Phytoremediation of LNAPLs and Residual Oils in the Vadose Zone and Capillary Fringe

This Thesis is submitted in partial fulfilment of the requirement for the degree of Doctor of Philosophy (PhD) in Engineering

By

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DECLARATION AND STATEMENTS

DECLARATION

This work has not been submitted in substance for any other degree or award at this or any other university or place of learning, nor is being submitted concurrently in candidature for any degree or other award.

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This thesis is the result of my own independent work/investigation, except where otherwise stated, and the thesis has not been edited by a third party beyond what is permitted by Cardiff University's Policy on the Use of Third Party Editors by Research Degree Students. Other sources are acknowledged by explicit references. The views expressed are my own.

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Acknowledgements

Dedication

To the Almighty God and to my wife Yetunde Fehintola, my mum Victoria Idowu, my late dad Jeremiah Oladoja and my children OreOluwa (Gift) Abimbola and David Olufemi without whom this thesis would not have been completed.

Abstract

The success of phytoremediation is dependent on the exposure of plants to contaminants, which is controlled by root distribution, physicochemical characteristics, and contaminant behaviour in the soil environment. Whilst phytoremediation has been successful in remediating hydrocarbons and other organic contaminants; there is little understanding of the impact of non-aqueous phase liquids (NAPLs) on plant behaviour, root architecture and the resulting impact of this on phytoremediation. The ability of plants to phytoremediate dissolved-phase contamination is well known, but the impact of Light NAPLs (LNAPLs) contaminants on plant growth and subsequent contaminant behaviour is largely unknown.

A review of current literature available on phytoremediation was conducted. Across the studies considered, sandy loam, loam, and silt loam appear to have a better organic contaminant removal than other soil types because of nutrient availability and water supply for plant growth and root development. The review shows that the NAPLs, in particular, have an effect, which suggests that there is a physical effect of NAPLs on plants rather than the chemical impact.

In this thesis, experimental works with ryegrass (*Lolium perenne*) grown under both hydroponic conditions and planted in artificial soils are presented, exploring the impact of the physical presence of an LNAPL (mineral oil) on plant growth, root distribution and oil removal. In the presence of LNAPL, a significant increase in root biomass yields and distribution, a decrease in shoot biomass and significant LNAPL removal were observed. Roots close to LNAPL sources were able to remove dissolved-phase contamination, and root growth through LNAPL sources suggest that direct uptake/degradation is possible, but any contribution from physical and direct interaction between root and NAPL has not been conclusively demonstrated here. Evidence of root redistribution in the case of LNAPL contamination across multiple adjacent pores is also presented. Although some impediment to root growth was seen at low oil contamination levels in general increased root biomass and also deeper root structures were observed as the coverage of the oil layer increased. The presence of plants corresponded to significant removal of the LNAPL in both hydroponic conditions and planted soil, whereas without plants only minimal oil loss was observed. The research has demonstrated the potential for plants to tackle NAPL contamination and shows that the phytoremediation of organic contamination is not limited to tackling only the dissolved phase, but that roots interacted with the NAPL which resulted in a significant indirect reduction in the presence of the LNAPL.

Glossary

NAPLs	Non-Aqueous Phase Liquids
LNAPLs	Light Non-Aqueous Phase Liquids
WFSS	Washed Fine Silica Sand
MOH	Mohs Scale of Mineral Hardness
LOI	Loss on Ignition
EC	Electrical Conductivity
BS	British Standards
KCL	Potassium Chloride
PLA	Polylactic Acid
3D	Three Dimensions
PVC	Polyvinyl Chloride
L/H	Left Hand Side
R/H	Right Hand Side
SCN	Scenario
CEC	Cation Exchange Capacity
ORO	Oil Red O

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Chapter 1   INTRODUCTION

Phytoremediation is the treatment of environmental contamination through the use of plants to clean up or contain contaminants in soil *in situ*. It has been used in the treatment of numerous organic contaminants, with a number of different mechanisms postulated, including plant-associated direct plant uptake and translocation into plant shoots and metabolism of the contaminants (Gobelius et al., 2017, Wang et al., 2004), volatilization of contaminants (Limmer and Burken, 2016) or directly through plant, microbe and contaminant interactions with plant's rhizosphere (dos Santos and Maranho, 2018). Phytoremediation techniques are easy to implement and cost-effective. This strategy for removing contaminants has been extensively investigated, and the initial phytoremediation research showed great promise as a cost-effective remedial strategy (Gerhardt, 2009). However, despite a good knowledge of the mechanisms of remediation, and successful laboratory studies, efforts to transfer research to the field have been challenging (Tomlinson et al., 2017). Improvements in practical implementation of this technology requires a greater understanding than currently exists concerning plants, soils, and plant-soil interactions involved in the degradation of organic contaminants. With the actual mechanisms of the multiphase interactions of plants with contaminants remaining relatively ill-defined and as such pose one of the more intriguing challenges in phytoremediation research today (Tomlinson et al., 2017).

A significant problem humanity is presently confronting is the degradation of our soils. Some disturbing facts are 24 billion tonnes of fertile or 12 million hectares topsoil are lost every year to contamination (Food and Nations, 2015), with greater consequences on food production, human and ecosystem health.

Phytoremediation success might be limited by (i) the depth of the plant's root systems, (ii) the bioavailability of the contaminants with degradation occurring mainly at the plant rhizosphere (Gent et al., 2007, Arslan et al., 2017) and (iii) the long time required for complete remediation, as a result of the toxicity effect of contaminants on plant growth and root development. Nonetheless, there are many advantages of phytoremediation. The consistency of reported positive reductions or loss of contaminant across most studies shows that phytoremediation is a worthwhile intervention for remediating or removing most organic contaminants from soil. In all cases, however, the interaction between contamination and the plant root system is central to the success of the treatment. Many organic

contaminant species are relatively insoluble in water, and so are commonly refered to as non-aqueous phase liquids (NAPLs), a separate liquid phase to groundwater which is relatively immobile, difficult to remediate and a persistent and recalcitrant source of dissolved phase contamination which pose serious management challenges (Tomlinson et al., 2017). Light non-aqueous phase liquids (LNAPLs), such as fuel oils, are less dense than water and so are commonly present in the capillary zone and around the phreatic surface. They are therefore likely to interact with plant root systems and so could be considered targets for phytoremediation but to date there has been little consideration of the impact of NAPLs on plant roots, or *vice versa*. Their physical distribution may be complex, with scenarios ranging from larger zones of continuous NAPL contamination to small unconnected individual ganglia isolated in single pore spaces with the latter becoming more common as the contaminant source ages.

Interaction between the plant rhizosphere and contaminants is essential for remediation – the potential for plants to clean up dissolved phase contamination is well established as these are mobile and easily taken up by roots or microorganisms. The ability of various species to phytoremediate oil contamination at levels where NAPLs would be expected has also been demonstrated (Hunt et al., 2018, Lu et al., 2010). However, the interaction of LNAPLs with roots, and their effect on root development and morphology, plant growth and subsequent contaminant behaviour is yet to be established. For example, NAPLs may hinder root development and instigate root avoidance of NAPL-contaminated pores or zones, but roots in close proximity to NAPLs may be able to reduce dissolved-phase contamination through mechanisms including uptake and rhizodegradation such that non-equilibrium conditions arise, causing relatively rapid dissolution of the NAPL. It may even be the case that roots and the rhizosphere interact with the NAPL to bring about its removal or breakdown directly. The impact of likely NAPL-forming contaminants on roots has been considered previously (Vázquez-Cuevas et al., 2018b), but the impact of the physical form of the chemical, and therefore the presence or absence of NAPL, was not addressed. Roots of plants in soil mixed with heavy oil were found to be coarse and injured (Naidoo, 2016, Franco et al., 2011) with increased root diameter commonly observed. Effects of oils or similar contaminants that are known

or likely to impact upon root morphology include decreased hydraulic conductivity due to heavy oil blocking flow paths (Khamehchiyan et al., 2007a), higher soil temperature due to darker soil causing increased absorption of heat (Balks et al., 2002), increased mechanical impedance (Merkl et al., 2005)[*], water deficiency causing drought stress (Merkl et al., 2005), or increased competition for nutrients such as phosphorus with microorganisms biodegrading the oil (Merkl et al., 2005). However, the actual mechanisms of the multiphase interactions of plant, soil minerals, soil pore liquid and soil pore gas with NAPL contaminants in the rhizosphere remain ill-defined. Part of this work have been reported in Oniosun et al. (2018).

1.1 Research aims and objectives

The overall research aim driving this project is to understand plant and NAPL interactions within the vadose zone. Phytoremediation of organic contaminants depends on the close interaction between plant and contaminant. For some phytoremediation treatments of contaminated soils, NAPLs may be present and prove detrimental to the process. A key question is whether the spatial distribution of roots is governed or correlated with the spatial distribution of NAPL contamination in the soil and what role does the form of contamination play in the remediation, and why this happens?. This work will explore the multiphase interactions of plant, soil minerals and soil pore liquid with NAPL contaminants at microcosm and macrocosm levels. By so doing, the investigations seek to develop an understanding of how phytoremediation can be employed for source zone treatment in the presence of NAPLs.

In addressing the project aims, some questions are to be answered:

- What is the performance and efficacy for current phytoremediation studies based on scientific findings?

- What is the impact of NAPL on physical distribution of roots at pore scale as a single plant exposed to oil?

- What is the impact of NAPL on physical distribution of root growth on a planted soil?

- How do plants impact the behavior and fate of NAPL?

Some objectives were established to address the research questions:

- Conduct a review of current literature available to compare a large number of phytoremediation studies with regards to their performance and efficacy of the studies. Compare broad trends in results focusing on scientific findings.

- Investigate and observe the impact of contaminant spatial distribution on the root development in individual plants in order to understand the interactions of roots and non-aqueous phase liquids at the small scale.

- Investigate and observe the spatial distribution of root development between contaminant spatially distributed in unsaturated, NAPL contaminated soils in order to understand the interactions of roots and non-aqueous phase liquids at the larger scale.

- Investigate and study the fate of NAPLs in order to understand whether plants clean up only dissolved contamination or if there is any effect on the NAPL itself.

In order to address the primary aims of the thesis, two series of experimental laboratory works were designed and undertaken:

Experiment 1 – Laboratory microcosm phytoremediation experiments.

The hydroponic microcosms mimicked the soil pore structure to explore the impact of NAPL on individual plant root systems with single seed. The principal aim of the hydroponic study is to explore how root growth and distribution is affected by physical proximity to an LNAPL in the pore space. Quantitative and semi-quantitative measurements for root growth, root morphology, NAPL loss, shoot height and root length were measured over time, and root and shoot biomass

determined. Preliminary results from a small part of this work have been reported in Oniosun et al. (2018).

Experiment 2 – Laboratory mesoscale phytoremediation experiment.

The larger scale mesocosms have explored phytoremediation and impact of oil on plants in artificial soil systems. The behaviour observed at the microcosm scale was explored at a larger scale in soil mesocosms to determine how the response of an individual plant could be extrapolated to the behaviour of a planted soil and its effect on LNAPL contamination. The primary aims of the mesocosms study are to observe the spatial distribution of root development in the presence of a spatially distributed contaminant in unsaturated soils and to explore how soil/plant/water relations are affected by direct physical contact with NAPLs. In particular, this study investigates the impact of an LNAPL on root growth, root distribution, and oil loss, in soil at the macroscopic scale. Also, preliminary results from a small part of this work have been reported in Oniosun et al. (2018).

1.2   Thesis overview

This thesis investigated the multiphase interactions of plant, soil minerals and soil pore liquid with LNAPL (mineral oil) contamination on two different studies. The project included the characterization of two soils modeled in the laboratory. Preliminary growth trials were conducted in the laboratory to test all the equipment and materials.

This thesis comprises of 6 chapters including chapter 1 (Introduction), dealing with the context and background of previous researches in the field of phytoremediation.

Chapter 2 – Literature Review: This chapter used an elaborate review methodology to compare a large number of phytoremediation studies with regard to their performance and efficacy. The study assessed 350 experimental (laboratory and field-based) articles concerning the success or otherwise of phytoremediation and by combining outcomes from multiple studies seeks to identify patterns and relationships not apparent at the level of individual investigations. The study compared broad trends in results focusing on variables such as site-specific soil

conditions, plant and contaminant type, soil pH and organic matter/organic carbon, plant survival, contaminant reduction and the fate of pollutants.

Chapter 3 – Materials and Methods: This chapter presents the materials used and the experimental methods adopted for carrying out the laboratory tests presented in this thesis, and the methods used to analysed and interpreted results.

Chapter 4 – Laboratory phytoremediation experiments on the impact of NAPLs on root distribution and growth, and oil Loss, at the pore-scale. This chapter presents the results of the experimental work with ryegrass *(Lolium perenne)* grown under hydroponic conditions, exploring how plant growth, root distribution and development, and oil removal are affected through direct physical contact with mineral oil, a light non-aqueous phase liquid (LNAPL), in pore-scale 3D-printed rhizoboxes. The results and analysis of root distribution and growth, and oil loss in the hydroponic system are presented.

Chapter 5 – Laboratory phytoremediation experiments on the impact of NAPLs on root distribution and growth, and oil loss, in soils at the macroscopic scale. This chapter presents the results of a series of experiments that studied the impact of NAPL contaminants on plant growth in artificial soils at the macroscopic level to identify how phytoremediation can be employed for source zones treatment in the presence of NAPLs. The results and analysis of root distribution and growth, and oil loss in the artificially prepared sandy loam and loam are presented.

Chapter 6 – Implications and conclusions. This chapter applied the knowledge gained from the pore-scale and the macroscopic experiments to discussed how plants impact the behaviour and durability of NAPLs and identify how plants can be employed for phytoremediation of source zone contamination where NAPLs are present.

Work conducted as part of this thesis has been presented at the 8th International Congress on Environmental Geotechnics in 2018. An article has also been submitted to the Journal of Environmental Management. All papers are presented in Appendix 2.

Chapter 2   Literature Review

2.1   Introduction

This chapter presents the results of a review to compare a large number of phytoremediation studies. Phytoremediation is a method for cleaning up a wide range of environmental contaminants, a technique by which plant root exudates trigger microbial activities, or induce microorganisms to produce specific enzymes to enhance rhizodegradation or a process where plants degrade pollutants through plant uptake and metabolic process. However, this technology still requires a greater understanding than is currently available concerning plant and soil components and interactions involved in the degradation of organic contaminants. The goal of this study was to compare a large number of phytoremediation studies with regard to their performance and efficacy. The study assessed 350 experimental (laboratory and field-based) articles concerning the success or otherwise of phytoremediation and by combining outcomes from multiple studies seeks to identify patterns and relationships not apparent at the level of individual investigations. The study compared broad trends in results focusing on variables such as site-specific soil conditions, plant and contaminant type, soil pH and organic matter/organic carbon, plant survival, contaminant reduction and the fate of pollutants.

The aim and objectives of the review undertaken here is to significantly extend work done in previous reviews by combining outcomes from multiple studies and identify patterns and relationships not apparent at the level of individual investigations. And to compare broad trends in results focusing on quantitative analysis of scientific issues.

2.2   Methodology

An elaborate review methodology was used to compare a large number of phytoremediation studies regarding their performance and efficacy. Analysis was used to merge results and compare broad trends in outcomes (Antman et al., 1992). The methodology has been used to review large number of phytoremediation studies on the multiphase interactions of plant, soil minerals, soil pore liquid and soil pore gas with LNAPL contaminants. A key objective of the elaborate review methodology is to minimise bias and ensure that all reasonable efforts are made to include

relevant research papers and not be restricted by language or publication status. Such bias is minimised by adopting elaborate, review search methods that seek to gather research evidence which meets pre-set selection criteria to address the specific research questions. Such a meta-analysis procedure was used to assess 350 experimental (laboratory & field-based) articles regarding the success or otherwise of phytoremediation to determined trends in outcomes.

2.2.1 Literature search and scope

Data sources on the effects and mechanism of phytoremediation of residual oil hydrocarbons on soil were collated from the scientific literature. The following sources which include electronic databases covering all available years were searched for relevant studies: Scopus, Google Advanced Scholar, Web of Science, Engineering Village, Intute, National and regional databases, Universities Websites, Dissertations and thesis databases. To enhance the search of various bibliographic databases a combination of search fields were used. In most of the databases, a search using the Regular Search Field, an Advanced Search Option or the Multi-Field Search Option was undertaken. The search was carried out with two or more search terms active, typically for each search term (loose phrase or separate words) the field where those terms appear was also defined (with "Title, Abstract, and Keywords" fields mainly used to retrieve results in the title and summary of the documents). The concept of phytoremediation was combined with descriptive terms defining the contaminant by using the 'OR' or 'AND' operator based on the database platform. Also, truncation was used for finding variants of search terms, e.g. phyto* - to find phytoremediation, phytovolatilisation, phytodegradation, etc. Truncation was also combined with wildcard symbols (e.g., '*' or '?' or '#', depending on the database), to find terms with alternative spellings, such as "phyto*volatili#ion". Proximity searching was also carried out to find relevant results if they appear in different order by using different operators, depending on the database platform, such as Scopus Pre/n and W/n. e.g., phyto Pre/2 remediation and phyto W/2 remediation or 'Remediation W/2 by the plant', etc.

The terms in Table 2-1 were searched using truncation, wildcard and proximity symbols depending on the database platform:

Table 2-1- Search terms from various bibliographic databases

Process search terms (Loose phrase or Separate words)	The combine operators	The descriptive terms
Phytoremediation of		Petroleum
Plant remediation of		Contaminated soil
Phyto* of		Residual oil
Natural phytoremediation of		Hydrocarbon
Phytovolatili?ion of		Bioremediation
Rhy?odegradation of		Oil
Rhi?oremediation of		Diesel oil
Rhi?ostimulation of	'AND' / 'OR'	Total Petroleum Hydrocarbon (TPH)
Phytorestoration of		Polycyclic Aromatic Hydrocarbon (PAH)
Phytoaccumulation of		Light non-aqueous phase liquid
Phytotransformation of		LNAPL
Plant-assisted bioremediation of		Dense non-aqueous phase liquid
Plant-assisted degradation of		DNAPL
Plant-aided in situ biodegradation of		Organic contaminant
		Chlorinated Organic
		Chloro ethylene

2.2.2 The data collected

Data were independently obtained from the selected studies to determine broad trends in outcomes based on quantitative analysis of scientific issues. Data were extracted on the following key elements: the nature, type and concentration of the contaminant, the types of remediation technique used, the success or otherwise of technique, remediation plant(s), analyses, and results summary. The properties of the soil were also collated such as organic matter, pH value and soil types. Outcomes such as plant viability & survival, contaminant reduction and the fate of contaminants were also gathered as outlined in the research aims and objectives.

The values of the added or estimated contaminants in the soil by the studies were normalized arbitrarily using the 'Dutch Values' - Earth/sediment (mg/kg dry matter) 'Intervention Values' for soil remediation (van Volkshuisvesting, 2000, Pronk, 2000).

2.2.3   Implementation of paper selection and data collection process

The study selection was made in the following steps: (i) merging search results and removing duplicate records (ii) examining title and abstracts to remove obviously irrelevant studies, (iii) reviewing full text reports to ensure relevance of studies, (iv) making final selection decision on study inclusion based on relevance of studies and (v) undertaking data collection. To help ensure that the decisions of which studies to add are reproducible, literature suggested that it is advantageous for more than one author to repeat parts of the selection or analysis process (Becker, 1998, Higgins and Green, 2011). In this review, the author and the PhD supervisors took part in the paper selection process. The data collection process, analysis and graphical plots were carried out by the author. The assessments of relevant papers were made by people who were masked to information about the article, such as the journal that published it, the authors, the institution, and the scope of the results. The final data set contained 350 papers. The majority of the selected studies are laboratory experiments (75%), followed by greenhouse studies (17%), with 8% corresponding to field research.

The studies have been classified using two different categories considering contaminant type and contaminant state. The contaminant type category defines studies as considering either 'heavy oil', 'total petroleum hydrocarbons' (TPH), 'polycyclic aromatic hydrocarbons' (PAH) or 'higher alkanes' (TPH has been separated from higher alkanes because TPH are measure of gross quantity of amount of petroleum-based hydrocarbons without identification of its constituents (Edwards et al., 1997)). These sub-categories are based on the methods for sampling and analysis of environmental media for the family of petroleum hydrocarbons as listed by the Total Petroleum Hydrocarbon Criteria Working Group Series (TPHCWG) who group hydrocarbon compounds into a small number of fractions having similar transport properties. i.e., compounds having similar leaching factor (LF) and volatilization factor (VF) values ranging one order of magnitude for both aromatics and aliphatics (Gustafson, 1997). The range represents the fractions in equivalent carbon number (EC). It is a reasonable level of accuracy, given the simplifying

assumptions and uncertainty inherent in modelling the behaviour of hydrocarbons in soils and is consistent with other approaches dealing with complex mixtures (Bischoff et al., 1991, Peterson, 1994). The second categorisation is related to state of the contaminants and groups the studies as considering DNAPL, LNAPL, or dissolved contaminants. The sub-categories were determined based on density and site concentrations compared to water solubility of the petroleum hydrocarbons contaminants for the actual study conditions. The two categories have been chosen because there has not ever been any consideration of phytoremediation tackling free-phase contamination explicitly. Mineral oil (light and heavy or white mineral oil (petroleum) or saturated hydrocarbons)) have been classified under TPH (for the contaminant type category) and under LNAPL (for the contaminant state category). The breakdown of studies for each category are shown in Table 2–2.

Table 2-2 - The reviewed studies contaminants grouping by the review

Contaminant Type	Percentage of Studies (%)
Heavy oil	44
Total petroleum hydrocarbons (TPHs)	27
Polycyclic aromatic hydrocarbons (PAHs)	21
Higher alkanes	8
Contaminant State	**Percentage of Studies (%)**
Light non-aqueous phase liquids (LNAPLs)	67
Dense non-aqueous phase liquids (DNAPLs)	26
Dissolved (Concentration below solubility limit)	7

The analysis of the collected data has been structured to begin with the study of the effects of LNAPLs and residual oils on physical, mechanical and chemical properties of the soils and then, the impacts of LNAPLs on the plant behaviour and survival. This is then followed by consideration of the phytoremediation and plant accumulation of LNAPLs and residual oil and the implications for practical application.

2.2.4   Limitations and exclusion criteria

The review does not cover intervention techniques focusing on metals pollutants and mathematical models. Across studies, the depth of hydrocarbon contaminants

in the soil varied but the effects of contaminant depth were not analysed in the review. Moreover, the results of current research regarding plants used are difficult to interpret, and causative relationships are hard to establish because many studies involve plant species with many phenotypic differences. Growth conditions are not reported because the condition varied widely between studies. Contaminant levels and concentration are not reported; however, they have been used in the review sub-categories which were determined based on density and site concentrations compared to water solubility of the petroleum hydrocarbons contaminants for the actual study conditions.

2.3   Impact of NAPLs and residual oil on soil properties

2.3.1   NAPLs and residual oil physical effects on soil properties

NAPL contaminated soil saturates more gradually when compared to similar uncontaminated soils because of the nonpolar characteristics of the oil which causes contaminated soils, especially with high viscosity contaminants to exhibit poor or restricted water infiltration (Boulding and Ginn, 2016, Alrumman et al., 2015). The clogging of the pore space restricts water from reaching the rhizosphere and blocks interchange of gases in the soil. Some studies observed aggregation of NAPL contaminated soil, while others reported soil structure disintegration and dispersion following contaminant release. The non-polarity of oil causes the observed effects. It was suggested that the non-polarity of oil further restricts the infiltration of water and oxygen into the soil profile (Fernandez and Quigley, 1985, Khamehchiyan et al., 2007b). The restricted infiltration of water and oxygen can also lead to a decrease in the Atterberg limits in clay soil with increasing LNAPL contamination and there is a reduction in maximum dry density and optimum water content of the soil (Ghasemzadeh and Tabaiyan, 2017). Therefore, with restricted water infiltration in contaminated soils, the ability of the soil particles to be densely compacted is usually inhibited by capillary forces. Moreover, with the increasing oil content, the capillary tension force will decrease, (Delage, 2013, Fernandez and Quigley, 1985) due to the hydrophobic nature of oil that can inhibit water from making contact with the soil particles, this can create a soil crust which can also restrict water and oxygen infiltration into the soil profile and hinder plant development.

Direct shear tests to investigate the impact of NAPL contamination on the strength properties of soil show an extreme reduction in cohesion with increasing oil addition, especially in fine soils (Delage, 2013, Alrumman et al., 2015). Studies suggested that during infiltration of an unsaturated soil only capillary forces are involved. Therefore, the reduction in shear strength following NAPL contamination is likely due to mechanical interactions caused by the viscosity of the pore fluid and the physical-chemical impacts caused by the reduced dielectric constant (Ratnaweera and Meegoda, 2005).

It has been reported that following a release or oil application to the soil, the moisture content may increase or decrease depending on the soil properties (Benka-Coker and Ekundayo, 1995, Khamehchiyan et al., 2007b). Phytoremediation studies suggested that residual oil will pool in poorly drained low areas in fine-grained soils, displacing pore gas and causing the soil to saturate (Marín-García et al., 2016). In contrast to clayey soils, sandy soil presented severe water repellency due to relatively small surface area and low organic content, and are more prone to develop soil water repellency than finer soil (Oostindie et al., 2017). Some well aerated NAPL contaminated soils have a propensity to dry out and be susceptible to erosion as a result of increase in water repellency which may result in very long infiltration times causing more run-off, soil erosion, and a reduced capacity of the soil to support vegetative growth for phytoremediation (Huang et al., 2018) .

Therefore, the adequate addition of organic residues leads to the synthesis of complex organic compounds that hold soil particles together which help to maintain a loose, open, granular condition (Meena et al., 2015, Mbuthia et al., 2015) . Water is then better able to infiltrate and percolate downward through the soil. Organic matter promotes soil granulation thus maintaining large pores through which water can enter and percolate downward which permit the better exchange of gases between soil and atmosphere and thus enhances plant growth (Senesi and Loffredo, 2005).

2.3.2   NAPLs and residual oil chemical effects on soil

Studies indicate that inhibition of the nitrification of ammonium-N to nitrate-N is one of the key effects of NAPLs and residual oil on the soil as a result of contaminants depressing the activity of the microorganisms, such as low

temperature and a deficiency of water leading to low oxygen and unfavourable conditions for nitrifying bacteria (*Nitrosomonas*) (van Kessel et al., 2015). The extractable potassium and phosphorus levels in the soil may also become inhibited after contamination due to the reduction in nutrients availability following oil contamination (Inglezakis et al., 2017, Ramadass et al., 2015). Usually, there is a high demand for oxygen by microorganisms following LNAPL addition to soil, and anaerobic conditions may arise as the oxygen levels in the oil contaminated soil become diminished (Marshall, 2012, Norris, 2017). Literature suggests that increased solubility of iron and manganese in NAPL contaminated soil may also contribute to anaerobic or reduced nutrients conditions (Alarcón et al., 2008) that causes inhibition to plant developments.

2.4 Impact of LNAPLs and residual oil on plant behaviour and survival

2.4.1 Effects of contaminants on plant growth and developments

Organic contaminants are known to inhibit plant growth (Buss et al., 2015, Franco et al., 2011, Vázquez-Cuevas et al., 2018c). The primary inhibiting factors are considered to be the toxicity of lower molecular weight compounds and the hydrophobic properties of the higher molecular weight compounds limiting the ability of plants to absorb water by decreasing the field capacity of soils and nutrient contents (Inglezakis et al., 2017, Norris, 2017).

The effect of contaminant presence on plant growth in the reviewed literature was categorised based on seed emergence and relative plant yield data, using the descriptors detailed in Table 2–3.

Table 2-3 – Categorisation of effects of LNAPLs and residual oil on plant growth *by fresh/dry weight of shoots and leaves, shoot height, root length, fresh/dry biomass of shoot, fresh/dry biomass of root, chlorophyll content or soluble protein content. The contaminant effect descriptor was based on the review statistical analysis.

Contaminant effect descriptor	Seed emergence relative to control (days)	Plant yield*: (%) of control
Significantly Promotes Plant's Growth	Over 5 days early	Over 120
Moderately Promotes Plant's Growth	3-4 days early	110 - 120
Slightly Promotes Plant's Growth	1-2 days early	100 – 110
No Reported Inhibition on Plant's Growth	The same as control	No apparent change
Slightly Inhibits Plant's Growth	1-2 days delayed	70 – 99.5
Moderately Inhibits Plant's Growth	3-4 days delayed	40 – 70
Significantly Inhibits Plant's Growth	Over 5 days delayed	0 – 40

The results of this meta-analysis are shown in Figure 2–1, subdivided by each contaminant state considered (as defined in Table 2-2).
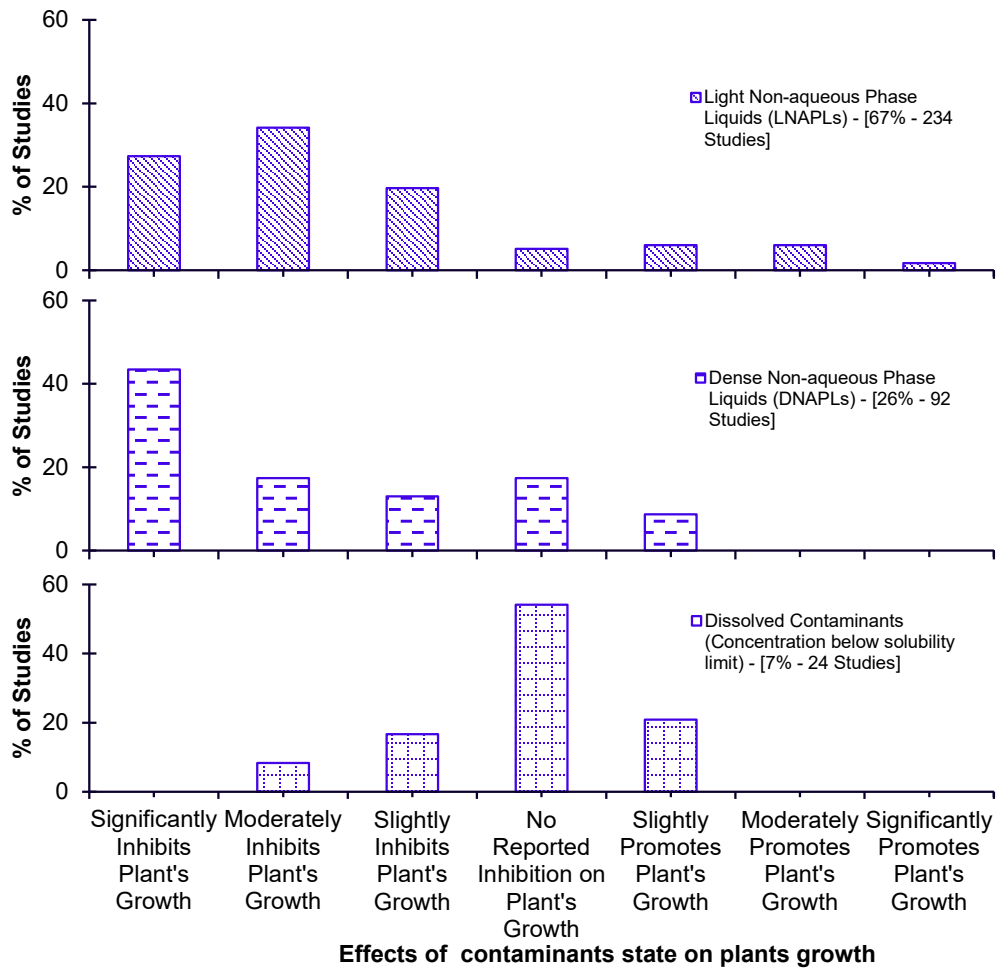
Figure 2-1 - The effect of contaminant states on plant growth and survival

Figure 2–1 indicates that both LNAPLs and DNAPLs have the potential to affect plant growth, with 81 % and 73 % of studies indicating some inhibition (i.e. this covers the three worst categories) of plant growth and 27 % and 43 % of studies indicating a significant impact from LNAPLs and DNAPLs respectively. Studies suggest that the disparity between solubilities of the constituents in NAPL has significant implications for the behaviour of these contaminants and their potential to contribute to plant development. First, some NAPLs compounds are toxic predominantly because of the relative solubility in water and volatility, e.g. xylenols, cresols and naphthols (Mateas et al., 2017, Malk et al., 2014). Literature suggests that the lower molecular weight (lighter fraction) hydrocarbons are more toxic than the medium and heavy fractions. For example, among the chlorinated

paraffins (CPs), the longer chains (LCCPs, C >17) are insoluble in water and are therefore metabolised slowly making them less toxic to plant growth (Parrish et al., 2006). Moreover, the soluble aromatic compounds appear to be more toxic because they degrade more slowly and are more volatile, and can move quickly through the cell membranes, causing harm to the plant and consequentially inhibit growth (Vázquez-Cuevas et al., 2018b). Second, the significant inhibition in plant growth has also been attributed to the presence of medium and higher molecular weight NAPLs in the soil, and even though they were reported to be less toxic, they did affect soil fertility and interchange of gases by clogging pore space. (Chen et al., 2013, Ramadass et al., 2015). This is because with low solubility and low volatility, the medium and higher molecular weight NAPLs are persistent in the environment (Vázquez-Cuevas et al., 2018b). Moreover, the exposure of the medium and higher molecular weight NAPLS to sunlight will increase their toxicity through acid and peroxide formation (Franco et al., 2011, Wolfe, 2013a). Compounds that are less soluble also tend to be more recalcitrant to phytoremediation and tend to cover the surface of the soil particles thereby reducing the electrostatic interaction with some essential nutrients (K^+, Ca^{++}, NH_4^+) for the plants (Atlas and Bartha, 2012, Wolfe, 2013a). Under these conditions, the plant will experience a metabolic imbalance caused by a condition of oxidative stress which hampers the ability of the cell to regulate the chemical processes (Romeh, 2017, Hou et al., 2015).

However, in Figure 2–1, the presence of dissolved contaminants with concentrations below the solubility limit appears to be less toxic to the plants with only 25% of studies report any noticeable inhibition on plant growth (i.e. this covers only two of the worst categories). This is possibly due to the fact that they degrade more rapidly (Wolfe, 2013a, Wenzl et al., 2006). Therefore, the NAPLs (lower, medium or higher molecular weight) have potential to contribute to the adverse effect on the plant growth due to high toxicity of their soluble components and can form a hydrophobic layer around root and seed that reduce nutrients absorption and oxygen. The NAPLs, in particular, may have an effect on phytoremediation, the review has indicated that both the medium and higher molecular weight NAPLs are persistent in the environment which suggests that there is a physical effect of NAPLs on soil and plant development rather than just the chemical impact.

70 % of studies reported a reduction in fresh or dry weight of shoots and leaves of plants with increasing contaminant concentration. Palmroth et al. (2006) and Zhang et al. (2010) found that the presence of high levels of contaminants negatively affect the growth and health of plants due to the reduced permeability of solutes induced by the introduction of pollutants. It was noted that clogging of some inter-particle spaces by contaminants, reducing the available inter-particle spaces for water seepage, is considered to be one of the reasons for this. Some physiological processes in plants, such as transpiration, respiration, and translocation are also affected adversely by oil contamination. It has been established for some time that heavy oils have herbicidal properties and direct effects on plants are usually very distinct (Baker, 1970). Leaves may display signs of phytotoxicity such as chlorotic or necrotic lesions and stunted growth (Adelusi Oyedeji et al., 2015, Agamuthu et al., 2010). Basumatary et al. (2012) and Feng et al. (2016), studying *Cyperus odoratus* and paddy rice in the presence of organic contaminants, also reported that there is a reduction in growth and development in terms of height, leaves and biomass production, which are usually characteristics of plant stress.

Plant roots can be directly or indirectly affected by the presence of heavy oil in the soil. Studies observed that roots of plants growing in pots after the soil was mixed with heavy oil were coarse and injured (Merkl et al., 2005, Phillips et al., 2012), with the roots of *Brachiaria brizantha* (Hochst. ex A. Rich.), *Stapf* (Poaceae), and *Cyperus aggregatus* (Willd.) were severely damaged and death resulted in contaminated pots. Root death or injury can be related to intermediate compounds – alkanoic acids, phenols and aromatic acids that can potentially form when heavy oil is biodegraded by microorganisms in soil (Atlas and Bartha, 2012, Vázquez-Cuevas et al., 2018b). Also, petroleum hydrocarbon-contaminated soil may become anaerobic and reducing conditions can result in increased solubility of iron and manganese to the level that the potentially phytotoxic elements are sorbed by roots and plants leading to growth deterioration in the form of thicker roots and decreased shoot height and mass (Alarcón et al., 2008, Dodangeh et al., 2018).

The results of the meta-analysis are also shown in Figure 2–2, subdivided by contaminant type (as defined in Table 2–2).
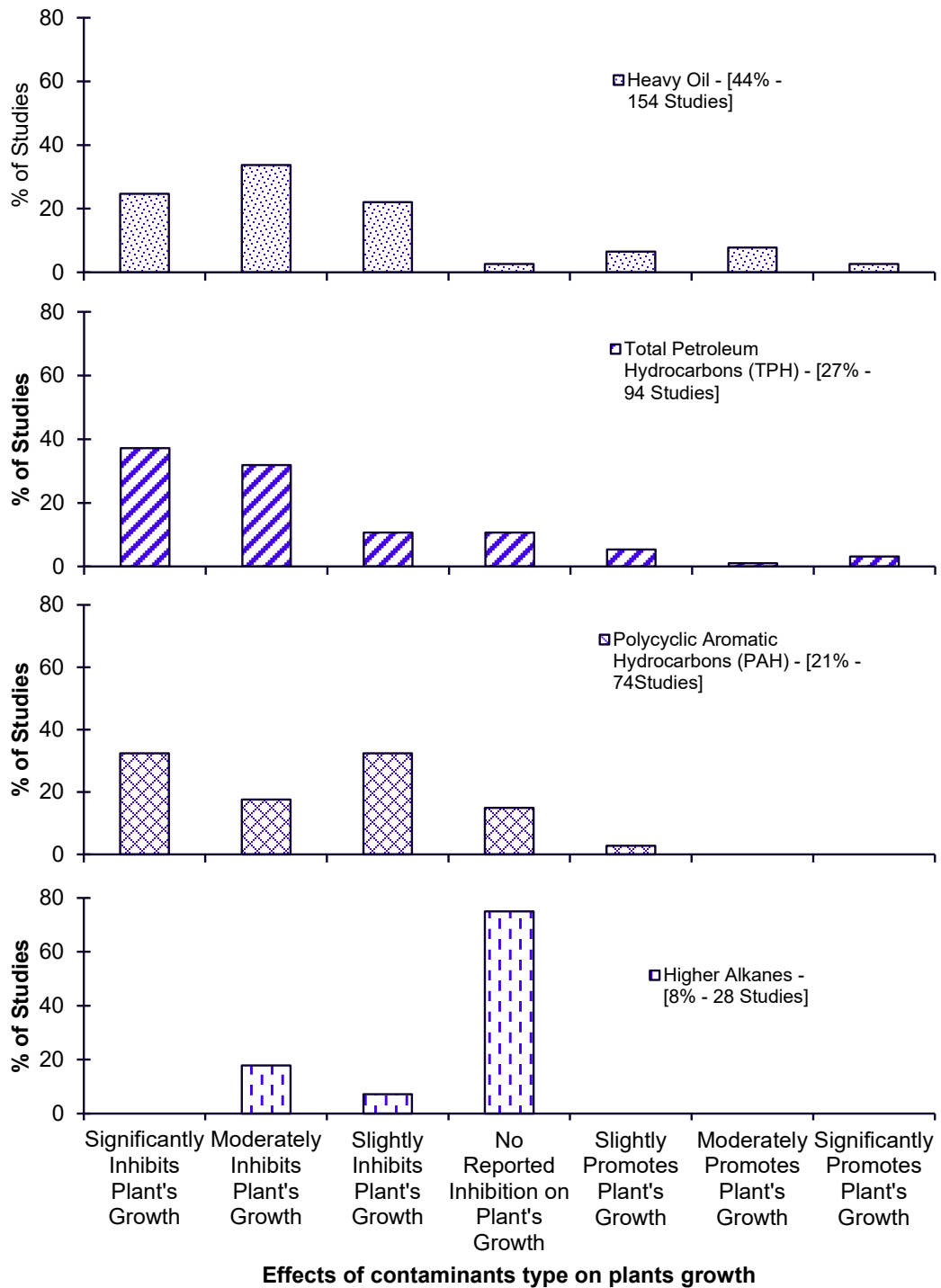
Figure 2-2 - The effect of contaminant type on plant growth and survival

Considering Figure 2-2 it is clear that heavy oil, total petroleum hydrocarbons (TPH) and polycyclic aromatic hydrocarbons (PAH) have potential to affect plant growth with 25, 37 and 32 % of studies respectively reporting a significant impact on growth

and 34, 32 and 18 % of studies respectively indicating moderately effects on growth and development of plants. The reports of significant and moderate impacts for the TPHs and PAHs are perhaps due to the toxicity of their soluble components on the plants (Rice et al., 1976, Wolfe, 2013a, Wenzl et al., 2006). Dissolved forms of these groups contain components that are toxic to the plant growth as water solubility determines the possible routes of its entry into the plant cells (Ramadass et al., 2015). Moreover, low solubility and low volatility of the heavy oil contaminants makes them persistent in the environment and more recalcitrant to phytoremediation.

From the reviewed literature, the reduced percentage growth and development for heavy oils, TPHs and PAHs may be due to a decrease in the soil organic carbon and total nitrogen after contamination (Al-Surrayai et al., 2009, Sutton et al., 2014). The loss of nitrogen could have resulted from the conversion of nitrate ions to gaseous forms of nitrogen through a series of subsequent biochemical reduction reactions as a result of denitrifying bacteria associated with the biodegradation process. Moreover, the hydrophobic nature of contaminated soil can also act as a physical barrier leading to a reduction in oxygen and water, and poor accessibility of nutrients especially nitrogen to the rhizosphere. The decrease in soil available organic carbon may be due to the facts that soil organic matter is capable of enhancing the adsorption of organic chemicals and reducing the bioavailability of pollutants which may lead to a decrease fraction of organic carbon available for microorganism degradation. (Chen et al., 2015).

In general, the higher alkanes have no impacts as reported by 75% of studies (Figure 2-2). These are chemicals which have been reported to be nearly non-toxic (Benelli et al., 2017, Esbaugh et al., 2016) and are insoluble in water and therefore metabolised more slowly from solution (Forth et al., 2017).

2.4.2 Toxicity effects of different types of LNAPLs and uptake mechanism

The toxicity of LNAPL components on plants and animals has been considered in many studies, with aromatic hydrocarbons and then naphthenes being reported as the most toxic (Rice et al., 1976, Wolfe, 2013b). Alkanes were recorded as the least toxic (Baker, 1971, Ottway, 1971) while pentadecane and higher alkanes are nearly non-toxic (Wolfe, 2013b). Moreover, the low molecular weight members of the alkanes are also most volatile, e.g., ethane. The interchange of the aromatic compounds with methyl increases their toxicity (Kauss et al., 1973, Rice et al., 1976), but also reduces their solubility in water and volatility meaning that toxicity can often be inversely correlated with water solubility. Joner et al. (2004) reported that monocyclic aromatics are the cause of serious injury to plants, and polycyclic aromatics cause long-term harm. Examples of polycyclic aromatic hydrocarbons in crude oil are 3,4-Benzopyrene, alkylbenzanthracenes, Benzo(d,e,f)chrysene and 1,2-Benzanthracene (Wenzl et al., 2006) and are classified as being carcinogenic by International Agency for Research on Cancer (IARC, 1972). Substituting nitrogen or oxygen in aromatic compounds in crude oil are also highly toxic and are quite hazardous because of the relatively high solubility in water, examples are pyridines, xylenols and cresols and their derivatives (Wolfe, 2013b). Toxic compounds with high water solubility can also occur as intermediate compounds of microbial degradation; examples are phenols, aromatic acids and alkanoic acids (Joner et al., 2001). Nonetheless, the intermediate products of the degradation are rapidly degraded further to carbon dioxide by various microbial species in the presence of oxygen (Siddiqui et al., 2001). Exposure to direct sunlight may also increase the toxicity of NAPLs through acid and peroxide formation (Kauss et al., 1973, Wolfe, 2013b).

The degradation process of organic contaminants by plant enzymes occurs both in root and shoot tissue. Usually, the movement of organic contaminants into plants occurs via the liquid phase of the soil via the following pathway as illustrated in Figure 2–3 (Simonich and Hites, 1995).
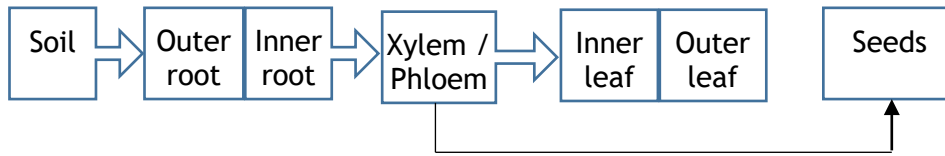
Figure 2-3 Uptake mechanism and translocation of organic contaminants

The plant enzymes can catabolize organic contaminants by wholly mineralising the organic pollutants to inorganic compounds, e.g., water and carbon dioxide, or by partly degrading them to stable intermediate compounds that are accumulated and stored in the plant tissues (Arslan et al., 2017, Chirakkara et al., 2016). The reviewed studies explained that the process of contaminants being taken up into plant roots consist of three parts. Firstly dissolved in the aqueous solution within the rhizosphere; secondly, adsorbed on the root (Li et al., 2014b); and thirdly penetrating into root tissues (Hurtado et al., 2016).

From the review literature, Vervaeke et al. (2003) observed a significant decrease of 79% in the mineral oil concentration in the planted disposal site, with the mineral oil degradation under willow was most pronounced in the root zone.

2.4.3   NAPLs and residual oil effects on seed emergence

In some phytoremediation studies, a delay in seed emergence and stunted growth was observed following oil contamination (Golan et al., 2016, Spiares et al., 2016). Adieze et al. (2012) suggested that contaminants can cause a film of oil to form around the seed which would act as a physical obstacle, prohibiting or reducing both water and oxygen transfer to the seed. As soil moisture conditions are affected by heavy oil contamination and due to hydrophobic nature of petroleum, water spreads inhomogeneously in the contaminated soil which can inhibit water from reaching the seed thereby adversely affecting seed emergence (Spiares et al., 2016, Xu et al., 2018).

2.4.4 Plant tolerance to NAPLs and residual oil contamination

Plant tolerance to organic contaminants is dependent on plant species, and also, on the type and concentration of the contaminants in the soil (Wu et al., 2015, Lv et al., 2016). The reviewed studies have assessed seed germination and plant yield as a means of assessing plants tolerance to organic contaminants. Most species with few stomata (i.e. tiny openings that allow plants to exchange gases necessary for cellular processes) and a thick cuticle (i.e. the water-impervious protective layer that limits water loss) have been reported to be more tolerant of NAPL (Edema et al., 2011, Heredia-Guerrero et al., 2018). The thick cuticle and few stomata are advantageous in reducing transpiration. Moreover, in extremely wet conditions, the few stomata and thick cuticle will prevent the excess water from leaching out nutrients (Fernández et al., 2017).

Literature also suggests that perennial plants with large food reserves are more tolerant than plants lacking underground reserves such as most annual plants (Barrutia et al., 2011, Bento et al., 2012). Plants with large underground food reserves in root systems (stolons and rhizomes) are more likely to; first, have an alternative supply of water and nutrient to mitigate the toxic effects and the inhibited supply of solutes and nutrients following oil contamination (Helga et al., 2018); second, plants are able to accumulate the organic contaminants into the storage system (Bonanno and Vymazal, 2017); third, enzymes are exuded (such as oxidoreductases) which may be able to metabolise organic contaminants (Hurtado et al., 2016); and fourth the enrichment of rhizosphere microorganisms which may be involved in the degradation processes (Macherius et al., 2014). Therefore, perennial plants survive contamination by underground rhizomes with studies suggesting that these species are the least sensitive when compared with annual vegetable crops that lack underground rhizomes or storage organs. These include amaranth, green pea, sorghum, maize, southern crabgrass, bermudagrass and alfalfa planted in oiled soil.

Tree species can also tolerate organic contaminants in soil. It was indicated that tropical woody plants, kiawe, milo, and kou showed less stress to organic contaminants as the seeds planted in contaminated soil did not suffer any adverse effects, and there was even, a decrease in the germination time (Pérez-Hernández et al., 2013, Bento et al., 2012). Studies suggest that this is linked to an increase in

the adsorption of water into the seminal cover of the seeds and the endosperm that causes enzymatic changes at the start of the germination (Zhang, 2013). Moreover, upon going through stress, the trees respond to organic contaminants in the soil by changes in rhizosphere exudates, which causes a modification in the rhizospheric activities and microflora composition which then results in the degradation of the contaminants, hence reducing the layer of contaminants around the roots (Pérez-Hernández et al., 2013). The tree species also tolerate organic contaminants due to the deep roots which can penetrate beyond contaminated layers after they are well established, their long life and biomass production (Rivera-Cruz et al., 2016).

2.5 Impact of soil on phytoremediation of organic contaminants

2.5.1 Impacts of organic matter and different soil types on phytoremediation

Optimal plant growth is a major factor influencing rhizodegradation of hydrocarbon contaminants. Soil organic matter content directly or indirectly affects the availability of nutrients for plants used in phytoremediation, by functioning as a source of N, P, S and many other substances through its mineralization by soil microorganisms. Organic matter promotes good soil structure, thereby improving tilth, aeration and retention of moisture and increasing buffering and exchange capacity of soils in the hydrocarbons contaminated soil rhizosphere and also, influencing the supply of nutrients from other sources, for example, as an energy source for N-fixing bacteria to reduce the adverse impacts of organic contaminants (He et al., 2015).

