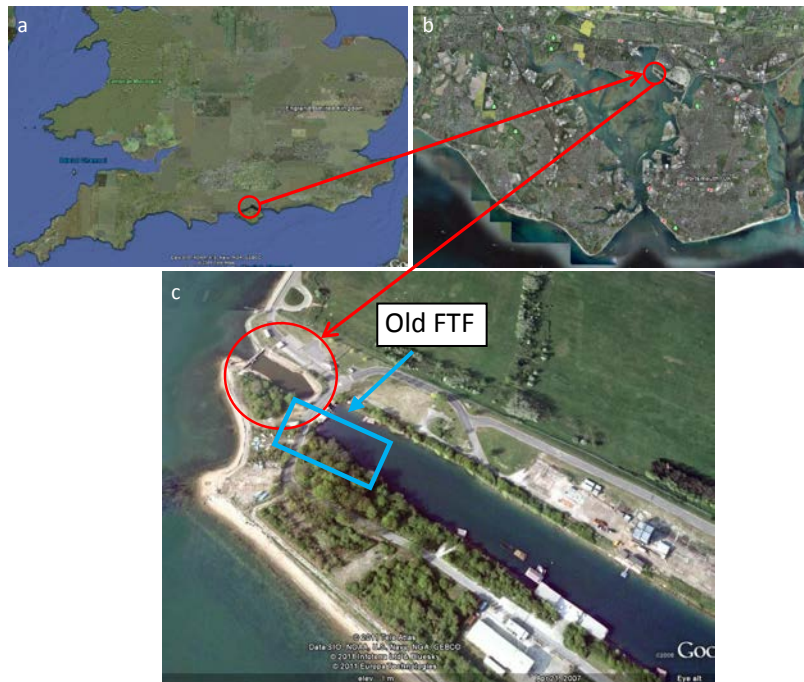
Plate 1. Horsea lagoon: South of England (a), Portsmouth Harbour (b) and Horsea Island (c) Google Earth, 2010

1.8.2 History of the site

Up until 2002, the lagoon received contaminated wastewater runoff from a large-scale naval fire fighting facility which had operated for approximately 50 years. The FTF (Plate 2) used DRO as a fire accelerant and large quantities of fire fighting foams. As well as burning DROs, many other combustibles were used including solvents, wood and paints. Wastewater was discharged into the lagoon, which is directly adjacent to Portsmouth Harbour. This discharge originally had a double rubble dam through which the lagoon waters were filtered into the harbour. In 1998, a double sheet piled dam with a concrete core with a sluice was constructed in place of the original dam, as a consequence water depth within the lagoon could be controlled by means of a mechanically operated sluice gate in the dam wall. In June 2000 the Environment Agency confirmed no further discharge of effluent would be made into the harbour unless discharge conditions were complied with.

Plate 2. The fire training facility based on Horsea Island decommissioned in 2002, Environmental Science Group, 2000

1.8.3 Ground conditions on the site

The UK MOD Environmental Science Group (ESG) carried out comprehensive site characterisation investigations in 2001 and 2003. Their findings noted that the sediment was generally comprised of black, fine-grained material. The underlying natural soil generally comprised a soft to firm grey clay with occasional chalk fragments. The sediment appeared to be anoxic from within a few millimetres of the surface. Hydrogen sulphide (H_2S) was between 3-58 ppm exceeding the occupational exposure limit of 15 ppm. This level is up to 4 times higher than the UK occupational exposure limit and, coupled with the proximity to residential lettings meant that an *in situ* method not requiring excessive mechanical disturbance, such as phytoremediation, was attractive.

The site investigations identified the only contaminant of concern to be petroleum hydrocarbons, in particular DRO. Sediment samples analysed at the Institute of Naval Medicine, Gosport determined the levels of DRO to be approximately 53,000 mg/kg wet weight. The samples tested at TES Bretby laboratory services, UK were slightly higher at 58,300 mg/kg wet weight. The remediation objective set by the MOD was to ensure the total DRO concentration was lower than 2,000 mg/kg.

2. SCOPE OF STUDY

2.1 Rationale

A large number of successful small-scale, *ex situ* studies have lead to the conclusion that plants and their associated microorganisms have the ability to increase the rate of hydrocarbon degradation in contaminated soils (Pradhan *et al.,* 1998; Schwab *et al.,* 1995). However, large full-scale studies have been less frequent and have given mixed results depending on the level of hydrocarbon contamination and the plants used. To date, no full-scale study has been carried out *in situ* using non-homogenised hydrocarbon-contaminated sediment, which would represent a 'real-life' phytoremediation operation to determine whether it is an appropriate technique for cleaning up *in situ* sediments.

Horsea lagoon represents an extremely contaminated site with a mixture of hydrocarbons comparable to the most contaminated sites ever investigated in phytoremediation studies. This study will investigate whether *ex situ*, homogenised experiments are representative of real life *in situ*, non homogenised experiments and also look at the sediment conditions over time and how they affect phytoremediation rates. The mechanisms affecting the numbers and types of microorganisms will also be investigated and compared between planted and unplanted environments.

2.2 Aims

The aims of this study were:

- To determine the extent of hydrocarbon contamination in Horsea lagoon.
- To assess whether the addition of plants can affect the degradation of DRO from non-homogenised sediment in a semi-aqueous environment compared to an unplanted control.
- To study the microbial populations capable of DRO degradation

- To determine whether phytoremediation is a suitable method for removing DROs from Horsea lagoon.

2.3 Objectives of the study

Three experimental systems were set up to investigate different aspects of the research, *ex situ* mesocosms; *ex situ* greenhouse microcosms and *in situ* full-scale study at Horsea lagoon.

The objectives of the study were:

- To determine the levels and variation of DRO contamination in Horsea lagoon

- To determine the most suitable native plant species for phytoremediation of the site.

- To investigate if nutrient supplements were required for the plant growth and the affect on the rate of growth of the plants.

- To assess if plant growth affects levels of DROs.

- To assess if the plants significantly affect the overall numbers of microorganisms, particularly the number and diversity of hydrocarbon-degrading bacteria.

- To investigate the environmental conditions which promote the hydrocarbon-degrading populations.

- To compare the findings from *ex situ* and *in situ* studies.

- To assess the optimum site conditions for increased DRO degradation.

- To make recommendations concerning the feasibility and strategy for phytoremediation of the site.

2.4 Research strategy

The three systems were used to address different objectives. The plant selection and growth characteristics were initially investigated in the *ex situ* mesocosms, the *ex situ* microcosms were primarily designed for investigations into microbial populations and the

in situ field evaluation focused on the DRO concentrations. However, there was some overlap between the monitoring parameters which allows comparison between the 3 systems.

2.4.1 *Ex situ* mesocosms

An important aspect of the study was to determine the species of plant to be used for the phytoremediation. It needed to be a native species in order to minimise its effect on the local ecosystem and able to tolerate both semi-saline water and extremely high concentrations of sediment DRO. The plants chosen for the initial trial were *T. latifolia* and *P. australis*. Both of these plants have previously demonstrated high decontamination rates of hydrocarbons in phytoremediation studies due to their rapid growth, high biomass, and a high tolerance for contamination (McDonald, 2006). Growth of both plants was evaluated in Horsea lagoon sediment, using mesocosms, with and without the addition of fertiliser. This was assessed in controlled conditions as adding fertiliser to the lagoon could cause algal blooms and affect the surrounding harbour, which is designated a SSSI, SPA and Ramsar site.

2.4.2 *Ex situ* greenhouse microcosms

An *ex situ* greenhouse microcosm study was set up to closely examine the microbial mechanisms of DRO degradation at a variety of depths in the highly contaminated, homogenised Horsea lagoon sediment. The identity of the hydrocarbon–degrading bacteria found in the planted and unplanted microcosms were determined and the results compared with those species found in the *in situ* study. The greenhouse setting allowed some control of environmental conditions comparable to the majority of previous phytoremediation studies. Dismantling of the microcosms also allowed sediment samples to be removed from the plant rhizosphere and also bulk soil for comparison after the routine monitoring had been completed.

2.4.3 *In situ* field study

An *in situ* study at Horsea lagoon was set up to determine whether phytoremediation was a viable method for cleaning up highly contaminated sites under real-life environmental conditions with non-homogenised sediment. The concentration of DRO in the planted and unplanted sites were routinely monitored at a variety of locations and depths to determine the optimum conditions for hydrocarbon degradation. The numbers of microbes present as well as their identity were investigated and compared to the bacteria identified in the greenhouse microcosms.

2.4.4 Monitoring methods

It was important to determine the fate of the total DRO concentrations over time in the homogenised sediment in environmentally controlled greenhouse microcosms and in the non-homogenised sediment at Horsea lagoon.

This was done using accelerated solvent extraction (ASE) and GC-FID. The height, shape and position of the UCM on the chromatogram produced can help determine how degraded the hydrocarbons present were. The total DRO was determined by drawing a line from the baseline at the retention time for C_{10} to the baseline at the retention time for C_{25} on the chromatogram incorporating the UCM. This method was advised by the Total Petroleum Hydrocarbon Criteria Working Group (1998) and was demonstrated in many published papers investigating environmental samples (Mujis & Jonker, 2009, Bregnard *et al.,* 2000, Wang *et al.,* 1995). This method of analysis was preferential to one involving the measuring of each peak height due to the lack of distinct peaks found in the Horsea lagoon sediment chromatograms. The results showed whether a small-scale study could accurately represent an *in situ* site study and whether plants significantly increased the degradation of DRO by comparing planted and unplanted sites. The GC-FID chromatograms could also be qualitatively analysed to determine changes in maximum UCM height, UCM shape,

start and end of the UCM and any resolved peaks to assess whether there had been a change in the DRO patterns over time and between studies.

Environmental conditions including; moisture content, pH, air temperature, air humidity, water salinity, sediment composition, submergence and distance from the waters edge were all used to investigate seasonal variability and determine the optimum conditions for future *in situ* phytoremediation studies.

To assess the influence of plants on microbial populations the total number of culturable bacteria and fungi were examined using the most probable number (MPN) method. When the ESG carried out their study of Horsea lagoon they noted that the sediment appeared to be anoxic within a few millimetres of the surface. However, as aerobic bacteria have been shown to be the primary hydrocarbon degraders, the aim of the study was to increase the oxygen levels within the sediment with the addition of plants to monitor whether this increased aerobic activity.

The hydrocarbon degradation ability of the bacteria, at various depths in the planted and unplanted sites was also investigated. Previous studies such as Kirk (2005) and Wrenn (1996) have defined presumptive 'hydrocarbon-degrading' bacteria as any bacteria able to grow in or on minimal media with a hydrocarbon added as the sole carbon source. In this study similar techniques were used but the results were referred to as 'hydrocarbon-tolerant' bacteria as they cannot be confirmed as to whether they are presumptive to be degrading hydrocarbons without a colorimetric indicator added. Colorimetric hydrocarbon degrading indicator plates allowed the identification of metabolic differences between organisms by the colour of the colonies they produce (Hopwood, 1970). In this study, the respiration activity of the bacteria was made visible by the redox stain 2,3,5-triphenyl tetrazolium chloride (TTC), which when reduced produces a clearly visible red triphenyl formazan (Olga *et al.,* 2008).

Once the hydrocarbon-degrading bacteria were detected they were characterised using an activity assay or molecular techniques. Previously reported species found in hydrocarbon contaminated environments include, *Pseudomonas, Flavobacterium* and *Alcaligenes* (Euliss *et al*., 2008). Characterising the culturable bacteria found in Horsea lagoon sediment allows the microbial population in these extreme levels of DRO to be compared with hydrocarbon degrading bacteria encountered in other phytoremediation studies at lower concentrations of contamination.

3. MATERIALS AND METHODS

3.1 Systems

Three different trials involved in this work are described in the following sections:

3.1.1 *Ex situ* mesocosm

This initial trial had the aim of selecting the preferred plant species for future experiments. In April 2007, approximately 450 L of the hydrocarbon-contaminated sediment was removed from Horsea lagoon. This sediment was mixed thoroughly with 300 L of Horsea lagoon water to provide a homogenous material both with respect to depth and location. It was then transported to the research facility belonging to the University of Portsmouth in Petersfield, Hampshire. It was divided equally into 15 large 100 L plastic bins and covered with 20 L of Horsea lagoon water to simulate the conditions found at the lagoon site. Using random number generation, six of these bins were then planted with equal numbers of newly dug *P. australis* and six with *T. latifolia* from a nearby freshwater lake believed to be marginally tainted with heavier hydrocarbon fractions. The remaining three bins contained no plants and acted as controls (Plate 3). Using random number generation again, three of the six bins containing each plant species received regular nutrient addition (Phostrogen) and all the bins were topped up with water on a weekly basis to maintain submerged conditions.

From April 2008 until October 2008, plant growth was monitored using bi-weekly shoot height measurements taken on ten randomly selected shoots (if that number was present) and counting the total number of shoots in each bin.

Plate 3. *Ex situ* mesocosms, H. Pinchin, July 2008

In August 2010, one mesocosm planted with *P. australis* was dismantled. Sediment samples were taken from three depths (0 cm, 15 cm and 30 cm) using an acrylic tube (1 m long x 53 mm diameter x 3 mm wall thickness from Clear Plastic Supplies, U.K) This process is shown in Plate 4a and b. In addition, samples were also taken from the same depths in a control mesocosm. These samples were analysed for DRO concentration and microbial counts.
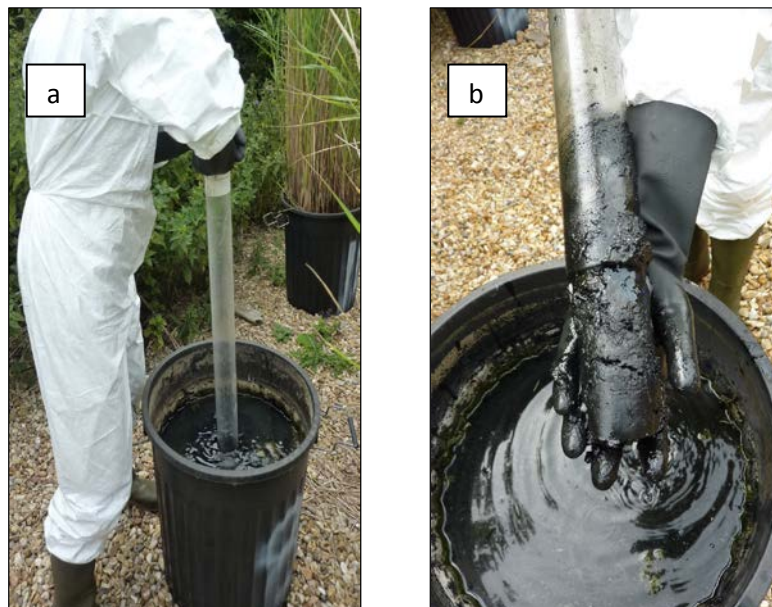
Plate 4. Extracting a mesocosm sediment core (a) the extracted core (b) H. Pinchin, July 2008

3.1.2 *Ex situ* microcosm

The microcosms were mainly set up to allow examination of microorganisms in environmentally controlled conditions. Three microcosms were set up in April 2009. Perspex cylinders of 1 m height x 200 mm diameter (Clear Plastic Supplies, U.K) had four 27 mm diameter holes cut into the side of them and these were sealed with neoprene plug stoppers (B&Q, U.K). These holes were cut at 72˚ angles from each other at 0 cm, 15 cm, 30 cm and 45 cm spiralling down from the surface of the sediment (Plate 5a). The cylinders were then fastened to flat perspex bases, filled with sediment from Horsea lagoon and then topped up with water weekly to simulate the conditions found at the lagoon site. Two microcosms were planted with a small bunch of *P. australis* from the Gosport fuel depot which is situated locally to Horsea lagoon and contains hydrocarbon-contaminated sediments (PL1 and PL2) and one was left unplanted as a control (UP3) (Plate 5b).
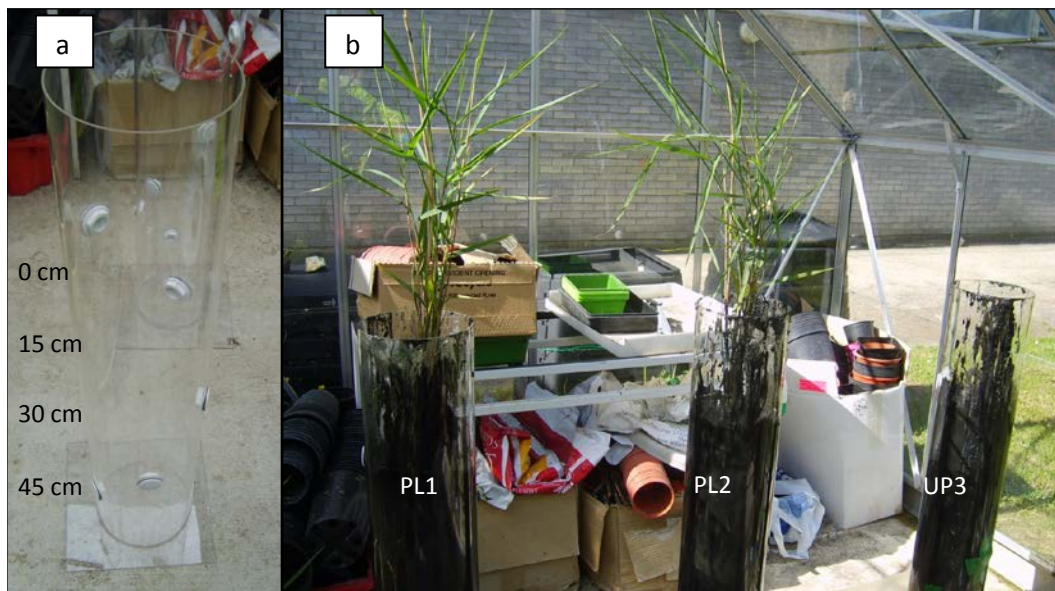
Plate 5. Greenhouse microcosms (a) Empty (b) August 2009, H. Pinchin

From August 2009, samples were taken monthly through the holes in the side wall of the cylinders using a sterile stainless steel 1 cm x 15 cm tube and used to investigate the

microbial numbers present in the sediment. From January 2010, the DRO concentration at each sample site was also determined. In January 2010, bacterial isolates were cultured from the MPN culture plates and grouped according to morphology. Bacterial isolates were then tested for their hydrocarbon degrading capabilities and identified using API, Biolog and molecular techniques.

In August 2010, 17 months after construction, planted microcosm, PL1, was dismantled. Sediment samples were then taken from four depths (10 cm, 20 cm, 40 cm and 60 cm) at the centre and edges. Samples were also taken from the unplanted microcosm, which was not dismantled. The DRO concentration and number of microorganisms was determined.

3.1.3 *In situ* Horsea lagoon field trial

An *in situ* trial was carried out to investigate the rates of hydrocarbon removal by plants in non-homogenised sediment in an uncontrolled environment.

A survey of the slope of the lagoon sides (Figure 2) determined that the South West side was best for planting. Here the bank slope into the water was gentler and the maximum depth of water was only 0.6 m at a 4 m distance from the surrounding wall.
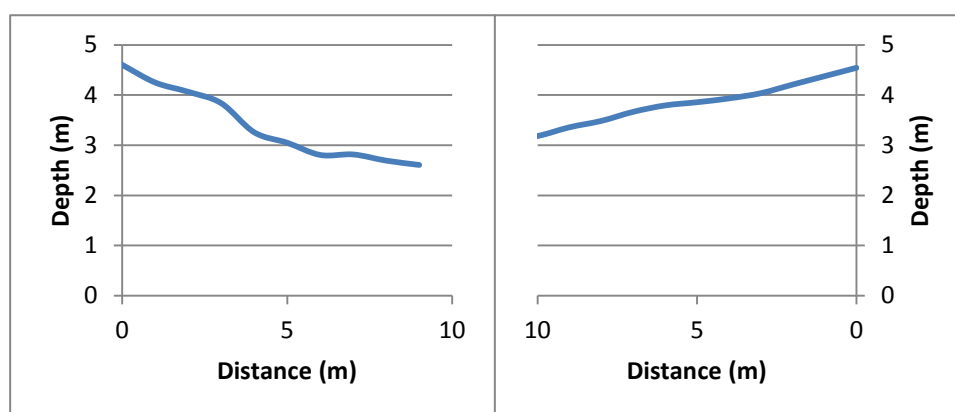
Figure 2. Slope gradients from the North East (left) and South West (right) banks of Horsea lagoon

In situ planting in Horsea lagoon took place in April 2009. Eight 1 m^2 quadrats were set up along the South West shore. Approximately 90 small bunches of *P. australis* were dug up from the Gosport fuel depot and planted at a rate of 11 clumps/m^2. The planted sites designated PL1 to PL4, descended down the slope, with site PL1 furthest from the edge of the water and site PL4 in the water. Sites UP1 to UP4 were located parallel to the planted sites but remained unplanted (Plate 6).
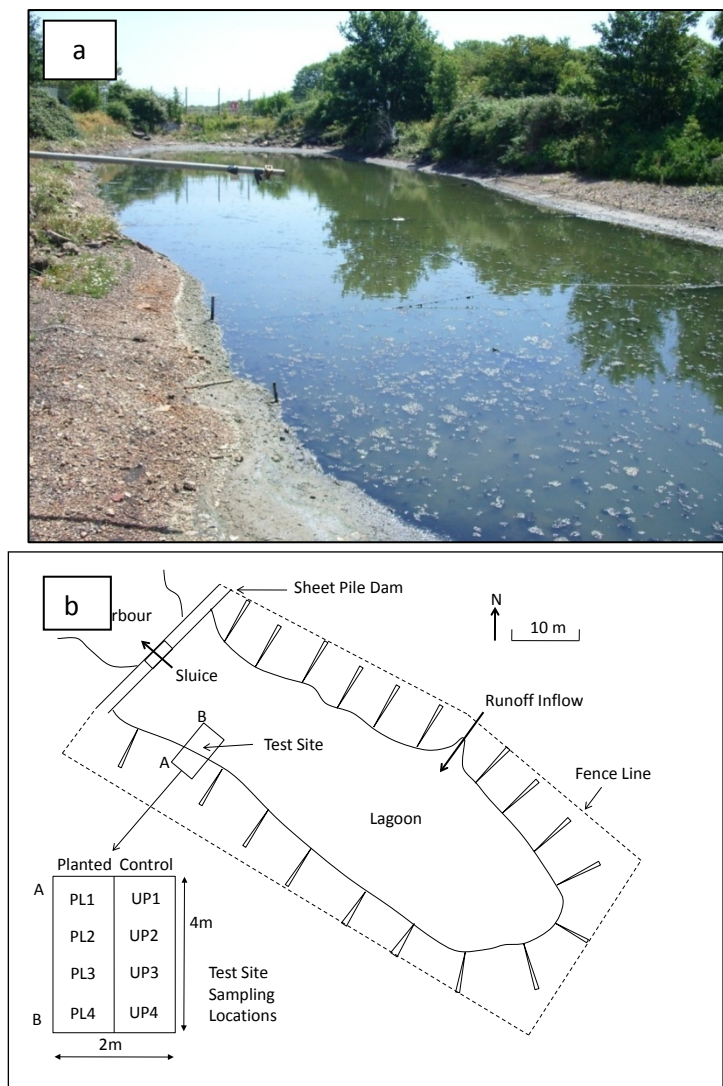
Plate 6. Horsea Lagoon site: (a) View from Dam, Environmental Science Group, 2009 (b) Schematic plan

From May 2009, sediment cores were removed bimonthly from all positions using a 1 m x 53 mm acrylic corer (Clear Plastic Supplies, U.K) (Plate 7a). Sediment was pushed into a new aluminium tray using a tight fitting plunger and sliced into depth ranges of 0-2 cm, 2-7 cm, 12-17 cm (Plate 7b)
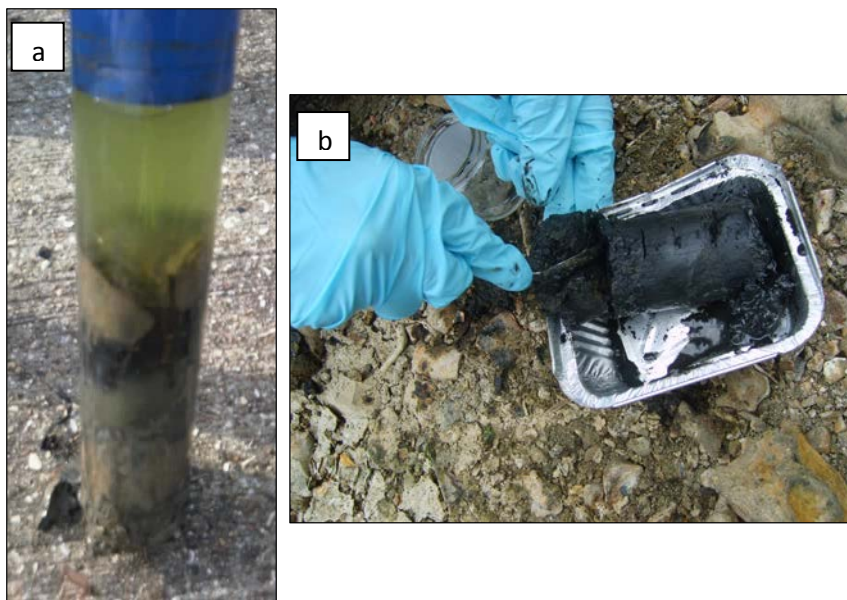
Plate 7. Collection of sediment cores from Horsea lagoon showing (a) the collection tube (b) sectioning up the extracted sediment core, H. Pinchin, 2009

The sediment was then packed into labelled glass jars with no headspace and tightly closed (Plate 8a). The jars were immediately wrapped in aluminium foil to block exposure to light (Plate 8b) and then transported in cold boxes packed with freezer packs to slow microbial growth rates (Plate 8c). They were then stored at 4˚C and analysed for DRO content within 48 hours.

From March 2010, the microbial numbers at each sample site were also determined. In March 2010, bacterial isolates were cultured from the MPN culture plates and grouped according to morphology. Bacterial isolates were then tested for their hydrocarbon degrading capabilities and identified using API, Biolog and molecular techniques.
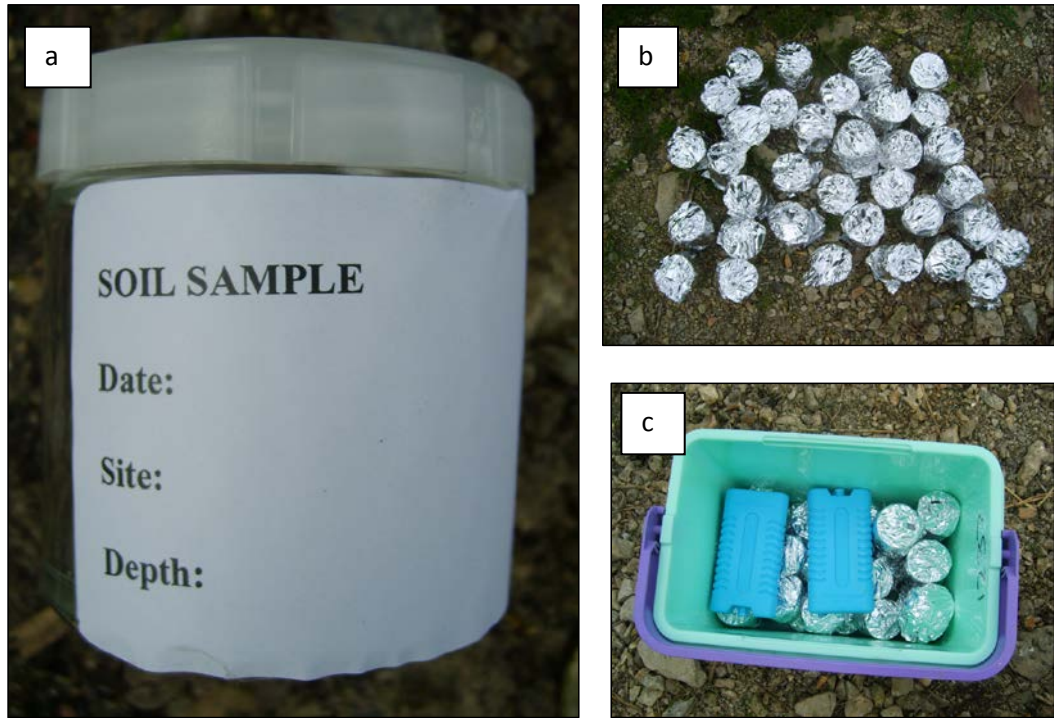
Plate 8. Sample collection bottles showing (a) a labelled collection jar (b) a jar wrapped in foil (c) jars being transported, H. Pinchin, 2009

The sediment at Horsea lagoon was predominately sand (58-85%) with relatively large amount of silt (10-38%) and a small proportion of clay (5-9%). PL1-PL3 and UP2-UP4 were classed as 'sandy loam' and PL4 and UP1 were classed as 'loam sand' (Figure 3). Planted sites PL1 to PL4 had almost exactly the same mean levels of sand (70%), silt (24%) and clay (7%) as the unplanted sites UP1 to UP4 (71%, 23% and 6% respectively) making the sites suitable for comparison.

The water in the lagoon and the surrounding sea water was measured for pH and salinity level to investigate whether this could be a limiting factor for the plants. The salinity of Horsea lagoon water was found to be 7 g/L and the sea water was 39 g/L, *P. australis* can tolerate salinity up to 65 g/L so these levels are well within this range. The pH of Horsea lagoon water was found to be 8.4 which is a little more alkaline than desired as *P. australis* requires a pH of between 4.8-8.2 for optimum growth.

(a) PL1
- 7%
- 23%
- 70%

(b) PL2
- 9%
- 20%
- 71%

(c) PL3
- 6%
- 36%
- 58%

(d) PL4
- 6%
- 15%
- 79%

(e) UP1
- 5%
- 10%
- 85%

(f) UP2
- 5%
- 35%
- 60%

(g) UP3
- 8%
- 26%
- 66%

(h) UP4
- 5%
- 21%
- 74%

Sand (0.05 mm- 2.00 mm)
Silt (0.002 mm-0.05 mm)
Clay (less than 0.002 mm)
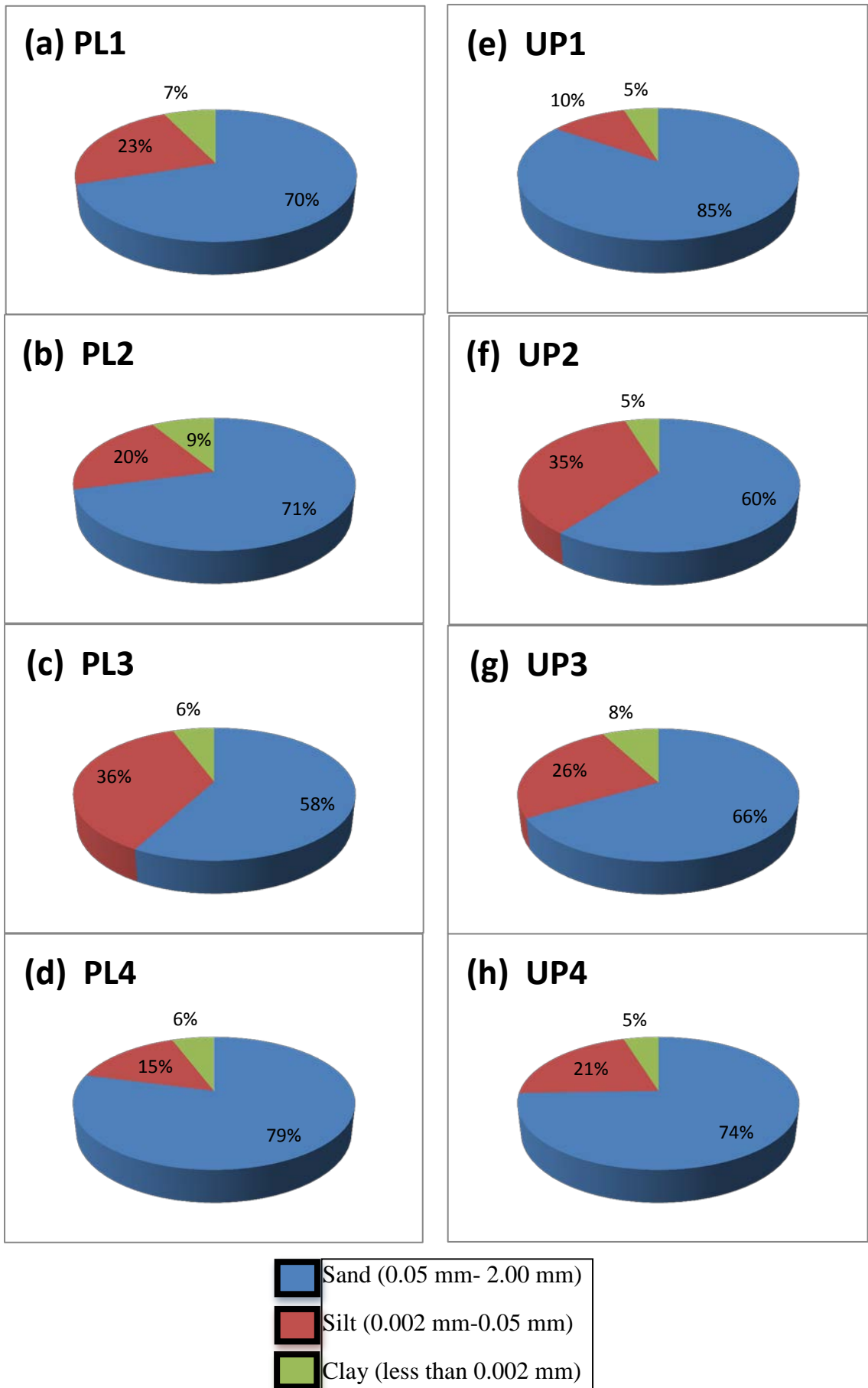
Figure 3. Sediment particle size in Horsea lagoon sample sites

3.2 Site characteristics

3.2.1 Sediment particle size

The International Organisation for Standardisation (ISO, 2003) method for determination of dry matter and water content on a mass basis-Gravimetric method was used. The sediments were spread thinly over foil trays and dried for 24 hours at 60˚C. 100 g of each mixed, dried sediment was weighed and sieved down to 2 mm. The sieved sediments were then placed in glass beakers containing 20 mL 1 M HCl (Fisher, UK). 5 mL 1 M HCl was then added until the frothing stopped. The sediments were then filtered using QL 100 filter paper (Fisher, UK). The sediments were again dried in a drying cabinet for 24 hours at 60˚C. Sediment was weighed and 25 g of each sediment added to glass litre beakers. Then 25 mL of 30% hydrogen peroxide was added to each sediment sample. A large petri dish was placed over the top to form a loose lid and samples were left for 12 h. The petri dishes were then removed and the beakers were put on a hotplate and boiled for 30 min but not allowed to dry out. Samples were all made up with 200 mL with distilled water and 10 mL 10% sodium hexametaphosphate (Fisher, UK) was added. Samples were poured into 1L glass measuring cylinders and made up to 1L with distilled water. Cylinders were then stirred thoroughly with plastic rods. After 46 s and 5 h, a soil hydrometer (model ASTM 152H, ERTCO, Cole-Palmer, UK) -5 to 60 Grams was placed into each suspension and a buoyancy reading and the temperature taken.

The sediment particle size was then determined using the following formula once the hydrometer readings were corrected by adding 0.3 units per 1˚C above 20˚C and subtracting 0.3 units for every 1˚C below 20˚C.

$$Sand\ (\%) =$$

$$100 - \left[\frac{first\ hydrometer\ reading\ (corrected\ for\ temperature)\ x\ 100}{Inorganic\ sample\ weight} \right]$$

$$Clay\ (\%) = \left[\frac{second\ hydrometer\ reading\ (corrected\ for\ temperature)\ x\ 100}{Inorganic\ sample\ weight}\right]$$

$$Silt\ (\%) = 100 - (\%\ sand + \%\ clay)$$

3.2.2 Calculating the dry weight of sediment

Ten grams of each sediment sample was dried for 24 hours at 40˚C in a drying cabinet (Leec, UK). The sediment was then re-weighed.

$$Sediment\ dry\ mass\ (\%) = \left|\frac{Dry\ sediment\ mass\ (g)\ x\ 100}{Wet\ sediment\ mass\ (g)}\right|$$

3.2.3 Sediment pH

Sediment pH was measured by a method developed by the Institute of Naval Medicine. 10 g of each sediment sample was dried for 24 h in a drying cabinet at 40˚C (Lec, UK), re-weighed and placed into centrifuge tubes containing 25 mL of distilled water and shaken for 15 min on a flat bed rocker (model L520, Gerhardt Schuttelmaschine). The tubes were then left to settle for one hour. The pH was read using a pH meter (Orion) after being calibrated for pH 7, 9 and 10.

The pH in the lagoon was also tested on site using a pH probe (model HI9125, Hanna, UK).

3.2.4 Measuring the amount of suspended solids of the waterlogged sediment

A suitable volume of mixed mesocosm sediment was vacuum filtered through a pre-weighed GF/C Whatman filter paper. It was then dried in an oven at 105˚C for 2 h, cooled in a desiccator and re-weighed to 4 decimal places. Suspended solids in mg/L was then calculated using the following formula:

$Suspended\ solids\ (mg/L)$

$$= \left[\frac{final\ filter\ paper\ weight\ (mg) -\ Initial\ filter\ paper\ weight\ (mg)}{Sample\ volume\ (mL)}\right] x\ 1000$$

3.2.5 Organic and inorganic material in the sediment

Filter paper from the suspended solids test was placed in a crucible and put in a furnace at

550˚C for 20 min. The filter paper was cooled in a desiccator and re-weighed.

Inorganic material was calculated using the following formula:

$Inorganic\ material\ (mg/L)$

$$= \left[\frac{final\ filter\ paper\ weight\ (mg) -\ Initial\ filter\ paper\ weight\ (mg)}{Sample\ volume\ (mL)}\right] x\ 100$$

Organic material was calculated using the following formula:

Organic material (mg/L) = suspended solids (mg/L) – inorganic material (mg/L)

3.2.6 Chemical Oxygen Demand (COD) of the sediment

COD measures the amount of an oxidising agent that is consumed when the organic matter in the water sample is oxidised completely to CO_2. Two mL of a suitable dilution of sediment was added to a COD vial (0-1500ppm) containing 86% sulphuric acid, mercury sulphate and chromium trioxide. The lids were tightly sealed and gently mixed. The vials were placed in a COD reactor block (DRB200, Hach, UK), preheated to 150˚C, for two hours, cooled then read in a colorimeter (DR890, Hach, UK). The colorimeter reading was then multiplied by the dilution factor.

