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Engineering Fungal-Mycelia for Soil Improvement

A Thesis presented for

The Degree of
Doctor of Philosophy in
Structural, Geotechnical and Seismic Engineering
(Cycle: XXXI)

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Declaration

This thesis is the result of the author's original research. It has been composed by the author and due acknowledgement must always be made of the use of any material contained in, or derived from, this thesis.

Signed: 

Date: 17/06/2019

Dedication

To my wife Atule Margaret
and our children Enyone Leonora & AnibeOjo Emmanuel

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Table of Contents

Publications

1. Gráinne El Mountassir, James M. Minto, Leon A. van Paassen, Emmanuel Salifu, Rebecca J. Lunn (2018) Applications of Microbial Processes in Geotechnical Engineering. *1st edn, Advances in Applied Microbiology.* 1st edn. Elsevier Inc. doi: 10.1016/bs.aambs.2018.05.001. (A section of this Book Chapter forms part of the Literature Review [chapter 2] of this thesis)

2. Gráinne El Mountassir and Emmanuel Salifu (2018) Experimental studies on the hydraulic effects of fungal-mycelia in sandy soil. *Book of Abstracts, InterPore 10th Annual Meeting and Jubilee, New Orleans, United States;14-17th May, 2018.*

3. Emmanuel Salifu and Gráinne El Mountassir (2019) Potential application of fungal biogeotechnology as irrigation canal lining material for agricultural water conservation*; Book of Abstracts, InterPore 11th Annual Meeting, Valencia, Spain; 6 – 10th May, 2019.*

4. Gráinne El Mountassir, Emmanuel Salifu, Alexandria Schellenger (2019) Investigation of the Erodibility of Fungal Treated Soils Using a Laboratory JET Apparatus*; Book of Abstracts, InterPore 11th Annual Meeting, Valencia, Spain; 6 – 10th May, 2019.*

5. Emmanuel Salifu and Gráinne El Mountassir (2019) Preliminary observations of the shear behaviour of fungal treated soil. *Proceedings of the 7th International Symposium on Deformation Characteristics of Geomaterials (IS-Glasgow)*, UK; 26 – 28th June 2019. To be published online in: the EPJ web of conferences.

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10. Applications of fungal-hyphal networks in ground engineering; part of text written up for publication on the H2020 EURAXES project website as part of the technical deliverables for the Project TERRE (https://cordis.europa.eu/project/rcn/198308/results/en; www.terre-etn.com). Authors: Emmanuel Salifu, Gráinne El Mountassir, Gianfranco Urciuoli, Alessandro Tarantino.

Abstract

Most of the conventional materials, processes and techniques used by geotechnical engineers for ground improvement are associated with the emission of greenhouse gases. Global targets for reducing carbon emissions therefore pose a direct challenge to research and practice in the field of geotechnical engineering and has led to the development of interdisciplinary approaches seeking alternative low carbon technologies that are resilient to climate change. This thesis presents a novel, potentially low-carbon technique involving the use of fungal hyphal networks of *Pleurotus ostreatus* (*P. ostreatus*) for ground engineering applications.

An investigation was carried out to understand the range of environmental conditions suitable for growth of *P. ostreatus* in sand. Subsequently, the influence of the growth of *P. ostreatus* on the hydraulic and mechanical behaviour of sand was explored, in particular the influence on soil wettability, soil water retention curve, infiltration behaviour, saturated hydraulic conductivity and the stress-strain behaviour of sands. In addition, the influence of growth of *P. ostreatus* on the erodibility of sand was assessed using the Jet erosion test.

The results presented in this thesis demonstrate that the treatment of sand with *P. ostreatus* (i) induced extreme water repellency, (ii) caused a shift of the soil water retention curve increasing the air entry value from ~0.6 to 6 kPa (iii) reduced the rate of infiltration of water into sand (iv) lowered saturated hydraulic conductivity by one order of magnitude from 1.3×10^{-4} to 3.0×10^{-5} ms^{-1} (v) inhibited the development of dilatancy during shearing with an associated loss of peak shear strength and (vi) significantly improved the resistance of sand to erosion.

These results provide for the first time, evidence of the influence of the growth of fungal hyphae on the hydro-mechanical behaviour of soils within a geotechnical engineering context. The findings of this thesis implies that there is the potential to deploy fungal hyphal networks as a low carbon technique in areas of ground improvement where resistance of surface erosion and/or the creation of a semi-permeable barrier is required.

Chapter 1

Introduction

1.1 Background

Global population witnessed an unprecedented increase by two billion, from 1990 to 2015, and is projected to continue to increase: from 7.3 billion in 2015 to 8.5 billion in 2030, 9.7 billion in 2050 and 11.2 billion by the year 2100 (United Nations, 2015). Major implications of this include: increasing demand for food and for civil infrastructure alongside rapid urbanisation. Soils are the base materials for agricultural production and the foundation for civil engineering infrastructure. DeJong *et al.* (2010) observed that in order to meet the envisaged societal demand for civil infrastructure, there is an underlying requirement for competent soils. However, it is a common occurrence, that the geotechnical characteristics of soils at a site selected for infrastructure construction do not meet the engineering requirements. As a result, ground improvement is carried out to increase the strength and stiffness of soil, to modify hydraulic behaviour or to improve resistance to erosion by wind/water.

The exploitation and processing of natural resources to provide for the needs of the global population since the industrial revolution has contributed to the increased rate of degradation of the environment. A practical consequence of this is global warming. According to the 2018 special report on the impacts of global warming released by the United Nation's Intergovernmental Panel on Climate Change (IPCC), human activities have caused a global temperature increase of approximately 1°C relative to pre-

industrial levels and consequences of this temperature increase will persist for centuries to millennia (UN, 2018). The IPCC warns that the implications of global warming exceeding 1.5°C will be catastrophic, encompassing climate change and climate-related risks for both humans and natural systems, resulting in a rise in ocean temperatures, increased magnitude of storm events, flooding, soil erosion and loss of land resources, drought and desertification amongst others, with huge cascading impacts on ecosystems and biodiversity, human health and security, water supply and built infrastructure, food security and livelihoods in general. A critical step in forestalling this global threat will be the full implementation of the net zero global anthropogenic CO_2 emissions (and other greenhouse gases) goal agreed at the Conference of the United Nations Framework Convention on Climate Change held in Paris in 2015.

Conventional ground improvement techniques contribute to global CO_2 emissions, directly and indirectly. It is estimated that there are approximately 40,000 soil and ground improvement projects, carried out annually worldwide, at a total value of over US$6 billion, (DeJong *et al.,* 2010). As such, it is imperative to review the impacts of conventional ground improvement techniques on the environment. Further, there is a clear need for the urgent development of sustainable low-carbon technologies for ground improvement.

Conventionally, the classical methods of ground improvement involve: (i) mechanical techniques, i.e. techniques that use mechanical energy such as, compaction, external loading, construction of drains or the introduction of reinforcement; (ii) chemical techniques that involve the introduction of chemical reactants to produce soil binding

products and or fill pore space, (iii) thermal techniques (heating/freezing) and (v) electrical techniques (e.g. electro-osmosis). These processes are often expensive, energy intensive, highly invasive and involve the use of heavy machinery which may contribute to air pollution. Chemicals (including cements) often used for soil improvement are toxic/hazardous to soil organisms and groundwater (e.g. resulting from high pH plumes induced by cements), and can impede the growth of vegetation (Karol, 2010). Furthermore, the production of cement, which is widely used in ground engineering, is the third largest source (estimated to contribute ~5%) of global anthropogenic CO_2 emissions (Reijnders, 2007; Andrew, 2017).

In a bid to find alternatives to traditional ground engineering techniques, geotechnical engineers have begun to actively collaborate with geochemists and microbiologists to investigate bio-geo-chemical approaches to enhancing soil characteristics (DeJong *et al.*, 2010). Of the emerging bio-mediated soil improvement techniques to arise at this new multi-disciplinary interface, bio-mineralisation is being studied widely, at present. Biomineralisation generally involves the use of selected species of microorganisms to precipitate minerals depending on the prevailing or induced environmental conditions (Mitchell and Santamarina, 2005; DeJong *et al.*, 2010; Dhami, Reddy and Mukherjee, 2013). The biomineralisation process most studied by geotechnical engineers to date is microbially induced calcite precipitation (MICP) via ureolysis, a bio-grouting technology that involves the precipitation of calcite using bacteria. It has been successfully studied for a wide range of applications requiring increased soil strength and reduced permeability (Mitchell and Santamarina, 2005; Whiffin, van Paassen and Harkes, 2007; Ivanov and Chu, 2008; DeJong *et al.*, 2010). This method has been shown to be successful in coarse-grained materials but is

difficult to apply in fine grained soils due to the relative size of pores vis-à-vis the microorganisms. MICP and other 'biogrouts' are also faced with the challenge of transporting microorganisms to target locations in the ground and achieving a homogenous treatment zone. Due to filtering of bacteria, in order to create homogenous treated zones, arrays of closely-spaced injection and extraction boreholes are required. In-situ application of MICP requires sophisticated equipment for monitoring of the chemical, geochemical and geophysical changes taking place in the soil during bio-grouting in order to determine the extent of treatment achieved. Furthermore, there is a lack of reliable predictive models for field scale applications. Finally, microbially induced precipitation of calcite via ureolysis still leaves a high concentration of ecologically non-desirable ammonium chloride in the soil.

Another biological technique which is employed for soil improvement is the use of plant roots. The roots of vegetation (trees, shrubs, grasses) have been found to possess desirable characteristics that can contribute to slope stabilization and soil strength (Stokes *et al.*, 2007; Stokes et al., 2009). Several studies have investigated the geotechnical implications of engineering plant roots to protect soils against landslides, slope failure and erosion. For instance, finer (smaller sized) roots have been found to possess higher tensile strength and contribute more to soil aggregate formation and erosion resistance compared to larger roots which primarily enhance soil stability by providing mechanical support as anchors for soil mass (Genet et al., 2007; Nyambane and Mwea, 2011; Zhang et al., 2012; Saifuddin and Osman, 2014). The shoots of plants also play key roles in evapotranspiration and soil-water-atmospheric moderation which directly affects the hydraulic behaviour of soils, altering soil suction and its mechanical behaviour (Ng *et al.*, 2013; Ni *et al.*, 2016). Variable plant

characteristics such as anatomy, life-cycle, climatic adaptation and size of roots are significant considerations and can limit utilisation of specific plant species in ground improvement works.

Studies suggest that the mutualistic symbiosis formed between plant roots and the hyphae of arbuscular mycorrhizal (AM) fungi can influence soil hydro-mechanical properties, contributing to erosion control, for instance (Tisdall *et al.*, 2012). AM fungi enmesh and bind soil micro (and smaller macro-) aggregates into larger aggregates thus improving soil structure and physical quality (Miller and Jastrow, 1992; Rillig and Mummey, 2006), whilst also promoting plant growth, as aggregates act as stores for nutrients and water (Tidsall & Oades, 1982). The effectiveness of the hyphal networks of mycorrhizal fungi is dependent on the length of the hyphae, surface area and fungal biochemical exudates (Tisdall, Smith and Rengasamy, 1997). AMFs are obligate biotrophs, meaning that they are only able to live in a plant host and would not complete their life-cycle when there is no host (Ferrol *et al.*, 2004). This is a critical limitation if these species were to be considered for deployment in engineering applications in locations where their host plants are unavailable or are undesirable.

There are thousands of species of fungi belonging to classes other than AM fungi that may possess similar characteristics. While some of these have been isolated and studied for application in the fields of mycology, biotechnology and soil science (for aggregation purposes), so far, no study has systematically attempted to investigate the potential hydro-mechanical contributions of the hyphal networks of non-mycorrhizal (saprotrophic) filamentous fungi to soils, for the purpose of modifying soil engineering behaviour from a geotechnical engineering perspective. The overall aim of this PhD

research was to investigate for the first time the potential use of fungal hyphal networks for ground engineering applications. It is hypothesized that if suitable species of fungi with desirable characteristics for soil modification are investigated, their growth could be engineered for ground improvement either as a stand-alone technique or in conjunction with other sustainable soil improvement technologies.

Pleurotus ostreatus, a saprotrophic, non-parasitic soil fungus whose fruiting body (mushroom) is the second most cultivated globally, was selected for use in this study, after initial screening of several potentially suitable species.

1.2 Research Objectives

The specific objectives of this research were to:

1. Investigate the influence of environmental conditions (temperature, moisture content, type and organic substrate content) on growth and biomass produced by fungal mycelia (*P. ostreatus*) in soils,
2. Investigate the extent of alteration caused to the wettability (water repellency) of sand treated with *P. ostreatus*, and to investigate the persistence of these changes with time.
3. Determine the (i) soil water retention curve, (ii) infiltration behaviour and (iii) saturated hydraulic conductivity of sand treated with *P. ostreatus.*
4. Investigate the stress-strain behaviour of sand treated with *P. ostreatus* subjected to direct shear tests, and the influence of organic content.

5. Assess the erodibility characteristics of sand treated with *P. ostreatus*. Moreover, to investigate the influence of inoculation methods and growth duration on the erodibility of fungal treated sand.
6. Outline potential applications of deployment of fungal hyphal networks in ground improvement based on the growth results and hydro-mechanical behaviour evaluated for fungal-treated sand in this research programme.

For the experimental objectives 2-5 above, assessment of the behaviour of fungal treated soils was made by direct comparison with untreated control specimens. As an entirely novel area of study in the field of geotechnical engineering, this research campaign combined knowledge, skills and techniques in microbiology, mycology and geotechnical engineering. This PhD research is experimental, and at laboratory scale represents proof of concept for the potential use of fungal hyphal networks in ground improvement.

1.3 Structure of thesis

This thesis is made up of nine chapters. Chapter 2 presents a review of the literature relevant to fungal hyphal networks in soil. Chapter 3 presents the investigation into optimal environmental conditions for hyphal growth. Chapter 4 presents the investigation into the extent of water repellency induced by fungal treatment in sands. Chapter 5 continues the investigation of the hydraulic behaviour of fungal treated sands, presenting experimental procedures and results for determining soil water retention and infiltration behaviour and saturated hydraulic conductivity. Chapter 6 presents an investigation into the stress-strain behaviour of fungal-treated sands subjected to direct shear tests. Chapter 7 presents an investigation into the erodibility

of fungal-treated sand conducted using a Jet Erosion Test apparatus developed at the University of Strathclyde. Chapter 8 outlines potential areas of application of this technology in ground engineering practice as well as the limitations of this study and some recommendations for future work; and Chapter 9 presents the main conclusions of this research with a list of specific topics suggested for future research.

All references cited are presented at the end of the thesis. This is followed by Appendices referred to within the thesis.

Chapter 2

Fungal Hyphal Networks in Soil[1]

2.1 Introduction

Traditional soil mechanics and geotechnical engineering as defined at the first conference of the International Society for Soil Mechanics and Foundation Engineering (ISSMFE) in 1936, was focused strictly on the application of the principles of hydraulics and mechanics to engineering problems involving earthen materials, regardless of the presence of organic matter. Hydro-mechanical studies of soil were initially based on the assumption that soil contained only solid minerals and water or air. In engineering designs and soil analyses, engineers conservatively assumed that soils were either completely dry or completely saturated, ignoring both the possibility of soil containing more than one fluid (water and air) and soil organic constituents at the same time. However, many phenomenon, for example the swelling of expansive soils could not be appropriately evaluated by considering soil as either strictly dry or saturated (Fredlund, 1996). Thus development occurred in the methods of measurement of soil suction and characterisation of the behaviour of unsaturated soils, and theoretical frameworks to predict partially saturated soil behaviour over the last sixty years (Fredlund and Rahardjo, 1993; Tarantino and Mongiovì, 2001;

[1] *Part of the contents of this chapter form part of the following book chapter: El Mountassir, G. et al. (2018) Applications of Microbial Processes in Geotechnical Engineering. 1st edn, Advances in Applied Microbiology. 1st edn. Elsevier Inc. doi: 10.1016/bs.aambs.2018.05.001.*

Fredlund, 2006; Lu and Likos, 2006). This understanding expanded the horizons of geotechnical engineering, providing better insight into more practical soil-related engineering problems. This is because much of the world's population live in arid areas where core human activities are carried out in direct contact with unsaturated soils (the vadose zone). Changes in soil water contents from completely dry to saturated conditions and vice-versa, impacts greatly on soil hydro-mechanical behaviour (Fredlund and Rahardjo, 1993), and by extension, affects stability of geo-infrastructures. Researchers in the field of unsaturated soil mechanics have also led the way in the development of interdisciplinary collaborations, with the fields of hydrology, soil physics and chemistry. However, until recently geotechnical engineers have largely ignored any possible influence of biological effects on the hydro-mechanical behaviour of soils, preferring to view the ground as a sterile engineering material.

With increasing awareness about environmental sustainability issues like global warming and the impacts of climate change, the geotechnical community is in search of ground improvement technologies which are resilient to climate change, involve low carbon emissions, are ecologically-friendly and cheap, yet effective (Mitchell and Santamarina, 2005; Shackelford, 2005; Abreu *et al.*, 2008; Burbank *et al.*, 2013). As such researchers have begun to turn their attention to the role of biological components in soils and their potential for ground improvement over the last 15 years (Mitchell and Santamarina, 2005), with the formation of a new sub-discipline: "biogeotechnics'. El Mountassir *et al.*, (2018) gives a detailed overview of the progress so far in the application of engineered microbial activity to geotechnical engineering. It is proposed here that engineering the growth of the vegetative part of fungi in the soil, creates a

hyphal network, with an architecture similar to that of plant roots, albeit at a micro-scale and that these hyphal networks have potential for deployment in ground improvement. This is a novel concept in the domain of geotechnical engineering and it is the crux of this study.

This chapter presents from the literature a brief description of fungi, their occurrence, classification, modes of growth and the main mechanisms by which fungi can influence soil structure. This is followed by a discussion of the fungal characteristics (arising from the mechanisms presented) which give support to the proposition that fungi can modify the hydro-mechanical behaviour of soil.

2.2 Fungi & fungi-soil interaction

2.2.1 Occurrence

Fungi account for one-quarter of the biomass on earth (Miller, 1992) and they can be found in almost all habitats on earth, wherever oxygen is present; in some cases, they also thrive in the absence of oxygen. Fungi make up three-quarters of the total microbial biomass in soils (Ritz and Young, 2004). Some species of fungi are oligotrophic, that is, they are capable of living in nutrient-poor environments such as in the soils of the Dry Valleys of Antarctica, on rock surfaces in caves while other species have been found in aquatic habitats ranging from ecosystems in small streams to ocean sediments in deep-sea environments (Sterflinger, 2000; Gorbushina, 2007; Shearer *et al.*, 2007; Biddle *et al.*, 2010; Cantrell *et al.*, 2011; Nagahama and Nagano, 2012; Vanderwolf *et al.*, 2013). There is still no consensus on total fungal diversity. Several studies have reported estimates ranging from a relatively conservative 660,000

different fungal species (Mora *et al.*, 2011) to 1.5 million fungal species in the UK alone (Hawksworth, 1991, 2001). Based on more detailed studies including molecular surveys and census of single plants, fungal species are estimated to range from ~6 – 10 million species (Cannon, 1997; O'Brien *et al.*, 2005; Taylor *et al.*, 2013). This shows the richness of fungal diversity globally. However, there are about 99,000 known fungal species, of which only ~10% are known to be pathogenic and/or parasitic to humans, animals and plants (Carris, Little and Stiles, 2012).