Organic matter has a profound effect on the structure of many soils. The soil organic constituents favourably influence the permeability, aeration and water-holding capacity of soil (Zhou et al., 2018). Usually, when compared with the phytoremediation of the contaminated soils with low organic content, soil with a high organic matter will have a higher oil holding capacity. The results of a laboratory phytoremediation experiment by Rowell (1976) on soils with different organic matter contents showed the fastest rate of oil decomposition in the soil

with high organic matter, while decomposition of oil was slow and low in soil with a low organic matter content.

A soil texture triangle analysis (Shirazi and Boersma, 1984) is presented in Figure 2–4 (overlaid the results from the 350 phytoremediation review studies) to explore if soil texture has any bearing on contaminant removal.
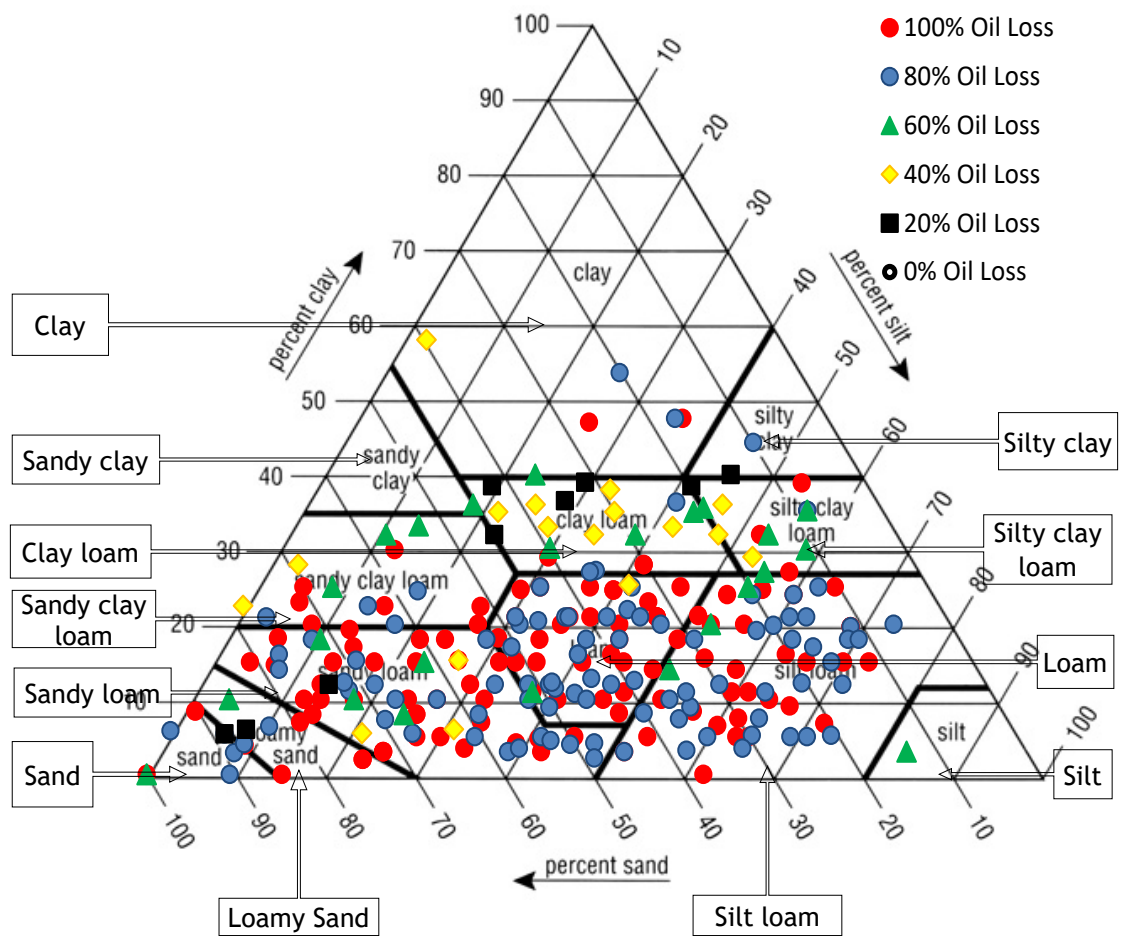
Figure 2-4 - The effect of soil types on contaminant removal based on the overlaid results from the 350-phytoremediation reviewed literature

Moreover, Figure 2–5 shows the effect of soil texture and the study duration to explore if soil texture and study time have any bearing on contaminant removal.
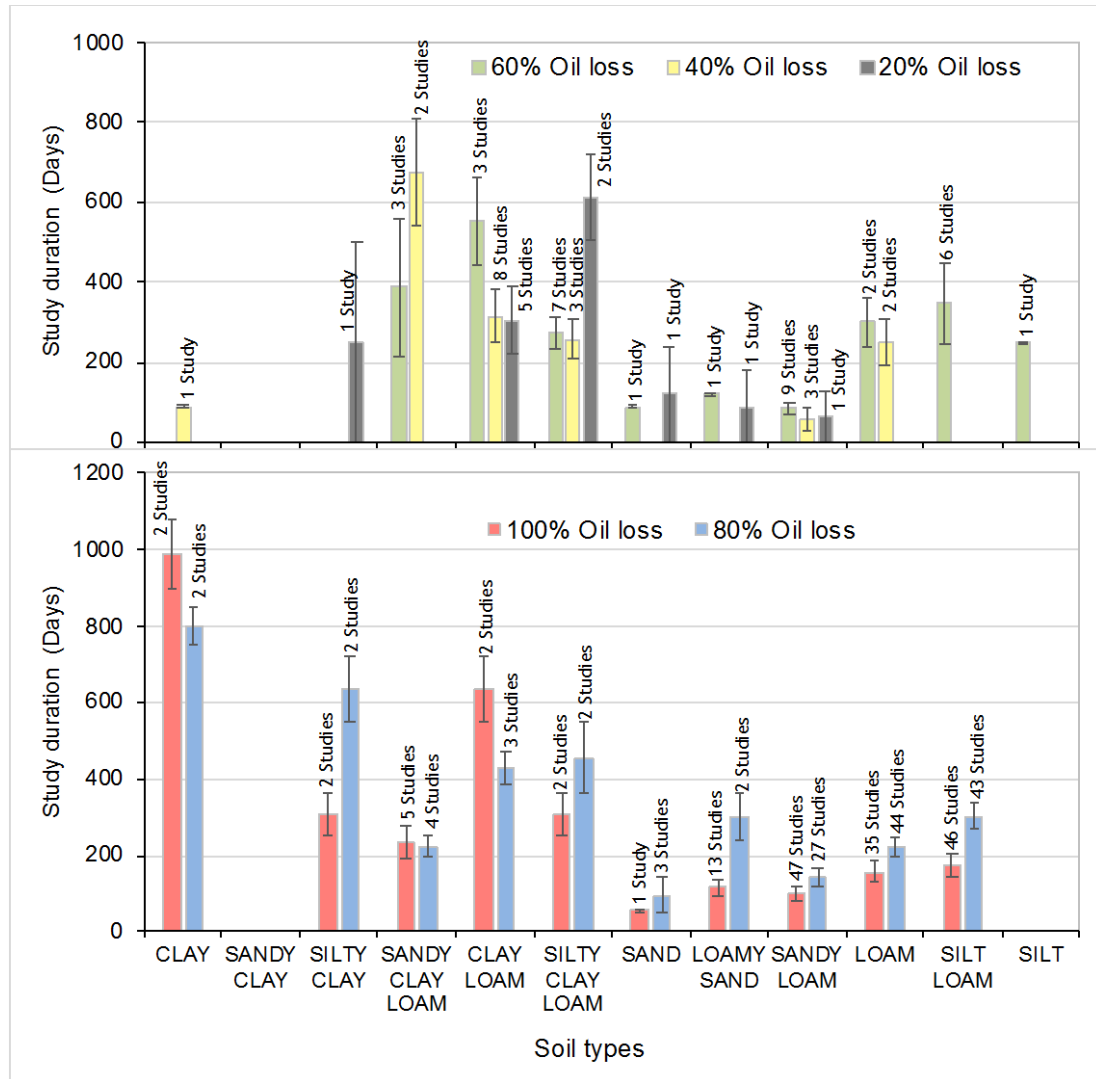
Figure 2-5 – The effect of soil types and study durations on contaminant removal based on the reviewed literature. Error bars represent ± one standard error of the mean.

It appears from Figure 2–4 that sandy loam, loam and silt loam have a better contaminant loss than other soil types, with 85% of studies reporting 80% or more oil loss having these soil types. Also, from Figure 2–5, the rate of contaminant removal is better for the loam soils (loamy sand, sandy loam, loam and silt loam) than clay soils, with 141 studies suggested that there was 100% contaminants removal in less than 200 days. Moreover, this also implies that remediation in clay is effective but slow, whilst in clay loam it's faster but not always as effective Figure 2–5. This observation indicates that there is better root development for the sandy loam, loam and silt loam soil types perhaps due to increased nutrient availability

and pore water supply for plant growth and root development when compared to more fine-grained soil. As noted by Gadi et al. (2016), optimum water retention in the root zone is considered to be one of the essential requirements for plant growth and yield. As suggested by the effects of plant root on soil-water retention curves (Leung et al., 2015), sandy loam and loam are at optimum suctions values, whereas a soil of high plasticity will only desaturate at high suctions. This implies that soil containing a higher percentage of fine particles will not favour the growth of the plant species of interest for phytoremediation.

The reviewed studies reported reduced seed germination and low biomass (shoot and root) production for plants grown in contaminated sandy soils as compared to contaminated loamy soils indicating a loam texture is a better soil for phytoremediation. For example, Afzal, (2011) and Al-Sanad, (1995) reported that during hydrocarbons phytoremediation, loamy soil provided the best medium for plant growth, and sandy soil the worst. In addition to loam soils having smaller pores when compared with sandy soils, which favours the optimum water retention in the rhizosphere, differences in organic carbon content and cation exchange capacity of the soil also have an impact. Loam soil that is high in organic matter has a high cation exchange capacity (about 250 to 400 cmol kg^{-1}). However, clay soil has a low CEC (about 3 – 15 cmol kg^{-1}), while sand and silt soils have a negligible CEC (because they are not charged particles). Therefore, loam soil usually demonstrates high nutrient levels that were possibly leading to better plant growth (Kaakinen et al., 2007, Sharma et al., 2015). Sandy soil texture is typically more porous, warmer, drier, and less fertile than soils with a medium texture thus limiting plant growth in phytoremediation studies (Ouzounidou et al., 2015).

Regarding the effect of clay content on oil phytoremediation and degradation, it was observed that reduction in oil content was inversely correlated with clay content because clay content influences the properties of soil and, a high clay content may reduce moisture permeation and aeration and consequentially slow down decomposition and impede phytoremediation (Ratnaweera and Meegoda, 2005, Nawab et al., 2016).

Moreover, soil water content, which is affected by the organic matter content and clay content, can influence the amount of oil that is held in the soil pore space and therefore affect oil phytoremediation. NAPLs must displace the existing pore fluids

to enter into the pore space; the air is displaced from the vadose-zone pores and water from saturated zone pores (Delage, 2013). Also, soil moisture conditions are affected by heavy oil contamination, and due to the hydrophobic nature of petroleum, water spreads nonuniformly in the contaminated soil, which can inhibit solutes and nutrients from reaching the rhizosphere. Therefore, the stress experienced by plants due to lower solutes and nutrients may inhibit plant growth, leading to an increase in root diameter and reduced root length and consequentially affects phytoremediation (Liu et al., 2014)

Figure 2–6 presents the overlaid results from the 350 phytoremediation review studies relating organic matter and soil pH with contaminant loss from the 350 reviewed studies. The Effect of NAPLs and variation between the chemicals were not analysed. The range of pH is found to be between 5.0 – 8.5 with 85 % of studies within a pH range of 6 - 8. For studies reporting ≥ 80 % contaminant loss, 20 % of these studies have pH less than 6.8. These findings suggest that within acceptable ranges for plant growth, the soil organic matter and pH have little impact on contaminant removal. Some of the reviewed studies also confirmed that oil phytoremediation was slow under acidic soil conditions, and an increase in pH of soil from 6.0 to 7.8 increases the decomposition rate and plant rhizosphere degradation were optimal at a soil pH of 6.0 – 7.5 (Malhi, 1990, Willscher et al., 2017).
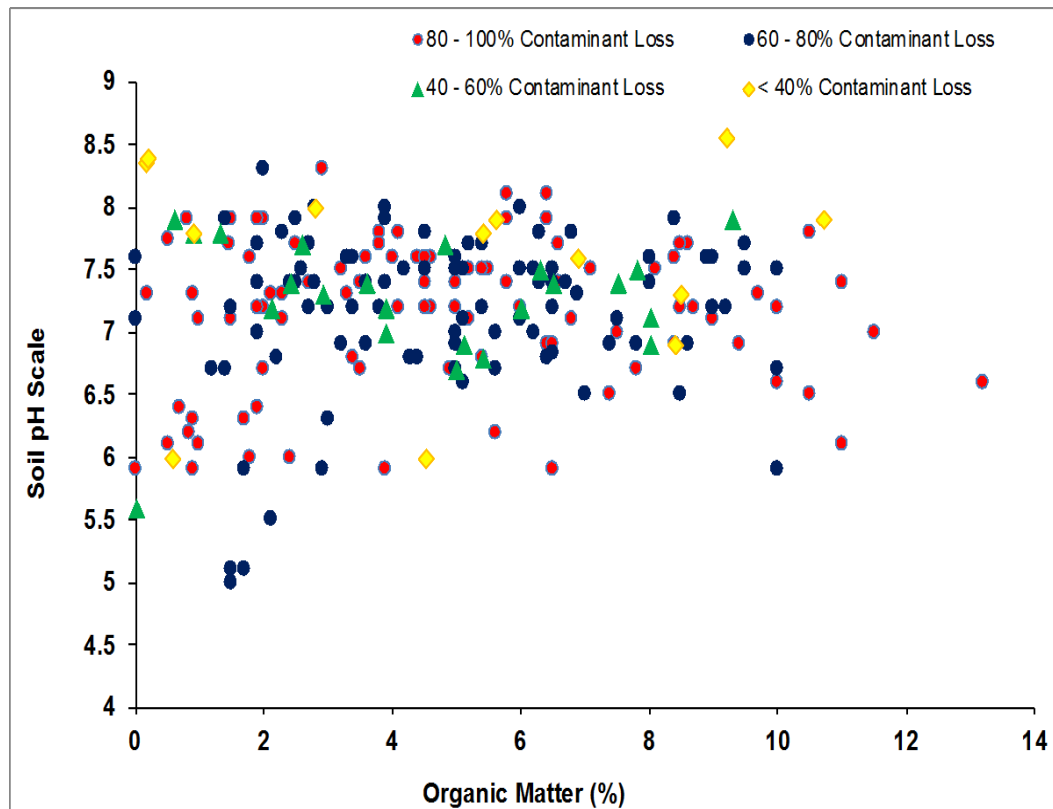
Figure 2-6 - The impacts of pH and organic matter on contaminant loss

2.6 Phytoremediation and plant accumulation of NAPLs and residual oil.

Phytoremediation of organic contamination in soils may arise as a result of contaminant uptake into plant tissues (Ucisik and Trapp, 2008, Hurtado et al., 2016), phytovolatilization (Limmer and Burken, 2016) or biodegradation of contaminants by rhizosphere-associated microorganisms (Ghattas et al., 2017). Less than 1% of the literature explores explicitly the impact of phytoremediation on NAPLs, therefore NAPL phytoremediation requires much more investigations than currently available.

The meta-analysis of contaminant removal by plants and microorganisms by contaminant state and contaminant type considered (as defined in Table 2-2) are shown in Figure 2-7 and Figure 2-8 respectively. In Figure 2-8, the heavy oil and TPH has been separated from higher alkanes because heavy oil and the TPH are measure

of gross quantity of amount of petroleum-based hydrocarbons without identification of its constituents. The heavy oil and TPH values still represent a mixture (Edwards et al., 1997, WHO., 1982).
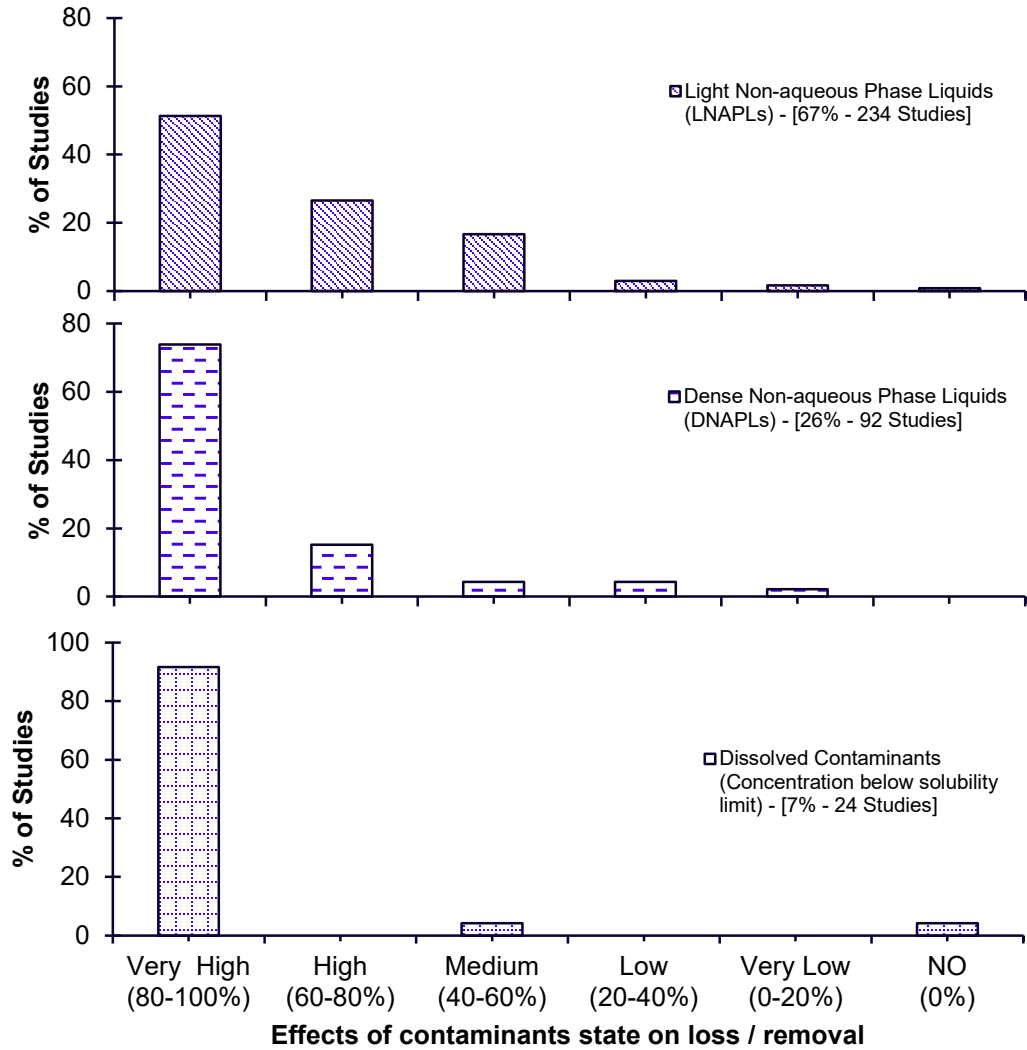
Figure 2-7 – Effects of contaminant state on LNAPLs and residual oil concentration loss or removal
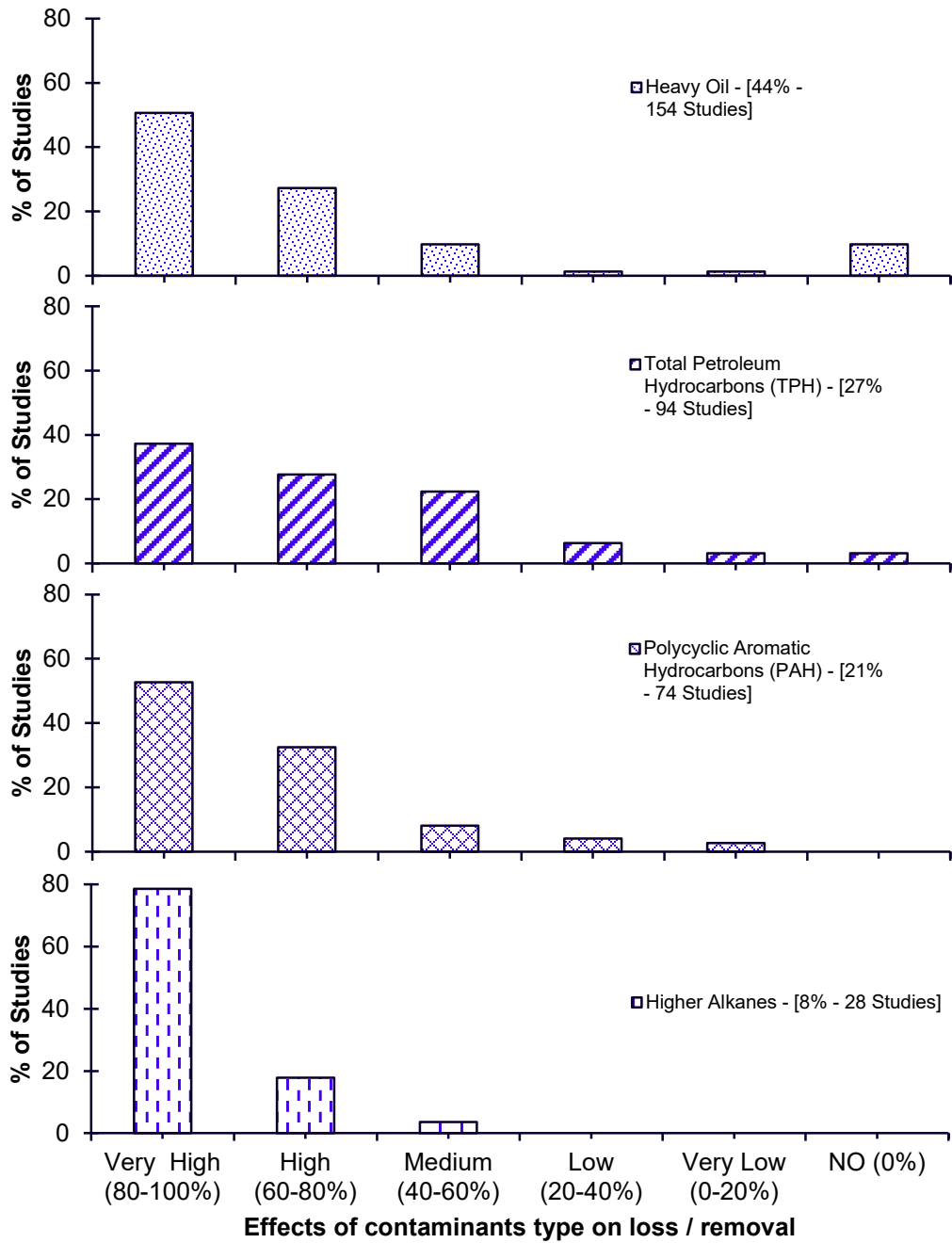
Figure 2-8 - Effects of contaminant type on LNAPLs and residual oil concentration loss or removal

On the above Figure 2‑7 and Figure 2‑8, it is fairly clear that DNAPLs and LNAPLs, and certain specific contaminant groups (Heavy oil, TPH and PAH) do appear to have

more of a spread-out profile (indicating less effective removal) than the dissolved contaminants and the higher alkanes. Both the higher alkanes group (100 %) and dissolved group (96 %) have the highest number of studies indicating a medium to very high contaminants loss. Moreover, Figure 2–1 and Figure 2–2, supported the results, with 75% of the studies indicating no reported inhibition and slightly promotes plant growth for the higher alkanes and the dissolved contaminants groups respectively. Therefore, the better plant growth and root development might have played a significant role in the significant phytoremediation success of the higher alkanes and the dissolved contaminant groups. The results suggest that the significant contaminant loss from the soil for these groups of contaminants may also be due to less toxicity. Phytoremediation studies showed that the potential for high phytoremediation of the organic contaminant in the soil could be due to good plant development and favourable root morphology such as greater root length and larger surface area (Franco et al., 2011, Merkl et al., 2005). It was noted that fibrous root systems provide a large specific surface area and root volume and a high number of root tips to interplay with microorganisms in the rhizosphere thereby promoting soil aeration by de-compacting the soil and creating new macropores and space for the pores containing water and oxygen for plant respiration (Colombi et al., 2017, Al-Kaisi et al., 2017). High root volume could enhance oxidative degradation of organic compounds (Liao et al., 2015), and this may also widen the way for "trapped" contaminants to become accessible to plant rhizosphere bacteria.

Figure 2–7 indicates that light non-aqueous phase liquids and dense non-aqueous phase liquids can also be phytoremediated with 95 % and 93 % of studies suggest a medium to very high contaminant loss. Likewise, 88, 87 and 93% of studies for heavy oils, TPHs and PAHs respectively report a medium to very high contaminant loss which also demonstrates apparent phytoremediation of NAPL contamination, despite their physical state and toxicity of their dissolved components on plants. It was reported that the dissolved components of these groups affect plant growth rate through reducing photosynthetic rates and photochemical efficiency, resulting in significant reduction in cell size, leaf area, stem elongation and overall plant mass production (Fatima et al., 2018, Gerhardt et al., 2017). The abiotic stress effects, may further limit the survival, growth and plant phytoremediation potential. Since soil polluted with organic contaminants have poor water holding capacity and high volatility (Agnello et al., 2016), plants are subject to high degree

of stress effects due to lack of access to solutes and nutrients (Oostindie et al., 2017).

240 out of 350 studies investigated the effects of contaminants state on plant accumulation of the contaminant by the plants, and 206 out of 350 studies investigated the effects of contaminants type on plant accumulation of the contaminant by the plants. 189 studies (contaminant state grouping) and 145 studies (contaminant type grouping), suggested that plant don't accumulate contaminant. Only 51 studies (contaminant state grouping) and 61 studies (contaminant type grouping), suggested that plant accumulate contaminant. Therefore, from 112 studies that observed contaminant accumulation from both categories. It was demonstrated in experiments to determine the residual levels of organic contaminant concentrations in the plant that shoots and leaves have a higher concentration of contaminant than the roots and stems following remediation as a result of soil-to-root transfer of organic contaminants followed by the subsequent upward translocation of the contaminant in the transpiration stream to shoots and leaves (Atagana, 2011, Bystrzejewska-Piotrowska et al., 2008, Li et al., 2014a). Moreover, the root uptake has been reported as the primary pathway for organic contaminants accumulation in the shoots and leaves (Waqas et al., 2014). The process of accumulation by the plant is believed to be by the presence of roots and the plant enzymes secreted in exudates into the soil; then the degradation occurs at oil-water interfaces of soil pores where degradative organisms are located before the uptake of the resultant compound or enzymes within the plant tissues (Kaakinen et al., 2007, Molnár et al., 2002).

The meta-analysis of contaminant accumulation by the plant as analysed by contaminant state and contaminant type considered are shown in Figure 2–9 and Figure 2–10 respectively.
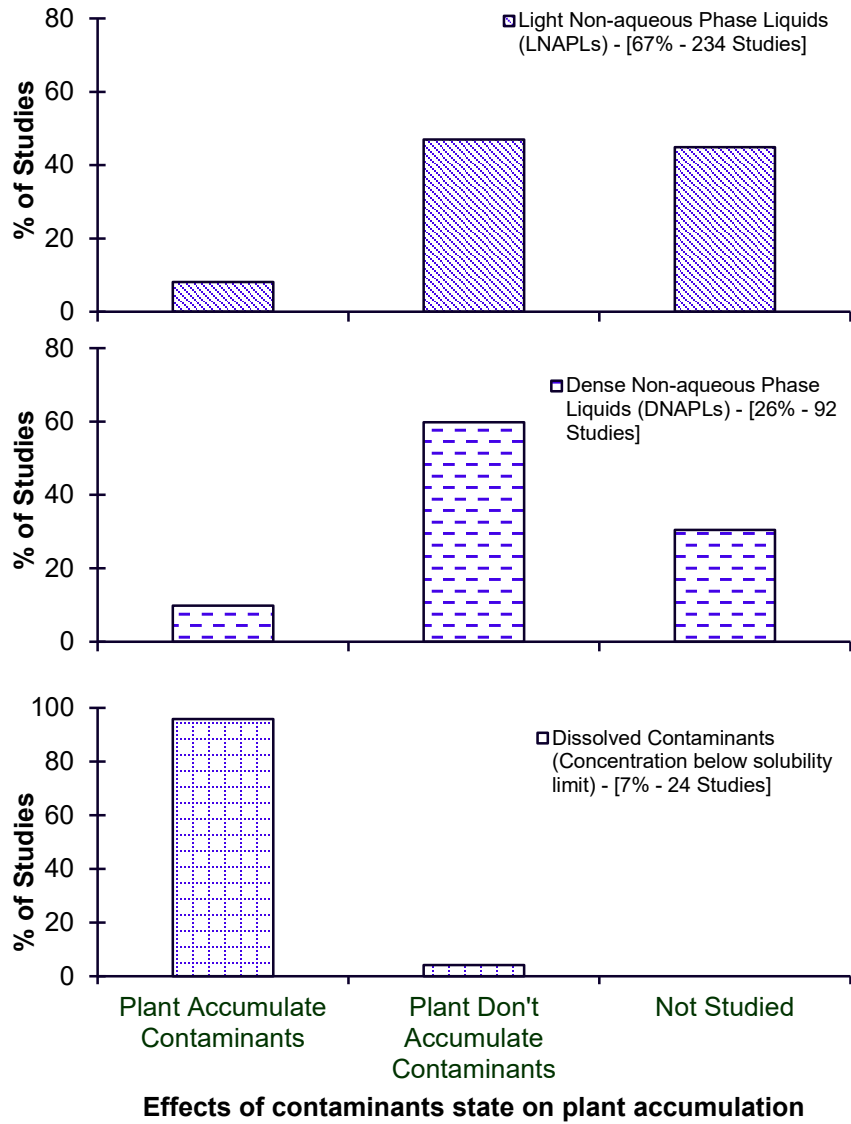
Figure 2-9 - Plant accumulations of LNAPLs and residual oil contaminants
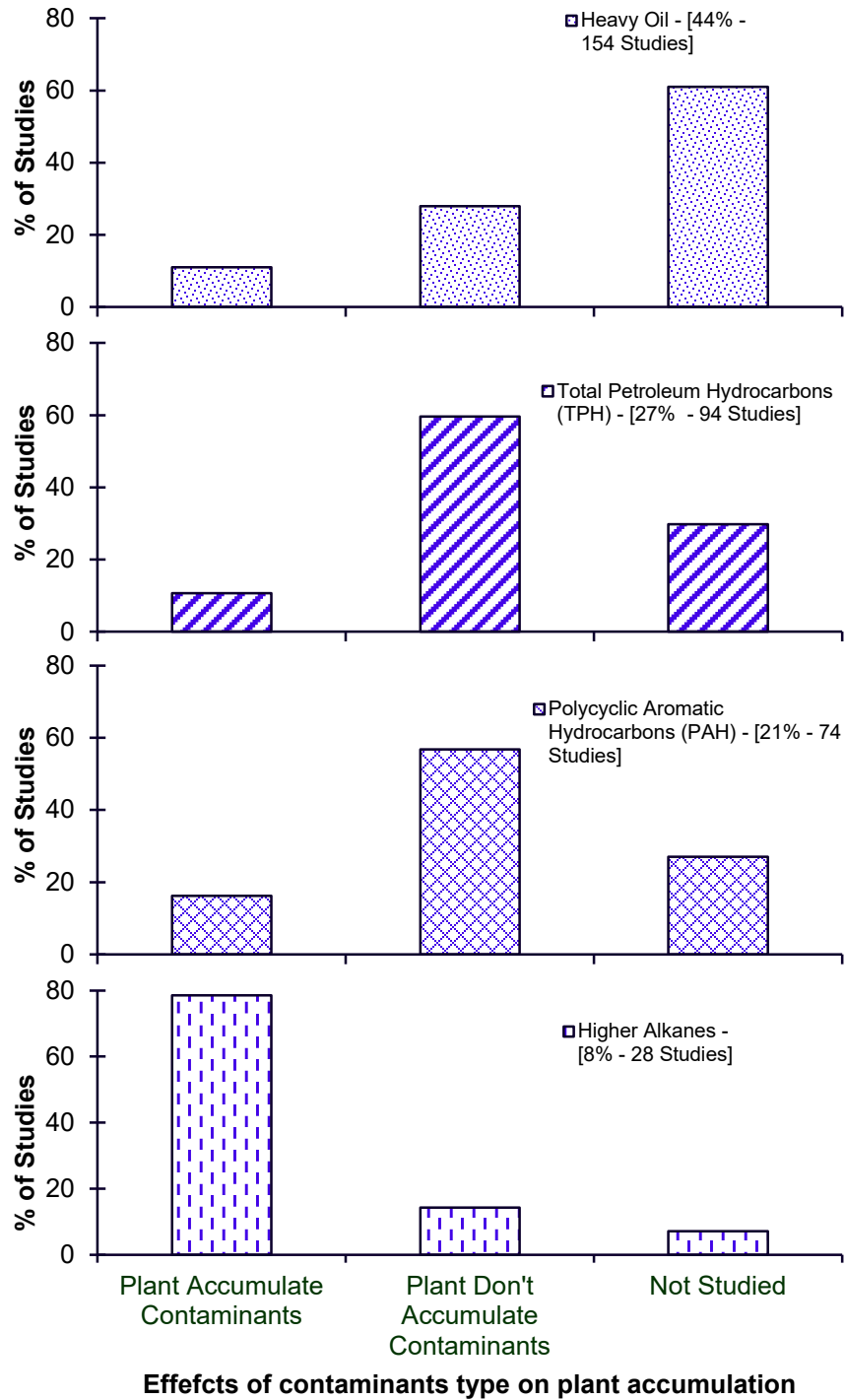
Figure 2-10 - Plant accumulations of LNAPLs and residual oil contaminants

The contaminants state in Figure 2-9 shows that 96 % of studies reported a significant plant uptake of contaminants for the dissolved phase and the low percentage number of studies for LNAPLs (8 %) and DNAPLs (10 %) accumulations in

the plant. Figure 2–10 illustrating contaminant types indicates that 79 % of studies for higher alkanes suggest plant accumulate these contaminants and the low number of studies for heavy oil (11 %), TPHs (11 %) and PAHs (16 %) uptake by the plants. The higher number of studies reporting accumulations of dissolved phase and higher alkanes can also be related to Figure 2–7 and Figure 2–8 which show that higher percentage of studies reported that the dissolved phase contaminants and the higher alkanes have no or little impacts on plant survival and growth because they have a low impact on plant. The results suggest that the extent of plant accumulation of contaminants is affected by contaminant toxicity. Therefore, the accumulation of organic contaminants could be more significant in an environmental condition which is characterised by the less toxic (i.e. low impact on the plant development) organic contaminants present in the soil.

The low percentage of studies reporting contaminant accumulation in the plant with LNAPLs and DNAPLs, or with heavy oil, TPH and PAHs, is possibly due to the high initial concentration and the inherent toxicity of their dissolved components (Li et al., 2014a, Empereur-Bissonnet et al., 2013). Perhaps, it may also be that these groups are dissolving at such a slow rate that microbes can degrade them before they get to the plant rhizosphere. Literature reports that though the roots were exposed to high concentrations of organic contaminants, the plant did not appear to translocate them into the roots, shoots or leaves probably because they are not in the non-aqueous form (Montiel-Rozas et al., 2016, Hurtado et al., 2016). Moreover, the plants' accumulation of organic contaminants was mostly reported with the low concentration of organic contaminants (Figure 2–9). Although most oil components appear to be degraded, the high molecular weight branched hydrocarbons, and polycyclic aromatic components are broken down quite slowly (Wang et al., 2012, Ma et al., 2012). The degradation of organic contaminants by plant enzymes occurs both in root and shoot tissue. The plant enzymes can catabolize organic contaminants by wholly mineralising the organic pollutants to inorganic compounds, e.g., water and carbon dioxide, or by partly degrading them to stable intermediate compounds that are accumulated and stored in the plant tissues (Arslan et al., 2017, Chirakkara et al., 2016). The reviewed studies explained that the process of contaminants being taken up into plant roots consist of three parts. Firstly dissolved in the aqueous solution within the rhizosphere; secondly,

adsorbed on the root (Li et al., 2014b); and thirdly penetrating into root tissues (Hurtado et al., 2016).

2.7 Implications for practise and conclusions

The understanding of the impact of NAPL on soil quality from the literature review has inform this thesis in many ways.

It is clear from the literature review that NAPLs and residual oil contamination can adversely alter the chemical and physical properties of the soil and consequently affect vegetation.

Sandy loam, loam, and silt loam have a better organic contaminant removal than other soil types because of the optimum suction values that favour water retention, nutrient availability and water supply for plant growth and root development. The literature suggested that high organic matter content leads to good removal but it is not crucial that it be present as long as organic matter is within the acceptable range for plant growth, the soil organic matter and pH have little impact on contaminant removal as suggested from the analysed data.

Laboratory studies have revealed that depending on the volume of contaminants released and the site properties, some of the LNAPLs or their water-soluble constituents will usually penetrate the surface soil and be transported as a separate phase in the vadose zone under the influence of gravity, capillary, and pressure forces. The migrating NAPLs might be leaving residual droplets such that the contaminated area is diffuse and comprise of many unconnected small contaminant sources, while others will migrate downward to the water table and coexist with water in the pore space. Lateral and radial migration can also occur, depending on the spill volume and the soil characteristics, and not only in the direction of the groundwater flow. As a result, the remedial action is challenging, and phytoremediation methods may offer the viable options for a successful cleaning; therefore, the potential for phytoremediation of NAPL contaminant sources needs to be established. For example, roots close to NAPLs may be able to reduce dissolved phase contamination through mechanisms including uptake and rhizodegradation, resulting in non-equilibrium conditions that cause relatively rapid dissolution of the NAPL. Moreover, it may even be the case that roots and the

rhizosphere interact with the NAPLs to bring about their removal or breakdown directly.

Plant tolerance to organic contaminants toxicity is species dependent, and the use of trees and perennial plants with vast food reserves, few stomata and thick cuticle for phytoremediation could be advantageous because of their deep roots, food reserves and long life.

Moreover, the residual NAPLs material left following the traditional ex-situ remediation techniques and evaporation of crude oil are toxic and contain potentially carcinogenic components which may, consequently, pose a public health hazard.

The effects of different hydrocarbon contaminants were explored in two ways. The first group was based on contaminant types, and the second categorised as contaminant states based on the actual study conditions. Using both categories in assessing impacts of contaminant types on plant behaviour and plant accumulation of contaminants. There is a minor effect of different contaminants, with 'dissolved' and 'higher alkanes' contaminants having little impacts on plant, while all other hydrocarbons significantly inhibit plant survival.

Chapter 3   Materials and methods

3.1   Introduction

This chapter presents the materials used and the experimental methods adopted for carrying out the laboratory tests presented in this thesis.

Non-aqueous phase liquids (NAPLs) are persistent sources of contamination in the ground, providing a long-term supply of dissolved phase contamination and taking significant periods to dissipate naturally. Even in the long term, light NAPLs (LNAPLs) take the form of a separate phase within the ground, often as individual ganglia in pore spaces within the capillary zone such that the contaminated region is diffuse and comprised of many unconnected small contaminant sources. Consequently, remedial action is challenging and success may be limited to ex-situ remediation techniques. The ability of plants to phytoremediate dissolved-phase contamination is well known, but the impact of LNAPLs on plant growth and subsequent contaminant behaviour is largely unknown.

Two series of laboratory experimental works were carried out based on the overall research aims and objectives as set out in Chapter 1.

The first series of experiments were carried out to study the impact of a LNAPL on plant root growth and distribution, and absorption of LNAPL by plants at pore-scale level. The tests were carried out in small-scale 3D-printed rhizoboxes with a single ryegrass plant (*Lolium perenne*) grown in each rhizobox under a soil-free and hydroponic conditions. The rhizoboxes provided a simplistic model of soil macropores allowing control of LNAPL distribution and precise monitoring of root growth and contaminant loss in individual pores.

The second series of experiments were carried out to investigate the impact of a LNAPL on root growth, root distribution, and oil loss, in soil at the macroscopic scale. The tests were carried out in a larger scale rhizoboxes with multiple ryegrass plants (*Lolium perenne*) grown in each rhizobox using laboratory prepared soils similar to that of naturally occurring soils.

In section 3.2, the soils used were characterised and the details of light aqueous phase liquids (LNAPL) used are discussed. In section 3.3, the properties of the soils were discussed. Section 3.4 presents materials and methodology for the single plant

experiments. In section 3.5 the materials and methodology for the larger scale experimental works with plant are described. A summary of the materials used and some salient features of the test set ups are presented in section 3.6.

3.2 Soils and light non-aqueous phase liquid (LNAPL) used

3.2.1 Soils used

Two soils were prepared and used in this study, such as a sandy loam and a loam. The compositions of the prepared soils were similar to that of naturally occurring sandy loam and loam soils. The soil texture triangle (Shirazi and Boersma, 1984) provided guidelines for determining the percentages of sand, silt and clay for preparing the two soils. The soils (i.e. sandy loam and loam) were prepared by mixing predetermined percentages of sand, silt and clay based on the dry weight. A portable electric mixer was used for obtaining homogenous mixtures. The sandy loam was composed of 70% sand, 20% silt and 10% clay, whereas the loam was composed of 40% sand, 40% silt and 20% clay. The soils were selected for this research because it appears from 350 phytoremediation studies (Figure 2–4 and Figure 2–5) that sandy loam, loam and silt loam have a better contaminant loss than other soil types, with 198 studies reporting 80% or more oil loss having these soil types (Vervaeke et al., 2003, Sung et al., 2004, Reza, 2008, Mohsenzadeh, 2009). Moreover, from Figure 2–5, the rate of contaminant removal is better for the loam soils (loamy sand, sandy loam, loam and silt loam) than other soils, with more than 140 studies indicated that there was 100% contaminants removal in less than 200 days. However, artificial soil was used with no organic carbon for this thesis, ensuring that the NAPL removal will be as a result of the presence of plant, and not related to the impact of organic matter. A quarter-strength Hoagland's solution was used for nutrient supply. The solution is essential for the growth of plants due to the lack of organic matter in the artificial soils. The nutrient solution contains nitrogen and potassium and other essential elements for plant growth (Hothem et al., 2003), making it best for the development of plant in low nutrient scenarios.

3.2.2   Light non-aqueous phase liquid (LNAPL) used

The light non-aqueous phase liquid (LNAPL) used in this investigation was a mineral oil (MSDS name: mineral oil, light and heavy or white mineral oil (petroleum) or saturated hydrocarbons). The mineral oil was supplied by Fisher Bio-Reagents (Fisher Scientific UK, 2018). All the laboratory tests involving LNAPL were carried out using this petroleum byproduct. The mineral oil used is a non-aromatic and slightly toxic hydrocarbon composed of a mixture of C_{16}-C_{25} carbon atoms. The density and viscosity of the oil are 0.83 Mg/m^3 and viscosity of 33.5 x 10^{-3} Pa.s. The other characteristics of the mineral oil are very low volatility and water solubility. It is considered to be non-miscible with water. The boiling point range is between 260 - 426 °C. The oil is stable at normal temperatures and pressures and has been used in the past in some phytoremediation investigations (Lee et al., 2008, Popp et al., 2006). From the literature review, Figure 2–1 and Figure 2–2, the LNAPL and the TPH categories (under which mineral oil was classified) do appear to have more of a spread-out profile indicating the negative effects of mineral oil on plants. Moreover, Figure 2–7 and Figure 2–8, results from literature also shows that the LNAPL and the TPH categories can be successfully phytoremediated. The chosen mineral oil has low toxicity to plants (Vervaeke et al., 2003), volatility and aqueous solubility, firstly to minimise the impacts of the toxicity of the NAPL on plant growth (ensuring the main impacts are due to physical presence), and secondly to ensure that any contaminant loss is down to phytoremediation and biodegradation rather than solubilisation or volatilisation.

The Oil Red O colorant, also called solvent red 27 (Sigma-Aldrich) was added to the mineral oil before preparing the soil-mineral oil mixtures or use in the hydroponic conditions. ORO is a fat-soluble, lipid sensitive, diazo (R–N=N–R′) dye (i.e. it stains fat and lipid components in biological samples) and Its also used to stain waxes and oil to a red tint (MF; et al., 2005). It has a molecular weight of 408.510. The structural confirmations of ORO prevent it from ionizing and therefore facilitate its solubility in lipids (Kutt and Tsaltas, 1959). ORO absorbs radiation having wavelength of 518 nm and stains the item red (Beaudoin, 2004). The red stain is attributed to the hydroxyl auxophore (Beaudoin, 2004). Laboratory preliminary growth trials show that the colorant have no impact on plant growth and development before it was used in the research.

The concentration of colorant used was 50mg/L. An addition of the colorant is expected to enhance oil visibility which in turn allows the movement and location of the LNAPL within the soil samples (Page et al., 2007). The oil was stored in dry and cool conditions.

3.3 Properties of the soils

The properties of the two soils were determined following British Standards Methods (British Standards Institution, 2009, British Standards Institution, 2010a, British Standards Institution, 2010b, British Standards Institution, 2010e, British Standards Institution, 2010d, British Standards Institution, 2010c, ASTM Standard C-1699-09, 2009).

3.3.1 Initial water content

The oven-drying method was used to determine the initial water content of the soils (British Standards Institution, 2010a). The initial water contents of sandy loam and loam were found to be similar (0.2%).

3.3.2 Particle size distributions

The soils used were mixtures of sand, silt and clay of various percentages. The sand used in the soils was a commercially available ProArena 100™ Leighton buzzard sand procured from Garside Sands, Leighton Buzzard, Bedfordshire, UK. Table 3–1 shows the size range of the particles present in the sand. The particle sizes of the sand in this study was controlled by carrying out dry-sieve analysis using a sieve size range of 2.63 to 0.063 mm. The silt-size fraction (< 0.06 mm) was derived from the sand by using a 63 μm sieve. The specific gravity, silica content, pH and loss on ignition at 100 °C of the sand fraction were found to be 2.65, 94.6 %, 7.6 and 1.04 % respectively. The specific gravity, silica content, pH and loss on ignition at 100 °C of the silt-size fraction were found to be 2.65, 94.8 %, 7.5 and 0.93 % respectively. Table 3–2 shows the chemical composition of the sand used. As can be seen in Table 3–2, the silica content in the sand was about 95%.

Table 3-1 – Particle size distribution of Garside Washed Fine Silica Sand (WFSS) (Source: Leighton Buzzard, Bedfordshire)

Sieve size (mm)	Average % passing
2.63	100
1.18	100
0.60	96
0.30	80
0.15	24
0.063	1

Table 3-2 – Chemical composition of Garside Washed Fine Silica Sand (WFSS) (Source: Leighton Buzzard, Bedfordshire)

Chemicals		%
Silica	SiO_2	94.6
Alumina	Al_2O_3	1.52
Titania	TiO_2	0.33
Iron	FE_2O_3	1.20
Magnesium	MgO	0.11
Calcium	Cao	0.07
Sodium	Na_2O	0.66
Potassium	K_2O	0.61
Loss on ignition @ 100℃	L on I	0.93

The clay used was Speswhite kaolin. Speswhite kaolin was procured from Imerys Performance Minerals, Par, Cornwall, UK. Table 3–3 shows the properties of the Speswhite kaolin. The specific gravity and pH of Speswhite kaolin were found to be 2.6 and 7.5 respectively. Beside the dominant kaolinite mineral, other minerals (about 0.2 %), such as mica, quartz, and feldspar or ilmenite were found to be present in the clay.

Table 3-3 – Properties of Speswhite kaolin (Source: IMERYS®; (IMERYS, 2008)).

Mineralogy	Kaolinite (main) and other minerals (mica, quartz, and feldspar or ilmenite)
Aspect ratio	20:1
Specific Gravity	2.6
Refractive Index	1.56
MOH Hardness	2.5
Moisture **content** (%)	1.5
pH:	5 - 7.5

3.3.3   Specific gravity of soil solids

The specific gravity values of the two prepared soils (sandy loam and loam) were determined by using pycnometer method (British Standards Institution, 2010a). The specific gravity of sandy loam and loam were found to be 2.68 and 2.66 respectively.

3.3.4   Liquid limits

The liquid limits of the soils were determined by cone penetrometer (British Standards Institution, 2010a). The soils were made to pass through a 425 µm sieve prior to performing the liquid limit tests. The values of liquid limits for sandy loam and loam were found to be 13 % and 15 % respectively.

3.3.5   Plastic limits

The plastic limits of the soils were determined by rolling method (British Standards Institution, 2010a). The soils were made to pass through a 425 µm sieve prior to performing the plastic limit tests. The values of plastic limits for sandy loam and loam were found to be 10.6 % and 10.3 % respectively.

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3.3.6   Organic contents

The organic contents present in the soils were determined by loss on ignition (LOI) method (British Standards Instituition, 1990). The samples of both soils were oven-dried at a temperature of 440 ℃. The organic content was determined based on the percentage loss in weight of the soil samples. The organic content in both soils were found to be 0 %.

3.3.7   pH values

The pH of samples was measured using a Mettler Toledo SevenMulti dual meter with specific pH and EC electrodes. Firstly, pH 4 and pH 7 were used to calibrate the pH electrodes. After the calibration process, the electrodes were tested with deionized water blanks before sample analysis was conducted to ensure that calibration solutions were removed from electrodes. The equipment was calibrated for every pack of samples analyzed. Further, Whatman Grade 40: 8 µm (medium flow) filter

paper was used to screen each liquid mixture before immersing the pH probes. The pH values of the sandy loam and loam averaged at 7.9 ± 0.1 and 6.6 ± 0 respectively.