3.2.7 Biochemical oxygen demand (BOD) of sediment

BOD is an empirical test for determining the amount of biochemically degradable organic material by measuring the oxygen used by the aerobic biological organisms to oxidise the

organic matter and inorganic matter such as sulfides and ferrous iron (Liu, Björnsson, & Mattiasson, 2000). A suitable dilution (1/F) of the sample was made with aerated distilled water containing 1 mL/L of ferric chloride, calcium chloride, magnesium sulphate, phosphate buffer and allythiourea. Initial dissolved oxygen concentration was taken using an oxygen meter (50B, YSI, UK). The samples were then incubated for five days at 20°C in the dark. Dissolved oxygen concentration was then re-taken.

BOD was then calculated using the formula:

BOD (mg/L) = $1/F [(S_1-S_2)-(B_1-B_2)]$

Where S_1 = Initial dissolved oxygen concentration of sample

S_2 = Final dissolved oxygen concentration of sample

B_1 = Initial dissolved oxygen concentration of blank control

B_2 = Final dissolved oxygen concentration of blank control

$1/F$ = Dilution factor

3.2.8 Measuring air temperature and humidity

Air temperature and humidity were measured hourly by the Solent weather station operated by the Meteorological Office, Exeter, UK. The data was supplied via personal communication (Jebson, 2010).

3.3 Analysis of Diesel Range Organics (DRO) in sediments

3.3.1 Extraction process

The amount of sediment sample used depended on the contamination. For these samples, 1 g sediment was mixed with equal parts Hydromatrix (Varian, UK) until a crumb like state was reached. This was then added to an extraction vial (Dionex, UK) sandwiched between acid washed sand (VWR, UK) and partitioned with disposable cellulose filters (Dionex,

UK). The extraction was performed on an accelerated solvent extractor (ASE) 200 (Dionex, UK) using the method adapted from Dionex method 324 'accelerated solvent extraction of hydrocarbon contaminants in soils' and gave a mean 91% extraction efficiency.

The ASE had the following settings:

- solvent 1:1 hexane: acetone (Fisher Scientific, UK)

- heat= 9 min

- static= 5 min

- flush= 60 % volume

- purge= 60 sec

- cycles= 1

- pressure= 103.4 bar

- temperature= 200˚C

For every eight sediment samples extracted, 0.6 g of a standard 1,060 mg/kg DRO contaminated clay loam (LGC Standards, UK) was extracted for quality control. The resulting extract was then made up to 50 mL in 1:1 hexane: acetone and 1.5 mL was pipetted into a gas chromatography (GC) auto sampler vial (Technopath, UK) and capped using crimp caps (Technopath, UK).

3.3.2 Analysis of extracts for DRO

Before each run of samples, the GC-FID was calibrated using freshly made standards of: 1000 mg/L, 5000 mg/L, 20,000 mg/L, 30,000 mg/L, 40,000 mg/L and 50,000 mg/L diesel in 1:1 hexane: acetone.

3.3.3 Measuring the levels of DRO in the sediment extract

The prepared extracts were analysed on a GC-FID using a method from Anatune (Turner, 2004). A GC-FID works by directing the gas phase output from the column into a

hydrogen flame. The increased current due to electrons emitted by burning carbon particles is then measured.

The GC (model 6890 Series, Hewlett Packard) had the following settings:

- Cycle time= 3.52 min

- column= 15 m x 0.32 mm i.d. x 0.1 µm film

- carrier gas= helium

- column flow= 5 mL/min

- inlet temperature = 300˚C

- split flow ratio= 50 mL/min

- gas save= on

- saver flow= 20 mL/min

- saver time= 2 min

The flame ionisation detector (model GCXLR8R, Anatune) had the following settings:

- Temperature= 300 ˚C

- Hydrogen flow= 40 mL/min

- Air flow= 400 mL/min

- Make up nitrogen= 30 mL/min

1 µL sample was injected using a cycle dual injection. The temperature cycle was: oven temperature was 50˚C (hold 0.6 min) to 250˚C at rate 120˚C/min, then to 300˚C at 100˚C/min, then to 350˚C at 90˚C/min. After every sediment sample, a blank comprising 1:1 hexane: acetone was run through the GC-FID to remove any remaining analyte from the column. After every eight samples extracts were injected with a standard of 12,700 mg/L and 36,900 mg/L for quality control along with the sediment standard that had been extracted by ASE (Figure 26). A chromatogram was produced for each sample (see appendix Figure 27) with the resolvable hydrocarbons appearing as peaks and the unresolvable hydrocarbons appearing as the area between the lower baseline and the curve

defining the base of the resolvable peaks (EPA, 2003). Before each run of samples, 1 µL of a hydrocarbon window-defining standard in chloroform (AccuStandard, UK) was run through the GC-FID under the same conditions as the sediment extracts. The sum of all the peaks eluting between C_{10} and C_{25} was found by projecting a horizontal baseline between these retention times (EPA, 2003). This area under the chromatogram including the UCM was used to identify the DRO in the injected sample.

Results were then expressed as DRO concentration/dry weight using the following calculation:

$$DRO\ dry\ mass\ (mg/kg)$$

$$= amount\ of\ DRO\ in\ 1\ \mu L\ injected\ sample\ (\mu g) x\ 50{,}000\ (\mu L)\ x\ \left[\frac{100}{Dry\ mass\ (\%)}\right]$$

3.3.4 Qualitative GC-FID chromatogram analysis

The chromatograms from the dismantled mesocosms and microcosms as well as day 0 and day 437 in PL1, PL4, UP1 and UP4 from Horsea lagoon were qualitatively analysed to determine the carbon number position of start of the UCM (Figure 4), the maximum height of the UCM and any resolved peaks using the retention times of the DRO standards. The total UCM area was calculated in pA^2 by tracing around the perimeter of the UCM hump in ImageJ (National Institutes of Health, USA).
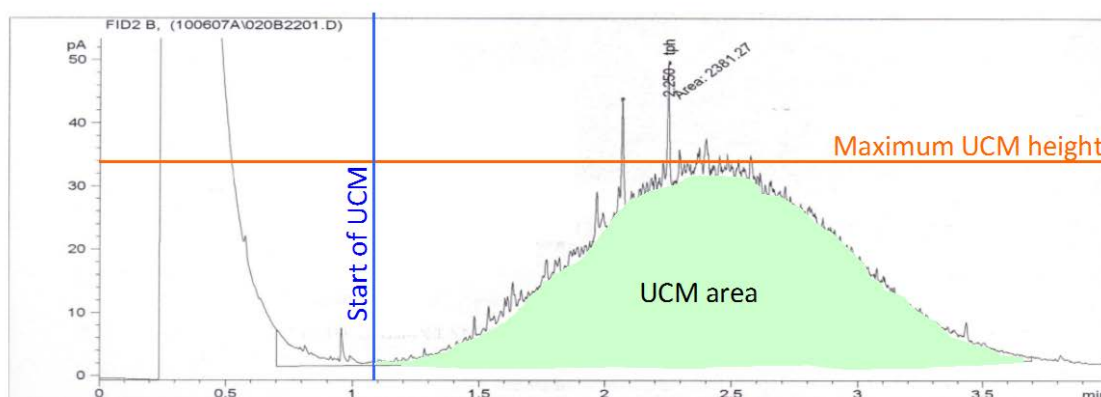
Figure 4. GC-FID chromatogram showing qualitative analysis

3.4 Microbial analysis

3.4.1 General microbial counts

3.4.1.1 Most Probable Number (MPN) method

MPN allows the enumeration of culturable microbial numbers without having to count every cell or colony. Numbers of bacteria and fungi were determined by culturing the sediment dilutions on specific agar and incubating them at different temperatures and for different lengths of time.

One gram of sediment was added to 50 mL Phosphate Buffered Saline (PBS). The MPN method of Harris and Sommers (1968) was then followed using four different agars (Table 2).

Table 2. Incubation times, temperatures and agars used to culture the microorganisms

Aerobic microbe cultured	Agar	Incubation temperature (˚C)	Incubation time (days)
Total Bacteria	TSA+75 ppm cycloheximide	25	7
Total Fungi	PDA	25	7
Hydrocarbon-tolerant bacteria	Bushnell Haas agar with 1% diesel	28	7
Hydrocarbon-tolerant fungi	Minimal salts agar+0.02% diesel+100 ppm chloramphenicol	28	21

The following media were used:

Trypticase soy agar (TSA) with 75 ppm cycloheximide

15 g casein peptone, 5 g soya peptone, 5 g sodium chloride and 15 g agar. 40 g TSA (Oxoid, UK) was mixed with 1 L distilled water and 75 ppm cycloheximide (Oxoid, UK) to inhibit fungal growth and autoclaved at 121˚C for 15 min at 15 psi according to the method of Kirk *et al.*(2005)

Potato dextrose agar (PDA)

Four grams potato starch, 20 g glucose and 15 g agar. 39g PDA (Oxoid, U.K) was mixed with 1 L distilled water and autoclaved at 121˚C for 15 min at 15 psi.

Bushnell Haas agar with 1% diesel

The agar used by Kirk *et al.* (2005) contained: 1 g KH_2PO_4, 1 g K_2HPO_4, 1 g NH_4NO_3, 0.2 g $MgSO_4.7H_2O$, 0.05 g $FeCl_3$, 0.02 g $CaCl_2.H_2O$, 15 g technical agar and 990 mL distilled water was autoclaved at 121˚C for 15 min at 15 psi. 10 mL diesel was syringed through Whatman 0.45 µm filters (Fisher Scientific, UK) into the agar when it had cooled to 60˚C.

Minimal salts agar with 0.02% diesel and 100 ppm chloramphenicol

From Kirk (2005) used the following agar: 250 mg KCl, 1 g NaH_2PO_4, 1 g NH_4NO_3, 0.5 g $MgSO_4$, 20 g technical agar (Oxoid, UK) and 1 L distilled water was autoclaved at 121˚C for 15 min at 15 psi. 0.2 mL diesel was syringed through Whatman 0.45 µm filters (Fisher Scientific, UK) into the agar with 100 ppm chloramphenicol when it had cooled to 50˚C.

3.4.2 Identification of isolates

3.4.2.1 Isolating of bacteria

Bacteria were isolated from the mixed colonies that grew on the Horsea and microcosm January 2010 sample MPN plates. These were streaked using a flamed loop onto corresponding agar plates and incubated (Table). These plates were then re-streaked to obtain single colonies until all plates contained pure isolated cultures. Bacterial plates were stored in sealed bags in labelled boxes in a 4˚C cold room and were re-streaked every two weeks to maintain a pure culture.

All isolates were screened using a set of three tests: Gram stain, Schaeffer-Fulton endospore stain and an anaerobic test.

3.4.2.2 Gram stain

Gram staining divides bacteria into two classes- Gram positive and Gram negative (Prescott, Hartley, & Klein, 1999). A few colonies of each bacterial isolate were heat fixed onto glass microscope slides. Each slide had crystal violet pipetted on to it for 30 sec then rinsed with distilled water. Gram's iodine, acting as a mordant was pipetted on and left for 1.5 min and rinsed off with 95% ethanol, to de-colourise the Gram negative bacteria, then rinsed with distilled water. Finally, safranin was pipetted onto the slide and left for 30 sec to colour the Gram negative bacteria then rinsed with distilled water. Slides were viewed at x1000 magnification with an oil immersion lens using a light microscope (Olympus). Gram positive bacteria were stained violet and Gram negative were stained pink.

3.4.2.3 Schaeffer-Fulton endospore stain

Many bacteria produce endospores and this test was to screen whether they did or not. The stain is called a Schaeffer-Fulton procedure and involved steaming the bacterial smear whilst staining it with malachite green (Prescott *et al*., 1999). A few colonies of each bacterial isolate found to be Gram positive rods were heat fixed onto glass microscope slides. The slides were placed over a steam bath until droplets of water formed under them. This allowed penetration of the dye to the endospores. Malachite green was pipetted onto each slide and left for 5 min, reapplying malachite green if some evaporated. The slides were placed on a staining rack and rinsed with distilled water. Safranin was pipetted onto the slides and left for two min to stain the remaining vegetative cells before being rinsed with distilled water. Slides were viewed at x1000 magnification with an oil immersion lens using a light microscope.

3.4.2.4 Anaerobic growth

Not all bacteria can grow under anaerobic conditions. This will assess which bacterial isolates are facultative anaerobes. A few colonies of each bacterial isolate were streaked

using a flamed loop onto two sets of corresponding agar plates. One set was placed in a 2.5 L anaerobic jar with an AneroGen sachet (Oxoid, UK) and an anaerobic indicator (Oxoid, UK). Both sets of plates were then incubated (Table) and checked for growth.

3.4.2.4 Grouping of bacteria

All culture plates were separated into groups based on morphology. These groups were then split into sub groups, to avoid duplication of plates, based on their Gram stain appearance and given a letter and number code for identification.

3.4.3 Hydrocarbon-degrading bacterial isolates

3.4.3.1 Determining the hydrocarbon-degrading bacterial isolates

Hydrocarbon degradation ability was assessed using a novel colorimetric indicator culture plate comprising mineral salts, 2,3,5-triphenyl tetrazolium chloride (TTC) and diesel as the sole carbon source. TTC acts as an electron acceptor for many dehydrogenase enzymes. When this compound is reduced by the catalytic effect of the soil dehydrogenase, it forms triphenyl formazan which has a characteristic reddish colour (Page *et al.,* 1982). Klüber *et al.,* (1995) determined that 90% of hydrocarbon-oxidising bacteria isolated showed the ability to reduce TTC and Bochner & Savageau (1977) stated that a cell could not grow without reducing the tetrazolium. However, previous studies have shown the growth of colourless colonies as well as colonies with a red centre on plates containing limited or no added carbon source and TTC (Olga *et al*., 2008).

A few colonies of each bacterial group were streaked using a flamed loop onto Bushnell Haas agar with 0.0025% TTC and 1% diesel. Plates were incubated at 25°C for five days. Hydrocarbon-degrading bacteria produced pink or red colonies, non hydrocarbon-degraders did not grow or appeared white.

The following media were used:

Bushnell Haas agar with 0.0025% tetrazolium chloride (TTC) and 1% diesel

Adapted from methods by Kirk *et al.* (2005) and Bochner & Savageau (1977), 1 g KH_2PO_4, 1 g K_2HPO_4, 1 g NH_4NO_3, 0.2 g $MgSO_4.7H_2O$, 0.05 g $FeCl_3$, 0.02 g $CaCl_2.H_2O$, 15 g technical agar and 990 mL distilled water was autoclaved at 121˚C for 15 min at 15 psi. 10 mL diesel was syringed through Whatman 0.45 µm filters (Fisher Scientific, UK) into the agar with 0.025 mL TTC (Oxoid, UK) when it had cooled to 60˚C.

3.4.3.2 Identifying the hydrocarbon-degrading bacterial isolates using API

API 20 NE (BioMerieux, UK) methodology is based on 20 biochemical tests was used as an initial method to identify hydrocarbon-degrading bacteria. It is routinely used in hospitals as a method of identifying bacteria responsible for an increasing number of opportunistic infections. Substrates include: potassium nitrate, tryptophan, glucose, arginine, urea, aeculin and gelatin. A few colonies of each hydrocarbon-degrading bacterial isolate were tested using API 20 NE plates To determine whether the bacteria was oxidase positive or negative, a few drops of oxidase reagent was pipetted onto a folded filter paper. Each bacterial isolate was individually streaked across the wet filter paper using a flamed loop. If the streak turned purple the bacteria was determined as oxidase positive.

The following media was used:

0.85% NaCl (isotonic saline)

8.5 g NaCl (Fisher, UK) was added to 1 L distilled water and autoclaved at 121˚C for 15 min at 15 psi in 2 mL vials.

API AUX medium

7 mL vials of API AUX medium were ordered pre-made from BioMerieux, UK. Each consisted of 2 g ammonium sulphate, 1.5 g agar, 10.5 mL vitamin solution, 10 mL trace

elements, 6.24 g monosodium phosphate, 1.5 g potassium chloride and 1 L demineralised water.

Oxidase reagent

A small amount of N,N,N',N'-Tetramethyl-p-phenylenediamine dihydrochloride (Sigma Aldrich, UK) was added to a 5 mL vial of distilled water until a light purple colour was reached.

One single colony of the hydrocarbon-degrading bacterial isolate was suspended into a 2 mL vial of 0.85% saline. Four drops of this suspension were then added to a vial of AUX medium. The AUX medium suspension was then pipetted into each of the 20 wells on the API 20 NE then incubated at 30˚C for 24 h. After this time it was determined whether the bacterial isolate had utilised NO_3 and TRP. The strip was placed back in the incubator at 30˚C for another 24 h and the remaining results determined. One single colony of the hydrocarbon-degrading bacterial isolate was streaked across Whatman filter paper (Fisher, UK) soaked in oxidase reagent. If the oxidase reagent turned purple the result was positive.

3.4.3.3 Identifying the hydrocarbon-degrading bacterial isolates using Biolog

Biolog plates have been developed to identify bacterial isolates based on their ability to oxidize 95 different carbon sources (Smalla *et al.,* 1998). The Biolog plates were placed into a colorimetric plate reader and the identification was based on the colour change in each plate well and is limited to the associated database.

A few colonies of each hydrocarbon-degrading bacterial group were streaked using a flamed loop onto agar plates. Plates were incubated at 25˚C for 24 h. A turbidity standard (Table) was inserted into a turbidity meter (Biolog, Technopath, Ireland) and a reading taken. A sterile swab was moistened in a new vial of inoculating fluid. The swab was rolled over the isolated colonies and then twirled into the inoculating fluid tube to release the colonies. The inoculating fluid was inserted into the turbidity reader and more colonies

were added until the turbidity was the same as the turbidity standard. The inoculating fluid was poured into a sterile trough (Biolog, Technopath, Ireland) and 150 µL was pipette into each well of a microplate (Biolog, Technopath, Ireland). The microplate was then sealed in a bag and incubated (Table 3) and read using the Biolog MicroStation spectrophotometer (Biolog, Technopath, Ireland) after 4-6 hours and 16-24 hours.

Table 3. Hydrocarbon-degrading bacterial isolate preparation for Biolog identification

Organism type	Turbidity standard (%)	Microplate	Culture media	Incubation temperature (°C)
Gram negative non-enteric	52	GN2	Biolog universal growth media+5% sheep blood	30
Gram negative enteric	61	GN2	Biolog universal growth media+5% sheep blood	37
Gram positive cocci and Gram positive rods (non-spore forming)	30	GP2	Biolog universal growth media+5% sheep blood	30
Gram positive rods (spore forming)	28	GP2	Biolog universal growth media +0.25% maltose+ swabbed with thioglycolate	30

The following media was used:

Biolog universal growth agar (BUG) with 5% sheep blood (B)

BUG+B plates pre-made from Biolog, Technopath, Ireland for the growth of Gram negative enteric and non-enteric bacterial isolates as well as Gram positive cocci and non-spore forming Gram positive rods.

Biolog universal growth (BUG) agar with 0.25% maltose (Ma) and thioglycolate (T)

BUG+Ma plates pre-made from Biolog, Technopath, Ireland. Eight drops of concentrated thioglycolate (Technopath, Ireland) was added to 3 mL sterile distilled water. A swab was dipped in and gently stroked across the whole surface of each BUG+Ma plate and allowed to dry. These were used to grow spore forming Gram positive rods.

Inoculating fluid

Inoculating fluid is ordered in pre-made from Biolog, Technopath, Ireland.

Biolog Turbidity standards

Biolog turbidity standards of 20%, 28%, 52%, 61% were pre-made from Technopath, Ireland.

3.4.3.4 Identifying the hydrocarbon-degrading bacterial isolates using molecular techniques

Molecular investigation was done on the hydrocarbon-degrading bacterial isolates to definitively identify them with the help of Dr Joy Watts and the School of Biological Sciences. Molecular identification is a reliable technique as it is based on extracting the DNA from the hydrocarbon-degrading isolate, amplifying the sequence through Polymerase Chain Reaction (PCR) and comparing the results to an extensive GenBank DNA database.

DNA was extracted from all the culturable hydrocarbon-degrading bacterial isolates using the Qiagen DNeasy blood and tissue kit using the protocols for Gram positive bacteria as per the instructions from the manufacturer. The resulting DNA is used in the PCR to amplify the 16S rRNA gene for sequencing and identification. After the PCR was complete, samples were examined by gel electrophoresis in a 0.8% tetrabutoxytitanium (TBT) gel containing ethidium bromide as a fluorescent tag dye to aid visualisation. The PCR tubes which yielded bands were sent to Functional Biosciences (MI, USA) for

sequencing. Sequences were then compared to those in the GenBank DNA database by using the BLAST algorithm and the closest phylogenetic relatives checked.

3.4.4 *P. australis* roots and rhizomes

3.4.4.1 Hydrocarbon degrading bacteria around P. australis roots and rhizomes

In August 2010, root and rhizome samples were removed from a planted microcosm using flamed tweezers and scalpel blade (Fisher Scientific, UK). Samples were transported in new, labelled petri dishes (Fisher Scientific, UK) and kept at 4˚C to be used within two hours of removal. The roots and rhizomes were separated using sterilised tweezers and scalpel blade and put into separate petri dishes. 60˚C Bushnell Haas agar with 0.0025% TTC and 1% diesel was poured over them and they were left to incubate at room temperature for five days. Hydrocarbon-degrading bacteria appeared pink or red, non hydrocarbon-degraders did not grow or appeared white.

3.4.4.2 Hydrocarbon degrading bacteria around intact P. australis

In August 2010, intact *P. australis* samples were removed from a planted microcosm using a serrated knife. Samples were transported in new, labelled bags and kept at 4˚C and used within two hours of removal. One of the reeds was held upright in a glass vase whilst 60˚C Bushnell Haas agar with 0.0025% TTC and 1% diesel was poured over it. Aluminium foil was wrapped around the top of the vase to prevent contamination. It was then left to incubate at room temperature for five days. Hydrocarbon-degrading bacteria appeared pink or red, non hydrocarbon-degraders did not grow or appeared white.

3.4.4.3 Preparation of P. australis roots and rhizomes for the scanning electron microscope (SEM)

In October 2009, root and rhizome samples were aseptically removed from the microcosms and stored at 4˚C to be used within two hours of removal. Samples were fixed in 5%

glutaraldehyde in 0.1 M HEPES buffer (Sigma, UK) and 0.25% sucrose for one hour at room temperature in a rotator (Agar scientific). The samples were then washed twice in 0.1 M Phosphate Buffered Saline (PBS) buffer at 4˚C on a rotator for five minutes. Samples were then dehydrated by progressively placing them in 30% ethanol, 50% ethanol, 70% ethanol, 90% ethanol, 100% ethanol and then 100% acetone for 15 min each. Samples were then covered in hexamethyldisilazane (HDMS) (Fisher Scientific, U.K) and left overnight. Samples were mounted onto metal stubs with double-sided carbon tape. Finally, a thin layer of metals (gold and palladium) was applied over the samples using an automated sputter coater for 15 min. Samples were viewed under a SEM and images taken.

4. MESOCOSM STUDY

4.1 Introduction

In April 2007, a mesocosm trial was set up to help determine which native reed would grow successfully in the highly contaminated Horsea lagoon sediment and would then be used in the *in situ* lagoon study. The growth, height and number of shoots of *P. autralis* and *T. latifolia* in Horsea lagoon sediment was monitored bi-weekly throughout the growing season of 2008 to determine whether plants were able to survive in these extremely high levels of contamination. From the appearance of new shoots in April to senescence in October (Plate 9a and b) the effect of nutrient addition to the water was studied.
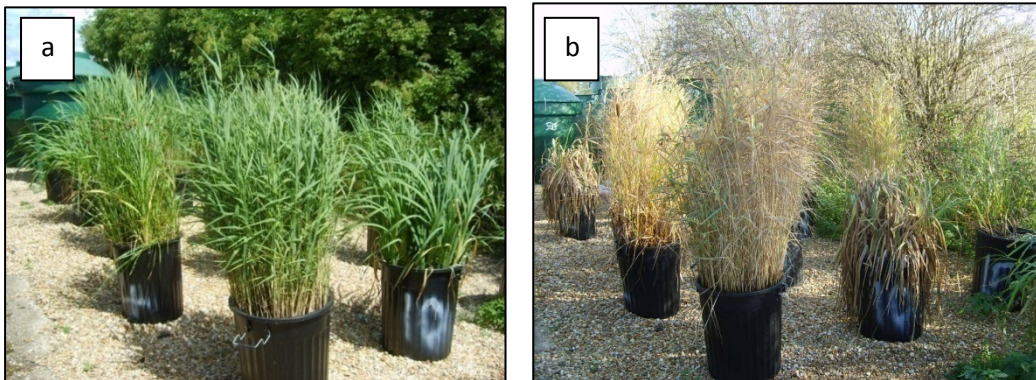
Plate 9. *Ex situ* **mesocosms (a) growing healthily in July 2008 (b) and in senescence due to cold weather in October 2008, H. Pinchin**

In August 2008 a homogenised sediment sample was extracted from an unplanted mesocosm and tested for suspended solid and inorganic matter content. COD and BOD were also determined on a submerged sediment sample. This was to determine the percentage of volatile organics present in the sediment and how readily they would be degraded by the native microorganisms.

In August 2010, one mesocosm planted with *P. australis* was dismantled. Final sediment

samples were taken from different depths throughout the mesocosm: 0, 15 and 30 cm. These sediment samples were then analysed to determine their DRO concentrations and microbial numbers after the three year study.

All statistical analysis was carried out using Minitab software v15. Data was tested for normality using the Ryan Joiner test. Microbial counts and DRO concentrations were found to be significantly different to normal and were Log10 transformed in order to normalise the data. Comparisons between two groups were made using a 2-sample t-test (reported as the t-statistic and the p-value) and when there were more than two groups one way analysis of variance (ANOVA) was performed (reported as the F-value with the degrees of freedom of the effect and error in brackets followed by the p-value) after checking for equal variance. Pearsons correlation was performed to identify any trends between variables and reported as the regression coefficient (r) and the p-value.

4.2 Results

4.2.1 Biodegradability of Horsea lagoon sediment

The sample of mixed sediment and water was found to have a 10.3% moisture content with 36% volatile suspended solids (VSS) (Table 4). The overall BOD: COD was approximately 1:20 indicating that the organics present in the sediment are extremely hard to biodegrade.

Table 4. Physical and chemical characteristics of Horsea lagoon sediment

Parameter	Concentration (mg/kg)
SS	102,890
VSS	36,440
COD	125,350
BOD	6,162

4.2.2 Growth of plants

Both *P. australis* and *T. latifolia* grew well in the homogenised Horsea lagoon sediment. None of the planted mesocosms failed to grow and the numbers of shoots and shoot height only decreased due to senescence in October.

4.2.2.1 Number of shoots in the mesocoms

P. australis produced a significantly higher number of shoots than *T. latifolia* (ANOVA F=165.37, p=0.000), which is indicative of the growth habit (Figure 5a and b). The initial mean number of shoots was 66 in the *P. australis* mesocosms and four in the *T. latifolia* mesocosms. New shoots emerged at a mean rate of two shoots per day to reach a maximum of 408 by 17th September in the *P. australis* mesocosms. This compared to an average increase of only 0.2 shoots per day in the *T. latifolia* mesocosms reaching a maximum of 41 by 2nd September. This number then slowed down due to senescence at a rate of 9.7 shoots per day to 70 in the *P .australis* mesocosms and 0.6 shoots per day to 12 in the *T. latifolia* mesocosms on the final monitoring occasion. There was no growth in the unplanted control mesocosms.

4.2.2.2 Effect of nutrients on shoot number

The addition of nutrients significantly increased the number of shoots in both *P. australis* (t(78) =-2.28, p=0.025) and *T.latifolia* (t(68) =-6.61, p=0.000) (Figure 5a and b). The rate at which *P. australis* shoots emerged increased from 1.9 shoots per day to 2.4 shoots per day when nutrients were added. An increase in the maximum total number of shoots was also seen with nutrient addition, rising from 349 to 461 shoots by 17th September. Nutrients also increased the shoot emergence rate for *T. latifolia* from 0.1 to 0.4 shoots per day with densities also rising from 19 to 62.
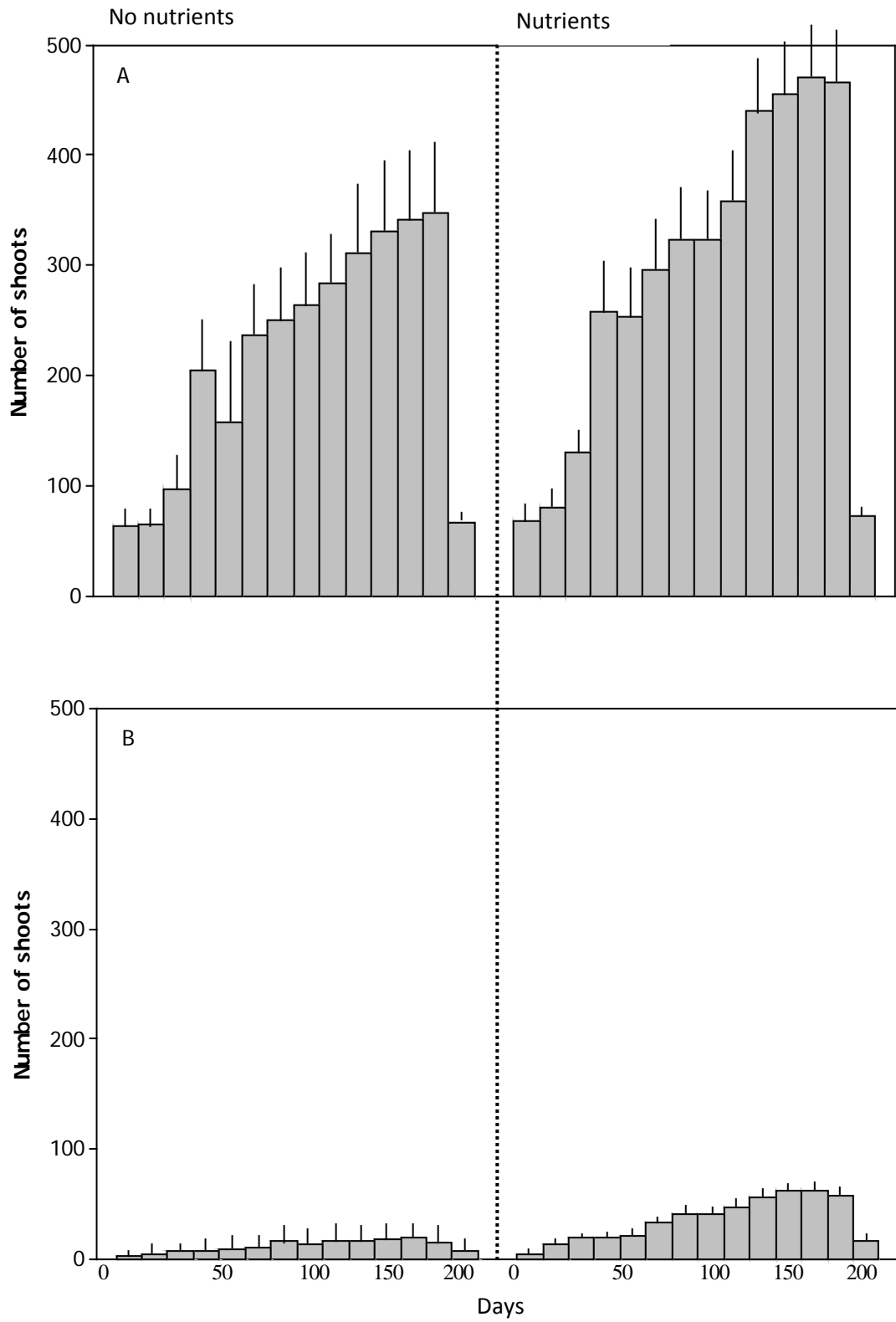
Figure 5. Mean number of shoots in (a) *P. australis* and (b) *T. latifolia* over time in the mesocosms (SEM shown by error bars)

4.2.2.3 Shoot height in the mesocosms

Not only did *P.australis* produce a greater number of shoots over time, they also grew significantly taller than *T. latifolia* (ANOVA F=5.58, p=0.019) (Figure 6a and b). The initial mean shoot height was 24.0 cm in the *P.australis* mesocosms and 17.3 cm in the *T. latifolia* mesocosms. The height of the *P. australis* increased to a maximum of 115.9 cm by 2nd September whereas *T. latifolia* reached a maximum of 93.4 cm by 21st August showing their different growth seasons and phenology. Their height then decreased to 78.9 cm for the *P. australis* and 76.6 cm for *T. latifolia* on the final count due to strong winds breaking off dead culms.

4.2.2.4 Effect of nutrients on shoot height

The effect of nutrient addition, with regard to shoot height, was varied. For *P .australis* there was no significant difference in maximum shoot height (Figure 6), both sets grew to 115.4 cm and 119.6 cm although the microcosm with added nutrients reached this height in only 152 days as opposed to 167 days without nutrients. However, the addition of nutrients significantly increased the mean shoot height in *T. latifolia* (t(69)=-3.84, p=0.000) (Figure 6b). Without added nutrients the mean shoot height increased from 14.1 cm at a rate of 0.4 cm per day to a maximum of 70.6 cm by 21st August and when nutrients were added the height increased from 20.4 cm at a rate of 0.6 cm per day to a maximum of 117.9 cm by 17th September.

In August 2010, a *P. australis* mesocosm was dismantled to examine microbial communities and DRO concentrations. Plate 10 shows the extensive growth of roots and rhizomes that *P. australis* produced. It also shows how *P. australis* has changed the physical appearance of the contaminated sediment. The top 20 cm of the core was tightly matted with roots and was light brown due to oxygenation. However, once the core had
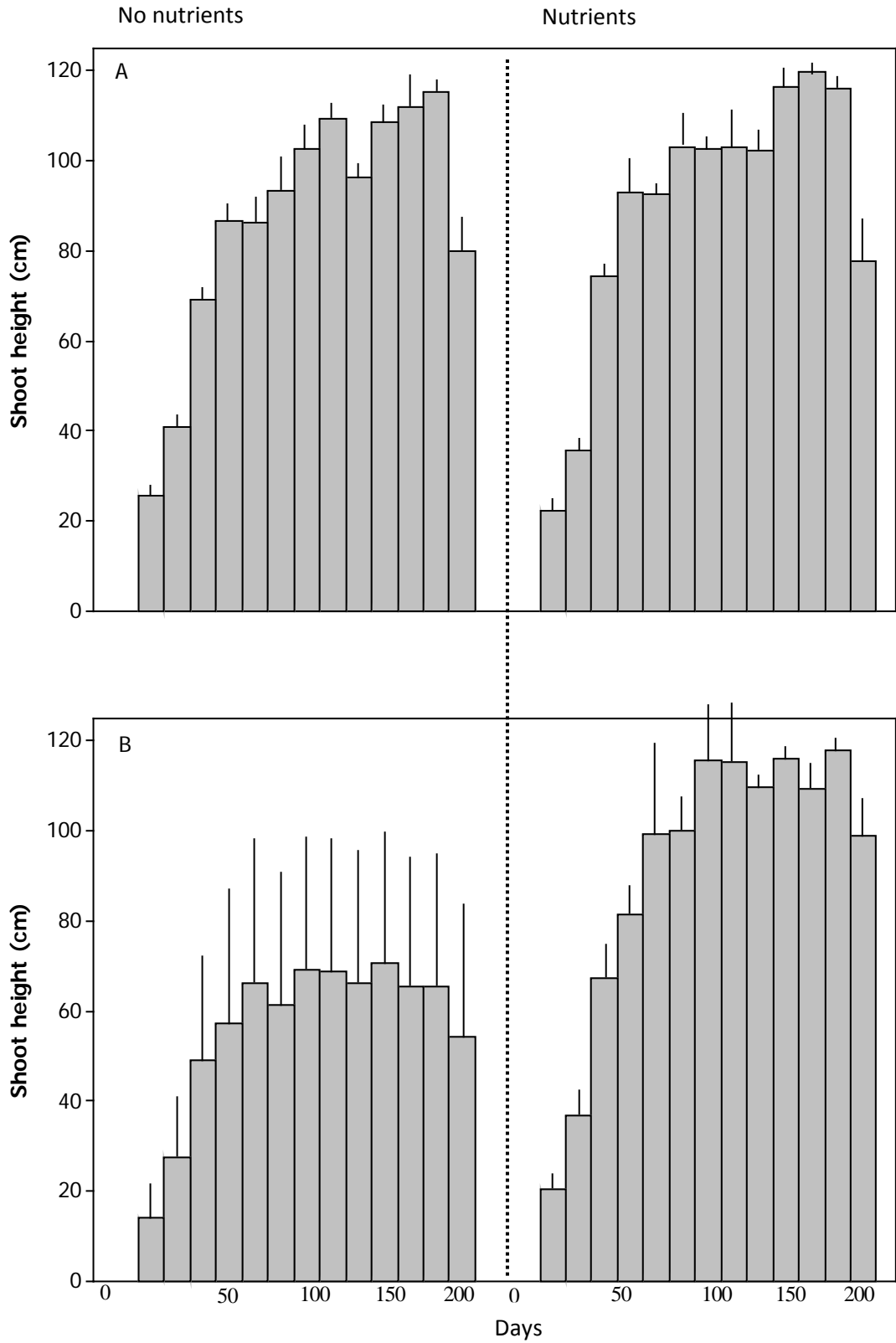
Figure 6. Mean shoot height of (a) *P. australis* **and (b)** *T. latifolia* **over time in the mesocosms (SEM shown by error bars)**

been sectioned, the inside was far less densely matted and was still black. The texture of the black sediment was much more crumbly due to the lack of roots holding it together.

4.2.3 Dismantling of a mesocosm

Plate 10. Dismantled mesocosm showing the brown oxygenated, densely rooted sides, H. Pinchin, August 2010.

4.2.3.1 DRO concentration

There was no significant difference between the levels of DRO in the planted mesocosm and the unplanted mesocosm $(t(15)=0.41, p=0.687)$ (Table 5). There was also no significant difference between the levels of DRO at different sample depths (ANOVA $F=0.86, p=0.444$).