2.2.2 Classification

The classification of fungi into phyla, historically considered to include Ascomycota, Basidiomycota, Chytridiomycota and Zygomycota (e.g. Webster and Weber, 2007) is continuing to change as research provides more evidence for further differentiation and expansion of the kingdom (introduction of Glomeromycota and Microsporidia phyla). The Glomeromycota phyla typically form mutualist symbiotic relationships with a significant number of plants as endomycorrhizas or arbuscular mycorrhizal fungi (AMF) and play a major role in terrestrial ecosystems (Smith and Read, 2008). About 98% of the fungal species that have been well described to date currently belong to the sub-kingdom Dikarya, which comprise of Ascomycota and Basidiomycota (Stajich *et al.*, 2009). Ascomycota have been extensively studied and comprise mycorrhizals and saprotrophs, and are commonly found in symbiotic relationships with algae and cyanobacteria (lichens) or as plant parasites (Bindschedler, Cailleau and Verrecchia, 2016). Basidiomycota are typically the mushroom forming fungi, although some species are ectomycorrhizals which are in mutualistic symbiosis with plants while others are saprotrophic and parasitic (Smith and Read, 2008).

Saprotrophic species of the Dikarya sub-kingdom have been described as significant for global biogeochemical cycles as they commonly explore resources from varied ecological niches ranging from environments with rich organic substrates to extreme oligotrophic (nutrient poor) environments (Bindschedler, Cailleau and Verrecchia, 2016).

2.2.3 Morphology, nutrition and growth

Fungi are capable of both sexual and asexual reproduction, producing numerous types of spores used for dispersion and protection in unfavourable conditions (Moore, Robson and Trinci, 2013). Morphologically, fungi can be unicellular (*e.g.* yeasts) or multicellular – exhibiting filamentous growth through the development of hyphae (Fig. 2-1). Hyphae are elongated, tubular structures, consisting of an outer cell wall made up of polysaccharides (chitin) which resists the internal tugor pressure exerted by the protoplast, thereby maintaining its shape and rigidity (Klein and Paschke, 2004; Deacon, 2007). Hyphae possess high mechanical resistance due to their structural configuration such that some species are capable of boring through rocks and hard mineral substrates (Jongmans *et al.*, 1997; Van Schöll *et al.*, 2008). Hyphal diameters range between 1 – 30 µm and lengths can span from several microns to several metres (Islam *et al.*, 2018). Hypha of filamentous fungi can branch into multiple hyphae (Fig. 2-1a) and can anastomose, creating a massive three-dimensional vegetative structure called the mycelium (Fig. 2-1b). It has been reported that hyphae can form into a densely packed hardened mass, which is called sclerotia, which serves as a long term food reserve to help the fungus survive extreme conditions (Money, 2015).

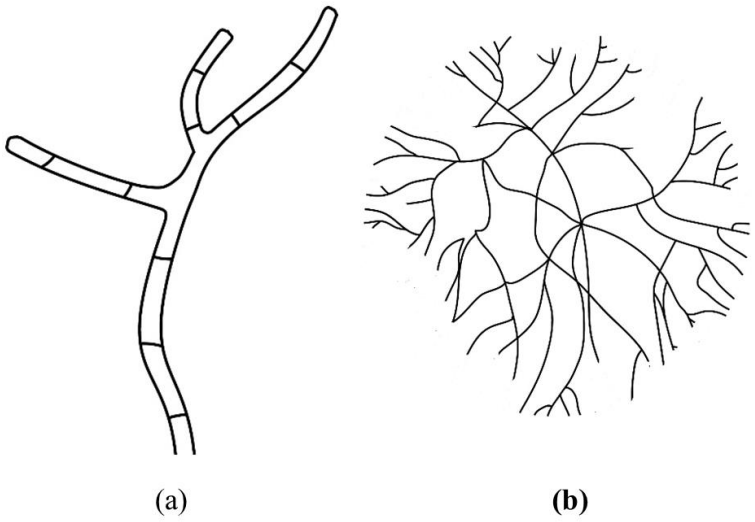


Figure 2-1:(a) structure of a filamentous hypha with sub-apical branching. (b) 2-dimensional mycelium with multiple branching hyphal network

Based on their mode of nutrition, fungi can generally be considered under the following categories: (i) saprotrophic (i.e., decomposers) – these fungi digest dead organic matter; (ii) pathogenic or parasitic fungi - they colonize their hosts, which may be plants or other organisms, and cause diseases in the process; and (iii) fungi which exist in symbiotic relationships: with plants as mycorrhizal fungi (ectomycorrhizal and arbuscular mycorrhizal) forming a mutually beneficial relationship increasing plant uptake of nutrients and water (e.g., nitrogen and phosphorus) and protecting against soil pathogens; and with algae and cyanobacteria as lichens (Jeffries *et al.*, 2003; Konhauser, 2007; Hoorman, 2011).

Nutrients for metabolic activity are absorbed and transported to fungal cells after substrates are pre-digested by exudates which are secreted by the fungus into the environment as hyphae develops. This process is directly linked to hyphal growth and extension. Hyphae exhibit apical growth (i.e. growth at hyphal tips) (Fig 2-1a). New apices (sub-apices) emerge with each branching hyphae. In general, as cell walls are formed using absorbed nutrients, they thicken overtime and become rigid, but the

hyphal tips remain flexible and capable of expansion. During metabolic activity, the cytoplasm is mobilised from 'older' parts of the hypha to the flexible tips by the action of the spitzenkörper (a collection of small vesicles which act as the organising centre for growth), resulting in the extension of the tip. (Gooday, 1995; Bartnicki-Garcia *et al.*, 2000; Money, 2008).

While older hyphae differentiate and begin sexual development and asexual spore production, the actively growing part of the hyphae explores its environment by penetrating potential substrates for nutrient acquisition, leading to exponential growth, as massive sub-apical branching networks are formed (see Fig. 2-2a). The hyphal-network structure provides an intimate linkage for facilitating resource transmission across the entire architecture of the fungus, thereby creating the unique resilient behaviour of fungi which enables them to survive poor environmental conditions, paucity of resources, competition, predation and even damage, in ways that are different from bacteria and other multicellular plants or animals (Heaton *et al.*, 2012; Fricker *et al.*, 2017). The explorative growth process of fungal hyphae can result in damage or rupturing of hyphal cell walls and sometimes even the cytoplasm of the hypha might leak out completely resulting in total inactivity or cell death (Plamann, 2009; Tegelaar and Wösten, 2017). Hyphae of filamentous fungi species are often compartmentalised by cross-walls or septa; and in some species, there exists perforations or septal pores to allow inter-compartmental flow of cytoplasm.

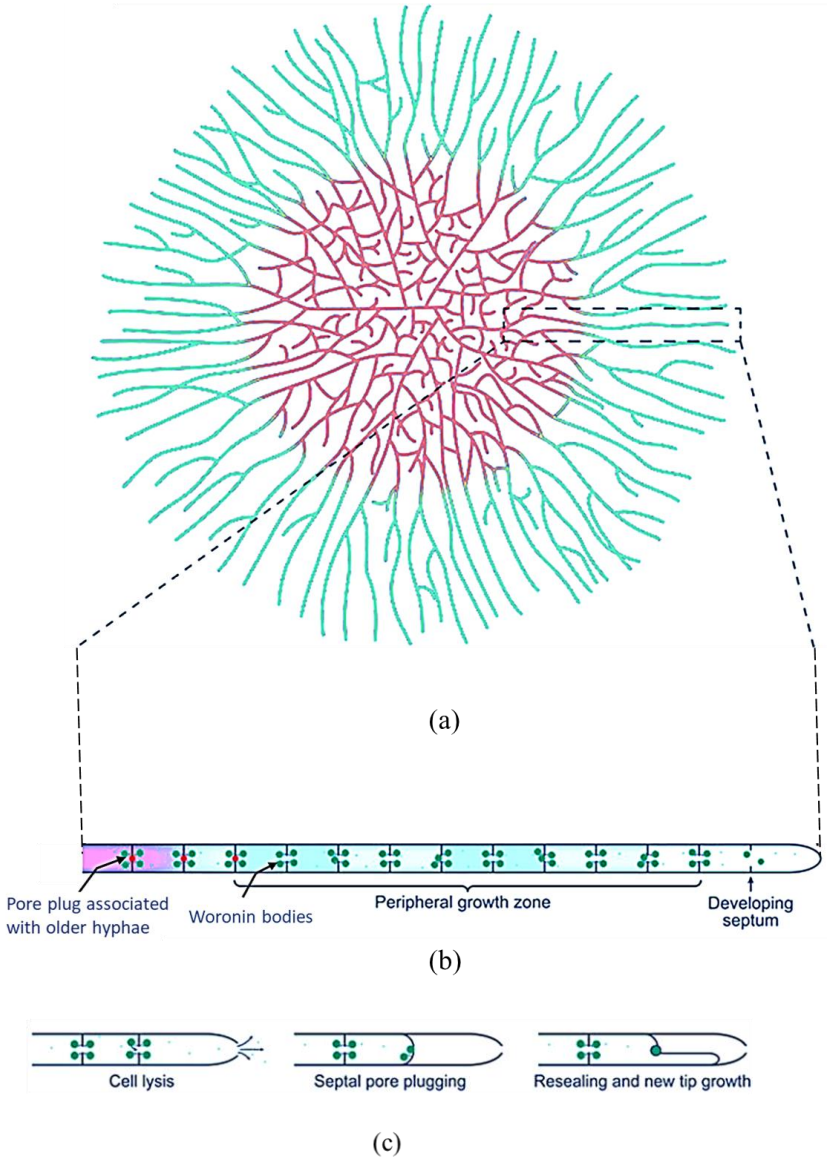


Figure 2-2: (a) Mycelium with actively growing part (green) and 'older' hyphae (red) (b) Magnified hypha showing septa with Woronin bodies and septa plugs for older part of hypha. (c) Shows a ruptured cell and the Woronin bodies being deployed to plug the adjoining septa while new tip emerges to continue hyphal growth process (Jedd and Pieuchot, 2012).

To systematically manage the risk of damage to hyphal cell walls, species of the Ascomycota division contain Woronin bodies (i.e. protein organelles found around the septal pores) to plug the pores when required (Markham and Annette, 1987; Jedd and Pieuchot, 2012). These septa plugs (Woronin bodies) are activated as an 'emergency response' to cellular wounding or loss of cytoplasm, they prevent hyphal desiccation, ensure tugor pressure is maintained and facilitate continued hyphal growth (Plamann,

2009; Tegelaar and Wösten, 2017). Basidiomycetes fungi possess septal pore caps (SPC) which are localised in their septal pore and are essentially equivalent to Woronin bodies (Müller *et al.,* 1998). There exists another type of pore plug (shown as red dots in Fig. 2-2b) - although details about this plug is not fully established in literature – which is associated with older hyphae, and is functional in the differentiation of cells and their subsequent sexual development and asexual spores production (Jedd and Pieuchot, 2012). There are rare cases where older hyphae in soils are left empty, due to rupture/wounding or movement of cytoplasm to apical/subapical tips. While this could potentially halt overall growth, it is believed that Woronin bodies close to apical tips are more efficient, offering significant resilience to hyphae and strategic ability to cope with stress arising from loss of cytoplasm in other compartments. Nonetheless, where the spitzenkörper is partially or fully disintegrated, growth may be halted even though a sufficient amount of cytoplasmic fluid may still be retained within the hyphae (Tegelaar and Wösten, 2017). These damage response mechanisms of hyphae are potentially significant for applications involving engineered fungal growth, such that even if damage were to occur, hyphae are in effect self-healing in the ground, thereby ensuring durability of the mycelium.

2.3 Interactions between fungal-hyphae and soils

Fungi are known to play an important role in soil aggregation, both in the formation of aggregates and in maintaining aggregate stability (Lynch and Bragg, 1985; Rillig and Mummey, 2006). From an agricultural perspective soil aggregate stability is important for maintaining transport of air, water and nutrients within the soil. From a geotechnical engineering perspective, the aggregation of soils influences their

hydraulic behaviour (i.e. permeability and water retention capability (e.g. Juang and Holtz, 1986; Barbour, 1998; Vanapalli, Fredlund and Pufahl, 1999) and their mechanical behaviour (Barden and Sides, 1970; Alonso, Gens and Whight, 1987). Although it is widely acknowledged that aggregated soils are encountered within geotechnical engineering (e.g. Collins and McGown, 1974; Alonso, Gens and Whight, 1987) little, if any, consideration has been given to the role of microorganisms in the formation or stability of aggregates in this context.

Studies by soil and agricultural scientists have observed increased size of aggregates formed in soils inoculated with fungi and enhanced resistance to breakdown upon wetting, for a range of different fungal species including mycorrhizal and saprotrophic species (e.g. Tisdall and Oades, 1979, 1982; Degens, Spading and Abbott, 1996; Caesar-TonThat and Cochran, 2000; Caesar-Tonthat, 2002; Peng, Guo and Liu, 2013). Rillig and Mummey, (2006) outline three categories of mechanisms by which fungi (focused on arbuscular mycorrhizal fungi, AMF) can contribute to soil aggregate stability (Fig. 2-3): (i) Biophysical, (ii) Biochemical and (iii) Biological mechanisms.

2.3.1 Biophysical mechanisms

The biophysical influence of fungal hyphae is similar to the action of plant roots (although at a smaller scale) where hyphae act to enmesh and entangle soil particles, binding micro-aggregates together (Tisdall and Oades, 1982).

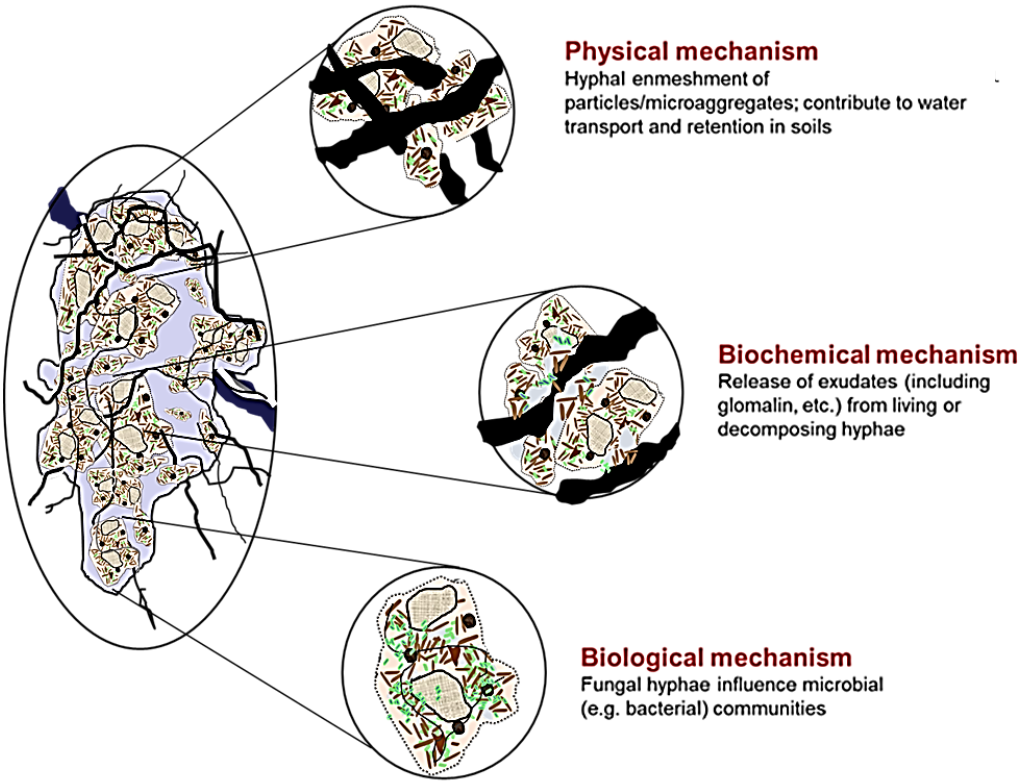


Figure 2-3: Mechanisms by which fungal hyphae contribute to soil aggregation (after Rillig and Mummey, 2006)

The effects of plant roots are well-studied, they bind soil particles and aggregates together providing an additional apparent cohesion against shearing (Stokes *et al.*, 2009). The level of reinforcement provided is dependent on root tensile strength and root architecture (e.g. root diameter, root length density). Greater shearing resistance is provided by many smaller diameter roots than by a smaller number of larger diameter roots, where the fraction of the soil plane occupied by the plant roots is the same (Stokes *et al.*, 2009). By drawing similarities with plant root reinforcement literature, the mechanism by which fungal hyphae bind particles and aggregates might also be expected to depend on the morphological properties of the fungal networks (e.g. hyphae diameter, density, and interconnectivity) and the tensile strength of the different strains of fungal hyphae (Rillig and Mummey, 2006). However, little is

known of how these properties vary between different species and strains. Hyphae may also be hypothesised to contribute to water transport and retention in soils, ultimately inducing wetting and drying cycles on a localised-scale (Rillig and Mummey, 2006) which may influence binding of soil particles to hyphae and influence mechanical behaviour of micro-aggregates; these effects remain largely unexplored. Additionally, the growth of fungal hyphae has been observed to influence soil structure by aligning clay particles along hyphae, due to the stress exerted on soil particles during growth, possibly even forming micro-aggregates (Rillig and Mummey, 2006).

In terms of synthetic fibres, it has been widely reported in geotechnical engineering that the addition of fibres increases soil strength (i.e. compressive, shear or tensile strength at failure) and increases strain to failure (i.e. increased ductile behaviour) (e.g. Ranjan, Vasan and Charan, 1996; Santoni, Tingle and Webster, 2001; Michalowski and Čermák, 2003). The reinforcing effect increases with increasing fibre content (up to a limit) and increasing aspect ratio (length/diameter) (e.g. Michalowski and Čermák, 2003). Fungal hyphae can be considered to be micro-scale roots with a very high aspect ratio. Furthermore, unlike synthetic fibres fungal hyphae may also exhibit anastomosis forming complex interconnected three-dimensional networks with further potential for entanglement and enmeshment of soil particles and aggregates.

2.3.2 Biochemical mechanisms

Soil aggregate formation and stability are also influenced by biochemical processes. Fungal hyphae are known to secrete biochemical products into their surroundings (exudates), as well as containing products in their hyphal walls, that may after

decomposition persist in the soil (Rillig and Mummey, 2006). Chenu, (1989) demonstrated that scleroglucan (a fungal polysaccharide) improved the stability of kaolinite and montmorillonite aggregates, and increased clay porosity. Glomalin-related soil protein has been correlated with soil aggregate stability for AMF amended soils (e.g. Wright and Upadhyaya, 1996, 1998; Rillig, 2004) and is thought to act as a 'glue-like' substance. Studies by Caesar-TonThat and Cochran, (2000) and Caesar-Tonthat, (2002) on a saprotrophic species highlighted the importance of insoluble extracellular polysaccharide compounds on the water stability of aggregates amended with the fungus. Comparing aggregate stability for soils inoculated with fungi with those inoculated with liquid media in which the microorganisms were grown, demonstrated that the binding agents remain in close association with the hyphae and are not excreted into the liquid/soil media (Aspiras *et al.*, 1971).

Filamentous or mycelia-forming fungi such as those belonging to the Ascomycota or Basidiomycota phyla are also known to secrete proteins called hydrophobins (Wessels *et al.*, 1991; Wessels, 1996). Hydrophobins play varied roles in the functional processes that occur throughout the growth and life cycle of fungi including, modification of environmental conditions to allow sporulation and aerial hyphae formation (Wessels, 1996; Wösten *et al.*, 1999; Van Wetter, Wösten and Wessels, 2000), mediation of hyphal attachment to surfaces, substrate colonisation (Wösten, Schuren and Wessels, 1994; Temple *et al.*, 1997) and involvement in the production of fruiting bodies (Lugones *et al.*, 1999). Hydrophobins self-assemble at surficial interfaces forming amphipathic (or amphiphilic) layers capable of altering surface wettability. Given the role of hydrophobins in aiding fungal hyphae attachment to

surfaces, and the role in altering surface properties, it is envisaged that these proteins may also play a role in soil aggregation (Rillig and Mummey, 2006).