3.3.8   Standard Proctor compaction tests

The standard Proctor compaction tests (BS light compaction effort) were carried out using a 2.5 kg rammer falling through a height of 300 mm. The volume of the compaction mold used was 1000 cm^3.

Figure 3-1 shows the standard Proctor compaction curves of the soils. The maximum dry density of the sandy loam was 2.0 Mg/m^3 at the optimum water content of 8.9 %. Moreover, the maximum dry density of the loam was 1.96 Mg/m^3 at the optimum water content of 9.8 %.
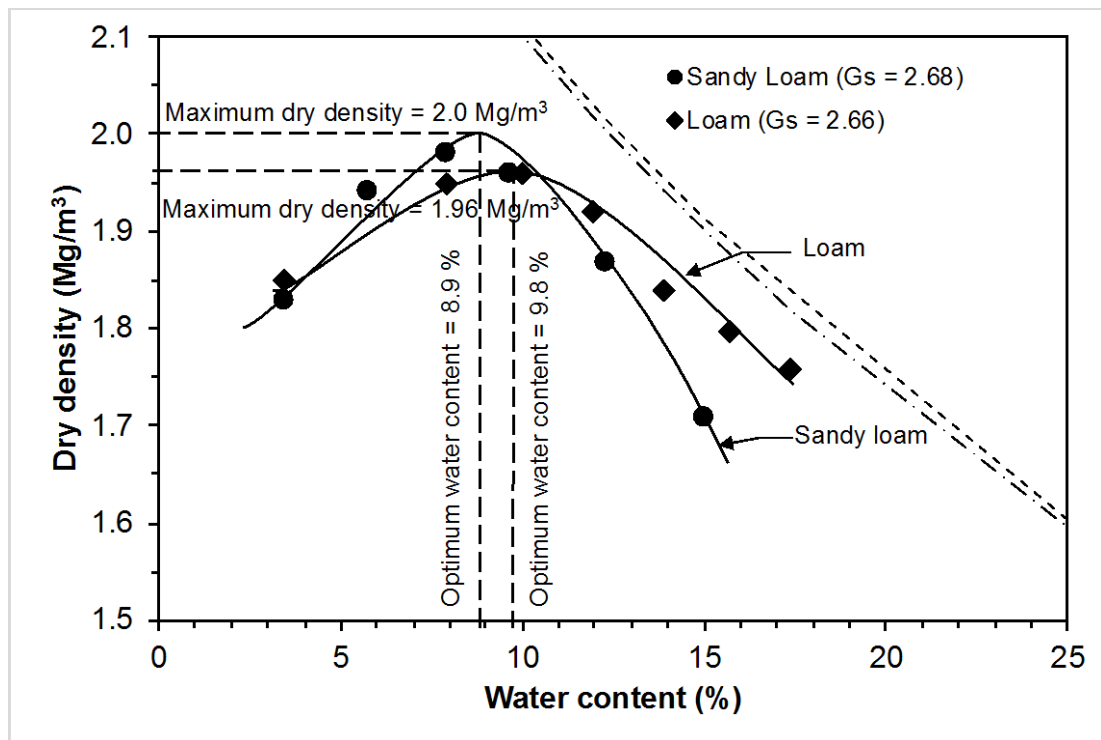
Figure 3-1 - Standard Proctor compaction test results for the soils used

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3.3.9   Permeability tests (constant and falling head methods)

The constant head and falling head methods (British Standards Institution 2010c) were used to determine the coefficient of permeability of the soil.

3.3.9.1   Procedure for Constant Head test

The constant head test method is suitable for soils having coefficients of permeability in the range of 10^{-2} to 10^{-5} m/s. The method was therefore used for sandy loam soil specimen only. The test procedures followed are outlined in BS 1377-5:1990 (British Standards Institution, 2010d). The flow volume of water passing through the soil in a known time is measured, and the hydraulic gradient is measured using manometer tubes. The specimen was connected through the inlet to the constant head reservoir. The bottom outlet was opened and a steady flow of water was established. The quantity of water flow for a time interval was recorded. The process was then repeated three times for the same interval.

3.3.9.2   Specimen preparation method for constant head test

The initial water content, dry density, initial void ratio and degree of saturation of compacted sandy loam soil specimen for the permeability test were 0.2 %, 0.98 Mg/m^3, 0.348 and 19.7 % respectively. A 2.5 kg sample was taken from a thoroughly mixed air-dried soil. The sample was stored in an airtight container. The required quantity of water was added to get the desired moisture content. The sample was then mixed thoroughly. The empty permeameter mould was weighed. The inside was greased slightly, and then the mould was clamped between the compaction base plate and extension collar. The assembly was placed on a solid base and filled with soil-water mixture and which was then compacted. Following the completion of compaction, the collar and excess soil were removed. The weight of mould with the sample was then measured. The mould with the sample was then placed in the permeameter, with drainage base and a cap. For observation and recording, the water flow was very low at the beginning, and gradually increased and then remains constant. Table 3–4 shows the testing details and the key information that are required for calculating the coefficient of permeability from constant head tests.

Table 3-4 Results for Constant Head Method for sandy loam

Experiment No.		1	2	3
Length of specimen	L (cm)	10	10	10
Area of specimen	A (cm^2)	42.45	42.45	42.45
Time	t (sec)	264	624	37
Discharge	q (cm^3)	50	100	10
Height of water	h (cm)	2.8	0.2	4.2
Temperature	(ºC)	25	25	25

The coefficient of permeability for a constant head was calculated from Equation 3−1.

$$k = \frac{qL}{Aht} \quad\text{...} \quad 3{-}1$$

Where k = coefficient of permeability,

q = discharge cm^3/sec

L = length of specimen in cm

A = cross-sectional area of specimen in cm^2

H = constant head causing flow in cm

t = time in sec

The coefficient of permeability of sandy loam was found to be 1.5 x 10^{-4} m/s at 20ºC.

3.3.9.3   Procedure for falling head test

The soil specimen was prepared following the procedure stated in section 3.3.9.4. The soil specimen was then saturated with the de-aired water. The permeameter was then assembled in a tank filled with water. The inlet nozzle of the mould was then connected to the standpipe. Some water was allowed to flow until a steady flow was obtained. The time interval 't' for a fall of a head in the stand pipe 'h'

was recorded. This step was repeated three times to determine 't' for the same head.

3.3.9.4   Specimen preparation method for falling head test

Falling head tests were carried out to measure the coefficient of permeability of the loam. The initial water content, dry density, initial void ratio and degree of saturation of the compacted sample tested were 0.2 %, 0.948 Mg/m^3, 1.81 and 22.0 % respectively. The loam sample was prepared using static compaction method. 1000 g of representative loam soil was weighed and mixed with distilled water corresponding to the optimum moisture content determined by standard proctor compaction test. The soil-water mixture was then stored in an airtight container for 24 hours. The permeameter was assembled for static compaction by attaching the 3 cm collar to the bottom end of 0.3 litres mould and the 2 cm collar to the top end. The mould assembly was then supported over 2.5 cm end plug, with 2.5 cm collar resting on the split collar and kept around the 2.5 cm end plug. Then, the inside of the 0.3 litres mould was lightly greased. The soil-water mixture was placed into the mould. The top 3 cm end plug was then inserted into the top collar while tapping the soil with hand. The entire assembly was then kept on a compressive machine and the split collar was removed. The compressive force was then applied until the flange of both end plugs touched the corresponding collars. The load was maintained for 1 minute and later released. Then the top 3 cm plug and collar were removed, and a filter paper was then placed on a fine wire mesh on the top of the specimen and the perforated base plate was secured. The mould assembly was then turned upside down and the 2.5 cm end plug and collar were removed. The sealing gasket was then inserted and the perforated top plate was placed on the top of the soil specimen. The top cap was fixed before the commencement of the test. Table 3-5 shows the testing details and the results obtained from the tests for calculating the coefficient of permeability.

Table 3-5 Results for Falling Head Method for loam

Experiment No.		1	2
Area of stand pipe (dia. 3.75 mm)	a (mm²)	11.045	11.045
Cross sectional area of soil specimen	A (mm²)	4185.39	4185.39
Length of soil specimen	L (mm)	203	203
Initial reading of stand pipe	h_1 (mm)	990	990
Final reading of stand pipe	h_2 (mm)	850	850
Time	t (sec)	659	659
Test temperature	(°C)	20	20

The coefficient of permeability from falling head test was calculated from Equation 3–2

$$k = \frac{2.3 \times a \times L \left(\log_{10} {h_1}/{h_2}\right)}{A \times t} \quad m/sec \quad \dots\dots\dots\dots\dots\dots\dots\dots\dots\dots\dots\dots\dots 3{-}2$$

The coefficient of permeability of loam was found to be 1.2 x 10^{-7} m/s at 20 °C.

3.3.10 Water retention behaviour of the soils

The water retention behaviour of the soils used in this investigation was studied by carrying out pressure plate tests and chilled-mirror dew-point potentiameter tests (British Standards Institution, 2009, ASTM Standard C-1699-09, 2009). For the pressure plate tests, soil specimens were prepared at initial water content of 15.6 % for sandy loam and 18 % for loam. The pressure plate tests were carried out using a 5-bar pressure plate extractor. The applied suctions in the pressure plate tests were 10, 25, 50, 100, 150, 200, 250, 300, 350 and 400 kPa. Multiple specimens were used for pressure plate tests. The mass of the soil specimens were monitored periodically until an equilibrium was reached under each applied suction. Specimens at each applied suction were dismantled after the equilibrium was reached and the water contents of the specimens were measured by oven-drying method.

3.3.10.1 Suction measurement by dewpoint potentiameter

A chilled-mirror dew-point device was used to indirectly determine the total suction (in the range 0 to 300 MPa to an accuracy of ± 0.05 MPa from 0 to 5 MPa and 1% from 5 to 300 MPa) of soil specimens. The device is commercially known as WP4C Dewpoint PotentiaMeter. The WP4C device is made up of a sealed chamber with a fan, a mirror, a photodetector cell, and an infrared thermometer (Deacagon Devices, 2010). The WP4C is provided with a drawer to place a specimen cup. For most soil specimen, the representative measuring time is from 10 to 15 minutes while in a precise mode of measurement.

The WP4C operating principles is by a chilled mirror measuring the dew-point of ambient water vapour in a sealed chamber. The measurement is recorded when the dew-point of the ambient water vapour is at equilibrium with water in the soil samples. The attached Peltier thermoelectric cool the chilled mirror. Condensation starts to occur on the mirror surface when the dew-point temperature is reached. The first dew-point at which condensation takes place is detected by a beam of light focusing on the mirror which reflects into a photodetector cell. A thermocouple records the dew-point temperature. The temperature of the soil specimen at which relative humidity measurement must be made is measured using a temperature control device that comes with the WP4C. A temperature equilibrium plate can also be used to bring the temperature of the specimen to the set-point temperature of the device. The dew-point with the set specimen temperature is then used to determine the relative humidity. Kelvin's law is used to calculate the total suction. The device software performs the calculation.

3.3.10.2 Sample preparation for chilled-mirror dewpoint tests

Five soil-water mixtures were prepared corresponding to the data on the compaction curve of each soil type. The chosen water contents were 2, 4, 6, 8 and 10% for both soils. Soil-water mixtures were prepared by adding predetermined quantities of distilled water. The mixtures were thoroughly mixed. The moist soil was then placed in double bags and stored in sealed plastic containers for at least 24 hours. Using a 1000 cm^3 mold, the standard Proctor compaction (BS light compaction effort) was carried out with a 2.5 kg rammer falling through a height of 300 mm to compact the soil. Five specimens were obtained by trimming the initially

prepared compacted samples to the recommended size (half the capacity of the specimen cup).

3.3.10.3 Suction measurement using chilled-mirror dewpoint potentiameter

Calibrations of the WP4C device was done before each test using standard solution to ensure proper operation. Potassium chloride (KCl) solution was used in calibrating the device. The sample cup containing the soil sample was first placed on the thermal equilibration plate for approximately ten minutes to bring the temperature of the sample cup to the set-point temperature of the device. The sample was then moved to the WP4C sample chamber. The measured final suction of any sample was indicated by a blinking green LED indicator located in the device. The duration of each test was found to vary between 10 to 15 minutes. The oven drying method was used to determine the water contents of the soil samples after the total suction measurements.

3.3.10.4 Suction of soil-mineral oil mixtures

For studying the effect of mineral oil on suction of the soils, suction measurements were carried out on compacted soil-mineral oil mixtures. The procedure adopted for preparing soil-mineral oil samples for chilled mirror dewpoint tests was similar to that mentioned in section 3.3.10.2. In this case, six soil-mineral oil samples were prepared with mineral oil instead of distill water. The oil content chosen were 2, 4, 6, 8, 10 and 12%.

The procedure adopted for measuring suctions of compacted soil-mineral oil mixtures using chilled-mirror dewpoint potentiameter remained the same as that adopted for soil-water mixtures (see section 3.3.10.3). The duration of each test was found to vary between 10 to 30 minutes. The oven drying method was used to determine the oil contents of the soil samples after the total suction measurements.

3.3.10.5 Influence of water and mineral oil on suction of the soils used

The experimental results from the pressure plate and chilled-mirror dewpoint potentiameter tests are shown in Figure 3–2. Figure 3–2 shows the water content

versus suction plots of the soils used. Figure 3–3 shows the suction versus fluid content (water or mineral oil) plots of the soils.

It can be seen from Figure 3–2 that the suction-water content plot of loam remains above that of the sandy loam up to a suction of about 1.0 MPa. At higher suctions (>1.0 MPa), the water retention behaviour of both soils are very similar. The experimental data presented in Figure 3–2 were analysed to determine the air entry values which showed that both soils desaturated at very low suctions.

Figure 3–3 (a) and (b) show that the interaction between the soils and mineral oil was not very distinct. For all mineral oil contents used, the suction of the soil remains at about 100 MPa. A greater suction in case of loam soil mixed with mineral oil as compared to the sandy loam with mineral oil is attributed to a higher clay content in the loam soil.

Mineral oils usually possess a very low vapour pressure. The magnitude of suction at a mineral content of 1% (that is, 100 MPa or higher) as compared to that of suctions of the soils at the same water content of 1% is due to the fact that the soils were oven dried prior to mixing the mineral oil. The suction of the soils at oven dried condition can be expected to be 1000 MPa. The suction of the soils at oven dried condition can be expected to be greater than 100 MPa. Additionally, mineral oil does not hydrate the surfaces of soil particles as that occurs in case of adding water to soils. The presence of LNAPL in oven-dried soils reduced the total suction from 1000 to about 100 MPa. However, an increase in the LNAPL content (i.e., the oil content) did not influence the suction of the soils. For both soils, the measured suctions remained at about 100 MPa for a range of oil content between 2 to 12% indicating that the trapped air within the soil systems did not allow a reduction of suction of the soils when the oil content was increased.
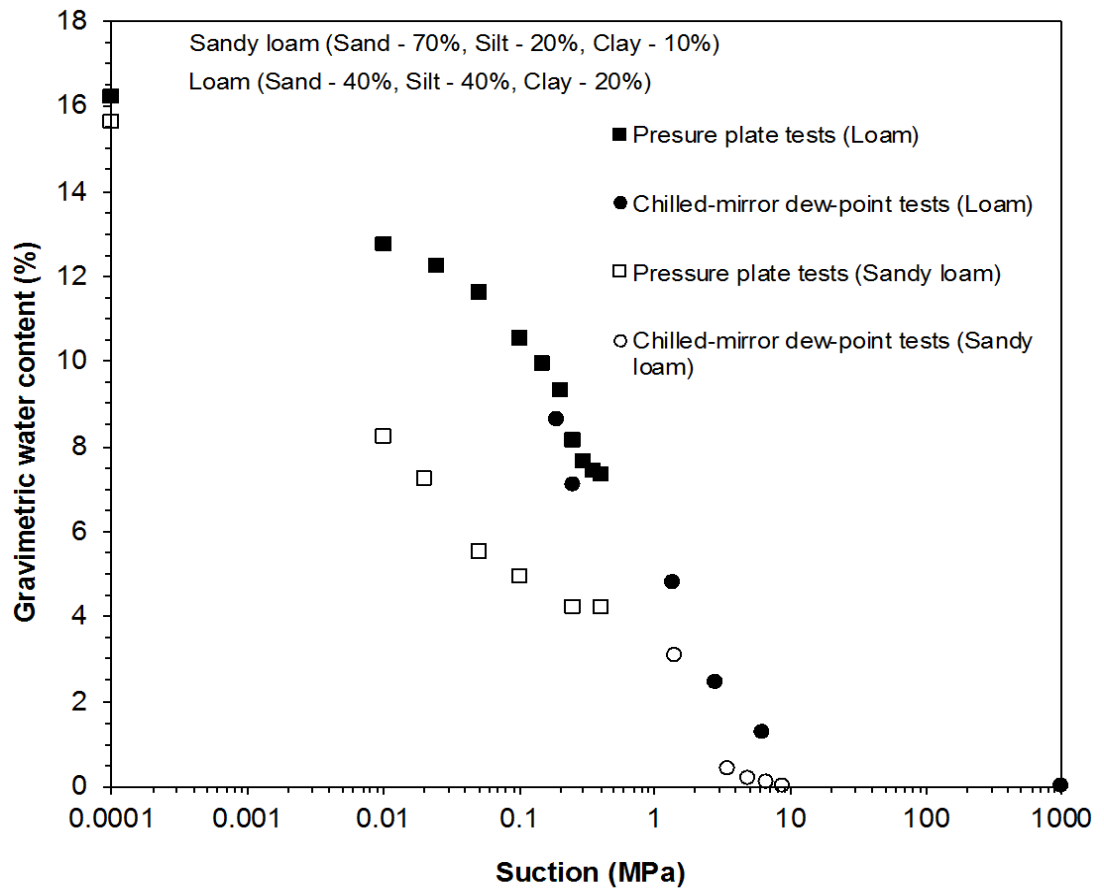
Figure 3-2 Water retention curves of the soils based on pressure plate and chilled-mirror dew-point potentiometer tests
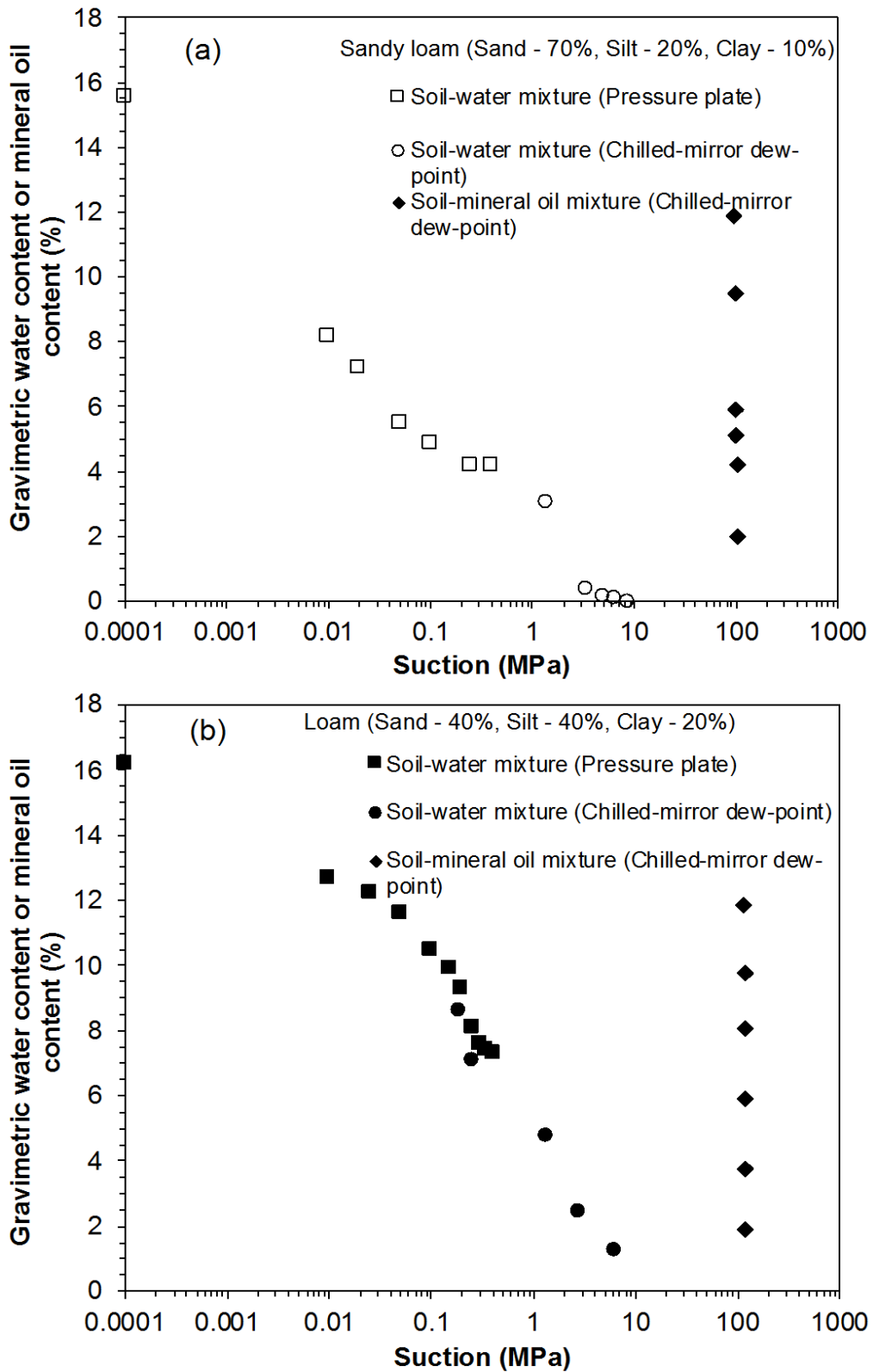
Figure 3-3 Soil-water and soil-mineral oil retention of the soils used in this study based on pressure plate and chilled-mirror dew-point potentiometer tests

The properties of the soils used are summarised in Table 3-6. The sandy loam and loam soil have very similar plasticity properties and both soils tend to desaturate at a lower suction which is very important in relation to the workability and efficiency of growing plants.

Table 3-6 Properties of soils used in this investigation

Soil type	Initial water content, w (%)	Specific gravity of soil solids, (G$_S$)	Liquid limit, w$_L$ (%)	Plastic limit, w$_P$ (%)	Initial total organic matter, (%)	Loss on ignition @ 100°C (LOI) (%),	Initial pH value	Initial void ratio of compacted soil for permeability test (e)	Permeability, k (m/s)
Sandy Loam (70% Sand; 20% Silt; 10% Clay)	0.2	2.68	13	10.6	0.065	0	7.9	0.348	1.5×10^{-4}
Loam (40% Sand; 40% Silt; 20% Clay)	0.2	2.66	15	10.3	0.074	0	6.6	0.399	1.2×10^{-7}

3.4   Laboratory Microcosm Phytoremediation Experiments

This section discusses the materials and methodology for the hydroponic experimental conditions with a single ryegrass (*Lolium perenne*) seed. The growth trial explores the impact of LNAPL (mineral oil) on root distribution and growth, and oil loss.

3.4.1   Microcosm phytoremediation tests

The response of single grass plants to oil in a soil-free, hydroponic system has been explored to understand the impacts on root growth and distribution, as well as oil removal, in a highly idealized scenario.

3.4.2   Apparatus for microcosm phytoremediation tests

For studying phytoremediation of LANPL with a single perennial ryegrass seed under hydroponic conditions, a pore-scale 3D-printed rhizobox was designed and fabricated in this study (Figure 3–4). The external dimensions of the box are 30 high x 15 long x 3 mm thick; the internal dimensions are 27.5 high x 10 wide x 2 mm thick; and a 19 x 5 x 2 mm thick base. These were designed using AutoCAD and printed from polylactic acid (PLA) on an Ultimaker 3D printer. Each box had PLA back and side walls, base and four equally spaced columns (1.75 mm wide by 2.0 mm thick).

A V-shaped seed housing was created to ensure the consistent location of seed germination and plant growth (Figure 3–4). To allow visual observation of plant development, acetate transparencies (26 x 15 x 1 mm) were bonded to the rhizobox front with superglue and further sealed with LS-X jointing compound and external leak sealer ensuring water and oil tightness (transparent front cover not shown in Figure 3–4 for clarity). Each transparency was placed to leave a 2mm gap at the base of the box, allowing nutrient solution movement to and from an external reservoir (Figure 3–4).

Rhizobox materials (PLA, glues/sealants and acetate) were tested for their potential impacts on experimental observations through oil absorption and permeability tests. The trial tests showed an average absorption of 1.14% (PLA and glues/sealants) and 0.02% (acetate). Moreover, the average permeability rate of 1.13 mg/min/m^2 was obtained for the PLA, glues/sealants and acetate. The results were found to be

appropriate for the test requirements. The pore-scale rhizoboxes were then systematically arranged in a plastic container as explained in section 3.4.7.
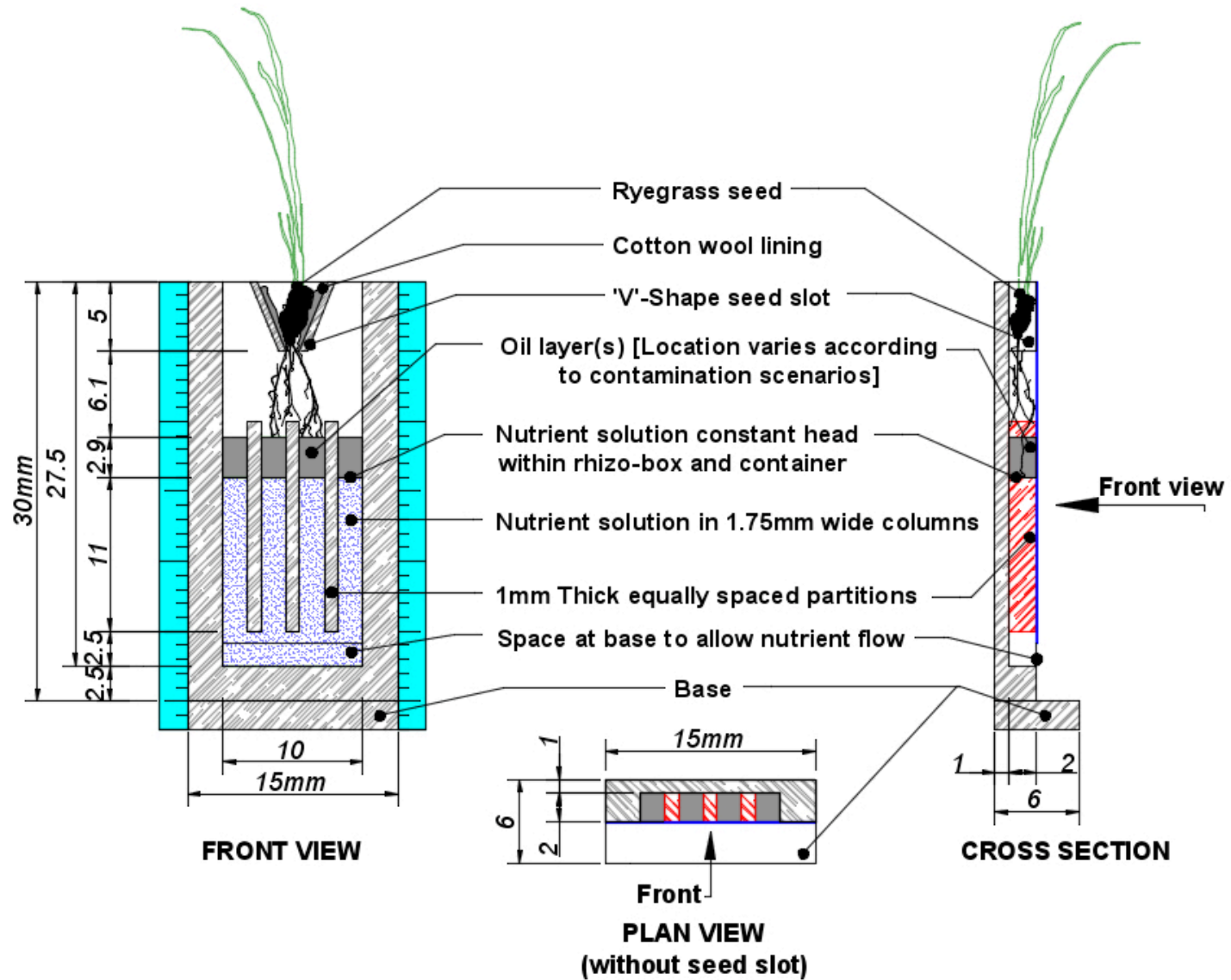
Figure 3-4 - Schematic of the pore-scale 3D-printed rhizobox

3.4.3   Contamination scenarios for microcosm phytoremediation tests

Ten contamination scenarios were considered in this study. The ten scenarios were considered to give a practical representation of the state of LNAPL in pore spaces in the capillary zone and could be considered as a continuous or semi-continuous phase, or as unconnected ganglia which act as individual contaminant sources. Table 3–7 presents the details of the scenarios considered. The ten scenarios comprise all possible combinations of oil contamination in the four columns (note that combinations that are 'reflections' of others, e.g. oil in columns 2 and 4 rather than 1 and 3, are considered to be identical and so were not tested. Scenario 10 is deemed to be a no-oil control to which other scenarios can be compared. Five replicates were tested for each of the ten scenarios. In each case, a 2.9 mm thick contamination zone was established on the nutrient solution constant head surface. The water table was maintained at the base of the contamination layer in all cases (Figure 3–4).

Table 3-7 - Mineral oil contamination scenarios inside the rhizoboxes

Contamination Scenarios	Column 1	Column 2	Column 3	Column 4
1	Oil			
2		Oil		
3	Oil	Oil		
4		Oil	Oil	
5	Oil			Oil
6	Oil		Oil	
7	Oil	Oil	Oil	
8	Oil	Oil		Oil
9	Oil	Oil	Oil	Oil
10				

3.4.4   Light non-aqueous phase liquid (LNAPL) used

The light non-aqueous phase liquid (LNAPL) used as a contaminant in this investigation was a mineral oil as discussed in section 3.2.2.

3.4.5   Plant used

Perennial ryegrass (LIBRONCO *"Lolium perenne"*) was used as a remediation plant. The plant was germinated in-situ from seeds obtained from Boston Seeds, UK. *Lolium perenne* was selected due to its well established capability to remediate organic contaminants and was found to reliably grow in the hydroponic experimental conditions employed (Gunther, 1996, Gurska et al., 2009, Hou, 1999, Kechavarzi et al., 2007, Rezek et al., 2008). The seeds were stored in a sealable bag and kept in a cool, dry storage.

3.4.6   Hydroponic solution and nutrients used

The plant growth was supported by using quarter strength Hoagland's solution (prepared as 2.5 g/L Hoagland's No.2 Basal Salt Mixture (Sigma-Aldrich, UK) in deionized water) as the hydroponic solution. The diluted solution was then stored in a fridge.

3.4.7   Rhizobox preparation and arrangements

The fifty rhizoboxes were affixed to the base of a 66 cm (W) x 65 cm (D) x 21 cm (H) plastic container with a raised back level and low front for easy access. The boxes were arranged in a uniform clockwise row as shown in Figure 3–5, with a centre-centre distance of 6 cm between adjacent boxes, and 18 cm distance between rows to allow monitoring and photography of root growth without disturbing the plant.

The reservoir container was filled with 3500 ml of the nutrient solution (Hoagland's No. 2 Basal Salt Mixture, Sigma Aldrich, UK), maintaining the height of nutrient solution in the rhizoboxes at 18 mm above the lowest point of the base with no oil present. When oil was present, the upper surface of the oil layer was at a height of 20 mm above the lowest point of the base. A Mariotte bottle supplied nutrient solution to the reservoir when necessary to maintain a constant fluid level within the rhizoboxes and surrounding reservoir. The reservoir fluid was pumped through

an ultraviolet water steriliser (Vecton 120 Nano) at around 5 ml per minute (one volume per 11.7 hours) to control microbial growth (Figure 3–6). The pH was checked daily to ensure that it was maintained between the range of 5.3 – 6.5 to maximise nutrient solubility. Airborne microbial contamination and water loss to evaporation were minimized by a purpose-made plastic cloche with vents to allow air circulation. The container and cloche were contained within a transparent PVC tunnel greenhouse.

(a)

(b)
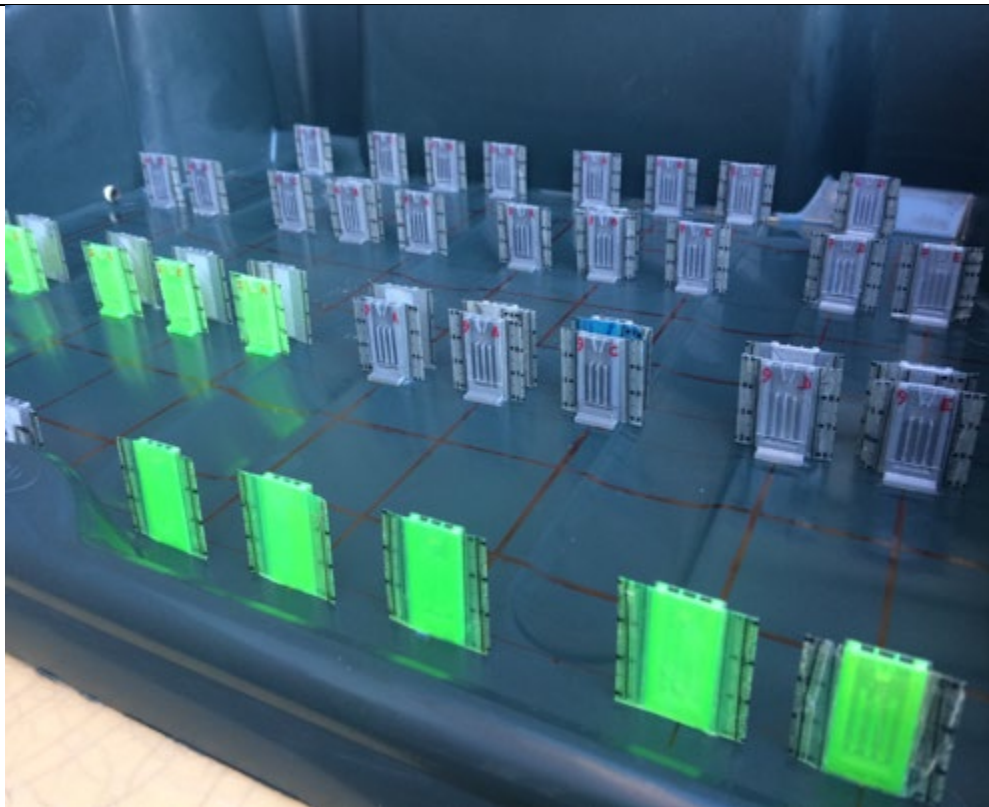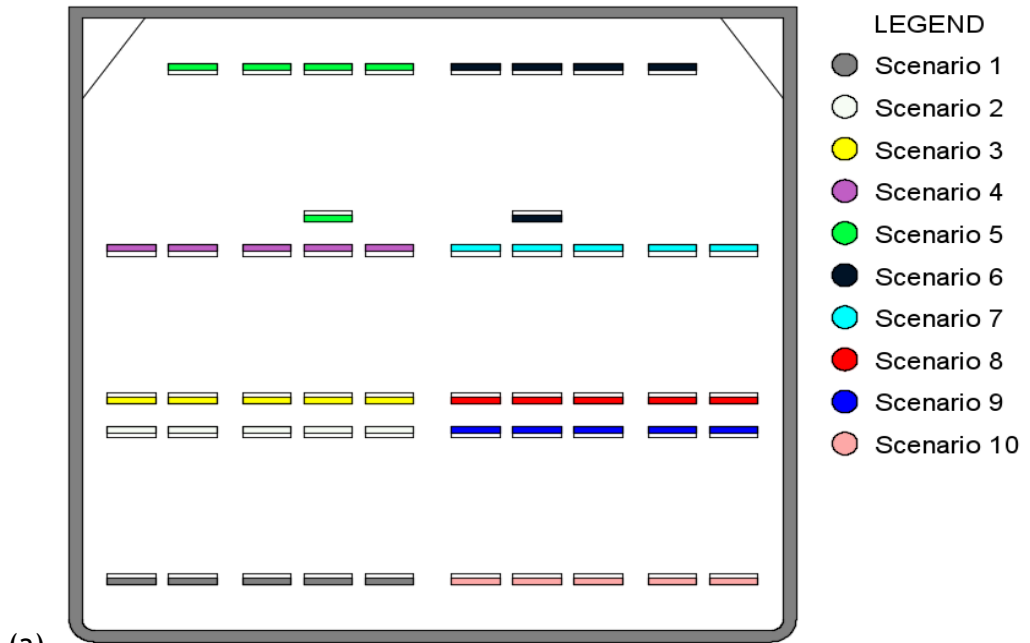
Figure 3-5 - Pore-scale rhizobox arrangement inside a plastic container. (a) plan view: each scenario is represented by a different colour, with white rectangles showing the location of the acetate film, i.e. the front of the box. (b) Physical arrangement of rhizoboxes. Variation in box colour is due to change in PLA batch used.

Ten microliters of coloured mineral oil was deposited on the nutrient solution surface in all the rhizobox columns designated as being contaminated by oil (Table 3–7) with a Hamilton 701RN syringe. This gave an oil layer within the column of depth 2.9 mm. One seedling of perennial ryegrass was placed into the seed housing, along with a small amount of cotton wool moistened with quarter-strength Hoagland's solution.

Figure 3–6 shows the growth trials set-up. Plant images were captured with a water resistant 12 MP wide-angle digital camera, placed on a small camera stand located 15 cm from the front of the rhizobox (Figure 3–6). A Softbox Twin-Head Continuous lighting kit, comprising 2 x continuous single lamp heads105W (5500K Daylight balanced Compact Fluorescent Light bulbs) with integrated 50 cm x 70 cm softboxes was used as a broader light source.
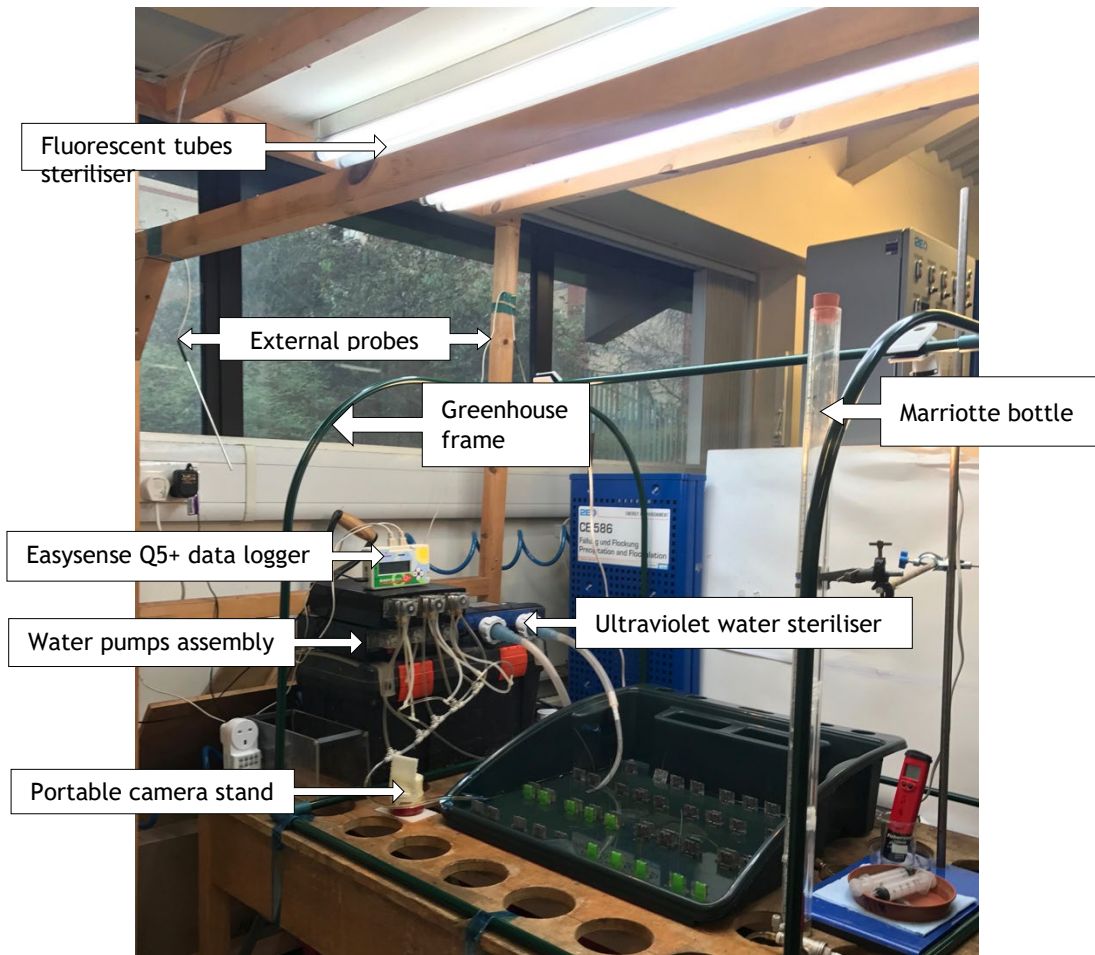
Figure 3-6 - Micro-scale rhizobox growth test set up. Plastic cloche and the transparent PVC greenhouse cover removed for clarity.

3.4.8 Environmental conditions

The container with the rhizoboxes were subjected to a 16-hour day length light exposure provided by four 58 W cool white daylight spectrum fluorescent tubes, placed 1500 mm above the rhizoboxes, Figure 3–6.

An Easysense Q5+ temperature data-logger (Data Harvest Group Ltd, 2018) was used to record the ambient temperature for the experiments (Figure 3-6). The data logger was placed in the middle of the growing platform. The temperatures are measured by three different probes: Two external probes placed at a different location across the planting platform and one internal thermometer within the logger (Figure 3–6). The logger also measured and recorded the humidity and air pressure, taking readings at every hour. All climatic data logged information can be found in Appendix 2.

3.4.9 Plant and mineral oil analysis

The microcosm phytoremediation experiments lasted four weeks, with day 0 defined as the time of seeding. At 7, 14, 21 and 28 days, images were taken and observations made of root and shoot growth patterns and oil levels in all rhizoboxes. Semi-quantitative measurements of root growth and distribution and oil loss were made during the experiment as fully quantitative and accurate data could not be obtained without disturbing the specimen.

The presence of roots in each column of each rhizobox was assessed as established (score = 1, where the root was observed to reach a depth of at least 14 mm below the seed housing (8 mm below the surface of the oil layer where present), limited (score = 0.5, where the root has penetrated the oil layer and/or water beneath but where the depth is less than 14 mm below the seed housing), and none (score = 0, where there is no root, or the root has not yet penetrated the oil and/or water layer); whilst oil loss was categorized as full (score = 1, where there was no visible oil left), partial (score = 0.5, where oil was visible but clearly reduced in thickness) or none (score = 0, where the oil has remained at or near its initial volume, i.e. approximately 2.9mm using the box side ruler as a reference).

The main shoot (vegetative parent tiller) height and main (primary seminal) root length were also measured over time. The root and shoot biomass were determined

at the end of the experimental growth trial by carefully washing the seedlings with de-ionized water and separating them into shoots and roots at the crown (growing point). The fresh root and shoot samples were dried at 75°C for 24 hours in accordance with British Standards Instituition (2007) and then weighed to determine the biomass production. Total root and shoot lengths were determined by summing the total length of all roots or shoots in a replicate.

The oil and root scoring data were statistically between scenarios and columns examined using non-parametric t-tests and the quantitative shoot and root data was statistically examined using an independent-sample t-tests (SPSS v25) at P<0.05 confidence level to compare the differences in the means.

3.5 Laboratory Mesoscale Phytoremediation Experiments

This section presents the materials and methodology for experimental work with ryegrass (*Lolium perenne*) grown in two prepared soils for exploring the impacts of LNAPL (mineral oil) on root distribution and growth, and oil loss in soil at the macroscopic scale.

3.5.1 Mesoscale phytoremediation tests

The behaviour observed at the microcosm scale was explored at a larger scale in soil mesocosms to determine how the response of an individual plant could be extrapolated to the behaviour of a planted soil and its effect on LNAPL contamination.

As with the microcosm experiments, mineral oil (coloured with Oil Red O) was used as a model LNAPL contaminant (section 3.2.2), perennial ryegrass (*Lolium perenne*) was used as the model plant (section 3.4.5) and quarter-strength Hoagland's solution was used for nutrient supply (section 3.4.6). The solution is essential for the growth of plants due to the lack of organic matter in the artificial soils used, as the nutrient solution contains nitrogen and potassium (Hothem et al., 2003), making it best for the development of plant in low nutrient scenarios.

3.5.2   Apparatus for mesoscale phytoremediation tests

For studying phytoremediation of LANPL with multiple plant perennial ryegrass seeds using artificially prepared soils, a specially constructed large-scale acrylic plant growth rhizobox was designed and fabricated in this study. The box internal dimensions are 350 high x 250 wide x 25 mm deep as shown in Figure 3–7.

This transparent box was constructed from 12 mm thick clear acrylic material on all sides to enable visual inspection of both contaminant transport and root growth during the experiment. The front plate of the box (not shown in Figure 3–7 for clarity purpose) was fixed in place using sealant and screws to allow removal after the test and to allow the sample extraction for post-test analysis. Some space was left at the top of the box for tolerance.

A 3mm acrylic sheet central partition separated the chamber into two segments, although the number of columns vary according to the experimental scenarios. A 40 mm gravel layer was placed at the bottom of the rhizobox interior to allow free drainage of the top layers of soil. This gravel fraction was prepared from thoroughly washed gravel passing the 12 mm test sieve but retained on 4 mm test sieve and covered with a layer of Whatman filter paper to prevent mixing with the soil.

Water levels within the chamber were controlled using an external, movable, plastic tube connected by flexible tubing to a port located in the gravel layer. The water level in this was maintained at a constant height, maintaining the 'groundwater' level within the soil.
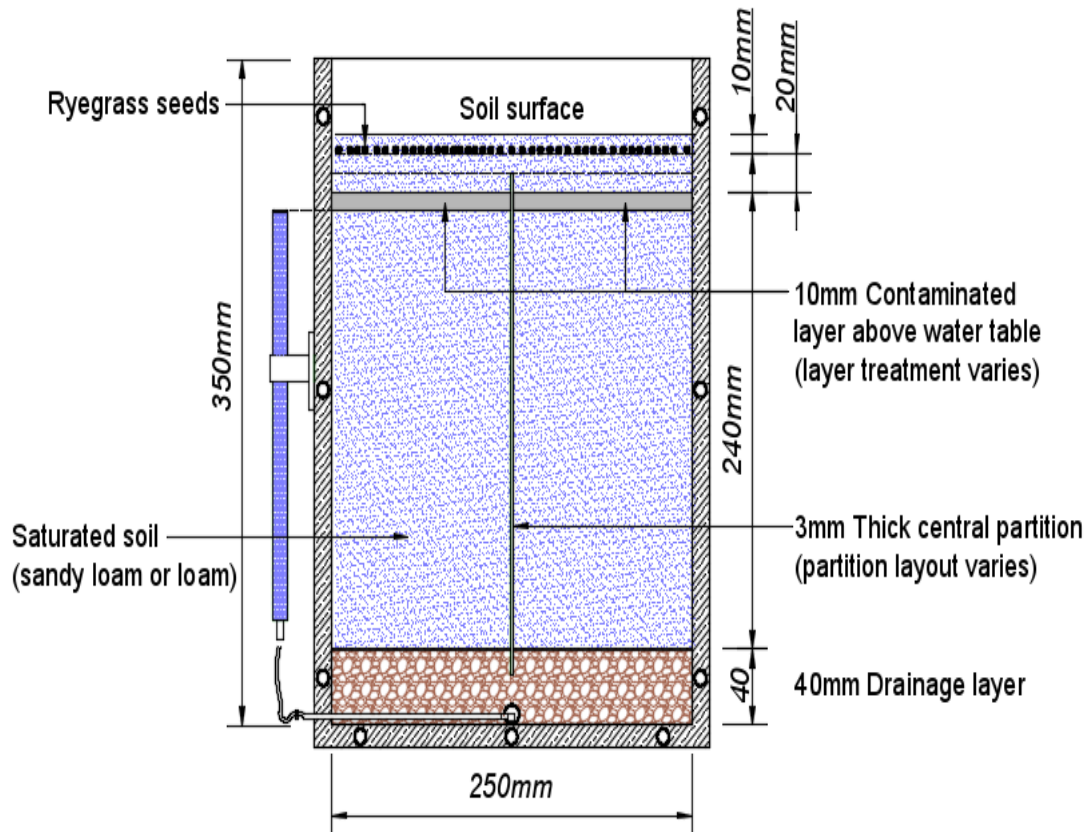
Figure 3–7 - A Schematic view of rhizobox section

3.5.3   Sample preparation and soil placement

Two soils (sandy loam and loam) were prepared as discussed in section 3.2.1. The properties of materials used in this investigation are summarised in Table 3–6.

For each test either sandy loam or loam was placed in four layers: a 230 mm thick soil layer, a 10 mm thick contamination layer, a 20 mm soil layer and a final 10 mm soil layer.