When the planted and unplanted mesocosms were examined individually the planted mesocosm showed a significant decrease in the DRO concentrations with depth (ANOVA $F=8.34, p=0.018$) (Figure 6a). Conversely, the DRO concentration in the unplanted mesocosm increased with depth, but not significantly (Figure 6b).

Table 5. The mean levels of DRO (mg/kg) in planted and unplanted mesocosms (SEM shown in brackets)

Depth (cm)	Mean DRO in planted mesocosm (mg/kg)	Mean DRO in unplanted mesocosm (mg/kg)
0	2.45×10^5 (5.5×10^4)	1.42×10^5 (2.5×10^4)
15	2.15×10^5 (5.8×10^4)	2.02×10^5 (1.1×10^5)
30	5.37×10^4 (1.7×10^4)	2.22×10^5 (6.0×10^4)

4.2.3.2 GC-FID chromatograms from the dismantled mesocoms

The chromatograms from dismantled mesocosms (see appendix and table 6) showed the composition of residual hydrocarbons to be in the C_{10}-C_{11} to C_{25} DRO range with a symmetric distribution centred at C_{17}-C_{19} and a high proportion of UCM.

Table 6. Summary of GC-FID chromatogram data analysis from planted (PL) and unplanted (UP) dismantled mesocosms

Day	Site	Depth	Total area (pA^2)	UCM area (%)	Maximum UCM height (pA)	Carbon number at UCM peak	Start of UCM
1218	PL	0	1605	83	22	C18	C10
1218	PL	0	592	39	10	C18	C11
1218	PL	0	1072	79	16	C18	C11
1218	PL	15	5228	73	55	C17	C10
1218	PL	15	4723	84	50	C17	C10
1218	PL	15	2472	85	28	C17	C10
1218	PL	30	5271	76	56	C17	C10
1218	PL	30	3788	87	40	C17	C10
1218	PL	30	6655	79	70	C17	C10
1218	UP	0	1662	88	22	C18	C11
1218	UP	0	2190	80	27	C18	C10
1218	UP	0	2993	70	38	C18	C10
1218	UP	15	6357	86	65	C17	C10
1218	UP	15	687	98	11	C19	C10
1218	UP	15	2389	89	32	C18	C11
1218	UP	30	3347	90	30	C17	C10
1218	UP	30	2946	88	30	C17	C10
1218	UP	30	2846	83	30	C17	C10

Overall, the planted mesocosm chromatograms showed a 19% total area and 12% UCM area increase when compared to the unplanted mesocosm chromatograms. However, at 0 cm the planted mesocosm showed a 52% lower total area and a 55% lower UCM area when compared to the unplanted mesocosm and then a significant increase in total area (p=0.014 ANOVA), UCM area (p=0.009 ANOVA) and UCM maximum height (p=0.021 ANOVA) with depth unlike the unplanted mesocosm (Figure 7).

There were no clearly resolved peaks before the start of the UCM and two clearly resolved peaks in all the chromatograms at C_{16} and C_{17}. The maximum UCM height was seen at C_{18} on all the 0 cm samples and C_{17} in all the 30 cm samples.
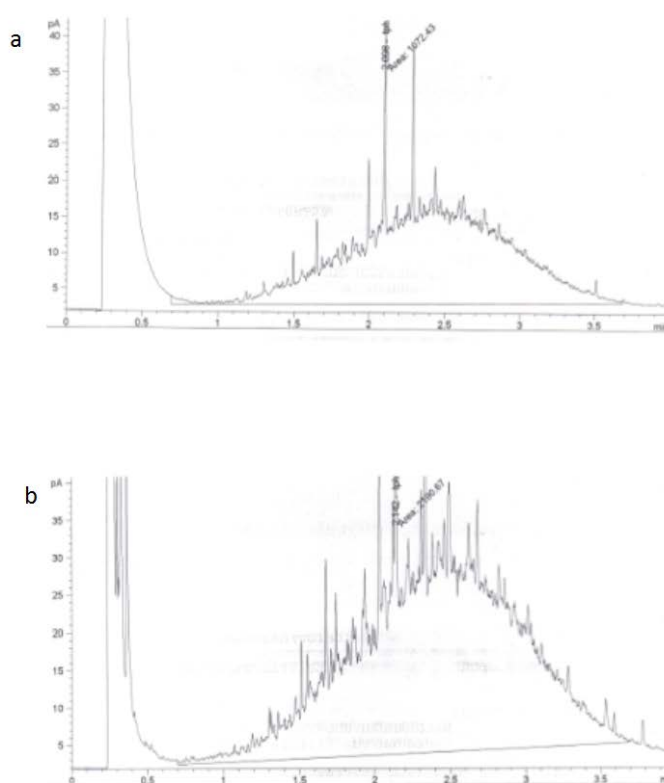
Figure 7. GC-FID chromatograms from the dismantled mesocosms at 0 cm (a) PT1 (b) UT1

4.2.3.3 Numbers of culturable microorganisms in the mesocosms

Overall, there were significantly higher numbers of total bacteria (t(15)=-5.21, p=0.000), hydrocarbon-tolerant bacteria (t(15)=-3.21, p=0.006) and hydrocarbon-tolerant fungi (t(14)=-2.67, p=0.018) in the planted mesocosm compared to the unplanted mesocosm. The number of total fungi was also higher in the planted mesocosm but was not significantly different to the control (t(15)=-1.35, p=0.179) (Table 7).

There was a significant increase in the number of total bacteria (ANOVA F=7.99, p=0.020), hydrocarbon-tolerant bacteria (ANOVA F=20.69, p=0.002) and total fungi (ANOVA F=51.49, p=0.000) with depth in the unplanted mesocosm. In contrast to this the planted mesoscosm had a significant decrease in the number of total fungi with depth (ANOVA F=23.06, p=0.002) (Figure 8).

Table 7. Microbial counts in a planted and an unplanted mesocosm 3 years after planting (SEM shown in brackets)

Microbial group	Mean number in the planted mesocosm (cfu/g)	Mean number in the unplanted mesocosm (cfu/g)
Total Bacteria	4.3×10^7	1.4×10^6
	(2.1×10^7)	(6.5×10^5)
Hydrocarbon-Tolerant Bacteria	1.1×10^7	2.2×10^6
	(3.5×10^6)	(1.6×10^6)
Total Fungi	6.5×10^6	1.1×10^6
	(3.3×10^6)	(1.1×10^5)
Hydrocarbon-Tolerant Fungi	3.2×10^4	1.3×10^4
	(7.4×10^3)	(4.9×10^3)
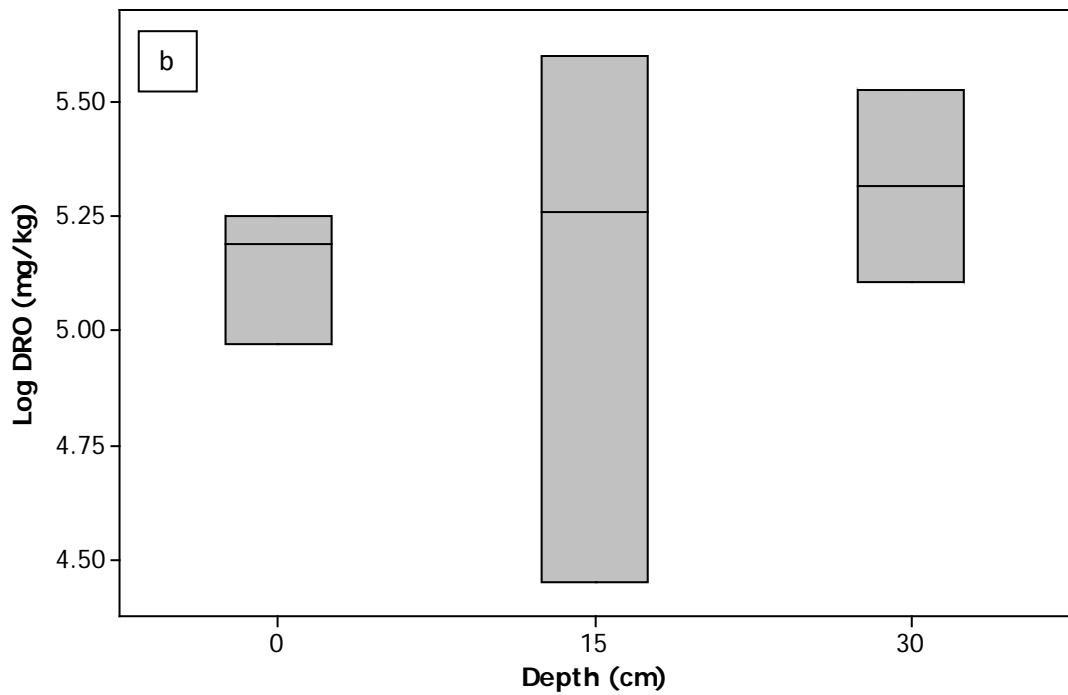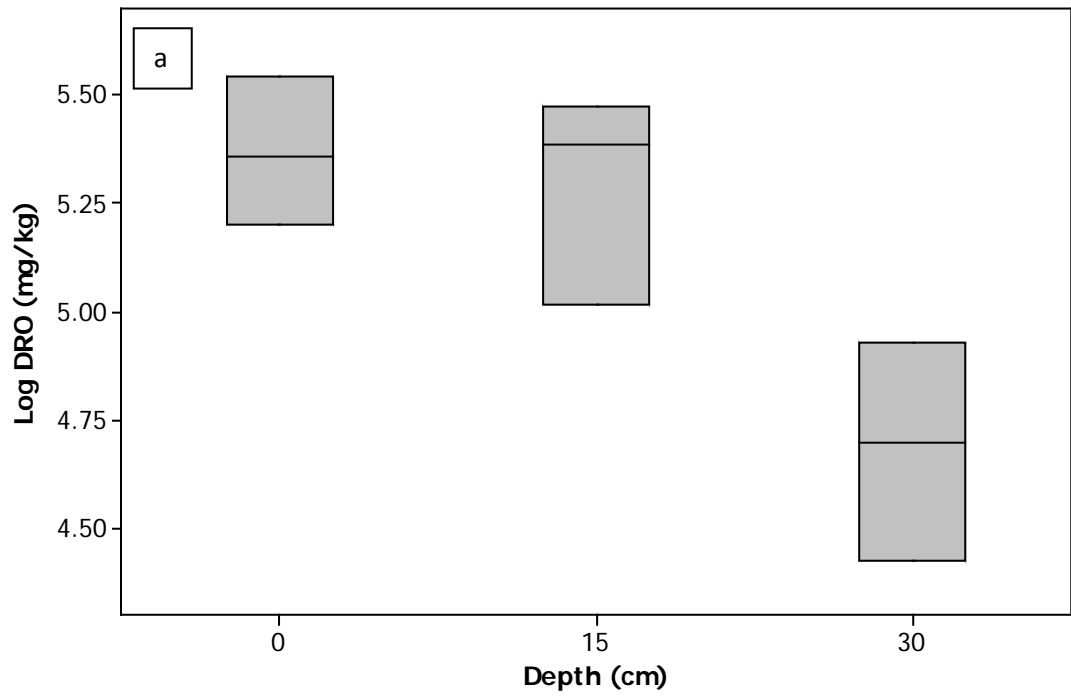
Figure 8. DRO concentration (mg/kg) with depth in the (a) dismantled planted and (b) unplanted mesocosms. The boxes represent the 1st and 3rd quartiles (the interquartile range) with the median shown as a horizontal line within the box.

4.3 Discussion

4.3.1 Biodegradability of organics in the sediment

The results showed that there were high levels of hydrocarbon in the Horsea lagoon sediment and the BOD: COD ratio was 1:20. The ratio of BOD: COD is used as a means of determining how easily organic matter is biodegraded in an environment (Samudro & Mangkoedihardjo, 2010). A high ratio of BOD to COD shows that the organics present are relatively biodegradable whereas a low BOD to COD ratio indicates that the organics present are either more slowly biodegraded or contain a significant fraction of unbiodegradable material (Clesceri *et al.,* 1998). This indicates that the organic compounds present in Horsea lagoon are not degraded easily in the sediment by the native microorganisms. This could be due to the concentration of hydrocarbons being too high for the degrading microorganisms to survive or there could be too few microorganisms in the sediment to degrade the large quantities of hydrocarbon.

4.3.2 Plant species selection

Encouragingly both plants in the trial grew successfully in the mesocosms. This was encouraging as no previous studies have investigated growth in such highly contaminated sediments although, *P. australis* has been shown to have the ability to penetrate through 3.5 cm of soil containing up to 350,000 mg/kg bitumen tar (Tischer, 2002). Although nutrients did stimulate growth, the reeds grew well without them. Adding nutrients to the sediment in the *in situ* study was felt to be costly and could also pose the risk of algal blooms in the lagoon which could increase the pH and hydrogen sulphide levels during decomposition (Jana *et al.,* 2001). Nutrient addition was therefore ruled out of the next stages of the study.

P. australis had significantly larger numbers and taller shoots, even in the trials with no nutrient application. *P. australis* has also been described as the 'prime temperate species'

for phytoremediation in sediments due to its high tolerance to a wide range of pollutants and high rate of oxygen transfer (Gawronski & Gawronska, 2007). It is also more robust for transplanting and handling and was therefore chosen as the plant species used in the microcosm and *in situ* studies.

4.3.3 The effect of *P. australis* on sediment 3 years after planting

The mesocosms containing homogenised Horsea lagoon sediment were set up to not only determine whether plants could grow in such contaminated conditions but also to replicate the large number of studies carried out using contaminated site sediment in *ex situ* environments (Euliss *et al*., 2008; Kirk *et al*., 2005). However, this study differs from most due to it being situated outdoors allowing the mesocosms to be exposed to environmental conditions. Phytoremedation of highly contaminated sediments such as those found at Horsea lagoon was not expected to be a fast process so the mesocosm trial, as the longest running experiment in this study, was deemed the most likely to show the effects of phytoremediation, including the influence on bacterial communities and the ability to enhance hydrocarbon degradation.

These elevated microbial counts and the changed physical appearance of the sediment indicate that *P. australis* rhizospheres modified conditions within the sediment and promoted the growth of microbes potentially able to degrade DRO.

Rhizosphere soil has been shown to contain 10-100 times more microbes per gram than unvegetated soil (Lynch, 1990). *P. australis* has an effective aeration system consisting of internal convective through-flow of gases which gives it outstanding capacities to vent its underground tissues (Brix *et al*., 1996) and highly developed gas aerenchymatous tissues (Armstrong, 1964) as well as a venturi-induced convection system (Armstrong *et al.*, 1992). The observed changes in microbial populations may therefore have been affected by

rhizosphere oxygen availability, although root exudates and physical changes may also be important factors.

There were significant differences in overall DRO concentrations between the planted and unplanted mesocosms. The GC-FID chromatograms for both had a large UCM baseline hump, which is common for sediments with historical or repeated contamination with TPH. The general pattern of n-alkane peaks found in the diesel standard chromatograms were not seen presumably because they were preferentially lost by microbial degradation or other weathering processes (Frysinger *et al.*, 2003). However, all sites showed two highly resolved peaks at C_{16} and C_{17} in all of the sites, which could be due to biomarkers. Biomarkers are highly resistant oxidation products formed during hydrocarbon degradation that can be grouped into sesqui-(C_{15}), di- (C_{20}), sester- (C_{25}) and triterpanes (C_{30}) and are collectively known as terpanes (Wang, 2004). Bycyclic sesquiterpanes including eudesmane and drimane (C_{15}) are ubiquitous in weathered sediments containing diesel and pristine (C_{17}) is a highly branched n-alkane and known biomarker often found in field studies due to its resistance to biodegradation (Mohanty & Mukherji, 2008).

Despite there being no significant difference in DRO concentrations and similar chromatogram patterns between mesocosms, there is evidence that the reeds were influencing sediment microbial populations. This was also observed in a 5 year phytoremediation study in El Dorado, Arkansas by White *et al*. (2006). In Horsea lagoon sediment, numbers of bacteria, hydrocarbon tolerant bacteria and hydrocarbon tolerant fungi were significantly higher as well as the number of fungi being higher in the planted mesocosm compared to the unplanted mescocosm (Table 5).

4.4 Summary

- The low BOD: COD ratio showed that the hydrocarbons in the Horsea lagoon sediment were extremely hard to biodegrade.

- Both *P. australis* and *T. latifolia* grew well in the contaminated sediment however, *P. australis* had a significantly higher number of shoots and shoot height.

- The addition of nutrients significantly increased the number of shoots in both *P. australis* and *T. latifolia* and significantly increased the shoot height in *T. latifolia.*

- After three years, a mesocosm containing *P. australis* had significantly higher numbers of total culturable bacteria, hydrocarbon-tolerant bacteria and hydrocarbon-tolerant fungi, however, the DRO concentration was not significantly different between a planted and unplanted mesocosm.

- The GC-FID chromatograms showed a symmetrical shape between C_{10}-C_{11} to C_{25} centred around C_{17}-$_{19}$.

- There was a large symmetrical UCM on all the GC-FID chromatograms.

- *P. australis* was chosen to be used in further phytoremediation studies.

5. MICROCOSM STUDY

5.1 Introduction

There have been a large number of successful greenhouse studies of phytoremediation of hydrocarbons using microcosms; some using spiked sediments (Aprill, 1990; Kaimi *et al*., 2006) and some using contaminated site sediments (Euliss *et al*., 2008; Liste & Felgentreu, 2006). All showed an increased rate of hydrocarbon degradation in the planted microcosms compared to the unplanted microcosms. All of these studies have involved the use of homogenised sediment and controlled environmental conditions. In April 2009, a microcosm study using homogenised Horsea lagoon sediment was undertaken to determine the effect of plants on microbial populations and hydrocarbon levels on a smaller scale with environmentally controlled conditions.

Two microcosms were planted with a small bunch of *P. australis* from the Gosport fuel depot (PL1 and PL2) and one was left unplanted as a control (UP3). From July 2009, sediment samples were taken monthly from: 0, 15, 30 and 45 cm depth for microbial analysis. The number of culturable, aerobic bacteria, fungi, hydrocarbon-tolerant bacteria and hydrocarbon-tolerant fungi were enumerated using the MPN method to determine the influence of plants on increased microbial numbers. In January 2010, bacteria were cultured from MPN plate counts and isolated into pure cultures, then held as stock cultures at 4˚C. Subsequently these isolates were screened using the Gram stain procedure and assessed for their capacity to grow with and without oxygen (into aerobes and facultative anaerobes). Bacteria found to be Gram positive rods were investigated for endospore production and bacteria found to be Gram negative rods were tested using the API method of identification to help identify the bacterial characteristics of the microbial populations throughout the microcosm. All of the isolated bacteria were grouped and tested for hydrocarbon-degrading potential along with a section of *P. australis* rhizome and a small

clump of *P. australis* roots (Plate 10). All bacteria found to be hydrocarbon degraders were analysed using Biolog and molecular techniques for species identification. From January 2010, sediment was also analysed for DRO concentration to determine whether planted microcosms had increased DRO degradation along with determination of moisture content and pH.

Plate 11. Planted microcosm showing roots and rhizomes, H. Pinchin, August 2009

In August 2010, 17 months after planting, one planted microcosm was dismantled and sediment samples were taken from the centre and edge at four depths (10, 20, 40 and 60 cm). Samples were also taken from the centre of the non-dismantled unplanted microcosm. The DRO concentration and microbial numbers in these samples were analysed to see if there was a difference between the planted and unplanted microcosms after 17 months in controlled environmental conditions.

All statistical analysis was carried out using Minitab software v15. Data was tested for normality using the Ryan Joiner test. Microbial counts and DRO concentrations were

found to be significantly different to normal and were Log10 transformed in order to normalise the data. Comparisons were made using one way analysis of variance (ANOVA) was performed (reported as the *F*-value followed by the p-value) after checking for equal variance. Pearsons correlation was performed to identify any trends between variables and reported as the regression coefficient (r) and the p-value.

5.2 Results

5.2.1 DRO concentrations in the microcosms

5.2.1.1 Changes in DRO over time in the microcosms

P. australis grew well in both planted microcosms (Plate 11). They had a large shoot and root density and grew to a similar height to the plants grown outside in the mesocosms.

Plate 12. Greenhouse planted (PL1 and PL2) and unplanted (UP3) microcosms, H. Pinchin, June 2010

DRO concentrations in the microcosms were determined from January 2010-June 2010 on 3 sampling occasions. Overall the DRO concentrations significantly decreased over time (r=-0.403, p=0.025) and comparison of the individual microcosms showed that the DRO

concentrations were significantly different to each other (ANOVA F=6.04, p=0.007). At the end of the study, the DRO concentrations were significantly higher in the planted microcosms, PL1 and PL2 (ANOVA F=9.50, p=0.004) although on day 1, UP3 contained lower levels of DRO (61,620 mg/kg) than the planted microcosm, PL1 (81,196 mg/kg). However, microcosm PL2 had the highest rate of decline in DRO concentration after sampling began (256 mg/kg per day) followed by microcosm UP3 (211 mg/kg per day) and then microcosm PL1 (200 mg/kg per day) although the differences between microcosms were very small (Table 8)

Table 8. DRO concentrations (mg/kg) in the microcosms (SEM shown in brackets)

Microcosm	Mean DRO (mg/kg)		
	day 261	day 352	day 415
PL1	8.3×10^4	5.7×10^4	5.3×10^4
	(1.5×10^4)	(5.3×10^3)	(3.9×10^3)
PL2	7.1×10^4	4.1×10^4	3.1×10^4
	(1.5×10^4)	(1.3×10^4)	(1.6×10^4)
UP3	-	3.4×10^4	2.0×10^4
		(1.7×10^4)	(4.1×10^3)

5.2.1.2 Factors affecting DRO concentration in the microcosms

The planted microcosms were regularly watered to keep the plants alive and the unplanted microcosm was periodically topped up when the sediment had dried out. During sampling it was noted whether each microcosm was submerged or not.

The concentration of DRO significantly increased with moisture content (r=0.555, p=0.001) (Figure 9) and there was significantly more DRO in submerged microcosms (ANOVA F=9.50, p=0.004). The planted microcosms, PL1 and PL2, were submerged 100% of the time on all sample dates and the unplanted microcosm, UP3, was submerged during only 57% of the sampling dates.
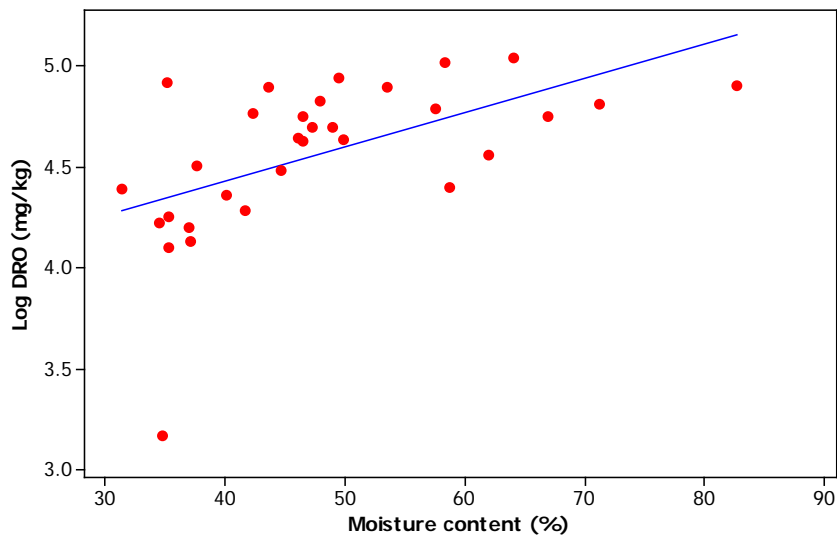
Figure 9. Relationship between DRO concentration (mg/kg) and sediment moisture content (%)

There was no correlation between DRO concentrations and the depth of the sediment. However, moisture content, which was shown to increase with DRO concentration, significantly decreased with sediment depth (ANOVA F=3.93, p=0.011) (Figure 10).
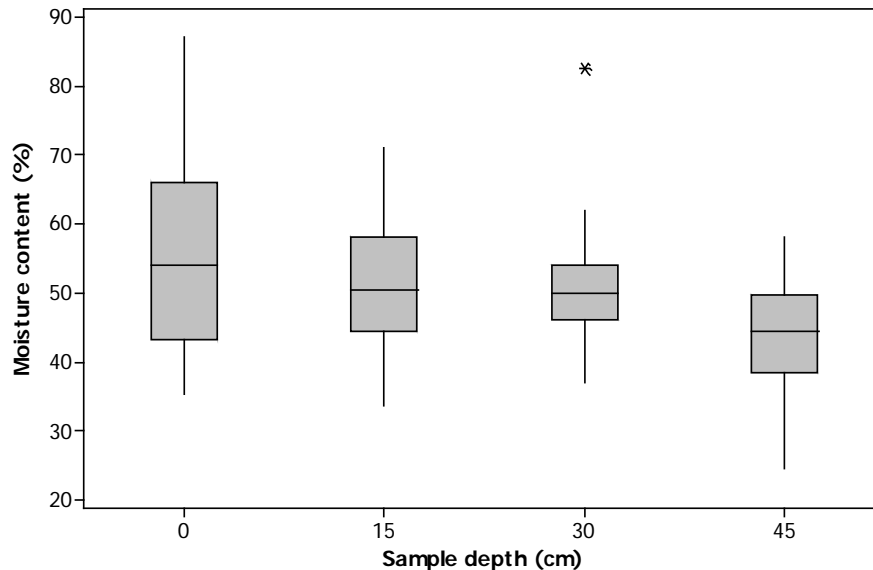
Figure 10. Moisture content of sediment at different depths in the microcosms. The boxes represent the 1st and 3rd quartiles (the interquartile range) with the median shown as a horizontal line within the box. The extreme values (1.5 times the interquartile range (IQR) from the upper or lower quartile) are the ends of the lines extending from the IQR

In August 2010, after the sampling had been completed, sediment samples were taken from the edge of microcosm PL1 where most of the roots were positioned and from the centre of the same microcosm away from the root masses (Plate 13).
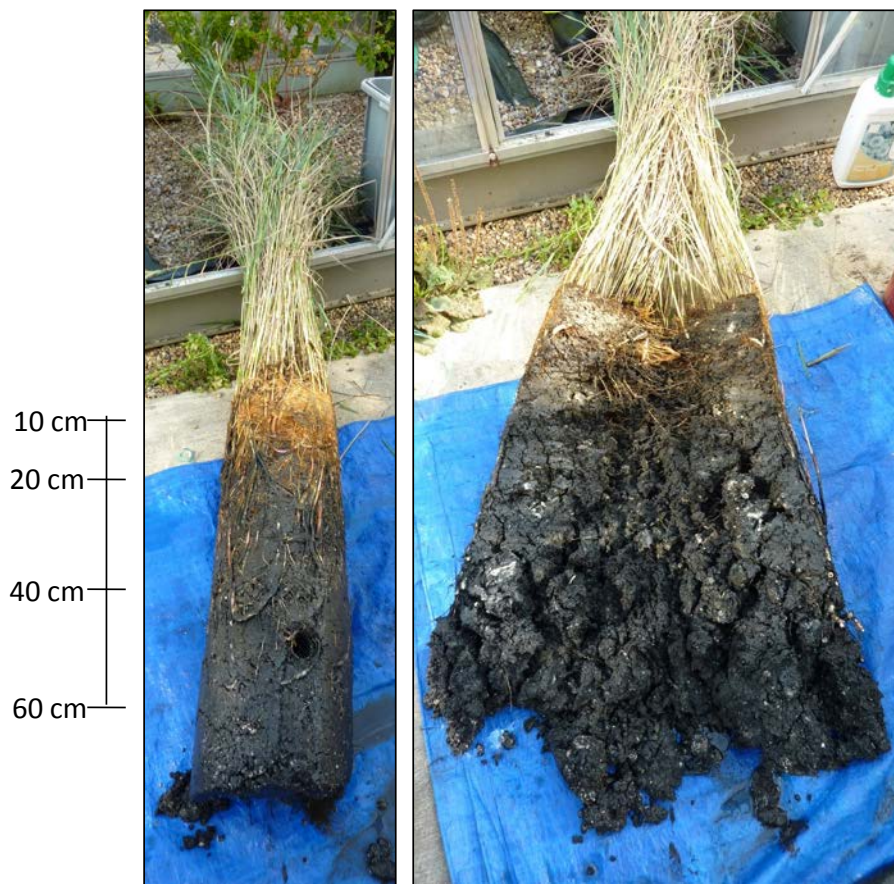
Plate 13. Dismantled microcosm showing a high density of roots at the edges, H. Pinchin, August 2010

The lowest DRO concentrations were seen in the upper layers of the microcosm (26,776 mg/kg DW and 29,173 mg/kg DW in the centre and edge samples respectively) where volatilisation of the hydrocarbons is most likely to occur. Different patterns were then observed with depth; DRO concentrations decreased in the centre samples but increased in the edge samples (Figure 11).
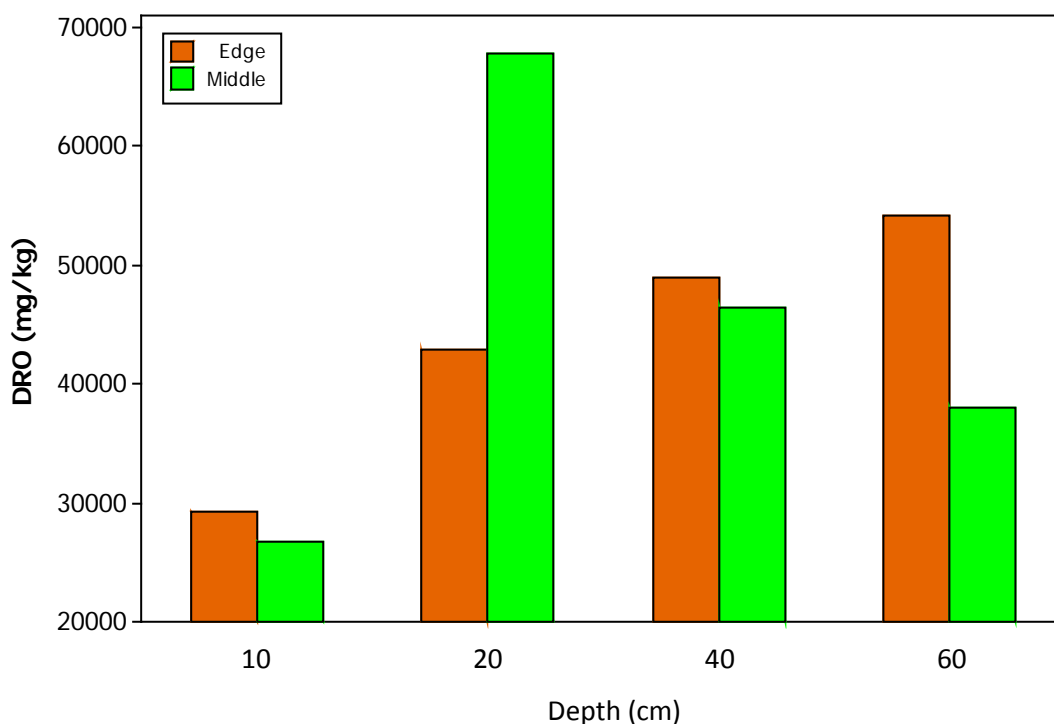
Figure 11. DRO concentrations at different depths in the middle and edge of the planted microcosm PL1 (16 months after construction)

5.2.1.4 GC-FID chromatograms from the dismantled microcosms

The chromatograms from dismantled microcosms (see appendix and table 8) showed the composition of residual hydrocarbons to be in the C_{10}-C_{12} to C_{25} DRO range with a symmetric distribution centered at C_{17}-C_{18} in the planted microcosm and C_{19} in the unplanted microcosm and a high proportion of UCM.

Overall, the planted edge and middle microcosm chromatograms showed a 59% total area and 61% or 53% UCM area increase when compared to the unplanted microcosm chromatograms. The unplanted microcosm chromatograms had a lower total area and UCM area at each depth except for 10 cm where the UCM area was lower in the planted middle (Figure 12).

The chromatograms all showed a resolved peak before the start of the UCM hump at C_{10}, which was larger in the unplanted microcosm. The two highest resolved peaks present

within the UCM were at C_{16}-C_{17} and $C_{17\text{-}18}$ in the planted microcosms and at C_{16}-C_{18} and C_{17}-C_{19} in the unplanted microcosms.
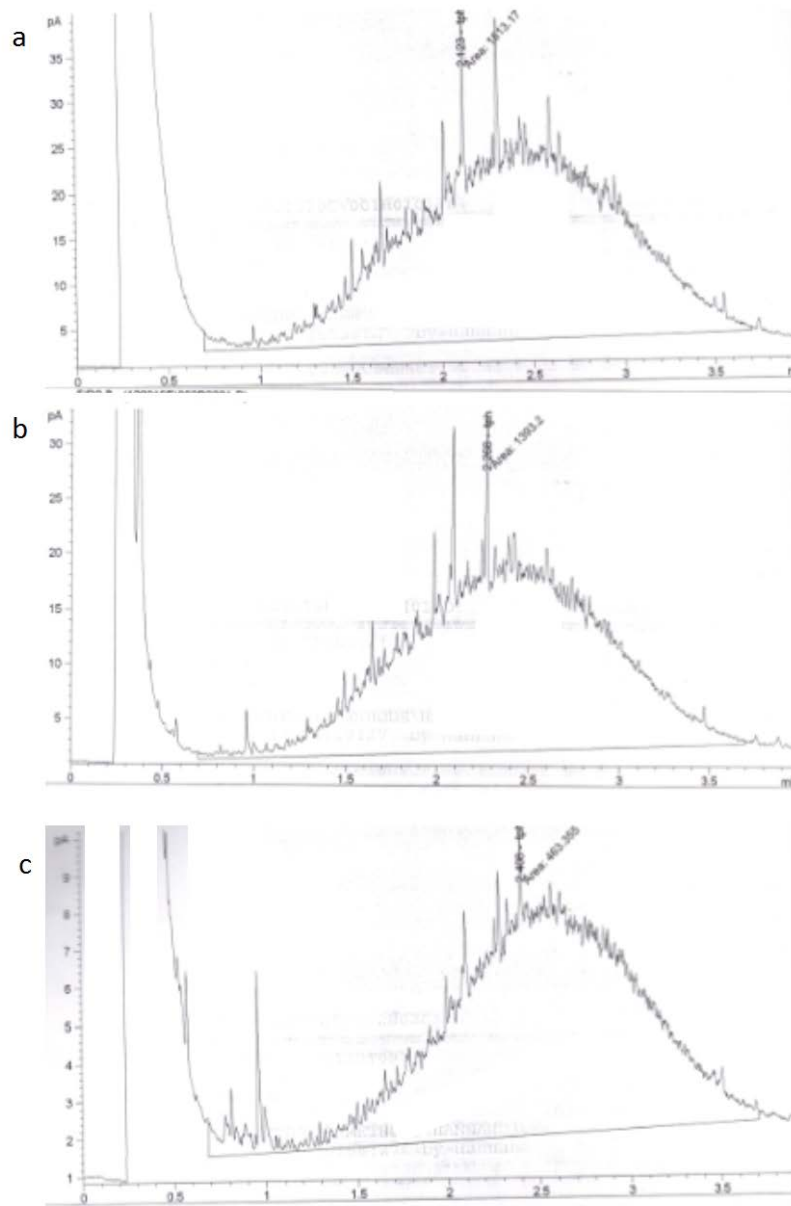
Figure 12. GC-FID chromatograms from the dismantled microcosms 60 cm (a) PL1 edge (b) PL1 middle (c) UP3

Table 9. Summary of GC-FID chromatogram data analysis from planted (PL) and unplanted (UP) dismantled microcosms.

Day	Site	Depth	Total area (pA^2)	UCM area (%)	Maximum UCM height (pA)	Carbon number at UCM peak	Start of UCM
478	PL1 Edge	10	1054	95	14	C18	C11
478	PL1 Middle	10	821	35	13	C18	C12
478	PL1 Edge	20	1702	78	24	C18	C11
478	PL1 Middle	20	2321	80	30	C18	C10
478	PL1 Edge	40	1657	75	21	C17	C10
478	PL1 Middle	40	1691	70	23	C18	C11
478	PL1 Edge	60	1813	80	24	C18	C10
478	PL1 Middle	60	1393	63	18	C18	C11
478	UP3	10	616	73	9.5	C19	C12
478	UP3	20	507	97	8	C19	C11
478	UP3	40	993	62	14	C19	C11
478	UP3	60	463	77	8	C19	C12

5.2.2 Number of culturable microorganisms

5.2.2.1 Factors affecting microbial counts in the microcosms

The microcosms were sampled approximately monthly (n=7) from August 2009 to June 2010. The number of culturable bacteria, hydrocarbon-tolerant bacteria, fungi and hydrocarbon-tolerant fungi were determined using the MPN enumeration technique. Overall, there was no significant difference between the total number of culturable microorganisms between the planted and unplanted microcosms.

The hydrocarbon-tolerant bacteria responded to environmental factors more than the other microorganisms. Over time, both the number of hydrocarbon-tolerant bacteria (r=0.355, p=0.000) and the number of hydrocarbon-tolerant fungi (r=0.357, p=0.001) increased. The

number of hydrocarbon-tolerant bacteria increased steadily slowly over time, whereas the number of hydrocarbon-tolerant fungi increased over the first three sampling occasions then remained at this level for the rest of the study.