2.3.3 Biological mechanisms

Finally, in terms of biological mechanisms, fungi may influence the location and density of microbial populations in the soil, for example exudates may act as substrates for bacterial growth, which could also impact on the formation or stability of soil aggregates (Rillig and Mummey, 2006).

The extent of the role played by each mechanism within a given soil will be highly dependent on the fungal type and species (or indeed community as a whole) and the soil composition, grain size and pore size distribution. For example, Aspiras *et al.*, (1971) demonstrated by sonicating fungal inoculated aggregates, that aggregate stability was not greatly reduced, despite the hyphal network being disrupted, concluding that the role of binding substances, (mainly polysaccharides) is more important than the physical entangling effect of the hyphae for clayey soils (where clay content was >25%). Whereas Degens, Spading and Abbott, (1996) demonstrated for sandy soils that aggregation could be attributed to increases in hyphal length, with hyphae observed via Scanning Electron Microscopy to cross-link sand grains together via short hyphal lengths. Furthermore, Degens, Spading and Abbott, (1996) observed no difference between the hot-water extractable carbohydrate carbon content of aggregated and non-aggregated soils, indicating that microbial polysaccharides were not in this case the dominant mechanism controlling aggregation. What is not yet clear

is how aggregations on a local scale, formed or maintained stable via fungal activity, may influence the bulk hydraulic and mechanical behaviour of soil.

2.4 Use of fungi in ground engineering

Fungi are ubiquitous in soils and the observations of fungi soil-interactions outlined above support the proposal that fungal growth could indeed be engineered for geotechnical engineering applications. To date, the use of fungi for soil improvement applications has been largely limited to the combined study of plant-mycorrhizal systems (Jeffries *et al.*, 2003; Graf and Frei, 2013; Mardhiah *et al.*, 2016a), in eco-engineering studies. The introduction of mycorrhizal fungi has mainly been considered as a means to enhance plant growth for successful re-vegetation of degraded soil systems following erosion, landslide or desertification (Requena *et al.*, 2001; Caravaca *et al.*, 2003). The presence of mycorrhizal fungi promotes the formation and stability of aggregates acting as stores for nutrients and water for plant growth (Tisdall and Oades, 1982), thus accelerating and aiding plant colonisation (Jeffries *et al.*, 2003; Graf and Frei, 2013; Peng, Guo and Liu, 2013). Furthermore, mycorrhizal have been shown to increase root production, root length density and for some species even enhance plant root tensile strength (Stokes *et al.*, 2009). Peng, Guo and Liu, (2013) demonstrated that independent of the involvement of plant roots, hyphal networks have a positive impact on the stability of soil aggregates. The mechanisms by which arbuscular mycorrhizal fungi may influence soil aggregations are expected to be similar for other types of fungi (Rillig and Mummey, 2006). Furthermore, considering that binding substances are known to be closely associated with hyphal surfaces for a range of fungal types (Aspiras *et al.*, 1971), it is proposed

that other fungal species could by themselves also be considered for soil improvement applications, for example to enhance resistance against water or wind-induced erosion (Tisdall *et al.*, 2012; Mardhiah *et al.*, 2016a).

2.5 Conclusion

Given the vast number of different fungal species and variations in their behaviour there is huge scope for their deployment in geotechnical engineering. It is envisaged that ground improvement technologies incorporating fungi could be relatively cheap given that treatment of soil surfaces could be conducted in a relatively easy manner over potentially large areas.

The use of fungal hyphal networks in ground improvement is a new avenue of research within biogeotechnics, with many research questions to be addressed. To begin to investigate the feasibility and limitations of their deployment from an engineering perspective, a better understanding of the possible changes to soil behaviour that can be induced by fungal inoculation is needed for a range of fungal species. This thesis is a first step in this direction and focuses on gaining an in-depth understanding of the growth behaviour of *P. ostreatus* in sand and the influence of this growth on the hydro-mechanical behaviour of sand.

Chapter 3

Optimisation of Environmental Conditions for Growth of *P. Ostreatus* Towards Applications in Soil Improvement

Abstract

Fungal hyphal/mycelial networks have been proposed as a potential low cost, low carbon and environmentally friendly soft geotechnical engineering technique for soil improvement. Being a novel idea in the area of biogeotechnical engineering, several knowledge gaps exist that must be addressed. One of these is the growth requirements of identified fungal species in soils. This study was undertaken to contribute to this understanding by assessing the influence of selected environmental factors on the growth of the fungus *Pleurotus ostreatus* (*P. ostreatus*) in soil, and determining specific ranges of factors required for optimum mycelium growth and biomass production. Preliminary tests to observe the influence of temperature, moisture content and organic substrate on fungal growth were carried out on soils inoculated with *P. ostreatus* in petri-dishes. Results showed that *P. ostreatus* can grow in sands at temperatures between 5 - 30°C, moisture contents of 3.1 – 66.7% and amended with 1 – 15% of respective organic substrates (spent coffee grounds, lignocel or guar gum). This initial assessment provided information for optimality tests using the Taguchi design of experiments coupled with Grey Relational Analysis. The radius of mycelium growth was determined by (1) imaging of hyphae stained with fluorescein di-acetate on the 6th day after inoculation of sterilised sandy soil with *P. ostreatus*, while dry

fungal biomass was determined, via ergosterol analysis, on the 6th, 18th and 24th day after inoculation. Optimal environmental conditions for radius of mycelium growth and/or fungal biomass on 6th day after inoculation were found to be 25°C, 5% moisture content and 3% guar gum. For biomass production up to 24 days, the optimal conditions were: 20°C, 10% moisture content and 1% spent coffee grounds. Results show that mycelia of *P. ostreatus* can grow in a wide range of environmental conditions. Recipes for laboratory based growth of mycelia of *P. ostreatus* can be developed based on information from this study. Such soil colonised mycelia grown based on this recipe can be used for further laboratory-based studies towards soil improvement applications or packaged as inoculants for deployment of fungi to treatment sites by techniques such as hydro-seeding or broadcast seeding. This study is a first key step in the understanding of requirements for fungal growth in soil towards the engineering of fungal hyphal/mycelial networks for soil improvement.

3.1 Introduction

Recently El Mountassir *et al.* (2018) proposed a novel technique involving the engineering of fungal hyphal/mycelial networks for soil improvement. Naturally occurring fungus (specifically, the mycorrhizal fungi species which form mutualistic symbiosis with plants) are known to enhance the formation and maintenance of stable soil aggregates via three main mechanisms: biophysical, biochemical and biological (Rillig and Mummey, 2006). It is proposed that these mechanisms can be stimulated by engineering fungal growth in soils in order to modify the hydraulic and mechanical behaviour of soils. The extent to which fungal growth may influence bulk engineering soil behaviour will depend on the amount/nature of growth obtained and the activity of the microorganisms with time. Furthermore, the growth and associated impacts of different fungal species are likely to be highly variable. Besides mycorrhizal fungi, there are other fungal species which are ubiquitous in soils, like the filamentous saprotrophs of the Ascomycota and Basidiomycota fungal divisions. These species do not require any living hosts for their growth and survival in soils, and some of them are neither parasitic to plants nor pathogenic to humans, making them potentially more preferable for sustainable deployment in soil improvement works.

In order to assess the potential of fungal-mediated soil improvement using saprotrophic species, the following areas need to be investigated: i) how environmental conditions influence the growth of specific fungi of interest for soils, in terms of growth rate and biomass produced over time, ii) the influence of specific fungi on soil bulk hydraulic and mechanical behaviour and iii) strategies for

deployment and engineering of the growth of suitable density of mycelia networks at field scale. This chapter addresses item i) for the fungal species *P. ostreatus*.

3.1.1 Growth of *P. ostreatus* in soil

P. ostreatus is a unicellular saprotrophic fungus species of the Basiodiomycota division. Its fruiting body, commonly called the 'oyster mushroom', is the second most cultivated edible mushroom in the world (Kues and Liu, 2000; Yamanaka, 2005; Rühl, Fischer and Kües, 2008). *P. ostreatus* has been selected for study as it is a cord-forming fungi, it is easy to cultivate in the laboratory, it grows from dispersed spores, and can form massive three-dimensional mycelia networks as its hyphae undergo anastomosis (Heaton *et al.*, 2012). *P. ostreatus* has been widely studied for a range of applications such as: production of its fruiting body - mushroom as food (Sánchez, 2010; Chae and Ahn, 2013; Hoa and Wang, 2015), production of laccase, peroxidases and ligninolytic enzymes relevant for solid waste fermentation and applications in biotechnological, brewing and food industries (Nyochembeng, Beyl and Pacumbaba, 2005; Mikiashvili *et al.*, 2006; Rühl, Fischer and Kües, 2008; Patel, Gupte and Gupte, 2009) as well as for bioremediation of contaminated soils (Purnomo *et al.*, 2010; Zebulun, Isikhuemhen and Inyang, 2011; Mohammadi-Sichani *et al.*, 2018). However, there are no studies involving growth of the hyphae/mycelia of this species of fungus for the purpose of improving soil strength or modifying soil hydraulic properties. To inform test methods and achieve the objectives of this work, below some studies are reviewed which investigate the influence of the growth of fungal hyphae of other saprotrophic species on soil properties, as well as hyphal/mycelial growth requirements in soils.

a) Influence of fungal hyphae growth on soil structure

Some authors have studied fungal behaviour in soils using other filamentous fungi species to investigate various soil properties that may be relevant for soil bio-engineering as proposed in this study. These include, the spatial distribution of fungal hyphae and colonisation of soils with respect to changes in soil structural characteristics like porosity, permeability, bulk density and water content of soils (Harris *et al.*, 2003; Otten and Grinev, 2008; Falconer *et al.*, 2012; Kravchenko *et al.*, 2016). Using a facultative parasite and air borne pathogen, *Rhizoctonia solani*, in an arable sandy loam soil, Harris *et al.*, (2003) found that there was more hyphal growth at lower soil bulk densities and in areas with higher porosities. This was further supported by findings from Otten *et al.*, (1999, 2001, 2004) who studied the same fungus and found greater and faster hyphal growth occurring in soils with greater macroporosity (pore size > 75 μm), while the converse was true for soils with higher bulk densities or containing fine particles densely packed together in the form of smaller aggregates. Furthermore, Falconer *et al.*, (2012) combined a model for liquid distribution in an unsaturated system with a model for fungal growth dynamics to study how the dynamics of water distribution and pore air in a sandy loam soil changes with fungal growth; their results showed that for fungal growth, the soil pore-size distribution had a greater effect on the water distribution in the soils than the porosity. Based on measurements of functional fungal biomass, they observed that the colonisation efficiency of fungi decreased with increasing soil water content; and that, this may have influenced the hyphal morphology, resulting in the growth of hyphae around water droplets as observed in one of the soil samples they investigated. Although their study aim and approach were epidemiological with reference to the

prevalence of the plant pathogen *R. solani* in agriculture, nevertheless, the outcomes provide some basic information that could be considered in the early stages of understanding conditions like soil water content required for fungal growth.

b) Environmental conditions relevant for fungal growth

Donnelly and Boddy, (1997) investigated the influence of temperature and water potential on mycelia systems of two basidiomycetes decomposers of woodland (*Stropharia caerulea* and *Phanerochaete velutina*) and found that changes in environmental conditions affected both the mycelia extent and biomass of the species investigated. A temperature of 25°C was found to be most suitable for the growth of both fungal species; while *S. caerulea* recorded better growth at suction values above 0.02 MPa, *P. velutina* was sensitive to suction > 1.3 MPa. Similar studies have been conducted by mycologists who are interested in the growth characteristics, interactions and behaviour of naturally occurring saprotrophic fungal species in woodland ecology (Donnelly and Boddy, 1998; Fricker, Bebber and Boddy, 2008; Hiscox *et al.*, 2016). Findings suggest that the growth of and biomass production by saprotrophic fungal hyphae/mycelia is sensitive to the prevailing environmental conditions like soil type and structure (Harris *et al.*, 2003; Ritz and Young, 2004), temperature (Donnelly and Boddy, 1997; Hiscox *et al.*, 2016), moisture conditions (Mihail, 2002; Ritz and Young, 2004), type and distribution of organic matter (Stack, Kenerley and Pettit, 1987; Donnelly and Boddy, 1998), as well as the presence of other microbial species or community (Worrich *et al.*, 2017). Based on these and lab-based studies of fungal growth in commercially available growth-media, as reported in literature, the key environmental factors affecting growth rate and biomass production for hyphae or

mycelia of *P. ostreatus* include: temperature, moisture content, type/amount/constituents of nutrients (substrates), availability of oxygen and pH (Frey, Elliott and Paustian, 1999; Hoa and Wang, 2015; Hoa, Wang and Wang, 2015). Most studies have grown *Pleurotus* species in various culture media (e.g. potatoe dextrose, malt extract, yeast extract) and investigated the significance of nutrient sources and optimal conditions for production of spawn and/or oxidative enzymes for application in food, medicinal and biotechnological industries (Mikiashvili *et al.*, 2006; Hoa and Wang, 2015). In general, it has been shown that the most suitable conditions for growth of the mycelia of *P. ostreatus* in spawns and culture media are temperature ranges of 24 – 32°C (Neelam, Chennupati and Singh, 2013; Hoa and Wang, 2015) and substrates or nutrient sources containing higher lignocellulose contents with carbon to nitrogen ratios (C/N) often > 20 (Alborés *et al.*, 2006; Patil *et al.*, 2010; Badu, Twumasi and Boadi, 2011). Significantly lower, or in some cases zero nitrogen contents favoured mycelia production while higher nitrogen contents were preferable for mushroom production (Alborés *et al.*, 2006; Hoa, Wang and Wang, 2015; Siqueira *et al.*, 2016; Cabrera, 2018). Some of the locally sourced substrates investigated for their suitability for use as spawn to grow mushrooms of *P. ostreatus* include: rice straw, wheat straw, saw dust, coffee grounds, soybean, and several other agro-industrial wastes (Shah, Ashraf and Ishtiaq Ch., 2004; Patil *et al.*, 2010; Badu, Twumasi and Boadi, 2011; Das *et al.*, 2015; Cabrera, 2018). To the best of the author's knowledge, there has been no reported results on the investigation of the influence of these or similar conditions and substrates on the growth rate and biomass of *P. ostreatus* grown in soils. Hence there is a need to investigate systematically, exploring suitable ranges and determining optimal environmental conditions for the growth of

P. ostreatus in soil, mimicking variable field conditions, for the aim of engineering its growth for soil improvement for geotechnical applications. The influence of variable soil pH on the growth of *P. ostreatus* has not been investigated in the study presented here, although in a preliminary experiment set up to determine the changes in the pH of sterile sandy soil (amended with lignocellulose) due to growth of *P. ostreatus* across 14 days, it was observed that there was minimal variation throughout the period, as average pH obtained was 5.9 and did not differ significantly from the pH of untreated controls.

The aim of this chapter is to investigate the influence of environmental conditions on the growth of *P. ostreatus* grown in soil which could then be used for the ultimate purpose of engineering mycelia growth for soil improvement. Specifically, this chapter seeks to determine optimal growth conditions in terms of temperature, moisture content and organic substrates for mycelium growth and biomass production of *P. ostreatus* in sands.

3.2 Methodology

3.2.1 Experimental design and set up

A preliminary experiment was initially performed to observe the effects of varied conditions on the growth of fungal mycelium and to provide information on most suitable ranges. Thereafter, selected ranges of these conditions were tested for optimality, following a Taguchi orthogonal design of experiments and grey relational analysis. This experiment was conducted in order to determine optimal environmental conditions for i) radius of mycelium growth at 6 days after inoculation (6DAI); ii)

fungal dry biomass at 6DAI; iii) combined radius of mycelium growth and fungal dry biomass at 6DAI and iv) fungal dry biomass over an extended period (up to 24 days). A chart describing the experimental plan carried out is presented in Fig. 3-1.

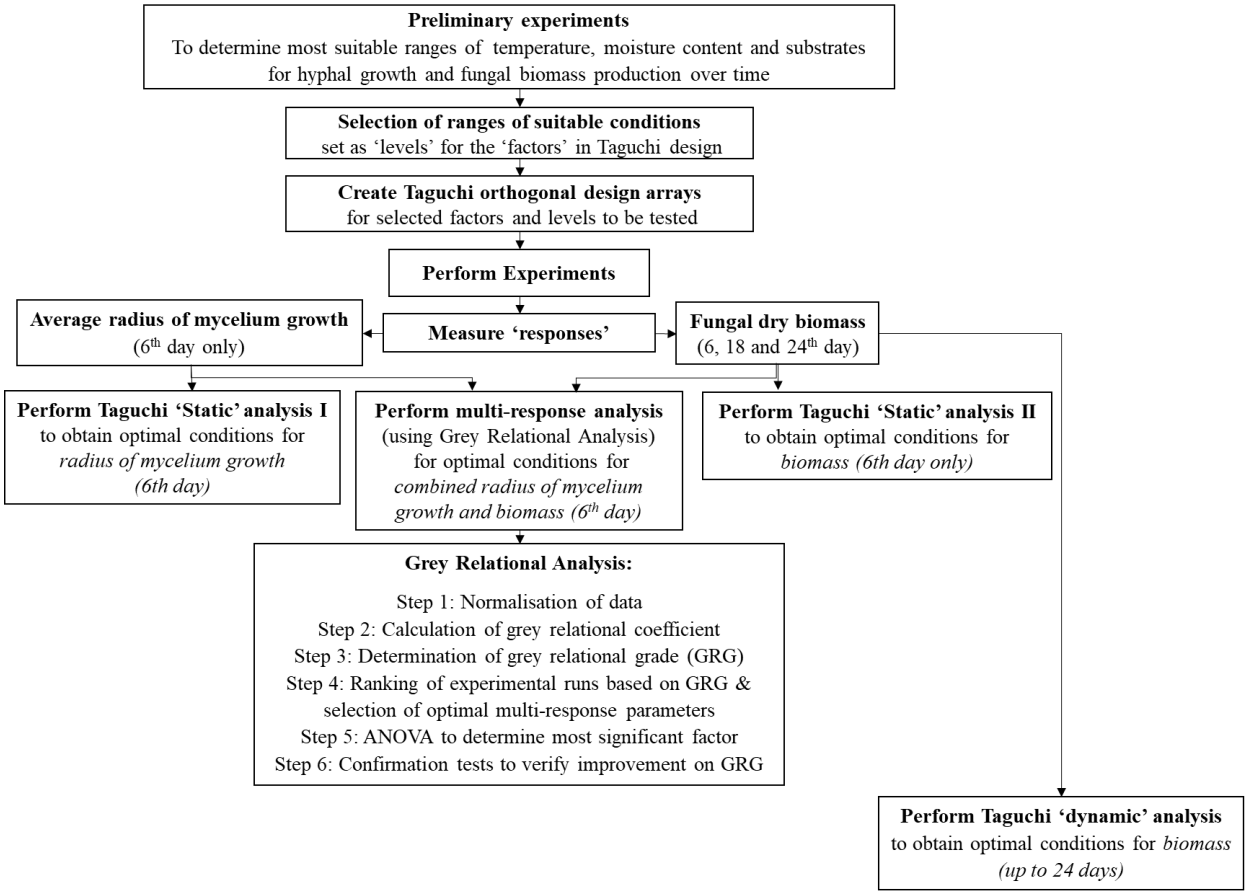


Figure 3-1: Chart showing experimental plan and layout

3.2.2 Materials

a) Soil

Kiln dried uniformly graded industrial fine silica sand was used for this study. The particle size ranged from 0.075 – 0.45 mm, with D_{60} = 0.33 mm, D_{30} = 0.38 mm and D_{10} = 0.2 mm. The sand was sterilised prior to inoculation to study the growth of *P. ostreatus* in the absence of other soil microorganisms. As this is the first set of

experiments investigating the growth of *P. ostreatus* in soils for soil improvement purposes, sand was chosen as the soil to study for fungal growth due to its high porosity, to ensure adequate aeration for fungal growth and also to assist with imaging of fungal growth. Furthermore, sand was selected to minimise fungal-soil particle biochemical interactions.