Required amounts of sandy loam or loam sample were taken from a thoroughly mixed air-dried sample. The samples were then stored in an airtight container. The sandy loam or loam soil was prepared in plant growth rhizoboxes by first of all wet-packing a 230 mm thick layer of soil at a water content equal to the plastic limit to a target density of 1.748 Mg/m^3 and 1.549 Mg/m^3 for sandy loam and loam respectively. The sample was mixed thoroughly and placed into the rhizobox before

compacted on a mechanical shaker for 2 seconds. The chosen initial water contents of the soils were corresponding to the plastic limits of the soils and for full saturation condition. Following the completion of compaction, the soil sample was then saturated via the external plastic tube (i.e. from the base of the soil) with quarter-strength Hoagland's solution to the top of the soil.

A 10 mm thick layer of soil was placed on top. In some scenarios' plant growth rhizobox, this had a moisture content equivalent to the plastic limit as before. In the other, the soil was mixed with mineral oil (coloured with Oil Red O) instead of water, to an oil content equivalent to the plastic limit. The placed soil was then compacted by gently tapping the box to the same target densities as before to produce a layer of a constant horizontal thickness of 10 mm.

Further, a 20 mm layer of uncontaminated moistened soil was prepared which had a moisture content equivalent to the plastic limit as before and placed above the 10 mm layer. Subsequently, the ryegrass seeds were spread on top of the 20 mm layer of uncontaminated soil to a density of 50 g/m². Finally, the seeds were then covered by a further 10 mm layer of moistened soil.

3.5.4   Contamination scenarios for mesoscale phytoremediation tests

Five contamination scenarios were considered in this study for the mesoscale phytoremediation tests. The five scenarios have been arranged to explore the response of plants to oil in artificial soils, as well as oil removal in persistent sources of contamination in the ground which probably may provide a long-term supply of dissolved phase contamination and taking significant periods to dissipate naturally. Figure 3–8 presents the details of the scenarios considered. In each case a 10 mm thick contamination zone was established 30 mm below the soil surface (termed hereafter as the contamination layer) and depending on the scenario this is either made up of contaminated or clean soil in each of the partitioned columns. The water table was maintained at the base of the contamination layer in all cases (Figure 3–7).

Control *scenario 1* for sandy loam or loam were control experiments which had two equal columns (12.35 mm) with LNAPL contaminated soil placed in the

contamination layer of the right-hand column but with no seed planted (Figure 3–8a). *Scenario 2,* also was a control experiment for the two prepared soils with seeds planted evenly across (Figure 3–8b). *Scenario 3* was a contaminated setup which had a continuous LNAPL contaminated soil placed in the contamination layers and seeds planted evenly across (Figure 3-8c). *Scenario 4* was an experimental setup which had two equal columns (12.35 mm) with LNAPL contaminated soil placed in the contamination layer of the right-hand column and clean soil in the left-hand column with seed evenly planted across (Figure 3-8d). *Scenario 5* had two main equal columns (12.35 mm), where the left side was entirely uncontaminated, and the right side was subdivided into five alternately contaminated and uncontaminated sub-columns with seeds evenly planted across (Figure 3–8e). In total there were 20 rhizoboxes with each scenario replicated twice for both soils. Two replicates were used for the soil experiments because the analytical work required was considerable for each specimen, and it was determined that two replicates was a manageable number. Using averaging across more than two replicates may increase the precision of root, shoot and oil loss measurements and permits minor changes to be distinguished.

(a) Scenario 1 – Oil in column 2 (No plant)   (b) Scenario 2 – Plant only (No Oil)

(c) Scenario 3 – Oil in column 1 and 2 (with plant)

(d) Scenario 4 – Oil in column 2 (with plant)

(e) Scenario 5 – Oil in columns A, C and E (with plant)
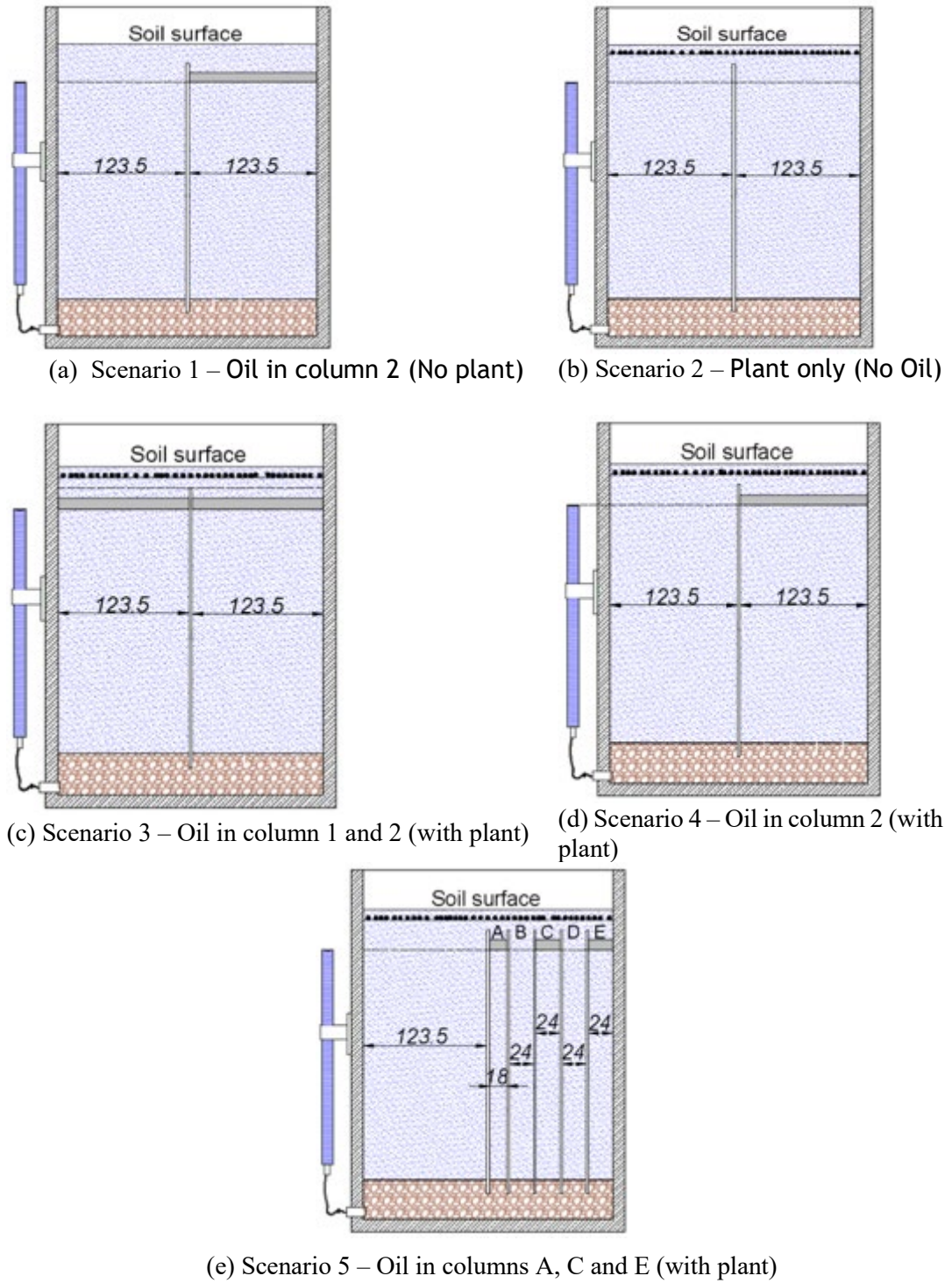
Figure 3-8 - Schematic of the five rhizoboxes treatments. (All Dimensions in 'mm')

3.5.5   Watering and environmental conditions for mesoscale tests

Over the ten weeks of testing period, quarter-strength Hoagland's solution was added via the supply tube every day to maintain the water table at the same position. The plant growth chambers were wrapped with aluminium foil to protect roots from light and subjected to lighting and environmental conditions as that occurred for the microscale experiments, Figure 3–9.
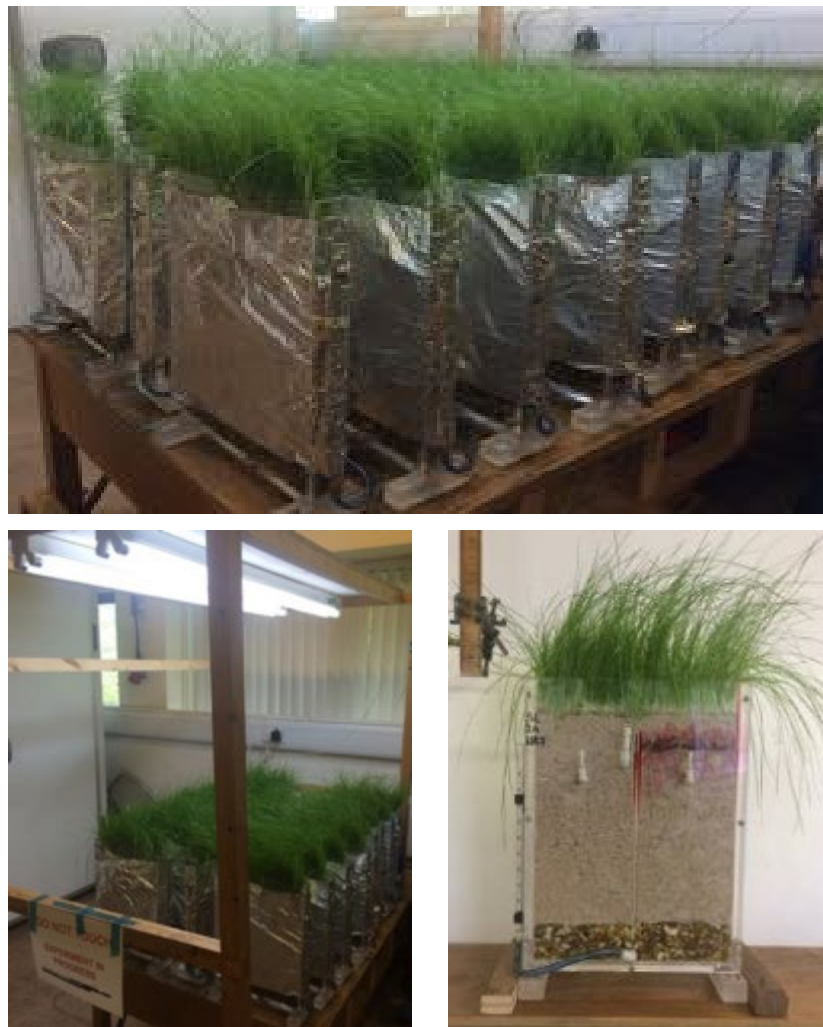
Figure 3-9 – Mesoscale phytoremediation growth test set up

3.5.6 Plant analysis

During the growth trials, the shoot growth was monitored by using the surface of the soil as a datum and the digital images of the plants were documented. The shoot growth was monitored on alternate days and for the entire duration of the tests, At the end of the experiment, the shoots were cut for each of the ten columns (A-E, left and right sides) for analysis using the soil surface as a cutting reference. The rhizoboxes were then stored in upright positions in a freezer at -20 °C to allow the soil to freeze. The front plate of the rhizobox was removed and the frozen soil was extracted to allow it to be cut along the predetermined vertical and horizontal boundaries. Samples were taken up to a depth of 10 cm from the soil surface because the analytical work required was considerable for each specimen, and it was determined that combining two specimens was a manageable number (Figure 3–10).

Figure 3–10 shows the individual sample cut boundaries. The frozen soil grid cutting was carried out using a 2mm tooth pitch Starrett high performance welded band saw blade. In Figure 3–10 the dashed lines indicate band saw blade cut centres and the redline boundaries show combined samples for analysis. The volumes of the samples within the rhizobox as indicated in Figure 3-10 are: Sample (i) – 3.2 cm^3; Sample (ii) – 6.4 cm^3; Sample (iii) – 10.97 cm^3; Sample (iv) – 21.94 cm^3. The samples were kept in individual resealed bags in a freezer at -20 °C prior to analysis.

The mineral oil in the frozen sawn soil cubes (samples) was extracted by dissolving the frozen soil samples in distilled water. Each sawn soil sample was mixed with distilled water at a ratio of approximately 1 to 4 by volume in Fisherbrand™ sterilin polystyrene petri dishes. The root was then separated from the soil by placing the mixed soil in a 200-micron sieve (with base). The sieve base was filled with distilled water, and the floating ryegrass roots above the water were collected with the aid of a wire mesh scoop. The plant materials (shoot and root) were dried at 75 °C for 24 hours in accordance with British Standards Instituition (2007) to avoid excessive drying. The root biomass content is expressed in mg/cm^3 of soil and shoot biomass expressed in mg. Sealed bags were used to store all dried plant materials in a cool and dry place in the laboratory.

The quantitative shoot and root data was statistically examined using an independent-sample t-tests (SPSS v25) at $P<0.05$ confidence level to compare the differences in the means.
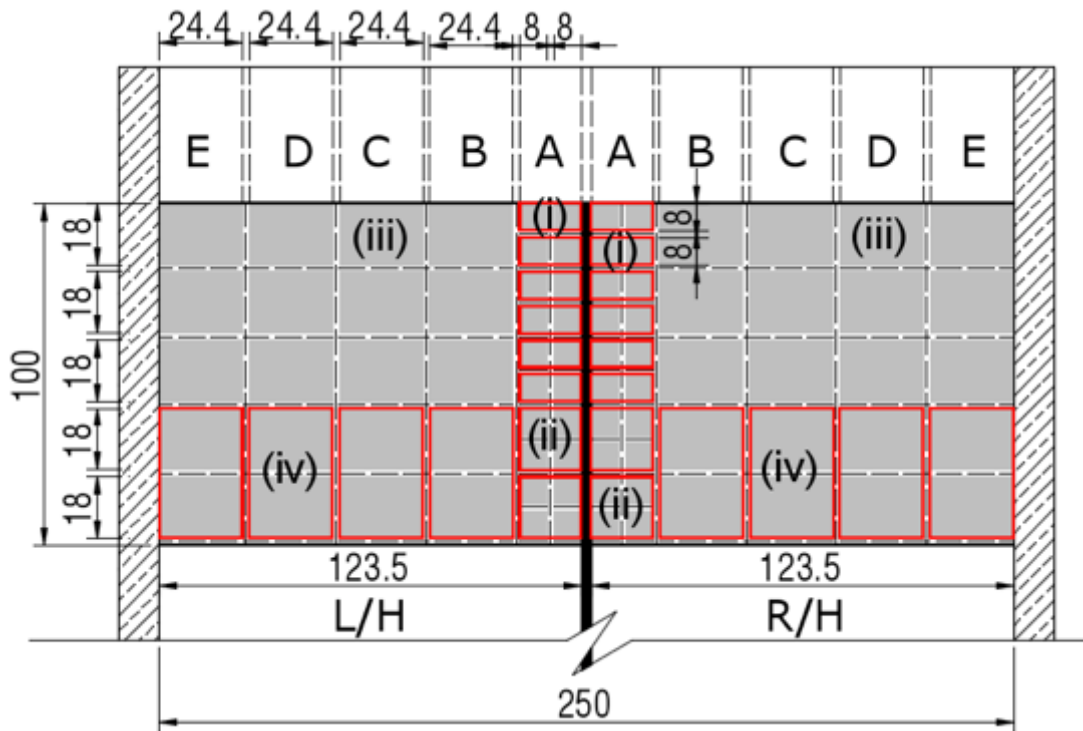
Figure 3-10 – Samples taken up to the depth of 10 cm from the soil surface. Individual sample cut boundaries (dashed line) and combined samples (red line). * (i) - represents 3.2 cm³ sample volume, (ii) - represents 6.4 cm³ sample volume, (iii) - represents 10.97 cm³ sample volume and (iv) - represents 21.94 cm³ sample volume – *L/H – Left Hand Side; R/H – Right Hand Side. All dimensions in millimetres (mm)

3.5.7 Oil Analysis

The mineral oil content was measured at the end of the experiment using the 'Oil Mat' method (Al-Ansary and Al-Tabbaa, 2007). Polypropylene oil absorbent mats (MAT440, New Pig Ltd) were used which had an absorption capacity of 0.043 L/cm² and exhibited 80 % and 99 % recovery at oil concentrations (by weight in soil) of

0.01 and 20% respectively in the preliminary tests (see appendix 4, calibration data for oil analysis). The concentration of mineral oil was determined by adding 40 g of distilled water to the already sawn frozen contaminated soil cubes in a petri dish (a ratio of approximately 1 to 4 by volume). An oil mat cut to the size of the inner diameter of the petri dish was then placed over the mixture of oil, soil, and water and covered to avoid evaporation. The petri dish container was then placed on an orbital shaker for 24 hours to dislodge trapped oil. The mat was taken out after 24 hours and weighed. The difference in weight of the matting over this period was reported as the mass of oil present in the sample.

The quantitative oil data was statistically examined using an independent-sample t-tests (SPSS v25) at P<0.05 confidence level to compare the differences in the means.

3.6 Summary

This chapter presents the materials used and the methods adopted in case of a series of experiments that studied the impact of LNAPL contaminant on plant growth in hydroponic conditions (microscale) and specially prepared soils (mesoscale).

The properties of the sandy loam used in this investigation were 2.68, 13 %, 10.6 %, 0.348, 7.9 and 1.5×10^{-4} m/s for the specific gravity of soil solid, liquid limit, plastic limit, the initial void ratio of compacted soil for permeability, pH and permeability respectively. The specific gravity of soil solid, liquid limit, plastic limit, the initial void ratio of compacted soil for permeability, pH and permeability for loam were found to be 2.66, 15 %, 10.3 %, 0.399, 6.6 and 1.2×10^{-7} m/s respectively.

The maximum dry density for sandy loam was 2.0 Mg/m³ at the optimum water content of 8.9 %, while 1.96 Mg/m³ maximum dry density was recorded for loam at the optimum water content of 9.8 %. The water content versus suction plots showed that both soils desaturated at lower suction. The chilled-mirror dew-point tests for soil-mineral oil mixtures showed very high value of suction (100 MPa) in the presence

of oil, however, varying the oil content in the soil has less impact on the suctions measured.

A pore-scale 3D-printed rhizobox was designed and fabricated for studying phytoremediation of LANPL with a single perennial ryegrass seed under hydroponic conditions. The boxes were designed to give a realistic representation of the effects of LNAPL contaminants spatially located in individual ganglia on the physical distribution of the root growth. Mineral oil (coloured with Oil Red O) was used as a model LNAPL contaminant, perennial ryegrass (*Lolium perenne*) was used as the model plant, and quarter-strength Hoagland's solution was employed for nutrient supply. Following four weeks of growth, the remaining oil levels were monitored and scored as the oil layer was not sufficiently thick to allow accurate measurement. Similarly, root growth in each of the four columns was observed and scored. Subsequently, the microcosms were dismantled before roots and shoots were separated and total weight and length recorded.

For the mesoscale phytoremediation study. A specially constructed large-scale rhizobox was designed and fabricated. The boxes were designed to give a highly idealised scenario to study the response of plants to oil in artificial soils, as well as oil removal in persistent sources of contamination in the ground. Similar to the microcosm experiments, mineral oil (with Oil Red O), perennial ryegrass (*Lolium perenne*), and quarter-strength Hoagland's solution was employed. Following the ten week growth period the shoot material was cut and divided into samples for each of the ten columns (left and right sides) and stored before freezing of the plant growth rhizobox. After removal of the front cover of the rhizobox the frozen soil was cut into the sections with a Starrett band saw blade. Individual samples were frozen in resealable bags prior to analysis. The mineral oil content of each sample was determined using hydrophobic oil-absorbent matting (MAT440, New Pig Ltd).

Chapter 4   Laboratory Phytoremediation Experiments on the Impact of NAPLs on Root Distribution and Growth, and Oil Loss, at the Pore-Scale

This chapter presents the results of the experimental work with ryegrass *(Lolium perenne)* grown under hydroponic conditions, exploring how plant growth, root distribution and development, and oil removal are affected through direct physical contact with mineral oil, a light non-aqueous phase liquid (LNAPL), in small-scale 3D-printed rhizoboxes. These rhizoboxes provided a simplistic model of soil macropores allowing control of oil distribution and accurate monitoring of root growth and oil loss in individual 'pores'. The main aim of the experiment was to explore the multi-phase interactions of plant, water, and LNAPL contaminants at the pore-scale level to identify how phytoremediation can be employed for source zone treatment in the presence of NAPLs. The research questions, the aims and the purpose of the growth trial are further explained in section 1.1. Growth trials were conducted in a series of small-scale 3D-printed rhizoboxes to study oil loss, root mass, root length, shoot growth, the biomass of roots and shoots as described in sections 3.5.2 to 3.5.7.

The light non-aqueous phase liquid (LNAPL) used is a mineral oil and was selected so that the experiment could be conducted with just one well defined petroleum by-product component as described in section 3.2.2. The oil and root scoring data were statistically examined using non-parametric t-tests and the quantitative shoot and root data was statistically examined using an independent-sample t-tests (SPSS v25) at P<0.05 confidence level to compare the differences in the means.

The results and analysis of root distribution and growth, and oil loss in the hydroponic system for the ten scenarios considered in this research are presented in the following sections: (4.1) the impacts of a NAPL location on the plant growth. (4.2) the plant effects and oil loss in a hydroponic system. (4.3) the general analysis, discussions and summary.

4.1 Impact of a NAPL zone on plant growth

Seedling germination and growth was found to be consistently good across all replicates in all scenarios, at least 80% of control as per OECD tests (Reuschenbach et al., 2003). There were no germination failures in any rhizobox. Examples of plant images to illustrate root growth and oil loss are shown in Figure 4-1.
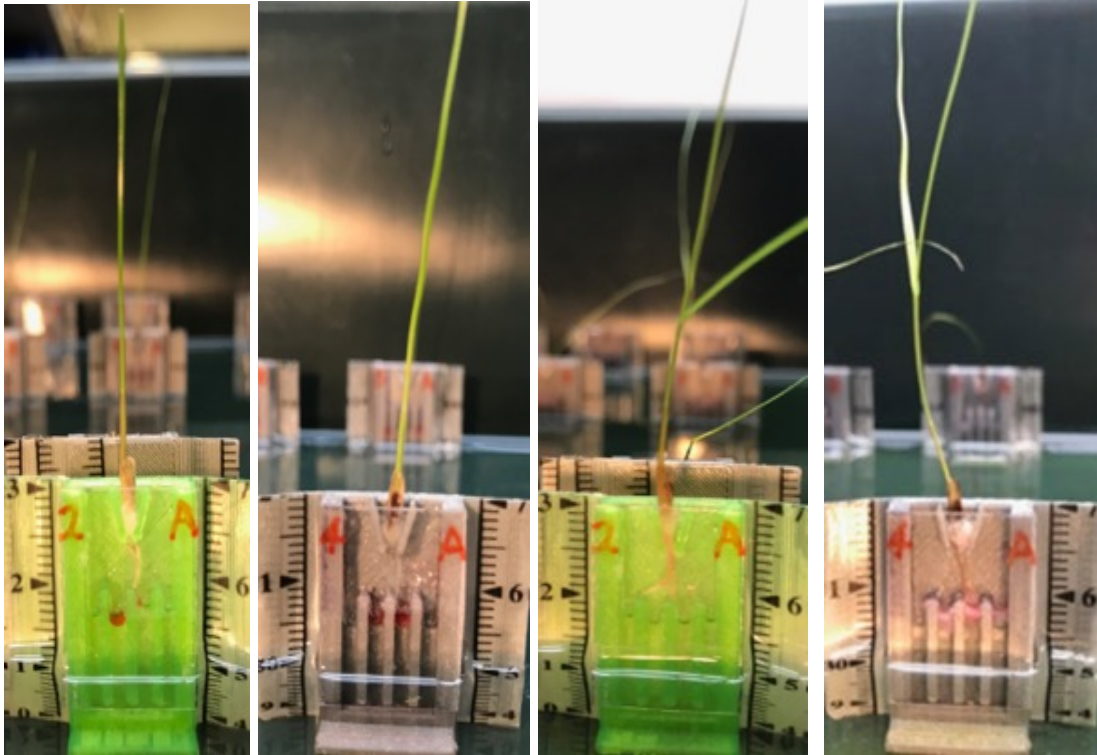
Figure 4-1. Rhizobox images illustrating examples of plant growth and oil loss for (from left): scenario 2 (day 14), scenario 4 (day 14), scenario 2 (day 28) and scenario 4 (day 28).

Figure 4-3, Figure 4-4 and Figure 4-5 show stacked root growth and oil loss scores – each 'stack' includes the scores from all five replicates, presented for each of the 4 columns in all ten scenarios, for days 14, 21 and 28 respectively. For example, if full root growth or complete oil loss (i.e. score = 1) was observed in a particular column in all five replicates, the bar will have a total index of 5. Stacked root growth bars are presented (in these and later figures) in order to graphically show the distribution of growth across all replicates as it was not found to be sufficiently informative to present data as, for example, averages with error bars given the limited number of possible scores in the raw data.

No significant growth was observed on day 7, nor was any oil loss observed as any roots were aerial and had not yet contacted the contaminant, and hence no results are presented for this time. At all three other times and in all scenarios, it is apparent that roots were spatially located primarily in the two middle columns (columns 2 and 3) regardless of contaminant location, indicating that roots tend to grow vertically downwards with little lateral spread initially, and that this is largely unaffected by the presence of individual oil 'ganglia' in these columns. The roots appear to coexist with the contaminants in oil-contaminated columns rather than avoiding them. Nonetheless, from day 21 onwards (Figure 4–4 and Figure 4–5), there was an apparent effect of mineral oil on root growth in the experimental scenarios 7, 8 and 9 where three or four columns had oil, with root growth being considerably more evenly distributed across the columns. In Figure 4–2, the standard deviation of the root growth score across each rhizobox was determined as a measure of root growth distribution across the different columns. For scenarios 1-6 and 10, at 28 days, this value was typically between 0.4 and 0.6 (n = 35, with one outlier at 0.3), which may be expected given the preponderance of growth in columns 2 and 3. For scenarios 7-9, this measure of root growth distribution was typically between 0 and 0.3 (n = 15, with two outliers at 0.48), demonstrating much more even growth.
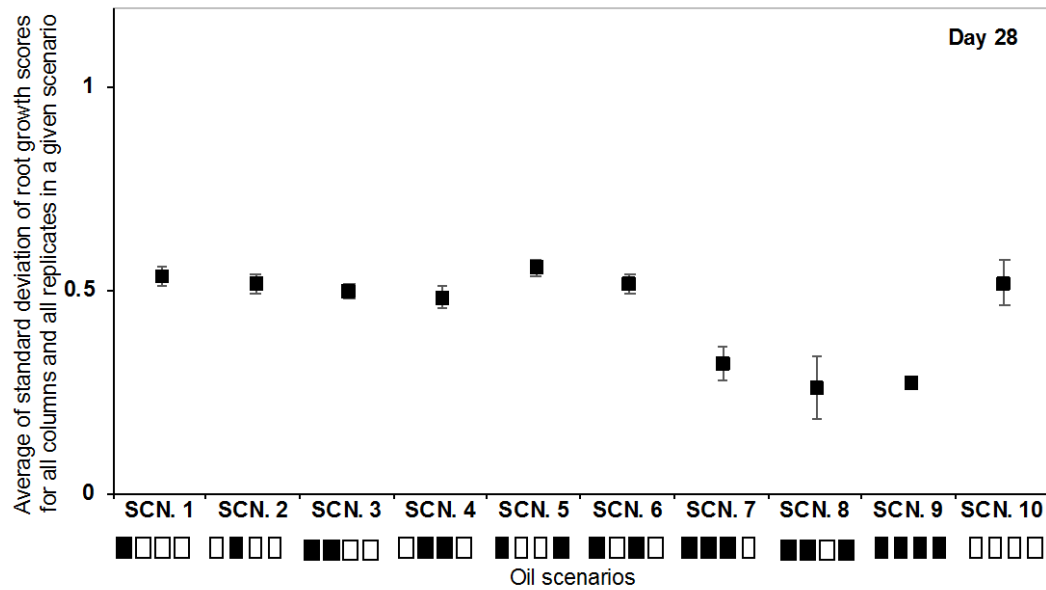
Figure 4-2 – The standard deviation of the root growth score across each rhizobox in different scenarios (Day 28). For each column, root growth is scored for all five replicates and all scenarios and the average of the standard deviation for these scores presented as a scatter plot (established root = 1; limited root growth = 0.5; no root growth = 0). Error bars represent ± one standard error of the mean.

However, it is not simply the presence of oil in columns 2 and 3 which subsequently caused the plant to seek out new routes to the nutrient medium, as scenario 4 had oil only in these columns and no diversion or spreading of roots was observed. Instead, it is hypothesized that the larger oil presence in scenarios 7 to 9 may probably led to higher levels of *dissolved* mineral oil (though dissolved mineral not measured in the study), at least transiently, and that it might be this that limited growth in columns 2 and 3 and therefore caused root spreading. There is some evidence for this in that the root growth in columns 2 and 3 of scenario 4 was found to be consistently higher than that in scenarios 7 to 9.
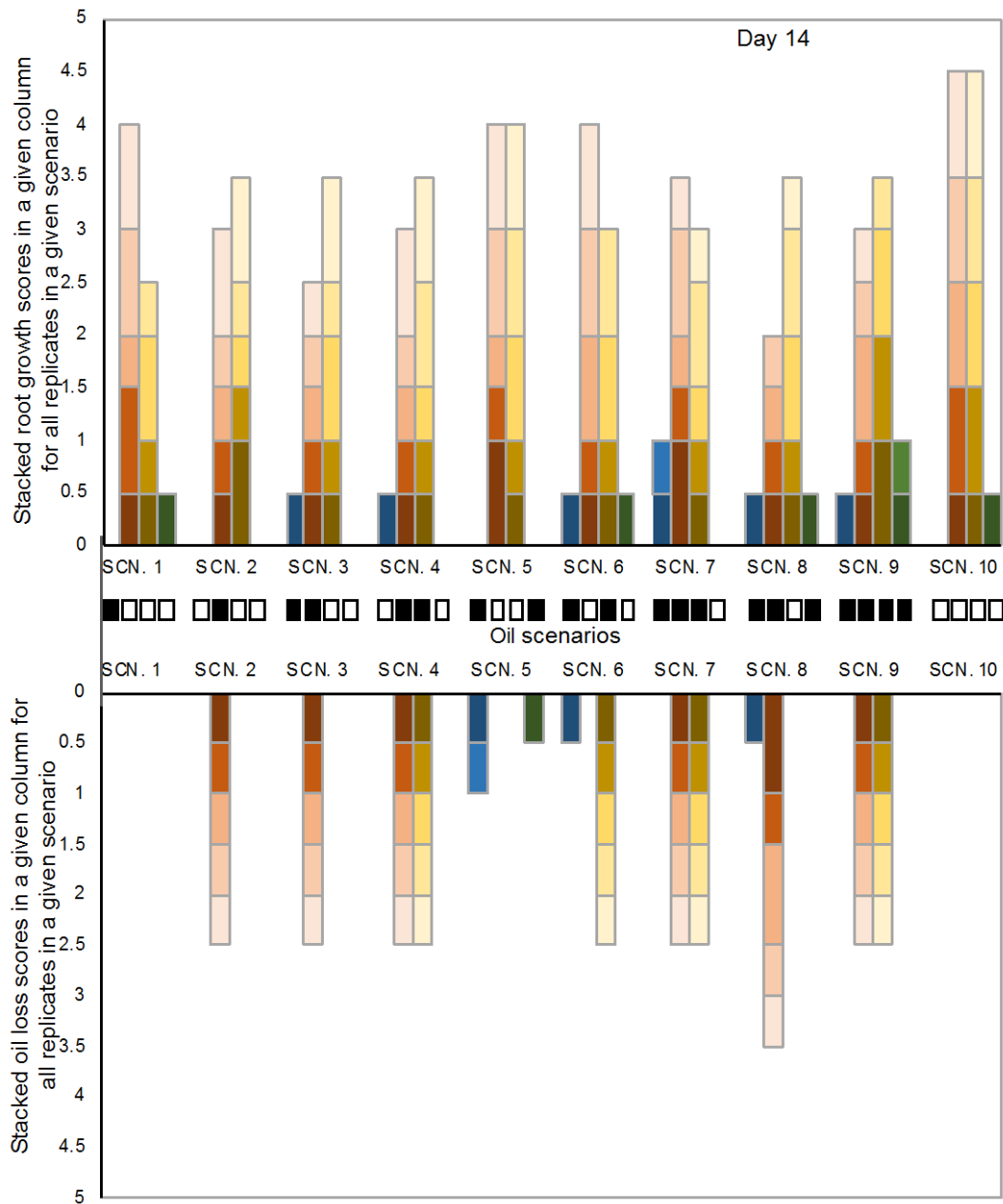
Figure 4-3. Root growth and oil loss in individual columns for all ten scenarios (Day 14) (blue – column 1; orange – column 2; yellow – column 3; green – column 4). For each column, root growth and oil loss are scored for all five replicates and these scores presented as a stacked bar (established root / full oil loss = 1; limited root growth / partial oil loss = 0.5; no root growth / no oil loss = 0).
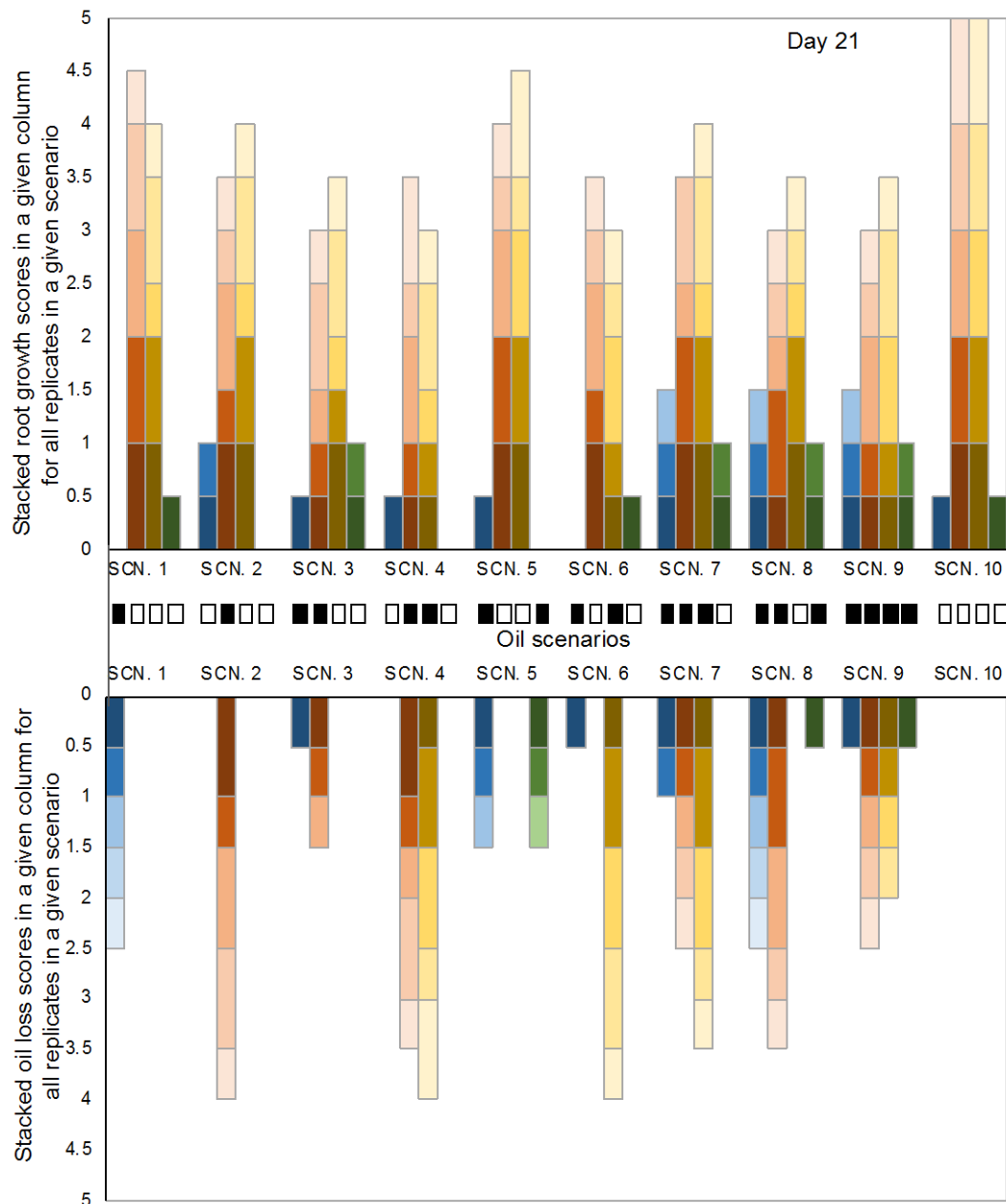
Figure 4-4. Root growth and oil loss in individual columns for all ten scenarios (Day 21) (blue – column 1; orange – column 2; yellow – column 3; green – column 4). For each column, root growth and oil loss are scored for all five replicates and these scores presented as a stacked bar (established root / full oil loss = 1; limited root growth / partial oil loss = 0.5; no root growth / no oil loss = 0).
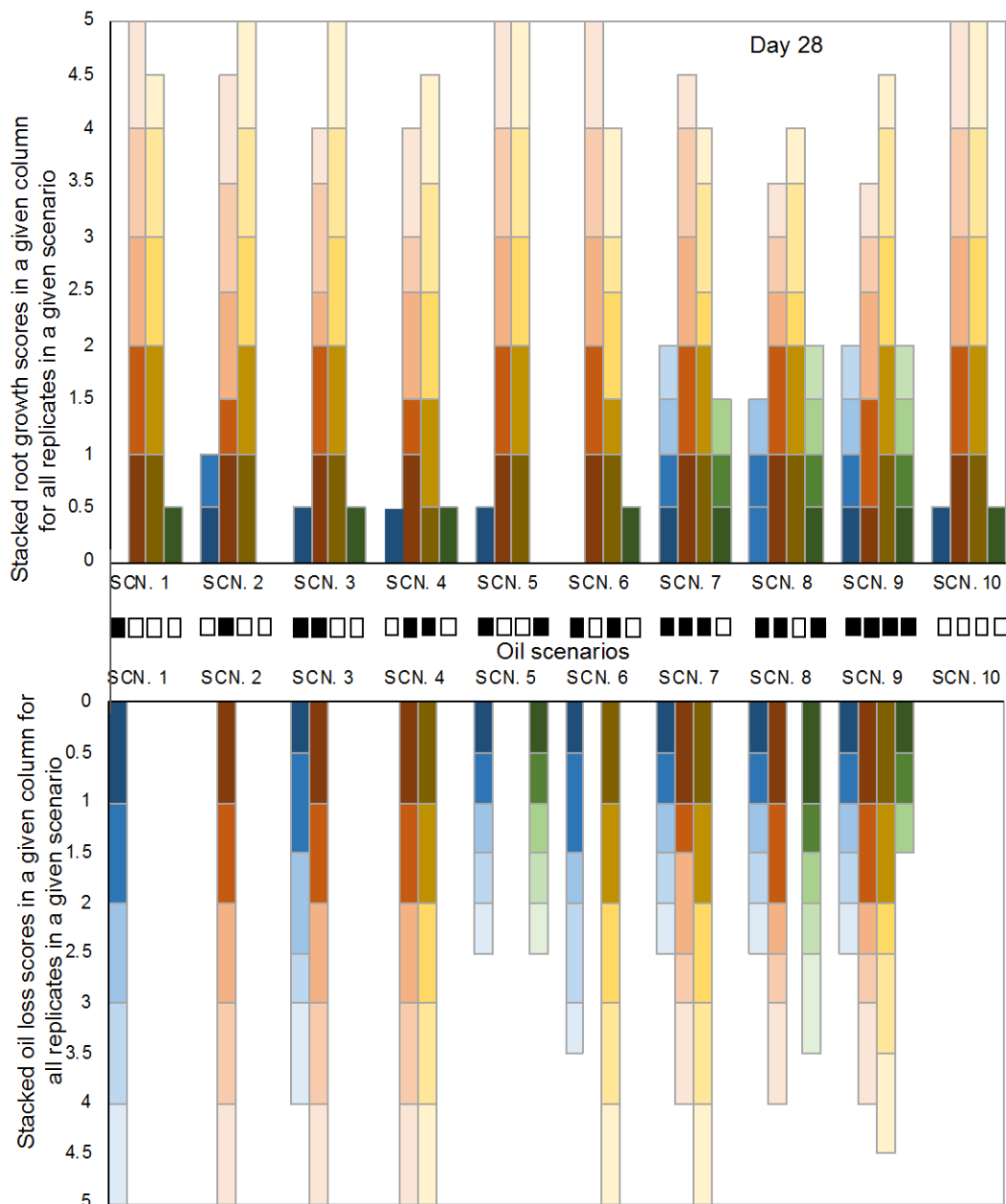
Figure 4-5. Root growth and oil loss in individual columns for all ten scenarios (Day 28) (blue – column 1; orange – column 2; yellow – column 3; green – column 4). For each column, root growth and oil loss are scored for all five replicates and these scores presented as a stacked bar (established root / full oil loss = 1; limited root growth / partial oil loss = 0.5; no root growth / no oil loss = 0).

4.2   Plant effects and oil loss in a hydroponic system

In oil contaminated columns, the presence of a root led to substantial oil loss as time progressed (Figure 4-3 – Figure 4-5) whereas in plant-free experiments little or no oil loss was observed (see section 5.1). Even where little or no root growth was observed in an oil-contaminated column, the oil still disappeared, albeit much more slowly than when a plant root was present. This suggests that oil removal was possible through the actions of roots in adjacent columns, potentially due to phytoremediation of the dissolved fraction of oil leading to increased rates of oil dissolution. Greater oil loss was generally observed in scenarios with less contamination overall, similar to the outcomes in other studies (Terzaghi et al., 2018, Vázquez-Cuevas et al., 2018a). This may also be related to the possible phytoremediation of dissolved phase oil, as if all roots contribute to remediation of all columns, a smaller amount of oil will generally be remediated more quickly.

4.3   General analysis and discussions

The effect of the presence of oil on the extent of root growth in individual columns across all scenarios is demonstrated in Figure 4-6. The average of the observed root growth indices for a given column with or without oil for all five replicates and all ten scenarios are scored and presented as a bar chart in Figure 4-6. The average root growth scores were presented because the total number of columns with and without oil are different and so a stacked plot (as in Figures 4-2, 4-3 and 4-4) would not suffice (e.g. there are more column 3s and 4s without oil than with it). Although the effect of oil is small, in columns 2 and 3 there is apparently a small negative effect of the presence of oil on root growth in individual columns. This was only statistically significant in column 2 however ($p = 0.008$, $p = 0.034$ and $p = 0.007$ for days 14, 21 and 28 respectively). It may be that the thickness of the oil layer was insufficient to affect the root growth significantly, and that greater amounts of oil would have a larger effect. In addition, ryegrass has some tolerance to mineral oil contamination (Zhu et al., 2018).
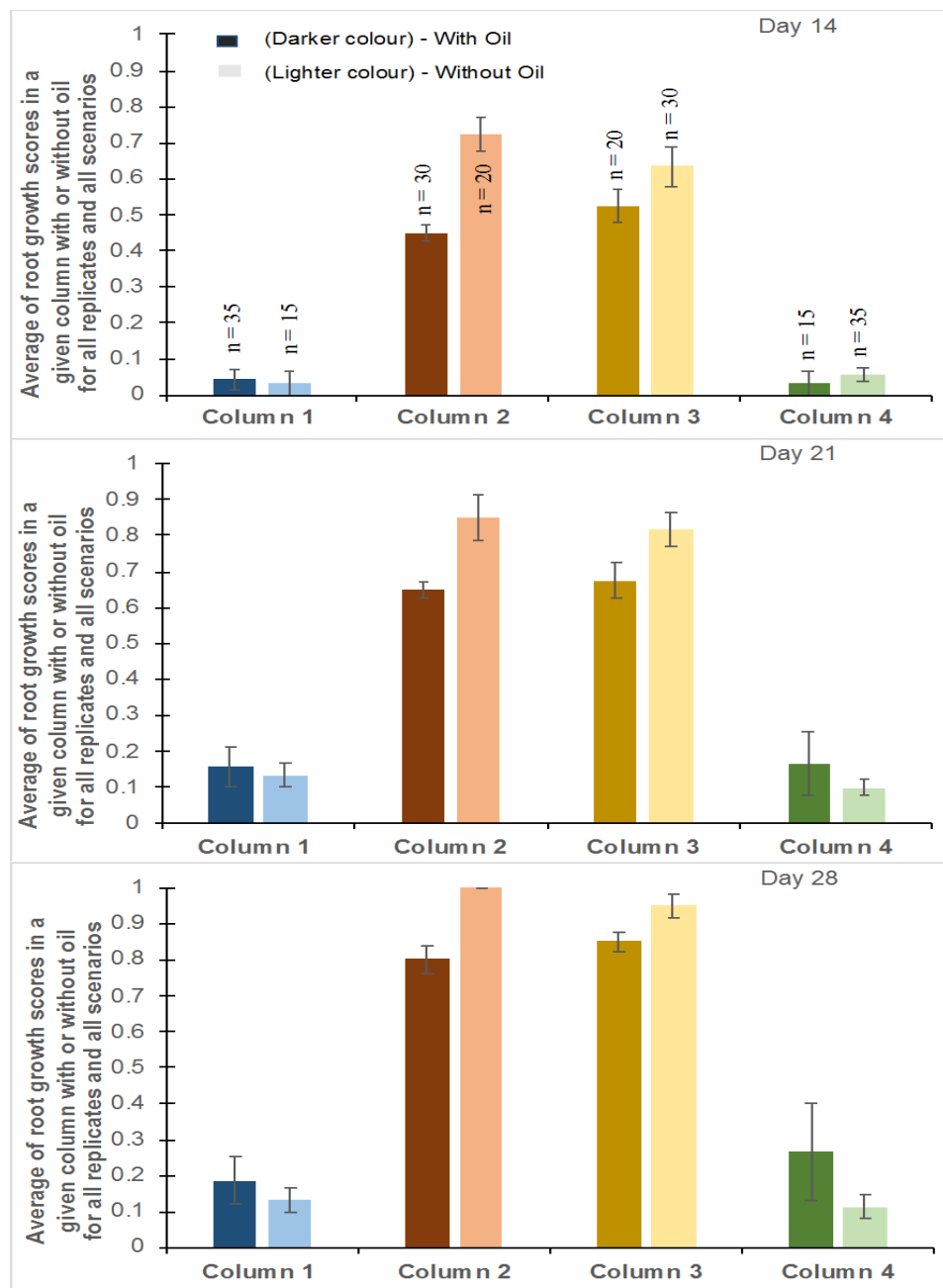
Figure 4-6. **Effect of presence of oil on average root growth score in individual columns at days 14, 21 and 28. For each column, root growth is scored for all five replicates and all scenarios and the average of these scores presented as a column bar (established root = 1; limited root growth = 0.5; no root growth = 0). The numbers of each column with and without oil (i.e. the number of readings used to calculate the averages) are presented on the figure. Error bars represent ± one standard error of the mean.**

Figure 4–7 illustrates how the root growth scores for all columns and all replicates in a given scenario (stacked bars including the growth scores from all four columns from all five replicates for each scenario – i.e. maximum score of 20) are affected by increasing oil coverage (represented by the number of columns with oil present). At day 14, the presence of oil had a negative effect on root growth, but the situation was slightly worse at the lowest oil coverage, but over time this effect decreased and may even reversed by day 28, with the highest oil coverage having slightly higher root growth than without any oil. It was observed that, in the oil-contaminated columns, plant roots were coarse and crooked, while those in uncontaminated columns were long, fine and smooth. Slightly increased root growth with increasing oil levels was observed in scenario 7 and 9 (oil in three or four columns), compared to the uncontaminated scenario 10, and this may be a response of the plants to environmental stress, increasing the spread of roots in an effort to find an uncontaminated route to nutrient supply. The mass increase was partially caused by an observed thickening of roots. Root injuries and changes in root architecture (length, thickness and branching) are commonly observed as a result of abiotic stresses such as drought, salinity or metal contamination (Franco et al., 2011) although the actual impact is highly species dependent.
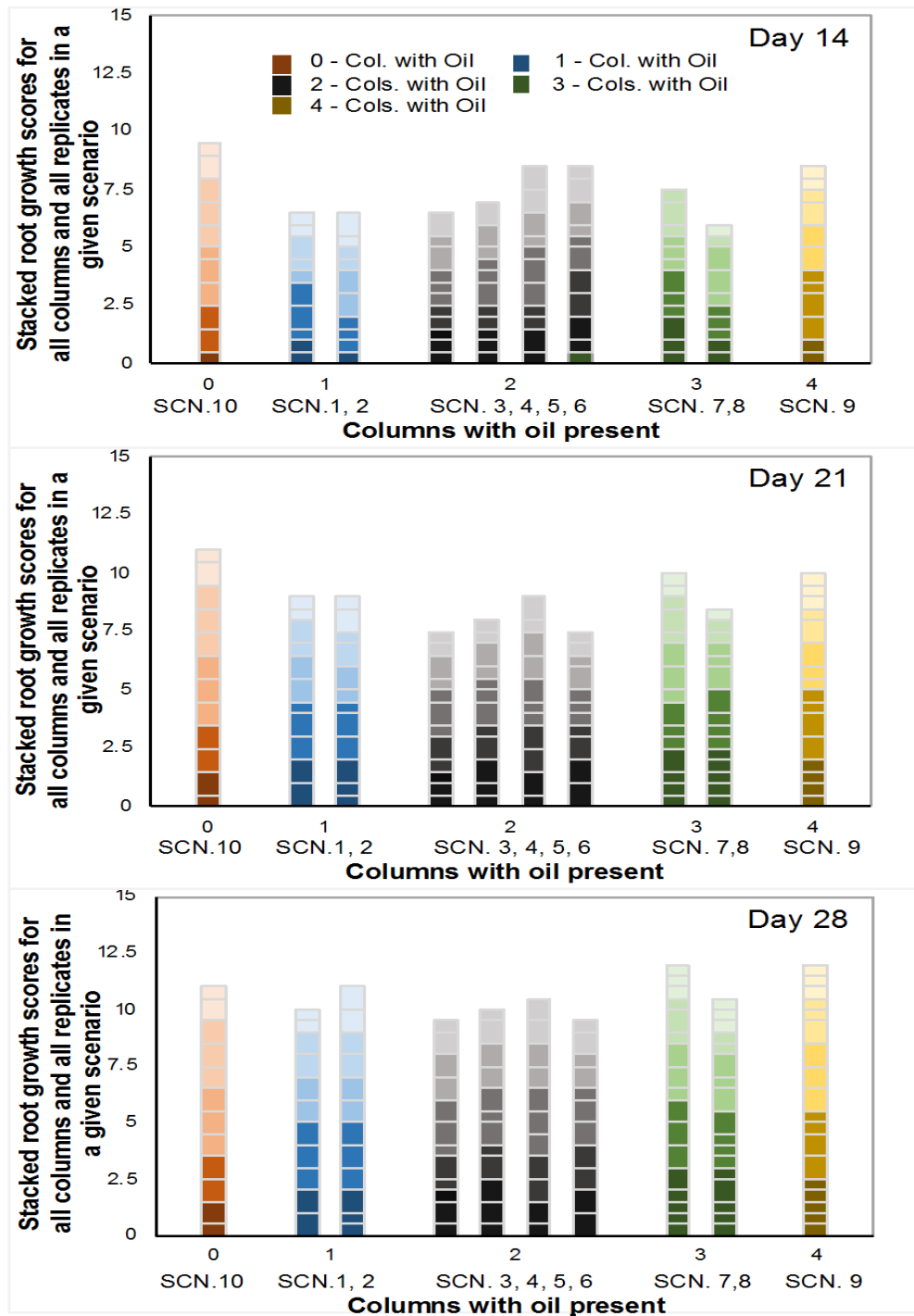
Figure 4-7. Effect of oil coverage (number of columns with oil present) on root growth (Days 14, 21 and 28). Root growth scores for all 4 columns in all 5 replicates are stacked for a given scenario (established root = 1; limited root growth = 0.5; no root growth = 0).