The number of culturable hydrocarbon-tolerant bacteria significantly decreased as the DRO concentration increased (r=-0.390, p=0.030) however, none of the other microorganisms had a significant linear relationship with DRO concentrations (Figure 13).
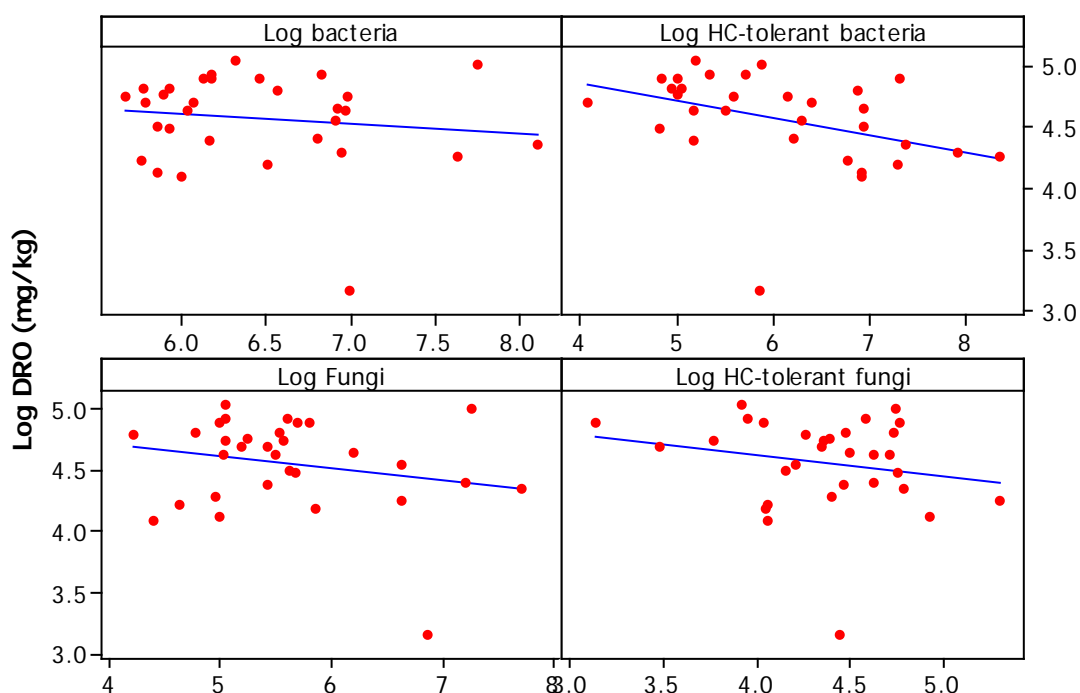
Figure 13. The effect of DRO concentration on microbial numbers in the microcosms

The number of hydrocarbon-tolerant bacteria (r=-0.335, p=0.004), total fungi (r=-0.412, p=0.000) and total bacteria (r=-0.621, p=0.000) significantly decreased with depth (Table 10).

This association was seen in all the microcosms and was most consistent for culturable hydrocarbon-tolerant bacteria (Figure 14) however, there was no correlation between the number of culturable hydrocarbon-tolerant fungi with sediment depth.

Table 10. The mean number of microorganisms at each depth in the microcosms (SEM in brackets)

Microcosm	Depth (cm)	Total bacteria (cfu/g)	Hydrocarbon-tolerant bacteria (cfu/g)	Total fungi (cfu/g)	Hydrocarbon-tolerant fungi (cfu/g)
PL1	0	9.8×10^6 (3.0×10^6)	1.7×10^6 (1.3×10^6)	8.6×10^5 (3.5×10^5)	1.7×10^4 (6.4×10^3)
PL1	15	8.6×10^6 (6.8×106)	1.3×10^6 (1.2×10^6)	1.8×10^5 (9.1×10^4)	1.5×10^4 (3.0×10^3)
PL1	30	1.1×10^6 (3.3×10^5)	3.2×10^5 (2.2×10^5)	1.5×10^5 (3.5×10^4)	3.1×10^4 (6.0×10^3)
PL1	45	9.0×10^5 (1.7×10^5)	5.4×10^5 (3.8×10^5)	2.0×10^5 (3.8×10^4)	3.6×10^4 (7.0×10^3)
PL2	0	2.5×10^7 (8.9×10^6)	3.7×10^7 (3.6×10^7)	7.6×10^6 (2.3×10^6)	6.6×10^4 (2.4×10^4)
PL2	15	7.7×10^6 (1.4×10^6)	3.6×10^6 (3.2×10^6)	3.3×10^6 (2.6×10^6)	2.1×10^4 (6.3×10^3)
PL2	30	2.9×10^6 (8.9×10^5)	3.5×10^6 (3.3×10^6)	2.7×10^5 (8.6×10^4)	4.6×10^4 (2.7×10^4)
PL2	45	2.7×10^6 (1.2×10^6)	1.9×10^6 (1.3×10^6)	1.9×10^5 (6.5×10^4)	1.2×10^5 (3.6×10^3)
UP3	0	2.8×10^7 (2.0×10^7)	1.8×10^7 (1.3×10^7)	9.1×10^6 (8.6×10^6)	2.9×10^4 (7.4×10^3)
UP3	15	2.7×10^6 (1.5×10^6)	2.3×10^6 (1.4×10^6)	1.4×10^6 (1.2×10^6)	1.4×10^4 (4.5×10^3)
UP3	30	1.1×10^6 (1.4×10^5)	1.4×10^6 (1.3×10^6)	1.9×10^5 (6.6×10^4)	1.9×10^4 (1.1×10^4)
UP3	45	1.3×10^5 (2.2×10^5)	1.5×10^6 (1.4×10^6)	2.4×10^5 (6.6×10^4)	2.6×10^4 (7.8×10^3)

Figure 14. Number of culturable hydrocarbon-tolerant bacteria at different depths over time (SEM shown by error bars)

The number of culturable hydrocarbon-tolerant bacteria was inversely related to moisture content (r=-0.306, p=0.009) (Figure 15). This was also apparent in submerged sediment samples (ANOVA F=9.26, p=0.003) and in the unplanted microcosm UP3 when the microcosms were looked at individually (r=-0.540, p=0.006). None of the other microorganisms showed any correlation with moisture.

5.2.2.2 Culturable microorganisms throughout the dismantled microcosms

In August 2010, sediment samples were taken from four depths from the root dominated edges and less dense root growth in the middle of PL1 as well as a combined middle and edge sediment sample from the unplanted microcosm, UP3. These samples were analysed for culturable microbial counts using the MPN enumeration method. There was no significant difference between the number of culturable microorganisms in samples taken at the edge of the planted microcosm and the centre (Table 11).
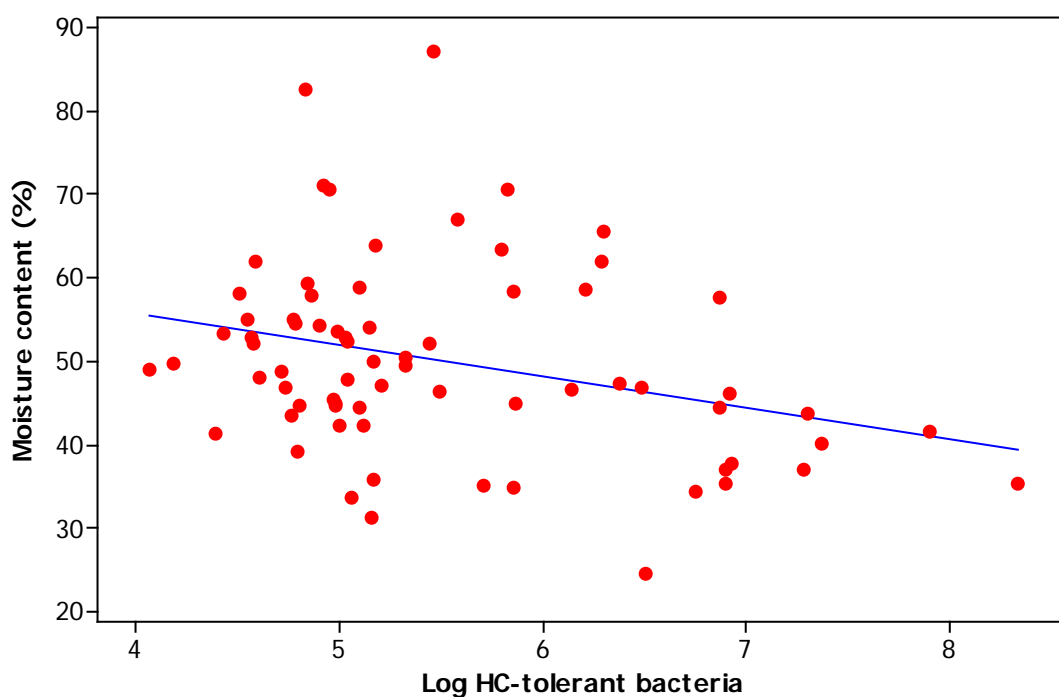
Figure 15. Relationship between sediment moisture content (%) and number of culturable hydrocarbon-tolerant bacteria

Table 11. Microorganisms in microcosms PL1 and UP3 with depth

Microcosm	Sediment depth (cm)	Site of sample collection	Total bacteria	Hydrocarbon-tolerant bacteria	Total fungi	Hydrocarbon-tolerant fungi
PL1	10	Edge	8.78×10^5	4.97×10^7	2.93×10^6	3.34×10^4
PL1	10	Centre	1.89×10^6	7.40×10^6	3.22×10^6	8.70×10^7
UP3	10	Combined	8.12×10^6	1.54×10^6	4.59×10^5	1.62×10^5
PL1	20	Edge	9.90×10^6	9.90×10^5	3.30×10^6	8.60×10^4
PL1	20	Centre	4.97×10^6	4.97×10^6	1.67×10^6	2.43×10^4
UP3	20	Combined	6.90×10^6	5.24×10^5	4.02×10^5	8.05×10^4
PL1	40	Edge	1.60×10^6	1.60×10^6	3.67×10^5	1.26×10^4
PL1	40	Centre	2.32×10^6	2.32×10^5	6.78×10^5	2.51×10^4
UP3	40	Combined	3.82×10^6	1.21×10^5	2.93×10^5	1.76×10^4
PL1	60	Edge	1.16×10^7	2.14×10^5	8.38×10^5	1.68×10^4
PL1	60	Centre	1.72×10^6	9.05×10^4	5.12×10^5	4.61×10^4
UP3	60	Combined	2.20×10^6	3.75×10^5	2.88×10^5	5.75×10^6

There was also no difference in the number of culturable microorganisms in the planted microcosm PL1 compared to the unplanted microcosm UP3 except for the number of culturable fungi, which were significantly higher in the planted microcosm PL1 (ANOVA F=7.45, p=0.021). However the number of culturable hydrocarbon-tolerant bacteria was higher in the planted microcosm PL1 than the unplanted UP3, except at the 60 cm depth where the roots were sparse. Overall, the number of culturable hydrocarbon-tolerant bacteria decreased with depth (r=-0.761, p=0.004) as did the number of culturable fungi in the planted microcosm PL1 (r=-0.830, p=0.011).

5.2.3 Culturable bacterial isolates

In January 2010, 256 culturable bacteria were isolated from the microcosm MPN enumeration plate counts. After they were all Gram stained and the positive rods were stain tested for endospores (Plate 13a and b), 64 visually different culturable bacterial isolates were identified and given a code letter and number (see appendix).

Of the 256 culturable bacteria isolates, 95 were found in PL1, 83 in PL2 and 78 in UP3 (Table 12 and Table 13). When investigating the diversity of these bacterial isolates, out of the 64 different bacterial isolates, 39 grew exclusively in the planted microcosms but only 10 grew exclusively in the unplanted microcosm. The highest number of total bacteria isolates was found at the surface and at 30 cm deep in all three microcosms and the lowest number were found in the deepest sample sites.

PL2 had the highest diversity of different bacterial isolates exclusively found in one microcosm (n=17) with UP3 having the lowest number (n=10) indicating that the addition of *P. australis* may have increased the number and diversity of the culturable bacterial communities (Table 12 and table 13).
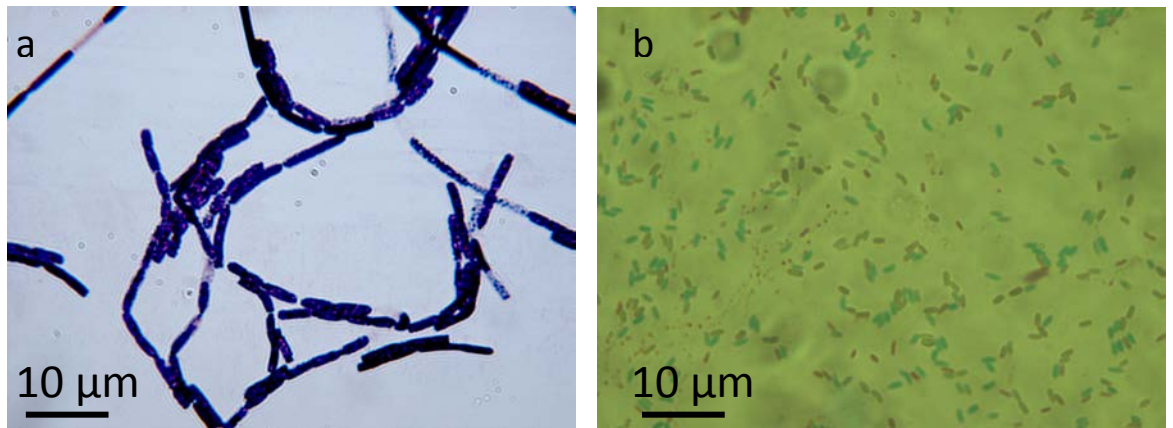
Plate 14. An example of (a) Gram stained culturable bacterial isolate (b) Endospores from a culturable bacterial isolate stained green, H. Pinchin

Nineteen out of the 64 bacterial isolates were facultative anaerobes. The overall number of facultative anaerobes was very similar in PL1 (n=31), PL2 (n=40) and UP3 (n=34). However there was a higher diversity of facultative anaerobes in the planted microcosms with PL2 (n=13) having twice the number of different facultative anaerobes found in UP3 (n=6).

There were 64 different bacterial isolates found in the microcosm sediment: of these 38 were positive rods and 21 positive rods capable of forming endospores. UP3 had the highest number of total endospore producing bacterial isolates (n=50) however PL1 had only slightly less (n=46). Although PL2 had the lowest number of endospore producing bacterial isolates (n=37) it had the highest diversity of different endospore producing bacterial isolates (n=12) with UP3 having only one fewer (n=11).

The majority of bacterial isolates were found to be Gram positive rods. Bacterial isolate A was the most abundant being found a total of 65 times across all the sampling sites. It was a facultative anaerobe and endospore producer allowing it to survive in both anaerobic and aerobic conditions and in times of unfavourable environmental conditions.

Table 12. Summary of bacterial isolates unique to the planted microcosms

Bacterial isolate code	Gram stain	Shape	Facultative anaerobe	Endospore producing	PL1 0 cm	PL1 15 cm	PL1 30 cm	PL1 45 cm	PL2 0 cm	PL2 15 cm	PL2 30 cm	PL2 45 cm
B	-	R	-		/		/					
C	+	C	+		/					/		
D	+	R	+	-			/			/		
F	+	R	+	-			/					
G	+	R	+	-			/		/			
H	-	R	-		/							
K	+	R	+	-					/			
L	+	R	-	-					/		/	
O	+	C	+								/	
P	+	R	-	+					/			
Q	+	R	-	+					/			
R	+	R	-	-				/				
S	+	R	-	+	/							
T	-	CB	+		/							
U	+	R	+	+		/			/			
W	+	C	-							/		
Y	+	R	+	-						/	/	/
Z	+	R	-	-				/				
B2	-	R	-								/	
T2	+	R	-	+	/		/					
C2	+	R	-	+				/				
D2	+	R	-	-							/	
F2	-	C	-			/						
I2	-	R	-							/	/	
K2	-	R	-		/							
L2	-	R	-						/	/		
P2	-	R	-		/		/			/		/
Q2	-	R	-				/	/				
S2	+	R	-	-	/							
U2	-	R	-			/			/			
E8	-	R	+								/	/
F8	-	R	+		/				/		/	
H8	-	R	-		/		/		/		/	
I8	+	C	+								/	/
J8	+	R	-	+							/	
J9	+	R	-	+		/						/
K9	+	R	-	-					/			
L9	+	R	-	+						/		
M9	+	R	+	+					/			

Colour	/	/	/	/	/	/	/
Frequency	1	2	3	4	5	6	7

Key: R= Rod C= Cocci CB= Cocci Bacilli

Table 13. Summary of bacterial isolates unique to the unplanted microcosm

Bacterial isolate code	Gram stain	Shape	Facultative anaerobe	Endospore producing	UP3			
					0 cm	15 cm	30 cm	45 cm
I	+	R	-	+	/		/	
N	+	R	+	-	/	/		
A2	-	R	-			/		
H2	+	R	-	-		/		
M2	+	R	-	+		/		
R2	+	R	-	+			/	/
V2	-	R	-				/	
W2	+	R	+	+			/	
X2	-	R	-		/			
K8	+	R	+	+	/			

Key: R= Rod

Colour	/	/
Frequency	1	2

5.2.2.4 Activity of hydrocarbon-degrading bacteria in the microcosm

Bacterial isolates grown from the microcosm MPN enumeration plate counts were tested for their ability to degrade hydrocarbons (n=256). A small colony was streaked onto an agar plate containing Bushell Haas agar with 0.0025% TTC and with or without the addition of 10 mL/L diesel as the sole carbon source and incubated for five days at 25˚C. The bacterial isolates that grew pink or red on the plates were deemed hydrocarbon-degrading (Plate 15).
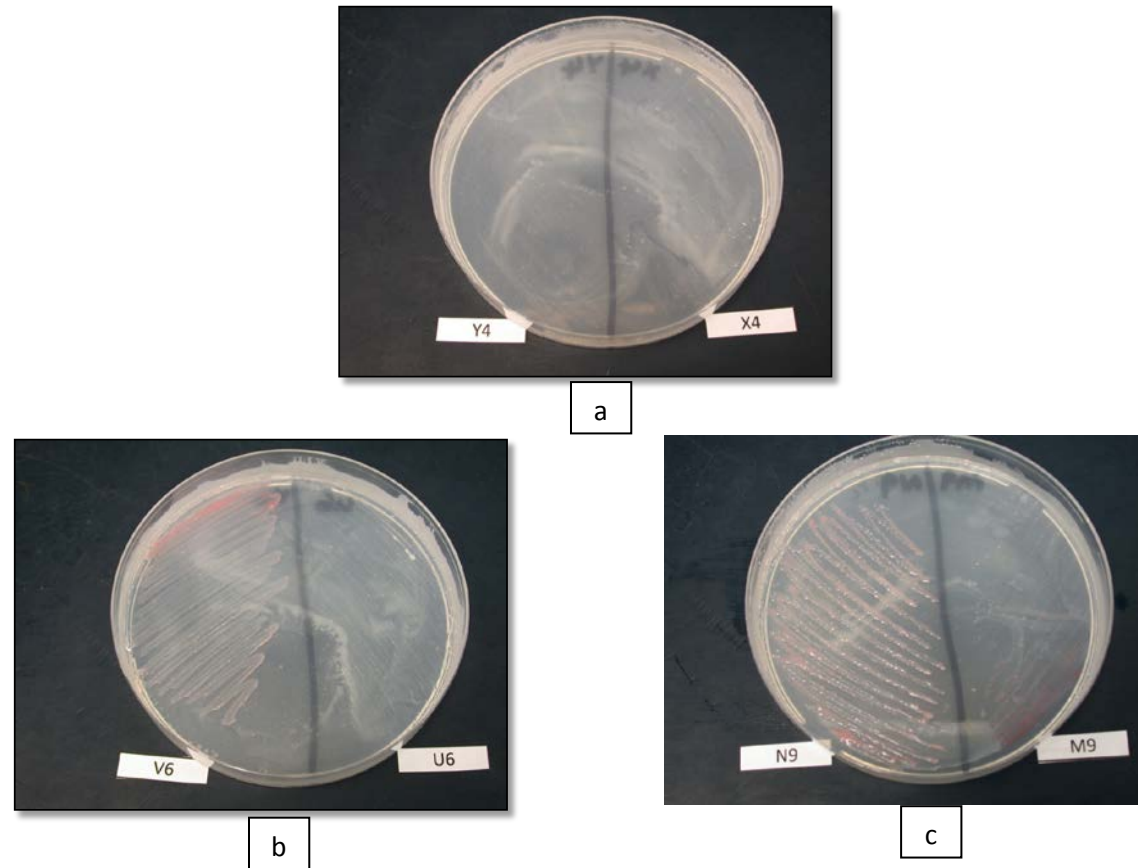
Plate 15. Bacterial isolates streaked onto hydrocarbon degrading indicator agar+10 mL/L diesel plates and incubated for 5 days at 25°C. Results: (a) No hydrocarbon degrading activity (b) One hydrocarbon degrading bacterium (left) (c) Two hydrocarbon degrading bacteria, H. Pinchin

Overall, 33% of the bacterial isolates were determined to be hydrocarbon-degraders according to the colorimetric plates (Table 14). Three times as many different hydrocarbon-degrading bacterial isolates were exclusively found in the planted microcosms (n=12) than in UP3 (n=4). Planted microcosm, PL1, had the most hydrocarbon-degrading bacterial isolates (n=39) as well as the highest diversity (n=12). However, the other planted microcosm, PL2, had the lowest number of hydrocarbon-degrading bacterial isolates (n=24) and the same number of different hydrocarbon-degrading bacterial isolates as UP3 (n=9).

Approximately one third of the hydrocarbon-degrading bacterial isolates were facultative anaerobes (29%) with the highest number of total hydrocarbon-degrading bacterial (n=6) and the highest diversity (n=3) found in PL2.

Out of the 21 different hydrocarbon-degrading bacterial isolates, there were 13 Gram positive rods, 54% of which produced endospores. The highest number of endospore-producing hydrocarbon-degrading bacterial isolates was found in PL1 (n=20) however, the highest diveristy (n=5) was found in UP3.

The three most commonly occurring hydrocarbon-degraders were L8 (n=29), E2 (n=14) and M8 (n=10). They were all found to be Gram positive rods but only L8 and M8 were able to produce endospores. L8 was found in all the depths in UP3 and three out of the four depths in the planted microcosms. It was found predominantly in the upper layers of the microcosms where oxygen may be more available, and was abundant in PL1.

The roots and rhizomes of the reeds are also sites for bacterial growth so these were cultured on Bushnell Haas with 0.0025% TTC indicator plates with and without 10 mL/L diesel. The plate containing diesel developed red areas around the *P. australis* roots and rhizome indicating that hydrocarbon-degrading microorganisms were present; whereas the plate

Table 14. The location and frequency of the hydrocarbon-degrading bacterial isolates in the microcosms at different depths

Bacterial isolate code	Gram stain	Shape	Facultative anaerobe	Endospore producing	PL1				PL2				UP3			
					0 cm	15 cm	30 cm	45 cm	0 cm	15 cm	30 cm	45 cm	0 cm	15 cm	30 cm	45 cm
L8	+	R	-	+	/	/	/		/		/	/	/	/	/	/
E2	+	R	-	-			/	/		/	/	/	/	/	/	/
M8	+	R	-	+	/			/	/	/			/		/	/
G8	-	R	-				/						/			/
O8	-	CB	-		/	/									/	
B	-	R	-		/		/									
K9	+	R	-	-					/							
C	+	C	+		/					/						
T2	+	R	-	+	/		/									
I8	+	C	+								/	/				
M9	+	R	+	+					/							
H	-	R	-		/											
R	+	R	-	-			/									
T	-	CB	+		/											
Z	+	R	-	-			/									
B2	-	R	-								/					
D2	+	R	-	-							/					
R2	+	R	-	+											/	/
W2	+	R	+	+											/	
H2	+	R	-	-										/		
K8	+	R	+	+									/			

Colour	/	/	/	/	/	/	/
Frequency	1	2	3	4	5	6	7

Key: R= Rod C= Cocci CB= Cocci Bacilli

without diesel did not develop any red colouring after five days at room temperature (Plate 16). On a larger scale a sterile glass vase was filled with warm Bushnell Haas with 0.0025% TTC indicator agar and 10 mL/L diesel and a small plant of *P. australis.* After five days the area around the roots and rhizomes had become red in colour (Plate 17) indicating hydrocarbon-degrading activity was concentrated around these areas.
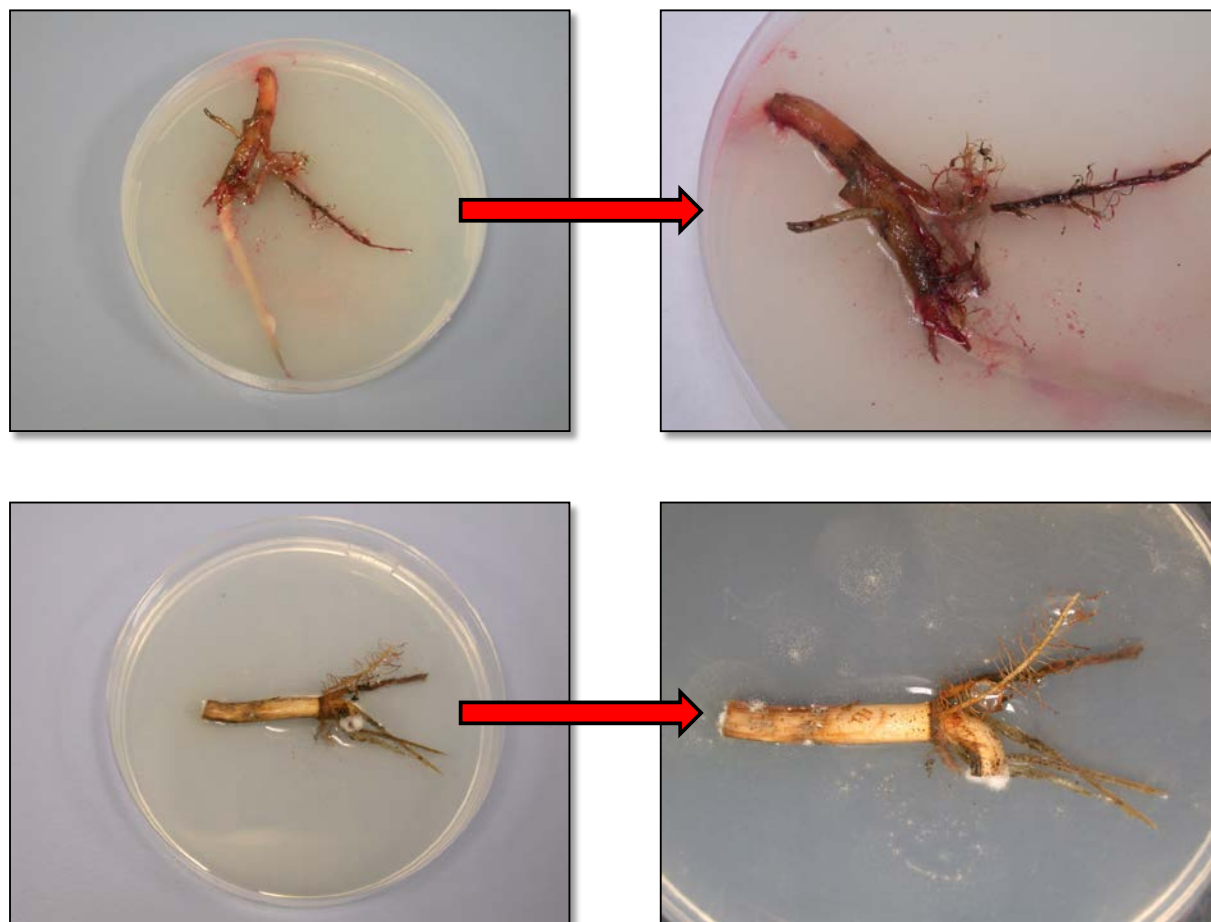
Plate 16. *P. australis* **rhizome and roots in hydrocarbon degrading indicator agar (a) with 10 mL/L diesel after 5 days at room temperature (b) without diesel after 5 days at room temperature, H. Pinchin**
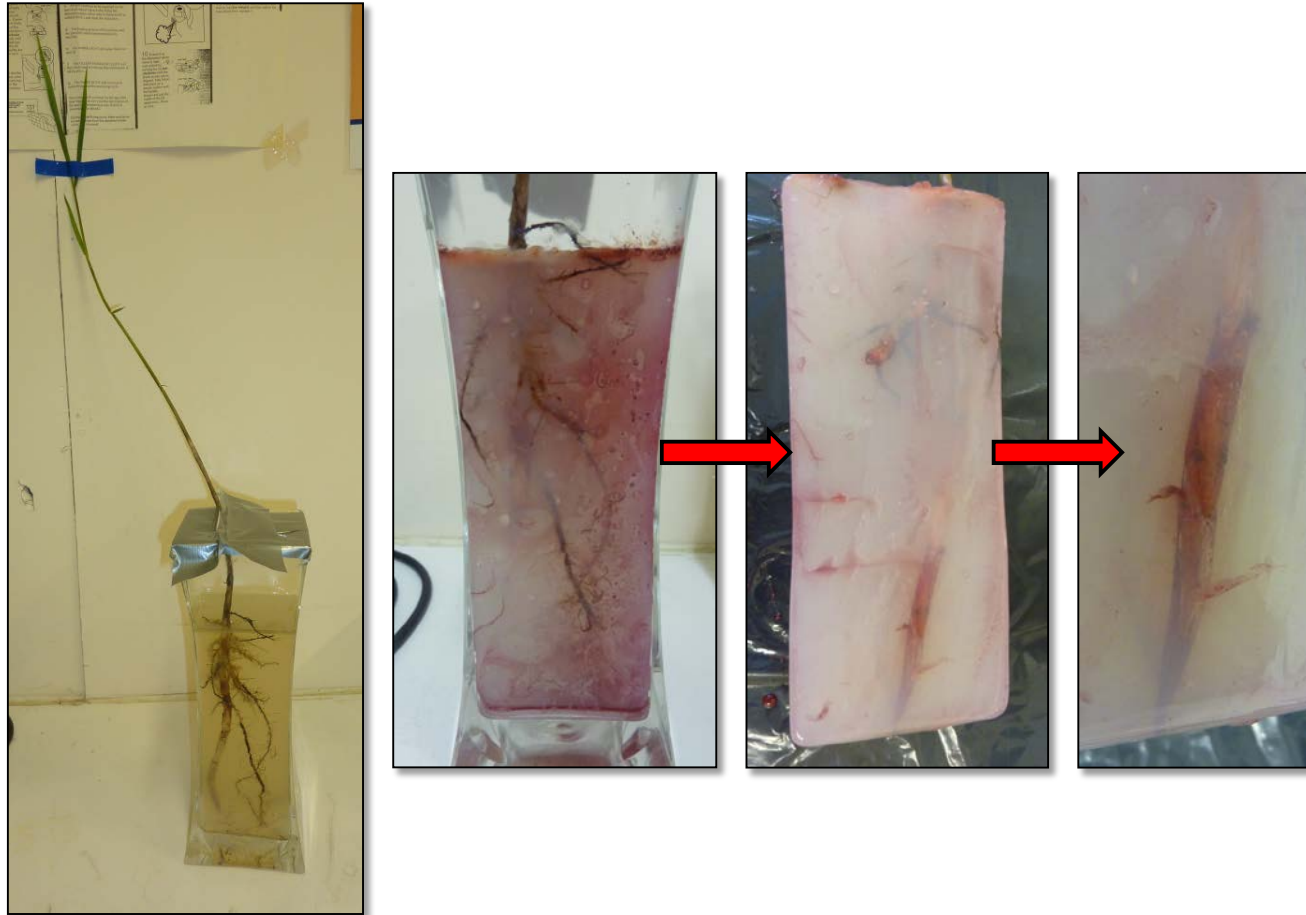
Plate 17. *P. australis* in hydrocarbon degrading indicator agar+10 mL/L diesel at room temperature (a) day 0 (b) day 5, H. Pinchin

5.2.2.5 Characterisation of the hydrocarbon-degrading cultural bacterial isolates from the microcosms

One bacterial isolate from each of the 21 hydrocarbon-degrading bacterial isolate groups was selected for genus and species identification and tested using the Biolog plate reader and molecular techniques. The four Gram negative rod culturable bacterial isolates were also API tested (Table 15)

Table 15. Selected hydrocarbon-degrading bacterial isolates found in the microcosms identified by API, Biolog and molecular methods

Bacterial isolate code	site	depth (cm)	API	id %	Biolog	id %	Molecular	id %
L8	PL1	0					*Pseudomonas asplenii*	100
E2	PL2	15						
M8	PL1	0					*Acidovorax* spp.	85
G8	PL1	30	*Aero.hydro./caviae*	99	*Aeromonas* spp.			
O8	PL1	15					*Pseudomonas stutzeri*	100
B	PL1	30	*Pseudomonas stutzeri*	99.4	*Pseudomonas stutzeri*	100	*Pseudomonas* spp	92
K9	PL2	0			*Tsukamurella inchonensis*	100	*Gordonia rubripertincta*	99
C	PL2	15			*Pediococcus urinaeequi*	95	*Trichococcus flocculiformis*	97
T2	PL1	30						
I8	PL2	30			*Tsukamurella inchonensis*	85	*Rhizobium* spp.	90
M9	PL2	0			*Rhodococcus coprophilus*	82	*Mycobacterium frederiksbergense*	98
H	PL1	0						
R	PL1	45						
T	PL1	0					*Exiguobacterium* spp.	95
Z	PL1	45			*Kurthia gibsonii*	99	*Bacillus* spp.	90
B2	PL2	30	*Pseudomonas stutzeri*	99.4	*Pseudomonas stutzeri*	100	*Pseudomonas stutzeri*	99
D2	PL2	30					*Bacillus cereus*	98
R2	UP3	45						
W2	UP3	30						
H2	UP3	15			*Bacillus* spp.			
K8	UP3	0						

There was a clear difference in bacterial identification results between the API, Biolog and molecular methods. After API testing, three out of the four Gram negative rod bacterial isolates were identified and two were found to be the same species, *Pseudomonas stutzeri*, although they appeared morphologically different during earlier examination. This species

identification (ID) was found with all the different methods for both isolate B and B2 indicating that these isolates were correctly identified and indeed the same species and genus. Hydrocarbon-degrading bacterial isolate O8 was also found to be *Pseudomonas stutzeri* and isolate L8 was found to be *Pseudomonas asplenii* making *Pseudomonas* spp. the most abundant hydrocarbon-degrading species found in the microcosms.

Biolog analysis identified seven hydrocarbon-degrading bacterial isolates to species level and two hydrocarbon-degrading bacterial isolates to genus level. Once again there were four hydrocarbon-degrading bacterial isolates that were found to be the same two hydrocarbon-degrading bacterial isolate: B and B2 were found to be *Pseudomonas stutzeri* and K9 and I8 were found to be *Tsukamurella inchonensis*. Using a minimum of 97% ID and above, the molecular analysis also identified seven hydrocarbon-degrading bacterial isolates to species level but using a range of 80-96% ID it also identified five hydrocarbon-degrading bacterial isolates to genus level. Seven hydrocarbon-degrading bacterial isolates were not identified by any of the techniques used.

5.3 Discussion

5.3.1 DRO concentration in the microcosm

P. australis was grown in greenhouse microcosms to assess the influence of the microbial communities on enhancing hydrocarbon degradation in environmentally-controlled conditions when compared to an unplanted control. Although the sediment was thoroughly homogenised before the start of the experiment, the DRO concentration varied considerably with UP3 having the lowest initial DRO and in all the microcosm sites throughout the study with the highest concentration being observed as 109,000 mg/kg in PL1 at 15 cm depth on January 2010 and the lowest concentration being observed as 1,458 mg/kg in UP3 at 15 cm depth in April 2010. This could be because insufficient sediment samples were analysed to confirm homogeneity or the possible migration of the hydrocarbons into the lower depths due to settlement or through water flushing. This lack

of homogeneity made determining the effect of *P. australis* on the contaminated sediment very difficult to interpret as samples taken from just a small distance away sometimes had very different DRO concentrations. It also appears that the microcosms did not start with the same initial DRO concentration although this again could be due to insufficient sampling. Campbell (1978) stated that the scale of special variability was an important consideration when designing an environmental survey intended to monitor the changes in contaminant concentrations. He also stated that the composition of natural materials is typically heterogeneous on a small scale and homogeneous on a large scale for organic contaminants in sediments which also appears to be the case in the Horsea lagoon microcosm study.

Overall the DRO concentration decreased over time in all the microcosms and although the planted microcosms had a significantly higher DRO concentration than UP3 it was PL2 that had the highest rate of decline. Many studies performed with soil microcosms have reported the incomplete degradation of diesel oil, the lowest mean DRO concentration in this study was 2.04×10^{4} mg/kg. It is difficult to then predict a possible time scale for complete DRO degradation especially as Marchal *et al.* (2003) claimed that commercial diesel oil was only 60-73% biodegradable. They stated that only the lighter hydrocarbons could be degraded and the heavier branches would be resistant to biodegradation. The phytotoxicity of the DRO could prevent the plants growing and thereby inhibiting the effects of phytoremediation (Banks *et al.,* 1999) but this was not the case in the greenhouse microcosm study. However Bystrom and Hirtz (2002) stated that theoretically all hydrocarbons could be biodegraded but the time it took to degrade the substances differed depending on treatment, microbial breakdown potential and molecule structure. Since there are hundreds of different compounds in petroleum products, with a varying rate of biodegradation, it could be difficult to predict the total rate of degradation. Furthermore the levels of the different compounds in a petroleum product can vary between different

manufacturers which make a prediction of the degradation rate even more difficult. Considerable time could be needed to achieve regulated levels depending upon the initial concentrations and the desired end point (Frick *et al.,* 1999). Overall phytoremediation has proven to be an effective method for cleaning up soils that have low or intermediate contamination of petroleum hydrocarbons (Bystrom & Hirtz, 2002), unfortunately Horsea lagoon sediment exceeds the concentration deemed 'toxic' by Riser-Roberts (1998b) of 100 µg/g soil, suggesting a very long time period will be required for remediation.

When looking at the factors affecting the DRO concentration, it was shown that DRO was significantly higher in sediments with higher water content and in submerged microcosms. Hutchinson *et al.,* (2003) noted that aerobic microbial activity in soil rhizospheres, increased from essentially zero at very low water content to an optimum level at 60%. In this work, the sediment moisture content was predominantly between 30 and 70%, therefore the range was suitable for enhanced aerobic activity if oxygen was available.

Moisture content tended to decrease with depth and although there was an association between DRO increasing with moisture content, the association between DRO and depth was not significant.

5.3.2 DRO in the dismantled microcosms

Fifteen months after planting, one planted microcosm (PL1) and the unplanted microcosm (UP3) were dismantled and the sediments analysed. There was found to be no difference in DRO concentration between edge samples extracted from an area with dense roots and rhizomes and central samples with less roots. Although there was again no significant correlation between DRO concentration and depth there was less DRO in the top sampling sites, possibly due to either increased microbial degradation in the dense root zones or hydrocarbon migration or volatilisation. There have been some reports on mobilisation of DRO into the soil column but in most cases there has been little evidence for the

significant downward leaching of DRO (Atlas, 1991). DRO generally increased with depth in the samples taken from the root-packed edge samples and vice versa in areas that had less roots. This seems to indicate there is an increased rate of DRO degradation in the areas with more roots and oxygen and therefore a rhizosphere effect from microorganism degradation, root exudates and oxygen transfer and sediment channels being created by the roots.