b) Organic substrates

Three organic materials (lignocellulose, spent ground coffee, guar gum) were used as nutrient amendments for growth of the fungus in the soil. These were each added to the sand in different proportions for respective treatments as described in the experimental details in section 2.3 below. **Lignocellulose (Lignocel®, LIG):** particles of natural wood fibres (size 0.5mm – 1mm; Grade HB 500 – 1000) obtained from J. RETTENMAIER & SÖHNE GmbH containing lignin, cellulose and hemicellulose. The Carbon to Nitrogen ratio (C/N) of LIG is ~450:1 and its elemental/biochemical composition are typical of decaying organic matter in natural soils with higher lignocellulosic contents (Imran *et al.,* 2016), hence its choice for this study. **Spent coffee ground (SCG)**: by-products from brewing of coffee was obtained from *Nourish*®, the University of Strathclyde catering services. SCG typically has a C/N of between 17:1 to 20:1 (Ballesteros, Teixeira and Mussatto, 2014; Campos-Vega *et al.,* 2015). SCG was selected in this study since it is readily available as a 'waste' material and could be a low cost substrate for growth of fungus. **Guar gum (GG) powder**: supplied from Intralabs, Plymouth-UK; this is a pure and natural powder processed from guar beans and used as thickener in the food and medical industry and as an environmentally friendly biopolymer for strengthening soils (Viswanath *et al.*,

2017; Dehghan *et al.*, 2019). GG powder is a polysaccharide compound that forms gel in water (hydrocolloid or 'hydrogel') and was chosen here for growth of *P. ostreatus* to test the fungal growth in a non-lignin based substrate.

c) P. ostreatus

The fungus species: *Pleurotus ostreatus*, strain Jacquin ex Fr. Kummer, supplied from DSMZ (DSM No. 3344), Germany was used.

d) Preparation of inocula

Appendix A details a preliminary experiment investigating a range of inoculants and inoculation methods for use in this PhD research. In this chapter, colonised beech wood was selected as the inoculant and they were prepared based on the method by Donnelly and Boddy, (1998) with slight modifications, based on outcomes of preliminary tests specific to *P. ostreatus*. Cubes of pre-cut beech wood ($1cm^3$ for preliminary tests, 0.5 x 0.5 x 0.5 cm^3 for other tests) supplied from *Timbercut4u UK* were frozen at -20°C until needed for the tests. The required number of the beech blocks were first defrosted by soaking them in deionised water overnight, and were then autoclaved at 121°C for 20mins. These were then aseptically placed in 500 mL conical flasks containing 7-day old cultures of *P. ostreatus* grown on malt extract agar, and then incubated at 25°C for colonisation of the blocks in the dark for 14 days. The inoculum was obtained by taking out a colonised beech block and scraping off any excess mycelium that was attached to it.

e) Preparation of specimens

Sand, LIG, SCG and GG used in this study were autoclaved at 121°C for 20mins. Specimens were prepared using 20 g of dry sand mixed with varying amounts of substrate and water. The mixture was then placed in a 90 mm diameter petri dish and gently compacted to provide a level surface. Proportions of organic substrate and water were dependent on the experiment and are specified in sections 3.2.3 and 3.2.4 below. In all tests performed, specimens were inoculated by placing a single inoculum centrally on the soil surface in the petri dish. Untreated controls were prepared in a similar manner but not inoculated.

3.2.3 Preliminary experiments

Preliminary tests were performed to investigate the influence of each environmental factor on mycelia growth. The three factors considered were: temperature, moisture content and organic substrate (LIG, SCG or GG). Specimens for the preliminary tests were prepared by keeping two factors fixed and varying the third one. For each specimen in all tests, sand formed 85% of total specimen mass.

Firstly, to observe the effect of temperature on the growth of mycelia, LIG (5% of total specimen mass) was used as the substrate and each specimen was made up to constant moisture content of 11.1%, where water content, w (%) is defined as the (mass of water/mass of solids) x 100. Specimens were prepared in triplicates with each set incubated at 7 different temperatures ranging from 5 - 35°C (with increments of 5°C).

A second set of specimens were composed of 5% LIG and were all incubated at a fixed temperature of 25°C to observe changes due to variable moisture content. In this case,

specimens were made up to moisture contents of 0, 1, 3.1, 5.3, 11.1, 17.7, 25, 42.9, 66.7 and 100%, going from completely dry to beyond saturated (or inundated) conditions.

Lastly, with fixed temperature (25°C) and moisture content (11.1%), specimens were set up with variable amounts of substrates of 1, 3, 5, 10 and 15% for each of LIG, SCG and GG respectively.

In all cases, the radius of mycelium growth was determined using a linear scale (ruler) at 3, 6 and 12 days after inoculation (DAI).

3.2.4 Optimality tests

The Taguchi design of experiment using orthogonal arrays was used to determine optimal combinations of temperature, moisture content and amount/type of substrates required for greater extent of mycelium growth and increased fungal biomass. This method was coupled with the Grey Relational Analysis (GRA) for optimising multi-response characteristics in order to determine the environmental conditions which give the best performance in terms of combined radius of mycelium growth and dry fungal biomass production.

a) The Taguchi orthogonal design of experiment

The pioneer efforts of Fisher (1920) in the development of the concept of design of experiments and extensive studies on optimal environmental conditions for agricultural crop production (Fisher, 1925, 1926), was improved upon by the Taguchi method (Taguchi, 1990) introduced in 1986. The Taguchi method aims to create robust

products and processes by optimising parameters using a log function of the desired output, known as the Signal-to-Noise (S/N) ratios. The Taguchi method is popularly used in manufacturing process, but has also shown positive outcomes when used for process and product optimisation in the fields of agricultural science, environmental studies, basic and medical sciences as well as business and management (Elshennawy, 2004; Wu and Chen, 2006; Daneshvar *et al.*, 2007; du Plessis and de Villiers, 2007; Houng *et al.*, 2007; Romero-Villafranca, Zúnica and Romero-Zúnica, 2007; Tasirin *et al.*, 2007). Compared to a full factorial design of experiments requiring a huge number of experimental runs, the Taguchi approach presents a simpler and less cumbersome alternative which uses orthogonal arrays to significantly reduce the number of experimental runs thereby saving time and costs with results highly comparable to those obtained from some factorial designs (Taguchi, Jugulum and Taguchi, 2004).

Factors and quality characteristics in Taguchi design

Taguchi design has two main types of factors: the control and noise factors. Control factors are controllable parameters in an experiment while noise factors are uncontrollable factors that cause variability of response in a product or process. The identified control factors in this study were selected ranges of temperature, moisture content and amount of varied organic substrates determined based on results of the preliminary tests. The respective ranges (or levels) of these factors were used to design the $L_9(3^3)$ Taguchi orthogonal array (section 3.3.2) adopted for this study. Available oxygen and relative humidity during fungal growth were not measured but were assumed to remain constant depending on the incubators used for experimental runs; these were considered as noise factors for the period of the study. The Taguchi method

performs optimisation by identifying control factors that minimize the effect of the noise factors on the performance or response of the product/process. The responses measured in this case were the growth of fungal mycelia in terms of (a) radius of mycelium growth and (b) dry fungal biomass.

Depending on the nature of a problem, the Taguchi optimisation design approach is either static or dynamic. (1) Static analysis is used when several control factors are identified as having direct influence on desired outputs and a fixed level of quality characteristic is implemented. Available quality characteristics of responses (or measured behaviours) for a static response design include:

larger-the-better: which maximises the response with S/N = $-10 * \log(\sum (1/Y^2)/\mathrm{n})$; *smaller-the-better:* which minimises response with S/N = $-10 * \log(\sum (Y^2/\mathrm{n}))$; and lastly, *target-the-best:* which targets desired response with S/N = $-10 * \log(\sigma^2)$.

[Where: *"Y = responses for the respective factor level combination; n = number of responses in the factor level combination and σ = standard deviation of the responses for all noise factors for the given factor level combination".*]

This study assumes that increased mycelia growth in soil (radius of mycelium growth and dry fungal biomass) would translate to increased potential effects on soil modification (Degens, Spading and Abbott, 1996), therefore, the *'larger-the-better'* quality characteristic was used in all static analysis to determine optimal conditions for each of the two responses. (2) Dynamic response design is used when there is a signal factor which directly decides the output. The signal factor is a factor that has a range of settings that is controlled by the user during the experiment to directly

influence the response. While the quality characteristic of interest is set at a fixed level in static design, it is flexible along a range of values in the dynamic design and is ultimately aimed at improving the slope, that is, enhancing the relationship between the change in output response and the signal factor. The sensitivity of the slope is therefore critical for determining optimal parameters in a dynamic design analysis. In this study, incubation period (or time) was taken as a signal factor and dry fungal biomass were measured at 6, 18 and 24 days after incubation to determine optimal conditions over an extended growth duration.

b) Grey relational analysis for multiple response characteristics

The Taguchi method was designed for optimisation of single-response characteristics and encourages the use of engineering judgement when multi-response characteristics are encountered (Lin, 2004; Balasubramanian, 2011). This could be subjective and lead to inconsistency and uncertainties in decision-making. For determination of optimal factors and their effects on combined multi-responses such as combined radius of mycelium growth (mm) and fungal biomass (mg g^{-1}), the Grey system theory or Grey relational analysis (Deng, 1989) is often used. Coupled Grey relational analysis and Taguchi method was proposed by Chen *et al.* (2000) and has been widely used for optimisation of problems involving multi-performance quality characteristics (Lin, Chang and Chen, 2006). The method has been found suitable where multiple response data are incomplete or uncertain, vary in terms of units or numerical orders of magnitude and are generally scarcely interrelated (Morán *et al.*, 2006). In this method, optimisation of a multi-response problem is transformed into optimisation of a single Grey relational grade (GRG).

For determination of optimal conditions for combined radius of mycelium growth and biomass in this study, the Grey relational analysis was implemented in the following 6 steps:

Step 1: experimental results were normalised to form a range between 0 and 1. This is also called Grey relational generation. Since the response is to be maximised, normalisation was achieved by using the *'larger-the-better'* linear data pre-processing method (Wang, Z., Zhu, L.I. and Wu, 1996):

$$x_i(k) = \frac{y_i(k) - min\ y_i(k)}{max\ y_i(k) - min\ y_i(k)} \quad (3\text{-}1)$$

Where: $x_i(k)$ is the value after the grey relational generation, $min\ y_i(k)$ is the smallest value of $y_i(k)$ for the *kth* response, and $max\ y_i(k)$ is the largest value of $y_i(k)$ for the *kth* response.

Step 2: the grey relational coefficient $\xi_i(k)$ was then calculated from the normalised data as follows:

$$\xi_i(k) = \frac{\Delta_{min} + \zeta\Delta_{max}}{\Delta_{oi}(k) + \zeta\Delta_{max}} \quad (3\text{-}2)$$

Where: $\xi_i(k)$ = Grey relational coefficient; Δ_{0i} is the deviation sequence given by: $\|x_0(k) - x_i(k)\|$, where $x_0(k)$ = reference sequence and $x_i(k)$ = comparability sequence. Δ_{min} and Δ_{max} are the minimum and maximum values of the absolute differences Δ_{0i} of all comparing sequences. ζ is the distinguishing or identification coefficient with value ranging between 0 to 1. $\zeta = 0.5$ in this analysis.

Step 3: the grey relational grade (GRG) was thereafter obtained as follows:

$$\gamma_i = \frac{1}{n}\sum_{k=i}^{n}(k) \quad (3\text{-}3)$$

Where: γ_i is the required grey relational grade for ith experiment and n = number of response characteristics.

Step 4: the mean response table for the GRG was generated and higher values of mean GRG were taken as the optimal combination of parameters for the multi-responses.

Step 5: analysis of variance (ANOVA) was generated to determine the parameters with most significant influence on the multi-response and to obtain the relative percentage contribution of each factor (Datta, Bandyopadhyay and Pal, 2008). The F-value is often used for judging statistical significance in this analysis.

Step 6: confirmatory test to verify the improvement of the GRG was performed. The predicted value of GRG for optimal level was obtained using:

$$\hat{\gamma} = \gamma_m + \sum_{i=1}^{q} (\bar{\gamma}_i - \gamma_m) \tag{3-4}$$

Where: γ_m is the total mean of the GRG, $\bar{\gamma}_i$ = mean of the GRG, and q = number of fungal growth parameters that show statistically significantly effect on the multi-response characteristics.

The Taguchi array design and analysis were carried out using Minitab 18.0 statistical software.

c) Determination of 'response I' – radius of mycelium growth

The average radius of mycelium growth in the sand specimens was determined by staining the mycelia following a method by Stahl and Parkin (1996) with slight modifications. Specimens were taken out of the incubator and stained on the 6th DAI using fluorescein diacetate, FDA, a cell viability stain that indicates the presence of

living fungi in a medium. The stain was prepared according to the protocol published in the Cold Spring Harbour protocols (Anonymous, 2008). 3 mL of the staining solution was sprayed onto samples using a fine mist spray bottle, thereafter specimens were left to stand for 4 hrs at room temperature to allow the stain to permeate the hyphae. Images of the stained mycelium in the specimens were then captured by placing them under ultraviolet light and using a Nikon D3200 DSLR camera set at f/5.6 and ISO-100. Images were imported into ImageJ Fiji software (Schindelin *et al.*, 2012) and the average distances from the edge of the inoculum to the ends of mycelial radial extension, representing the radius of the mycelium were estimated. Six measurements were made for each specimen and the average obtained. The same procedure for image analysis was used to estimate the radius of mycelium growth for at least one unstained replicate of each specimen. This was intended to capture any sections of non-living hyphal/mycelial extent that would typically not be stained by FDA, and is complemented with observations from stained specimens in case of any less dense mycelia growth front that may not be clearly visible to the eye if unstained with FDA.

d) Determination of 'response II' – dry fungal biomass

Ergosterol is a fungus specific lipid and bio-marker indicating the presence of living fungus. It has been established that a relationship exists between fungal biomass and the concentration of ergosterol in the fungal mycelia (Matcham, Jordan and Wood, 1985; Djajakirana, Joergensen and Meyer, 1996; Stahl and Parkin, 1996). This relationship has been used to quantify fungal biomass in given media such as soil (Grant and West, 1986; West, Grant and Sparling, 1987; Montgomery *et al.*, 2000;

Gong, Guan and Witter, 2001). In this study, the rapid technique for ergosterol determination by physical disruption was used (Gong *et al.*, 2001), while a relationship between ergosterol concentration and fungal biomass: 2.2 µg ergosterol mg^{-1} fungal dry biomass (Matcham, Jordan and Wood, 1985; Montgomery *et al.*, 2000) was used to estimate biomass. Details are given in the following sub-sections.

i) Ergosterol extraction and quantification

Acid-washed glass beads (10 g, sized 212 – 1180 µm diameter) were introduced into a 50 mL scintillator vial containing 10 g of specimen (sand, organic substrate, water & mycelium) with fungal growth. 15 mL of methanol was added. The mixture was vortexed for 10 s and placed on a shaker set at a speed of 3200 rpm for 1 hr. Thereafter, the mixture was left to stand for 15mins. 5 mL aliquots of supernatant were transferred into a 15 mL centrifuge tube and subjected to centrifugation for 10 min at speed of 1100 rpm and temperature of 5°C. 3 mL of the supernatant was then filtered through a 0.2 µm syringe filter and 1 mL of the filtrate was transferred into a 1.5 mL micro-centrifuge tube. This portion of the extract was loaded into the auto sampler of the High-Performance Liquid Chromatography (HPLC) for analysis.

Ergosterol analysis was achieved using a Dionex UltiMate 3000 standard HPLC system comprising of a UV detector, a pump, an autosampler and a C_{18} reverse-phase column. Ergosterol was eluted with 100% methanol (mobile phase liquid) at a flow rate of 1 mL min^{-1}, UV detector wavelength set at 282 nm and column pressure maintained at 660 – 700 psi. The sample injection volume was 20 µL. Linear calibration curves were determined for ergosterol standards prepared using pure

ergosterol (Sigma Aldrich). At least five different concentrations were used and linearity was checked for each day of specimen analysis.

Fig. 3-2a presents stacked chromatograms for different orders of magnitudes of standard ergosterol concentrations used for calibration; retention time ≅21 mins. This is for the 6th day ergosterol determination and is typical of what was obtained for the 18th and 24th DAI. Typical linearity plot is also shown in Fig. 3-2b and was used for converting the peak areas (mAU*min) to ergosterol concentrations (µg/L) for the test specimens.

ii) Estimation of mycelia biomass

Fungal biomass (FB) is often estimated using established relationships between ergosterol concentration and biomass. This relationship depends on the age of mycelium, incubation temperature and ergosterol extraction procedures (Bermingham, Maltby and Cooke, 1995). Conversion factors (CF) for ergosterol to fungal biomass typically range between 0.35 – 31 µg ergosterol mg^{-1} fungal biomass (Matcham, Jordan and Wood, 1985; Bermingham, Maltby and Cooke, 1995; Djajakirana, Joergensen and Meyer, 1996; Stahl and Parkin, 1996; Montgomery *et al.*, 2000). The CF for mycelia of *P. ostreatus* is rare in literature, however, Matcham, Jordan and Wood (1985) found the CF for *Agaricus bisporus*, a fungal species that bears similar characteristics as *P. ostreatus* to be 2.2 µg ergosterol mg^{-1} dry mass of mycelium (of fungal biomass). This was therefore selected for use in this study; however, it should be noted that this is used in the absence of any values in literature for *P. ostreatus* and to give an estimation of the biomass of mycelium of *P. ostreatus* in sands.