Figure 4–8 combines the root growth and oil loss data for columns where oil was present, for each time point, and shows that root growth appears to be correlated to oil loss at days 14 and 21 – increased root growth in columns 2 and 3 is matched by increasing levels of oil loss. It should be noted that there is overlap of data points on this figure, because of the limited number of possible values for both parameters. However, as time progressed there was some root growth and concomitant oil loss in columns 1 and 4, though the oil loss was large for relatively small root growth and in certain cases, oil was lost without any root growth in a column. This indicates that there is another mechanism of oil loss – as noted above, it is suggested that enhanced removal of the low levels of dissolved phase mineral oil by established roots in columns 2 and 3 disrupts the equilibrium causing further mineral oil in all columns to dissolve, which in turn is removed by the action of the roots and possibly attendant microorganisms.
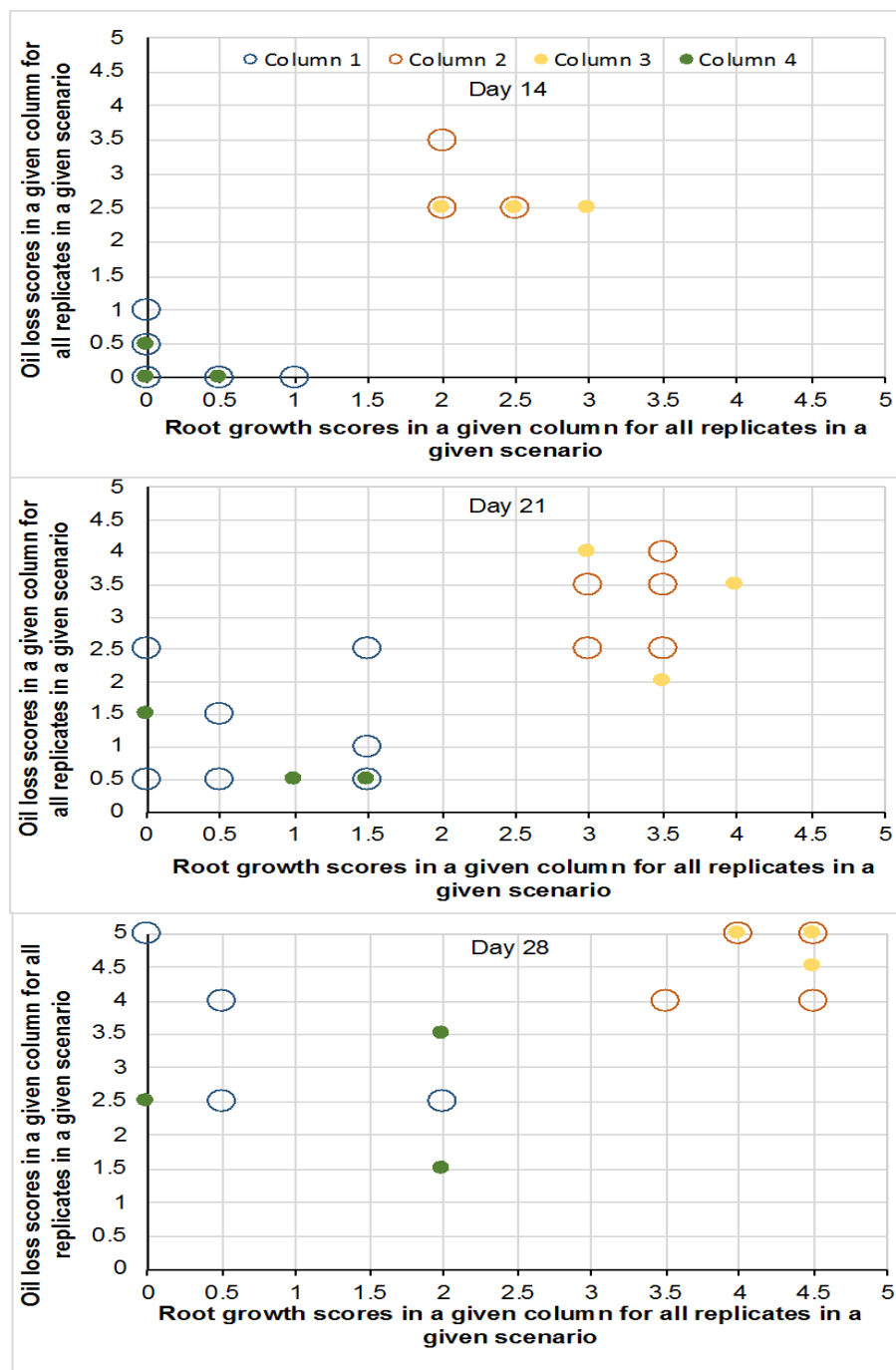
Figure 4-8. Relationship between root growth and oil loss in individual columns for all ten scenarios, (Days 14, 21 and 28). Shape outlines indicate the plotted points for each column to account for some points overlapping. (blue – column 1; orange – column 2; yellow – column 3; green – column 4). For each column, root growth and oil loss are scored for all five replicates and the total counts of these scores presented as a scatter plot (established root / full oil loss = 1; limited root growth / partial oil loss = 0.5; no root growth / no oil loss = 0).

Figure 4–9 shows the total root and shoot length for each scenario, averaged across all replicates, whilst Figure 4–10 shows root and shoot biomass in a similar manner. There are trends in the average values in each scenario which may be of relevance but these must be viewed with caution given the high variability. Scenario 10, without any contamination, had the highest average total shoot length and mass as might be expected, whilst the average values from scenarios 4, 7 and 9 were significantly lower (shoot length: scenario 4 - p = 0.042; scenario 7 - 0.041; scenario 9 - 0.030. Shoot mass: scenario 4 - p = 0.032; scenario 7 - 0.0026; scenario 8 - 0.038; scenario 9 - 0.005). The largest average root masses (Figure 4–10) were also found in these scenarios, which are the ones with oil present in both central columns, so given the prevalence of root growth in these columns it is perhaps not surprising that this has impacted upon plant shoot development. With roots, the average total length in scenarios 7, 8 and 9 approached that of largest values in scenario 10 whilst others were lower, which is indicative of the greater distribution of root growth across all columns in these scenarios as noted earlier. It is in these scenarios also that the root mass (Figure 4–10,) was significantly larger than in scenario 10 (scenario 7 - p = 0.041; scenario 8 - p = 0.0025; scenario 9 - p = 0.019) due to a combination of the thickening noted previously and increased root spread. It is perhaps surprising that the greatest root growth was observed with either no oil or the highest levels of oil contamination, similar to the earlier root growth scoring in Figure 4-6 – the greatest inhibition was seen with low oil contamination. The observed effects could be related to the reported results from the literature on the mechanisms of plant toxicity (Simonich and Hites, 1995) and the physical impacts of NAPL. It was suggested that the contaminants will first dissolved in the aqueous solution within the rhizosphere (dissolved phase not measured in the thesis) and secondly adsorbed on the root (Li et al., 2014b); and thirdly penetrating into root tissues (Hurtado et al., 2016), causing harm to the plant and consequentially inhibit growth (Vázquez-Cuevas et al., 2018b). The significant inhibition in plant growth has also been attributed to the presence of NAPLs in the soil. They did affect soil fertility and interchange of gases by clogging pore space. (Chen et al., 2013, Ramadass et al., 2015), thereby reducing the electrostatic interaction with some essential nutrients (K^+, Ca^{++}, NH_4^+) for the plants (Atlas and Bartha, 2012, Wolfe, 2013a). Under these conditions, the plant will experience a metabolic imbalance caused by a condition of oxidative stress which hampers the ability of the cell to

regulate the chemical processes (Romeh, 2017, Hou et al., 2015). This has impacted upon plant shoot development and thickening and the increased root spread noted.

The increase in root biomass, compared to the corresponding decrease in shoot biomass, in response to increasing mineral oil might suggest the plants put more energy into root growth than shoot growth due to stress induced by oil contamination. Oil can not only reduce the amount of water and oxygen available for plant growth (Kaur et al., 2017) but also can interfere with plant-water relations by direct physical contact (coating of root tissues) thus negatively affecting shoot growth (Razmjoo and Adavi, 2012). Such phenomena affect the local biogeochemistry, for example changing nutrient dynamics (Xu and Johnson, 1997) which in turn cause changes in root morphology similar to those observed here (Franco et al., 2011, Hermans et al., 2006).

It has previously been found that mineral oil negatively affects plant root architecture (thickness, length and branching) as a result of injuries caused by contamination (Vervaeke et al., 2003). Studies have observed increased root biomass in mineral oil-treated soil, attributed to a typical plant response to the reduced rhizosphere mycorrhiza and nutrient deficiency due to oil contamination (Heinonsalo et al., 2000). Poorter and Nagel (2000) concluded that plants respond to a decrease in below ground nutrients with increased allocation of biomass to roots and a reduction in above-ground resources (e.g. sunlight) with increased allocation of biomass to shoots. This effect resulted in coarser roots, expressed in increased average root diameter with a reduction in specific root length, but a larger surface area. Greater phytodegradation of organic contaminants has previously been related to higher specific root surface area (Ahmad et al., 2012, Merkl et al., 2005).
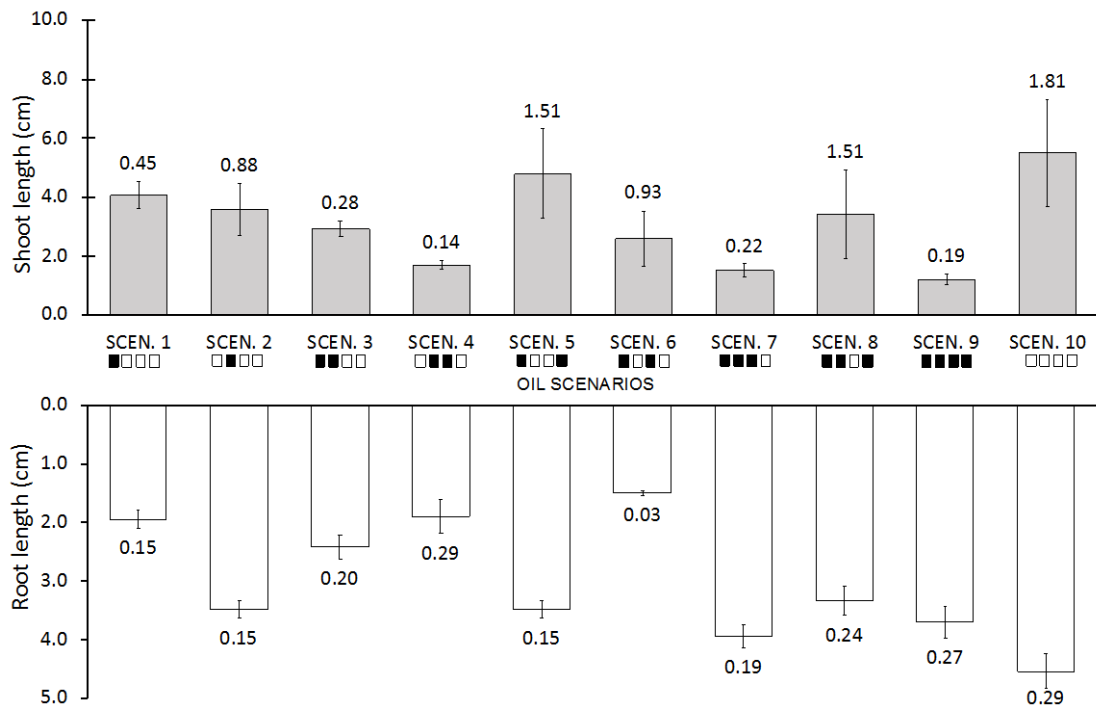
Figure 4-9 - Effect of the presence of oil on root and shoot length for all scenarios. For each scenario, root and shoot lengths are measured and totalled for each replicate, and the average of this total for all five replicates is presented for all ten scenarios. Error bars represent ± one standard error of the mean.
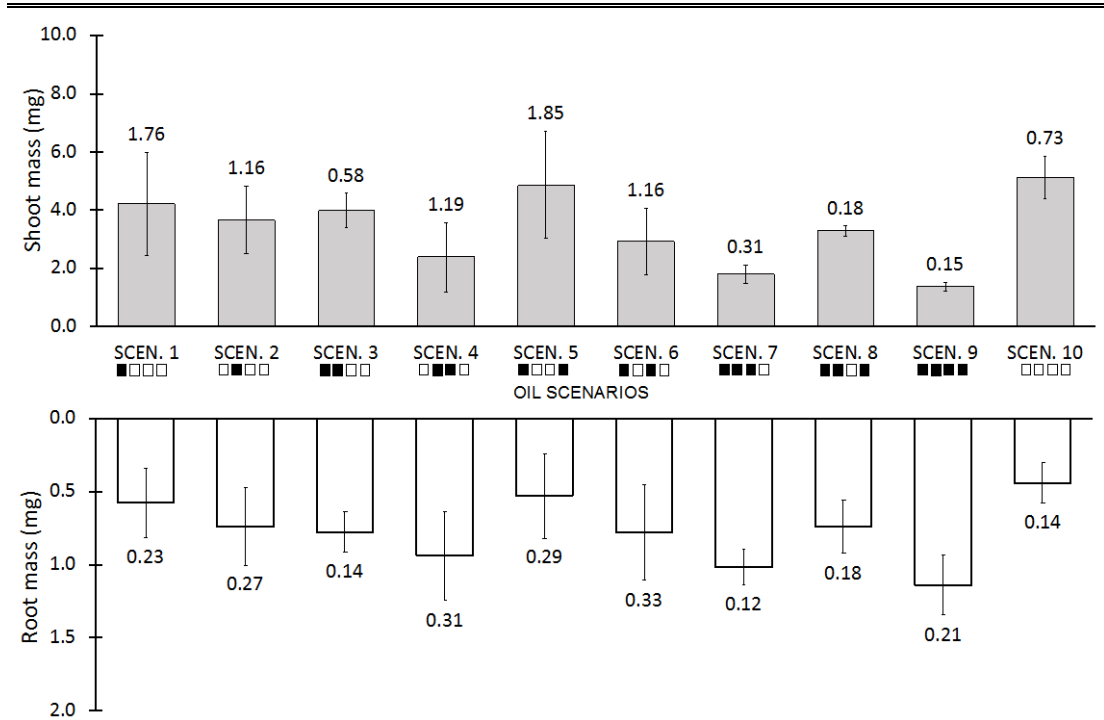
Figure 4-10 - Effect of the presence of oil on root and shoot biomass for all scenarios. For each scenario, root and shoot mass are totalled for each replicate, and the average of this total for all five replicates is presented for all ten scenarios. Error bars represent ± one standard error of the mean.

4.3.1   Limitations

Only plant control hydroponic scenario has been used, and it was determined that the oil-free plant control specimen was sufficient. Moreover, a plant-free control was planned for the mesoscale experiment. The specific column where the seminal root was located has not been ascertained due to the small scale rhizobox. Moreover, quantitative root length and root biomass can only be measured for the whole box (not in the columns) after the rhizobox has been dismantled due to the small size of the rhizobox. Scenarios have been arranged together for ease of data collection rather than using a randomised block design to allow statistical comparison across treatments. No characterisation tests performed on the mineral oil on the composition and properties such as solubility in water tests before and after the experiments as the analytical work required was considerable for each

specimen, Therefore, the dissolution of mineral oil in the hydroponic setup could not ascertained.

4.3.2   Summary

A laboratory experiment to provide a simplified simulation of the scenario believed to be familiar at many contaminated sites where the non-aqueous phase liquids are persistent sources of contamination was performed. Light NAPLs may be present in pore spaces in the capillary zone as a continuous or semi-continuous phase, or as unconnected ganglia which act as individual contaminant sources, providing a long-term supply of dissolved phase contamination. The degradation of the source zone and the root growth was monitored on a regular basis, and the resulting contaminant loss, root morphology, root, and shoot biomass analyzed. These pore-scale experiments have demonstrated consistent effects of LNAPLs on the growth and development of ryegrass, and also the removal of mineral oil by the plant. The root growth patterns recorded and observed indicate that physical presence of the root does not correlate to the individual contaminant ganglia degradation which suggests that the removal of the oil due to plants may be due to the removal of dissolved phase contamination, which in turn causes more oil to dissolve and be taken up by the plant. The presence of the plant resulted in an increased amount of oil loss over time from the system, and more loss was observed where there is less oil.

The root development and patterns for all the scenarios are consistent, regardless of contaminant locations. The same pattern of root distribution was also observed for the control scenarios. Therefore, the presence of oil does not prevent the roots from growing directly downward into the oil layers. Hence, the roots coexist with the contaminants within these columns rather than avoiding the contaminants which suggest that the thickness of the contaminant layers might be insufficient to affect root growth. There is, however, a substantial lateral spread of root in all four columns in the experimental scenarios where three or all four columns had oil suggesting that high oil contamination may cause root diversion whereby the plant increased the spread of roots in an effort to find an uncontaminated route to nutrient supply.

The impact of NAPL on root architecture is clear, with greater distribution with more extensive NAPL (thought to be caused by increased access to dissolved phase oil) and changes to individual root morphology. There was evidence of negative impacts on root development regarding changes to root architecture within the contaminated layers which may be attributed to the toxicity of mineral oil and a reduced supply of moisture and nutrients, which will stress root development. Root death or injury has been linked to oil toxicity. Hence, increased root biomass observed in the presence of oil contamination, indicating that the plant was putting more energy into root growth to mitigate the oil toxicity effects and degrade the mineral oil contaminant. The presence of oil is also associated with reduced shoot biomass production as compared to the uncontaminated controls with much more significant shoot biomass due to its inherent toxicity. This suggests that the stress induced by oil toxicity causes the plant to exert more drive into root growth than shoot growth.

This hydroponic microcosm experiment has provided evidence of the impact of the presence of an LNAPL (mineral oil) on plant growth, root distribution and oil removal. The study has demonstrated the potential for plants to tackle NAPL contamination and shows that the phytoremediation of the contaminant is not limited to the dissolved phase but that roots and the rhizosphere interacted with the NAPL which resulted in significant indirect reduction in the presence of the LNAPL. Apart from direct root contact with NAPL, roots close to NAPLs were able to remove dissolved-phase contamination through uptake and rhizodegradation resulting in the rapid dissolution of NAPL source. The study indicates the potential for phytoremediation of LNAPLs by perennial ryegrass in a hydroponic system, and in soils that are accessible by plant root systems.

Chapter 5 Laboratory Phytoremediation Experiments on the Impact of NAPLs on Root Distribution and Growth, and Oil Loss, in Soils at the Macroscopic Scale

This chapter presents the results of a series of experiments that studied the impact of NAPL contaminants on plant growth in an artificial soil at the macroscopic level to identify how phytoremediation can be employed for source zones treatment in the presence of NAPLs. The main aim of the experiment was to explore the impact of LNAPLs on plant growth and subsequent contaminant behaviour at macroscopic levels. By so doing, an understanding of how phytoremediation can be employed for source zone treatment in the presence of NAPLs would be developed. The research questions, the aims and the purpose of the growth trial are further explained in section 1.1. Growth trials were conducted in a series of rhizoboxes to study root mass, root length, shoot growth, the biomass of roots and shoots as described in sections 3.5.1 to 3.5.7.

The light non-aqueous phase liquid (LNAPL) used is a mineral oil and was selected so that the experiment could be conducted with just one well defined petroleum by-product component as described in section 3.2.2. The oil loss, shoot and root data were statistically examined using an independent-sample t-tests (SPSS v25) at the $P < 0.05$ confidence level to compare the differences in the means.

The results and analysis of root distribution and growth, and oil loss in the artificially prepared sandy loam and loam for the five scenarios considered in this research are presented in the following sections: (5.1) control scenario 1, plant-free oil contaminated soils. (5.2) control scenario 2, the response of grass plants to oil-free soils. (5.3) scenario 3, the response of grass plants to continuous zones of oil contamination in soils. (5.4) scenario 4, the response of grass plants to a discontinuous zone of oil contamination in soils. (5.5) scenario 5, the response of grass plants to small unconnected zones of oil contamination in soils. (5.6) the general analysis, discussions and summary.

5.1 Control scenario 1 – Oil and water interaction with artificial soils

Scenario 1 tests were control experiments for sandy loam and loam. The experimental setups had two equal columns with LNAPL contaminated soil placed in the contamination layer of the right-hand column but with no seed planted in either (Figure 5–1). There are two replicates for each soil. The results for scenario 1 tests which considered oil and water interaction with artificial soils are presented and discussed.

| (a) – Sandy loam | (b) - Loam |
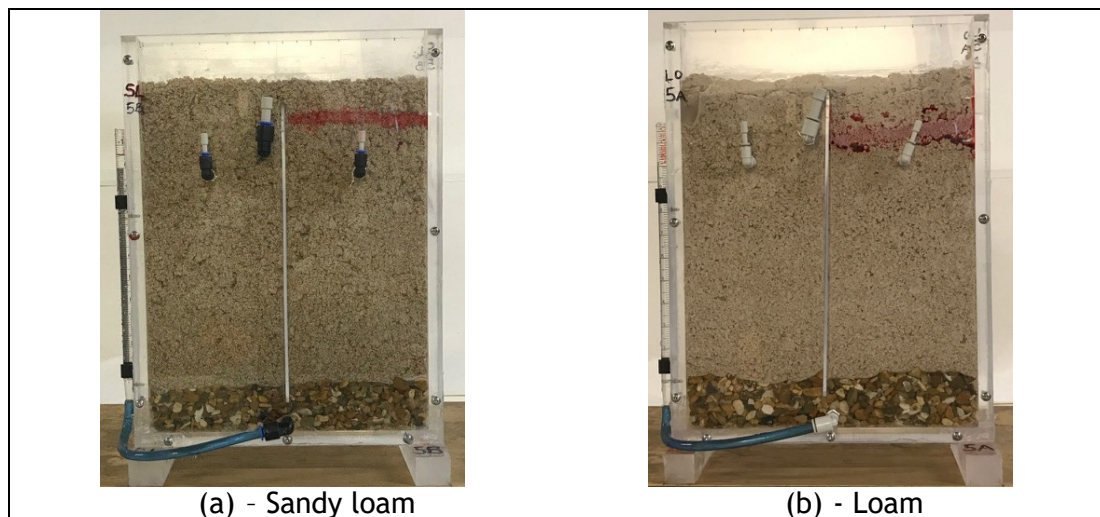
Figure 5-1 - Control scenarios 1 - LNAPL contaminated soil placed in the contamination layer of the right-hand column but with no seed planted.

The scenario 1 tests recorded low oil loss at the end of the experiment with total average oil loss for sandy loam and loam measured at 5.19 % and 3.59 % respectively, which suggested that there is little oil loss in the absence of plants for both soils. The results also provide confidence in the experimental protocols by recovering the majority of the oil present. Studies have shown that higher level of contaminant degradation in the planted soil may be explained by the rhizosphere effects supported by root system (Afzal et al., 2011, Alrumman et al., 2015). The presence of plants may favourably enrich the rhizosphere microbial flora by providing enzymes, exudates, and oxygen through its roots. The plant roots and mycelium can also create pores in the structure of the soil which can improve connectivity and

diffusion (Colombi et al., 2017). Therefore, the oil loss in the unplanted controls could take place by volatilization, photooxidation or the activity of microflora in the soil as a result of nutrient additions (Peng et al., 2009). Under this experimental condition, it would be expected that the natural breakdown would occur very slowly. In the soil that was supplied with nutrient solution, but without plants, the addition of nutrients and water may stimulate microbial activity, resulting in some crude oil breakdown compared to what can occur in the treatments without plant nutrients. The results from the analysed samples from the uncontaminated left side columns show zero oil recovery as measured at the end of the experiment using the 'Oil Mat' method.

5.2 Control scenario 2 – Plants and water interaction with artificial soils

Scenario 2 tests were control experiments for the two artificial soils with seeds planted evenly across (Figure 5–2). There are two replicates for each soil.

| (a) – Sandy loam | (b) - Loam |
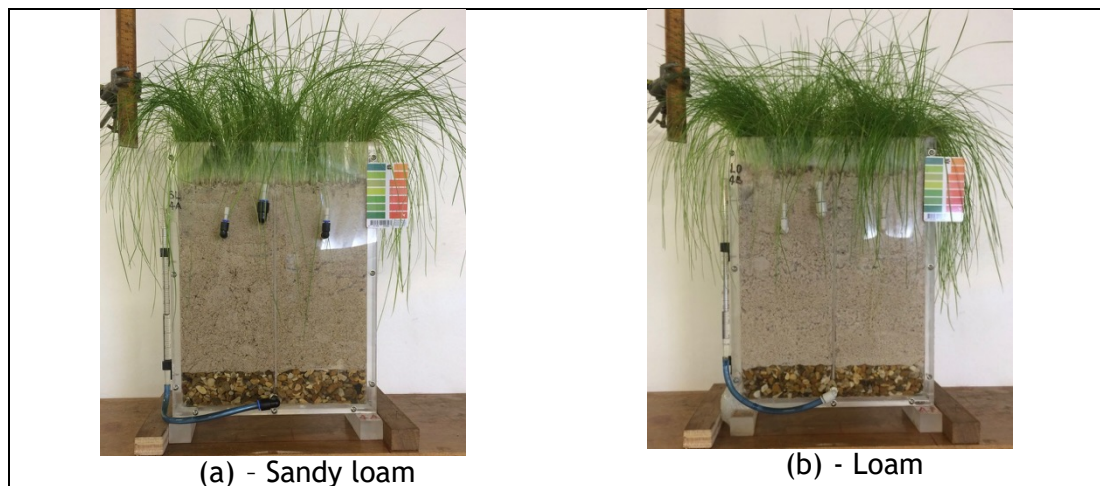
Figure 5-2 - Control scenarios 2 - Clean soil placed in both columns with seeds planted evenly across at a depth of 1 cm

Results of scenario 2 tests, which acted as a control experiment exploring plant and water interaction with uncontaminated sandy loam or loam, are shown in Figure 5–3, Figure 5–4 and Figure 5–5. Figure 5–3 and Figure 5–4 present root biomass with depth data for sandy loam and loam respectively, while Figure 5–5 presents shoot

and root biomass data for sandy loam and loam soils. Data are presented for both the left hand and right side of the central partition. Each side was further subdivided into five columns A - E ('A' closest to partition on both sides). The subdivision was done to study the difference in root development in different zones.

Root biomass decreases with depth in all samples. The discrepancy between the A samples and the B - E samples is because the former were taken every centimetre rather than every two centimetres as the remainder were. However, from Figure 5–3 and Figure 5–4 plots of root mass with depth, and Figure 5–5 plots of total root and shoot biomass for each of the columns, it is clear that there is a very uniform growth across all columns. The average total root biomass production for the whole box (across all sampling columns A – E) between a depth of 0 - 10 cm are similar for both sandy loam (600 mg) and loam (580 mg). Figure 5-3 shows the root biomass dropped below 2 cm depth in both columns. Also, in Figure 5–3, the average overall shoot biomass production is similar for both soils, with an average total of 3990 mg for sandy loam and 4100 mg for loam for the whole box. Though plant growth is dependent on many factors, the observed similarity in average root and shoot biomass for both soils may be due to the fact that sandy loam and loam soil have an almost identical initial water content of 10.6% and 10.3% respectively, and both desaturate easily at a lower suction as shown in section 3.3.10.5, Figure 3–2. Typically, at lower air entry value, soil with a lower proportion of fine soils will start desaturating, whereas soil with a high proportion of fine soils will desaturate at an air entry value of several hundreds of kPa (Barbour, 1998). Therefore, it may be that sandy loam and loam which contain almost similar lower percentages of fine particles induce lower suction which favours plant growth.
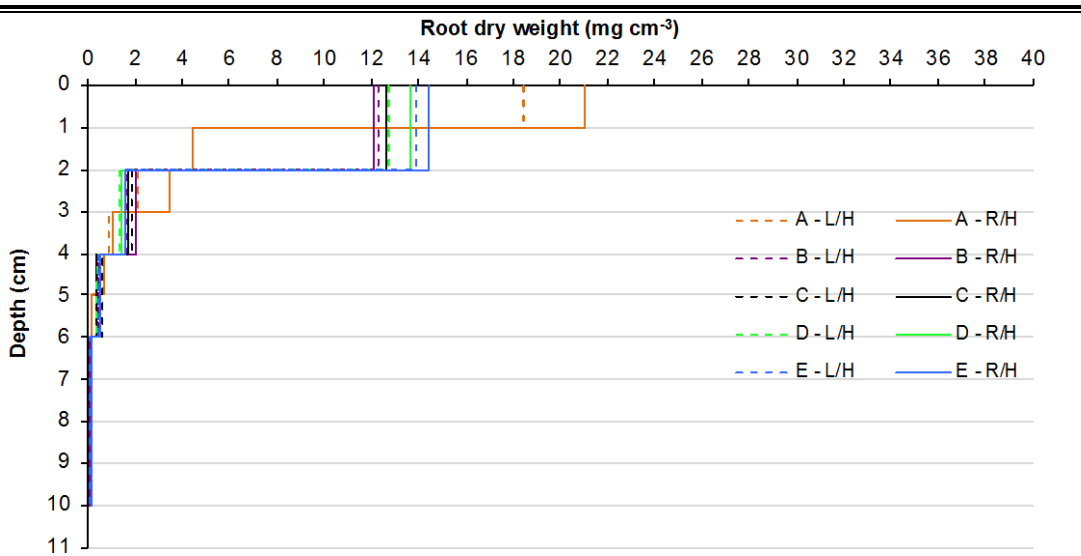
Scenario 2 – Replicate 1 - Root biomass with depth for sandy loam
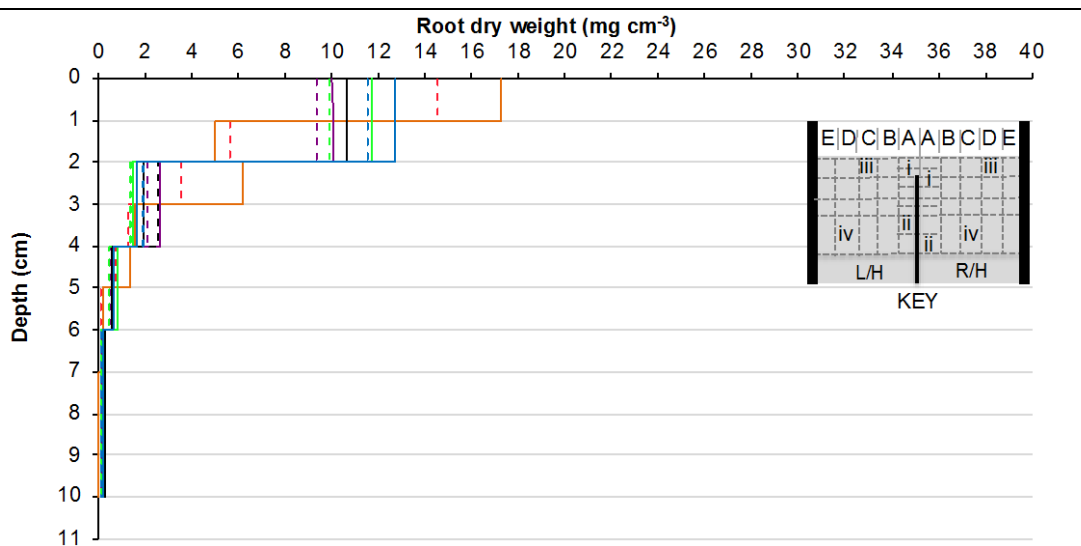
Scenario 2 – Replicate 2 - Root biomass with depth for sandy loam

Figure 5-3 - Control Scenario 2 – Replicates 1 and 2 - Sandy loam root biomass with depth.

Note: Refer to Figure 3–10 for samples' sizes and volumes. * L/H – Left Hand Side; R/H – Right Hand Side.

Scenario 2 – Replicate 1 - Root biomass with depth for loam

Scenario 2 – Replicate 2 - Root biomass with depth for loam

Figure 5-4 - Control Scenario 2 – Replicates 1 and 2 - Loam root biomass with depth.

Note: Refer to Figure 3–10 for samples' sizes and volumes. * L/H – Left Hand Side; R/H – Right Hand Side.

Scenario 2 – Root and shoot biomass for Sandy loam

Scenario 2 – Root and shoot biomass for loam

Figure 5-5 - Control Scenario 2 – Sandy loam and loam soils root and shoot biomass

Note: Refer to Figure 3–10 for samples' sizes and volumes. * L/H – Left Hand Side; R/H – Right Hand Side

5.3   Contamination scenario 3 – Impact of a continuous NAPL zone on plant growth

Scenario 3 experiments were contaminated setups for sandy loam and loam. These had a continuous LNAPL contaminated soil placed in the contamination layers and seeds planted evenly across (Figure 5-6). Scenario 3 was arranged to explore the response of plants to oil in artificial soils, to understand the impacts on root growth and distribution, as well as oil removal in persistent sources of contamination in the ground which provide a long-term supply of dissolved phase contamination and taking significant periods to dissipate naturally. There are two replicates for each soil.

| (a) – Sandy loam | (b) - Loam |
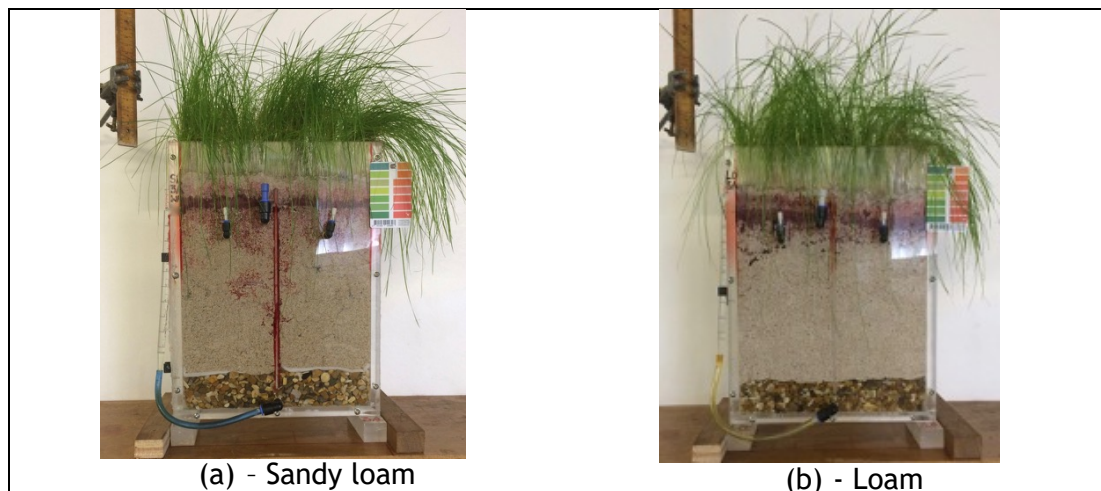
Figure 5-6 - Scenarios 3 - LNAPL contaminated soil placed in the contamination layers of both columns with seeds evenly planted in both at a depth of 1 cm.

The biomass present in the contaminated scenarios 3 for sandy loam and loam after 10 weeks are shown in Figure 5-7 and Figure 5-8. The figures are presented in a similar way to Scenario 2, although here the grey bar in Figure 5-7 and Figure 5-8 show the location of the contaminated zone. Again, there is generally uniform growth across the mesocosm, both above and below the soil surface.
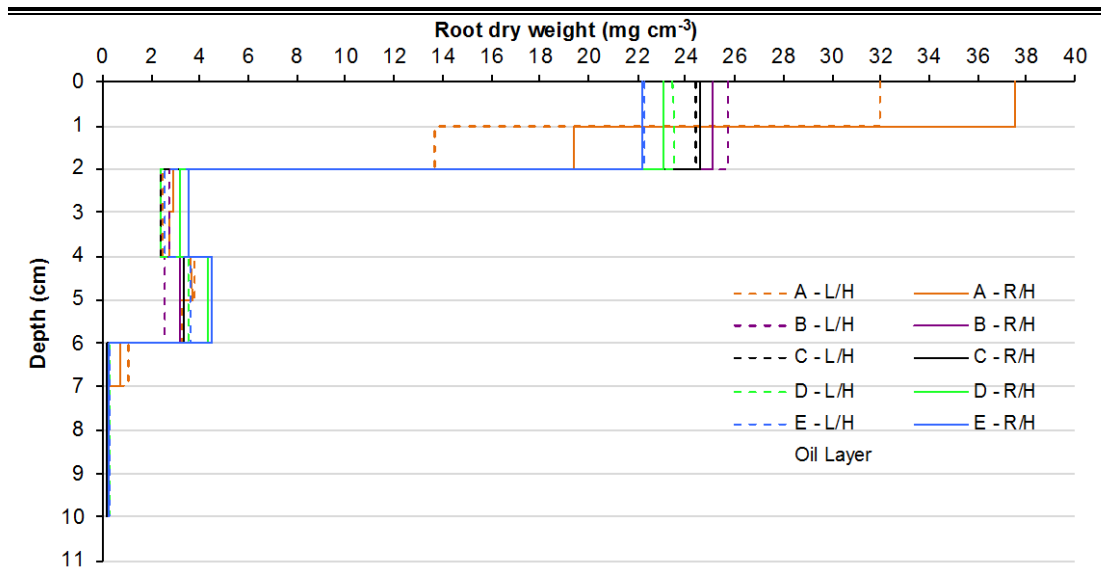
Scenario 3 – Replicate 1 - Root biomass with depth for sandy loam
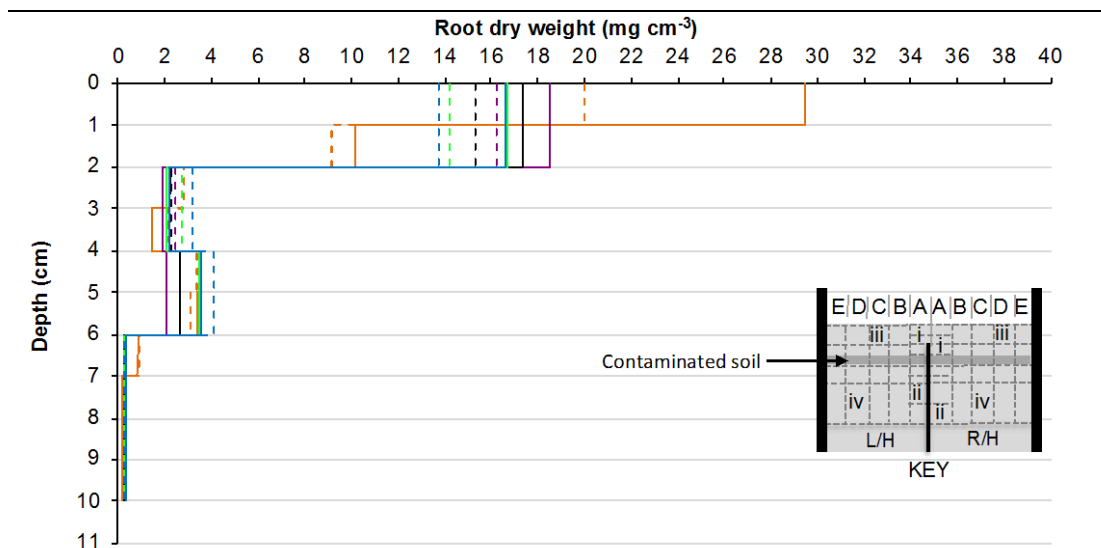
Scenario 3 – Replicate 2 - Root biomass with depth for sandy loam

Figure 5-7 - Scenario 3 – Replicates 1 and 2 - Sandy loam root biomass with depth.

Note: Refer to Figure 3–10 for samples' sizes and volumes. * L/H – Left Hand Side; R/H – Right Hand Side.
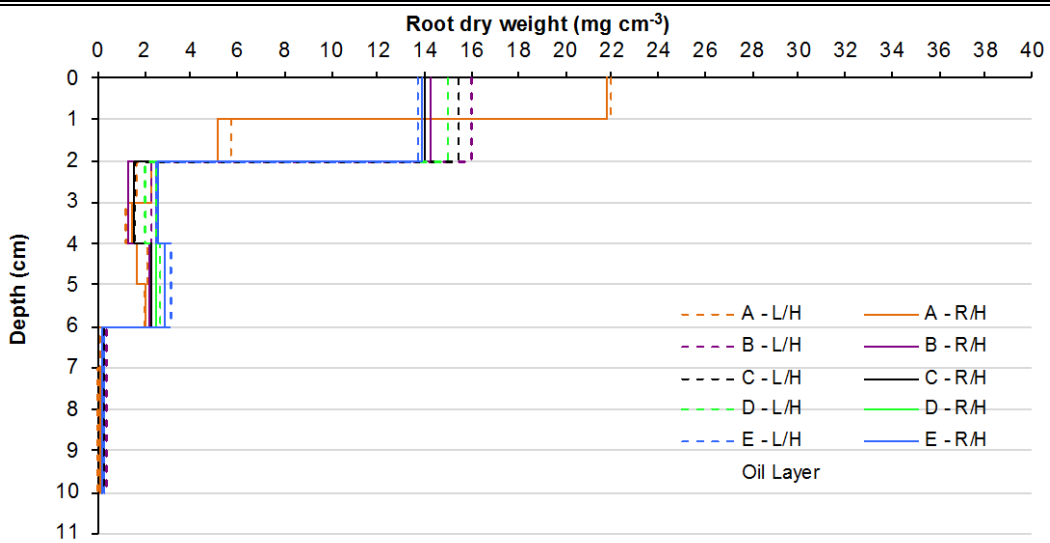
Scenario 3 – Replicate 1 - Root biomass with depth for loam
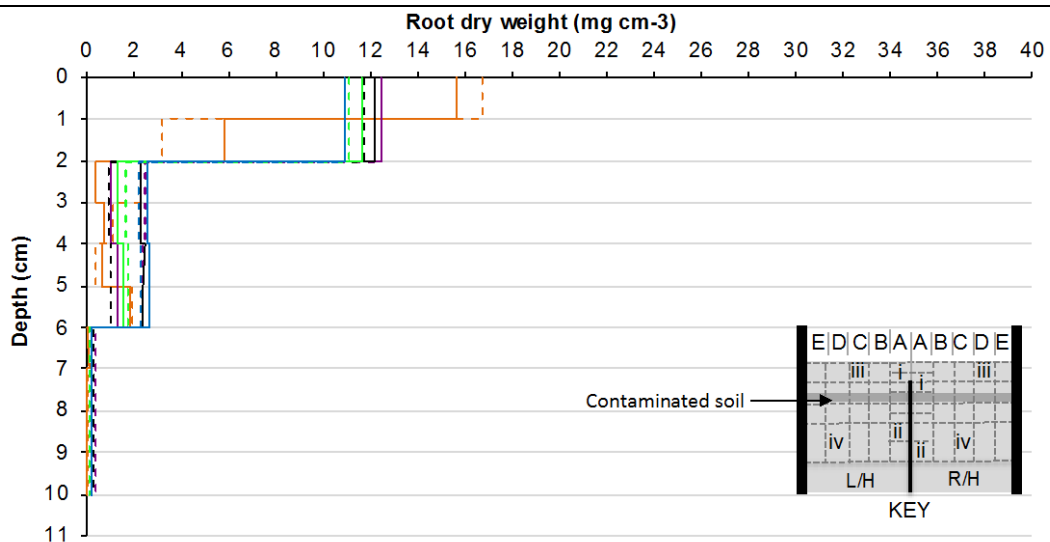
Scenario 3 – Replicate 2 - Root biomass with depth for loam

Figure 5-8 - Scenario 3 – Replicates 1 and 2 - Loam root biomass with depth.

Note: Refer to Figure 3–10 for samples' sizes and volumes. * L/H – Left Hand Side; R/H – Right Hand Side.

5.3.1 Scenario 3 - Impact of NAPL on shoot growth

Comparing the shoot mass in the control scenario 2 with scenario 3 for both soils, there is a significant difference in shoot growth in both soils as shown in Figure 5–9.
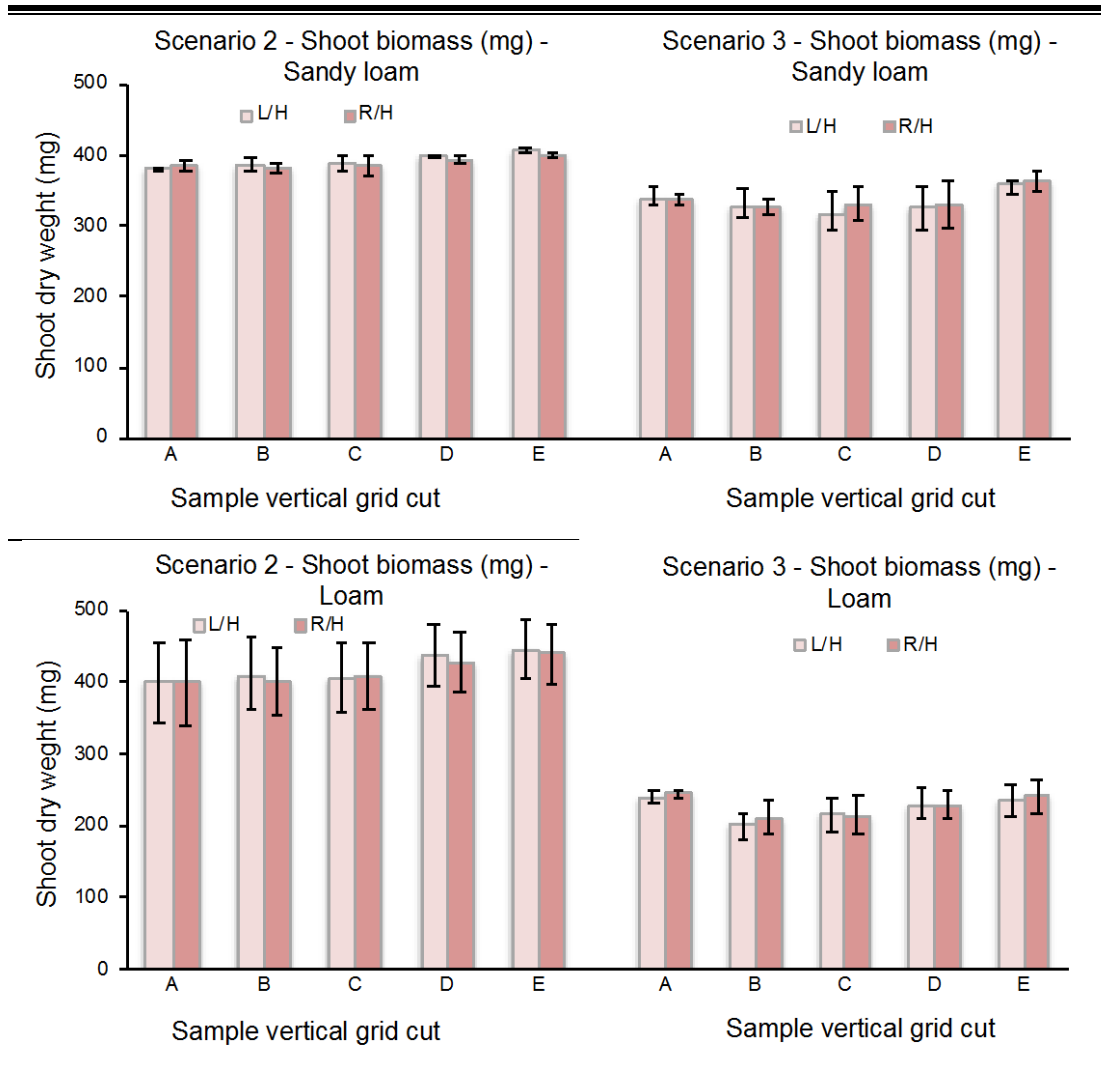
Figure 5-9 Scenarios 2 and 3 – Shoot biomass for sandy loam and loam soils. Error bars represent ± one standard error of the mean.