The GC-FID chromatograms showed a large symmetrical UCM hump which differed form the diesel control graphs that remained horizontal to the baseline with clearly defined peaks. This symmetrical hump shape can be an indicator of weathering and other environmental contaminants present in the sediment (Tam *et al*., 2005). The chromatograms all showed a definite peak at C_{10} before the start of the UCM. This could be due to a cyclic biomarker called adamantane, which belongs to the "diamondoid" hydrocarbon group. Diamondoids are rigid, three-dimensionally fused cyclohexane-ring alkanes that have a diamond-like (cage-like) structure and a high resistance to biodegradation (Wang, 2004).

5.3.3 Microbial numbers in the microcosms

Analysis of the total heterotrophic counts and specific hydrocarbon-tolerant microbial counts in the contaminated sediment can provide useful information on the biological activities of the sediment and the extent to which the indigenous microbial population has acclimatised to the site conditions (Hutchinson *et al*., 2003). These counts could also indicate whether the soil contains a healthy indigenous microbial population capable of supporting bioremediation and whether the addition of *P. australis* enhances the numbers and degradation affect.

Overall, the number of hydrocarbon-tolerant bacteria and hydrocarbon-tolerant fungi increased over time. Leahy and Colwell (1990) stated that hydrocarbons in the

environment were biodegraded primarily by bacteria although the concept of a maximum or threshold concentration for microbial degradation of hydrocarbons may apply in sediment ecosystems. The DRO concentration could have reached a maximum threshold for the microorganisms unable to tolerate hydrocarbons and hence they did not significantly increase allowing the better adapted microorganisms to flourish. It was important to determine whether this increase in microbial numbers was due to a rhizosphere effect by comparing the numbers found in planted and unplanted microcosms. During a study by Phillips (2009), rhizosphere heterotrophic communities in the planted treatments maintained significantly higher levels than those in the control soil. This was also shown in a study by Kirk *et al.* (2005) using Ryegrass and investigating the numbers of bacteria, fungi, hydrocarbon 'degrading' bacteria and hydrocarbon 'degrading' fungi in the rhizosphere and bulk soil at a decommissioned petroleum storage site. They found elevated microbial numbers in the rhizosphere samples apart from hydrocarbon 'degrading' fungi compared to bulk soil. However, only the number of bacteria and fungi increased significantly. In sediment from Horsea lagoon there was no significant difference between the number of microorganisms found in the planted microcosms and those found in UP3, although there was shown to be a rhizosphere effect on DRO concentration. However, Hickman and Novak (1989) concluded that total microbial numbers were a poor predictor for biodegradation potential because they did not take into account species diversity or metabolic regimes.

In the unplanted microcosm, only the number of hydrocarbon-tolerant bacteria had any associations with DRO levels. The number of hydrocarbon-tolerant bacteria was inversely related to DRO concentration indicating the possibility of an increased degradation effect or signifying a toxic effect of high DRO concentrations on microorganisms (Balba *et al.*, 1998).

Microbial populations in unplanted sediment are usually found to be approximately $\sim 1 \times 10^6$ cells/g, whereas planted rhizosphere sediment usually has microbial populations of approximately $\sim 1 \times 10^8$ cells/g (Shimp *et al*., 1993). During this study, mean microbial numbers for all the microorganisms were in the order of $\sim 1 \times 10^6$ cells/g except for the hydrocarbon-tolerant fungi which had only 2.8×10^4 cells/g. In soils containing large volumes of roots, microbial populations can reach $\sim 1 \times 10^{12}$ cells/g (Lynch, 1990) which is much higher than the values seen here. When comparing planted and unplanted microcosm numbers, Kirk *et al*. (2005) found numbers of bacteria in ryegrass-planted petroleum contaminated soils of up to 3.5×10^9 cells/g and only 4.5×10^7 cells/g in the unplanted soils after a 7 week trial. In the Horsea lagoon sediment, bacterial numbers were similar in both planted and unplanted sediment with a maximum of 5.7×10^7 cells/g and 1.3×10^8 cells/g respectively. The same was found with the numbers of hydrocarbon-tolerant bacteria with 2.2×10^8 cells/g in planted sediment and 8.0×10^7 cells/g in the unplanted sediment. These results are still not as high as the ryegrass planted soil possibly due to sediment structure or the high DRO content.

The number of bacteria, hydrocarbon-tolerant bacteria and fungi significantly decreased with depth. This may be due to the upper horizon containing better conditions for microbial growth due to oxygen diffusion and warming from solar radiation (Shimp *et al*., 1993).

Hydrocarbon-tolerant bacteria were the only type of microorganism affected by environmental conditions. They were inversely related to moisture content and were also found in significantly lower levels in submerged microcosms. All biochemical processes require water. For microorganisms, water provides the essential medium for colony formation and is a primary ingredient in a variety of cell processes such as hydrolysis and hydroxylation (Tate, 1995). In soil systems moisture content is an important factor for microbial activity generally increasing up to 60% (Hutchinson *et al*., 2003). When the

sediment is very saturated the transfer of oxygen and other nutrients can be inhibited. In this study, hydrocarbon-tolerant bacterial numbers were seen to increase at lower levels of moisture content. This could be due to increased availability of oxygen especially when the sediments were exposed. However, there were also lower levels of DRO with low moisture content which could perhaps influence bacterial numbers due to decreased toxicity.

5.3.4 Microbial numbers in the dismantled microcosms

After 17 months there was no significant difference between microbial numbers found in the densely root-filled edge site samples and the sparsely rooted samples taken from the middle of the microcosm suggesting that there had only been a limited rhizobial effect in the planted microcosm. When comparing the number of microbes in the planted microcosm and the unplanted microcosm there was no significant difference except for the number of fungi, which were higher in the planted microcosm. Extracting and diluting fungi from sediment samples for enumeration can cause them to produce a number of spores and fragment hyphae, giving an inaccurate result so this needs to be taken into account (Curl & Truelove, 1986). Another explanation could be that the fungi have attached themselves to the plant roots to take advantage of the exudates and increased oxygen in the rhizosphere. The number of hydrocarbon-tolerant bacteria was also higher in the planted microcosm at all but the deepest sample depths of 60 cm, the site furthest away from the surface and root mass indicating that these bacteria had increased in areas with dense roots and suggesting that there was a rhizobial effect on some of the microorganisms.

Although overall there was a lower total area and UCM area seen on the unplanted GC-FID chromatograms, the planted sites appeared to have had a bigger effect on breaking down hydrocarbon chain lengths. This is shown in the planted microcosm by the start of the UCM, the maximum UCM height and the two clearly defined resolved peaks all having lower carbon numbers than those seen in the unplanted microcosm.

This could be due to the *Pseudomonas* spp. found in the isolated planted sediment samples. *Pseudomonas* spp. are the best known bacteria capable of utilizing hydrocarbons as carbon and energy sources by producing biosurfactants. Biosurfactants are a heterogeneous group of surface-active chemical compounds produced by a wide variety of micro-organisms that enhance solubilization and bioavailability and in turn, the removal of contaminants. The biosurfactants produced in *Pseudomonas* spp. are called rhamnolipids. Rhamnolipids cause greater dispersion of water-insoluble n-alkanes in the aqueous phase due to their amphipathic properties and the molecules consist of hydrophilic and hydrophobic moieties, which reduce the interfacial tension of oil-water systems. This results in higher interaction of cells with solubilized hydrocarbon droplets much smaller than the cells and rapid uptake of hydrocarbon into the cells (Das & Chandran, 2011).

5.3.5 Bacterial isolates in the microcosms

A higher range and variety of culturable bacterial isolates were found in both of the planted microcosms compared to the unplanted microcosm. This indicated that *P. australis* had a positive effect on the diversity of bacteria found in the microcosms.

Out of the 64 different bacterial isolates, 30% were facultative anaerobes able to survive in both aerobic conditions near the sediment surface and the rhizosphere and also in areas that are likely to be anaerobic (e.g. the deeper sediment depth, areas of fine sediment particle size such as clay or areas with waterlogged airspaces). Both aerobic and anaerobic bacteria have been shown to degrade hydrocarbons although the predominant type are aerobic (Ward & Brock, 1978). Although the overall number of culturable facultative anaerobes found was very similar in each microcosm, there was a much higher diversity in the planted microcosm, PL2 indicating again that the addition of *P. australis* increased bacterial diversity.

Gram positive rods made up about 60% of the distinct bacterial isolates and about 1/3 produced endospores, enabling them to survive during extreme environmental conditions such as very high contamination levels encountered at Horsea lagoon. Although sediments are thought to be colonised by a predominately Gram negative microbial community, this view is changing with the more frequent use of molecular tools (Cattelan *et al.,* 1998). There were similar numbers as well as a similar diversity of endospore-producing bacterial isolates in the planted and unplanted microcosms indicating that the addition of *P. australis* had no effect on this category. Overall, no identification method could identify all the hydrocarbon-degrading bacterial isolates so further analysis needs to be done on the remaining unidentified isolates.

5.3.6 Hydrocarbon-degrading bacterial isolates in the microcosms

Hydrocarbon-degrading bacteria can be found in virtually all soils (Singer *et al.*, 2004) and they can adapt to degrade diesel (Wang *et al*., 2010). Communities exposed to hydrocarbons adapt through selective enrichment and genetic changes resulting in increased proportions of hydrocarbon-degrading bacteria and bacterial plasmids encoding hydrocarbon catabolic genes. In unpolluted environments, hydrocarbon degraders generally constitute less than 1% of the microbial community, whereas in hydrocarbon polluted ecosystems hydrocarbon-degraders often represent 1-10% of microorganisms (Atlas, 1981). In this study 30% of bacterial isolates were found to be capable of degrading DRO according to the colorimetric plate test. *P. australis* was shown to increase the diversity of the culturable hydrocarbon-degrading bacterial isolates in the planted microcosms and this in turn could increase the rate of remediation. There were three times as many different culturable hydrocarbon-degrading bacterial isolates uniquely found in the planted microcosms compared to UP3. PL1 also had the most culturable hydrocarbon-degrading bacterial isolates as well as the highest diversity of different culturable

hydrocarbon-degrading bacterial isolates out of all the microcosms, and these were found predominantly in the upper soil horizon.

The Biolog system was able to identify almost as many culturable hydrocarbon-degrading bacterial isolates as the molecular techniques (43% and 57% respectively). However the Biolog system is based on the ability of the bacterial isolate to oxidize 95 different carbon sources and is limited by the range of bacterial species in the database. Molecular techniques are based on extracted DNA and have a much larger database that is constantly updated and is therefore more reliable.

Four of the different culturable hydrocarbon-degrading bacterial isolates from the microcosms were identified as *Pseudomonas* spp. Two different hydrocarbon-degrading bacterial isolates were found to be *Pseudomonas stutzeri* and one was found to be *Pseudomonas asplenii.* Bacteria of the genus *Pseudomonas* are widely distributed in soil and water (Rossello-Mora *et al.,* 1994) and have been shown to degrade a variety of hydrocarbons including phenanthrene (Zhao *et al.,* 2009), naphthalene (Bosch *et al.,* 1999), toluene (Arenghi *et al.,* 2001), quinoline (Shukla, 1989), fluoranthene, pyrene (Ben Said *et al*., 2008) ethylbenzene, o-xylene, n-tetradecane, octanol, decanol (Ghazali *et al.,* 2004) as well as polychlorinated biphenyls (Ahamad & Kunhi, 1996; Tandlich *et al.,* 2001). A study by Rahman *et al.* (2002) showed that *P. stutzeri* degraded 1% Bombay High crude oil by 66% in 5 days and was found to be the best degraders out of the microorganisms tested. *Pseudomonas asplenii,* also found in this experiment has been isolated from PAH-contaminated soils before (Reed & Glick, 2005) and also been shown to degrade phenol (Fuji *et al*., 2000*).*

Two of the different culturable hydrocarbon-degrading bacterial isolates from the microcosms were identified as *Bacillus* spp. Bacteria of the genus *Bacillus* spp. are primarily found in soil and are able to produce spores that are resistant to heat and

desiccation and capable of remaining dormant for long periods (Madigan *et al.,* 2008). Bacillus have been found to degrade a variety of hydrocarbons including a 59% degradation of both a 1% Bombay High crude oil (Rahman *et al*., 2002) and oily sludge components of chain length C_{12}–C_{30} and aromatics (Verma *et al.,* 2006). Diesel-degrading *Bacillus* spp. have been isolated from soil samples collected from the Ilaje coastal area, Nigeria (Boboye *et al.,* 2010) and *B. cereus* was found to degrade 5% diesel in soil (Nwaogu *et al.,* 2008) and both 500 ppm and 1000 ppm by 87% and, 80% respectively when isolated from oil refinery field soil samples in Tehran, Iran (Yousefi *et al.,* 2009). *Bacillus cereus* is a known pyrene degrader (Kazunga & Aitken, 2000).

Rhizobium spp. was only found in the planted microcosm PL2. *Rhizobium* spp. infects legume roots and forms nodules which fix atmospheric nitrogen (Atlas, 1984). They have been isolated from heavily PAH contaminated soil (Vinas *et al.,* 2005) in previous studies. *P. australis* is not a legume so they are likely to have come from sediment attached to the *P. australis* or Horsea lagoon sediment during microcosm set up.

The remaining different hydrocarbon-degrading bacterial isolates that were identified were also found to be known hydrocarbon-degraders. *Gordonia rubripertincta* has been shown to degrade hydrocarbons (Pizzul *et al.,* 2006) such as n-alkanes (Lo Piccolo *et al.,* 2011), *Acidovorax sp.* has been shown to degrade phenanthrene (Singleton *et al.,* 2009), *Exiguobacterium sp.* can degrade diesel range n-alkanes (Mohanty & Mukherji, 2008), *Mycobacterium frederiksbergense* can degrade polycyclic aromatic hydrocarbons (Willumsen *et al.,* 2001) and *Trichococcus flocculiformis* can degrade monometric and diametric carbon sources (Scheff *et al.,* 1984).

5.4 Summary

- Both planted microcosms grew well.

- The concentration of DRO in the sediment significantly decreased over time with the highest rate of decline being in the planted microcosm, PL2.

- The DRO concentration in the sediment significantly increased as the moisture content increased and in submerged microcosms.

- There was no difference in microbial numbers between the planted and unplanted microcosms.

- The number of hydrocarbon-tolerant bacteria significantly increased with DRO concentration and significantly decreased with moisture content and depth.

- The GC-FID chromatograms showed a symmetrical shape between C_{10}-C_{12} to C_{25} centred around $C_{17\text{-}18}$.

- Qualitative analysis showed the planted sites appeared to have had a bigger effect on breaking down hydrocarbon chain lengths.

- The number of hydrocarbon-tolerant bacteria and of hydrocarbon-tolerant fungi significantly increased over time in the microcosms.

- A total of 64 different bacterial groups were isolated from the microcosms based on their morphology.

- The majority of the bacterial isolates found were Gram positive rods.

- There was an increased incidence and diversity of bacterial isolates in the planted microcosms.

- A novel colorimetric media for determining hydrocarbon-degrading potential was developed and shown to identify known hydrocarbon-degrading bacteria.

- There were three times as many hydrocarbon-degrading bacterial isolates in the planted microcosms compared with the unplanted microcosm.

- The most common hydrocarbon-degrading bacterial genus found in the microcosms was *Pseudomonas* spp.

6. HORSEA LAGOON STUDY

6.1 Introduction

Although phytoremediation of hydrocarbon-contaminated sediments has been studied extensively in laboratory and microcosm demonstrations with encouraging findings (Euliss *et al.*, 2008; Palmroth *et al.,* 2002; Paquin *et al.,* 2002), only a few field-scale applications have been tested (EPA, 2001). In 1997, a phytoremediation study of water-saturated sediments contaminated with hydrocarbons from a vehicle wash facility was carried out in Fort Riley, Kansas, USA. This involved removing the contaminated sediment, homogenising it and then spreading it as a 30 cm deep layer over mowed grassland. Fertiliser was then added to the field along with a grass mixture, a legume mixture and one area was kept unvegetated as a control. Initial motor oil range total petroleum hydrocarbon (TPH) concentrations found in the sediment were 904 mg/kg, 712 mg/kg and 846 mg/kg respectively (Karthikeyan *et al*., 2000). After 24 months the TPH had decreased by 76% in the grass mixture, 59% in the legume mixture and 73% in the unplanted site with no significant differences between the treatments. The lack of clear evidence of the effects of the plants was suggested as being due to low initial hydrocarbon conditions and that treatment differences were more likely to be observed in sediments with higher initial contamination levels.

All of the full scale experiments involving hydrocarbon contaminated sediments to date have included removing and homogenising contaminated sediment before the initiation of the study (King *et al.,* 2006; Vervaeke *et al*., 2003), meaning the next phase in advancing acceptance of phytoremediation as a regulatory alternative must be to demonstrate sustained contaminant removal *in situ* (Williams, 2002), with non homogenised sediment and a high initial TPH concentration.

Horsea lagoon was chosen as an on-site study location due to the high levels of hydrocarbon contamination acquired from the run-off from a FTF. A site assessment was carried out to determine the most suitable location in the lagoon to be planted and whether the lagoon water was of a suitable pH and salinity to allow plant growth. Sediment particle size was measured and air temperature and humidity was also monitored to determine whether environmental factors might affect sample results.

Sediment cores were taken from eight positions on the experimental site and from four different depths approximately bi-monthly from April 2009 until July 2010 to determine whether the addition of plants reduced DRO concentration compared to paired unplanted control sites. The planted sites PL1 to PL4, descend down the bank, with site PL1 furthest from the water's edge and site PL4 in the water. Sites UP1 to UP4 are the paired unplanted controls. Sediment sample chromatograms were qualitatively analysed to determine whether there were any shifts in pattern over time and difference between planted and unplanted sites.

From January 2010 these cores were also analysed for microbial numbers using the MPN method to determine whether the addition of plants increased the number of microbes. In March 2010, bacteria were cultured from MPN plate counts and isolated into pure cultures and then held as stock cultures at 4˚C. Subsequently they were Gram stained and assessed for their capacity to grow aerobically and anaerobically. Bacteria found to be Gram positive rods were investigated for endospore production and bacteria found to be Gram negative rods were tested using the API method of identification to help identify the bacterial characteristics of the microbial populations. All of the isolated bacteria were grouped and tested for hydrocarbon-degrading potential to discover where they are found, what they are and whether the addition of plants has had any effect. All bacteria found to be hydrocarbon-degraders were analysed using Biolog and molecular techniques for species identification.

6.2 Results

6.2.1 Plant establishment

The plants grew well during the first season (Plate 18a) however, the lagoon suffered damage from rabbits during season two (Plate 18b) as well as extreme flooding (Plate 19) during October 2009 which damaged some plants and stunted growth. A rabbit exclusion fence was constructed in May 2010.

Plate 18. *P. australis* **(a) growing well in Horsea lagoon July 2009 (b) and showing stunted flat tops caused by rabbits in May 2010, H. Pinchin**

6.2.2 Horsea lagoon DRO concentrations

The mean DRO concentrations at each site over time are shown in Figure 16. All sites were highly contaminated with visible black deposits of hydrocarbons and a noxious smell, mean values by sampling occasion and site ranged from 1,292 to 145,365 mg/kg DRO DW. The sites furthest down the bank (PL4 and UP4) had the highest concentrations and these were almost an order of magnitude higher than the other sites. Generally there was a great deal of variation between sampling occasions at each site, indicating that "hot spots" of contamination existed over small distances in the sediment.
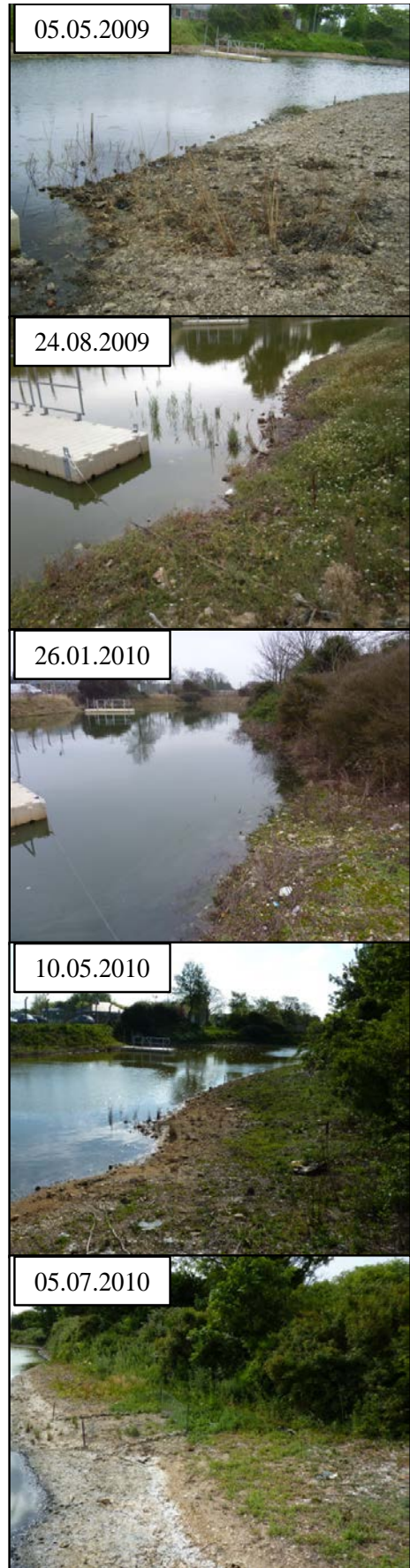
Plate 19. Horsea lagoon plant growth during the phytoremediation study showing the high water levels in October 2009, H. Pinchin
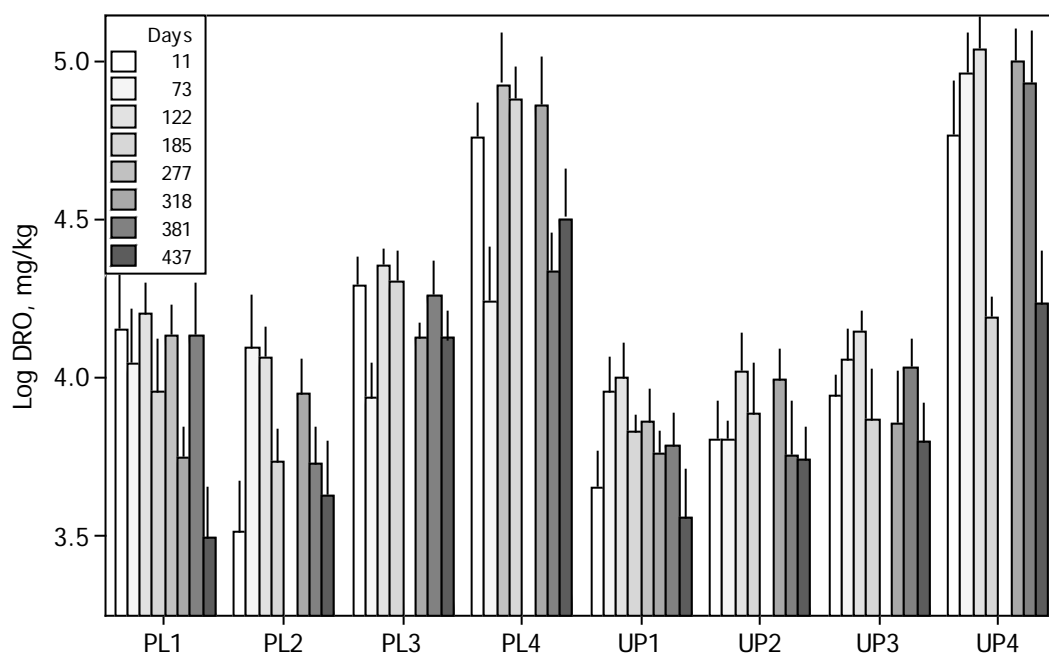
Figure 16. Mean Log DRO at each site over time (SEM shown by error bars)

All sites had lower DRO concentrations at the end of the study compared to the start. However PL1 was the only site to show a significant decrease (r=-0.455, p=0.009). An overall comparison of planted and unplanted sites showed no significant difference in DRO concentration (ANOVA F=0.70, p=0.404): DRO decreased by 56% in the unplanted sites and 43% in the planted sites, although the heterogeneous nature of the DRO in the sediment makes establishing a baseline for each site difficult. Even paired comparison of planted and unplanted sites at the same distance down the bank (e.g. PL1 and UP1, PL2 and UP2) gave different results with either the planted or unplanted being higher or no difference being seen.

6.2.3 GC-FID chromatograms from Horsea lagoon

The chromatograms from day 0 for planted sites PL1 and PL4 and unplanted sites UP1 and UP4 (see appendix and table 16) showed the composition of residual hydrocarbons to be in the $C_{12\text{-}16}$ to C_{25} DRO range with a symmetric distribution centred at $C_{19\text{-}20}$ and a high proportion of UCM.

Table 16. Summary of GC-FID chromatogram data analysis from planted (PL) and unplanted (UP) sites at Horsea lagoon.

Day	Site	Depth	Total area (pA^2)	UCM area (%)	Maximum UCM height (pA)	Carbon number at UCM peak	Start of UCM
0	PL1	0-2	939	50	25	C20	C13
0	PL1	2-7	296	97	10	C19	C12
0	PL1	7-12	187	51	5.5	C19	C13
0	PL1	12-17	190	86	6	C19	C13
0	PL4	0-2	1148	96	34	C19	C13
0	PL4	2-7	1821	96	51	C19	C12
0	PL4	7-12	815	91	23	C19	C12
0	PL4	12-17	878	92	24	C19	C12
437	PL1	0-2	453	42	6.5	C19	C13
437	PL1	2-7	184	37	1.7	C19	C13
437	PL1	7-12	114	48	1.5	C19	C13
437	PL1	12-17	75	97	0.6	C19	C13
437	PL4	0-2	960	33	13	C18	C11
437	PL4	2-7	643	98	7	C18	C12
437	PL4	7-12	2381	53	32	C18	C11
437	PL4	12-17	1670	79	22	C18	C10
0	UP1	0-2	213	96	7	C20	C14
0	UP1	2-7	120	97	4	C20	C14
0	UP1	7-12	69	99	2.5	C20	C16
0	UP1	12-17	103	97	3.5	C20	C14
0	UP4	0-2	452	97	14	C19	C13
0	UP4	2-7	1467	44	38	C19	C12
0	UP4	7-12	1446	99	38	C19	C12
0	UP4	12-17	1539	84	48	C20	C13
437	UP1	0-2	291	99	3.7	C18	C13
437	UP1	2-7	368	100	3.8	C18	C13
437	UP1	7-12	274	100	2.4	C18	C13
437	UP1	12-17	91	98	0.5	C18	No distinct hump
437	UP4	0-2	537	61	7	C18	C13
437	UP4	2-7	1995	80	24	C18	C11
437	UP4	7-12	770	73	8	C17	C11
437	UP4	12-17	495	99	6.5	C17	C11

However, the chromatograms from day 437 for planted sites PL1 and PL4 and unplanted

sites UP1 and UP4 showed the composition of residual hydrocarbons to be in the $C_{10\text{-}13}$ to

C_{25} DRO range with a symmetric distribution centred at C_{17-19} and a high proportion of Unresolved Complex Mixture (UCM) (Figure 17).
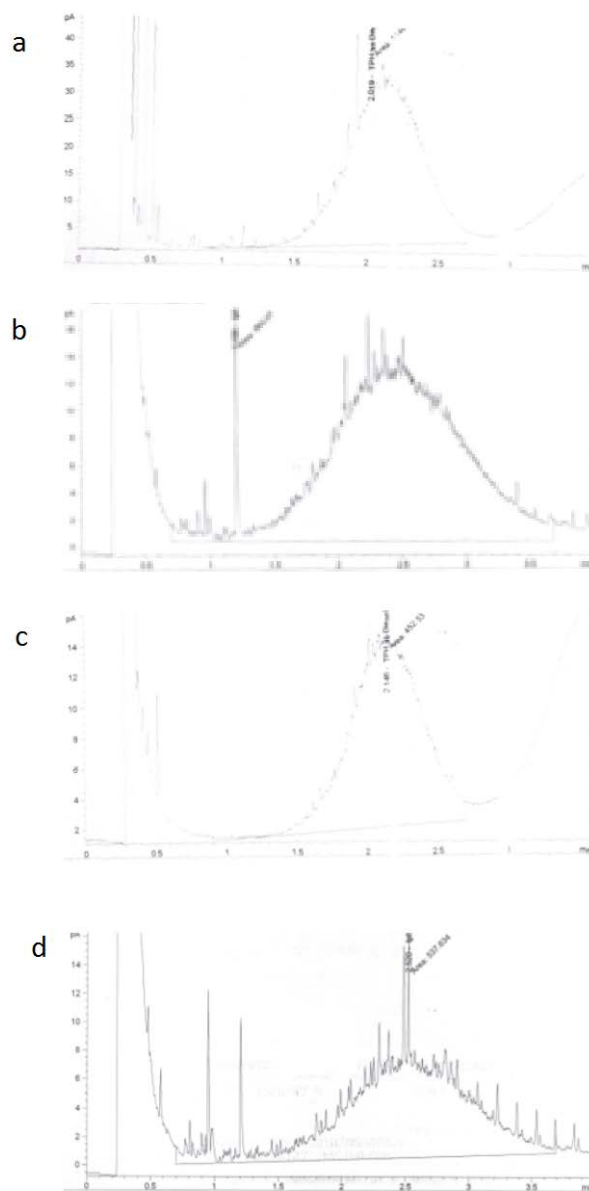

Figure 17. GC-FID chromatograms for Horsea lagoon 0-2 cm PL4 day 0 (a) day 437 (b) and UP4 day 0 (c) and day 437 (d)

Overall, the unplanted sites UP1 and UP4 decreased equally in total area by 11% and in UCM area by 11% however, in the planted sites PL1 and PL4 the UCM area decreased more than the total area over time (12% and 3% respectively).

Over time the maximum height of the UCM decreased at every site with a similar decline in the planted (53%) and unplanted (64%) sites and when looking at the sites separately, the largest UCM height decrease over time was in UP4 (67%). There was also a clear shift in the maximum UCM height carbon numbers in all of the sites over time. The sites furthest down the bank, PL4 and UP4, had a greater decrease in UCM area when compared to the sites at the top of the bank, PL1 and UP1.

The chromatograms all showed a resolved peak before the start of the UCM hump at $C_{10\text{-}11}$ and the two highest resolved peaks present within the UCM were at C_{17} and C_{18} on day 0 and $C_{16\text{-}18}$ and $C_{17\text{-}18}$ on day 437 in the planted sites and at $C_{17\text{-}21}$ on day 0 and $C_{15\text{-}18}$ on day 437 in the unplanted sites indicating a shift in the UCM hump. This shift is also clearly seen in all the sites in the start of the UCM hump from day 0 to day 437 in all of the sites; PL1 shifted from C_{12}-C_{13} to C_{13}, PL4 shifted from C_{12}-C_{13} to $C_{10\text{-}12}$, UP1 shifted from C_{14}-C_{16} to C_{13} and UP4 shifted from C_{12}-C_{13} to C_{11}-C_{13}.

6.2.4 Factors affecting DRO concentration in Horsea lagoon

The DRO concentration in the sediment significantly increased with sediment depth (r=0.233, p=0.001) (Figure 18 and Figure 19) increased moisture content (r=0.549, p=0.000) and when the sites were submerged (ANOVA, F=60.19, p=0.000). Moisture content was shown to increase with sediment depth (r=-0.176, p=0.009) and followed a similar pattern to DRO concentration with the highest values seen further down the bank (Figure 20).
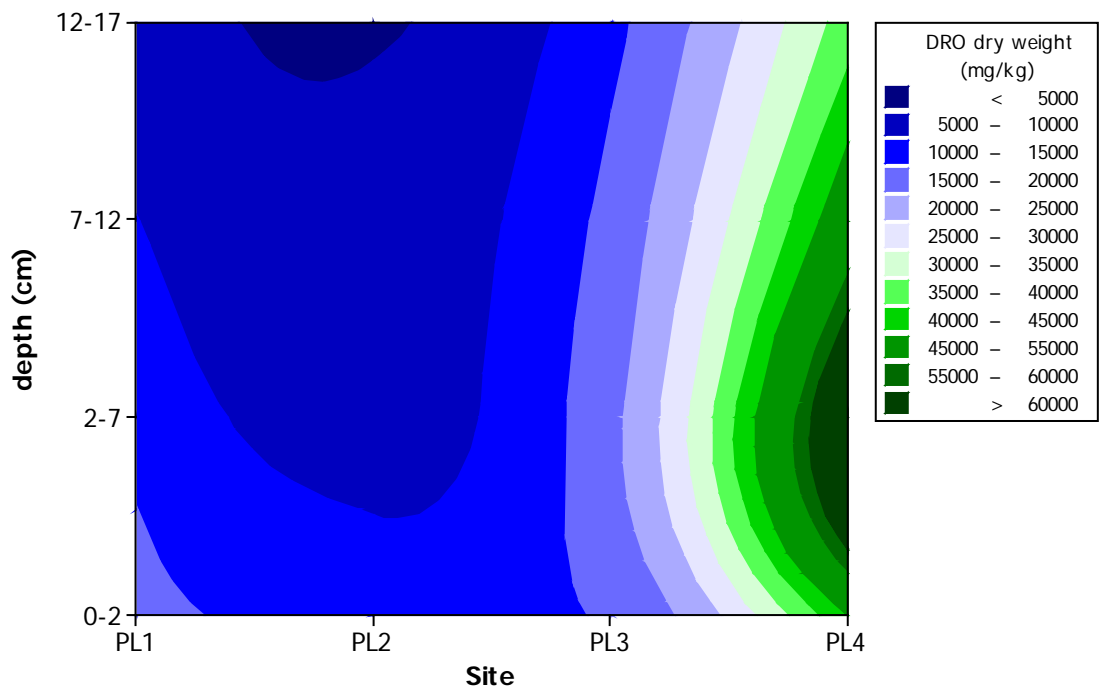
Figure 18. The DRO concentration at different sediment depths in the four planted sites at Horsea lagoon
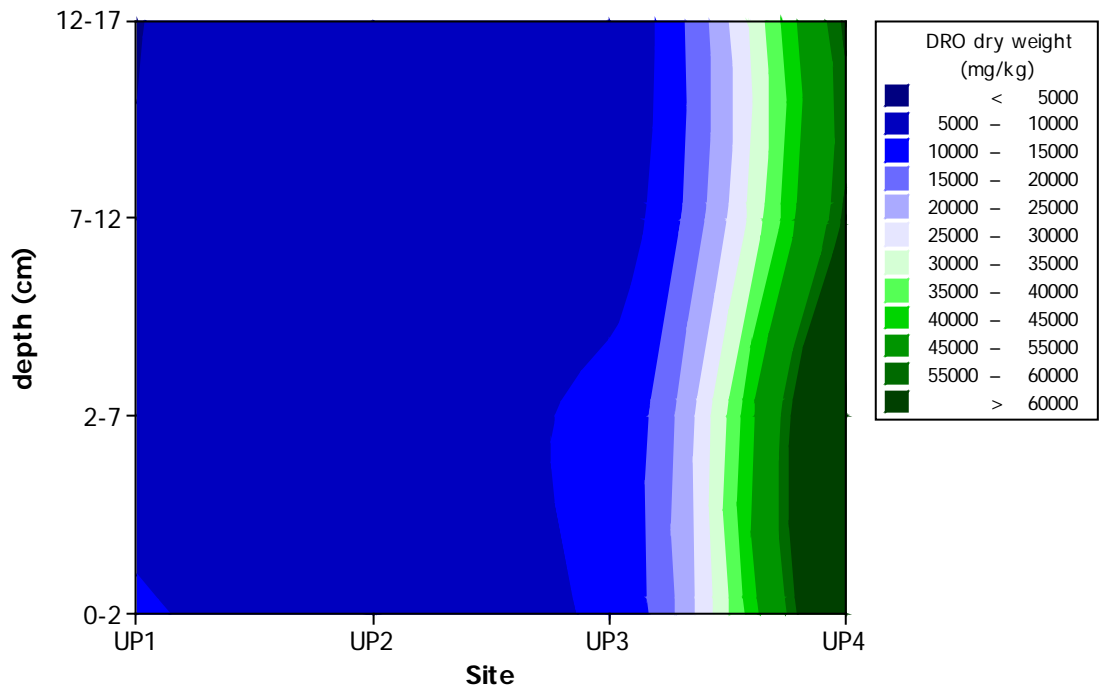

Figure 19. The DRO concentration at different sediment depths in the four unplanted sites at Horsea lagoon

Sites PL1 and UP1, the two sites furthest up the lagoon bank, were submerged 25% of the time and sites PL3 and UP3, the two sites midway up the lagoon bank, were submerged for about half of the time (43%) both showed no significant difference between DRO concentrations and whether the site was submerged or not. However, sites PL4 and UP4, the two sites furthest down the lagoon bank, were submerged for the most amount of time (86%) and showed a significant increase in DRO when the sites were submerged (ANOVA F=6.37, p=0.015).
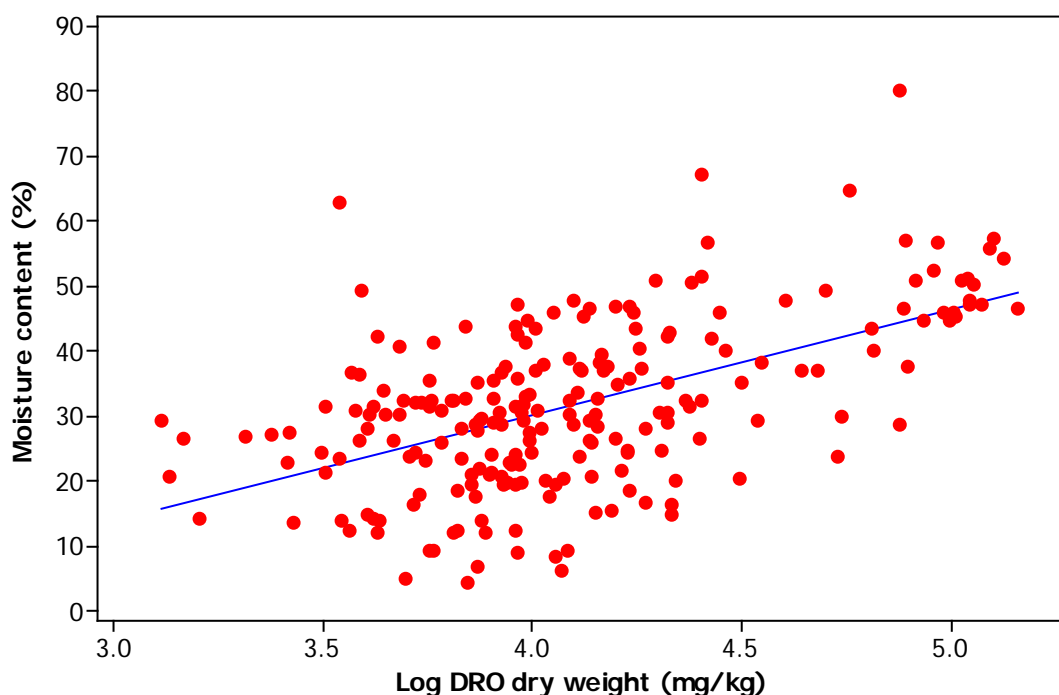