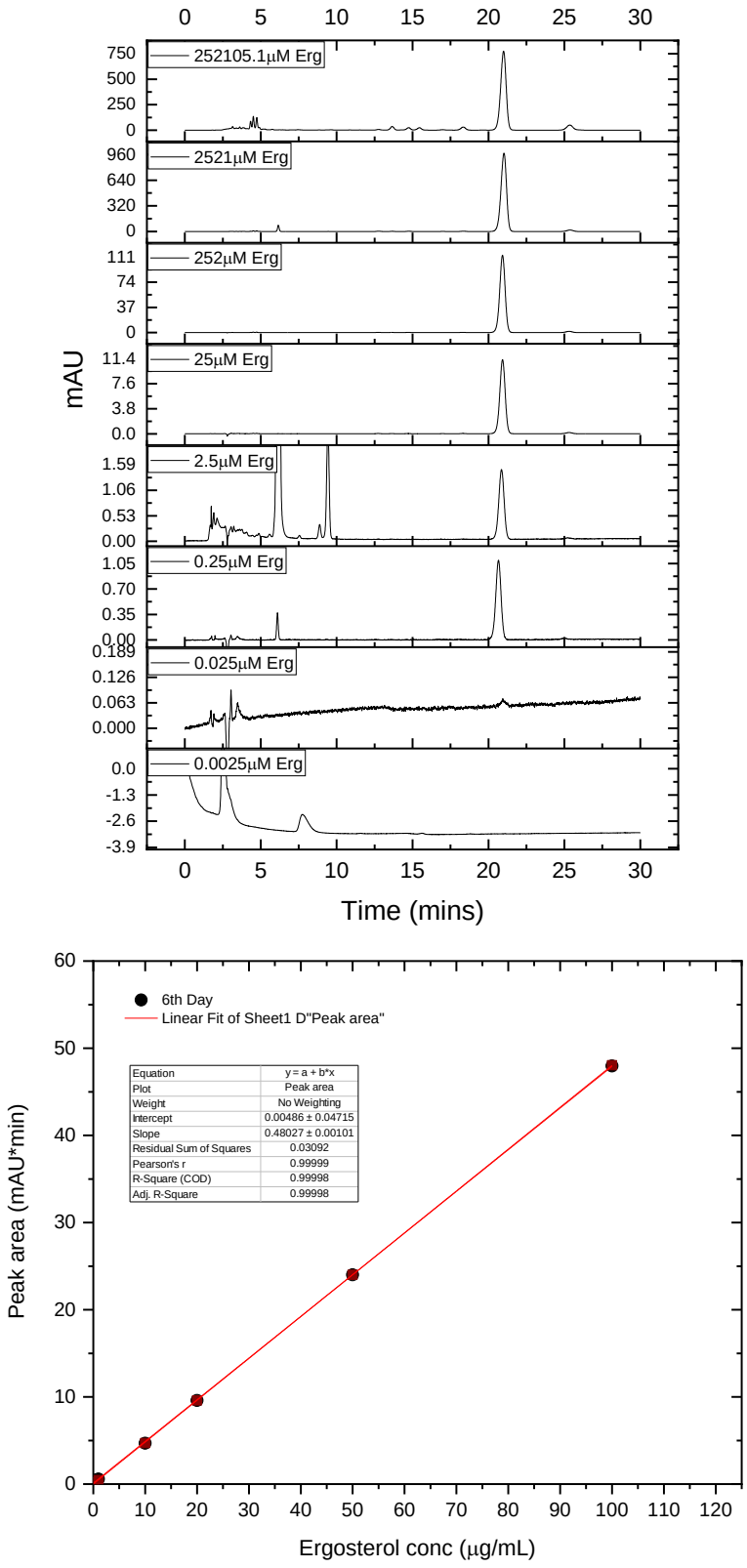


Figure 3-2: Typical profile of stacked chromatograms showing peaks for pure ergosterol at different concentrations used as standard for the calibration plot shown in (b).mAU = milli-Absorbance Units, a measure of intensity of the absorbance

3.3 Results and discussions

3.3.1 Influence of environmental conditions on mycelium growth for *P. ostreatus*

The results from the preliminary tests provided information on the influence of each factor on the radius of mycelium growth and some indication of relative biomass produced by *P. ostreatus*. Mycelium growth densities, assumed to give an approximate indication of fungal biomass, were visually inferred. In this discussion, higher mycelium density refers to the presence of massive concentration of hyphae/mycelium while comparatively lesser mycelial density implies faint and sometimes isolated mycelium spreading radially from the inoculant. Fig. 3-3 presents a typical specimen showing treated soil in a petri-dish with dense mycelium growing radially from the inoculant. Representative images of all other specimens captured for the preliminary tests are presented in Appendix B.

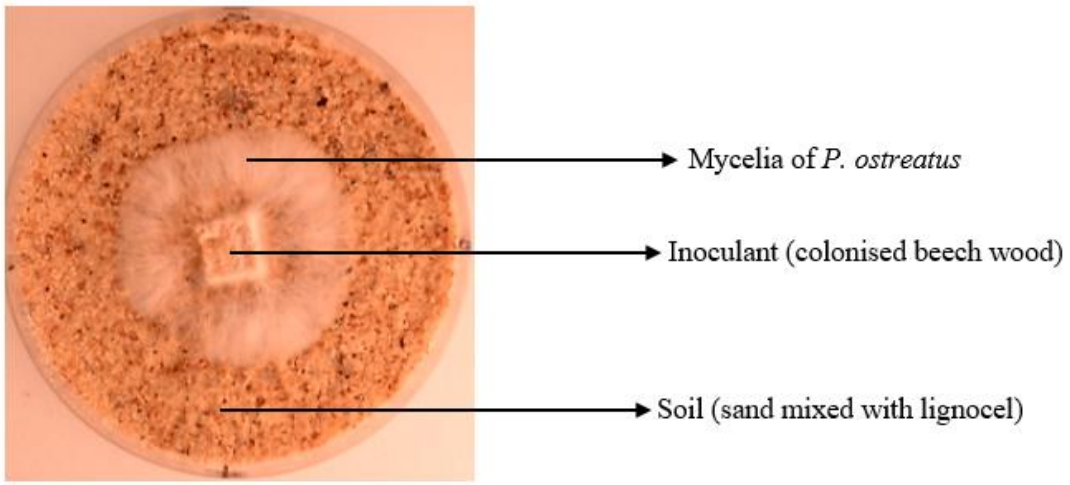
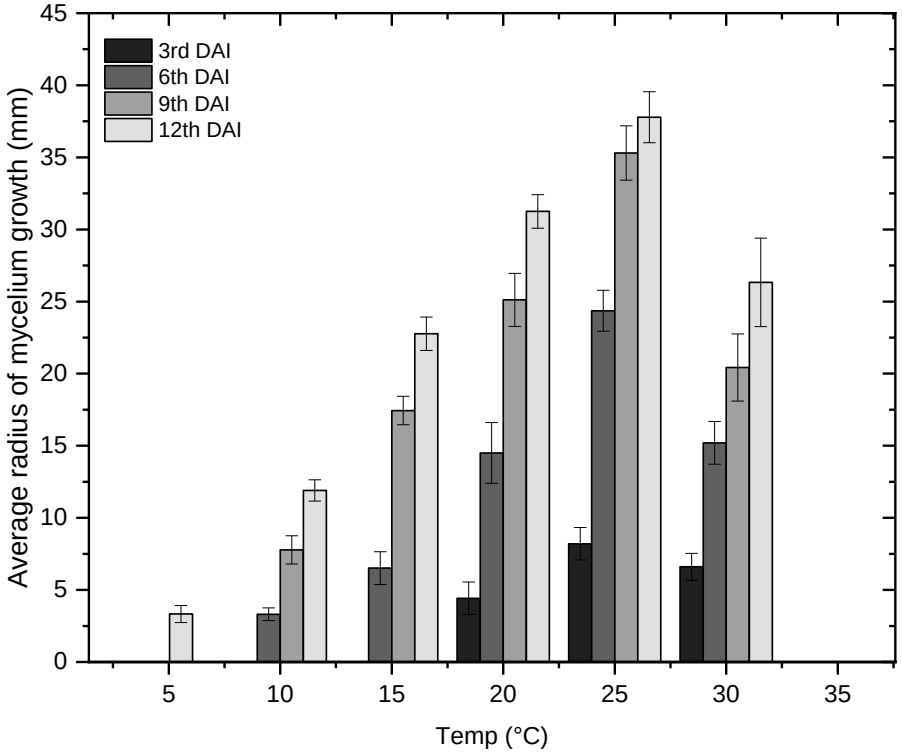


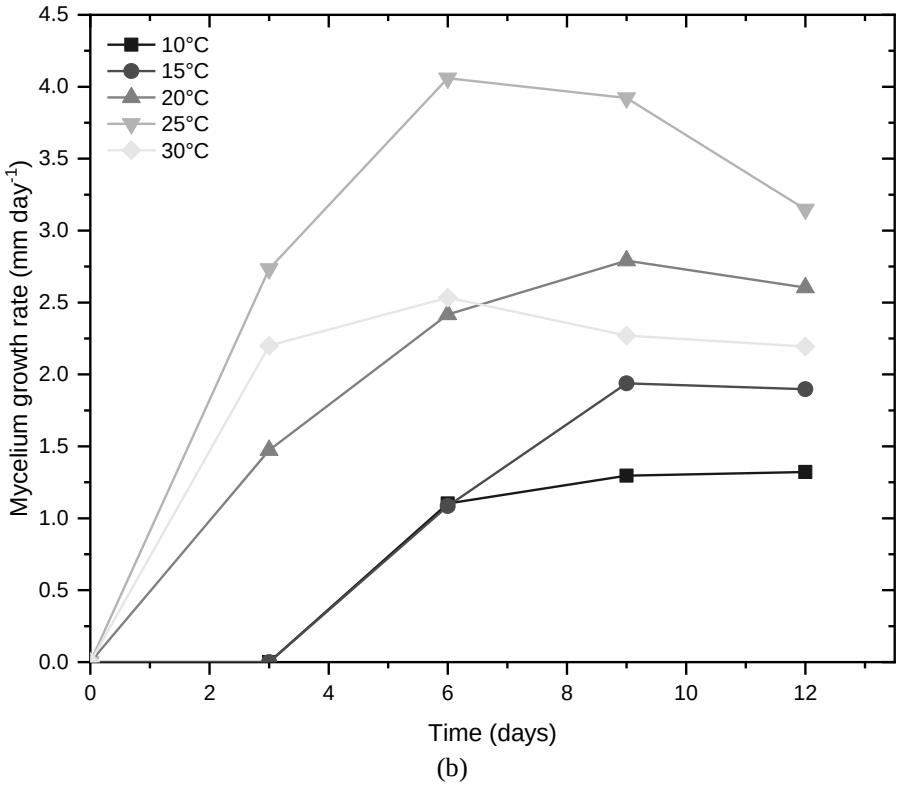
Figure 3-3: A typical treated specimen showing mycelia growing radially from the colonised beech wood inoculant in a 90 mm diameter petri-dish

a) Influence of temperature:

Fig. 3-4a presents the average radius of mycelium growth (cumulative) and Fig 3-4b the average mycelium growth rates (cumulative) measured from 3 to 12 DAI for specimens incubated at temperatures between 10°C and 30°C. It is evident that the maximum radius of mycelium growth and growth rate were recorded for specimens incubated at 25°C. Average radius of mycelium growth increased steadily with increasing temperature, attaining highest value of 37.78 mm at 25°C on the 12th DAI. There was a marked decline in growth at 30°C where the greatest extent of mycelium growth achieved was 26.33 mm. A similar trend is observed in the growth rate, with specimens grown at 30°C showing a reduced growth rate beyond 3rd DAI (Fig. 3-4b). In Fig. 3-4b, the decline seen in the growth rate recorded at 25°C beyond 6 DAI may be attributed to boundary conditions as the radius of mycelium growth was already >35 mm by the 9th DAI whereas maximum radius of the petri dish was 45 mm. According to Boddy *et al.*, (2009), some fungal species forage for resources by first deploying thin peripheral exploratory networks of hyphae as a less aggregated growing front ensuring transport and growth efficiency. It is possible that the mycelium growth front in this case may have already reached the edge of the petri-dish and upon encountering the 'obstruction' (boundary), initiated a morphological response 'communicated' across the highly coordinated hyphal network (Crowther, Boddy and Hefin Jones, 2012) leading to a reduction in growth rate. This mycelial front may not have been clearly visible for accurate measurements during the final days of data collection.



(a)



(b)

Figure 3-4: (a) Average radius of mycelium growth at different temperatures 3-12 DAI and (b) mycelium growth rate for temperatures between 10 – 30°C

Growth at 5°C was not obvious until the 12th day and no growth was recorded at 35°C throughout the period of observation. Mycelial densities seemed to decrease steadily from 10 to 30°C (based on the observations made on the 12th DAI) and also decreased over time, for specimens incubated at 20°C and 25°C. This is in agreement with what is reported in the literature and may in part be attributed to increased rates of bio-mineralisation as temperature increases (Vasconcellos, 1998), although no mineralisation was specifically observed/investigated in this study; and on the other hand, it could be mycelial morphological response to depleting resources as is observed in other species where hyphae regresses over time due to reduced activity in nutrient transport links as resources deplete, resulting in less dense mycelia systems (Donnelly, Wilkins and Boddy, 1995; Boddy *et al.*, 1999; Bebber *et al.*, 2007). This is based on the assumption that increased temperature results in depleting resources because: (i) there is increased fungal activity or usage of available (unreplenished) resources for hyphal development, and (ii) moisture content reduces due to drying with increasing incubation temperatures, since the relative humidity in the incubator was not maintained at 100%.

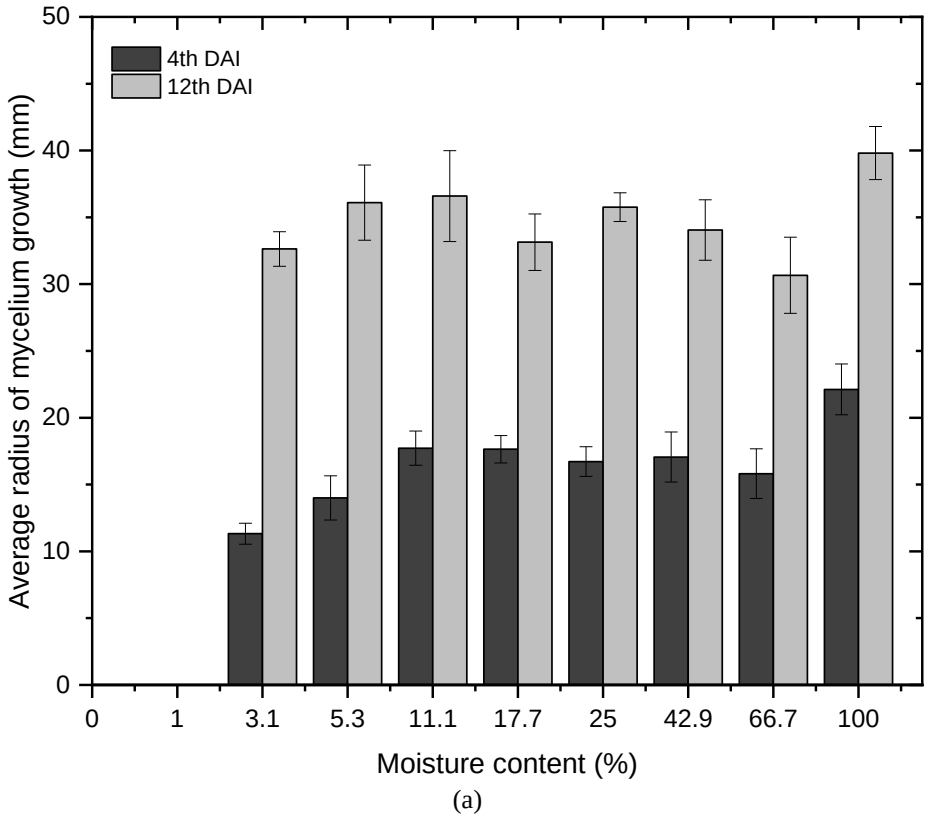
Temperatures of 15, 20 and 25°C were selected as the levels to be tested in Taguchi design for optimality. 15°C temperature was selected instead of 30°C for two reasons; first, to retain the potential of higher biomass production, compared to 30°C; and secondly, as representative level for 'lower' range of soil temperatures in consideration of temperate locations around the world where this technology may be deployed for soil improvement.

b) Influence of moisture content

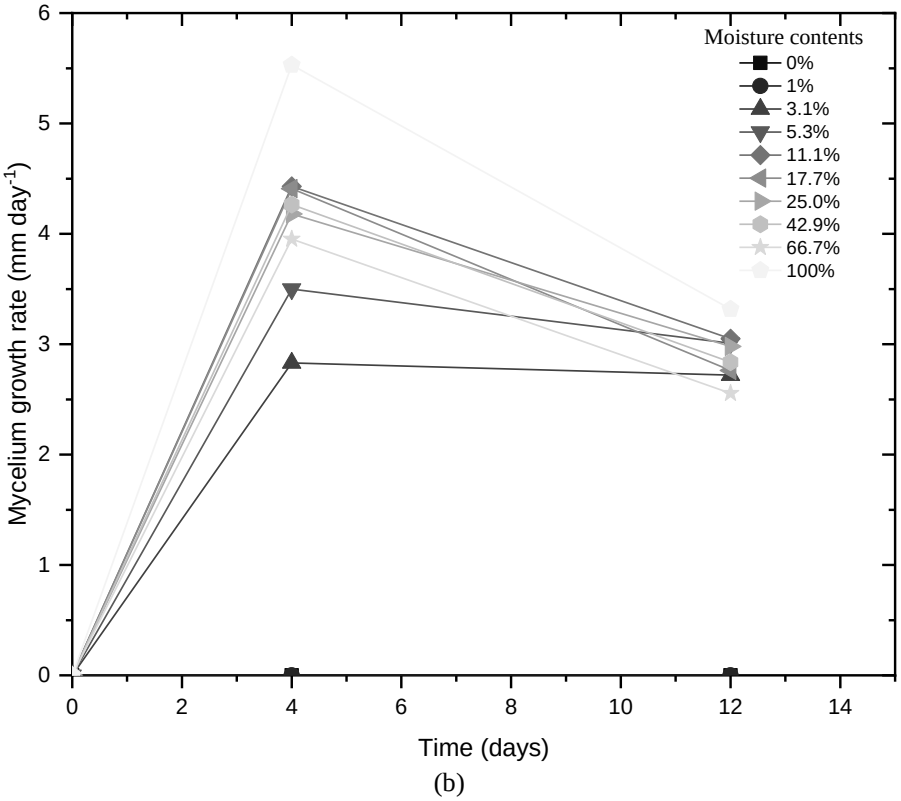
Figures 3-5a and 3-5b show respective stacked radius of mycelium growth and growth rates determined at 4 and 12 DAI for specimens at different moisture contents all grown at 25°C and 5%LIG. No growth was observed at 1% moisture content while the maximum radius of mycelium growth was recorded at 100% where specimens were inundated. At this moisture content (which is greater than saturated moisture content), no growth was observed within the soil, as grains were submerged, instead, mycelium growth was seen only to occur on the air-water interface (Fig. 3-6c). *P. ostreatus* is an aerobic microorganism and requires oxygen for respiration and hence to grow, thus with soil pores fully saturated, hyphae could not penetrate into the soil. Additionally, it was observed for the 100% moisture content specimens that the denser soil particles settled to the bottom of the dish and the less dense LIG particles floated on the surface, providing a nutrient rich surface for hyphal/mycelium growth at the air-water interface. Although no mycelium growth was observed at 1% moisture content, once additional moisture was introduced into the specimen, the extent of growth was similar in all specimens from 3.1% - 66.7% with radii between 11.32 – 15.81 mm at 4 DAI and 32.63 – 36.6 mm at 12 DAI. The density of the mycelium appeared to be fairly homogeneous, extending radially and increased with increasing moisture content on the 4th DAI (Fig. 3-6a &b). However, the mycelia appeared to 'thin out' at 12 DAI with hyphae becoming translucent. This may be due to absorption of water by the hyphae or deployment of hyphae further into the soil matrix as moisture content reduced overtime within the growth region. The growth at 100% moisture content is only suitable if interface growth is to be promoted. Where fungal growth extending into the soils is desirable, a lower moisture content would be required. For

lower moisture contents the greatest radius of mycelium growth was observed at 11.1% moisture content (average radius of mycelium and growth rates of 36.6 mm and 3.05 mm day^{-1} respectively). There seemed to be no significant difference in radius of mycelium growth recorded for moisture contents of 5.3, 17.7, 25 and 42.9% (overall mean radius of mycelium growth of 34 mm by 12^{th} day). However, mycelium growth rates (Fig. 3-5b) show a decline for all specimens after the 4^{th} day, with the decline being gradual for 3.1 and 5.3% but steeper for higher moisture contents. As data was only recorded at two time points, it is not clear when the decline in growth rates begins.

From the foregoing discussion, moisture levels of 5.3, 11.1 and 17.7% were therefore selected as levels for moisture content to be tested in the following Taguchi experiment.