The scenario 2 sandy loam shoot mass is significantly higher compared to scenario 3 (p = 0.0005), and also scenario 2 loam shoot mass is significantly higher compared to scenario 3 (P = 0.0003). The decreased shoot biomass in both soils in scenario 3 suggests the presence of oil inhibits shoot growth as a result of the physical exclusion of moisture and nutrients, and the oil toxicity. Therefore, these results

indicate that mineral oil does have an effect on shoot growth. As with the microcosm experiments (section 4.1), there is a decrease in the overall mass of shoots in the presence of oil, but there is a significant increase in the mass of roots, although this is particularly clearly illustrated with sandy loam (Figure 5-9). This latter effect was observed at all depths where roots were found, and root mass was also discovered at slightly greater depth.

5.3.2 Scenario 3 - Impact of NAPL on root growth

The comparison of root growth in the control scenario 2 and scenario 3 for sandy loam and loam shows there is a significant difference in root growth in both soils as indicated in Figure 5-10.
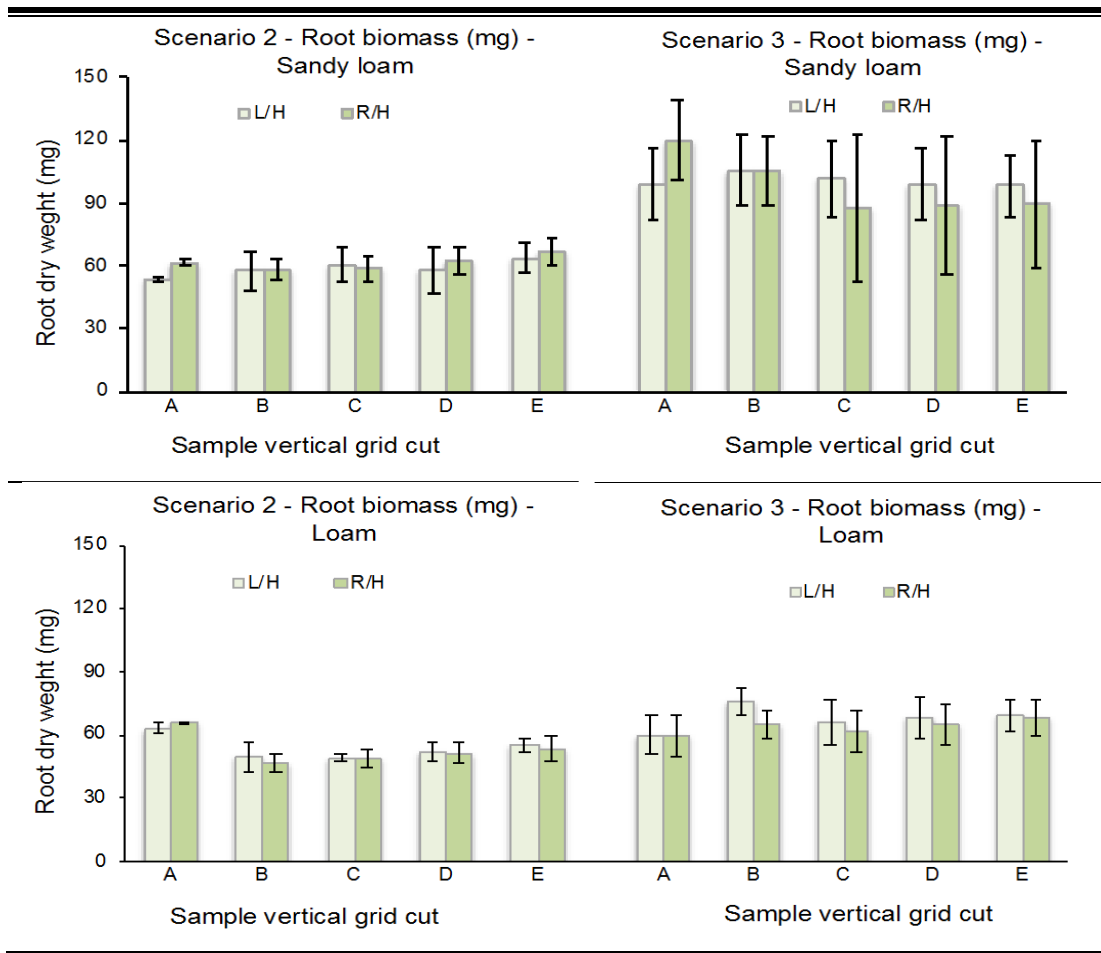
Figure 5-10 - Scenarios 2 and 3 – Root biomass for sandy loam and loam soils. Error bars represent ± one standard error of the mean.

The scenario 3 sandy loam root mass is significantly higher compared with scenario 2 (p = 0.0006), and also scenario 3 loam root mass is significantly higher compared with scenario 2 (P = 0.0005), perhaps because of the oil presence clogging the pore space thereby preventing oxygen and nutrients from reaching the rhizosphere which can lead to the observed injury to the roots.

The increased root growth may be a response of the plants to environmental stress, increasing the spread of roots in an effort to find an uncontaminated route to nutrient supply, as was observed in the microcosms, see section 4.3. The mass increase was partially caused by an observed thickening of roots (again also observed in microcosms), see section 4.1. Comparison of the results shows that the root biomass production for sandy loam is 36% greater than in the loam soil whose biomass production 15% higher than both samples of the uncontaminated controls (scenario 2). The higher root biomass for sandy loam might be because of an impact of the oil, which apparently has more effect in the sandy loam, perhaps because sandy loam has 30% less fine particles than loam. NAPL more easily displaces water to occupy the pores in sandy soil which holds water less tightly. Fine soil holds water more tightly making the movement of NAPL into and out of water-saturated soil pores difficult, which can lead to a decrease in their bioavailability and transport in the ground (Eibes et al., 2006, El-Tarabily, 2002) , this might give plants greater accessibility to larger pores meaning accessibility to nutrients and moisture. In the sandy loam, the contaminants are not bound up in the soil material and can clog the pore space leading to a decrease in solutes and pore water availability compared to more fine-grained soil. Hence, the reduced access to nutrients and moisture and the toxic oil compounds will affect the plants (Mitton et al., 2014). In the sandy loam soil, the ratio of the shoot to root mass is 3:1 in comparison to 6:1 for the control (scenario 2). The loam soil shoot to root mass ratio is 3:1 in comparison to 7:1 for the control (scenario 2), indicating the plant put more energy into root rather than shoot growth. The stress on root development may be because of the presence of high concentrations of mineral oil, which leads to increased toxicity and reduced access to moisture and nutrients.

5.3.3   Scenario 3 - Impact of plants on oil loss

Between 55 % and 60 % (sandy loam) and 45 % and 57 % (loam) of the oil was removed across all columns, when compared with scenario 1 where the total average oil loss for sandy loam and loam measured at 5.19% and 3.59% respectively (Figure 5-11). The results demonstrate the phytoremediation effect of ryegrass in non-aqueous phase contamination.
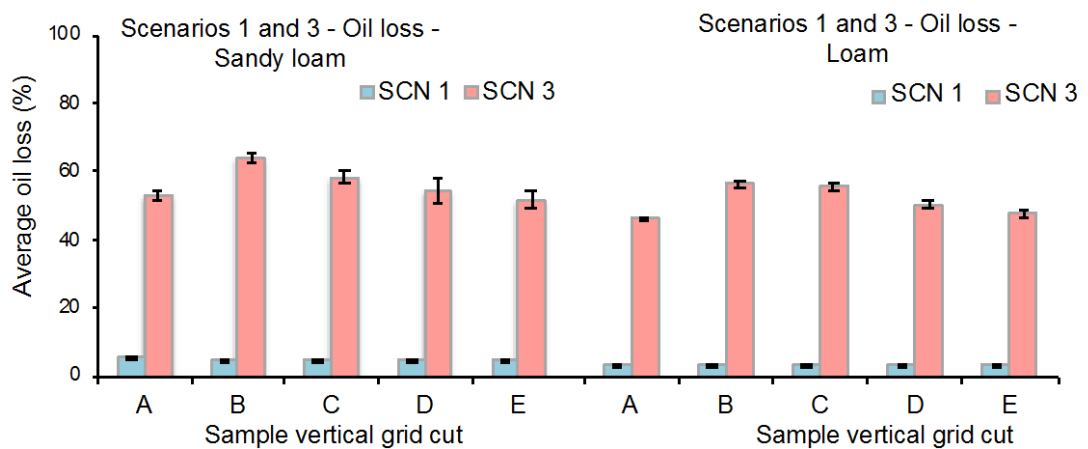
Figure 5-11 - Scenarios 1 and 3 – Oil loss for sandy loam and loam soils. Error bars represent ± one standard error of the mean.

In Scenario 3, sandy loam where greater amounts of root biomass were observed has a higher contaminant loss. The increased mineral oil loss in sandy loam, might suggest an increase in contaminant bioavailability and transport in the sandy loam. NAPLs will preferentially enter larger pores (easier to move water out of the larger pore in the sandy loam) resulting in higher residual saturation in the pores. Therefore, this might give plant roots greater accessibility to degrade the contaminants.

5.4 Contamination scenario 4 – Impact of a discontinuous NAPL zone on plant growth

Scenario 4 tests were experimental setups for both artificial soils. The scenarios had two equal columns with LNAPL contaminated soil placed in the contamination layer of the right-hand column and clean soil in the left-hand column with seed evenly planted across both (Figure 5–12). Scenario 4 arrangements had been tested to understand the response of root growth and distribution to discontinuous zones of oil contamination as well as oil removal. The physical distribution of NAPLs may be complex, with scenarios ranging from larger zones of continuous NAPL contamination to small unconnected individual ganglia. This scenario explored whether the plants may increase the spread of roots in an effort to avoid toxicity effects and environmental stress from oil contamination. Perhaps, the plants may increase the spread of roots in an effort to find an uncontaminated channel to nutrient supply in response to the toxicity effects and environmental stress from oil contamination. Again, there are two replicates for each soil.

(a) – Sandy loam   (b) - Loam

Figure 5-12 - Scenarios 4 - LNAPL contaminated soil placed in the contamination layer of the right-hand column and clean soil in the left-hand column with seed evenly planted across both at a depth of 1 cm.

Figure 5–13 and Figure 5–14 are presented in a similar way to both Scenario 2 and Scenario 3, although here the grey bar also shows the location of the contaminated zone on the right-hand side. Also, data are presented for both the left hand and right side of the central partition. Each side is further subdivided into five columns A - E. There are differences in growth between the right-hand and the left-hand columns, both above and below the soil surface for all the replicates. Comparing the overall total for the whole box for scenario 4 and scenario 2 shows that the root mass and shoot mass difference are statistically significant for both soils (Figure 5–15, Figure 5–16, Figure 5–17 and Figure 5–18).
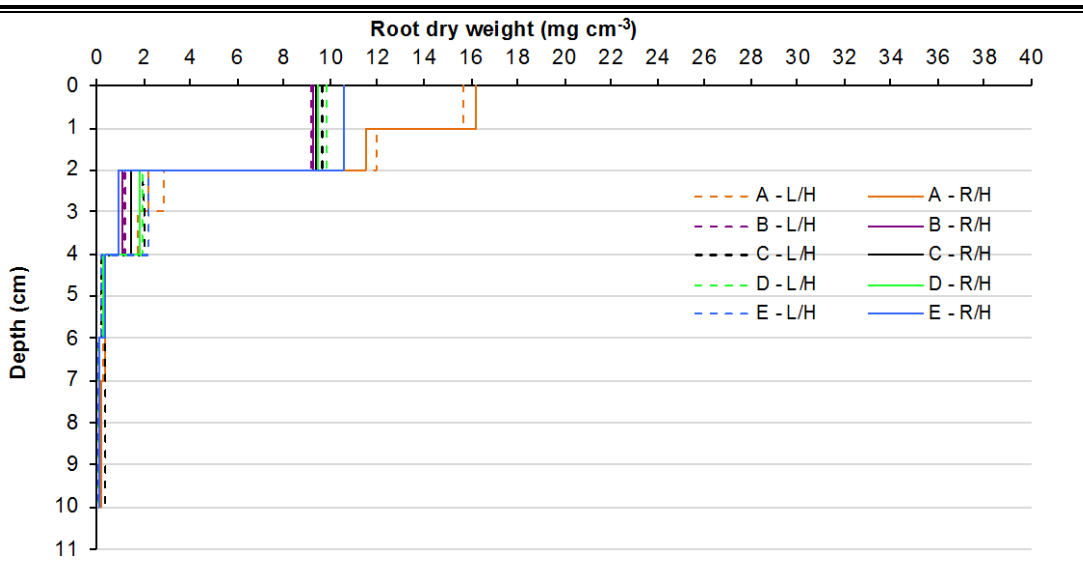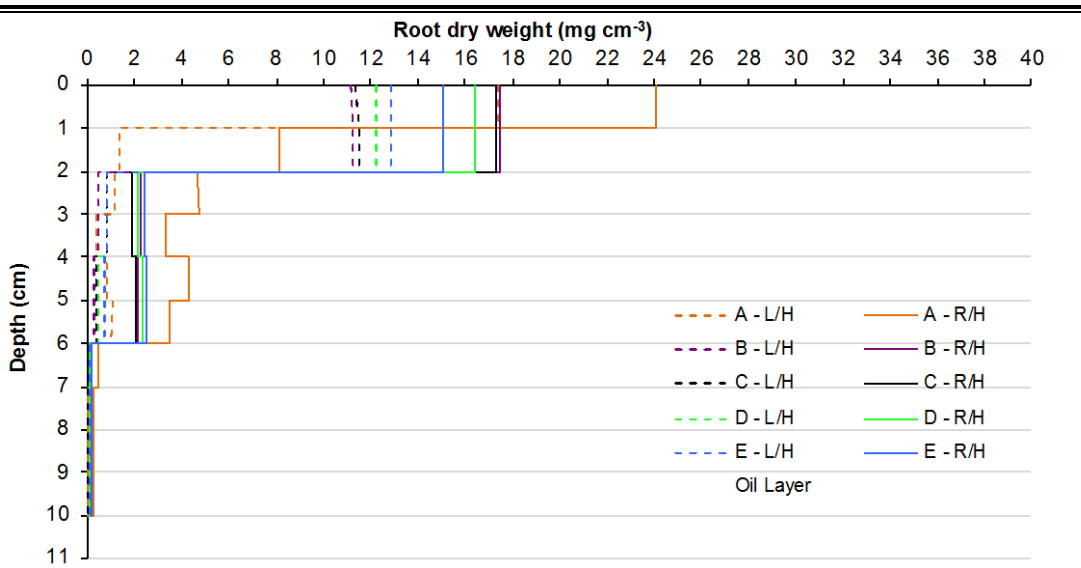
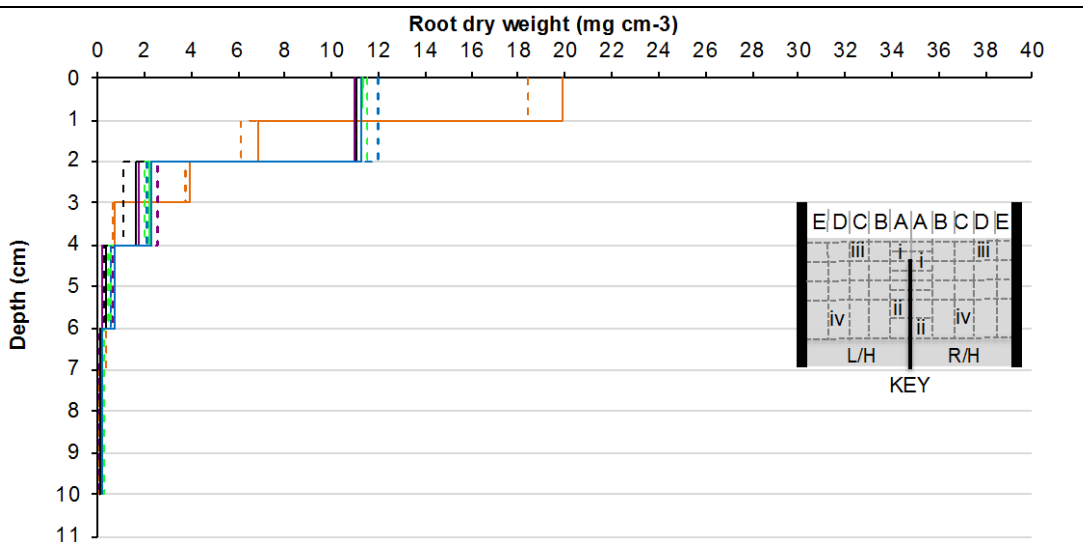Scenario 4 – Replicate 1 - Root biomass with depth for sandy loam
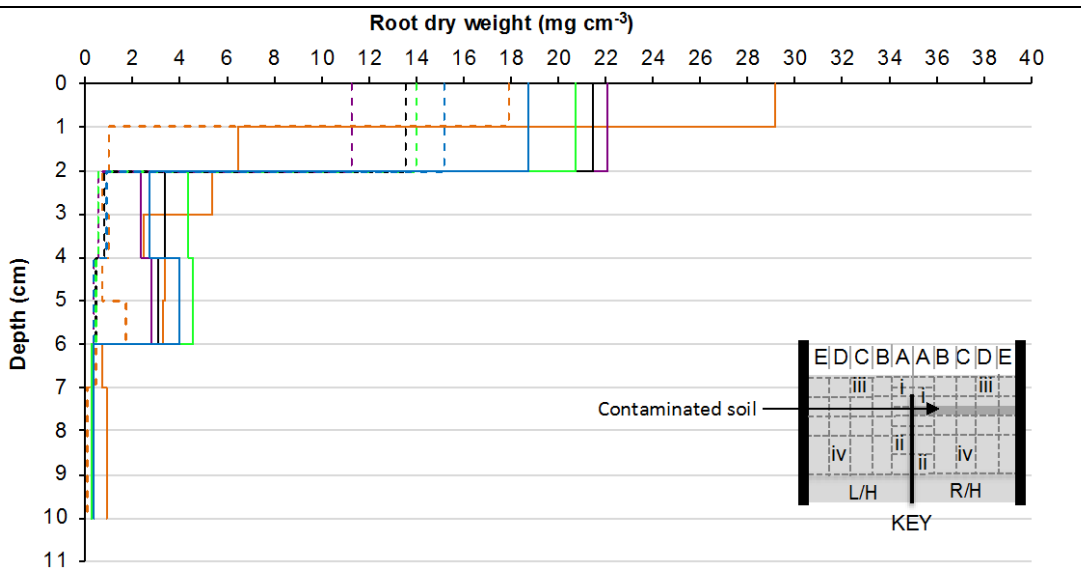
Scenario 4 – Replicate 2 - Root biomass with depth for sandy loam

Figure 5-13 - Scenario 4 – Replicates 1 and 2 - Sandy loam root biomass with depth.

Note: Refer to Figure 3–10 for samples' sizes and volumes. * L/H – Left Hand Side; R/H – Right Hand Side.
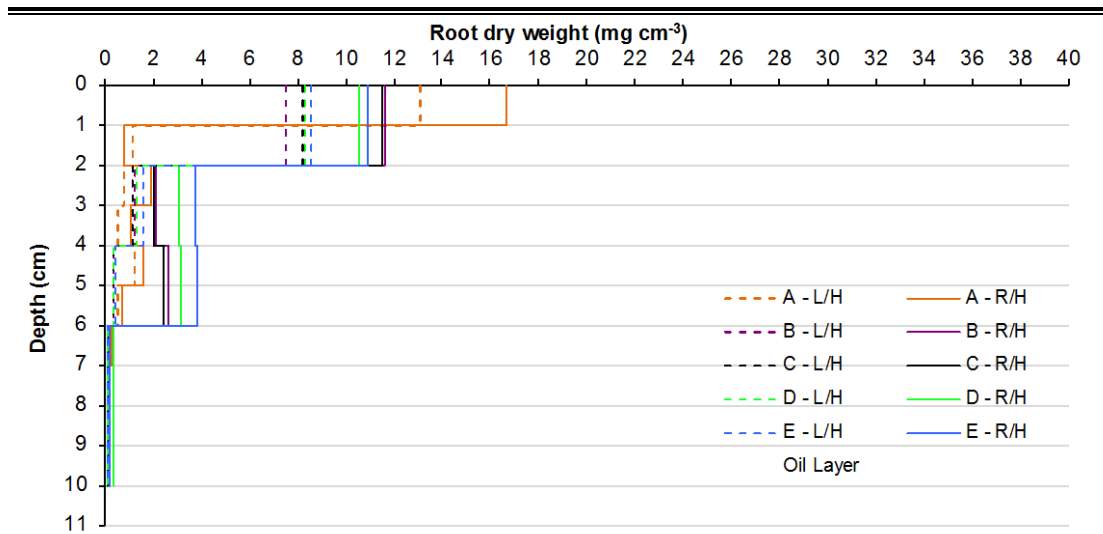
Scenario 4 – Replicate 1 - Root biomass with depth for loam
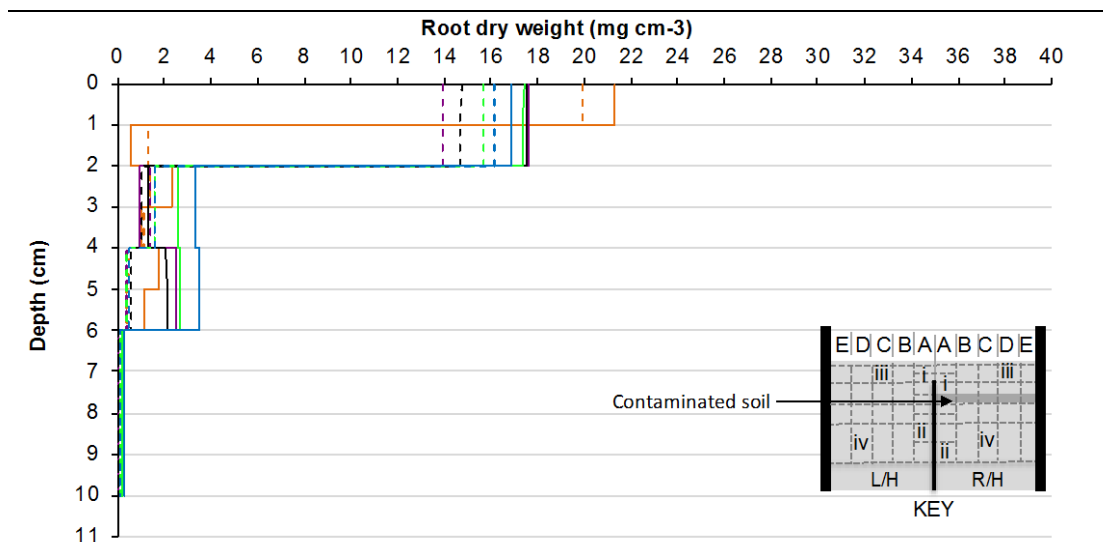
Scenario 4 – Replicate 2 - Root biomass with depth for loam

Figure 5–14 - Scenario 4 – Replicates 1 and 2 - Loam root biomass with depth.

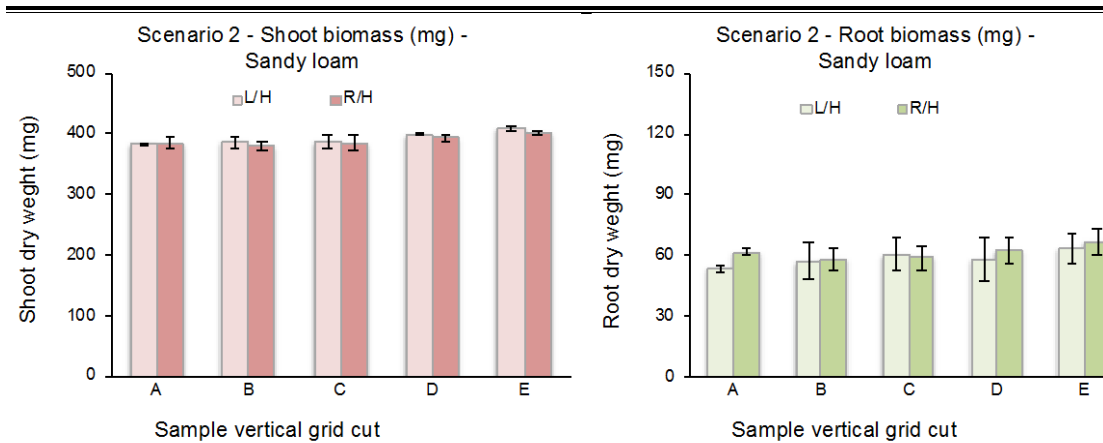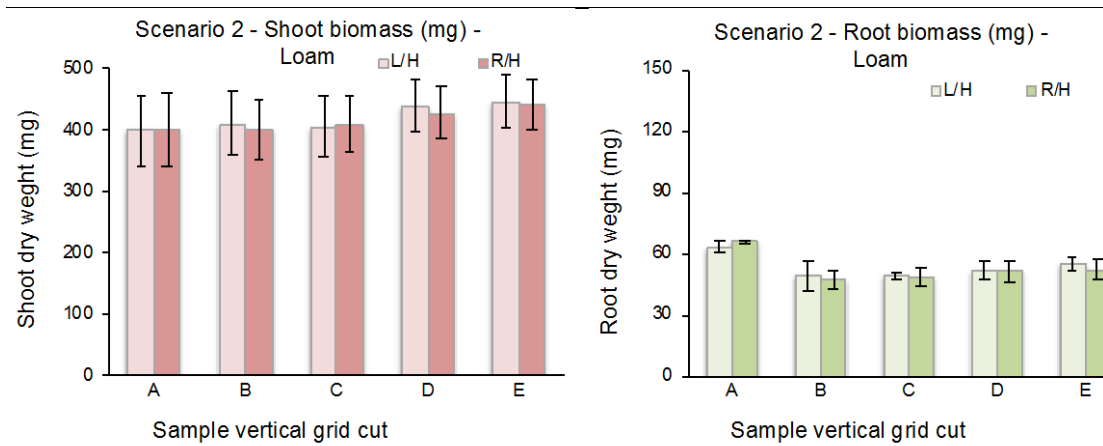Note: Refer to Figure 3–10 for samples' sizes and volumes. * L/H – Left Hand Side; R/H – Right Hand Side.

5.4.1   Scenario 4 - Impact of NAPL on shoot growth

The scenario 3 sandy loam shoot mass is significantly lower than scenario 4 (p = 0.015), and also scenario 3 loam shoot mass is significantly lower than scenario 4 (p = 0.017) as shown in Figure 5–15.

Figure 5-15 Scenarios 3 and 4 – Shoot biomass for sandy loam and loam soils. Error bars represent ± one standard error of the mean.

The higher shoot mass in scenario 4 also suggests the adverse effects of high levels of contaminant in scenario 3 which affects shoot growth when compared with

scenario 4. The results further confirmed that the presence of contaminant does affect plant root and shoot growth in both soils.

The scenario 4 sandy loam shoot mass is significantly lower compared with scenario 2 (p = 0.005), and also scenario 4 loam shoot mass is significantly lower compared with scenario 2 (p = 0.0002) as illustrated in Figure 5-16, the reduced shoot mass was also observed in scenario 3 as a result of the presence of oil (Figure 5-9).

Figure 5-16 Scenarios 2 and 4 – Shoot biomass for sandy loam and loam soils. Error bars represent ± one standard error of the mean.

Also, comparing shoot mass in scenario 4 right-hand with scenario 2 right side (Figure 5–16), as with the scenario 3, there is a decrease in the overall mass of shoots in scenario 4 right-hand column in the presence of oil, but there is a very significant increase in the mass of roots (Figure 5–16 and Figure 5–17).

Moreover, the combination of contaminated and uncontaminated zones within the same box has had an effect. The plant growth is affected by adjacent NAPL contamination. In scenario 4, the average total shoot mass for the right hand is 1657 mg and left hand 1908 mg. Therefore, comparison of scenarios 4 and 2 left side total shoot mass (1908 mg) is lower compared with scenario 2 (1945 mg) as shown in Figure 5–16. Moreover, comparison of scenarios 4 and 3 right side total shoot mass shows that scenario 4 right side shoot mass (1657 mg) is similar to scenario 3 (1661 mg), Figure 5–15. The results suggest that the fact that the contaminated and uncontaminated zones were in the same box has led to a decrease in the overall mass of shoots in scenario 4 compared with scenario 2, perhaps because of oil and reduced access to nutrients.

5.4.2   Scenario 4 - Impact of NAPL on root growth

Moreover, comparing the overall experiment total for scenario 3 and scenario 4 shows that the root mass difference is significant for sandy loam and loam (Figure 5-17).
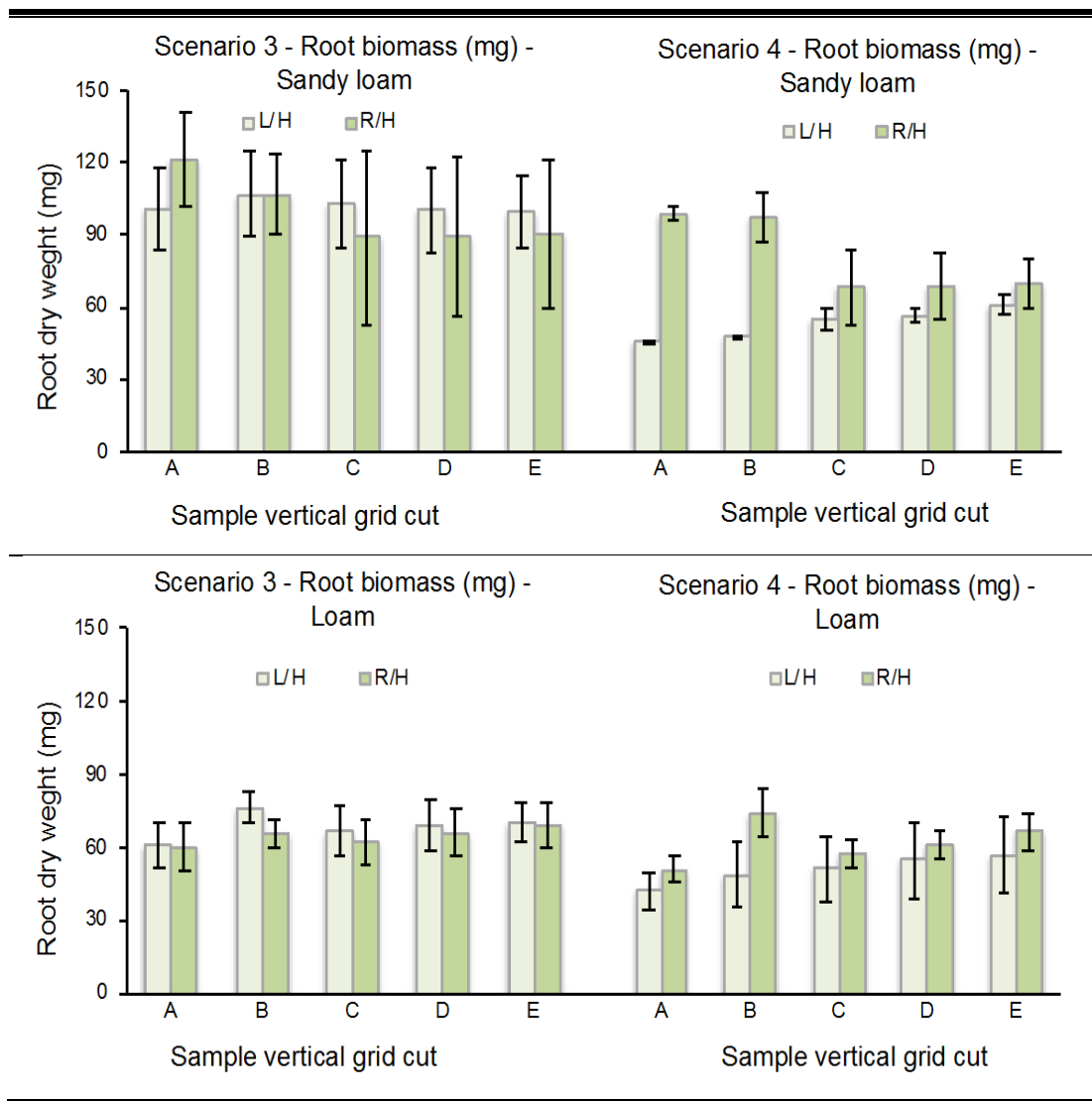
Figure 5-17 Scenarios 3 and 4 – Root biomass for sandy loam and loam soils. Error bars represent ± one standard error of the mean.

The scenario 3 sandy loam root mass is significantly higher compared with scenario 4 (p = 0.0005), and also scenario 3 loam root mass is significantly higher compared with scenario 4 (p = 0.0033) as illustrated in Figure 5–17. The increased root mass observed in scenario 3 in both soils may be because of the high levels (continuous zones) of oil contamination in scenario 3 resulting in more damaged roots as plant increased the spread of roots in an effort to find an uncontaminated route to nutrient supply.

Comparison of the results for the scenario 4 right side with scenario 3 also shows that the root biomass production for scenario 4 sandy loam is 25 % greater than in the loam, an effect that was also observed in the scenario 3 results. The increase in root biomass for the sandy loam soil might be as result of damaged roots in response to the increased contaminant bioavailability and transport in the sandy loam larger pores, resulting in less accessibility to oxygen, nutrients and moisture.

The scenario 4 sandy loam root mass is significantly higher compared with scenario 2 (p = 0.016), Figure 5–18 and also scenario 4 loam root mass is significantly higher when compared with scenario 2 (p = 0.005), Figure 5–18, the increased root also shows the adverse effects of oil on the root growth.
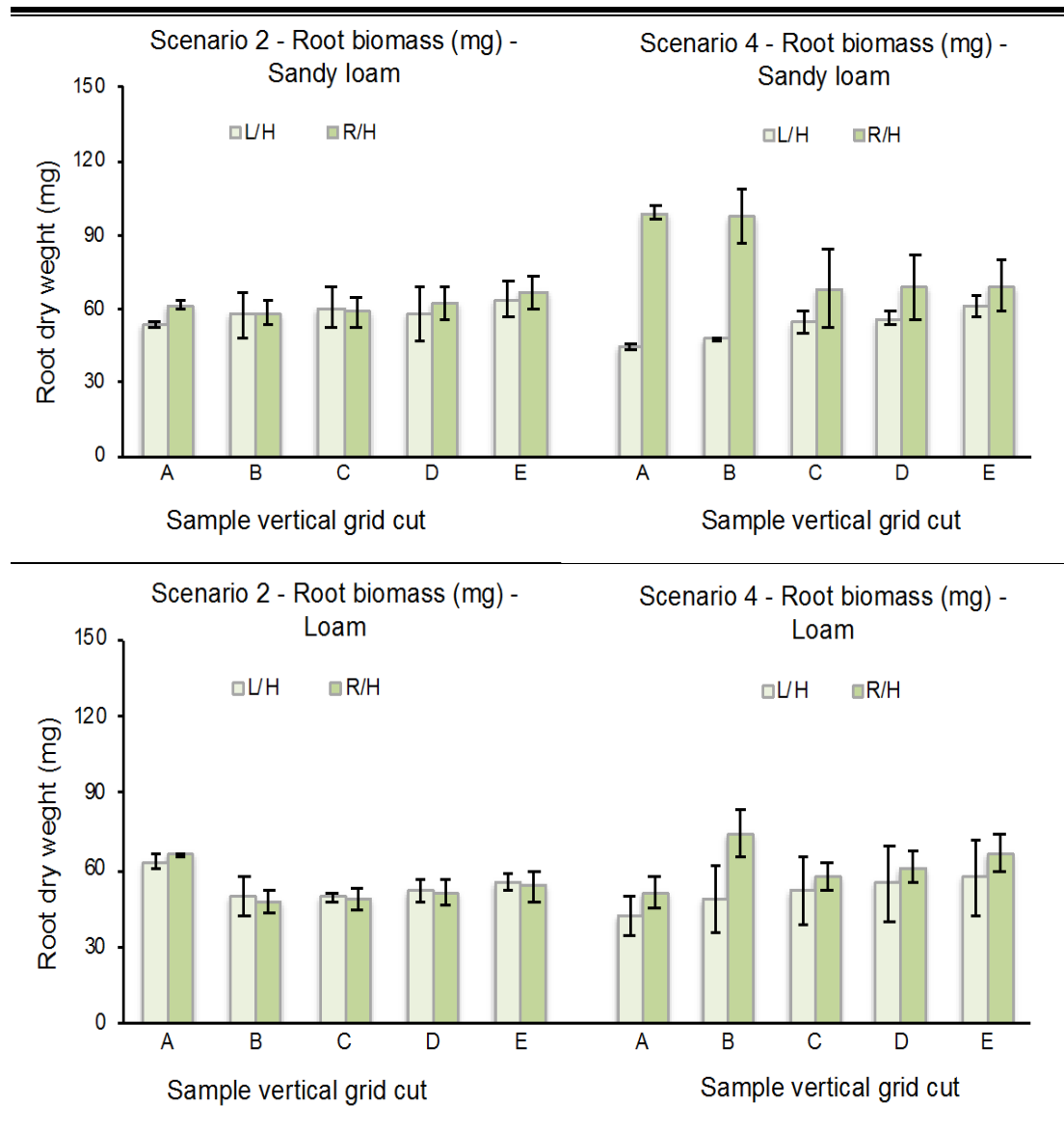
Figure 5-18 Scenarios 2 and 4 – Root biomass for sandy loam and loam soils. Error bars represent ± one standard error of the mean.

Also, comparing root mass in scenario 4 right-hand with scenario 2 right side, as with the scenario 3, there is a decrease in the overall mass of shoots in scenario 4 right-hand column in the presence of oil, but there is a very significant increase in the mass of roots (Figure 5-18). This is particularly clearly indicated in the scenario 4 sandy loam (Figure 5-18), where root biomass was increased at all depths compared to the scenario 2 control in locations where an oil layer was present.

Moreover, higher root biomass was recorded in the scenario 4 left side columns when compared to the control scenario 2 left side, as illustrated in Figure 5–18. It is probable that the roots responded to the diffusion of the dissolved phase contamination diffusing to the left, hence the changes in the root architecture. Moreover, the combination of contaminated and uncontaminated zones within the same box has had an effect. The plant growth is affected by adjacent NAPL contamination. In scenario 4, the total root mass for the right hand is 560 mg and left hand is 366 mg. Therefore, comparison of scenarios 4 and 2 left side total root mass indicates that scenario 4 left side root mass (366 mg) is higher compared with scenario 2 (270 mg), Figure 5–18. Moreover, comparison of scenarios 4 and 3 right side total root mass shows that scenario 4 right side root mass (560 mg) is similar to scenario 3 (558 mg), Figure 5–17. The results suggest that the fact that the contaminated and uncontaminated zones were in the same box has led to a decrease in the overall mass of shoots in scenario 4 and a very significant increase in the mass of roots (Figure 5–17, Figure 5–18) when compared with scenario 2, perhaps because of oil and reduced access to nutrients.

5.4.3   Scenario 4 - Impact of plants on oil loss

Comparing oil loss in scenario 4 with scenario 3 as shown in Figure 5–19, between 67 % - 76 % (sandy loam) and 58 % - 66 % (loam) of the oil were removed on the right-hand of scenario 4, while between 55 % – 60 % (sandy loam) and 45 % – 57 % (loam) of oil were lost across all columns (totals for the whole box) of scenario 3 (Figure 5–11).
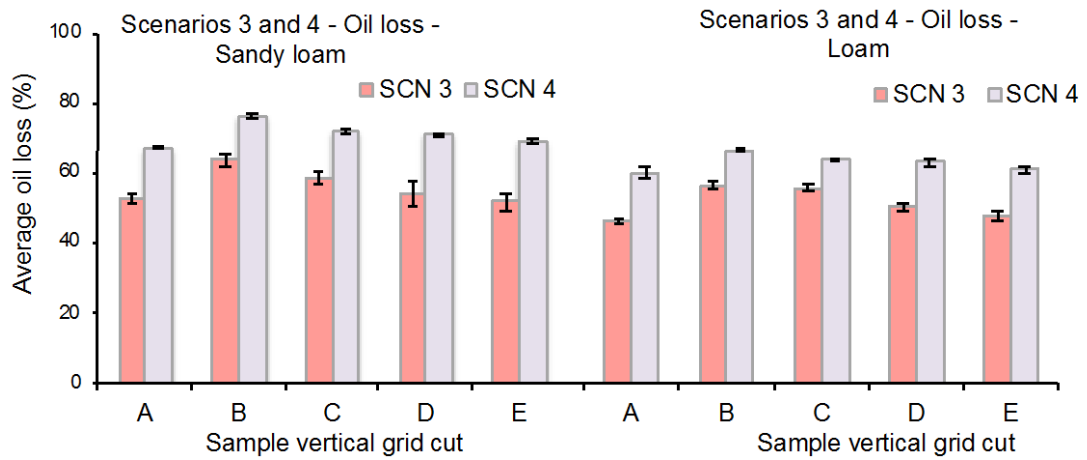
Figure 5-19 - Scenarios 3 and 4 – Oil loss for sandy loam and loam soils. Error bars represent ± one standard error of the mean.

This demonstrates apparent phytoremediation of NAPL contamination and the adverse effects of persistence sources of dissolved phase contamination. The total average oil loss for sandy loam is higher compared with loam in scenario 4, Figure 5-19. The lower overall percentage of contaminant loss in scenario 3, when compared to scenario 4 contaminated right-hand, might be because of the high levels (continuous zones) of oil in scenario 3 (Figure 5–19). The removal or loss of organic contaminants by the plant generally depends on the volume or its concentration in the soil (Zengel et al., 2016, Lim et al., 2016). The results suggest that more contamination might mean less contaminant loss due to the high oil content clogging the pore space, thereby reducing the supply of oxygen, moisture and nutrients which may be responsible for shoot growth inhibition and the observed injury to the roots and the subsequence less contaminant loss. The analysed samples from the scenario 4 left side show zero oil recovery.

5.5   Contamination scenario 5 – Impact of small unconnected NAPL zones on plant growth

Scenario 5 tests had two main equal columns, where the left side was entirely uncontaminated, and the right side was subdivided into five alternately contaminated and uncontaminated sub-columns with seeds evenly planted across both. (Figure 5‑20). The five sub-columns represent small unconnected contaminant sources which is more common as the contaminant source ages. There are two replicates for each soil.

| a – Sandy loam | b – Loam |
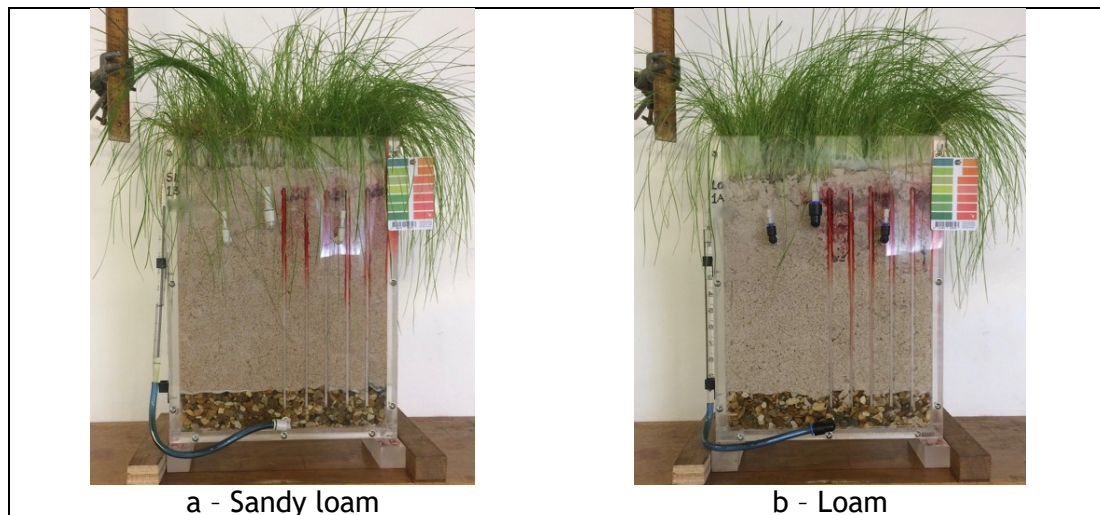
Figure 5-20 - Scenarios 5 – LNAPL contaminated soil placed in the contamination layer of the right-hand column (further subdivided) and clean soil in the left-hand column with seed evenly planted across both at a depth of 1 cm.

Results for scenario 5 are shown in Figure 5‑21 and Figure 5‑22, that show the root biomass with depth for the two soils as recorded at the end of the experiment. The effects observed in Scenario 4 right-hand column (Figure 5‑13 and Figure 5‑14) are replicated here but in smaller alternate unconnected zones. However, the introduction of five sub-columns has a negative effects of plant shoot and root biomass production.
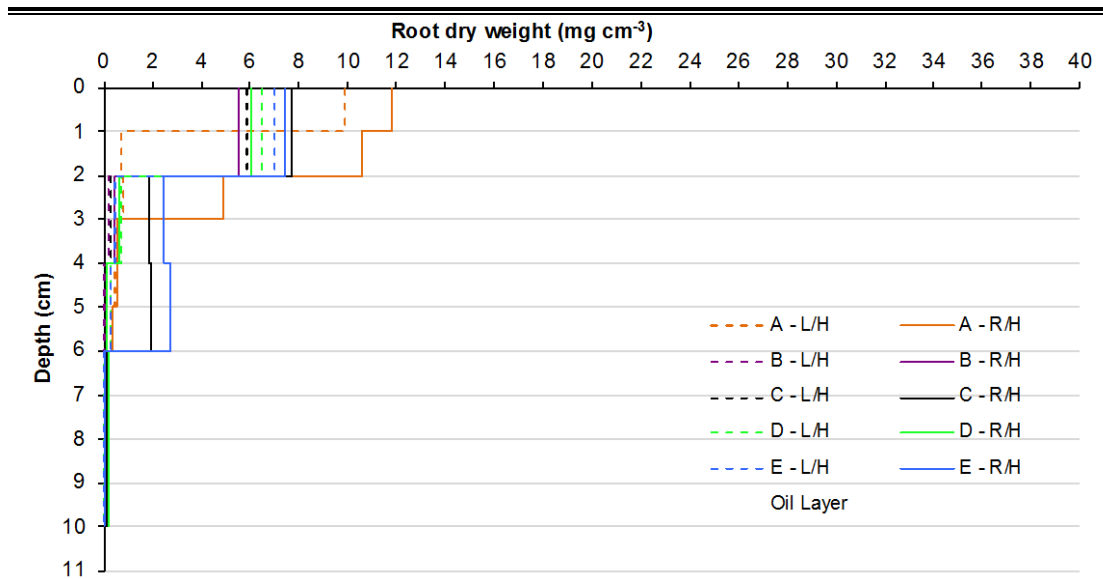
Scenario 5 – Replicate 1 - Root biomass with depth for sandy loam
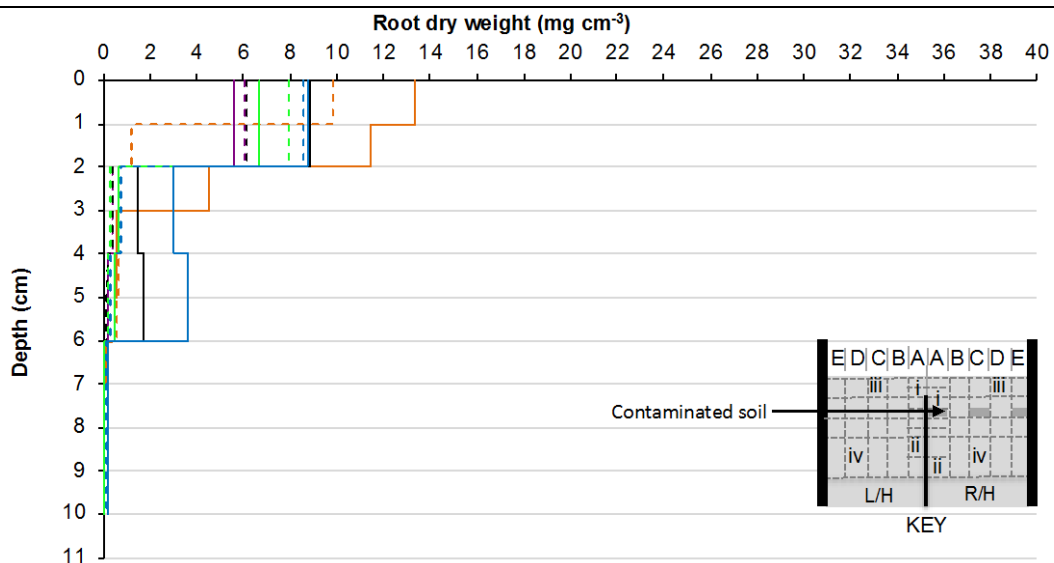
Scenario 5 – Replicate 2 - Root biomass with depth for sandy loam

Figure 5-21 - Scenario 5 – Replicates 1 and 2 - Sandy loam root biomass with depth.