Figure 20. DRO concentration (mg/kg) verses moisture content (%) in Horsea lagoon

The pH of the sediment samples was generally alkaline, with mean values at each site ranging from 7.8 to 8.5 (Figure 21). The pH tended to increase down the bank as sample sites become more waterlogged giving significantly different pH values at the various sites (ANOVA F=11.24, p=0.000).
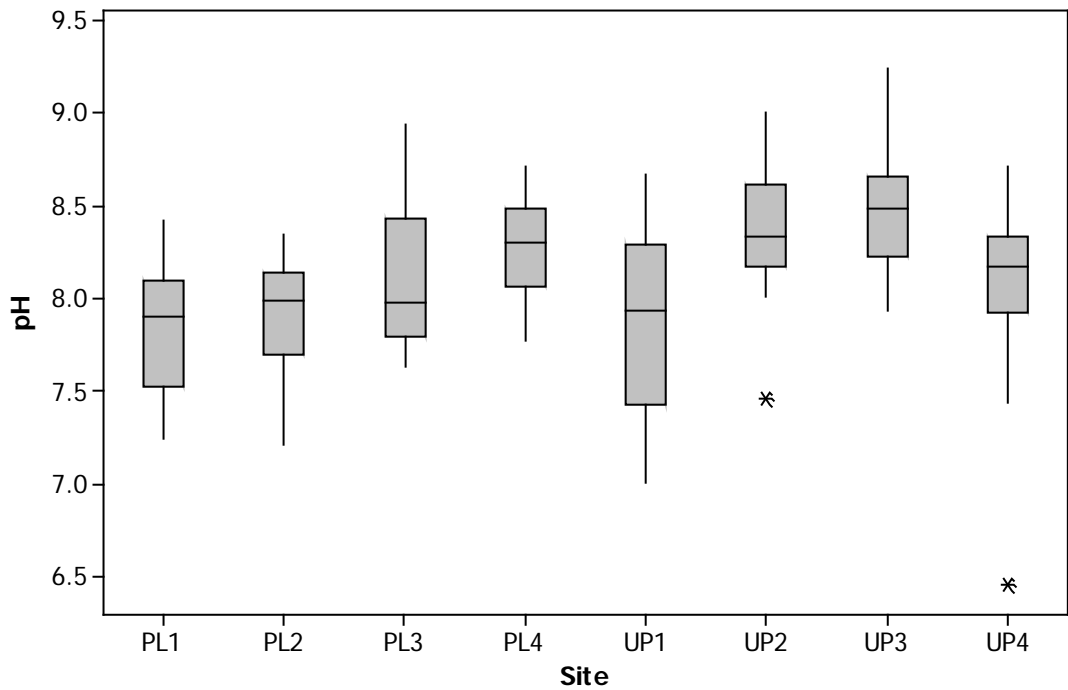
Figure 21. The pH of the sediment at each sampling site at Horsea lagoon. The boxes represent the 1st and 3rd quartiles (the interquartile range) with the median shown as a horizontal line within the box. The extreme values (1.5 times the interquartile range (IQR) from the upper or lower quartile) are the ends of the lines extending from the IQR

The mean air temperature on sampling days is shown in Figure 22 reflecting typical values of the temperature climate in Southern England. There was a range between 2.2°C in January 2010 to 19.7° C in July 2010.
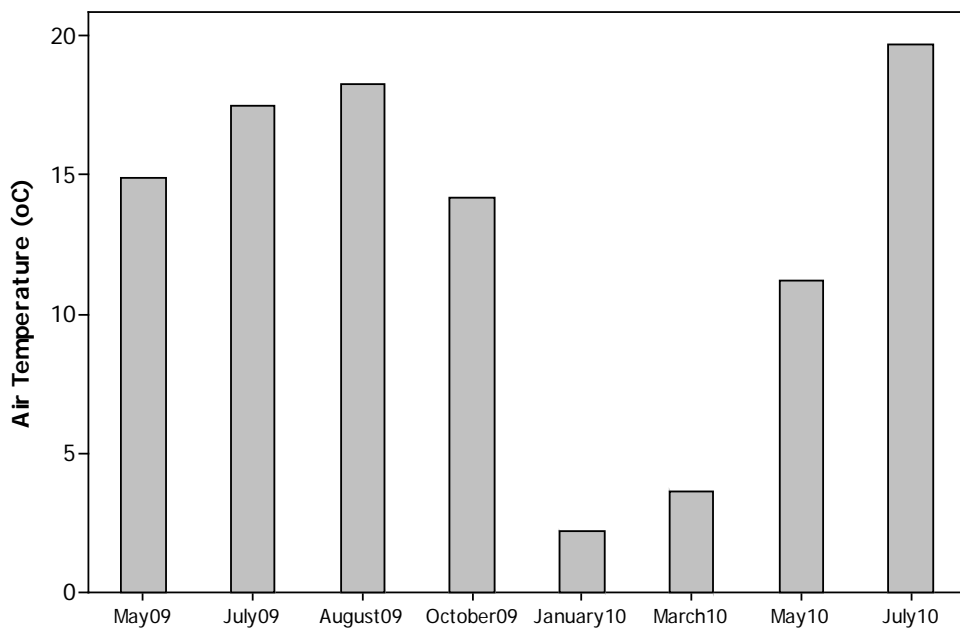

Figure 22. The air temperature at each sampling occasion

6.2.5 Number of culturable microorganisms

6.2.5.1 Microbial counts in Horsea lagoon

Horsea lagoon was sampled bi-monthly from March 2010 to July 2010. The number of total culturable bacteria, culturable hydrocarbon-tolerant bacteria, total culturable fungi and culturable hydrocarbon-tolerant fungi were determined using the MPN enumeration technique. The results are shown in Figure 23.

Different patterns were seen in the planted and unplanted sites. In the planted sites the total culturable bacteria significantly increased down the bank (r=0.336, p=0.022) corresponding to the higher levels of DRO and moisture content. The total culturable fungi numbers and hydrocarbon-tolerant bacteria also increased down the bank, however total culturable hydrocarbon-tolerant fungi counts showed variations between sites in the planted sites. The unplanted sites showed a mixed response with a small increase in total culturable bacteria but general decreases in the other microorganisms down the bank.

There were significantly higher counts of total culturable hydrocarbon-tolerant fungi in the unplanted sites compared to the planted sites (ANOVA F=6.70, p=0.011) however, there were no significant differences between any of the other microorganisms. On the last sample, the number of culturable hydrocarbon-tolerant bacteria had significantly decreased (r=-0.601, p=0.000). This was the case in every individual site (Figure 24) but this could be influenced by the dry conditions on the last sampling occasion.
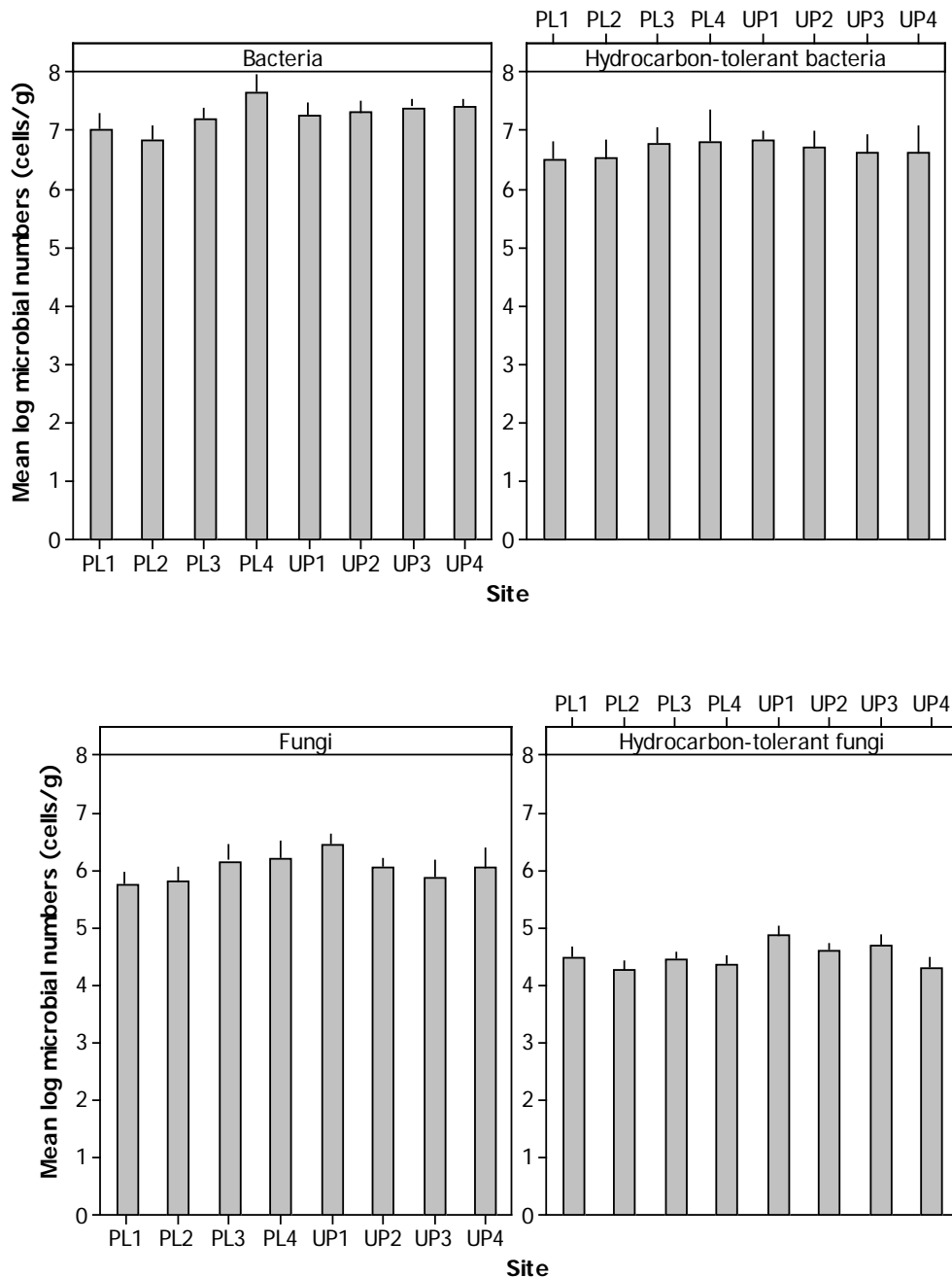
Figure 23. Mean microbial numbers at each site in the lagoon (SEM shown by error bars)

The number of total culturable bacteria and culturable hydrocarbon-tolerant bacteria significantly increased with DRO concentrations (r=0.267, p=0.010 and r=0.280, p=0.007 respectively). Culturable hydrocarbon-tolerant bacteria also significantly increased with moisture content (r=0.365, p=0.000) (Figure 25) whereas the number of culturable hydrocarbon-tolerant fungi significantly decreased (r=-0.320, p=0.002)
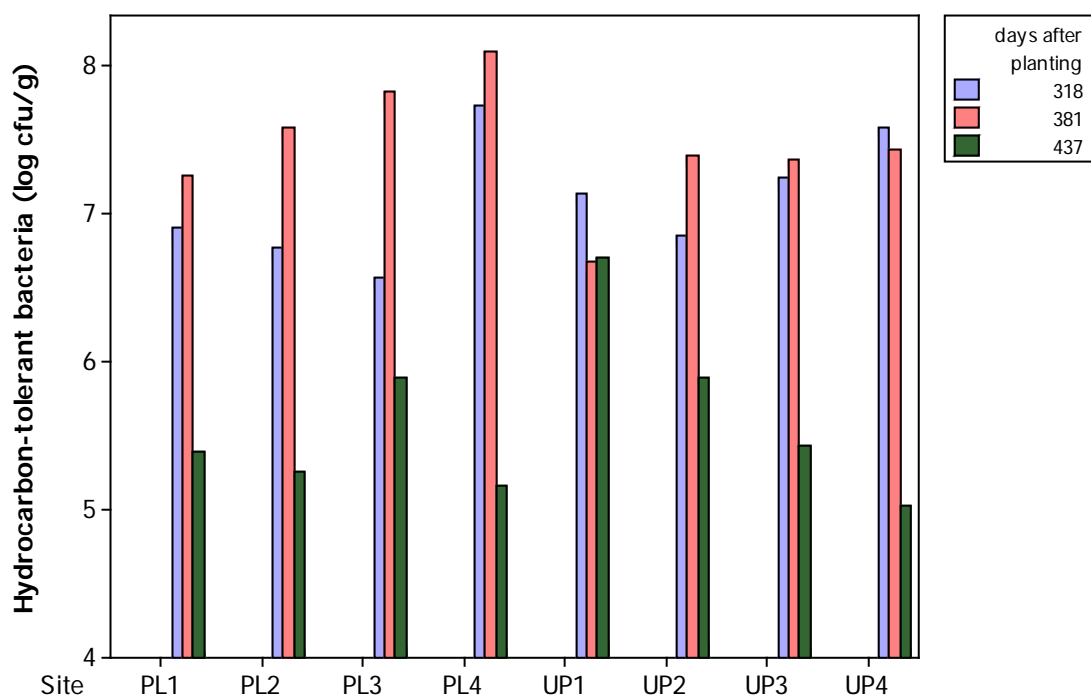
Figure 24. The Log number of culturable hydrocarbon-tolerant bacteria at each site on each sampling occasion

6.2.5.2 Culturable bacterial isolates

In March 2010, 504 cultures of bacteria were isolated from the Horsea lagoon MPN enumeration plate counts. After Gram staining all the isolates and endospore staining the positive rods, 157 visually different bacterial isolates were identified and given an identifying code letter and number.

Of the 504 total bacteria isolates, 62 were found in PL1, 72 in PL2, 65 in PL3, 45 in PL4, 73 in UP1, 64 in UP2, 60 in UP3 and 63 in UP4. Both the planted and unplanted sites had similar numbers of total bacterial isolates and no correlation with depth (Table 17 and Table 18).
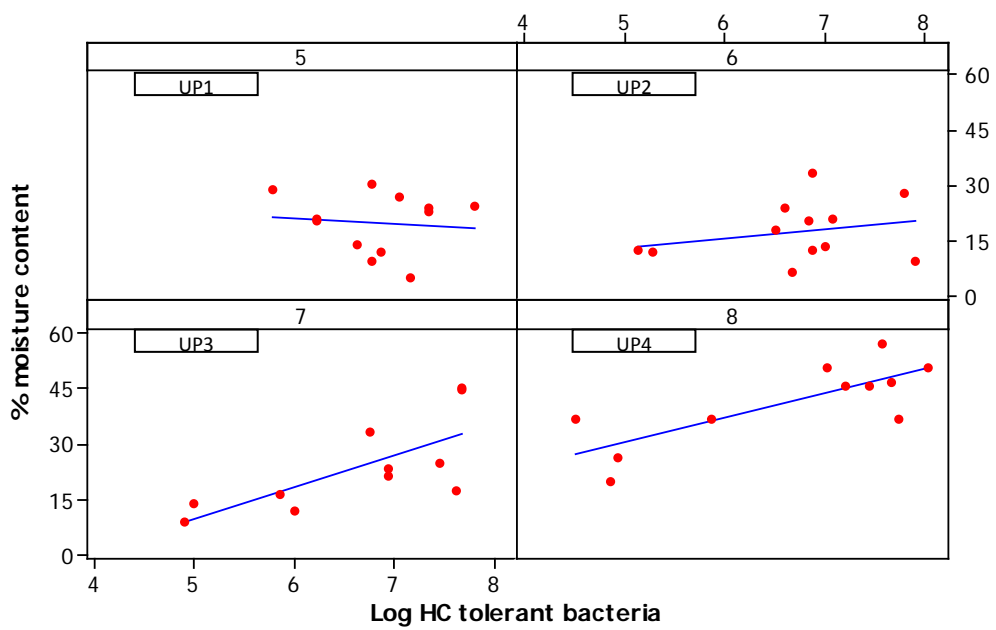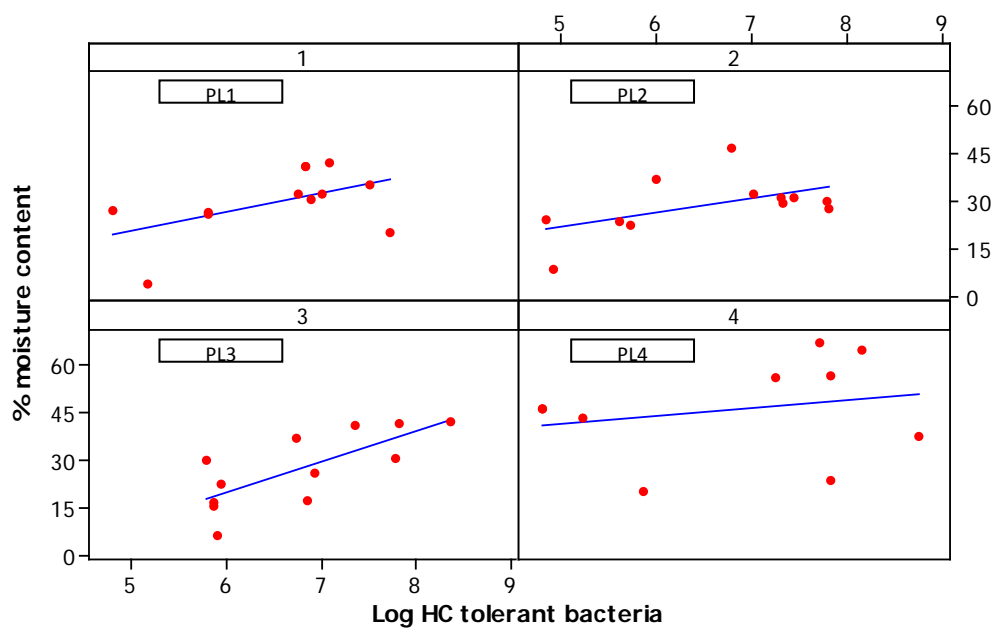
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Figure 25. Number of culturable hydrocarbon-tolerant bacteria with different sediment moisture contents

Table 17. The occurrence of different bacterial isolates in Horsea lagoon sediment sections in planted sites

Table 18.The occurrence of different bacterial isolates in Horsea lagoon sediment sections in unplanted sites

Looking at the diversity of these bacterial isolates, out of the 157 different bacterial isolates, 50 grew exclusively in the planted sites and 55 grew exclusively in the unplanted sites. When the sites were looked at individually, the number of different bacterial isolates was very similar in all the sites indicating that the addition of *P. australis* had no influence on either the total number of bacterial isolates or the diversity.

Thirty two out of the 157 bacterial isolates were facultative anaerobes. The overall number of culturable facultative anaerobes found was very similar in all the sites (PL1=10, PL2=12, PL3=7, UP2=11, UP3=11, UP4=10) except for PL4 (n=18) and UP1 (n=25) where the number was higher. There was a no significant difference in the diversity of culturable facultative anaerobes between the planted sites (n=29) and the unplanted sites (n=32).

There were 86 Gram positive rods out of the 157 different culturable bacterial isolates, 11 of which formed endospores. The overall number of total culturable endospore-producing bacterial isolates was very similar in all the sites (PL1=3, PL2=5, PL3=7, PL4=3, UP1= 7, UP2=9, UP3=6, UP4=3) as was the number of different culturable endospore producing bacterial isolates although the diversity was slightly higher UP1 (n=4) and UP2 (n=5)

The majority of bacterial isolates were found to be Gram positive rods. However, Gram negative culturable bacterial isolate X8 was the most abundant being found a total of 46 times across all the sampling sites.

6.2.5.3 Activity of culturable hydrocarbon-degrading bacteria

The 157 morphologically different bacterial isolates were tested for their ability to degrade hydrocarbons. A small colony was streaked onto an agar plate containing hydrocarbon-degrading indicator agar with and without the addition of 10 mL/L diesel as the sole carbon source and incubated for five days at 25°C. The bacterial isolates that grew pink or red on the plates were assumed to be hydrocarbon degraders (Table 19 and table 20).

Twenty seven morphologically different bacterial isolates were identified as hydrocarbon-degraders according to the colorimetric plates. Twice as many different hydrocarbon-degrading bacterial isolates were exclusively found in the planted sites (n=13) than were found exclusively in the unplanted sites (n=5). Although the highest number of hydrocarbon-degrading bacterial isolates was found in UP1 (n=17), the planted sites generally had higher numbers (PL1=16, PL2=14, PL3=16, PL4=8, UP2=10, UP3=3, UP4=10). The planted sites had a higher number of different hydrocarbon-degrading bacterial isolates (n=34) than the unplanted sites (n=24). When the sites were looked at individually the numbers were all similar to each other (PL1=9, PL2=8, PL4=6, UP1=7, UP2=9, UP4=6) except for adjacent sites PL3 (n=11) which had almost four times as many different hydrocarbon-degrading bacterial isolates as UP3 (n=3)

It was determined that 37% of the hydrocarbon-degrading bacterial isolates were facultative anaerobes. The highest number of facultative anaerobes were found in UP1 (n=12) although there were only 2 morphologically different hydrocarbon-degrading bacterial isolates present. The facultative anaerobes were evenly spread throughout the planted sites (PL1=5, PL2=7, PL3=7, PL4=5) but were concentrated in the upper layers of sediment in the unplanted sites (UP1=12, UP2=4, UP3=1, UP4=1).

Out of the 27 different hydrocarbon-degrading bacterial isolates, there were nine Gram positive rod producing hydrocarbon degraders, two of which produced endospores.

Table 19. The location and frequency of the hydrocarbon-degrading bacterial isolates in Horsea lagoon planted sites at different depth

Table 20. The location and frequency of the hydrocarbon-degrading bacterial isolates in Horsea lagoon unplanted sites at different depth

The two most abundant hydrocarbon-degrading bacterial isolates were E9 (n=29) and S8 (n=10). They were both found to be Gram positive rods but only E9 was found to be a facultative anaerobe able to survive in areas with no or little oxygen. It was found in all the sites but predominantly in the top layers of UP1.

6.2.5.4 Characterisation of the hydrocarbon-degrading bacterial isolates from Horsea lagoon

One of each of the 21 morphologically different hydrocarbon-degrading bacterial isolates were selected for genus and species identification and tested using the Biolog plate reader and molecular techniques. The four Gram negative rod bacterial isolates were also API tested. After API testing, one of the two Gram negative rod bacterial isolates were identified (Table 21). Biolog analysis identified eight hydrocarbon-degrading bacterial isolates to species level. Three of these were identified as the same bacteria, *Pseudomonas stutzeri* and one was identified as *Pseudomonas fluorescens*. Using a minimum of 97% ID and above, the molecular analysis also identified eleven hydrocarbon-degrading bacterial isolates to species level but using a range of 80-96% ID it also identified eleven hydrocarbon-degrading bacterial isolates to genus level. Five hydrocarbon-degrading bacterial isolates were not identified by any of the techniques used. Four of the eleven hydrocarbon-degrading bacterial isolates were found to be *Pseudomonas* spp. and another identified as *P. stutzeri*. Three were also identified as different *Bacillus* spp. making these two species the most commonly occurring in the Horsea lagoon samples.

6.2.5.5 SEM images

Scanning Electron Microscopy (SEM) images were taken of *P. australis* root and rhizome to determine their surface structure. The SEM images of the root and rhizome show the root has a much more textured, rough surface where as the rhizome has a smooth, ridged surface (Plate 20 and Plate 21). The rough root surface would allow for easier bacterial attachment as well as acting as a surface for fungi to coil around. These attached bacteria

and fungi would benefit from the exudates and higher oxygen concentrations found in the rhizosphere.

Table 21. Selected hydrocarbon-degrading bacterial isolates found in Horsea lagoon identified by API, Biolog and molecular methods

| Bacterial isolate	API	ID %	Biolog	ID %	Molecular	ID %
E9					*Pseudomonas* spp.	89
S8					*Stenotrophomonas terrae*	100
V8			*Rhodoccus coprophilus*	67	*Ancylobacter dichloromethanicus*	97
F3			*Pseudomonas stutzeri*	100	*Trichococcus* spp.	93
M3			*Tsukamurella inchonensis*	99	*Bacillus hwajinpoensis*	100
B4					*Paenisporosarcina quisquiliarum*	99
B5			*Cellulomonas flavigena*	100		
Q8			*Pseudomonas fluorescens biotype A*	90	*Pseudomonas* spp.	85
T4						
U4					*Exiguobacterium aestuarii*	99
D4					*Bacillus* spp.	95
E4					*Exiguobacterium* spp.	92
G4			*Pseudomonas stutzeri*	100	*Pseudomonas* spp.	95
S5						
U5					*Pseudomonas stutzeri*	99
V5					*Citricoccus* spp.	82
W5	*Pasteurella* spp.	94.2			*Paenisporosarcina macmurdoensis*	98
Y5					*Exiguobacterium aurantiacum*	98
V6					*Rhodococcus erythropolis*	98
W6						
I7			*Tsukamurella inchonensis*	100	*Bacillus cereus*	100
U8					*Pseudomonas* spp.	85
B3					*Oerskovia* spp.	82
P4						
M5					*Arthrobacter* spp.	96
N9					*Stenotrophomonas maltophilia*	99
Q3			*Pseudomonas stutzeri*	100	*Pseudomonas* spp.	92
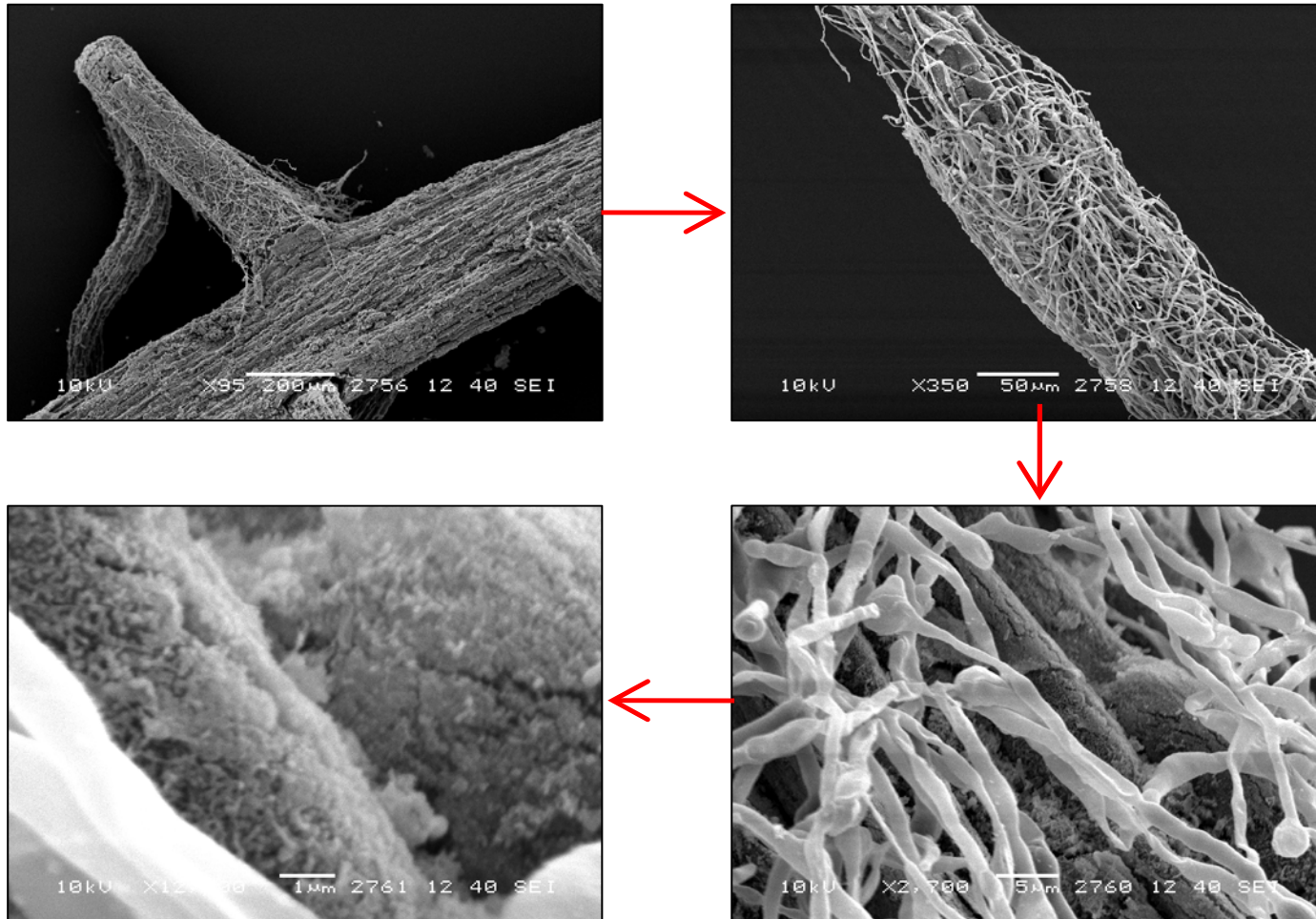
Plate 20. *P. australis* **roots surrounded by fungal hyphae under a SEM, H. Pinchin**
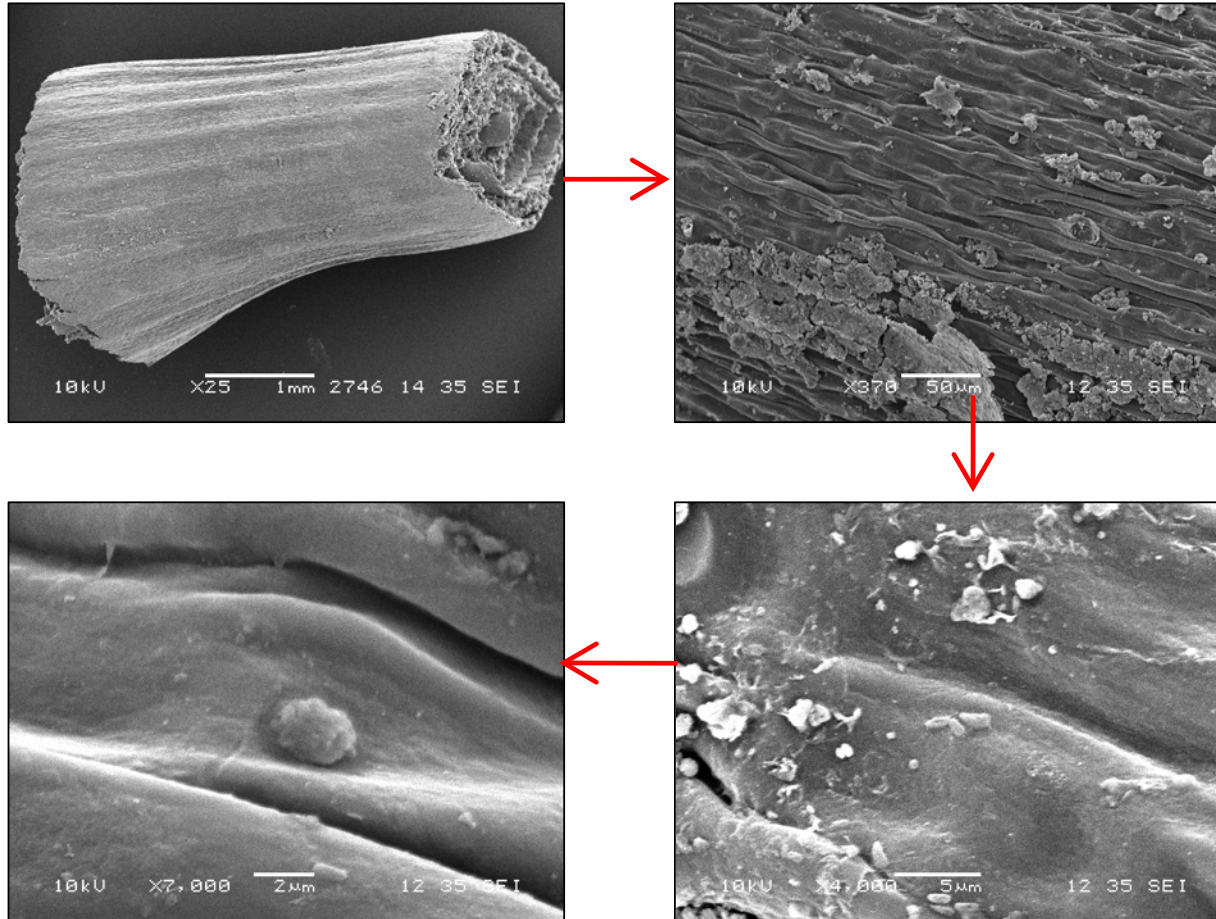
Plate 21. *P. australis* rhizome under a SEM, H. Pinchin

6.3 Discussion

6.3.1 DRO concentration

During DRO analysis of Horsea lagoon sediment, the sum of all the peaks found between C_{10}-C_{25} was generated by projecting a horizontal baseline between the retention times. It was found to be one of the most polluted sites to be assessed in phytoremediation studies reaching DRO concentrations of 145,364 mg/kg. The highest concentrations of hydrocarbon contamination reported in previous studies are 40,000 mg/kg (Shirdam *et al.*, 2005) and 147,000 mg/kg (Greenberg *et al.*, 2006).

The GC-FID chromatographs also showed a clear shift in chromatogram shape from a symmetrical hump centred at $C_{19\text{-}20}$ at day 0 to $C_{17\text{-}19}$ at day 437 and all of the sampling sites showed lower DRO concentrations at the end of the study, with an overall decrease of 56% in the unplanted sites and a 43% decrease in the planted sites. This result is not unique, as other studies into the phytoremediation of sediments contaminated with oil have also reported no significant differences in removal rates between planted sites and controls (King *et al.,* 2006; White *et al.,* 2006). King (2006) investigated homogenised, dredged TPH contaminated sediments in Warrington, UK. Sites planted with Willow, Poplar and Alder were compared to unplanted sites that were weeded and left untouched. After 32 months the overall levels of TPH had significantly decreased but there was no significant different between treatments. In Horsea lagoon, the monitoring may have disturbed the sites but also the last sampling occasion corresponded to a hot summer day and low water levels resulting in drying of the sediments which may have affected the pattern as DRO tended to decrease with moisture content. This raises a question as to whether less saturated sediments promote DRO removal processes i.e. volatilisation of lower molecular weight hydrocarbons or increased aeration increasing microbial biodegradation or if the association is an artefact of sediment deposition patterns. The lowest values at most sites were seen on the last sampling occasion when all sites were exposed to the air, suggesting

that exposure to air and low moisture content does influence DRO concentrations independently of any underlying pattern of deposition. This could be due to hydrocarbon volatilisation and the higher oxygen levels increasing microbial numbers and degradation rates.

The pollutant levels at Horsea lagoon are highly variable, even within the 1 m^2 sampling sites. Generally the unplanted sites had a lower DRO concentration than the planted sites, even at the start of the study although the chromatograms showed that UP1 and UP4 had hydrocarbons with a higher chain length than those found in PL and PL4, which makes interpretation of the treatment performance difficult. Many *ex situ* studies use homogenised samples that overcome this difficulty (King *et al*., 2006; Phillips *et al*., 2009), but this risks disturbing the sediment structure and therefore the results may not be representative of site conditions.

The varying water levels, caused by the need to gain regulatory consent for discharges, is another factor that makes interpretation of this study difficult as sites could either be submerged or exposed depending on the balance of runoff, inflow and discharge. The distribution of DRO also showed significant variation with position, with a general trend for higher pollutant levels down the bank. This may either be due to the historic pattern of sediment deposition or because these sites were submerged more often.

pH is an important factor affecting adsorption and desorption of contaminants in soils, the availability of contaminants to plant roots and the transport of contaminants into root cells (Huang & Chen, 2003). The majority of fungi and bacteria extracted from UK soil, grow in an optimum pH range of 5-9 and bacteria 6-9 (Atlas, 1984). During this study there was a significant difference between the pH at each site ranging from pH 6.5 to pH 9.2, all within this optimum microbial range. The pH of the sediment tended to increase as the sites became more waterlogged, site UP4 was the exception. The alkaline pH found in Horsea lagoon sediment, could be caused by the large quantities of chalk in the lower sediments

used to join together Great and Little Horsea to form Horsea Island in 1889. A highly alkaline sediment means nutrients are less available to the plants possibly hindering their growth.

Although biodegradation of hydrocarbons has been shown to decrease with increasing sediment depth, the relationship between plant enhanced bioremediation of hydrocarbons in sediment and depth has not been fully assessed (Keller *et al.*, 2008). In Horsea lagoon, the DRO concentration increased with depth indicating that there is possibly downward leaching of the hydrocarbons or that there is increased volatilisation in the upper horizons of the sediment due to the clay-silt layers drying out, cracking and exposing more sediment.

Most *ex situ*, microcosm experiments have been performed at moderate temperatures between 20 and 30˚C, which reflects the situations on shorelines and beaches (during the summer period) or in some tropical habitats (Berthe-Corti & Nachtkamp, 2010). However, *in situ* environments such as Horsea lagoon, have periods of moderate temperature in the summer months (20˚C) and periods of low temperature during the winter months (2˚C). Temperature influences both the rate of biological activity and the physco-chemical behaviour of hydrocarbons. In Horsea lagoon, when the air temperature is low, generally the DRO concentrations are high although the DRO concentrations were highest in August 2009 when the air temperature was at its second highest in both planted and unplanted sites. Increased temperatures can lead to an increase in hydrocarbon degradation, explained by increasing oxygen diffusion in the sediment improving microbial activity and increased surface volatilisation of the hydrocarbons. The increased temperatures can also lower moisture content in sediment like on the last sample date dramatically reducing the levels of DRO. Stainken *et al.* (1983) found that hydrocarbon contamination was highest during fall-winter and lowest in spring-summer. This could be explained by there being higher values of contaminated sediment particulate matter in winter resulting from re-suspension

of surface sediment due to increased turbulence in the lagoon due to high winds and long periods of rain as found in a study by Iturriaga (1979).