(a)



(b)

Figure 3-5: (a) Stacked column plot of average radius of mycelium growth at different moisture contents for 4th and 12th DAI (b) mycelium growth rates for specimens at different moisture contents up to 12th DAI

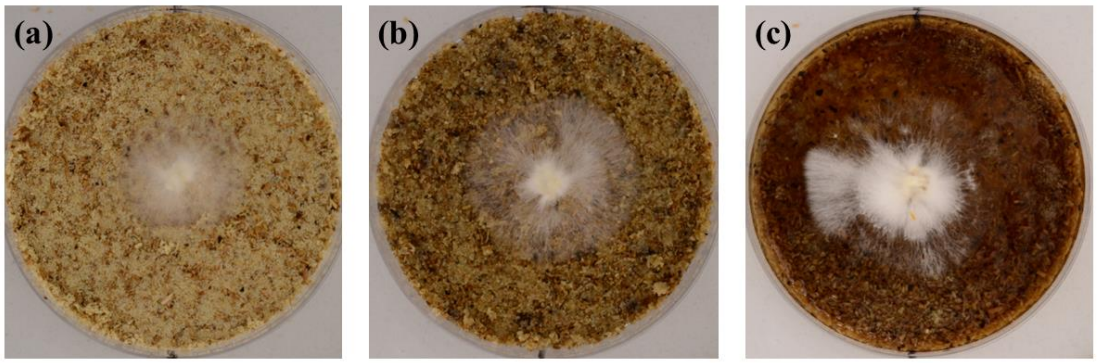
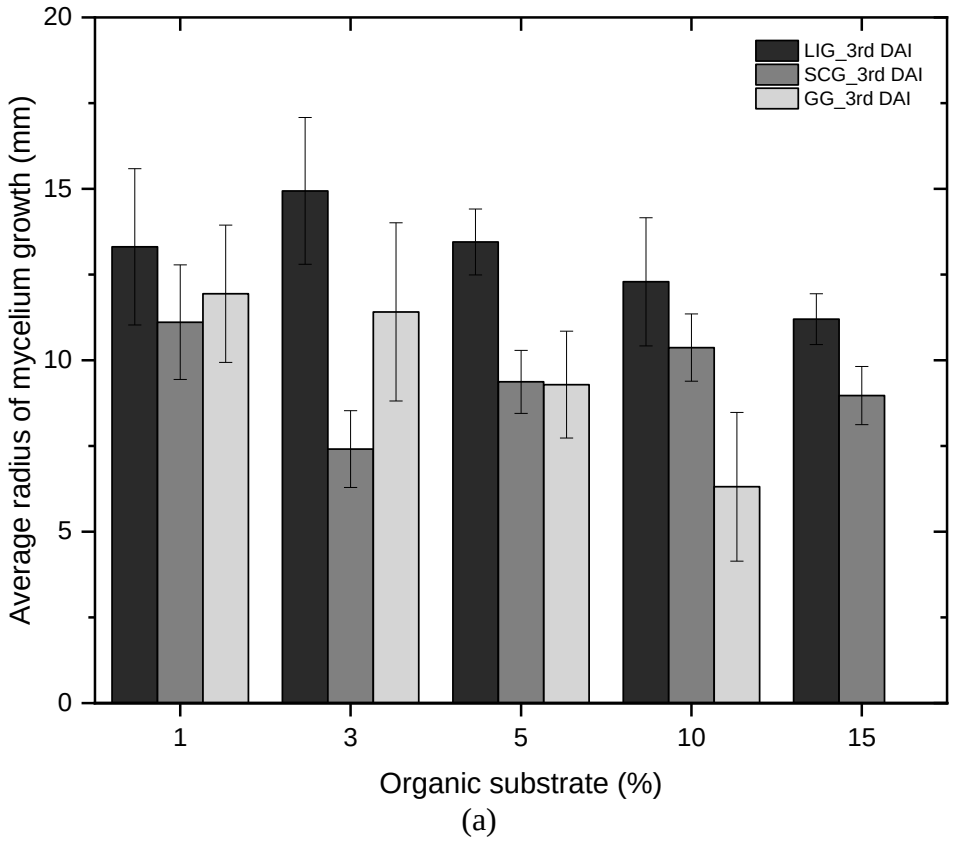


Figure 3-6: Mycelia of P. ostreatus growing in soils with moisture contents of (a) 5.3% (b) 42.9% (c) 100% all on 4th DAI.

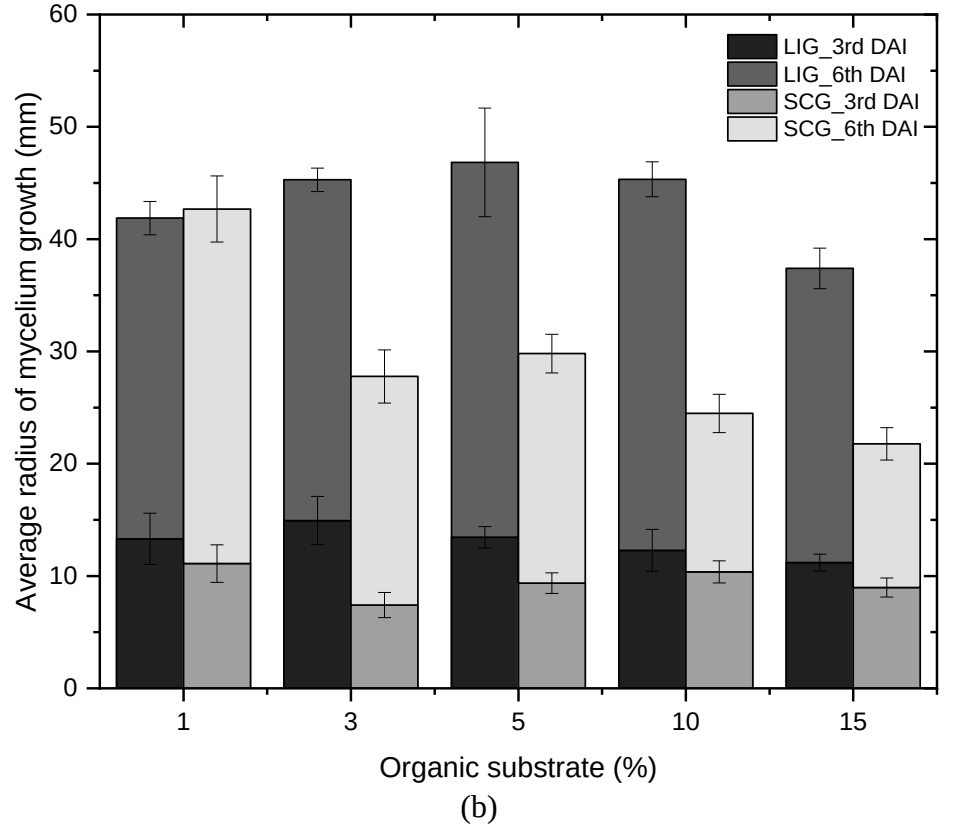
c) Influence of organic substrates

Average radii of mycelia for the respective organic substrates measured on the 3rd DAI is presented in Fig. 3-7a. It was observed that for specimens amended with LIG and GG, the radius of mycelium growth decreased with increasing amount of substrate

(above 3%), though LIG recorded highest radius of mycelium growth of approx.15mm when 3% LIG was used. Figure 3-7b presents a stacked plot of the radius of mycelium growth for specimens amended with LIG and SCG at the 3rd and 6th DAI. Fig. 3-7b shows that the total radius of mycelium growth (stacked 3rd and 6th DAI) increased from 42 mm to 47.5 mm moving from 1 to 3% LIG, and thereafter decreased with additional LIG. While SCG recorded its greatest radius of mycelium growth (stacked 3rd and 6th DAI) of 43 mm at 1%SCG which decreased to 22 mm at 15% SCG. GG was not tested on the 6th DAI as specimens and lids of petri-dishes were covered with massive wool-like mycelium, posing a challenge to determine or record accurate radius of mycelium growth. Generally, overall mycelium growth behaviour for the different amounts and types of substrates tested showed that the relatively higher amount of organic matter resulted in less mycelium growth in terms of distance but greater mycelium density. This is most likely due to the fact that with sufficient resource (especially nutrients) in abundance and homogeneous within close proximity, mycelia of *P. ostreatus* preferred to grow locally, finding no need to deploy hyphae further away from the inoculant in search of resources. Species exhibiting this kind of behaviour are described as 'short-range foragers' (Boddy *et al.*, 2009). Substrate levels (Fig. 3-8) which showed equivalent performance in terms of mycelium radius and density were selected for the optimality tests.



(a)



(b)

Figure 3-7: Influence of organic substrates on radius of mycelium growth for (a) LIG, SCG & GG 3DAI and (b) LIG & SCG 3rd and 6th DAI

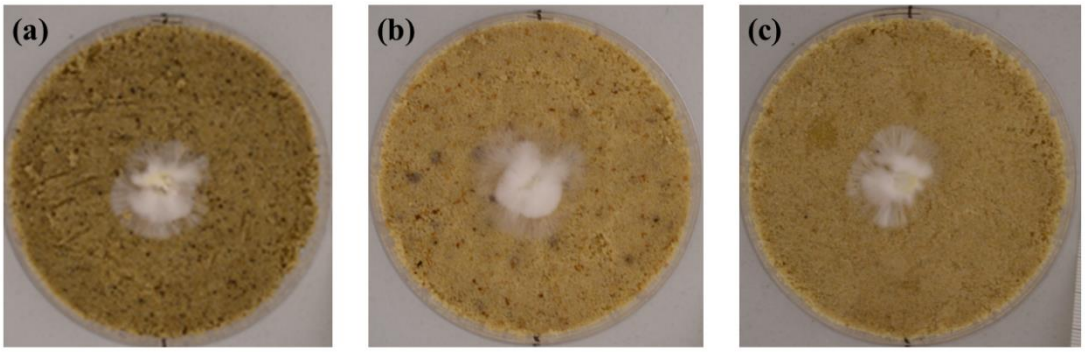


Figure 3-8: Organic substrate levels selected for Taguchi design were: 1%SCG, 3%LIG and 3%GG.

For all environmental factors investigated in the preliminary tests, with the exception of specimens with 100% moisture content, the overall mycelium growth rates ranged between 1.08 mm day^{-1} (specimen incubated at 15°C day 6) to 5.51 mm day^{-1} (for specimen amended with 10% GG, day 6); while radius of mycelium growth ranged from ~3.3 mm (specimens incubated at 10°C, day 6 and 5°C, day 12) to 37.78 mm (specimen incubated at 25°C, day 12). These ranges give a preliminary indication of the order of significance of the influence of controlled or combined factors for mycelia growth in soil in the laboratory. The statistical significance and optimal levels for best performing ranges of conditions were investigated using the Taguchi design of experiment. Suitable ranges of environmental conditions used for Taguchi design analyses were also selected based on observations from these tests.

3.3.2 Optimal conditions for fungal growth and biomass in soils

a) Factors, levels and Initial conditions selected for Taguchi design of experiment

Table 3-1 presents the factors and levels selected based on the preliminary experiments discussed above. These were used to design the orthogonal array/matrix as shown in Table 3-2. It was also observed from the preliminary tests that mycelium densities changed with time for specimens monitored on different days. To account for this

effect, time was therefore chosen as a 'signal' factor in the design of a Taguchi dynamic array in order to determine the optimal conditions for maximum biomass production up to 24th DAI (Table 3-3).

The initial conditions that were hypothesised and tested for best combined mycelium growth and biomass performances at 6th DAI were: temperature of 25°C, 10% moisture content and 3% lignocel. This hypothesis was based on the best performing levels in the preliminary tests and its results were used to measure improvements due to the Taguchi-Grey optimisation presented in sub-section 3.3.2(g).

Specimens for each treatment were prepared as described in section 2.2, with compositions as shown in Table 3-2. They were placed in sealed plastic storage boxes along with wet paper towels (to maintain humidity levels). The boxes were kept in incubators set at respective temperatures depending on the treatment conditions. Performance measurements were done on the 6th DAI (radius of mycelium and biomass), and on the 18th and 24th DAI (biomass only).

Table 3-1: Factors and levels for Taguchi design of experiments

Levels	**Factors**		
	Temperature (°C)	Moisture content (%)	Organic substrate (% of total mass)
1	15	5.3	SCG (1% Spent Ground Coffee)
2	20	11.1	LIG (3% Lignocel)
3	25	17.7	GG (3% Guar Gum)

Table 3-2: Taguchi orthogonal design of experiments array

Treatment	Design matrix			Compositions		
	Temperature	Moisture content	Organic substrate	Temperature (°C)	Moisture content (%)	Organic substrate (% of total mass)
T1	1	1	1	15	5.3	1% SCG
T2	1	2	2	15	11.1	3% LIG
T3	1	3	3	15	17.7	3% GG
T4	2	1	2	20	5.3	3% LIG
T5	2	2	3	20	11.1	3% GG
T6	2	3	1	20	17.7	1% SCG
T7	3	1	3	25	5.3	3% GG
T8	3	2	1	25	11.1	1% SCG
T9	3	3	2	25	17.7	3% LIG

Table 3-3: Dynamic array with time as signal factor for performance optimisation up to 24 days

Treatment	Factors/compositions			Signal
	Temperature	Moisture content	Organic substrate	Incubation period (days)
T1	15	5.3	1%SCG	6
	15	5.3	1%SCG	18
	15	5.3	1%SCG	24
T2	15	11.1	3%LIG	6
	15	11.1	3%LIG	18
	15	11.1	3%LIG	24
T3	15	17.7	3%GG	6
	15	17.7	3%GG	18
	15	17.7	3%GG	24
T4	20	5.3	3%LIG	6
	20	5.3	3%LIG	18
	20	5.3	3%LIG	24
T5	20	11.1	3%GG	6
	20	11.1	3%GG	18
	20	11.1	3%GG	24
T6	20	17.7	1%SCG	6
	20	17.7	1%SCG	18
	20	17.7	1%SCG	24
T7	25	5.3	3%GG	6
	25	5.3	3%GG	18
	25	5.3	3%GG	24
T8	25	11.1	1%SCG	6
	25	11.1	1%SCG	18
	25	11.1	1%SCG	24
T9	25	17.7	3%LIG	6
	25	17.7	3%LIG	18
	25	17.7	3%LIG	24

b) Experimental results or 'responses' obtained

i) Radius of mycelium at 6DAI

Figure 3-9 presents images of the untreated, treated unstained and treated FDA-stained specimens for all treatments T1 to T9. The fungal growth in the unstained images is clearly visible as the white mass extending radially from the central inoculation point. The fungal growth in the stained specimens is visible in green. Mycelium growth of *P. ostreatus* was estimated from stained and unstained specimens at 6DAI and can be seen in Figs. 3-9 & 3-10. Since there was no significant difference between radius of mycelium growth estimated via image analysis of FDA-stained specimens compared with those obtained via image analysis of unstained specimens (Fig. 3-10), therefore, results of the FDA-stained samples were used in the analysis to obtain optimal conditions for radius of mycelium growth. This is in consonance with findings in literature (Shen *et al.*, 2016) where no significant difference was found between mycelium growth extent estimated using image analysis for stained and unstained specimen. The average radius of mycelium growth for each treatment measured on the 6^{th} DAI is presented in Table. 3-4.

Table 3-4: Average radius of mycelium growth measured 6DAI

Treatments	**Factors**			**Responses**
	Temperature	Moisture content	Organic substrate	Average radius of mycelium growth (mm)
T1	15	5.3	1%SCG	10.55
T2	15	11.1	3%LIG	21.85
T3	15	17.7	3%GG	20.02
T4	20	5.3	3%LIG	27.50
T5	20	11.1	3%GG	20.08
T6	20	17.7	1%SCG	16.85
T7	25	5.3	3%GG	36.39
T8	25	11.1	1%SCG	19.50
T9	25	17.7	3%LIG	23.71

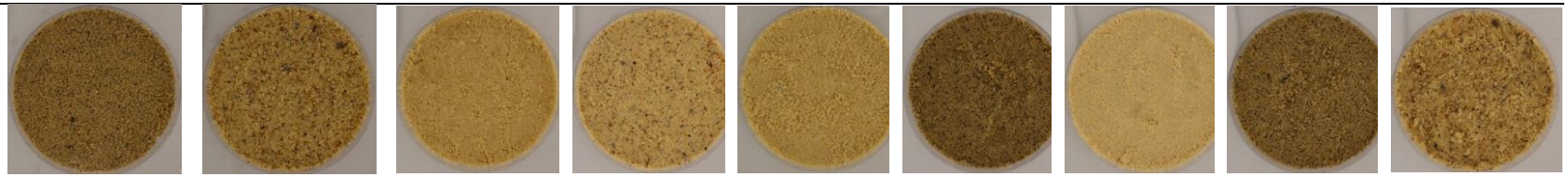
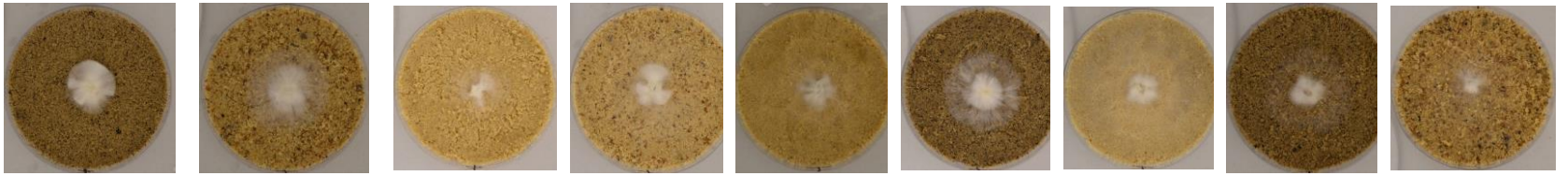
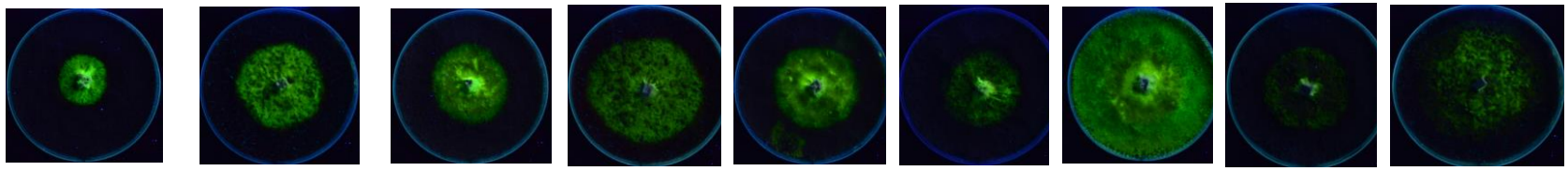
	T1	T2	T3	T4	T5	T6	T7	T8	T9
Temp	15	15	15	20	20	20	25	25	25
Moisture Content	5.3	11.1	17.7	5.3	11.1	17.7	5.3	11.1	17.7
Organic substrate	1% SCG	3% LIG	3% GG	3% LIG	3% GG	1% SCG	3% GG	1% SCG	3% LIG
Untreated									
Treated (unstained)									
Treated (Stained)									

Figure 3-9: Images of fungal growth extent 6DAI for all treatments showing untreated, treated unstained and treated-FDA-stained specimens

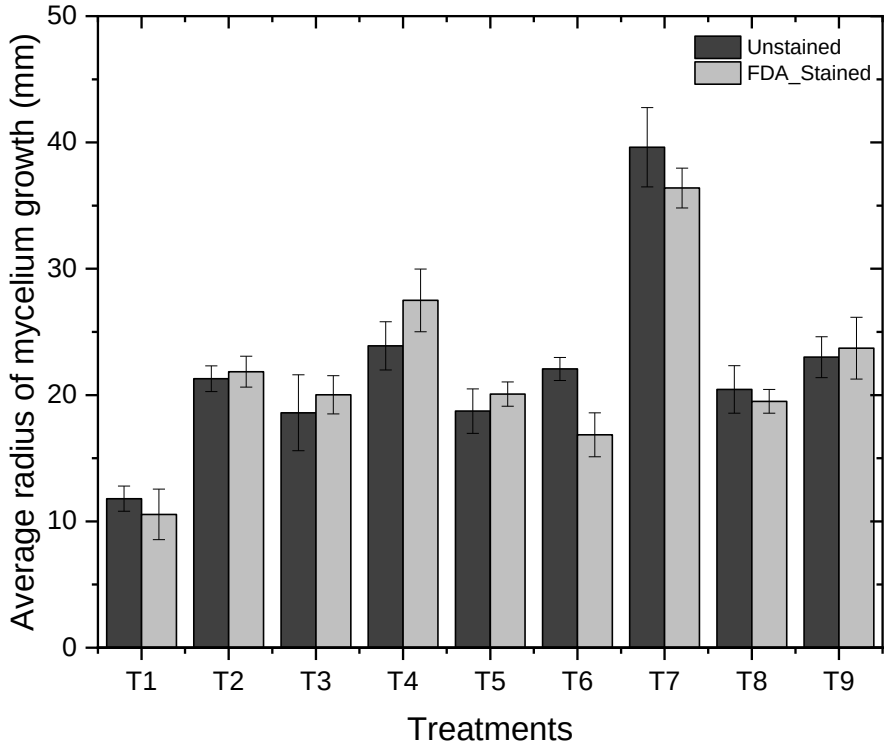


Figure 3-10: Comparison of average radii of mycelium growth per treatment for stained and unstained specimens measured on the 6th DAI using imaging techniques

ii) Fungal biomass at 6, 18 and 24 DAI

Extracted specimens used for HPLC tests showed ergosterol peaks at retention time of approximately 20.8mins (Fig. 3-11). Using the CF of 2.2 μg mL^{-1}, ergosterol concentrations were converted to fungal biomass in mg g^{-1} soil. These are shown in the 'response' column in Table 3-5.