Note: Refer to Figure 3–10 for samples' sizes and volumes. * L/H – Left Hand Side; R/H – Right Hand Side.
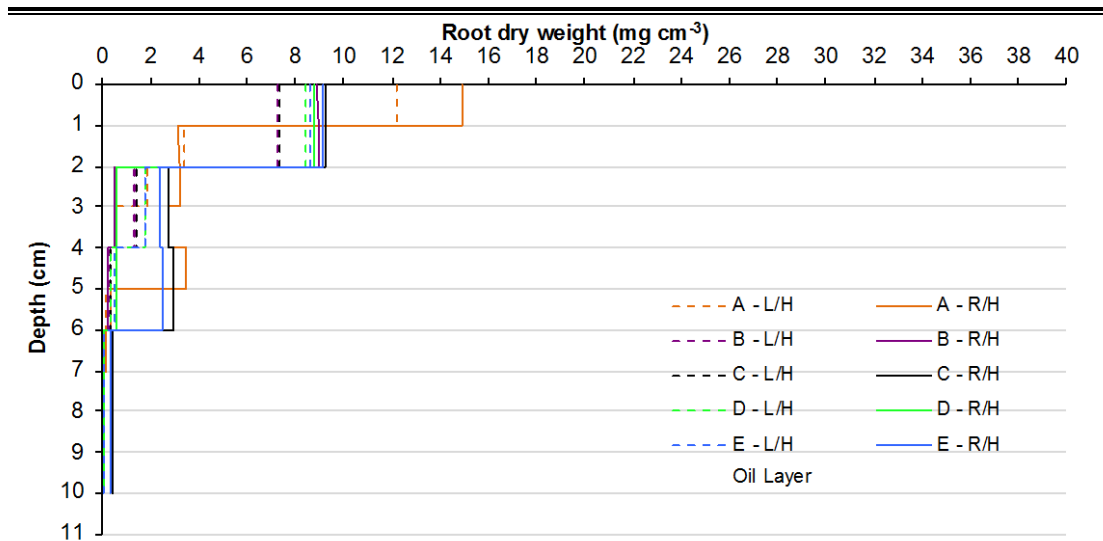
Scenario 5 – Replicate 1 - Root biomass with depth for loam
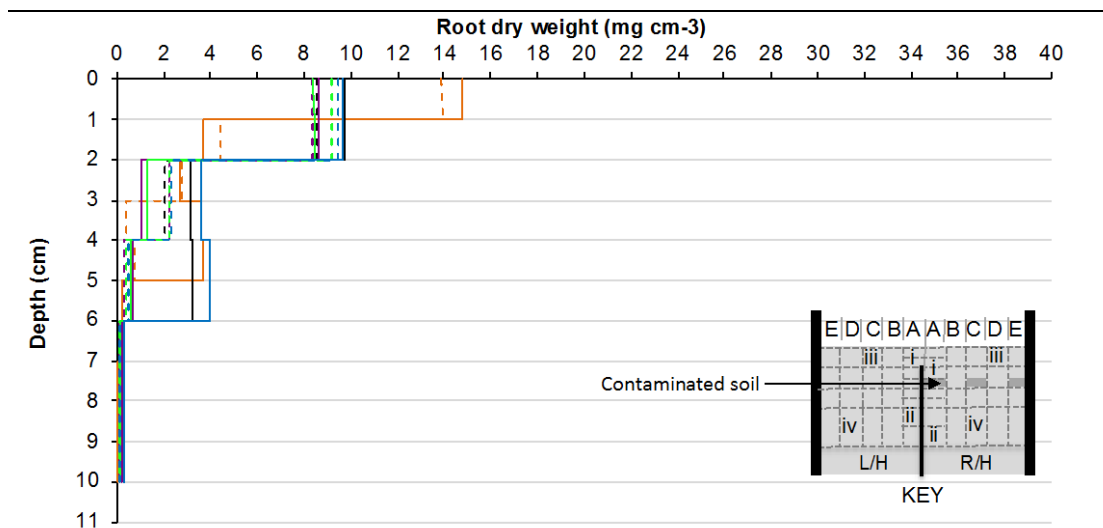
Scenario 5 – Replicate 2 - Root biomass with depth for loam

Figure 5-22 - Scenario 5 – Replicates 1 and 2 - Loam root biomass with depth.

Note: Refer to Figure 3–10 for samples' sizes and volumes. * L/H – Left Hand Side; R/H – Right Hand Side.

5.5.1 Scenario 5 - Impact of NAPL on shoot growth

In Figure 5-23, the comparison of the total root mass and shoot mass for the whole box for scenario 4, and scenario 5 shows that the scenario 4 root mass is significantly higher for sandy loam (p = 0.006) and loam (p = 0.0061) compared with scenario 5 as a result of root damage caused by the increased oil contamination.
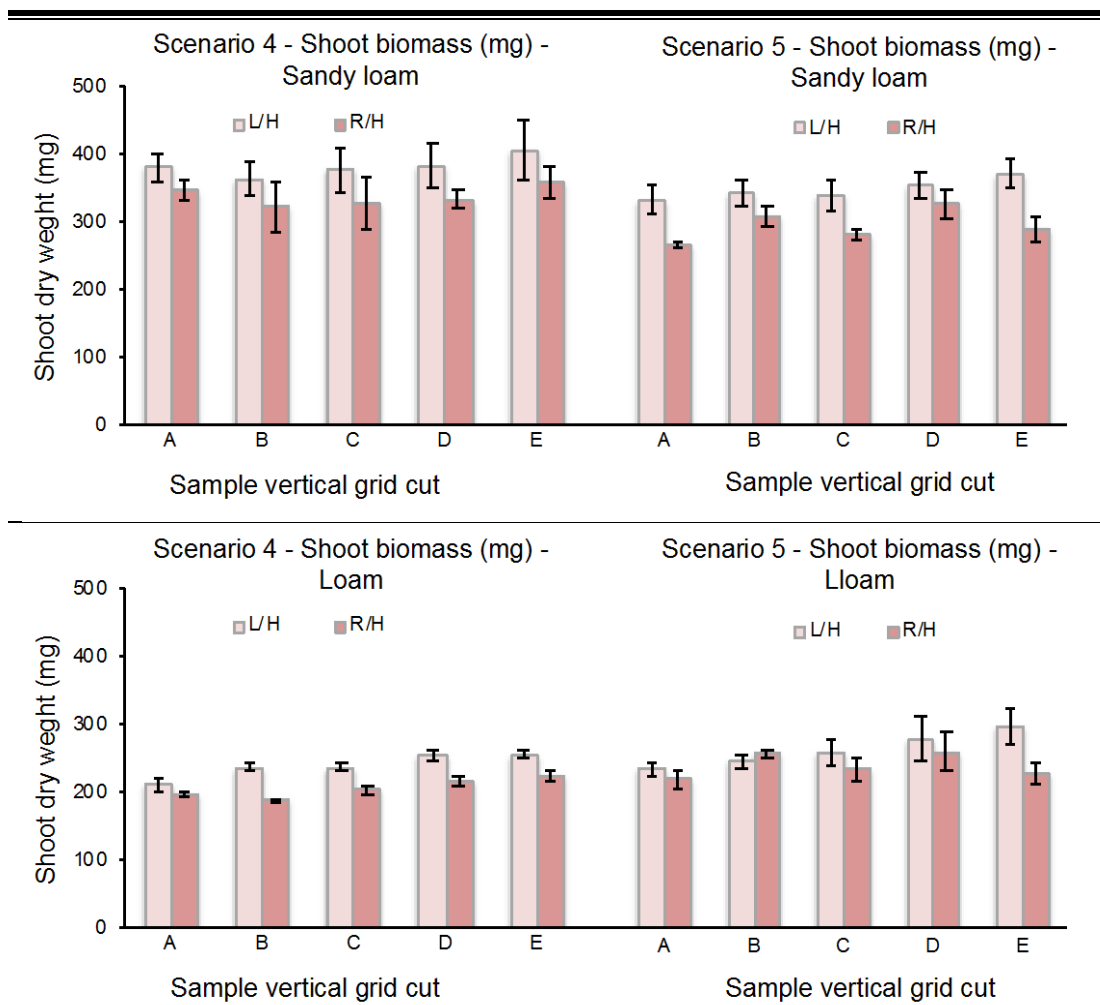
Figure 5-23 Scenarios 4 and 5 – Shoot biomass for sandy loam and loam soils. Error bars represent ± one standard error of the mean.

The increased oil contamination also leads to a significant reduction in the shoot mass in the loam for scenario 4 (p = 0.0068), Figure 5–23.

Moreover, the scenario 5 shoot mass is also statistically significantly lower (p = 0.0013, sandy loam; p = 0.0016, loam) compared with scenario 2, Figure 5–24. The results suggest that low contamination levels with the presence of non-continuous contamination reduce root mass as well as shoot mass. Perhaps, the smaller extent of the contamination regions in scenario 5 may have caused a fluctuation in nutrient availability, with small regions providing a very different condition of nutrient input and root competition which will negatively affect plant growth.

The inhibition to shoot growth and root growth was also observed with either no oil or the highest levels of oil contamination with multiple partitions in the microcosm experiments. This is similar to the earlier root growth scoring in Figure 4–6, microcosm experiments – the greatest inhibition was seen with low oil contamination and a significant root damage with higher levels of oil contamination. The observed effects could be related to the reported results from the literature on the mechanisms of plant toxicity (Simonich and Hites, 1995, Lim et al., 2016) and the physical impacts of NAPL. The contaminants will dissolved in the aqueous solution (not measured in the thesis) and adsorbed on the root (Li et al., 2014b); before penetrating into root tissues (Hurtado et al., 2016), causing harm to the plant and consequentially inhibit growth (Vázquez-Cuevas et al., 2018b). The significant inhibition in plant growth has also been attributed to the presence of NAPLs in the soil. They did affect soil fertility and interchange of gases by clogging pore space. (Chen et al., 2013, Ramadass et al., 2015), thereby reducing the electrostatic interaction with some essential nutrients for the plants (Atlas and Bartha, 2012, Wolfe, 2013a). The plant can experience a metabolic discrepancy caused by a condition of oxidative stress which impedes the ability of the cell to regulate the chemical processes (Romeh, 2017, Hou et al., 2015). This has impacted upon plant shoot development noted.
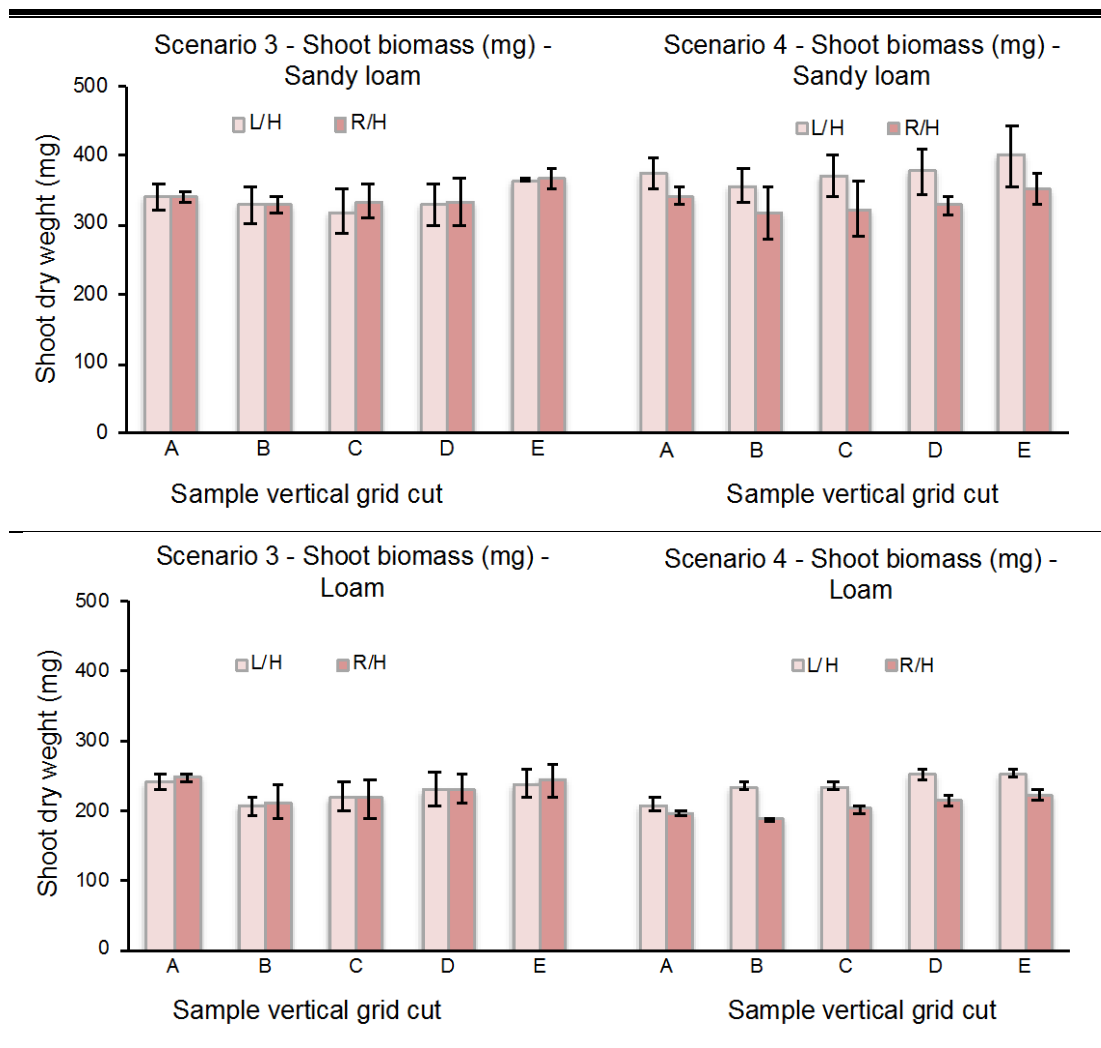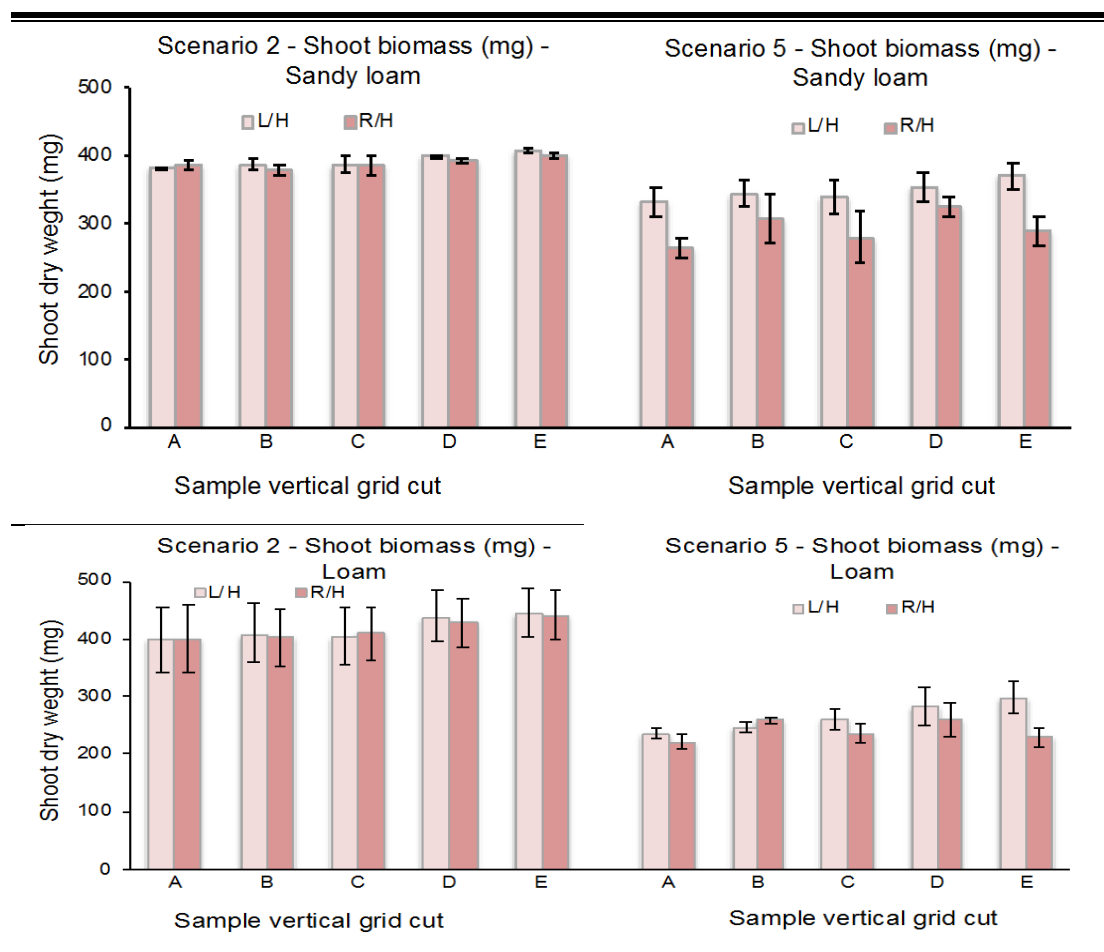
Figure 5-24 Scenarios 2 and 5 – Shoot biomass for sandy loam and loam soils. Error bars represent ± one standard error of the mean.

5.5.2   Scenario 5 - Impact of NAPL on root growth

The scenario 5 left side root biomass seems to be quite low when compared with the control scenario 2 left side, as clearly shown in sandy loam (Figure 5–25).
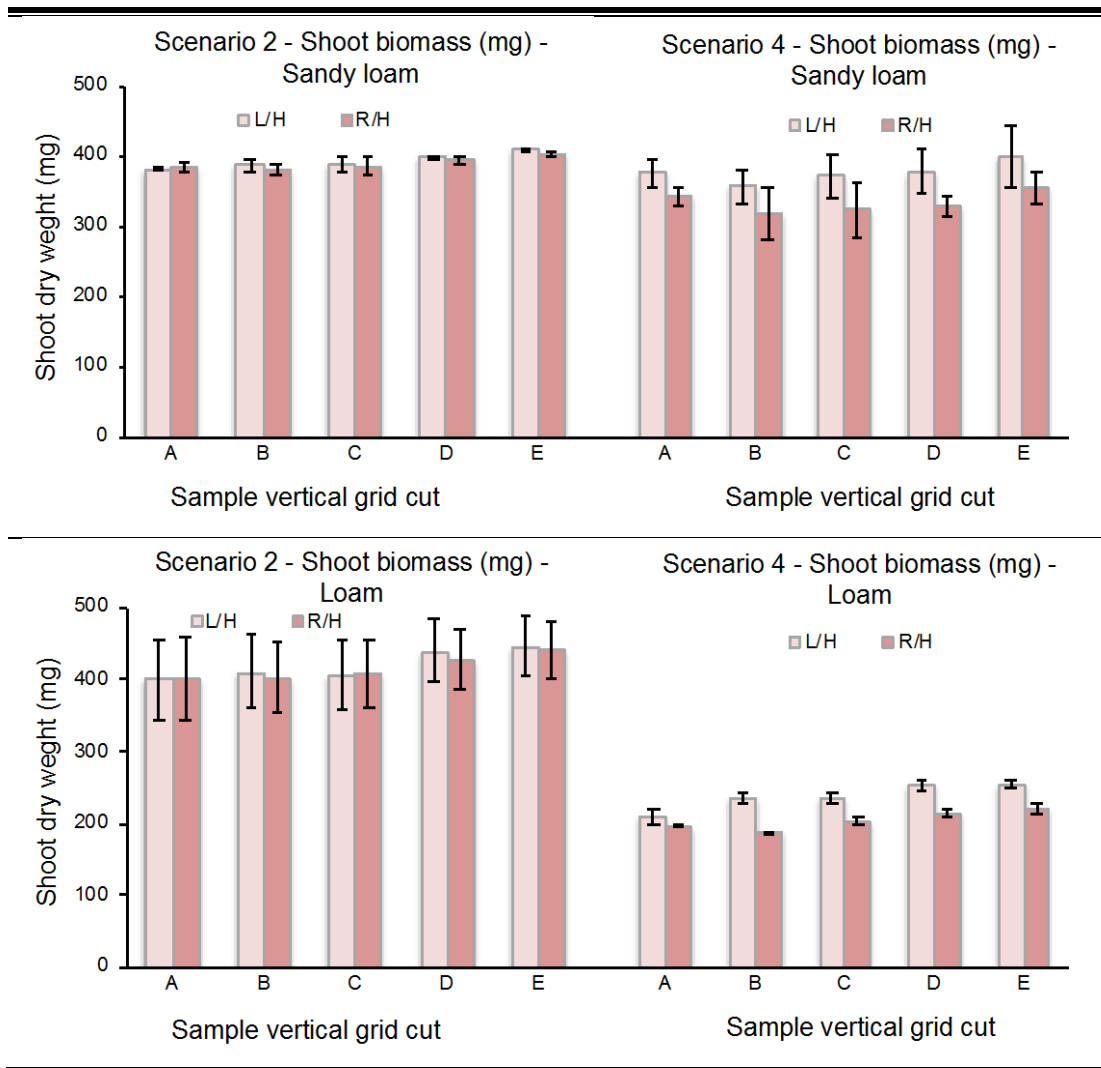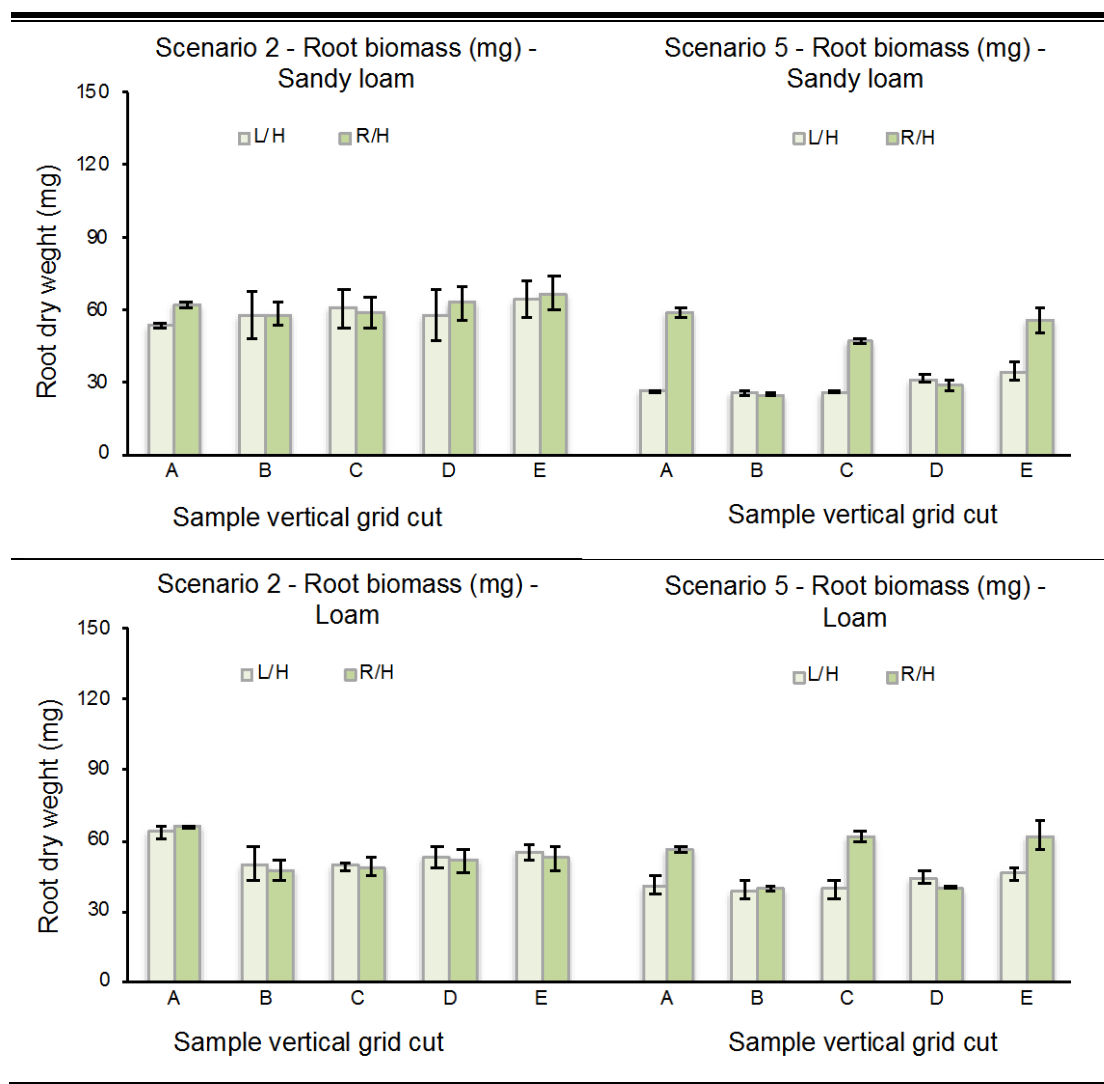
Figure 5–25 Scenarios 2 and 5 – Root biomass for sandy loam and loam soils. Error bars represent ± one standard error of the mean.

Although the root architecture for both scenario 5 and scenario 2 left side are similar (roots were fine, long and smooth), the lower root mass in scenario 5 left side suggests that there is a significant inhibition on the root growth in the low oil contamination, while injury to root was observed with high oil concentration as seen in all high-level oil contaminated layers. Moreover, the combination of alternate contaminated and uncontaminated zones within the same box has had an effect. Overall, root biomass for the whole box seems to be quite low in scenario 5, also particularly clearly illustrated in the sandy loam (Figure 5–25) when compared with the control scenario 2.

Therefore, the combination of alternate contaminated and uncontaminated zones within the same box has had an effect. These results show that the scenario 5 root mass is significantly lower (p = 0.0051, sandy loam; p = 0.0023, loam) compared with scenario 2. Moreover, the scenario 5 shoot mass is also statistically significantly lower compared with scenario 2, Figure 5–24. The results suggest that low contamination levels with the presence of non-continuous contamination reduce root mass as well as shoot mass. Perhaps, the smaller extent of the contamination regions in scenario 5 may have caused a fluctuation in nutrient availability, with small regions providing a very different condition of nutrient input and root competition which will negatively affect plant growth (McConnaughay and Coleman, 1998, Lavorel et al., 1997). Moreover, the significant inhibition in plant growth has also been reported at low oil concentration in the soil, and even though low contamination levels were considered to be less toxic, contaminants can affect soil fertility and interchange of gases by clogging pore space (Chen et al., 2013, Ramadass et al., 2015).

5.5.3   Scenario 5 - Impact of plants on oil loss

A higher proportion oil loss was observed in scenario 5 probably because of the reduced extent of contamination in the contamination layer, compared to other contaminated scenarios (Scenario 1 – R/H, Scenario 3, and Scenario 4 – R/H), Figure 5-11, Figure 5-19, Figure 5-26, and Figure 5-27. The sandy loam columns exhibited more oil loss compared to loam columns as shown in Figure 5–26 and Figure 5–27.

Figure 5–26 and Figure 5–27, decreasing amounts of oil loss were observed as the coverage of the oil layer increased. Although the improved performance is largest at low contaminant levels (as seen in scenario 5), as oil contamination increases, the oil recovery from the two soils becomes similar. The observed reduced oil loss with increasing oil levels might be because the removal of oil by the plant will generally depends on the levels in the soil (Zengel et al., 2016, Lim et al., 2016). The observed results might be because of the damaged root in the high-level contaminated scenarios, Figure 5-26, Figure 5-27 (Naidoo, 2016, Merkl et al., 2005) or the plant root branching and avoidance of contaminated zones in an effort to

find an uncontaminated route to nutrient supply as seen in the microcosm (section 4.2).
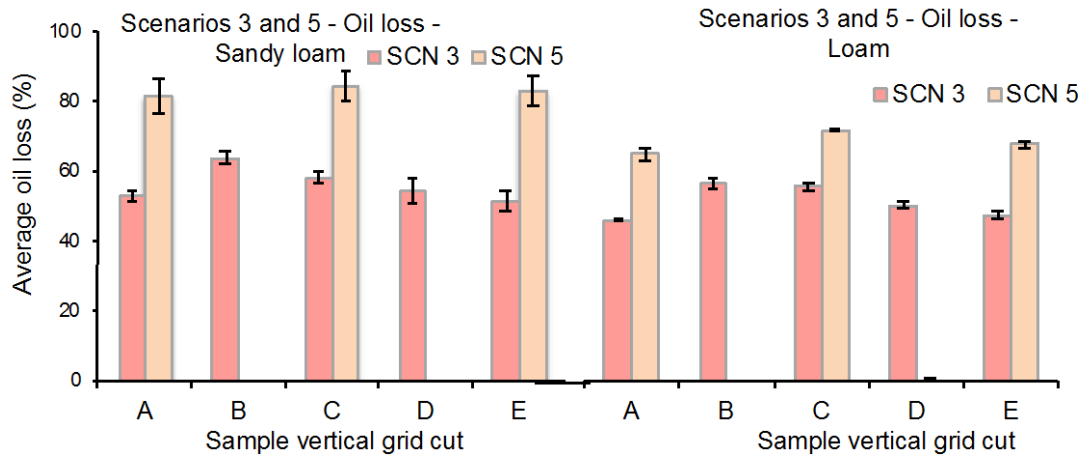
Figure 5-26 - Scenarios 3 and 5 – Oil loss for sandy loam and loam soils. Error bars represent ± one standard error of the mean.
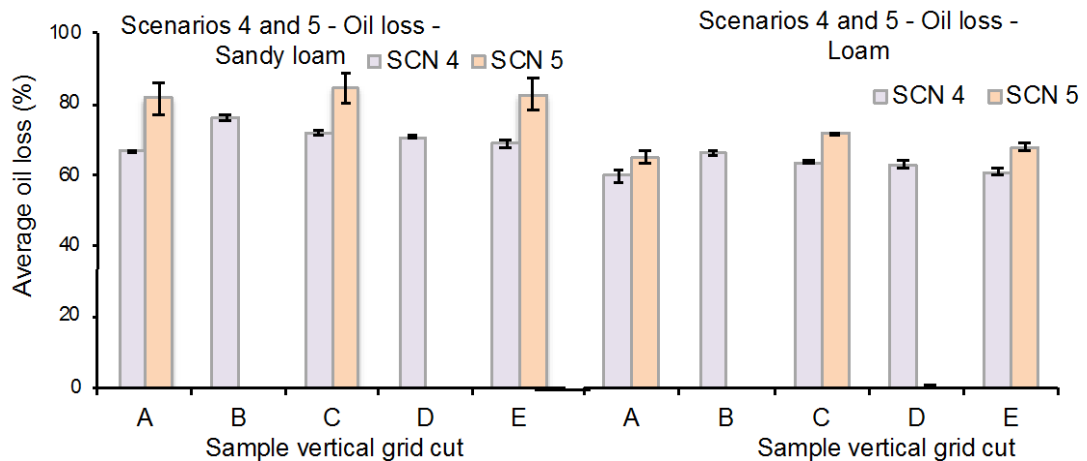
Figure 5-27 - Scenarios 4 and 5 – Oil loss for sandy loam and loam soils. Error bars represent ± one standard error of the mean.

5.6 General analysis and discussions

5.6.1 Plant root and shoot biomass production relative to oil coverage

The behaviour observed at the microcosm scale was explored at a larger size in soil mesocosms to determine how the response of an individual plant could be extrapolated to the behaviour of a planted soil and its effect on NAPL contamination. Plant root biomass was measured for each experimental setup to verify the impact of spatially distributed mineral oil on physical distribution of root growth and at the interface between plant root and oil. Similar to the microcosm experimental design (section 3.4.1), in all scenarios, the 3 cm uncontaminated surface soil layer allows germination and initial establishment of ryegrass which has been reported to be affected by phytotoxicity of mineral oil (Adam and Duncan, 2002). The presence of deeper contaminated layers has been found to result in enhanced initial root density and longer root system from preliminary investigations (Kechavarzi et al., 2007). In the reported tests, the introduction of the mineral oil contaminant in planted experiments corresponds to a decrease in the overall mass of shoots in the presence of oil, but there is a very significant increase in the biomass of roots (Figure 5–28).
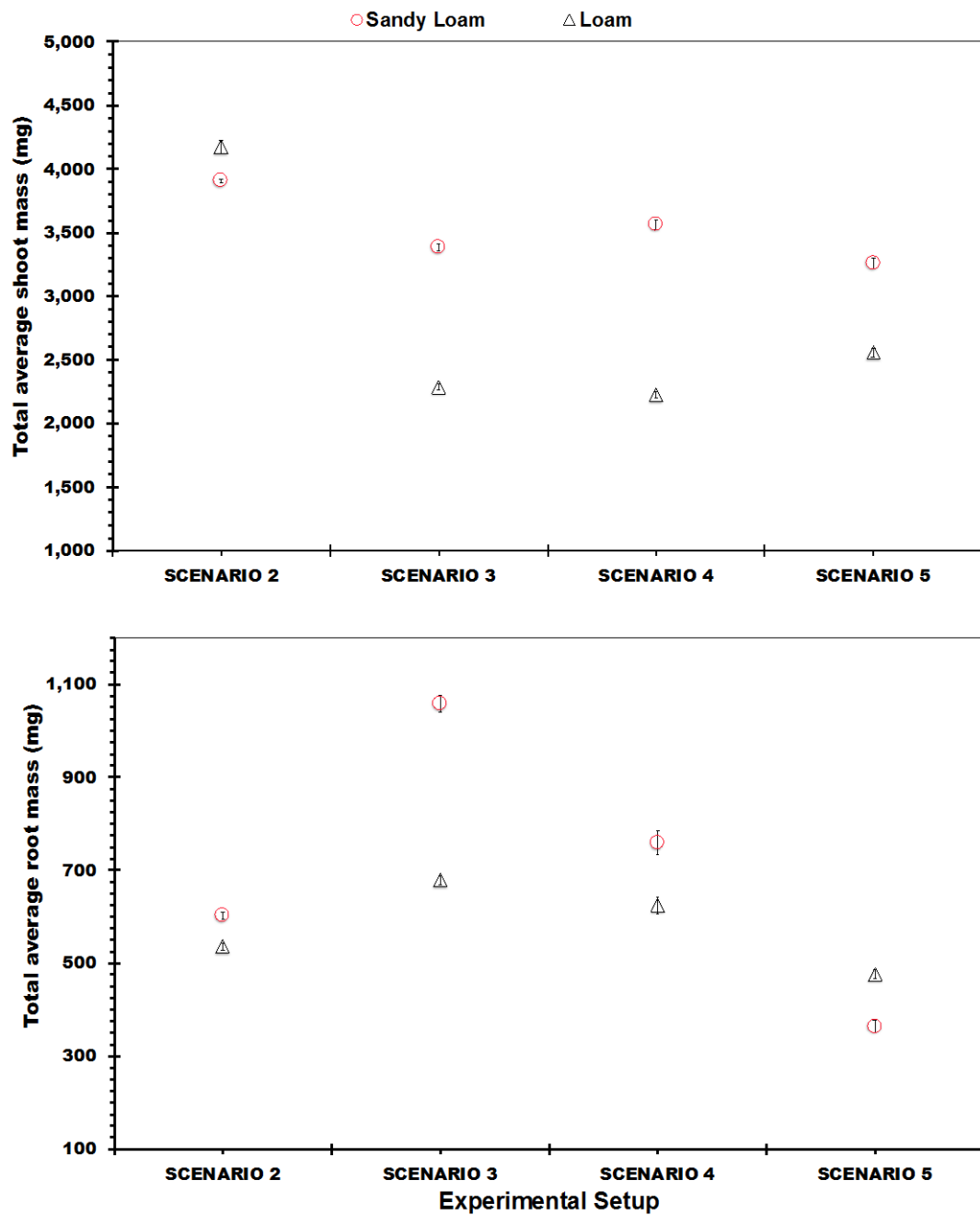
Figure 5-28 - Effect of the presence of oil on root and shoot biomass for all scenarios with plants. For each scenario, root and shoot dry mass are totalled up for each replicate, and the average of this total for the two replicates is presented for scenarios 2, 3, 4 and 5. Error bars represent ± one standard deviation.

The latter effect was observed at all depths where roots were found, and root mass was also observed at slightly greater depth (Figure 5–3, Figure 5–4, Figure 5–7, Figure 5–8, Figure 5–13, Figure 5–14, Figure 5–21, Figure 5–22). In particular, there appears to be an increase in root mass in all contaminated zones. Moreover, Increased root biomass was observed as the coverage of the oil layer increased (Figure 5–28), hence there is a decrease in root biomass across scenarios 3 - 5 for both soils as oil contamination levels reduced, this is particularly clearly illustrated in the sandy loam (Figure 5–28). The decrease root mass across scenarios 3 - 5 demonstrates the adverse effects of high sources of dissolved phase contamination and possibly the presence of non-continuous contamination layer which might have played a role in impact on the plants. The result, therefore, suggests that the response of plant root to environmental stress, in the form of changes to the root architecture by the plant may be dependent on the contaminant volume or its concentration in the soil and restrictions or non-continuous contamination layer. The non-continuous contamination layer may cause a variability in root development, as partitions provide a very different condition for root competition which will negatively affect plant growth. Moreover, high oil content will clog the pore space, thereby reducing the supply of oxygen, moisture and nutrients, especially in the sandy loam with low fine-grained contents. In the contaminated scenarios 3 and 4 with high oil contents, the root biomass production for sandy loam is greater than loam soil but the reverse is the case in the low oil contents scenario 5 with loam having a higher root mass than sandy loam, however, in the uncontaminated controls (scenario 2), the biomass production is similar for both samples (Figure 5–28). The higher root mass for loam in scenario 5 might be because the low oil content is bind up in the fine soil material, which can lead to a decrease in their bioavailability and transport in the ground, this might give plants greater accessibility to larger pores meaning accessibility to nutrients and moisture. The higher root biomass for sandy loam in the scenarios 3 and 4 might be because of an impact of the oil, which apparently has more effect in the sandy loam, perhaps because sandy loam has 30% less fine particles than loam. There is an increase contaminant bioavailability and transport in the sandy loam larger pores when compared with soils with high clay contents; therefore the toxic compounds can migrate in the soil and inhibit water and nutrients from reaching the rhizosphere which will affect the plants (Mitton et al., 2014).

Also, 50% and 36% more root biomass for sandy loam and loam respectively were recorded at all depth in the high contaminated scenarios 3 and 4 as a result of root damage. However, 65% and 55% less root biomass was recorded for the sandy loam and loam respectively at all depth in the lower contaminated scenario 5 when compared with the control scenario 2, suggesting that the greatest impediment to root growth was seen with high oil contamination levels (or possibly the presence of smaller non-continuous contamination layer) reduces root mass as well as shoot mass, but without non-continuous contamination layer or as the levels of NAPL increase, there is a threshold at which root mass starts to increase. The negative impact of NAPL and non-continuous contamination layer is of greater magnitude in the sandy loam. However, the higher root mass observed for loam might be because the low oil content having low inhibitory impacts on plant growth due to a decrease in their bioavailability in the rhizosphere. The higher root biomass recorded compared to the uncontaminated control is likely due to the toxicity of the inert or immobile mineral oil clogging the pore space and the physical exclusion of oxygen, moisture and nutrients required by the plant for growth and developments. The environmental stress effect on root development as a result of the contamination may be attributed to the presence of high concentrations of mineral oil; leading to increased toxicity and reduced access to moisture and nutrients, which will strain root development in the contaminated scenarios. The increased root growth may be a response of the plants to environmental stress, increasing the spread of roots in an effort to find an uncontaminated route to nutrient supply, as was observed in the microcosms. The mass increase was partially caused by an observed thickening of roots (again also observed in microcosms). Changes in root architecture such as length, thickness and branching (branching has been seen previously in the microcosms, see section 4.1 but not observed or measured in this experiment) are commonly observed as a result of abiotic stresses such as drought, salinity or metal contamination (Franco et al., 2011) although the actual impact is highly species dependent. In the presence of oil, plant roots were coarse and looked injured, while the uncontaminated control plant roots were longer, fine and smooth (Figure 5–29).

| (a) Root image in uncontaminated clean soil scenarios | (b) Root image in mineral oil contaminated scenarios |
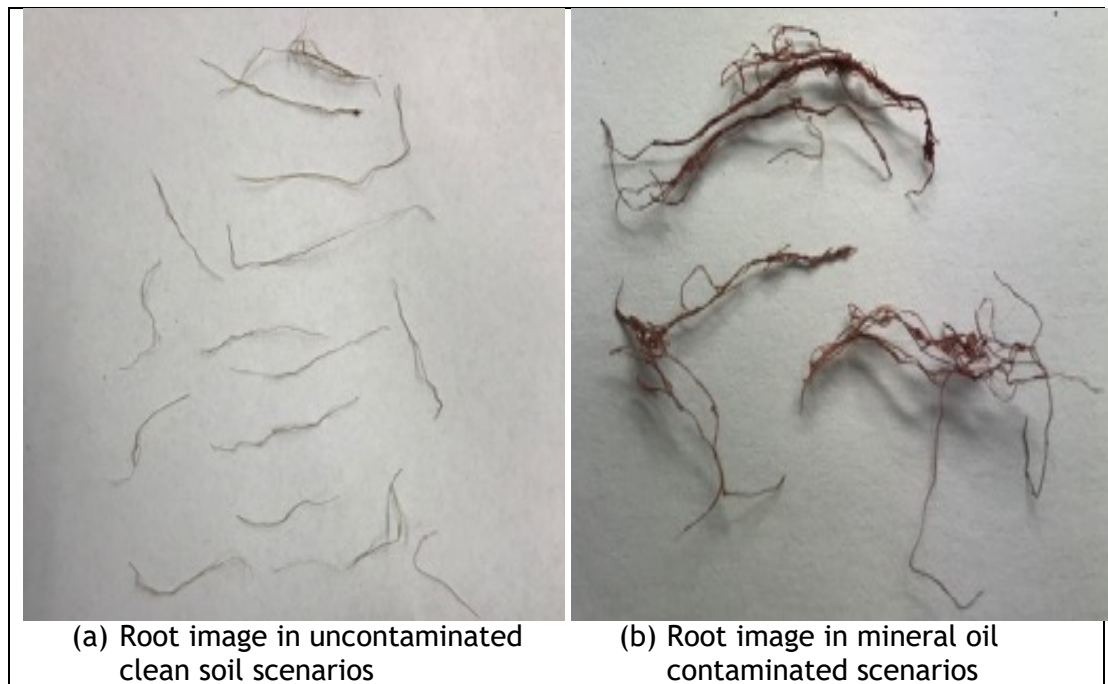
Figure 5-29 Root images in contaminated and uncontaminated soils scenarios

Plant roots can be directly or indirectly affected by the presence of oil in the soil. Merkl et al. (2005). Merkl et al. (2005) observed that roots of plants growing in pots after the soil mixed with heavy oil were coarse and injured, as roots of *Brachiaria brizantha* (Hochst. ex A. Rich.) *Stapf* (Poaceae), and *Cyperus aggregatus* (Wild.) were severely damaged, and death resulted in contaminated pots. Root death or injury can be related to intermediate compounds like aromatic acids, phenols and alkanoic acids that can form when oil is biodegraded by microorganisms in soil (Hutchinson and Freedman, 1978). Nonetheless, oil contamination affects soil moisture conditions, and due to the hydrophobic nature of petroleum, water spreads in-homogeneously in the contaminated soil which can inhibit water from reaching the rhizosphere. Therefore, the stress experienced by the plants due to less permeability may lead to an increase in root diameter and reduced root length (Zhang et al., 2003) .

As with the microcosm experiments, there is a decrease in the overall mass of shoots in the presence of oil (Figure 5–28). Contaminated scenarios relative masses of shoot and root is 8:3 when compared to the 10:1 for the control scenario 2. Scenarios 3 -

5, with LNAPL contaminants recorded the reduced shoot biomass when compared with control scenario 2. These results suggest that mineral oil does have an effect on shoot growth.

There is a decrease in the overall mass of shoots in the presence of oil (Figure 5–28) with scenarios 3 - 5 recording reduced shoot biomass when compared with control scenario 2. In particular, there appears to be a decrease in shoot mass in all contaminated zones when compared with control scenario 2 (Figure 5–28). Moreover, there is an increase in shoot biomass across scenarios 3 - 5 for both soils as oil contamination levels reduced, clearly illustrated in the loam soil (Figure 5–28). Lower shoot biomass was recorded in scenario 4 left-hand columns when compared to the control scenario 2 left hand especially in the loam soils, see Figure 5–18. It is probable that the roots responded to the diffusion of the dissolved phase contamination diffusing to the left, hence the changes in the shoot growth. The results also confirm that the presence of contaminant in adjacent zones does affect plant shoot growth in both soils. It may be because the plant put more energy into root growth than shoot growth due to stress induced by oil contamination. The adverse impacts on plant shoots might be because of high oil concentrations in the soil. This will not only reduce the amount of water and oxygen available for plant growth (Kaur et al., 2017) but also can interfere with soil-plant-water interactions by direct physical contact (coating of root tissues) thus negatively affecting shoot growth (Razmjoo and Adavi, 2012).

5.6.2   Plant effects and oil loss in artificial sandy loam or loam soils

The presence of plants corresponded to significant oil loss (between the average of 60 – 80 % for sandy loam and 50 – 70 % for loam), whereas without plants only minimal oil loss was noted (5.19% and 3.59% for sandy loam and loam respectively) as shown in Figure 5–30.
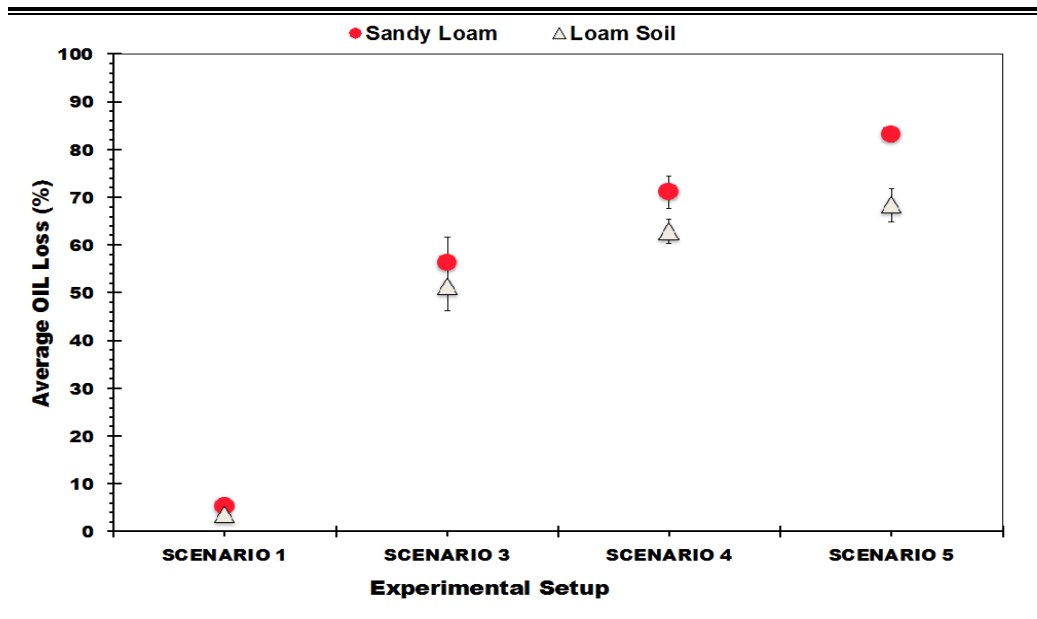
Figure 5-30 – Scenarios 1, 3, 4 and 5 – sandy loam and loam - Total average oil loss. Error bars represent ± one standard error of the mean.

Within planted experiments, decreasing amounts of oil loss were observed as the coverage of the oil layer increased. Although the improved performance is largest at low contaminant levels, as oil contamination increases, the oil recovery from the two soils becomes similar. Therefore, the results show that the success of phytoremediation is because of the presence of the plant which resulted in an increased amount of oil loss from the system (Figure 5-30). The observed reduced oil loss with increasing oil levels is because the removal of oil by the plant generally depends on the volume or its concentration in the soil (Zengel et al., 2016, Lim et al., 2016). The observed results might be because of the damaged root in the high-level contaminated scenarios (Naidoo, 2016, Merkl et al., 2005) or the plant root branching and avoidance of contaminated zones in an effort to find an uncontaminated route to nutrient supply as seen in the microcosm (section 4.2.10) and plant growth and root morphology in an oil polluted soil experiment by Langer et al. (2010). Perhaps, it may also be because all scenarios have the same mass of plants, and they can only deal with oil at a fixed rate, thereby taking longer to remove a similar percentage.

The sandy loam which produced higher amounts of root biomass has a higher contaminant loss, and it is perhaps not surprising that this has affected the plant shoot development. The increase in root biomass (the observed injury to roots) in response to increasing mineral oil loss, might suggest an increase in contaminant bioavailability and transport in the sandy loam. Oil will preferentially enter sandy loam larger pores, thereby resulting in higher residual saturation in the pores. Under these conditions, plant roots will have greater accessibility to degrade the contaminants; however, high oil content in the pore space will result in less accessibility to oxygen, nutrients and moisture and the consequential damaged roots. Loam soil with high fine-grained content holds water more tightly making the movement of oil into and out of water-saturated soil pores difficult, which can lead to a decrease in the oil bioavailability and transport in the ground and the resulting lower loss.

5.6.1   Limitations

Samples up to a depth of 10 cm has been analysed. It is possible that there was an impact of oil on root growth deeper than 10 cm, but the evidence presented in the results section suggests this will be minimal. There is a lack of oil free control for scenario 5, because the analytical work required was considerable for each specimen, and it was determined that two specimens was a manageable number. The uptake mechanism for LNAPL loss was not tested.

5.6.2 Summary

Phytoremediation of organic contaminants depends on the close interaction between plant and contaminant. For some scenarios, non-aqueous phase liquids (NAPLs) may be present and prove detrimental to the process. This work explores the impact of NAPL contaminant on plant growth in an artificial soil at the macroscopic scale to identify how phytoremediation can be employed for source zones treatment in the presence of NAPLs.

The multiple plant systems grown in artificial sandy loam and loam soils demonstrate the effects of the presence of an LNAPL (mineral oil) on the development of perennial ryegrass, and subsequently the removal of the oil by the plant. Plant growth and metabolism resulted in the removal of significant quantities of oil from the system, indicating that perennial ryegrass, and potentially other plant species too, is capable of the phytoremediation of non-aqueous phase liquids.