P. australis was able to grow well in the highly contaminated sediment. This was encouraging as phytotoxins (such as acetic, propionic, n- and iso-butyric and n-caproic acids) have been shown to significantly inhibit *P. australis* growth (Armstrong & Armstrong, 2001) and hydrocarbons in particular have been reported to reduce nutrient mobility (Kirk *et al*., 2005) and pass across cell walls and into plant roots inhibiting growth (Adam & Duncan, 2002; Kaimi, 2007).

The ability of *P. australis* to grow in these highly contaminated conditions has many benefits for the sediment; the incorporation of organic plant matter and development of passageways for aeration of the sediment is likely to provide long term improvements for pedogenisis and prevent re-suspension of sediments. Reeds have also been effectively used to dry off sewage sludge by maintaining drainage routes and evapotranspiration (Cooper *et al.,* 2004), this may have remediation benefits due to the link seen between moisture content and DRO during this study. The reeds also provide habitat improvement and can aesthetically enhance areas that had limited amounts of wildlife and had become unsightly.

6.3.2 Microbial numbers in Horsea lagoon

Despite there being a decrease in DRO concentrations in Horsea lagoon, the number of microorganisms did not increase over time and the numbers of hydrocarbon-tolerant bacteria significantly decreased. This could be due to a combination of lack of substrate and low water levels during the last sampling occasion as numbers of hydrocarbon-tolerant bacteria significantly increased with DRO concentration and moisture content. There was no correlation between microbial numbers and depth due to regular flooding preventing

oxygen transfer and volatilisation and tidal motions possibly leading to sediment deposition and erosion of the lagoon bank.

However, when the GC-FID chromatographs were analysed the sediment samples were all found to have a large UCM baseline hump that decreased after 437 days. The prominent UCM humps result from the chromatographic overlap of thousands of compounds (Frysinger, 2003) often representing the majority of the petroleum hydrocarbons present in sediments (Scarlett, 2007).

Although petroleum hydrocarbon compounds bind to soil components and become hard to remove or degrade, many bacteria, such as *Pseudomonas fluorescens* that was isolated from the planted sites, are capable of emulsifying hydrocarbons in solution by producing surface-active agents such as biosurfactants (Barathi, 2001). Biosurfactants reduce surface tension by accumulating at the interface of immiscible fluids, increasing the surface area of insoluble compounds, which leads to increased bioavailability and subsequent biodegradation of the hydrocarbons. During microbial biodegradation the hydrocarbon molecules are destroyed by sequential oxidation reactions that start with the formation of alcohols and proceeds to various organic acids, and ultimately to CO_2 and water; intermediate metabolites are phenols, aldehydes and ketones (Zemo & Foote, 2003). These polar compounds are structurally different from the hydrocarbons and have different chemical properties but are very water-soluble, especially compared to the precursor hydrocarbons (Melbye *et al.,* 2009). The total area and UCM area was found to be lower in the last four samples once the lagoon water had receded after extreme flooding. This flooding may have removed some of the UCM polar compounds due to their high aqueous solubility.

6.3.3 Bacterial isolates in Horsea lagoon

There were 157 different bacterial isolates found to be morphologically different from a total of 504 bacterial isolate culture plates from Horsea lagoon sediment. 55% were found to be Gram positive rods of which 11 were able to produce endospores enabling them to survive during extreme environmental conditions such as very high contamination levels encountered at Horsea lagoon. 20% of the bacterial isolates were facultative anaerobes able to survive in both aerobic conditions near the sediment surface and the rhizosphere and also in areas that are likely to be anaerobic.

Both the planted and unplanted sites had similar numbers of total bacterial isolate plates and diversity of total bacterial isolates. There was no correlation with depth indicating that the addition of *P. australis* had little influence on the sediment microbial communities possibly due to the roots not penetrating very deeply.

6.3.4 Hydrocarbon-degrading bacterial isolates

In this full scale *in situ* study only 15% of the total number of bacterial isolates were found to be capable of degrading hydrocarbon. Only 6% were able to form endospores but a much higher number were facultative anaerobes (37%) with the remaining bacterial isolates being strict aerobes. These were found predominantly in the upper sediment layers of UP1.

All of the hydrocarbon-degrading bacterial isolates that were identified were found to be known hydrocarbon degraders (Table 22).

Six of the different culturable hydrocarbon-degrading bacterial isolates from Horsea lagoon were identified as *Pseudomonas* spp. One was identified to species level as *Pseudomonas stutzeri*. Three of the different hydrocarbon-degrading bacterial isolates from the microcosms were identified as *Bacillus* spp. Two were identified to species level as

Bacillus cereus and *Bacillus hwajinpoensis.* Both of these species are also known hydrocarbon-degraders confirming that the isolated bacteria identified as hydrocarbon-degraders using the novel Bushnell Haas agar with 0.0025% TTC and 10 mL/L diesel plates were reliable.

Table 22. Examples of hydrocarbon-degrading ability in the identified bacterial isolates

Species	Reported to degrade	Reference
Stenotrophomonas terrae	phenol and PAHs	(Verma et al., 2011)
Ancylobacter dichloromethanicus	dichloromethane	(Muller, Bringel, & Vuilleumier, 2011)
Trichococcus **spp.**	hydrocarbons	(Aislabie, Saul, & Foght, 2006);
Paenisporosarcina quisquiliarum	variety of organic compounds	(Shivaji *et al.*, 2011)
Paenisporosarcina macmurdoensis	variety of organic compounds	(Shivaji *et al.*, 2011)
Exiguobacterium aestuarii	phenanthrene	(Tan, Qu, Zhou, Li, & Gou, 2009);
Citricoccus **spp.**	alkaliphilic and halophilic hydrocarbons	(Al-Awadhi, Sulaiman, Mahmoud, & Radwan, 2007)
Exiguobacterium aurantiacum	diesel range n-alkanes	(Mohanty & Mukherji, 2008)
Rhodococuccus erythropolis	range of hydrocarbons	(de Carvalho & ds Fonseca, 2005)
Oerskovia **spp.**	range of hydrocarbons	(Lazaroaie, 2010)
Arthrobacter **spp.**	range of hydrocarbons	(Efroymson & Alexander, 1991)
Stenotrophomonas maltophilia	PAHs	(Boonchan *et al.*, 1998)

6.4 Summary

- *P. australis* grew well in year one, but grew poorly in year two due to flooding and predation.

- All the sites had lower DRO concentrations at the end of the study but only PL1 had a statistically significant decrease with time.

- The highest DRO concentrations corresponded to the deeper water levels found at the sites further down the bank and were found to significantly increase with depth.

- In the planted sites the numbers of bacteria and hydrocarbon-tolerant bacteria increased down the bank corresponding to the higher DRO and moisture levels.

- In the unplanted sites the numbers of bacteria increased down the bank but the other microorganisms generally decreased.

- Numbers of hydrocarbon-tolerant bacteria were found to significantly increase with DRO and moisture levels

- The GC-FID chromatograms showed a symmetrical shape between C_{12}-C_{16} to C_{25} centred around C_{19}-C_{20} at day 0 and between C_{10}-C_{13} to C_{25} centred around C_{17}-C_{19} at day 437

- The UCM hump was much smaller in both planted and unplanted systems over the last few sampling occasions. This coincided with a very dry period and the exposure of sediments. Aqueous phase transport of polar compounds, with non-polar compounds perhaps mobilised by microbial biosurfactant production may be the predominant process affecting this observation.

- There were 504 bacteria isolated from Horsea lagoon, 157 were morphologically different bacterial isolates with similar amounts found in the planted and unplanted sites

- The majority of the bacterial isolates found were Gram positive rods.

- 27 out of the 157 morphologically different bacterial isolates were found to be hydrocarbon-degraders.

- There were twice as many different hydrocarbon-degrading bacterial isolates in the planted sites and had higher total numbers compared to the unplanted sites.

- Facultative anaerobes were found throughout all the depths in the planted sites but were concentrated in the upper horizons of the unplanted sites.

- The most common hydrocarbon-degrading bacterial species found in Horsea lagoon were *Pseudomonas* spp. and *Bacillus* spp.

- The molecular identification technique identified the most hydrocarbon-degrading bacterial isolates.

- SEM observation confirmed that fungal hyphae attach themselves to plant roots.

7. GENERAL DISCUSSION

The study lagoon is one of the most polluted sites that has been examined in phytoremediation studies. The pollution levels studied previously have been up to 40,000 mg/kg (Shirdam *et al.,* 2008) and 147,000 mg/kg (Greenberg *et al.*, 2006). The pollutant levels at Horsea lagoon are highly variable, even within the 1 m^2 sampling quadrats which makes interpretation of the performance difficult. Many *ex situ* studies use homogenised samples to overcome this problem (Euliss *et al* 2008, Liste & Felgentreu, 2006) but this risks disturbing the sediment structure and results may therefore not be representative of site conditions. The varying water levels caused by the need to gain regulatory consent for discharges is another factor that makes interpretation of this study difficult as sites could be submerged or exposed depending on the balance of runoff, inflow and discharge. The distribution of DRO also showed significant variation with position, with a general trend for higher pollutant levels down the bank. This may either be due to the pattern of sediment deposition or because these sites were submerged more frequently.

7.1 Plant growth and nutrient addition

Both wetland plant species were able to grow in the highly contaminated sediment. *P. australis* grew well in the greenhouse microcosm, outdoor mesocosms and in Horsea lagoon. This was encouraging as phytotoxins have been shown to significantly inhibit *P. australis* growth (Armstrong & Armstrong, 2001) and hydrocarbons in particular have been reported to reduce nutrient mobility (Kirk *et al*., 2005) and pass across cell walls and into plant roots inhibiting growth (Adam & Duncan, 2002; Kaimi, 2007). The only factors that reduced the growth of *P. australis* during the Horsea lagoon sediment studies was herbivory and site flooding. Competition between microorganisms and plants for nutrients such as phosphorus has been implicated in similar outcomes in other studies (Unterbrunner *et al.,* 2007). The microorganisms maximising hypothesis states that when microorganisms

and plants are competing for nutrients the microorganisms allocate sufficient inorganic nutrients to maximise their population biomass and leave the remainder for plant uptake (Harte & Kinzig, 1993). Although nutrients were shown to increase shoot density in the microcosms, it was felt that the risk of algal blooms and their consequences in an area surrounded by an SSSI was too great so the addition of nutrients in to Horsea lagoon was rejected.

7.2 Concentrations of DRO

All the GC-FID chromatograms showed a clear UCM hump even at the end of the studies making the interpretation of results difficult. UCM can be an indicator of hydrocarbon degradation with the UCM accounting for nearly all of the total mass of a highly degraded oil depending on the amount of high molecular-weight polar compounds and asphaltenes that were initially present in the non-degraded petroleum (Prince & Walters, 2007). In environmental sediments, the UCM can be made up of biogenic organic compounds (BOC) such as alkanes, sterols, sterones, fatty acids, fatty alcohols, waxes and wax esters which have been biosynthesized by living organisms such as vascular plants, algae, bacteria and animals. Many solvent-extraction methods for assessing soil hydrocarbons do not differentiate between DROs and BOCs. The naturally occurring organics present in soils and wet sediments can be easily misidentified and quantified as regulated DROs during analysis using such methods. In some cases, biogenic interferences can exceed regulatory levels resulting in remediation of petroleum impacts that are not actually present (Wang *et al*., 2009). Run–off from areas with banked soil or end of pipe sources like those seen in Horsea lagoon, have been shown to have the largest sources of anthropogenic background hydrocarbon contamination due to the deposition of dust, dirt, particulate matter, soot and solid wastes.

The GC-FID chromatograms from the dismantled mescosoms, dismantled microcosms and Horsea lagoon all showed a large amount of UCM present in the sediment with a symmetrical shape starting at C_{10-13} and ending at C_{25} indicating that all the hydrocarbon contamination in Horsea lagoon remained unaltered from the diesel range. A similar symmetrically humped chromatogram was seen in previous sediment studies between C_{13} and C_{25} (Tolosa *et al.*, 2009) and between C_{14} and C_{26} (Tam *et al.,* 2005) indicating partially weathered diesel fuel. Stiver & Mackay (1984) noted that typically all hydrocarbons below C_{15} are removed by evaporation with hydrocarbons below C_{12} being evaporated within 24 hours of release into the environment. However, when hydrocarbons are released into waters and emulsified, this can severely hinder their ability to evaporate (Fingas, 1995), which may be what has happened in the Horsea lagoon sediment.

In both the microcosms and in Horsea lagoon, there was a decrease in DRO concentrations over time, however, only the decrease in the microcosms was significant. This may be due to the sediment sampling disturbing the sites in Horsea lagoon allowing an increase of hydrocarbon volatilisation. It could also be due to the last sampling occasions corresponding to a hot summer and low water levels as the results showed a relationship between DRO concentration and moisture content.

Results from Horsea lagoon and the microcosm studies showed no significant difference in DRO concentrations between the planted and unplanted controls. This is not unique as other studies into phytoremediation of sediments contaminated with oil have also reported no significant differences in removal rates between planted sites and controls (King *et al.,* 2006; White *et al.,* 2006). However, a qualitative review of the GC-FID chromatograms showed the planted microcosm had an increased effect at breaking down the hydrocarbon chains present compared to the unplanted microcosm. This was indicated by the UCM hump being centred at a lower carbon chain number in the planted microcosms (C_{17}-C_{18}) compared to the unplanted microcosms (C_{19}).

The only factor shown to affect the DRO concentration was moisture content. The DRO concentration was significantly higher in sediment with high water content and in submerged sites in both the microcosms and Horsea lagoon. This raises a question as to whether less saturated sediments promote DRO removal processes (i.e. volatilisation or increased aeration and therefore microbial activity) or if it is an artefact of sediment deposition patterns in Horsea. However the lowest values at most sites were seen on the last sampling occasion when all sites were exposed to the air, suggesting that exposure to air and low moisture content does influence DRO concentrations.

7.3 Microbial numbers

At the end of the microcosm and Horsea lagoon study there were no differences between microbial numbers in planted and unplanted sites. However, there is evidence that the reeds do affect sediment microbial populations if given a longer time period. The dismantled mesocosms that had been growing for three years showed significantly higher numbers of bacteria, hydrocarbon-tolerant bacteria and hydrocarbon-tolerant fungi as well as elevated numbers of fungi in the planted container compared to the unplanted container. The factors affecting microbial numbers were DRO and moisture although they affected the microcosm and full scale Horsea lagoon trial in different ways. In the microcosm the number of hydrocarbon-tolerant bacteria decreased with an increased DRO concentration and increased moisture content. In Horsea lagoon, the number of hydrocarbon-tolerant bacteria increased with increasing DRO concentration and increased moisture content indicating that homogenised and non-homogenised sediment can act in a different manner. The decision was made to investigate only aerobes and facultative aerobes due to their increased ability to degrade hydrocarbons when compared to anaerobic microorganisms however this does affect the total microbial counts.

7.4 Hydrocarbon-degrading bacterial isolates

The types of bacterial isolates cultured from Horsea lagoon and the microcosms showed some similarities. Both contained Gram positive rods as the predominant bacterial type and identified *Pseudomonas* spp. as the most frequently occurring hydrocarbon-degrading bacterial isolate. This supports the findings of Karrick (1977), who stated that *Pseudomonas* spp. was the most dominant and widespread hydrocarbon-utilising microbe found in the marine environment.

There were twice as many different hydrocarbon-degrading bacterial isolates as well as a higher diversity of hydrocarbon-degrading bacterial isolates in planted sites of Horsea lagoon compared to the unplanted sites. This increased number of hydrocarbon-degrading bacterial isolates was also found in the microcosms where there were three times as many in the planted compared to the unplanted sites. This indicates that the addition of *P. australis* to the sediment increases the number of hydrocarbon-degrading bacteria and therefore may increase the rate of hydrocarbon degradation given a longer period of time.

8. RECOMENDATIONS FOR HORSEA LAGOON

If phytoremediation is to be applied at Horsea lagoon it is likely to require very long time periods in the flooded site. It is recommended that:

a. The surface runoff entering the lagoon is redirected to the harbour avoiding contamination of the water from the sediment. This is because the DRO concentration was shown to be significantly higher in sediment with a high water content and in submerged sites in both the microcosms and Horsea lagoon. It will also lower water levels in the lagoon drying out the sediment and increasing volatilisation and oxygen transfer.

b. The exposed sediments are planted with a large number of reeds. The extensive roots and rhizome growth were shown in both the mesocosms and the microcosms and will help to promote development of soil structure.

c. Rabbits are excluded from the vegetation until it is established due to the damage they were seen to cause to the young reeds in the second growing season.

d. Regular monitoring of the site is then undertaken to determine whether a longer period of time will show increased effects of phytoremediation. The mesocosms that were planted for the longest period, showed significantly increased numbers of microorganisms indicating that this will be the case.

9. CONCLUSIONS

- The pollutant levels in Horsea lagoon are highly variable, even within the 1 m^2 sampling sites which makes interpretation of the performance difficult. It is recommended that multiple sample repeats are taken with in a small area and time is taken to fully homogenise them before analysis.

- Both wetland plant species were able to grow in the highly contaminated sediment without nutrient addition making them both suitable for future phytoremediation studies in areas of high contamination even in ecologically sensitive areas where the addition of nutrients is not appropriate.

- DRO was affected by moisture content. The DRO concentration was significantly higher in sediment with a high moisture content and in submerged sites in both the microcosms and Horsea lagoon.

- In both the microcosms and in Horsea lagoon, there was a general decrease in DRO concentrations over time however, only the decrease in the microcosms was significant and there was no difference between the planted and unplanted plots indicating that phytoremediation does not have an effect on highly contaminated sediment in only 15 months and more time may be required.

- Another indicator that more time could be needed is the significantly higher numbers of bacteria, hydrocarbon-tolerant bacteria and hydrocarbon-tolerant fungi as well as elevated numbers of fungi in the planted sites compared to the unplanted sites in the mescosms that had been growing for three years.

- The factors affecting microbial numbers were DRO and moisture although they affected the microcosm and full scale Horsea lagoon trial in different ways indicating that these both factors should be considered when devising a phytoremediation plan.

- Using the novel colormetric indicator plates developed during this study, there were found to be twice as many different hydrocarbon-degrading bacterial isolates as well as a higher diversity of hydrocarbon-degrading bacterial isolates and almost four times as many hydrocarbon-degrading bacterial isolates found exclusively in the planted sites at Hosea lagoon and the microcosms respectively compared to the unplanted sites. This shows that although *P. australis* had a positive effect on the hydrocarbon-degrading bacterial community however this did not effect the DRO concentration and the novel plates can be used in future studies as a rapid screening method.

- Both Horsea lagoon and the microcosms contained Gram positive rods as the predominant bacterial type and identified *Pseudomonas* spp. as the most frequently occurring hydrocarbon-degrading bacterial isolate which will provide useful for future bioaugmentation experiments. It also increases the knowledge base on hydrocarbon-degraders found in highly contaminated sediments.

- The GC-FID chromatograms from the dismantled mescosoms, dismantled microcosms and Horsea lagoon all showed a UCM starting at C_{10}-$_{13}$ indicating that the short chain hydrocarbons had been lost due to volatilisation. The large symmetrical UCM hump indicated that the hydrocarbons present had suffered from extensive weathering processes.

10. FUTURE WORK

Future studies carried out on Horsea lagoon sediment could include:

- Determining the amount of DRO lost via volatilisation to discover how much degradation is by sediment microorganisms. This could be done in a laboratory study using GC analysis of the head space above a sealed container containing contaminated sediment as demonstrated by Zhou & Crawford (1995).

- Determining the optimum sediment moisture content for volatilisation of DRO and microbial degradation. This is because the moisture content was found to significantly affect the amount of DRO in the sediment. This could be done using uncontaminated sediment of different sediment moisture contents, spiked with a known amount of DRO. Samples of each sediment could then be analysed using a GC-FID over time.

- Determining and comparing which hydrocarbons are being degraded in planted sites and in unplanted control sites using GC-MS.

- Using micro-electrodes to determine the radial distribution of oxygen from the *P. australis* roots and whether this corresponds to the area that contains hydrocarbon-degrading bacteria. A small plant of *P. australis* can be placed in a flask containing Bushnell Haas agar with 0.0025% TTC and 1% diesel. The agar will turn red in the areas around the roots containing the hydrocarbon-degrading bacteria. A variety of distances from the roots can be monitored over time using a micro-electrode to measure the oxygen levels present as shown in a study by Armstrong *et al.,* (1994).

11. REFERENCES

Adam, G., & Duncan, H. (2002). Influence of diesel fuel on seed germination. *Environmental Pollution, 120*(2), 363-370.

Agency, E. (2007). *How to find out if waste oil and wastes that contain oil are hazardous*. Bristol: Environment Agencyo. Document Number)

Ahamad, P. Y. A., & Kunhi, A. A. M. (1996). Degradation of phenol through ortho-cleavage pathway by Pseudomonas stutzeri strain SPC2. *Letters in Applied Microbiology, 22*(1), 26-29.

Aislabie, J., Saul, D., & Foght, J. (2006). Bioremediation of hydrocarbon-contaminated polar soils. *Extremophiles, 10*(3), 171-179.

Al-Awadhi, H., Sulaiman, R., Mahmoud, H., & Radwan, S. (2007). Alkaliphilic and halophilic hydrocarbon-utilizing bacteria from Kuwaiti coasts of the Arabian Gulf. *Applied Microbiology and Biotechnology, 77*(1), 183-186.

Alloway, B. J. (1997). *Chemical Principles of Environmental Pollution* (Second edition ed.). London: CRC Press Inc.

Anderson, T. A., Guthrie, E. A., & Walton, B. T. (1993). Bioremediation. *Environmental Science and Technology, 27*(13), 2630-2636.

Aprill, W. (1990). Evaluation of the use of prairie grasses for stimulating polycyclic aromatic hydrocarbon treatment in soil. *Chemosphere 20*(1-2), 253-265.

Armstrong, J., & Armstrong, W. (2001). Rice and Phragmites: Effects of Organic Acids on Growth, Root Permeability, and Radial Oxygen Loss to the Rhizosphere. *American Journal of Botany, 88*(8), 1359-1370.

Armstrong, J., Armstrong, W., & Beckett, P. M. (1992). Phragmites australis: Venturi- and Humidity-Induced Pressure Flows Enhance Rhizome Aeration and Rhizosphere Oxidation. *New Phytologist, 120*(2), 197-207.

Armstrong, W. (1964). Oxygen Diffusion from the Roots of Some British Bog Plants. *Nature, 204*, 801-802.

Armstrong, W., Strange, M. E., Cringle, S., & Beckett, P. M. (1994). Microelectrode and Modelling Study of Oxygen Distribution in Roots. *Annals of Botany, 74*(3), 287-299.

Atlas, R. M. (1984). Microbiology: Fundamentals and Applications. In (pp. 92-97): Macmillan.

Baird, C., & Cann, M. (2004). *Environmental Chemistry* (Third edition ed.). New York: W.H.Freeman & Co Ltd.

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

Barathi, S, Vasudevan, N. (2001) Utilization of petroleum hydrocarbons by Pseudomonas fluorescens isolated from a petroleum-contaminated soil. *Environment International, 26*(5-6):413-6.

Ben Said, O., Goñi-Urriza, M. S., El Bour, M., Dellali, M., Aissa, P., & Duran, R. (2008). Characterization of aerobic polycyclic aromatic hydrocarbon-degrading bacteria from Bizerte lagoon sediments, Tunisia. *Journal of Applied Microbiology, 104*(4), 987-997.

Bendix, M., Tornbjerg, T., & Brix, H. (1994). Internal gas transport in Typha latifolia L. and Typha angustifolia L. 1. Humidity-induced pressurization and convective throughflow. *Aquatic Botany, 49*(2-3), 75-89.

Berset, J. D., Ejem, M., Holzer, R., & Lischer, P. (1999). Comparison of different drying, extraction and detection techniques for the determination of priority polycyclic aromatic hydrocarbons in background contaminated soil samples. *Analytica Chimica Acta, 383*(3), 263-275.

Berthe-Corti, L., & Nachtkamp, M. (2010). Bacterial Communities in Hydrocarbon-Contaminated Marine Coastal Environments. In K. N. Timmis (Ed.), *Handbook of Hydrocarbon and Lipid Microbiology* (Vol. 1, pp. 2349-2359). Berlin Heidelberg: Springer.

Boboye, B., Olukunle, O. F., & Adetuyi, F. C. (2010). Degradative activity of bacteria isolated from hydrocarbon-polluted site in Ilaje, Ondo State, Nigeria. *Degradative activity of bacteria isolated from hydrocarbon-polluted site in Ilaje, Ondo State, Nigeria, 4*(23), 2484-2491.

Bochner, B. R., & Savageau, M. A. (1977). Generalized indicator plate for genetic, metabolic, and taxonomic studies with microorganisms. *Appl Environ Microbiol, 33*(2), 434-444.

Boonchan, S, Britz, M, L., Stanley, & G, A. (1998). *Surfactant-enhanced biodegradation of high molecular weight polycyclic aromatic hydrocarbons by stenotrophomonas maltophilia* (Vol. 59). Hoboken, NJ, ETATS-UNIS: Wiley.

Bosch, R., García-Valdés, E., & Moore, E. R. B. (1999). Genetic characterization and evolutionary implications of a chromosomally encoded naphthalene-degradation upper pathway from Pseudomonas stutzeri AN10. *Gene, 236*(1), 149-157.

Bouwer, E. J. (1993). Bioremediation of organic contaminants in the subsurface. In R. Mitchell (Ed.), *Environmental Microbiology* (Vol. 11, pp. 424). New York: Wiley-Blackwell.

Bregnard, T. P., Höhener, P., Zeyer, J. (2009) Bioavailability and biodegradation of weathered diesel fuel in aquifer material under denitrifying conditions. Environmental Toxicology and Chemistry, *17* (7), 1222-1229.

Brix, H., Sorrell, B. K., & Orr, P. T. (1992). Internal Pressurization and Convective Gas Flow in Some Emergent Freshwater Macrophytes. *Limnology and Oceanography, 37*(7), 1420-1433.

Brix, H., Sorrell, B. K., & Schierup, H.-H. (1996). Gas fluxes achieved by in situ convective flow in *Phragmites australis*. *Aquatic Botany, 54*(2-3), 151-163.

Bystrom, A., & Hirtz, M. (2002). *Phytoremediation of Petroleum Hydrocarbon-.Contaminated Soil with Salix viminalis*. Chalmers University of Technology, Goteborg.

Callaway, R. M., & King, L. (1996). Temperature-driven variation in substrate oxygenation and the balance of competition and facilitation *Ecology, 77*(4), 1189-1195.

Campbell, J. B. (1978). Spatial Variation of Sand Content and pH Within Single Contiguous Delineations of Two Soil Mapping Units1. *Soil Sci. Soc. Am. J., 42*(3), 460-464.

Cattelan, A. J., Hartel, P. G., & Fuhrmann, J. J. (1998). Bacterial Composition in the Rhizosphere of Nodulating and Non-Nodulating Soybean. *Soil Sci. Soc. Am. J., 62*(6), 1549-1555.

Ciria, M. P., Solano, M. L., & Soriano, P. (2005). Role of macrophyte Typha latifolia in a constructed wetland for wastewater treatment and assessment of its potential as a biomass fuel. *Biosystems Engineering, 92*(4), 535-544.

Clesceri, L. S., Greenberg, A. E., & Eaton, A. D. (1998). *Standard Methods for the Examination of Water and Wastewater* (20 ed.). Washington: American Public Health Association.

Comin, F. A., Romero, J. A., Astorga, V., & Garcia, C. (1997). Nitrogen removal and cycling in restored wetlands used as filters of nutrients for agricultural runoff. *Water Science and Technology, 35*, 255-261.

Cooper, P., Willoughby, N., & Cooper, D. (2004). The Use of Reed-beds for Sludge Drying *Water and Environment Journal, 18*(2), 85-89.

Cunningham, S. D., Berti, W. R., & Huang, J. W. (1995). Phytoremediation of contaminated soils. *Trends in Biotechnology, 13*(9), 393-397.

Curl, E. A., & Truelove, B. (1986). *The Rhizosphere*. Berlin: Springer-Verlag.

de Carvalho, C. C. C. R., & ds Fonseca, M. M. R. (2005). Degradation of hydrocarbons and alcohols at different temperatures and salinities by Rhodococcus erythropolis DCL14. *FEMS Microbiology Ecology, 51*(3), 389-399.

Das, N, Chandran, P. (2011) Microbial degradation of petroleum hydrocarbon contaminants: an overview. *Biotechnology research international*, 2011:941810. Epub 2011/02/26.

Dix, H. M. (1980). *Environmental Pollution: Atmosphere, Land, Water and Noise*. Chichester: Wiley-Blackwell.

Dowty, R. A., Shaffer, G. P., Hester, M. W., Childers, G. W., Campo, F. M., & Greene, M. C. (2001). Phytoremediation of small-scale oil spills in fresh marsh environments: a mesocosm simulation. *Marine Environmental Research, 52*(3), 195-211.

Duke, J. A. (1979). Ecosystematic data on economic plants. *Quart. J. Crude Drug Res., 17*(3-4), 91-110.

Dworian, P. (1997). Interferences by natural organics in diesel analysis. Journal of Cold Regions Engineering, 1(2), 71-81/

Efroymson, R. A., & Alexander, M. (1991). Biodegradation by an Arthrobacter Species of Hydrocarbons Partitioned into an Organic Solvent. *Appl. Environ. Microbiol., 57*(5), 1441-1447.

EPA. (2001). A citizens guide to phytoremediation. *Office of Solid Waste and Emergency Response*(EPA-542-F-01-002), 1-2.

EPA. (2003). Nonhalogenated Organics Using GC-FID Method 8015D (EPA SW-846). EPA, Washington D.C., USA

Erickson, L. E., Davis, L. C., & Narayanan, M. (1995). Bioenergetics and bioremediation of contaminated soil. *Thermochimica Acta, 250*(2), 353-358.

Euliss, K., Ho, C. H., Schwab, A. P., Rock, S. A., & Banks, M. K. (2008). Greenhouse and field assessment of phytoremediation for petroleum contaminants in a riparian zone. *Bioresource Technology, 99*(6), 1961-1971.

Federation, I. T. O. s. P. (1987). *Response to Marine Oil Spills*. London: Witherby Seamanship International Ltd.

Fingas, M. F. (2001). *The Basics of Oil Spill Clean Up* (Second edition ed.). London: Environmental Emergency Branch.

Fingas, M. A. (1995). A literature review of the physics and predictive modelling of oil spill evaporation. *Journal Hazardous Materials*, 42, 157-175.

Flathman, P. E., & Lanza, G. R. (1998). Phytoremediation: Current views on an emerging green technology *Journal of Soil Contamination 7*(4), 415-432.

Foth, H. D. (1984). Fundamentals of soil science. In (8th Edition ed., pp. 1-10): Wiley.

Frick, C. M., Farrell, R. E., & Germida, J. J. (1999). *Assessment of Phytoremediation as an In-Situ Technique for Cleaning Oil-Contaminated Sites*. Saskatchewan: University of Saskatchewano. Document Number)

Frysinger, G.S, Gaines, R. B, Li, X, Reddy, C. M. (2003). Resolving the unresolved complex mixture in petroleum-contaminated sediments *Environ. Sci. Technol.*, 37(8), 1653-1662.

Fuji, K., Urano, N., Ushio, H., Satomi, M., Iida, H., Ushio-Sata, N., et al. (2000). Profile of a Nonylphenol-Degrading Microflora and Its Potential for Bioremedial Applications. *Journal of Biochemistry, 128*(6), 909-916.

Gawronski, S. W., & Gawronska, H. (2007). Plant taxonomy for phytoremediation. In N. Marmiroli, B. Samotokin & M. Marmiroli (Eds.), *Advanced Science and Technology for Biological Decontamination of Sites Affected by Chemical and Radiological Nuclear Agents* (Vol. 75, pp. 79-88). Netherlands: Springer

Gerhardt, K. E., Huang, X.-D., Glick, B. R., & Greenberg, B. M. (2009). Phytoremediation and rhizoremediation of organic soil contaminants: Potential and challenges. *Plant Science, 176*(1), 20-30.

Gessner, T. P., Kadlec, R. H., & Reaves, R. P. (2005). Wetland remediation of cyanide and hydrocarbons. *Ecological Engineering, 25*, 457-469.

Ghazali, F. M., Rahman, R. N. Z. A., Salleh, A. B., & Basri, M. (2004). Biodegradation of hydrocarbons in soil by microbial consortium. *International Biodeterioration & Biodegradation, 54*(1), 61-67.

Grace, J. B. (1988). The effects of nutrient additions on mixtures of Typha latifolia L. and Typha domingensis pers. along a water-depth gradient. *Aquatic Botany, 31*(1-2), 83-92.

Grace, J. B. (1989). Effects of Water Depth on Typha latifolia and Typha domingensis. *American Journal of Botany, 76*(5), 762-768.

Greenberg, B. M., Huang, X. D., Gurska, J., & Gerhardt, K. E. (2006). *Development and sucessful field tests of a multi-process phytoremediation system for decontamination of persistent petroleum and organic contaminants in soils*. Paper presented at the CLRA 2006 Reclamation and Remediation: Policy to Practice. from http://www.waterlooenvironmentalbiotechnology.com/pdfs/clra-06.pdf

Groudeva, V. I., Groudev, S. N., & Doycheva, A. S. (2001). Bioremediation of waters contaminated with crude oil and toxic heavy metals. *Int. J. Miner. Process, 62*, 293-299.

Gunther, T., Dornberger, U., & Fritsche, W. (1996). Effects of ryegrass on biodegradation of hydrocarbons in soil. *Chemosphere, 33*(2), 203-215.

Harris, R. F., & Sommers, L. E. (1968). Plate-Dilution Frequency Technique for Assay of Microbial Ecology. *Appl. Environ. Microbiol., 16*(2), 330-334.

Haslam, S. M. (1972). Biological flora of the British Isles. Phragmites communis Trin. . *Journal of Ecology, 60*, 585-610.

Hickman, G. T., & Novak, J. T. (1989). Relationship between subsurface biodegradation rates and microbial density. *Environmental Science & Technology, 23*(5), 525-532.

Hopwood, D. A. (1970). The Isolation of Mutants. In J. R. Norris & D. W. Ribbons (Eds.), *Methods in Microbiology* (Vol. 3A, pp. 363-433). New York: Academic Press Inc.

Huang, J. W., & Chen, J. (2003). Role of pH in phytoremediation of contaminated soils. In Z. Rengel (Ed.), *Handbook of Soil Acidity* (pp. 449-472). New York: Marcel Dekker Inc.

Hutchinson, S. L., Schwab, A. P., & Banks, M. K. (2003). Biodegradation of Petroleum Hydrocarbons in the Rhizosphere. In S. C. McCutcheon & J. L. Schnoor (Eds.), *Phytoremediation* (pp. 355-386). New Jersey: John Wiley & Sons Inc.

Ingham, E. R. (2000a). *The Living Soil: Bacteria*. Iowa: Soil and Water Conservation Societyo. Document Number)

Ingham, E. R. (2000b). *The Living Soil: Fungi*. Iowa: Soil and Water Conservation Societyo. Document Number)

Investigations, E. G. (2011). Generic notes- Encia Geoenvironmental Investigations. Retrieved 11.11.11, 2011, from http://www.burnley.gov.uk/planx_downloads/10_0430_General_Notes.pdf

ISO. (2003). *Soil Quality –Determination of dry matter and water content on a mass basis – Gravimetric method*. Retrieved. from http://www.icp-forests.org/pdf/Chapt_3a_2006%282%29.pdf.

Iturriaga, R. (1979). Bacterial activity related to sedimenting particulate matter. *Marine Biology, 55*(3), 157-169.

Jana, B. B., Chakraborty, P., Biswas, J. K., & Ganguly, S. (2001). Biogeochemical cycling bacteria as indices of pond fertilization: importance of CNP ratios of input fertilizers. *Journal of Applied Microbiology, 90*(5), 733-740.

Jebson, S. (2010). Air temperature and humidity. In H. Pinchin (Ed.). Port Solent: Met office.

Jenkins, A. (2005). *Soil Fungi*. NSW: NSW Department of Primary Instituteso. Document Number)

Ji, G. D., Yang, Y. S., Zhou, Q., Sun, T., & Ni, J. R. (2004). Phytodegradation of extra heavy oil-based drill cuttings using mature reed wetland: an in situ pilot study. *Environment International, 30*(4), 509-517.

Justice, S. f. (2011). Find your local Fire and Rescue Service. Retrieved 05 April 2011, from http://www.skillsforjustice.com/template01.asp?PageID=793

Juwarkar, A. A., & Jambhulkar, H. P. (2008). Phytoremediation of coal mine spoil dump through integrated biotechnological approach. *Bioresource Technology, 99*(11), 4732-4741.

Kaimi, E. (2007). Screening of twelve plant species for phytoremediation of petroleum hydrocarbon-contaminated soil. *Plant Production Science, 10*(2), 211-218.

Kaimi, E., Mukaidani, T., Miyoshi, S., & Tamaki, M. (2006). Ryegrass enhancement of biodegradation in diesel-contaminated soil. *Environmental and Experimental Botany, 55*(1-2), 110-119.

Kaplan, I. R., Galperin, Y., Lu, S-T., Lee, R-P., (1997) Forensic Environmental Geochemistry: differentiation of fuel-types, their sources and release time. Org Geochem, 27(5-6):317.

Karrick, N.L. (1977) Alterations in petroleum resulting from physio-chmical and microbiological factors. *Effects of petroleum on arctic and subartic marine environments and organisms.* Edited by Malins, D. C., 1, (pp. 225-228). New York, Academic Press.

Karthikeyan, R., Kulakow, P., Leven, B., Pierzynski, G., & Erickson, L. E. (2000). *Vegetated treatment of vehicle wash sediments: a field demonstration.* Paper presented at the Conference on Hazardous Waste Research, GP/RM Hazardous Substance Research Center, 101 Ward Hall, Manhattan, KS.