The results of the dry fungal biomass obtained for T1 – T9 suggest that biomass of *P. ostreatus* increased with time, as the final biomass on the 24th day were all greater than the initial biomass determined on the 6th day. Since ergosterol is only found in living hyphae, this implies that *P. ostreatus* continued to grow up to the 24th day, although at different rates. It is also clear from Table 3-5 that the amount of dry fungal biomass reduced between the 18th and 24th days for all specimens incubated at 15°C (i.e., T1, T2 & T3), but this was not the case for specimens incubated at 20°C and above, except

for T9. It is unclear why the biomass reduced after the 6th day and increased after the 18th day as observed in T9. Furthermore, most of the specimens containing GG (T3, T5 & T7) recorded the highest amounts of dry final biomass while the least amounts were found in specimens containing LIG (T2, T4 & T9). These results seem to indicate that an increased growth period (time), temperatures of 20 and 25°C, as well as addition of GG would provide increased amounts of fungal biomass.

Analysis of these factors and determination of specific optimal levels and degrees of significance of respective factors for mycelium growth and/or biomass production are presented in the following sections.

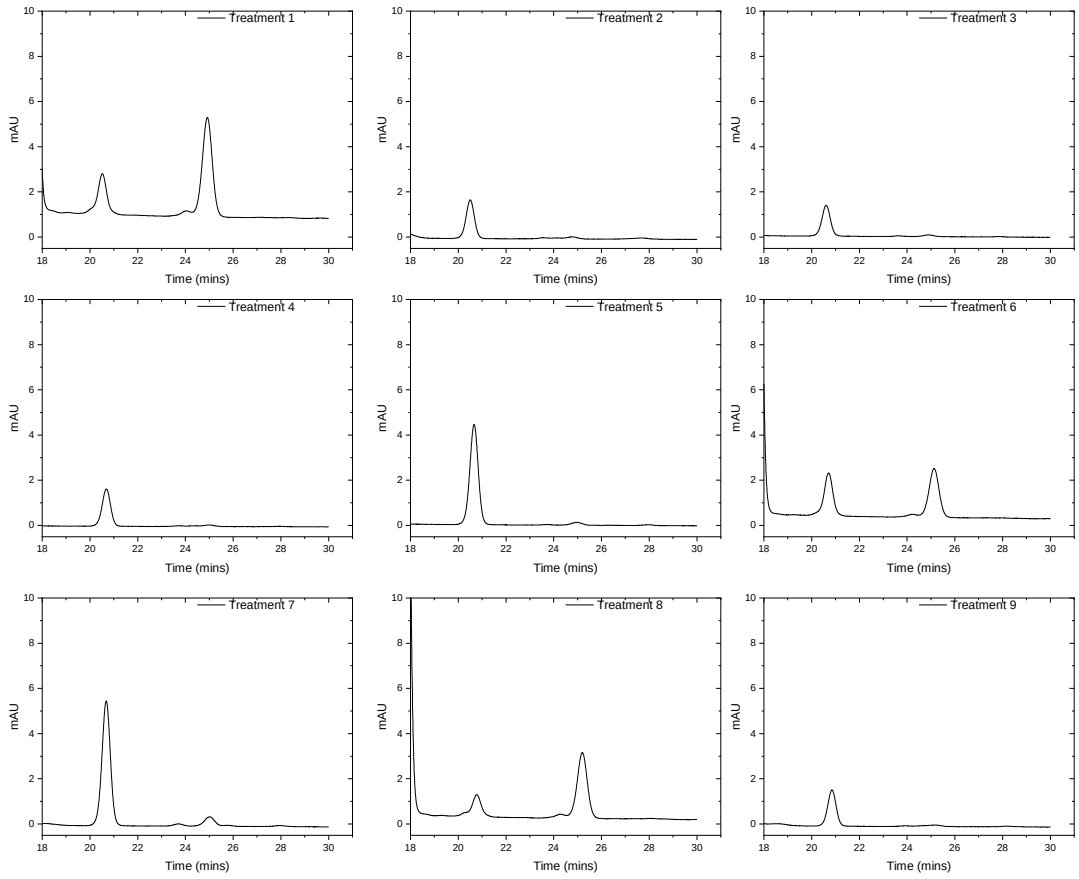


Figure 3-11: Panel of typical chromatograms showing ergosterol peaks at retention time of approx. 20.8 mins for T1-T9

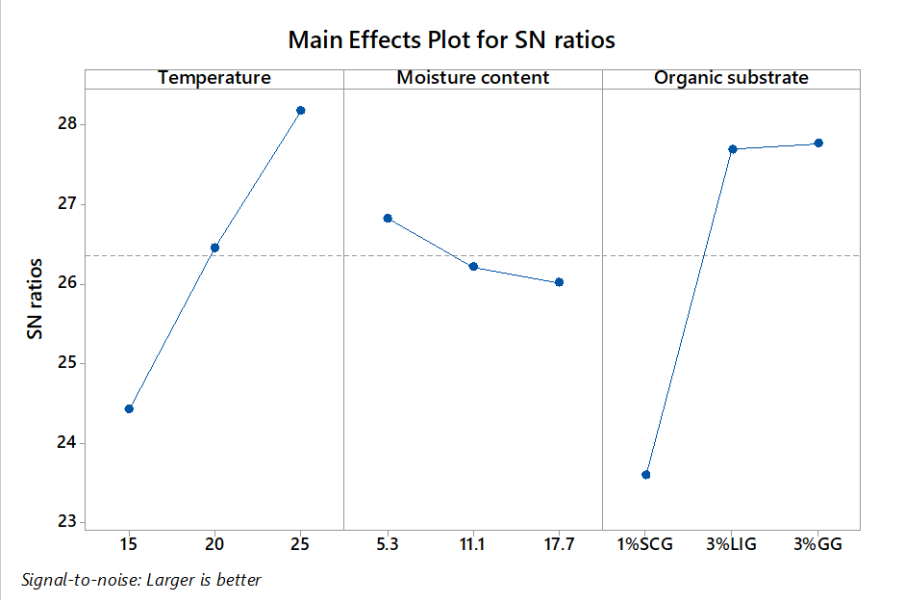
Table 3-5: Fungal dry mass measured for all treatment runs after incubation periods of 6, 18 and 24 days

Treatment	Factors			Signal	Response
	Temperature	Moisture content	Organic substrate	Incubation period (days)	Fungal dry biomass mg/g soil
T1	15	5	1%SCG	6	0.516
	15	5	1%SCG	18	1.137
	15	5	1%SCG	24	0.912
T2	15	10	3%LIG	6	0.440
	15	10	3%LIG	18	0.657
	15	10	3%LIG	24	0.435
T3	15	15	3%GG	6	0.350
	15	15	3%GG	18	2.398
	15	15	3%GG	24	2.047
T4	20	5	3%LIG	6	0.428
	20	5	3%LIG	18	0.538
	20	5	3%LIG	24	0.578
T5	20	10	3%GG	6	1.167
	20	10	3%GG	18	2.278
	20	10	3%GG	24	2.412
T6	20	15	1%SCG	6	0.517
	20	15	1%SCG	18	0.892
	20	15	1%SCG	24	1.235
T7	25	5	3%GG	6	1.444
	25	5	3%GG	18	1.835
	25	5	3%GG	24	2.257
T8	25	10	1%SCG	6	0.281
	25	10	1%SCG	18	0.786
	25	10	1%SCG	24	1.112
T9	25	15	3%LIG	6	0.412
	25	15	3%LIG	18	0.252
	25	15	3%LIG	24	0.403

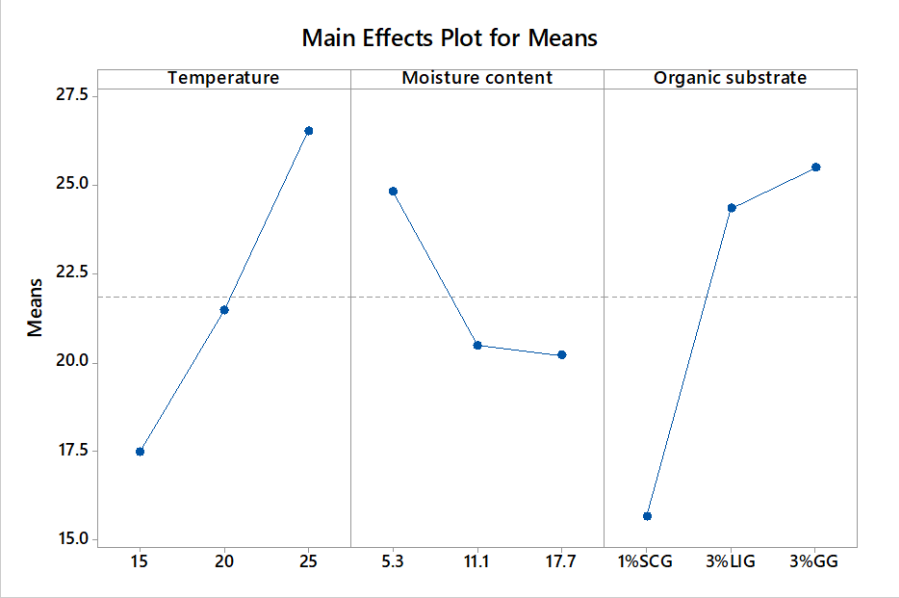
c) Optimal conditions for mycelium growth 6DAI

Using Taguchi static analysis, no single factor was found to be statistically significant (at $P<0.05$; 6DAI) based on the ANOVA. Meaning that there was no factor with the most significant effect on the measured responses (mycelium growth). However, from the ranking on the mean response tables, the relative strength of each factor's influence on S/N ratios and Means for the radius of mycelium growth is in the order: Moisture content < temperature < organic substrate. Therefore, of the three factors tested, the organic substrate had relatively greater influence on mycelium growth extent at 6DAI.

Fig. 3-12 show the main effects plots for the S/N ratios (Fig. 3-12a) and the individual level Means (Fig. 3-12b). It can be seen from these plots that the optimal levels for a *'larger-the-better'* outcome are: temperature level 3 (25°C), moisture content level 1 (5.3%) and substrates level 3 (3% GG). This combination of treatment will produce the optimal mycelia growth extent for a specimen incubated to grow for 6 days.



(a)



(b)

Figure 3-12: Main effects plots for (a) S/N ratios and (b) Means for radii of mycelium growth of all levels tested using the 'larger-is-better' quality characteristics on 6DAI

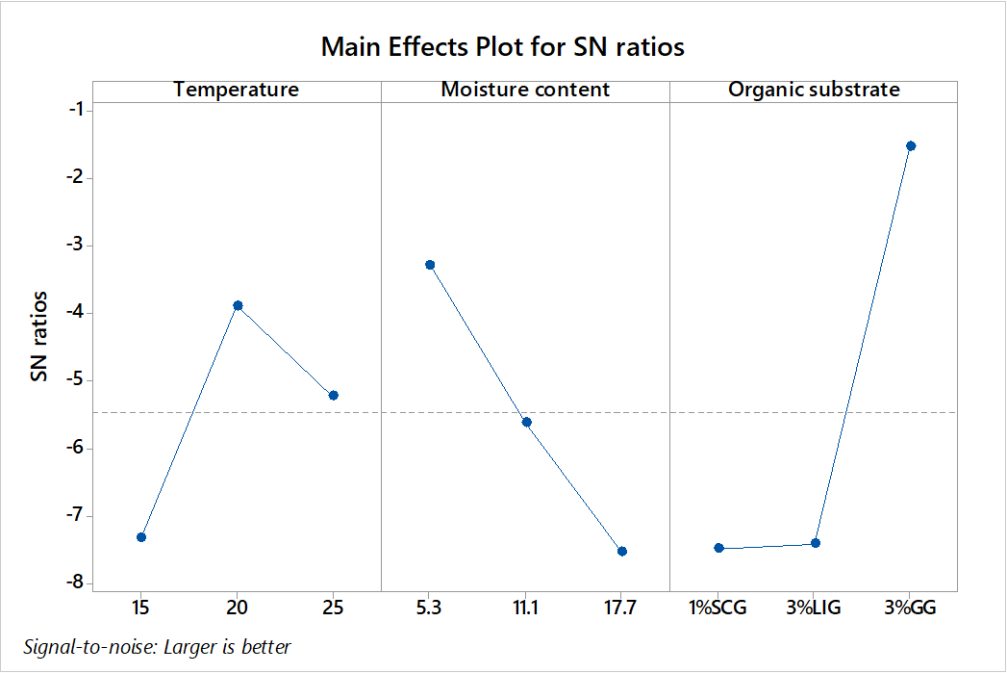
d) Optimal conditions for biomass production 6DAI

Taguchi static analysis for biomass production at 6th DAI also showed that no factor was statistically significant at $P<0.05$. The relative strength of each factor was in the order: temperature<moisture content<organic substrate

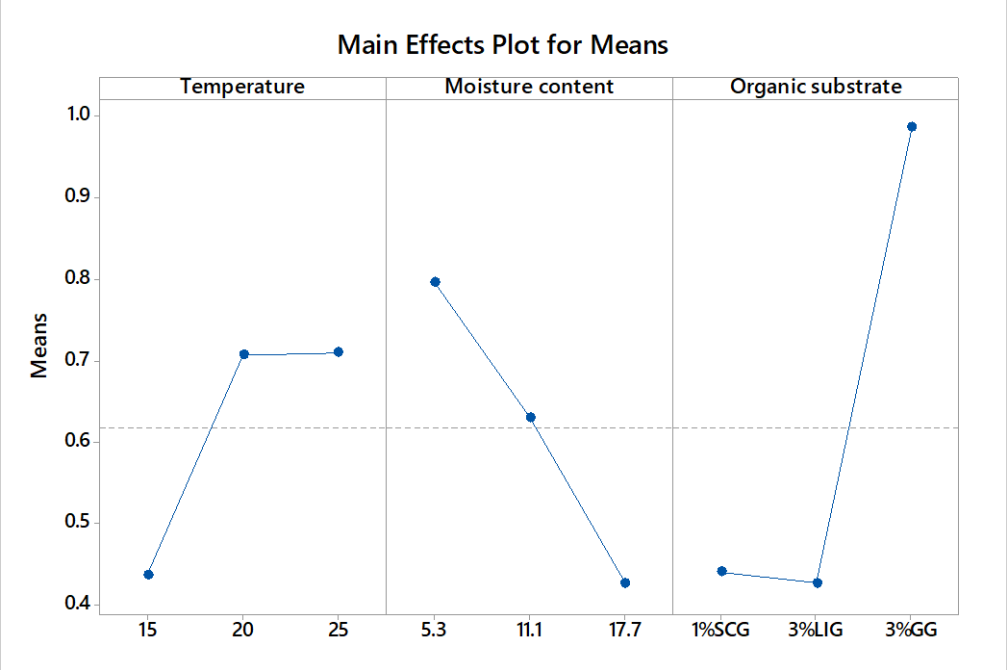
Both S/N ratios and means (Fig. 3-13) show that 5.3% moisture content (level 1) and 3% GG (level 3) were optimal conditions necessary for biomass production. In terms of temperature however, while temperature level 2 (20°C) clearly has greater S/N ratio, there existed a marginal difference between the Means for temperature level 2 and 3 (0.706 and 0.712 respectively) as seen in the main effects plot for means (Fig. 3-13b).

e) Optimal conditions for biomass production over time

From the Taguchi dynamic analyses, it was observed that the most statistically significant factors at $P<0.05$ were: (i) the organic substrates and (ii) the interaction between incubation period and organic substrates (See ANOVA Table Appendix C). Since the *'larger-is-better'* quality characteristic was implemented for this test, the ranking of the order of influence of factors was based on maximisation of the S/N ratios and slopes, whilst minimising the standard deviations. The ranking orders obtained were: for S/N ratios, Temperature<moisture content<Organic substrate; and for Slopes, Moisture content<Temperature<Organic substrates. The optimal levels that favoured highest biomass production for growth period of up to 24 days were: temperature level 2 (20°C), moisture content level 2 (11.1%) and organic substrate level 1 (1%SCG).



(a)



(b)

Figure 3-13: Main effects plots for (a) S/N ratios and (b) Means for dry biomass of all levels tested using the 'larger-is-better' quality characteristics on 6DAI

f) Optimal conditions for combined radius of mycelium growth and biomass production

Using Eqns. 3-1 to 3-4 and following steps 1 – 6 described in section 3.2.4(b), the grey relation analysis for multi-response characteristics (radius of mycelium growth, mm and fungal dry biomass, mg g^{-1}) was done to obtain optimal conditions for the combined responses. Table 3-6 shows the grey relational grades (GRG) and ranks. Factor combination with highest GRG value, ranked 1, represents the most optimal condition (Datta, Bandyopadhyay and Pal, 2008).

From Table 3-6, it can be seen that treatment T7 incubated at 25°C, with 5.3% moisture content and 3%GG ranked as 1, and therefore represents the optimal conditions of parameters for radius of mycelium growth and biomass production 6DAI. This is also corroborated by the mean GRGs in the response table for main effects on grey relational grade and graphically represented by the main effect GRG plot in Fig. 3-14. The dashed line in Fig. 3-14 is the mean of the GRGs. Temperature level 3, moisture content level 1 and organic substrate level 3 with highest mean values represent the optimal factor levels. The rankings obtained based on the attendant delta statistics (Appendix D) shows that the order of influence of each factor is moisture content<temperature<organic substrate.