In conclusion, the introduction of the contaminant in planted experiments was found to significantly impact upon vertical root biomass distribution, including zones of increased root growth compared to uncontaminated controls, with increased root biomass observed as the coverage of the oil layer increased. The increase in root biomass is seen through increased root thickening as a result of exposure to the LNAPL. There were injuries on root development in terms of changes to root thickness within the contaminated layers which may be attributed to the toxicity of mineral oil and a reduced supply of moisture and nutrients, which will stress root development in this zone. Root death or injury has been linked to oil toxicity in soil and reduced access of the plant to moisture and nutrients which strain plant growth. The effect on the root system architecture is consistent with effects observed in other species due to various environmental stresses.

The presence of oil is also associated with reduced shoot biomass production as compared to the uncontaminated controls with much more significant shoot biomass than contaminated soils due to its inherent toxicity. This suggests that the stress induced by oil toxicity causes the plant to exert more drive into root growth than shoot growth.

Significant removal of LNAPL was recorded in the planted soil, but minimal oil loss was noted in unplanted soils. Although sandy loam with greater root biomass removed 80% - 90% oil at lower concentrations, the improved removal was similar for both soils as concentration increases.

Chapter 6   Implications and conclusions

6.1   Introduction

This chapter discusses the outcomes of a review of current literature available on phytoremediation and two sets of experiments with ryegrass (*Lolium perenne*) grown in hydroponic conditions as well as planted soil that are presented in this thesis. The goal of the literature review was to use an elaborate review to compare a large number of phytoremediation studies with regard to their performance and efficacy. The study assessed 350 experimental (laboratory and field-based) articles concerning the success or otherwise of phytoremediation and by combining outcomes from multiple studies seeks to identify patterns and relationships not apparent at the level of individual investigations. The two sets of experiments explored the impact of the physical presence of an LNAPL (mineral oil) on plant growth, root distribution and oil removal. The use of hydroponic systems has investigated the response of single grass plant to oil in a soil-free, hydroponic system to understand the impacts on root growth and distribution, as well as oil removal, in a highly idealized scenario, whilst larger scale experiments (mesocosms) have investigated phytoremediation and effects of oil on plants in artificial soil systems. The knowledge gained has been used to discuss how plants impact the behaviour and durability of NAPLs. This thesis aimed to understand plant and NAPL interactions within the vadose zone. Phytoremediation of organic contaminants depends on the close interaction between plant and contaminant. For some phytoremediation treatments of contaminated soils. A key question is whether the spatial distribution of roots is governed or correlated with the spatial distribution of NAPL contamination in the soil and what role does the form or state of contamination play, and why this happens?. This work aimed to investigate the multiphase interactions of plant, soil minerals and soil pore liquid with NAPL contaminants at microcosm and macrocosm levels. By so doing, the investigations seek to develop an understanding of how phytoremediation can be employed for source zone treatment in the presence of NAPLs.

Based on the research questions in section 1.1, the elaborate review has given the research into phytoremediation an opportunity of a higher chance of detecting an effect and improve precision in the application of phytoremediation techniques by identifying patterns and relationships not apparent at the level of individual

investigations. Moreover, this has also allowed the degree of conflict to be assessed, and reasons for different results explored and explained. Also, the smaller scale hydroponic and the larger scale soils experiments that explored how plant growth, root distribution and development, and oil removal are affected through direct physical contact with LNAPL in pore-scale has established that LNAPLs may hinder root development and instigate root spread of NAPL-contaminated pores or zones and roots in close proximity to NAPLs may be able to reduce dissolved-phase contamination through mechanisms including uptake and rhizodegradation such that non-equilibrium conditions arise, causing relatively rapid dissolution of the NAPL.

This research has added to the understanding of the use of plants for remediating NAPL contaminants in many ways based on the initial aims and objectives in section 1.1. In particular, from the literature review, the study has:

- established from the review that sandy loam, loam, and silty loam have a better organic contaminant removal than other soil types because they favour water retention, nutrient availability and water supply for plant growth and root development.

- identified from the review that high organic matter leads to good removal but its presence is not crucial, and that within acceptable ranges for plant growth, the soil organic matter and pH have little impact on contaminant removal.

- indicated from the review that the physical presence of NAPLs, in particular, may have an effect, which suggests that there is a physical effect of NAPLs on plant rather than just chemical effects. The uptake mechanism indicates that the contaminants will dissolve in the aqueous solution within the rhizosphere and then adsorbed on the root before penetrating into root tissues.

- observed a reduction in the concentration of contaminants after intervention with plant species. Longer-term studies from the review showed that these positive trends were either maintained or improved with time.

Moreover, from the microcosms and mesocosms experiments, the study has:

- identified from the experiments that spatial distribution of NAPL contamination loss is related to the spatial distribution of roots.

- suggested, from interpretation of the experiments data, that phytoremediation of dissolved phase contamination accelerates the dissolution of LNAPLs into adjacent groundwater and thus can indirectly destroy these persistent contaminant sources considerably more rapidly than by natural attenuation alone.

- established from the experiments that the presence of NAPL does not prevent the growth of a root within a pore, allowing co-existence and therefore more rapid NAPL removal (either directly, indirectly or both) than might otherwise be the case.

- identified from the experiments that the impact of NAPL on root architecture is clear; it is not merely the presence of oil which subsequently caused the plant to seek out new routes to the nutrient medium. However, there was greater root distribution and spreading with more extensive NAPL (thought to be caused by increased access to dissolved phase oil) and changes to individual root morphology.

- suggested from the experiments that any contribution from direct interaction between root and NAPL has not been conclusively demonstrated but direct uptake of hydrocarbons is known to be possible (Hunt et al., 2018) although likely to be slower than dissolved phase effects.

- shown from the experiments that NAPL impact on the plant as a whole was detrimental, with considerably reduced above ground biomass (shoots) as well as the changes to the roots.

- indicated significant removal of LNAPL was recorded in the planted experimental scenarios, but minimal oil loss was noted in unplanted scenarios.

- identified from the experiments that the adverse effects of high sources of dissolved phase contamination and the presence of non-continuous contamination layer in the contamination layer played a role in variations in the impact of contamination on plant growth.

- established that soil type influenced the water retention characteristic curves of the soils from the experiments. Due to the presence of significant percentages of sand, both soils desaturated at suction less than 1.0 kPa. The loam (clay-size = 20%) was found to have higher water holding capacity than the sandy loam (clay-size = 10%) for suctions less than 1.0 MPa. At higher suctions, both soils had similar water retention characteristics, which may be one of the factors that lead to a similar average overall root and shoot biomass production for both uncontaminated soils.

- identified that the presence of LNAPL in oven-dried soils reduced the total suction from 1000 to about 100 MPa. However, an increase in the LNAPL content (i.e., the oil content) did not influence the suction of the soils. For both soils, the measured suctions remained at about 100 MPa for a range of oil content between 2 to 12% indicating that the trapped air within the soil systems did not allow a reduction of suction of the soils when the oil content was increased. Although plant growth is dependent on many factors, the fact that sandy loam and loam soils desaturate relatively easily at lower suctions would favor plant growth (Ni et al., 2016).

- established from the experiments that, of the soils considered, plants grown in sandy loam removed the most significant amounts of LNAPL, which suggest an increase in contaminant bioavailability and transport in the sandy loam. However, the improved contaminant removal was similar for sandy loam and loam soils as the concentration of contaminant increases.

6.2   Impact of NAPL on root growth.

Organic contaminants are known to inhibit plant growth (Buss et al., 2015, Franco et al., 2011, Vázquez-Cuevas et al., 2017). The primary inhibiting factors from many studies are considered to be the toxicity of low molecular weight compounds and the hydrophobic properties of the higher molecular weight compounds limiting the

ability of plants to absorb water by decreasing the field capacity of soils and nutrient contents (Inglezakis et al., 2017, Norris, 2017).

Generally, root matter decreases with depth across the mesocosms (Figure 5–3, Figure 5–4, Figure 5–7, Figure 5–8, Figure 5-13, Figure 5-14, Figure 5-21, Figure 5-22). This is because perennial grasses are fibrous-rooted (Schenk and Jackson, 2002) and root profiles of grass communities tend to be as shallow as possible and as deep as required to meet the evapotranspiration (ET) demand, also factors related to reduced probability of oxygen deficiency, and high water and nutrient availability in the upper soil layers (Schenk, 2008) are at play. At the mesoscale, it is clear that there is varying root growth across all sampling columns from the plots of root dry weight with depth, and also the plots of total shoot mass and root mass for each column. At least 80% of control as per OECD tests (Reuschenbach et al., 2003). Seedling germination and growth was found to be good and dense in all replicates in all scenarios in the mesocosms (example images in Figure 5-2, Figure 5-6, Figure 5-12 and Figure 5-20). Moreover, in the microcosm experiments, seedling germination and growth was found to be consistently good across all replicates in all scenarios (example images in Figure 4-1). Germination occurred in all pore-scale rhizoboxes.

In this investigation, there was an apparent effect of LNAPL on root growth in the microcosm experiments, it is hypothesized that high oil concentrations were leading to high levels of dissolved mineral oil contents that caused root growth to be evenly distributed across the columns. Moreover, in the planted larger scale experiments (mesocosm), high oil concentrations lead to changes in the root architecture. Mineral oil has been shown to have an adverse effect on the plants (Zhu et al., 2018). Furthermore, increased root mass (as a result of root damage) at high oil concentrations suggests environmental stress experienced by the plant. The environmental stress effect on root development as a result of the contamination may be attributed to the presence of high concentrations of mineral oil; leading to increased toxicity and reduced access to oxygen, moisture and nutrients, which will strain root development in the contaminated scenarios.

Moreover, root growth occurs at deeper locations (below the NAPL contamination) compared to uncontaminated samples resulting in changes in both root biomass quantity and location. More root biomass was recorded at all depths in the highly

contaminated scenarios as a result of root damage for sandy loam and loam soils. However, reduced root biomass was recorded for the sandy loam and loam at all depths in the lower contaminated compared with the uncontaminated control scenario, suggesting that the most significant impediment to root growth was seen with more damaged root mass. The increased root growth may be a response of the plants to environmental stress, increasing the spread of roots to find an uncontaminated route to nutrient supply, as was observed in both microcosm and mesoscale experiments. Marked thickening of roots partially caused the mass increase. In the presence of oil, in both microcosm and mesoscale experiments, plant roots were visibly damaged and injured, while the uncontaminated control plant roots were longer, fine and smooth (Figure 5–29).

6.3   Impact of NAPL on shoot growth.

Some of the functions of the root are to absorb moisture, nutrients and firmly hold the plant in the ground. The root development and metabolism can directly affect the growth of the plant parts above the ground (Davies and Zhang, 1991). In some studies, it is evident that under environmental stress, and during seed imbibition, a decrease in water and nutrients uptake takes place which causes a reduction in germination ability of seeds (Pirasteh-Anosheh et al., 2011, Ashraf et al., 2008). Environmental stress can cause a permanent loss in shoot yield potential because it inhibits seed germination (Ashraf et al., 2013, Sharif et al., 2007). The microcosm recorded an overall decrease in the mass of shoots (Figure 4–3, Figure 4–4 and Figure 4-5) at high oil levels. Moreover, in the presence of oil in the mesocosms, there was an overall decrease in the mass of shoots. Therefore, the behaviour observed at the microcosm scale and the larger size experiments in soils (sandy loam or loam) mesocosms shows there is a very significant increase in the mass of roots and a decrease in the overall mass of shoots in the presence of oil (Figure 5–28).

Both experiments suggest that due to stress induced by oil contamination, the plants put more energy into root growth than shoot growth. Therefore, the decrease in overall mass and length of the shoot after oil contamination suggests environmental stress experienced by the plant as a result of some physiological process in plants such as respiration, translocation and transpiration being affected adversely by LNAPL contamination.

6.4 Impact of spatial NAPL location on plant growth.

Plant growth is affected in areas adjacent to LNAPL contamination in all scenarios that combined contaminated and uncontaminated zones when compared to uncontaminated experiments without oil and the experiments with complete oil coverage. Hence, the combinations of contaminated and uncontaminated zones (separated by partitions) in the same box has led to a decrease in the overall mass of shoots and a very significant increase in the mass of roots for both microcosm and mesocosm experiments (see microcosm Figure 4-7 and mesoscale). The decreased shoot biomass and the increased root growth in all scenarios that combined contaminated and uncontaminated zones suggest the presence of oil and perhaps, the presence of the different zones (separated by partitions) inhibits shoot growth and causes root damage. The observed adverse effects suggest physical exclusion of moisture and nutrients as a result of oil toxicity and the presence of partitions. There was observed injury, characterized by a thicker and reduced length, to the roots. Specifically, there is an increase in the root mass in all locations. It is probable that the oil presence in adjacent columns led to dissolved mineral oil and that it might be this that inhibited root growth in the uncontaminated columns and therefore caused root damage. There is some evidence for this in that the root growth in contaminated columns soils was found to be consistently higher than the uncontaminated columns. See Figures 4-5, 4-6, 5-9, 5-10, 5-12 and 5-13.

However, the adverse effects of alternating contaminant locations on the plant growth are most significant as oil concentration levels increase (Figure 5–28, scenarios 3 - 5), indicating that the highest levels of LNAPL contamination and partitions have the most significant adverse impact on the root architecture. The increased in root mass as oil contamination levels increased also explains the adverse effects of high sources of dissolved phase contamination transport from the adjacent LNAPL zones and the presence of partitions which might have played a part in impact on the plants. The combinations of contaminated and uncontaminated zones (separated by partitions) may cause root competition with each other for space which will affect the plant. The result, therefore, suggests that the response of plant root to environmental stress, in the form of changes to the root architecture by the plant may be dependent on the contaminant

concentration levels in the soil and the physical restrictions imposed by the partitions.

6.5   Impact of root location on NAPL removal

In this study, it is apparent that the presence of NAPL does not prevent the growth of a root within a pore in the early stages regardless of contaminant location, indicating that roots tend to grow vertically downwards with little lateral spread initially and that this is mostly unaffected by the presence of individual oil 'ganglia'. The roots appear to coexist with the contaminants within the NAPL contaminated layers and therefore more rapid NAPL removal (either by direct interaction between root and LNAPL, indirectly by dissolved phase effects or both) than might otherwise be the case. In all oil-contaminated scenarios, the presence of a root led to substantial LNAPL loss (Figure 4–5) whereas in plant-free experiments little or no oil loss was observed (Figure 5–30). Even where little or no root growth was observed in an oil-contaminated pore, the oil was still removed, although more slowly than when a plant root was present. The analysed data from microcosm experiments show that root growth appears to be correlated to oil loss as increased root growth is matched by increasing levels of oil loss (Figure 4–8). Though, in some instances, the oil loss was substantial for relatively small root growth and oil was lost without any root growth. This suggests that enhanced removal of the low levels of dissolved phase mineral oil by established roots in the adjacent pores disrupts the equilibrium causing further mineral oil in all pores to dissolve, which in turn is removed by the action of the roots. Also, in the mesocosm experiments, this led to a decrease in the overall mass of shoots and a very significant increase in the mass of roots in the adjacent columns when compared with the oil-free experiments. The result suggests the probability of the roots responding to dissolved phase contamination diffusing to the adjacent uncontaminated columns causing LNAPL phytoremediation and changes in the root architecture, see Figure 5–17 and Figure 5–18.

6.6   Impact of different levels of LNAPL on plant growth

Studies reported a reduction in fresh or dry weight of shoots and leaves of plants with increasing contaminant levels. Palmroth et al. (2006) and Zhang et al. (2010) found that the presence of high levels of contaminants negatively affects the growth and health of plants due to the reduced permeability induced by the introduction

of pollutants. As soil moisture conditions are affected by heavy oil contamination and due to hydrophobic nature of petroleum, water spreads inhomogeneously in the contaminated soil which can inhibit water from reaching the rhizosphere thereby adversely affecting plant growth (Spiares et al., 2016, Xu et al., 2018).

In this investigation, scenarios without any contamination had the highest average total shoot mass and length as might be anticipated. In the microcosm experiments, it is not merely the presence of NAPL contamination (oil) which caused the plant to seek out new routes to the nutrient medium. It is postulated that the high oil presence led to higher levels of *dissolved* mineral oil, and that it was this that limited growth which subsequently caused the plant to seek out new routes to the nutrient medium.

In the higher oil content contamination scenarios, there was an increase in root mass as a result of root damage (Figure 5–29) for both soils and a decrease in shoot mass. The adverse effect of high oil contents on the root and shoot biomass is of greater magnitude in the sandy loam. It is expected that this is because of lower clay content in the sandy loam mixed with mineral oil. Oil displaces water more easily to occupy the pores in sandy loam soil which holds water less tightly when compare with loam soil. This means the contaminant can clog the pore space more easily in the sandy loam because the oil is not bound up in the soil material, leading to a decrease in nutrients and pore water availability compared to more fine-grained soil. Hence, the reduced access to solutes and moisture and the toxic oil compounds as a result of oil clogging the pore space in sandy loam will significantly affect the plants (Mitton et al., 2014).

For lower oil levels, loam soil had a higher root mass than sandy loam (Figure 5–25). There is higher suction in the case of contaminated loam soil when compared with sandy loam soil, Figure 3–3 (a) and (b). The higher root mass for loam in the lower oil level might be because loam soil holds water more tightly making the movement of oil into and out of water-saturated soil pores difficult. The low oil content is bound up in the fine soil material, which leads to a decrease in their bioavailability and transport in the ground, this might give plants greater accessibility to larger pores meaning accessibility to nutrients and moisture (oil recovered from loam soil is lower than sandy loam, Figure 5–30). However, with the increase in levels of oil concentrations, there appears to be a threshold at which root mass starts to

increase in both soils as a form of environmental stress (Figure 5–28). In the mesocosm experiments, the small unconnected oil contaminated zones scenarios average total root mass was lower when compared with the oil-free scenarios (clearly illustrated in the sandy loam, Figure 5–28). However, as the layer of contamination starts to increase, as shown in the discontinuous and continuous LNAPL zones scenarios, the root mass starts to increase for both soils in proportion to the contamination levels (Figure 5–28).

Moreover, there was a reduced oil loss (by proportion) as the levels of contamination increased. The observed reduced oil loss with increasing oil levels in both microcosm and mesoscale experiments might be because of the damaged root in the high-level contaminated scenarios. Also, the reduced oil loss may be because of plant root branching and avoidance of contaminated zones to find an uncontaminated route to nutrient supply. Perhaps, it may also be because all scenarios have the same number of plants, and they can only deal with oil at a fixed rate, thereby taking longer to remove a similar percentage.

6.7 Implications for phytoremediation

The hydroponic microcosm and mesocosm experiments have provided evidence of the impact of the presence of an LNAPL (mineral oil) on plant growth, root distribution and oil removal. The research has demonstrated the potential for plants to tackle NAPL contamination and shows that the phytoremediation of organic contamination is not limited to tackling only the dissolved phase, but that roots interacted with the NAPL which resulted in a significant indirect reduction in the presence of the LNAPL. It has been shown that roots close to NAPLs can remove dissolved-phase contamination through uptake and rhizodegradation resulting in the rapid dissolution of NAPL source, and that roots are able to tolerate direct contact with NAPLs, although whether LNAPL removal took place as a result of this direct contact is unclear. The study indicates the potential for phytoremediation of LNAPLs in a hydroponic system, and soils accessible by plant root systems. Although the grass species may only be suitable for shallow depth NAPL contamination, however, the successful phytoremediation of dissolved phase contamination that accelerates the dissolution of LNAPLs source suggests that other plants too may be employed. Studies have shown that deep-rooted tree species can also tolerate organic contaminants due to their deep roots (Pérez-Hernández et al., 2013, Rivera-Cruz et al., 2016).

The general limitation of the techniques is that plants have to be alive with water, nutrients and oxygen adequately available to the roots. Moreover, the soil texture (organic matter content, pH and clay content), contaminant levels and concentrations and other toxic elements must be within the limits of plant tolerance. NAPL contaminants may leach outside the rhizosphere and require containment if they have higher water solubility. The technique also may need to be considered as a remediation strategy in the long-term because of the time it takes the plants to be well established. However, despite these limitations, the technique may be appropriate in situations where significant surface areas of relatively NAPL contaminants exist in the surface soils. For example, in remediation of aged NAPLs, with scenarios ranging from larger zones of continuous NAPL contamination to small unconnected individual ganglia which act as individual contaminant sources. It has been shown that the plants may increase the spread of roots to find an uncontaminated channel to nutrient supply in response to the

toxicity effects and environmental stress from oil contamination. The research has demonstrated the phytoremediation effect of aged NAPLs contamination with complex physical distribution. In the case of different contaminants, the success of phytoremediation requires the bioavailability of the pollutants for uptake or absorption to, and metabolism by the plant or associated plant microbial systems.

6.8 Further work

The experimental design, materials and methods generated data that demonstrates the impact of non-aqueous phase liquids (NAPLs) on plant behavior, root architecture and the resulting impact of this on phytoremediation. While phytoremediation of mineral oil by ryegrass has been successful in this investigation, the robustness of the technique requires exploration, for example, changes to NAPL constituents and degree of contamination may adversely affect ryegrass to the extent that phytoremediation may not be possible. Only one well-defined petroleum by-product component (mineral oil) has been used as LNAPL. It is possible that a mixture of organic chemicals may result in only some of the compounds being removed or that the combination of chemicals might cause difficulties in plant growth or the soil structure. Thus, research into mixed organic contaminants is required. Similarly, while ryegrass is appropriate for shallow contamination, deeper contamination problems would require more deep-rooted plants, with different root architectures and densities, for example, hybrid poplars and willow trees (Reilley et al., 1996, Limmer et al., 2018), *Cedrela odprata* and *Tabreburia rosea* (Pérez-Hernández et al., 2013) and Acacia species (Bento et al., 2012).

Moreover, the contaminant level or concentration has not been phytotoxic; therefore, experimental work with extensive NAPL spill would have to be conducted to supplement the findings of this study. Although roots are able to tolerate direct contact with NAPLs, direct LNAPL removal as a result of this direct contact should further be investigated. Moreover, further investigations into whether the phytoremediation of dissolved phase contamination accelerates the dissolution of LNAPL source into adjacent groundwater should also be considered. Nonetheless, this study has highlighted the effect of LNAPL on root distribution and spreading with more extensive NAPL as a result of increased access to dissolved phase oil. While the fundamental underpinning science of the multi-phase interactions of

plant, soil minerals, soil pore liquid with NAPL contaminant has been investigated in part, further work is required to establish source zone phytoremediation of NAPLs and long-term predictive NAPL remediation models.

Additionally, in the microcosm experiments, semi-quantitative measurements of root growth and distribution and oil loss were made during the experiment as fully quantitative and accurate data could not be obtained for either measurement without disturbing the specimen. Thus, future experimental setup and equipment could be improved to allow fully quantitative and accurate data measurement. Also, in the mesocosm experiments, the set up could be improved so that the root growth can be physically monitored and measured over time. Additionally, the study only investigated soil properties before the experiment, so changes in soil conditions concerning the impact of NAPL and plant were not monitored. Therefore, the soil characteristics after NAPL phytoremediation will require more investigations. Ultimately, the underpinning science and attention to soil chemistry could improve the techniques of NAPL phytoremediation.

Phytoremediation is a technology that seeks to exploit the growth habits and metabolic abilities of the plants. There is a significant need to pursue both fundamental and applied research to understand the limiting factors in plant tolerance, increasing uptake and translocation of soils and groundwater contaminants. Moreover, the fate of many contaminants and the mechanisms of sequestration within the plant is not well understood. It will be difficult to exploit many of the recent advances in plant assisted remediation without this basic understanding. There is a need to provide research tools, for example by grouping plants that are different in only a single trait, such as a specific metabolic pathway or root morphology. The results of current research are difficult to interpret, and causative relationships are hard to establish because many studies involve plant species with many phenotypic differences. Also, research cooperation between the engineering, agronomic and chemical-remediation professionals to produce integrated hybrid technologies is needed, e.g. applied research into plants with higher biomass hyperaccumulators and higher translocation rates. Exploring the cooperation between various disciplines may eventually provide low-impact and environmentally sound remediation technologies.

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APPENDIX 1: LITERATURE REVIEW REFERENCE LIST

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APPENDIX 2: CONFERENCE AND JOURNAL PAPERS

The 8th International Congress on Environmental Geotechnics, 2018.

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Phytoremediation of Light Non-Aqueous Phase Liquids

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Abstract. Non-aqueous phase liquids (NAPLs) are persistent sources of con-
tamination in the ground, providing a long-term supply of dissolved phase
contamination and taking significant periods to dissipate naturally. Light NAPLs
(LNAPLs) take the form of a separate phase within the ground, often as indi-
vidual ganglia in pore spaces within the capillary zone such that the contami-
nated region is diffuse and comprised of many unconnected small contaminant
sources. Consequently, remedial action is challenging and success may be
limited to ex-situ remediation techniques. The ability of plants to phytoreme-
diate dissolved-phase contamination is well known, but the impact of LNAPLs
on plant growth and subsequent contaminant behaviour is largely unknown.
Experimental work with ryegrass *(Lolium perenne)* is presented, exploring the
impact of the physical presence of an LNAPL (mineral oil) on plant growth, root
distribution and oil removal. The presence of the oil was found to significantly
impact root biomass and distribution, leading to zones of increased root growth
alongside decreased shoot growth. Significant removal of the LNAPL was noted
in both hydroponic conditions and planted soil.

Keywords: Phytoremediation · Ryegrass · Mineral oil
Non-aqueous phase liquids

1 Introduction

Phytoremediation of organic contamination in soils may arise as a result of contaminant
uptake into plant tissues [1, 2], phytovolatilization [3] or biodegradation of contami-
nants by rhizosphere-associated microorganisms [4]. Success has been reported in the
remediation of a range of organic contaminant families - for example fuel oils [5],
chlorophenols [1] and polyaromatic hydrocarbons [6]. However, phytoremediation
studies typically do not report the form of the organic contamination, i.e. is it present as
a non-aqueous phase liquid (NAPL) or in the dissolved phase? It is to be expected that
NAPLs (particularly light, or LNAPLs) might impact on plant growth and surviv-
ability, not just through their toxicity but also through their physical presence in the
pore space and their ability to act as a barrier to moisture and solutes.
    The impact of NAPLs on plants, and the potential for plants to tackle NAPL
contamination, is unknown. Zhang et al. [7] explored uptake of trichloroethylene,
introduced in NAPL form, by alfalfa although the impact of NAPL on the plant is

unknown and impacts on the contaminant may have been limited to the dissolved phase. Previous studies on organic contaminants may have involved sufficient concentrations that NAPLs were present but this is not specifically reported. NAPLs are challenging to remediate [8] but if phytoremediation were capable of treating this form of contamination, then this may potentially be a valuable mechanism of remediation for LNAPLs in soils accessible by plant root systems. This work has explored the impact of the presence of LNAPL on plant growth, alongside determining the degree of oil removal due to the presence of the plant. Two studies are presented -hydroponic microcosms mimicked the soil pore structure to explore the impact of NAPL on individual plant root systems, whilst larger scale mesocosms have explored phytoremediation and impact of oil on plants in artificial soil systems.

2   Impact of NAPLs on Root Distribution and Growth, and Oil Loss, at the Root Scale

The response of single grass plants to oil in a soil-free, hydroponic system has been explored to understand the impacts on root growth and distribution, as well as oil removal, in a highly idealized scenario.

2.1   Experimental Design and Methodology

Perennial ryegrass *(Lolium perenne)* is capable of phytoremediation of organic contaminants [9] and grew reliably in the experimental conditions. Quarter strength Hoagland's solution (2.5 g/L Hoagland's No.2 Basal Salt Mixture (Sigma-Aldrich, UK) in deionized water) was used as a hydroponic solution. Mineral oil (Fisher BioReagents) was chosen as the model LNAPL. Its low toxicity, volatility and aqueous solubility minimised the impact of NAPL toxicity on plant growth (ensuring the main impacts are due to physical presence), and ensured that any contaminant loss was down to biodegradation rather than solubilisation or volatilisation. The oil was coloured with 50 mg/L Oil Red O (Sigma-Aldrich) to enhance visibility.

Plant growth microcosms (Fig. 1) were 3D-printed from polylactic acid. Each microcosm comprised four vertical columns to represent soil pore channels, above which a 'v' shaped housing held a plant seed whilst allowing roots to progress downwards through the open apex of the housing. The front face of the microcosm was covered with a thin sheet of acetate film for visual monitoring, apart from over a small area at the base. The construction materials only minimally absorbed the oil.

Experiments explored root growth in two scenarios with and without mineral oil. Each scenario had five replicates, with the ten microcosms secured to the base of a plastic reservoir filled to a depth of 20 mm with the Hoagland's solution - the opening at the base of the acetate film allowed this level to be maintained within the microcosm. This level was maintained using a Mariotte bottle system, whilst the reservoir fluid was pumped through an ultraviolet steriliser at approximately one volume per twelve hours to control algal growth. In the scenario with oil, each column had 10 µl mineral oil placed on the surface of the fluid by syringe. A single seed of ryegrass was placed in the seed housing and secured with a small amount of cotton wool soaked in Hoagland's
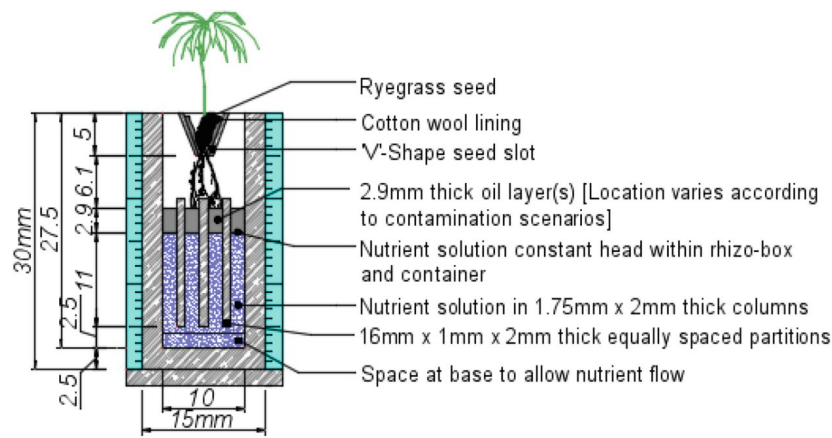
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Fig. 1. Plant growth microcosm schematic diagram.

solution. The planted microcosms were subjected to an artificial daylight regime from 58 W cool white daylight spectrum fluorescent tubes, located 1.5 m above the microcosms, for 16 h per day over four weeks.

Following this period, oil levels were observed visually and scored (all oil remaining = 1, partial oil = 0.5, no oil = 0) as the oil layer was not sufficiently thick to allow accurate measurement. Similarly, root growth in each of the four columns (numbered 1–4 from left to right) was observed and scored (established root growth = 1, partial root growth = 0.5, no root = 0). Subsequently, the microcosms were dismantled then roots and shoots separated and total weight and length recorded.

2.2   Results and Discussion

Root growth was observed to be non-uniform, with significant growth in the central columns (2 and 3, directly below the seed housing) when no oil was present whilst that for the peripheral columns (1 and 4) was much smaller, with only one microcosm in each showing partial growth (Fig. 2a). With oil present, the roots were better distributed across all columns, although there was still greater presence in the centre. This corresponds to typically greater oil loss in columns 2 and 3 with lower oil removal in the peripheral columns. Oil loss without plants was found to be minimal (results not presented), inferring that the process of oil loss is biological.

Root and shoot masses and lengths are shown in Fig. 2b. The presence of oil has a detrimental effect on both shoot mass and length. Root lengths are, on average, comparable but there is larger root mass with oil present. This may be explained by observations of thicker roots in the presence of oil, suggesting that this causes the plant to put increased effort into root development at the expense of shoot growth.
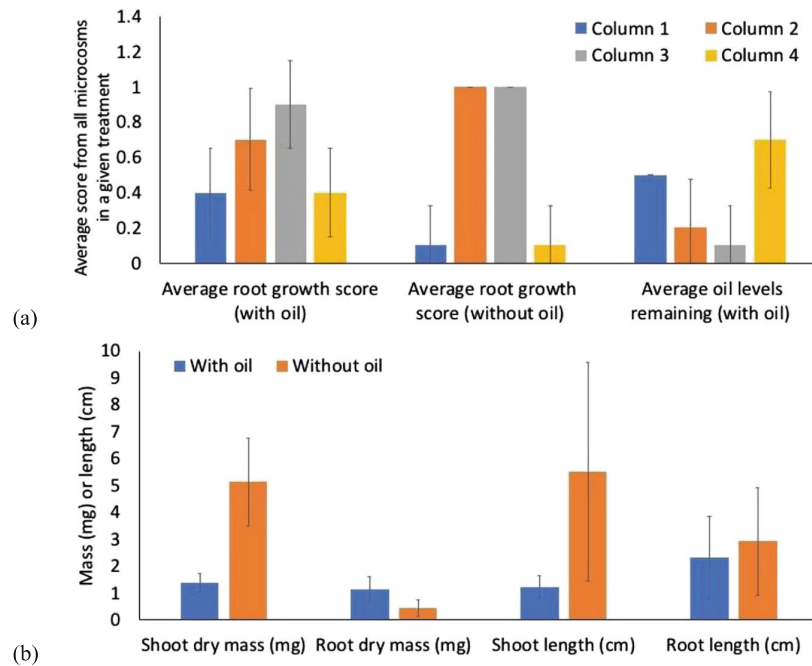
(a)

(b)

Fig. 2. (a) Scoring of root growth and oil loss in individual columns. (b) Final shoot and root masses and lengths.

3 Impact of NAPLs on Plant Growth in Soil at the Meso-Scale

The behaviour observed in microcosms was explored at a larger scale in soil meso-cosms to determine behaviour of a planted soil and effect on NAPL contamination.

3.1 Experimental Design and Methodology

A sandy loam was prepared by mixing sand (70% by mass Leighton Buzzard Gar- side medium sand, 0.18–2.00 mm), silt (20% by mass Leighton Buzzard Garside fine sand, < 0.06 mm) and clay (Speswhite kaolin, Imerys) in the laboratory. The components were thoroughly mixed using an electric mixer and the resulting soil characterized following British Standard methods [10] to determine liquid (13.0%) and plastic limits (10.6%), hydraulic conductivity (1.5×10^{-4} m/s) and pH (7.9).

Mineral oil (with Oil Red O) was again used as a model LNAPL, perennial ryegrass *(Lolium perenne)* was used as the model plant and quarter-strength Hoa- gland's solution was employed for nutrient supply. Experiments were conducted in acrylic plant growth chambers with internal dimensions 25 × 250 × 350 mm (Fig. 3). A central partition separated the chamber into two segments, although in these

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experiments conditions on either side were identical. A 40 mm thick free-draining gravel layer (4–12 mm) was placed at the bottom of the chamber and covered with a layer of Whatman filter paper to prevent mixing with the sandy loam. W ater levels within the chamber were controlled using an external plastic tube connected by flexible tubing to a port located in the gravel layer. The water level in this was maintained at a constant height, maintaining the 'groundwater' level within the soil.
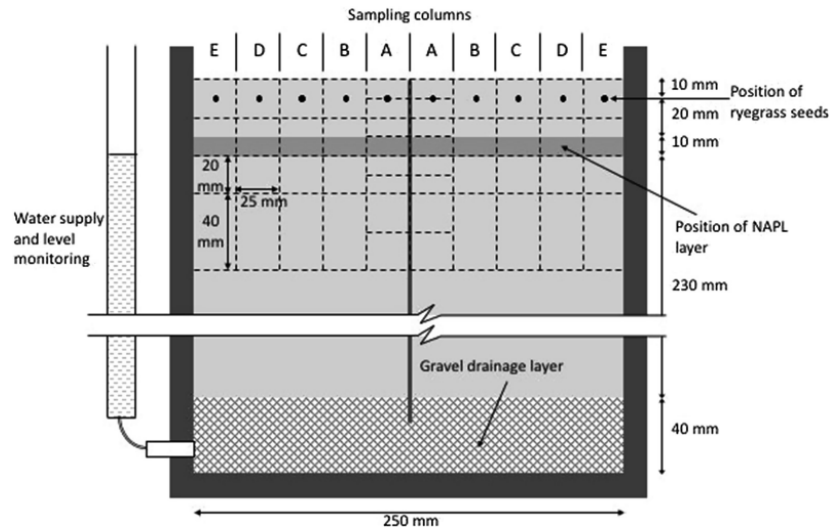
gram. Dashed lines represent sample cutting locations.

Sandy loam was placed in two plant growth chambers by firstly wet-packing a 230 mm thick layer with the moisture content at the plastic limit to a density of 1.75 g/cm^3 before shaking on a mechanical shaker for 2 s. This was then saturated *via* the plastic tube (i.e. from the base) with quarter-strength Hoagland's solution.

Next, a 10 mm thick layer of soil was placed on top, then lightly compacted to the target density. In one plant growth chamber this had a moisture content equivalent to the plastic limit as before. In the other, the soil was mixed with mineral oil to an oil content equivalent to the plastic limit. A further 20 mm of uncontaminated moistened soil was placed above this, with ryegrass seeds spread on top to a density of 50 g/m^2. This was then covered by a further 10 mm layer of moistened soil.

Over the ten weeks of the experiment, quarter-strength Hoaglands solution was added *via* the supply tube every day to maintain the water table at the same position. The plant growth chambers were wrapped with aluminum foil to protect roots from light and subjected to lighting and environmental conditions as for the microcosm experiments. Following the experiment the shoots were cut for each of the ten columns (A-E, left and right sides) and stored before freezing of the plant growth chambers at −20 °C. After removal of the front cover of the chamber the frozen soil was cut into the

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sections (see Fig. 3) with a Starrett band saw blade (2 mm tooth pitch). Individual samples were frozen in resealable bags prior to analysis.

Frozen soil samples were mixed with distilled water (approximately 1 to 4 by volume) in petri dishes. The mineral oil content of each sample was determined using hydrophobic oil-absorbent matting (MAT440, New Pig Ltd) [11] - a piece of mat was placed in the dish, covered and the sample shaken on an orbital shaker for 24 h. The difference in weight of the matting over this period was reported as the mass of oil present in the sample. In preliminary tests the matting was found to recover 80 and 99% of the oil present in soil samples with 0.01 and 20% oil by mass using this method. Root material was collected by placing the soil/water mixture in a sieve (200 pm
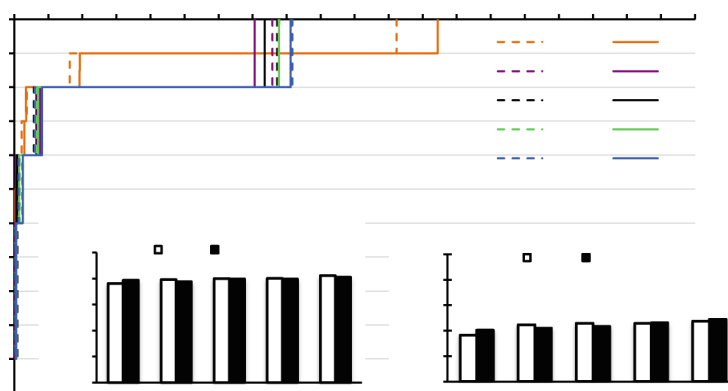
t oil, and (b) with oil.

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aperture) and submerging this in water such that the floating root matter could be collected and dried before weighing.

3.2   Results and Discussion

The biomass present in the oil-free mesocosm after 10 weeks is shown in Fig. 4a. Data are presented for both the left and right side of the central partition, in columns A-E (A closest to partition). Root matter decreases with depth, which is particularly clearly illustrated with the A samples. The discrepancy between the A samples and the B-E samples is because the former were taken every centimetre rather than every two centimetres as the remainder were. From the plot of root mass with depth, as well as the sub-plots of total root and shoot mass for each column, it is clear that there is very uniform growth across all sampling columns.

Figure 4b is presented similarly, although the grey bar shows the location of the NAPL and oil loss is presented alongside the plot of total root biomass. Between 50 and 70% of the oil was removed, demonstrating substantial phytoremediation of non-aqueous phase contamination. Once more, there was generally uniform growth across the mesocosm, both above and below the soil surface. As with the microcosm experiments, there is a decrease in the overall mass of shoots in the presence of oil, but there is a significant increase in the root mass, particularly in the uppermost two cm and just below the NAPL. The latter effect was observed at all depths where roots were found, and root mass was also found at slightly greater depth. The increased root growth may be a response of the plants to environmental stress, increasing the spread of roots in an effort to find an uncontaminated route to nutrient supply, as was observed in the microcosms. The mass increase was partially caused by an observed thickening of roots (again also observed in microcosms). Changes in root architecture (length, thickness and branching) are commonly observed as a result of abiotic stresses such as drought, salinity or metal contamination [12] although the actual impact is highly species dependent.

4   Conclusions

Two experiments at different scales have demonstrated consistent effects of the presence of an LNAPL (mineral oil) on the development of perennial ryegrass, and subsequently the removal of the oil by the plant. Both hydroponically grown single plants and multiple plant systems grown in artificial sandy loam soil demonstrate an increase in root biomass (both through increased root numbers and distribution, as well as root thickening) and a decrease in shoot biomass as a result of exposure to the LNAPL. The effect on the root system architecture is consistent with effects observed in other species due to various environmental stresses. Plant growth and metabolism resulted in removal of significant quantities of oil from the system, indicating that perennial ryegrass, and potentially other plant species too, is capable of the phytoremediation of non-aqueous phase liquids.

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APPENDIX 3: OIL ADSORPTION TESTS

1. Acetate Transparency Absorption Analysis by Sample Weight Gained

The absorption ability of acetate material was tested in the laboratory. Acetate film is a clear and flexible plastic sheet that will accept printing ink. It is also known for its wrinkle resistance, grease-proofness, water resistance, dimensional stability, high gas permeability, good electrical insulation properties, resistance to fogging and medium water vapor transmission (online ref., www.lairdplastics.com/product/materials/acetate).

The absorption ability was tested with a flat 2.3cm long x 3.0cm wide x 0.1cm thick cut out acetate materials fully immersed in mineral oil for 24hrs. The size was chosen to represents the front cover for the small rhi-box design for the plant root observation experiments. The sample was then taken out after 24hrs, thoroughly wiped out of oil and weigh. The process was repeated four times and the results are shown in table 1 below. The average mineral oil absorption to reach equilibrium is 0.02%.
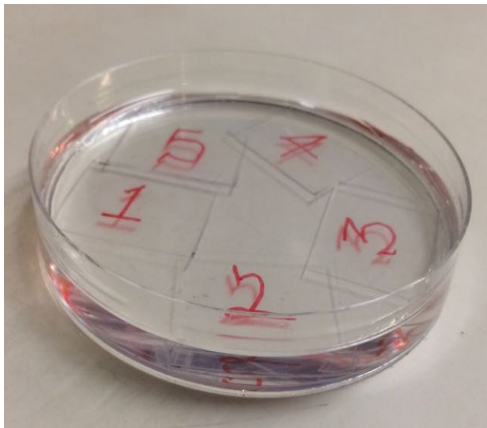

Fig. 1 – Acetate cut-outs fully immersed in mineral oil

Table 1: Acetate Transparency Material Oil Absorption Test

Material Sample	Sample Weight (g)	1st Reading Sample + Oil (after 24hrs). Weight (g)	2nd Reading Sample + Oil (after 24hrs). Weight (g)	3rd Reading Sample + Oil (after 24hrs). Weight (g)	4th Reading Sample + Oil (after 24hrs). Weight (g)	Average	Sample Oil Absorption / Weight Gained (g)	Percentage Absorption (%)
1	0.2201	0.2201	0.2201	0.2201	0.2201	0.2201	0.0000	0.00
2	0.1976	0.1976	0.1976	0.1976	0.1977	0.1976	0.0000	0.01
3	0.2113	0.2114	0.2114	0.2114	0.2113	0.2114	0.0001	0.04
4	0.1985	0.1986	0.1985	0.1986	0.1985	0.1986	0.0000	0.03
5	0.1932	0.1932	0.1933	0.1933	0.1933	0.1933	0.0001	0.04
								0.02

2. **3D Printing - PLA Filament Mineral Oil Permeability & Absorption Analysis by Rhizo-box Weight Loss**

This is a non-standard procedure that has been used to measure the permeation of mineral oil through PLA material between 0.1cm – 0.45cm thick. The rhizo-box exposed areas are 1.35cm high x 1.5cm long x 0.1cm thick, back cover; 1.35cm high x 0.2cm wide x 2.5mm thick, sides and 1.5cm long x 0.2cm wide x 0.45cm thick base. The acetate transparency is cut to shape and bonded to the rhizo-box front with super glue and further sealed with LS-X jointing compound, an external leak sealer to make it water and oil tight. The test method then involves filling the 3D rhizo-box with mineral oil. The test sample is then sealed by placing it an air tight container to prevent evaporation. The weight loss was then measured over time (in this case 24hrs). Permeation rates are then calculated from the rate of weight loss and the total areas of the four exposed sides of the PLA in (mg/min/m2). The average permeation rate is 1.13mg/min/m^2 as shown in table 2.
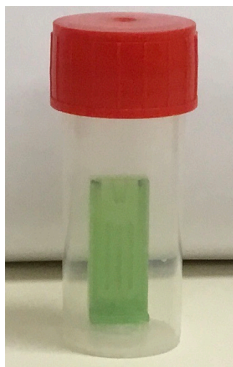
(a)   (b)

Figure 2 – (a) Rhizo-box Sample, (b) Sample in air tight container

3D Printing – PLA Filament Mineral Oil Absorption Analysis by Weight

Table 2: 3D Printing PLA Plastic Material Oil Permeability Test 1

3D Rhizo-box	3D Rhizo-box Weight (g)	3D Rizo-box + Oil (Before) Weight (g)	3D Container + Oil (after 24hrs). Weight (g)	3D Container + Oil (After 24hrs) Weight Loss (g)	Permeation Rates (mg/min /m^2)
1	1.625	1.9739	1.9721	0.0018	1.1990
2	1.6496	1.9959	1.9945	0.0014	0.9326
3	1.5682	1.9293	1.9267	0.0026	1.7319
4	1.655	2.0029	2.0011	0.0018	1.1990
5	1.6302	1.9841	1.9822	0.0019	1.2657
				Average	**1.2657**

Table 3: 3D Printing PLA Plastic Material Oil Permeability Test 2

3D Rhizo-box	3D Rhizo-box Weight (g)	3D Rizo-box + Oil (Before) Weight (g)	3D Container + Oil (after 24hrs). Weight (g)	3D Container + Oil (After 24hrs) Weight Loss (g)	Permeation Rates (mg/min /m^2)
1	1.6627	2.0031	2.0011	0.0020	1.3323
2	1.7092	2.0468	2.0449	0.0019	1.2657
3	1.687	2.0172	2.0152	0.0020	1.3323
4	1.6903	2.0111	2.0101	0.0010	0.6661
5	1.7906	2.1414	2.1398	0.0016	1.0658
				Average	**1.1324**

APPENDIX 4: CALIBRATION DATA FOR OIL ANALYSIS

The extraction ability of oil mat technique on mineral oil was measured. The oil mat used was the 'New Pig Ltd'. Oil-Only Mat; product number MAT440, which is made of 100% polypropylene which absorbs and retains oils and oil-based liquids without taking in a drop of water. The mat floats to clean up oil on water with absorption capacity of $0.043L/cm^2$. The technique was tested with known mineral oil concentrations ranging between 0.01% and 20% by weight. The range was chosen because 10.6% and 10.3% of mineral oil were added to the rhizo-box growth experiments for sandy loam and loam respectively.

20g of dry soil was mixed thoroughly with known mineral oil concentration by weight and placed in a petri dish container. 40g of water was then added to the mixture of mineral oil and soil and stir with spatula. Oil mat cut to the size of the inner diameter of the petri dish was then placed over the mixtures of oil, soil and water and covered to avoid evaporation. The container was then placed on orbital shaker for 24hrs to dislodge probable trapped oil. The mat was then taken out after 24hrs and weigh.

The recovery level of this technique is shown in the tables below, the result is consistence with the test result observed by ((Al-Ansary and Al-Tabbaa, 2007). [®]

Table 1: Oil Mat Technique Test – Sandy Loam Soil

% Oil Concentration added to 20g of Soil (Sandy Loam)	Oil Concentration by Weight (g)	Oil Mat Weight (g)	Oil Mat + Oil (g)	Extracted Oil (g)	% Extracted
0.01	0.002	2.1404	2.1421	0.0017	85
0.05	0.01	2.0556	2.0643	0.0087	87
0.1	0.02	2.1872	2.2054	0.0182	91
0.5	0.1	2.0818	2.1764	0.0946	94.6
1.0	0.2	2.1453	2.3364	0.1911	95.55
5.0	1.0	2.0558	3.0170	0.9612	96.12
10.0	2.0	2.2950	4.2434	1.9484	97.42
15.0	3.0	2.0105	4.9573	2.9468	98.23
20.0	4.0	2.0417	6.0242	3.9825	99.56

Table 2: Oil Mat Technique Test – Loam Soil

% Oil Concentration added to 20g of Soil (Loam)	Oil Concentration by Weight (g)	Oil Mat Weight (g)	Oil Mat + Oil (g)	Extracted Oil (g)	% Extracted
0.01	0.002	2.1199	2.1215	0.0016	80
0.05	0.01	2.1591	2.1676	0.0085	85
0.1	0.02	2.1021	2.12	0.0179	89.5
0.5	0.1	2.2478	2.3398	0.092	92
1.0	0.2	2.1446	2.3335	0.1889	94.45
5.0	1.0	2.2190	3.1756	0.9566	95.66
10.0	2.0	2.1878	4.1304	1.9426	97.13
15.0	3.0	2.2281	5.1702	2.9421	98.07
20.0	4.0	2.2920	6.2342	3.9422	98.555

APPENDIX 5: COMPLETE DATA SETS

Electronic copy, available from author.