Kazunga, C., & Aitken, M. D. (2000). Products from the Incomplete Metabolism of Pyrene by Polycyclic Aromatic Hydrocarbon-Degrading Bacteria. *Appl. Environ. Microbiol., 66*(5), 1917-1922.

Keller, J., Banks, M. K., & Schwab, A. P. (2008). Effect of soil depth on phytoremediation efficiency for petroleum contaminants. *J Environ Sci Health A Tox Hazard Subst Environ Eng, 43*(1), 1-9.

King, R. F., Royle, A., Putwain, P. D., & Dickinson, N. M. (2006). Changing contaminant mobility in a dredged canal sediment during a three-year phytoremediation trial. *Environmental Pollution, 143*(2), 318-326.

Kirk, J. L., Klironomos, J. N., Lee, H., & Trevors, J. T. (2005). The effects of perennial ryegrass and alfalfa on microbial abundance and diversity in petroleum contaminated soil. *Environmental Pollution, 133*(3), 455-465.

Klüber, H. D., Lechner, S., & Conrad, R. (1995). Characterization of populations of aerobic hydrogen-oxidizing soil bacteria. *FEMS Microbiology Ecology, 16*(2), 167-176.

Lazaroaie, M. M. (2010). Multiple responses of gram-positive and gram-negative bacteria to mixture of hydrocarbons. *Brazilian Journal of Microbiology, 41*, 649-667.

Leahy, J. G., & Colwell, R. R. (1990). Microbial degradation of hydrocarbons in the environment. *Microbiol Rev, 54*(3), 305-315.

Liste, H.-H., & Felgentreu, D. (2006). Crop growth, culturable bacteria, and degradation of petrol hydrocarbons (PHCs) in a long-term contaminated field soil. *Applied Soil Ecology, 31*(1-2), 43-52.

Liu, J., Björnsson, L., & Mattiasson, B. (2000). Immobilised activated sludge based biosensor for biochemical oxygen demand measurement. *Biosensors and Bioelectronics, 14*(12), 883-893.

Lynch, J. M. (1990). *The Rhizosphere*: John Wiley & Sons Inc.

Madigan, M. T., Martinko, J. M., Dunlap, P. V., & Clark, D. P. (2008). *Biology of microorganisms* (Twelth edition ed.). San Francisco: Pearson.

Marchal, R., Penet, S., Solano-Serena, F., & Vandecasteele, J. P. (2003). Gasoline and Diesel Oil Biodegradation. *Oil & Gas Science and Technology, 58*(4), 441-448.

Massacci A, P. F., Lannelli MA (2001). Remediation of wetlands by Phragmites australis. *MINERVA BIOTECNOLOGICA, 13*, 135-140.

Massacci, A., Pietrini, F., & Lannelli, M. A. (2001). Remediation of wetlands by Phragmites australis. *MINERVA BIOTECNOLOGICA, 13*, 135-140.

McDonald, S. (2006). *Phytoremediation of lead-contaminated soil using Typha latifolia.* Humboldt State University, Humboldt State.

McNaughton, S. G., Folsom, T. C., Lee, T., Park, F., Price, C., Roeder, D., et al. (1974). Heavy metal tolerance in Typha latifolia without the evolution of tolerant races. *Ecology, 55*, 1163-1165.

Melby, A.G., brakstad, O. G., Hokstad, J. N., Gregerson, I. K., Hansen, B. H., Booth, A. M., Rowland, S. J., Tollefsen, K. E. (2009) *Environmental Toxicology and Chemistry*, 28 (9), 1815-1824.

Mohanty, G., & Mukherji, S. (2008). Biodegradation rate of diesel range n-alkanes by bacterial cultures Exiguobacterium aurantiacum and Burkholderia cepacia. *International Biodeterioration & Biodegradation, 61*(3), 240-250.

Moore, B. J., Ross, S. D., Gibson, D. T., & Hardisty, P. E. (1999, May 31-June 1). *Constructed wetlands for treatment of dissolved phase hydrocarbons.* Paper presented at the Phytoremediation Technical Seminar, Calgary, AB.

Moorhead, K. K., & Reddy, K. R. (1998). Oxygen transport through selected aquatic macrophytes. *J.Environ. Qual., 17*, 138-142.

Muijs, B. Jonker, M. (2009). Evaluation of clean-up agents for total petroleum hydrocarbon analysis in biota and sediments. *Journal Chromatography A. 1216* (27), 5182-5189.

Muller, E. E. L., Bringel, F., & Vuilleumier, S. (2011). Dichloromethane-degrading bacteria in the genomic age. *Research in Microbiology, In Press, Corrected Proof.*

Muratova, A. Y. (2003). Studies of the Efficacy of Alfalfa and Reed in the Phytoremediation of Hydrocarbon-Polluted Soil. *Applied biochemistry and microbiology, 39*(6), 599-605.

Nichols, T. D., Wolf, D. C., Rogers, H. B., Beyrouty, C. A., & Reynolds, C. M. (1997). Rhizosphere microbial populations in contaminated soils. *Water, Air, and Soil Pollution, 95*, 165-178.

Nwaogu, L. A., Onyeze, G. O. C., & Nwabueze, R. N. (2008). Degradation of diesel oil in a polluted soil using Bacillus subtilis. *African Journal of Biotechnology, 7*(12), 1939-1943.

Olga, P., Petar, K., Jelena, M., & Srdjan, R. (2008). Screening method for detection of hydrocarbon-oxidizing bacteria in oil-contaminated water and soil specimens. *Journal of Microbiological Methods, 74*(2-3), 110-113.

Overbeek, J. v., & Blondeau, R. (1954). Mode of Action of Phytotoxic Oils. *Weeds, 3*(1), 55-65.

Page, A. L., Miller, R. H., & Keeney, D. R. (1982). *Methods of Soil Analysis, part II* (2nd edition ed.). Wisconsin: ASA-SSSA.

Palmroth, M. R. T., Pichtel, J., & Puhakka, J. A. (2002). Phytoremediation of subarctic soil contaminated with diesel fuel. *Bioresource Technology, 84*(3), 221-228.

Paquin, D., Ogoshi, R., Campbell, S., & Li, Q. X. (2002). Bench-Scale Phytoremediation of Polycyclic Aromatic Hydrocarbon-Contaminated Marine Sediment with Tropical Plants. *International Journal of Phytoremediation, 4*(4), 297 - 313.

Phillips, L. A., Greer, C. W., Farrell, R. E., & Germida, J. J. (2009). Field-scale assessment of weathered hydrocarbon degradation by mixed and single plant treatments. *Applied Soil Ecology, 42*(1), 9-17.

Pizzul, L., Pilar Castillo, M., & Stenström, J. (2006). Characterization of selected actinomycetes degrading polyaromatic hydrocarbons in liquid culture and spiked soil. *World Journal of Microbiology and Biotechnology, 22*(7), 745-752.

Potter, T. L., & Simmons, K. E. (1998). Composition of petroleum mixtures. *Total petroleum Hydrocarbon criteria working group series 2*, 1-12.

Pradhan, S. P., Conrad, J. R., Paterek, J. R., & Srivastava, V. J. (1998). Potential of Phytoremediation for Treatment of PAHs in Soil at MGP Sites. *Journal of Soil Contamination, 7*(4), 467-480.

Prescott, L. M., Hartley, J. P., & Klein, D. A. (1999). *Microbiology* (Fourth edition ed.). Boston: WCB McGraw-Hill.

Prince, R. C., Walters, C. C. (2009). Biodegradation of oil hydrocarbons and its implications for source identification. *Oil Spill Environmental Forensics*, Elsevier Publishers. 11, 349-380.

Rahman, K. S. M., Thahira-Rahman, J., Lakshmanaperumalsamy, P., & Banat, I. M. (2002). Towards efficient crude oil degradation by a mixed bacterial consortium. *Bioresource Technology, 85*(3), 257-261.

Reed, M. L. E., & Glick, B. R. (2005). Growth of canola (Brassica napus) in the presence of plant growth-promoting bacteria and either copper or polycyclic aromatic hydrocarbons. *Canadian Journal of Microbiology, 51*, 1061-1069.

Reid, G., & Wong, P. (2005). *Soil Biology Basics*. NSW: NSW Department of Primary Industrieso. Document Number)

Riser-Roberts, E. (1998a). *Remediation of petroleum contaminated soils: biological, physical, and chemical processes*. Florida: Lewis publishers.

Riser-Roberts, E. (1998b). *Remediation of Petroleum Contaminated Soils: Biological, Physical, and Chemical Processes* Florida: Lewis Publishers.

Rodwell, J. S. (1995). *British plant communities* (Vol. 4). Cambridge: Cambridge University Press.

Rook, E. J. S. (2002, 26 February 2004). Typha latifolia. *Aquatic plants* Retrieved 18 September 2008, 2008, from www.rook.org/earl/bwca/nature/aquatics/typhalat.html

Rossello-Mora, R. A., Lalucat, J., & Garcia-Valdes, E. (1994). Comparative biochemical and genetic analysis of naphthalene degradation among Pseudomonas stutzeri strains. *Appl. Environ. Microbiol., 60*(3), 966-972.

Rushton, D. G., Ghaly, A. E., & Martinell, K. (2007). Assessment of Canadian Regulations and Remediation Methods for Diesel Oil Contaminated Soils. *American Journal of Applied Science, 4*(7), 465-478.

Salmon, C., Crabos, J. L., Sambuco, J. P., Bessiere, J. M., Basseres, A., Caumette, P., et al. (1998). Artificial Wetland Performances in the Purification Efficiency of Hydrocarbon Wastewater. *Water, Air, & Soil Pollution, 104*(3), 313-329.

Salt, D. E., Baylock, M., Kumar, N. P. B. A., Dushenkov, V., Ensley, B. D., Chet, I., et al. (1995). Phytoremediation: A novel strategy for the removal of toxic metals from the environment using plants. *Biotechnology, 13*, 468-474.

Samudro, G., & Mangkoedihardjo, S. (2010). REVIEW ON BOD, COD AND BOD/COD RATIO: A TRIANGLE ZONE FOR TOXIC, BIODEGRADABLE AND STABLE LEVELS. *INTERNATIONAL JOURNAL Of ACADEMIC RESEARCH, 2*(4), 235-239.

Sandercock, P. M. (2008) Fire investigation and ignitable liquid residue analysis--a review: 2001-2007. Forensic science international, 176(2-3):93-110. Epub 2007/10/24.

Scheff, G., Salcher, O., & Lingens, F. (1984). Trichococcus flocculiformis; gen. nov. sp. nov. A new gram-positive filamentous bacterium isolated from bulking sludge. *Applied Microbiology and Biotechnology, 19*(2), 114-119.

Schnoor, J. L. (1997). *Phytoremediation*. Iowa: The University of Iowao. Document Number)

Schwab, A. P., Banks, M. K., & Arunachalam, M. (1995). Biodegradation of polycyclic aromatic hydrocarbons in rhizosphere soil. In R. E. Hinchee, D. B. Anderson & R. E. Hoeppel (Eds.), *Bioremediation of Recalcitrant Organics* (pp. 23-29). Columbus: Battelle Press.

Serrano, A., Gallego, M., Gonzalez, J. L. Tejada M. (2008) Natural attenuation of diesel aliphatic hydrocarbons in contaminated agricultural soil. Environmental Pollution, 151(3):494-502. Epub 2007/06/09.

Shimp, J. F., Tracy, J. C., Davis, L. C., Lee, E., Huang, W., & Erickson, L. E. (1993). Beneficial effects of plants in the remediation of soil and groundwater contaminated with organic materials. *Environmental Science and Technology, 23*(1), 41-77.

Shivaji, S., Kumari, K., Kishore, K. H., Pindi, P. K., Rao, P. S., Radha Srinivas, T. N., et al. (2011). Vertical distribution of bacteria in a lake sediment from Antarctica by culture-independent and culture-dependent approaches. *Research in Microbiology, 162*(2), 191-203.

Shukla, O. P. (1989). Microbiological degradation of quinoline by Pseudomonas stutzeri: the coumarin pathway of quinoline catabolism. *Microbios, 59*(238), 47-63.

Singer, A. C., Thompson, I. P., & Bailey, M. J. (2004). The tritrophic trinity: a source of pollutant-degrading enzymes and its implications for phytoremediation. *Current Opinion in Microbiology, 7*(3), 239-244.

Singleton, D. R., Guzman Ramirez, L., & Aitken, M. D. (2009). Characterization of a Polycyclic Aromatic Hydrocarbon Degradation Gene Cluster in a Phenanthrene-Degrading Acidovorax Strain. *Appl. Environ. Microbiol., 75*(9), 2613-2620.

Smith, P. (2011, 12.01.11). Petroleum Production. *U.S. Energy Information Administration* Retrieved 05.10.11, 2011, from http://www.eia.gov/ipm/supply.html

Solano, M. L., Soriano, P., & Ciria, M. P. (2004). Constructed wetlands as a sustainable solution for wastewater treatment in small villages *Biosystems Engineering, 87*(1), 109-118.

Stainken, D. M., Gray Multer, H., & Mirecki, J. (1983). Seasonal patterns of sedimentary hydrocarbons in the raritan bay-lower new york bay. *Environmental Toxicology and Chemistry, 2*(1), 35-42.

Stiver, W., Mackay, D. (1984). Evaporation rates of spills and hydrocarbons and petroleum mixtures. *Environmental Science Technology*. 18, 834-840.

Tam, N. F., Wong, T. W., Wong, Y. S., (2005) A case study on fuel oil contamination in a mangrove swamp in Hong Kong. Mar Pollut Bull., 51(8-12):1092-100. Epub 2005/07/19.

Tan, L., Qu, Y.-y., Zhou, J.-t., Li, A., & Gou, M. (2009). Identification and Characteristics of A Novel Salt-Tolerant <i>Exiguobacterium</i> sp. for Azo Dyes Decolorization. *Applied Biochemistry and Biotechnology, 159*(3), 728-738.

Tandlich, R., Brezná, B., & Dercová, K. (2001). The effect of terpenes on the biodegradation of polychlorinated biphenyls by Pseudomonas stutzeri. *Chemosphere, 44*(7), 1547-1555.

Tate, R. L. (1995). *Soil Microbiology*. New York: John Wiley and Sons Ltd.

Taylor, G. J., & Crowder, A. A. (1984). Copper and nickel tolerance in *Typha latifolia* clones from contaminated and uncontaminated environments. *Canadian Journal of Botany 62*, 1304-1308.

Tischer, S. (2002). Model trials of phytoremediation of hydrocarbon-contaminated sites by the use of different plant species. *Int J Phytoremediation, 4*(3), 187-203.

Tolosa, I., Mesa-Albernas, M., Alonso-Hernandez, C. M., (2009) Inputs and sources of hydrocarbons in sediments from Cienfuegos bay, Cuba. Mar Pollut Bull., 58(11):1624-34. Epub 2009/08/12.

Totten, G. E. (2004, 01.06.11). A timeline of highlights from the histories of ASTM committee DO2 and the petroleum industry.   Retrieved 09.11.11, 2011, from http://www.astm.org/COMMIT/D02/print_version.html

Turner, D. (2004) Cutting down Analysis Times with the Anatune GC XLR8R. *Chromatography Technical Note No 51.* Anatune Ltd, Cambridge, UK

Tyler-Walters, H. (2003, 07 October 2003). Common reed - Phragmites australis *Marine Life Information Network: Biology and Sensitivity Key Information Sub-programme* Retrieved 11 September 2008, 2008, from http://www.marlin.ac.uk/species/Phragmitesaustralis.htm

Verma, V., Raju, S. C., Kapley, A., Kalia, V. C., Kanade, G. S., Daginawala, H. F., et al. (2011). Degradative potential of Stenotrophomonas strain HPC383 having genes homologous to dmp operon. *Bioresource Technology, 102*(3), 3227-3233.

Vervaeke, P., Luyssaert, S., Mertens, J., Meers, E., Tack, F. M., & Lust, N. (2003). Phytoremediation prospects of willow stands on contaminated sediment: a field trial. *Environ Pollut, 126*(2), 275-282.

Vinas, M., Sabate, J., Espuny, M. J., & Solanas, A. M. (2005). Bacterial Community Dynamics and Polycyclic Aromatic Hydrocarbon Degradation during Bioremediation of Heavily Creosote-Contaminated Soil. *Appl. Environ. Microbiol., 71*(11), 7008-7018.

Volkman, J. K., Revill, A. T., Murray, A. P., (1997). Applications of Biomarkers for Identifying Sources of Natural and pollutant hydrocarbons in aquatic environments. *Molecular Markers in Environmental Geochemistry*. 36, 611-618.

Walworth, J. L., Woolard, C. R., Braddock, J. F., & Reynolds, C. M. (1997). Enhancement and inhibition of soil petroleum biodegradation through the use of fertilizer nitrogen: An approach to determining optimum levels. *Journal of Soil Contamination, 6*(5), 465 - 480.

Wang, Z., Fingas, M., Sergy, G. (1995) Chemical Characterization of Crude Oil Residues from an Arctic Beach by GC/MS and GC/FID. *Environ. Sci. Technology, 29* (10), 2622-2631.

Wang, Z.-z., Liu, X.-y., Zhang, X.-y., Wang, J., Cao, Z.-n., Zhong, C.-l., et al. (2010). Wetland bacteria isolated from Huangpu River-Yangtze River estuary and its degradation on diesel. *Journal of Shanghai University (English Edition), 14*(4), 292-296.

Ward, D. M., & Brock, T. D. (1978). Anaerobic metabolism of hexadecane in sediments. *Geomicrobiology Journal, 1*(1), 1 - 9.

Weis, J. S., & Weis, P. (2004). Metal uptake, transport and release by wetland plants: implications for phytoremediation and restoration. *Environment International, 30*(5), 685-700.

White, P. M., Wolf, D. C., Thoma, G. J., & Reynolds, C. M. (2006). Phytoremediation of Alkylated Polycyclic Aromatic Hydrocarbons in a Crude Oil-Contaminated Soil *Water, Air, and Soil Pollution, 169*(1-4), 207-220.

Wild, A. (1993). Soils and the Environment: An Introduction. In (pp. 69-187).

Williams, J. B. (2002). Phytoremediation in Wetland Ecosystems: Progress, Problems, and Potential. *Critical Reviews in Plant Sciences, 21*(6), 607-635.

Willumsen, P., Karlson, U., Stackebrandt, E., & Kroppenstedt, R. M. (2001). Mycobacterium frederiksbergense sp. nov., a novel polycyclic aromatic hydrocarbon-degrading Mycobacterium species. *International Journal of Systematic and Evolutionary Microbiology, 51*(5), 1715-1722.

Wrenn, B. A., & Venosa, A. D. (1996). Selective enumeration of aromatic and aliphatic hydrocarbon degrading bacteria by a most-probable-number procedure. *Can J Microbiol, 42*(3), 252-258.

Zemo, D. A., & Foote, G. R. (2003). The Technical Case for Eliminating the Use of the TPH Analysis in Assessing and Regulating Dissolved Petroleum Hydrocarbons in Ground Water. *Ground Water Monitoring & Remediation*, 23(3), 95-104.

Zhou, E., & Crawford, R. L. (1995). Effects of oxygen, nitrogen, and temperature on gasoline biodegradation in soil. *Biodegradation, 6*(2), 127-140.

12. APPENDIX

Figure 26. QC charts from GC-FID during Horsea lagoon sampling

The following are reproduced from the hard copy out puts which have been manually adjusted to be on similar X and Y scales so they can be visually compared.
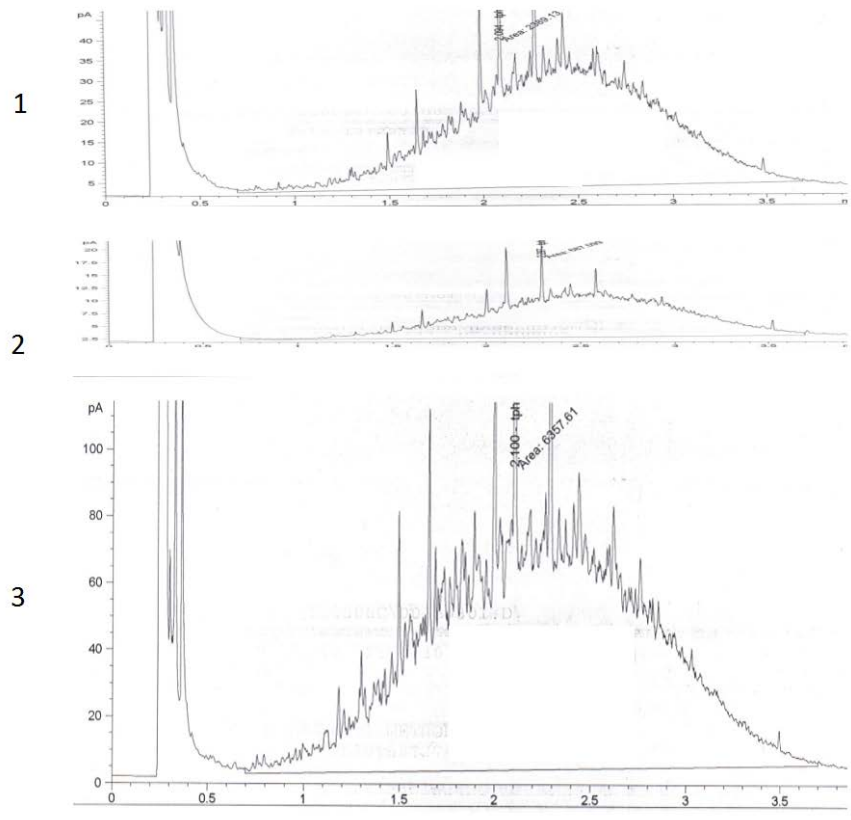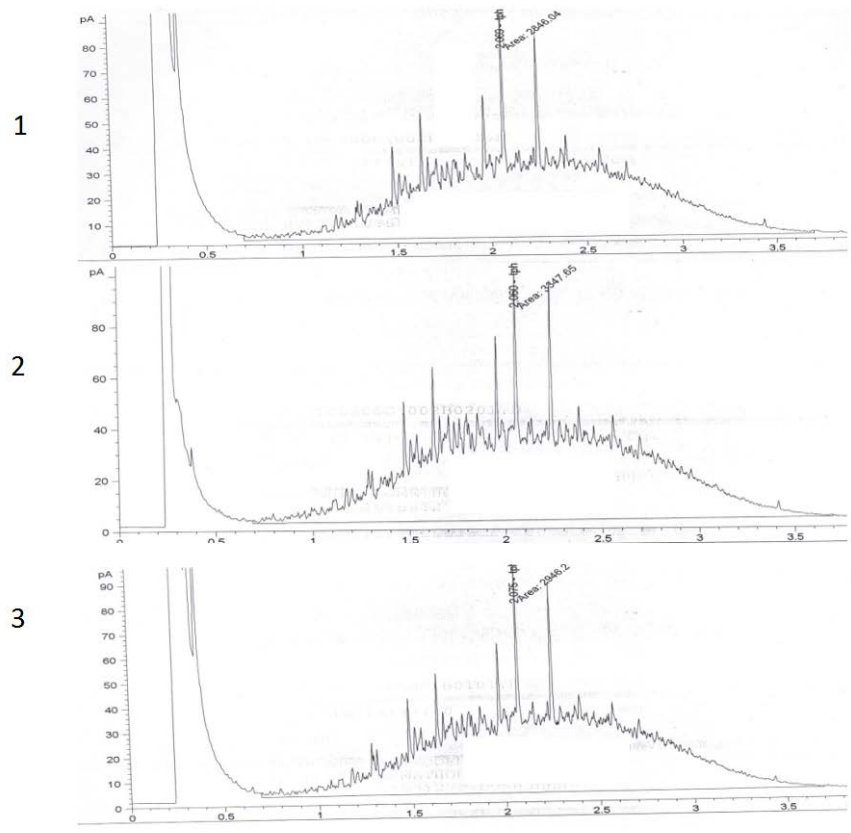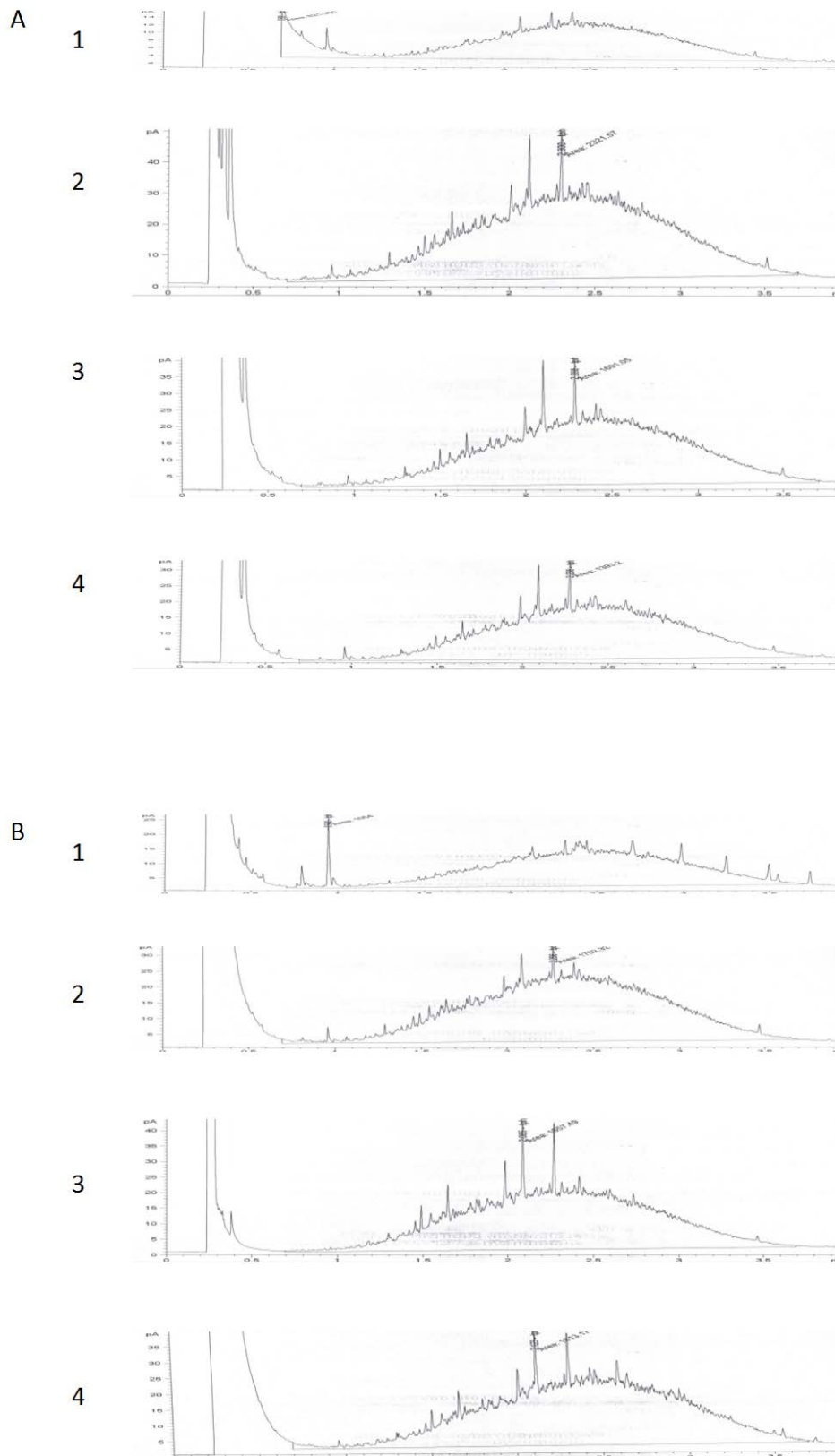
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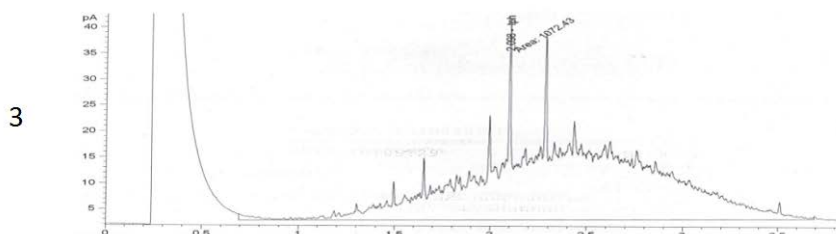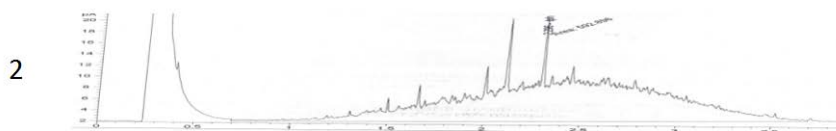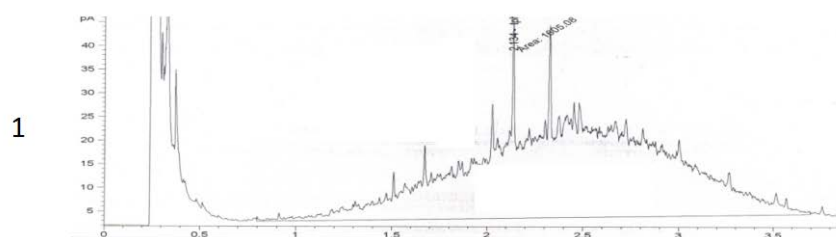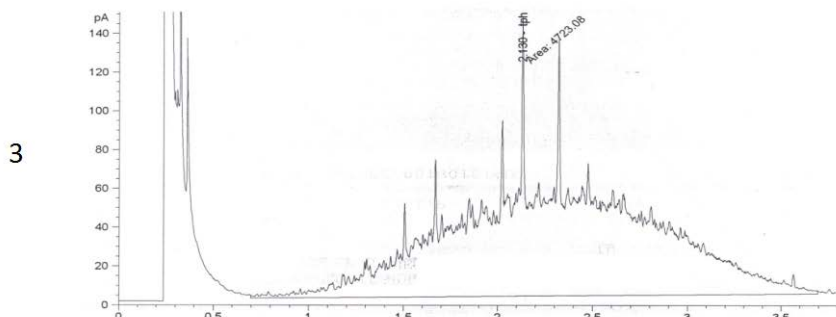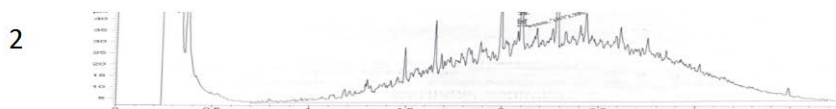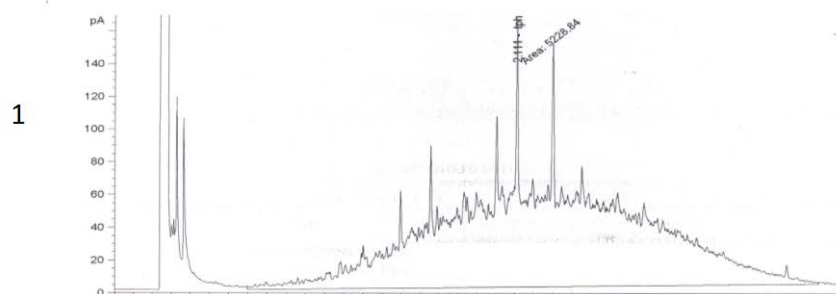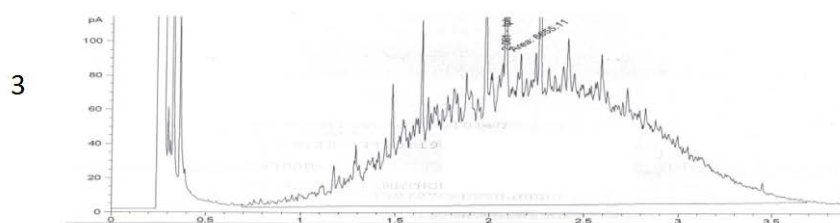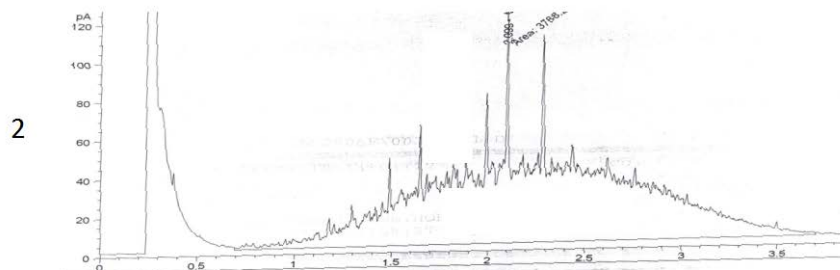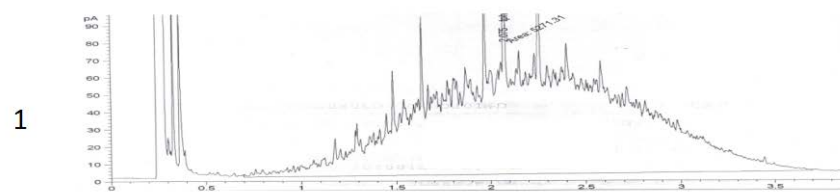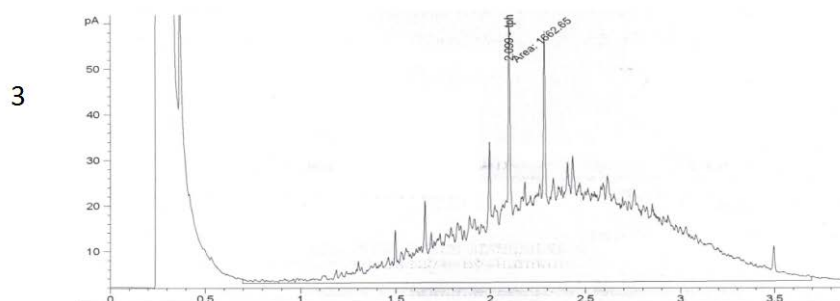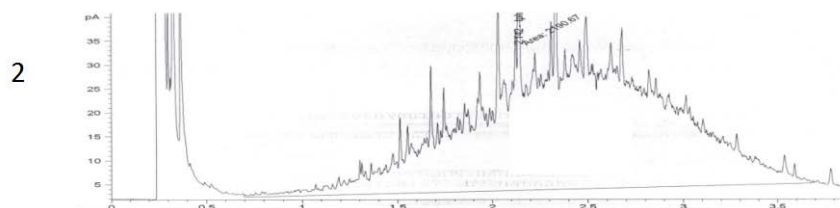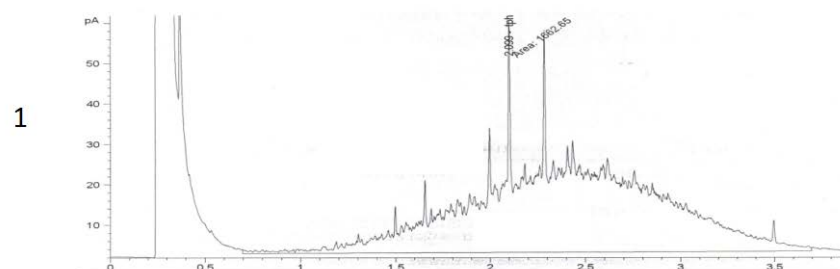
Figure 27. Dismantled mesocosm chromatograms showing UCM hump and n-alkane peaks in planted A (0 cm), B (15 cm), C (30 cm) and unplanted D (0 cm), E (15 cm), F (30 cm)
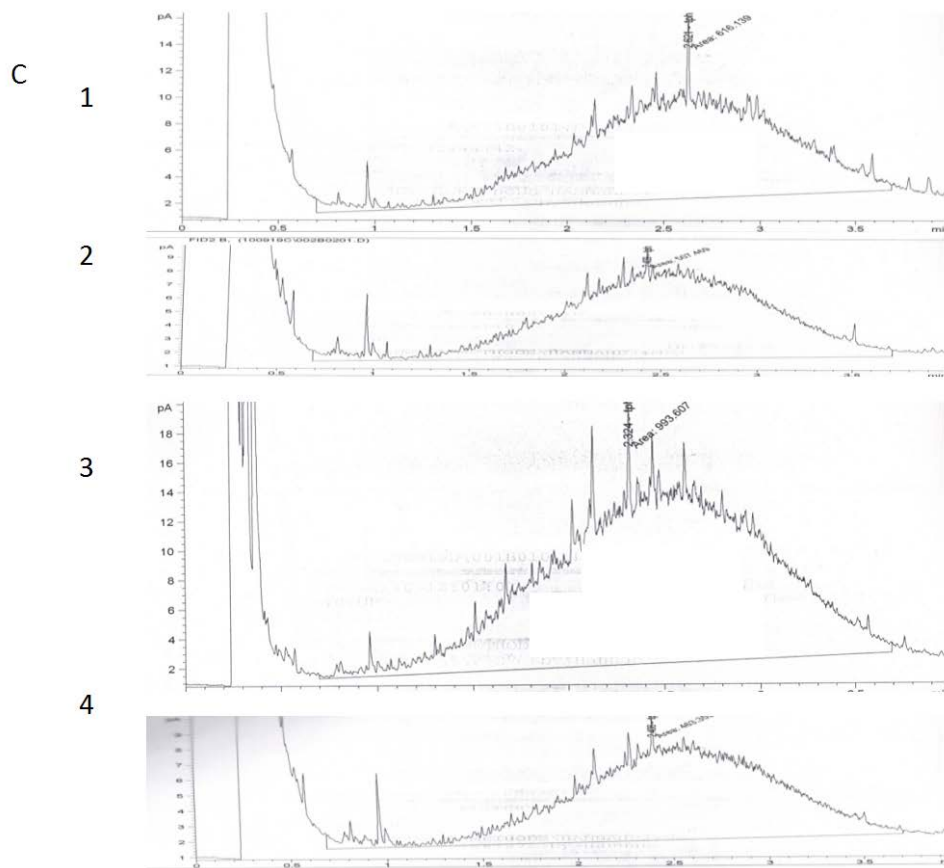
Figure 28. Dismantled microcosm chromatograms showing UCM hump and n-alkane peaks in planted edge A 10 cm (1), 20 cm (2), 40 cm (3), 60 cm (4), planted middle B (0 cm), B 10 cm (1), 20 cm (2), 40 cm (3), 60 cm (4) and unplanted C 10 cm (1), 20 cm (2), 40 cm (3), 60 cm (4)

Figure 29. Bacterial isolates from the microcosms

Figure 30. Bacterial isolates from Horsea lagoon