Table 3-6: Calculation of the grey relational grades for the multi-response behaviours measured at 6 DAI

Treatment	Experimental results		Step 1. Data pre-processing/Grey relational generation (larger-the-better) $x_i(k) = \frac{y_i(k) - min\ y_i(k)}{max\ y_i(k) - min\ y_i(k)}$***		Step 2: Grey relational coefficients $\xi_i(k) = \frac{\Delta_{min} + \zeta\Delta_{max}}{\Delta_{oi}(k) + \zeta\Delta_{max}}$**		Step 3: Grey Relational Grade (GRG) $\gamma_i = \frac{1}{n}\sum_{k=i}^{n}(k) *$	Step 4
	Average radius of mycelium growth (mm)	Fungal dry biomass (mg/g soil)	Average radius of mycelium growth (mm)	Fungal dry biomass (mg/g soil)	Average radius of mycelium growth (mm)	Fungal dry biomass (mg/g soil)		Ranks
Ideal sequence	1	1	1	1	1	1		
T1	10.55	0.52	0.000	0.207	0.333	0.387	0.360	9
T2	21.85	0.44	0.437	0.138	0.471	0.367	0.419	5
T3	20.02	0.35	0.366	0.060	0.441	0.347	0.394	6
T4	27.50	0.43	0.656	0.129	0.592	0.365	0.479	3
T5	20.08	1.17	0.369	0.767	0.442	0.682	0.562	2
T6	16.85	0.52	0.244	0.207	0.398	0.387	0.392	7
T7	36.39	1.44	1.000	1.000	1.000	1.000	1.000	1
T8	19.50	0.28	0.346	0.000	0.433	0.333	0.383	8
T9	23.71	0.41	0.509	0.112	0.505	0.360	0.433	4

*** $x_i(k)$ is the value after the grey relational generation, min $y_i(k)$ is the smallest value of $y_i(k)$ for the *kth* response, and max $y_i(k)$ is the largest value of $y_i(k)$ for the *kth* response.

** Grey relational coefficient = $\xi_i(k)$. Δ_{0i} is the deviation sequence, i.e., absolute value of the difference between the reference sequence and the comparability sequence; $\Delta_{oi} = \|x_0(k) - x_i(k)\|$ where $x_0(k)$ = reference sequence and $x_i(k)$ = comparability sequence. $\triangle_{min}$ and $\triangle_{max}$ are the minimum and maximum values of the absolute differences ($\triangle_{0i}$) of all comparing sequences. ζ is distinguishing or identification coefficient with value ranging between 0 to 1. $\zeta = 0.5$ in this analysis.

*γ_i is the required grey relational grade for *ith* experiment and *n* = number of response characteristics.

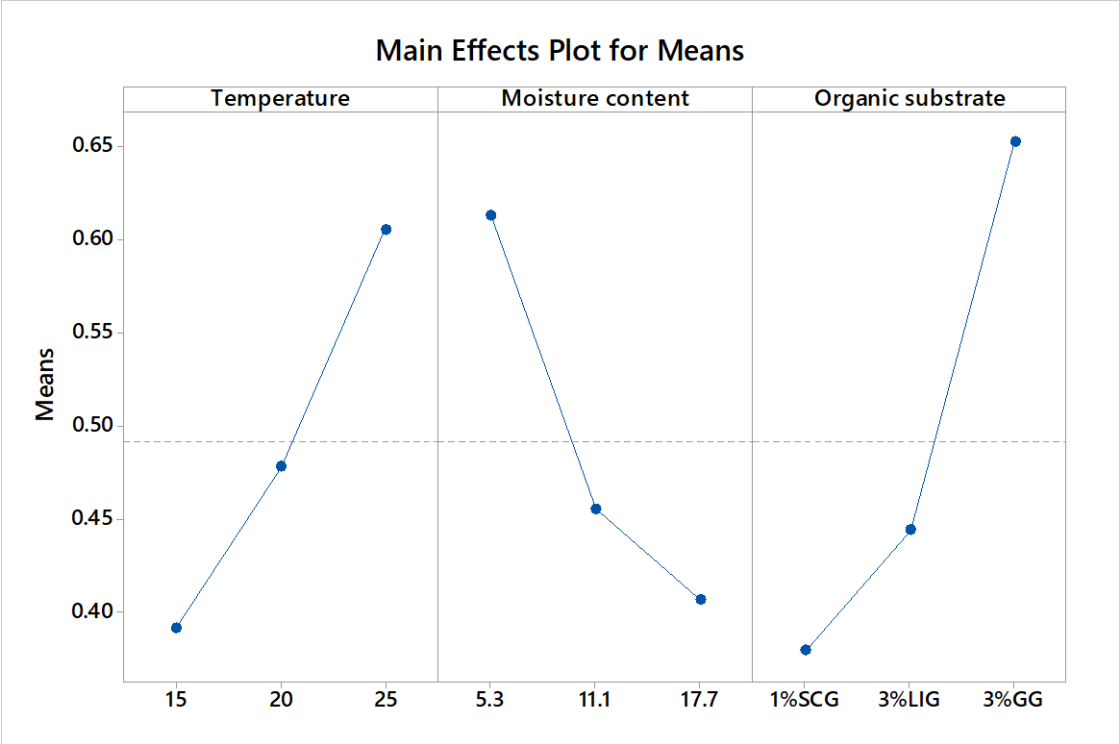


Figure 3-14: Main effects plot of grey relational grade

The F-test analysis of the ANOVA for overall GRG (Appendix E) shows that the most significant factor for radius of mycelium growth and dry fungal biomass is the type/amount of organic substrate. The percent contribution shows the relative influence of a factor on the mycelium growth and biomass. The major factor of influence (Appendix E) is clearly organic matter (with 38.23%) while temperature and moisture content (approx. 22% each) are relatively minor factors affecting the multi-response characteristics of fungal growth.

g) Confirmatory tests of improvements due to the Taguchi-Grey optimisation

After obtaining the optimal parameters, the prediction and verification of the enhancement of quality characteristics by the grey relational analysis was then performed. Using the optimal levels of radius of mycelium growth and biomass, the

estimated GRG prediction value $\hat{\gamma}$ was calculated using Eqn. 3-4. Table 7 shows a comparison of the predicted optimal conditions with the actual initial conditions tested (see sub-section 3.3.2(a)). It can be seen that the average radius of mycelium growth and fungal dry biomass both increased from 26.7 to 36.39 mm (36% increase) and 0.42 to 1.44 mg g^{-1} (~240% increase) respectively, with an overall GRG improvement of 0.479.

Table 7: Confirmatory test results using the initial and optimal factors and levels

	Initial conditions for radius of mycelium growth and biomass	Optimal conditions for mycelium growth and biomass production	
		Prediction	Experimental
Levels	25°C, 10%WC,3%LIG	25°C, 5%WC, 3%GG	25°C, 5%WC, 3%GG
Average radius of mycelium	26.7		36.39
Fungal dry biomass	0.42		1.44
Grey relational grade	0.52074	0.88764	1

Improvement in grey relational grade = 0.47926

3.3 Discussions

Selected key findings from the preliminary experiments and the Taguchi-Grey analysis are hereby highlighted with regards to practical implications for fungal growth in varied environmental conditions and some recommendations for further studies.

3.3.1 Temperature effect

Results of the preliminary tests showed that mycelium growth in sand at 5°C was not obvious until the 12th day and no growth was recorded at 35°C throughout the period of observation. This has implications for the locations and seasons in which field inoculations may be carried out. There are predictions of changes in soil temperatures induced by climate-change at given locations (Grillakis *et al.*, 2016; Oni *et al.*, 2017) with attendant implications for microbial activity in soils. The findings of this study

seem to suggest that a fungal species like *P. ostreatus* may show appreciable level of adaptability under a wide range of varied soil temperature conditions over time.

3.3.3 Moisture content

Though mycelia growth was recorded at soil moisture content of ~3 – 100%, it was observed that lower moisture contents favoured the growth of *P. ostreatus* in the soil. This is plausible since fungal hyphae would require oxygen present in soil pores to grow. Higher soil moisture content means less available oxygen and less available partially saturated pores for fungal growth. These have been found in previous studies to be significant limitations to growth of the hyphae of other saproptrophic fungi in soils (Harris *et al.*, 2003; Falconer *et al.*, 2012). In this present study, the optimal soil moisture content for mycelium growth and biomass production was found to be 5.3% (for short-term growth of 6 days) and 11.1% (for growth up to 24 days). It is arguable that the starting moisture content of 11.1% may have gradually decreased within the 24 days' incubation period, due to temperature and fungal growth, and this subsequent lower moisture meant an increase in partially saturated pores enhancing hyphal growth. In other words, as near surface soils are subjected to drying, lowering moisture content closer to the optimal soil moisture content for the fungus, there would be increased mycelia growth and biomass production in available soil pores. It is believed that the filling up of soil pores by fungal mycelial biomass and attendant biochemical exudates would have significant implications for soil hydro-mechanical properties. Further studies are required to support this proposition for growth of *P. ostreatus* in soil.

3.3.4 Organic substrates

The organic substrates were found to have the greatest influence on both mycelium growth and biomass production. As expected for a saprotrophic fungus, organic matter with higher C/N ratios is required for hyphal and cellular activities to ensure growth, survival and development. The three organic substrates used in this study differed in two key characteristics which may have contributed to the respective levels of influence they had on mycelium growth and biomass production; these are (i) particle size and (ii) C/N.

In terms of particle sizes, GG was supplied as a much finer powdery material in texture (particle size was not determined) compared to SCG which was obtained from an espresso coffee machine with its average particle sizes expected to range between 0.01 – 1 mm (Malvern, 2012; Choi *et al.*, 2017). LIG is derived from soft woods and was supplied as fibres with particle size range of 0.5 – 1.25 mm according to the supplier's data sheet (Appendix F). It is therefore expected that GG would be most easily accessible and digested by the fungi than SCG and LIG, in that order. This explains why specimens amended with GG recorded higher mycelium growth and biomass after 6 days of incubation. However, the hydration or dissolution of GG in water changes over time, depending on concentration, temperature, particle size (Mudgil, Barak and Khatkar, 2014). After initial dissolution in water, GG gradually forms into a higher viscous material binding soil particles together and retaining available soil water, making it difficult for hyphae to grow into soil pores. This would restrict mycelium growth to the soil surface only.

Although the C/N of LIG is higher than GG and SCG, its particle size would be more difficult for hyphae to absorb and digest, more so with depleting moisture content overtime, hence its limited influence on overall fungal growth and biomass production. Furthermore, addition of 1% SCG showed appreciable mycelia growth in the preliminary test and ended up as the optimal substrate level for biomass production up to 24 days. With the limitations of GG and LIG as resources depleted overtime, SCG probably performed better since its nitrogen content would have been gradually degraded in the initial growth period, leaving the opportunity for more growth during the later days. This interpretation is based on report by Cabrera, (2018) who observed initial slower growth of the hyphae of *P. ostreatus* in media amended with higher amounts of caffeine-nitrogen derived from SCG; and the specimens recorded a reduction in the amount of caffeine-nitrogen as mycelia grew over time. This explains why 1% SCG performed better than higher amounts in the preliminary tests in this present study. It is therefore plausible that mycelia experienced a relatively slower growth during the initial period (6 days) as a result of this nitrogen content.

It was also observed that when the organic substrate content is higher, growth is more localised and denser. This could have some implications for future engineering of fungal growth for soil improvement. If more distributed growth is desired it would be logical to use lower percentages of nutrients. In this study, lower respective substrate levels corresponding to the greatest extent of mycelium were chosen for optimisation thus targeting more distributed growth rather than densest fungal growth. Further studies may investigate the implications of distributed or denser mycelium growth on soil hydro-mechanical properties.

For the rest of the experiments reported in subsequent chapters of this thesis, LIG was used, despite its relatively poor performance in this present study. This was mainly due to the following reasons: (i) GG is cohesive and adhesive in nature and as such would not be ideal in terms of attempting to evaluate the hydraulic and mechanical effects of fungal growth alone in soils as the influence of GG on its own would first need to be fully characterised; (ii) SCG are difficult to standardise, as there are a plethora of coffee varieties and coffee making processes globally. The SCG used in this study was sourced from the University café with no guarantee of obtaining the exact same material another time. It is however recommended that for future studies, LIG fibres could be ground into powdery form to reduce its particle size and make it more digestible for hyphae.

Lastly, in this study, ergosterol concentrations was used to estimate fungal biomass even though ergosterol measures only live fungal biomass. There is a possibility that older fungal biomass exists, which may have senesced or began the process of cell differentiation and reproduction (Jedd and Pieuchot, 2012) and these were not captured in the biomass estimated as they are not expected to contain ergosterol. These may not be active in growth, but being present in soil, they may still potentially contribute to hydro-mechanical behaviour of soils. Moreover, as they produce spores or transform into sclerotia or mushrooms, they further ensure the sustenance of the life-cycle of the fungus in the soil.

3.4 Conclusion

The potential of engineering fungal hyphal/mycelial networks for soil improvement was the motivation behind this study. In order to provide information for further

laboratory studies, such as in the development of appropriate recipes for deployment of fungal mycelia to field sites, the influence of selected environmental conditions on fungal growth in the laboratory was investigated and the optimal conditions required for mycelium growth and fungal biomass production were determined for *P. ostreatus*. The findings of this study are as follows:

1. The hyphae of *P. ostreatus* showed average mycelium growth rates of 1.08 – 5.51 mm day^{-1} and average mycelium radii of 3.3 – 37.8 mm after 6 days growth period in sandy soils. These were recorded for specimens tested under varied environmental conditions ranging from temperature of 5 - 30°C, moisture contents of 3 – 66.7% and in soils amended with lignocel, spent coffee grounds and guar gum ranging from 1% to 15% respectively. This implies that *P. ostreatus* can grow even under extreme environmental conditions where nutrient supply may be low, and at low moisture contents.
2. In this study, the Taguchi design of experiment coupled with grey relational analyses were used to determine optimal conditions for fungal growth in soil, within the range of conditions investigated in this study. It was also shown that fungal growth characteristics are greatly enhanced by this approach, with both the radius of mycelia growth and dry fungal biomass improved by 36% and ~240% respectively.
3. The optimal environmental conditions for either radius of mycelium growth only, dry fungal biomass only or combined radius of mycelium growth and dry fungal biomass 6 days after inoculation were found to be 25°C, 5.3% moisture content and 3% guar gum as organic substrate.

4. For growth up to 24 days, where time factor is considered, the optimal conditions found were: 20°C, 11.1% moisture content and 1% spent coffee grounds.

Chapter 4

Fungal-induced Water Repellency in Soils

Abstract

Water repellent soils may be deployed in ground engineering works for applications requiring impermeable or semi-permeable barriers. Recent research has focused on investigating the suitability of chemical agents (e.g. silanes and organic acids) for inducing water repellency in soils. However, these compounds are sensitive to the presence of residual water and organic matter in soils, requiring increased amounts of chemical compounds to be added with increasing water content and losing the water repellent effect at critical water contents < 10%. Moreover, some of these chemicals are hazardous to humans and environmentally harmful; for instance, silanes react vigorously with water to produce hydrochloric acid, or with ammonia to produce a self-igniting compound while organic acids have long lasting effects on aquatic life. Furthermore, research has shown that soils treated with these chemicals exhibit increased erosion.

This chapter explores an alternative biological treatment for creating water repellent soils: fungal growth. *Pleurotus ostreatus* was grown in sterile sands and natural soils and the extent and persistency of water repellency induced was investigated. Three methods were used to assess the level of water repellency induced: (i) water drop penetration test (WDPT), (ii) Molarity of ethanol drop test (MEDT), (iii) modified sessile drop with contact angle determination via image analysis. Fungal induced

water repellency was found to be 'extreme' (contact angles >110°) after only one week of fungal growth. Water repellency was maintained in the fungal treated sand for 3 months, with contact angles remaining above 105°, even with no further supply of moisture or nutrients to the soil. Furthermore, water repellent layers were formed in sands with high initial moisture content, even up to saturated conditions, which is not possible in chemically treated sands. These results indicate that inducing fungal growth in soils is a promising treatment for creating water repellent soils and barriers for ground engineering.

4.1 Introduction

When liquid droplets form on hydrophobic or water repellent soils, the droplet forms a contact angle (CA) greater than 90° at the solid, liquid, air contact. In water repellent soils the water-entry pressure (that is required to initiate infiltration) becomes positive and thus contributes to a reduction in soil water infiltration, diversion of water from soil surfaces and an associated increase in runoff and accelerated erosion (Brandt, 1969; Bauters *et al.*, 2000; Doerr, Shakesby and Walsh, 2000; Letey, Carrillo and Pang, 2000; Feng, Letey and Wu, 2001). The extent of water repellency (WR) in soils is often described using two key terms: 'the degree (sometimes referred to as severity or magnitude or intensity)' and 'the persistence (or stability)' (King, 1981; Letey, Carrillo and Pang, 2000; Roy and McGill, 2002; Chau *et al.*, 2014). The degree of WR indicates how strongly water is repelled by the soil and is typically interpreted based on the magnitude of the CA. Persistence of WR is described by the duration that a soil remains water repellent when in contact with water, and is determined using the water drop penetration time (WDPT) test (Doerr, 1998). The persistence of WR using the WDPT test is often quantified over a relatively short time, that is, the time taken for a droplet of water to infiltrate into the soil; this does not necessarily imply a prolonged sustenance of WR in bulk soil for extended spatio-temporal durations (e.g. for many weeks, months or years). Some authors have classified the persistence of WR based on WDPT into slight, moderate, severe or extreme for ranges of WDPT between 6 – 60s, 61 – 600s, 601 – 3600s and >3600s respectively (Bisdom, Dekker and Schoute, 1993). Lourenço *et al.*, (2015) described the persistence of WR for some soils as 'permanent' when the WDPT exceeded 3 hours.

The extent of WR obtained for a given soil may depend on the process by which water repellency is induced. Surfaces of soil particles can become water repellent if they are 'coated' with hydrophobic compounds (Bisdom, Dekker and Schoute, 1993; Doerr, Shakesby and Walsh, 2000; Diehl and Schaumann, 2007). This may happen in several ways: (i) by the deposition of plant-derived organic matter such as plant oils, waxes or humic substances on soils (Debano, 2000) (ii) when soils are subjected to high temperatures (e.g. wildfires, smouldering) leading to vaporisation of organic materials and deposition of organic solutes which coat soil grains (Dekker, 1998; Doerr *et al.*, 2006; Rye and Smettem, 2015), (iii) from microbial and fungal secretions containing natural hydrophobic compounds (Rillig, 2005) and (iv) by the use of chemically-based hydrophobising agents such as silane compounds or organic acids to produce synthetic water repellent soils (Bachmann, Ellies and Hartge, 2000; Leelamanie, Karube and Yoshida, 2008a; Lee *et al.*, 2015; Lourenço, Wang and Kamai, 2015; Ng and Lourenço, 2015; Chan and Lourenço, 2016).

Induced WR alters the hydraulic (and thus mechanical) behaviour of unsaturated soils such that both water infiltration and evaporation are reduced (Shokri, Lehmann and Or, 2009; Beckett, Fourie and Toll, 2016). Although detailed characterisation of the hydro-mechanical behaviour of WR sands is still a topic of research in the geotechnical engineering community (Beckett *et al.*, 2018), current understanding suggests that the creation of water repellent soils hold the potential to be deployed in the formation of impermeable or semi-permeable barriers for a range of applications within ground engineering including to divert water away from expansive soils, to minimise infiltration into cut slopes, to reduce seepage through flood embankments, to create

barriers suitable for use in landfill caps, or canal/trench liners as outlined by Lourenço *et al.*, (2018).

4.1.1 Background: state of the art

Recent studies in geotechnical and geo-environmental engineering have focused on the chemical treatment of soils using silane compounds like dimethyldichlorosilane (DMDCS) and organic acids like stearic acids (SA) to induce water repellency for several purposes at laboratory scale (Komatsu *et al.*, 2012; González-Peñaloza *et al.*, 2013; Lourenço *et al.*, 2015; Ng and Lourenço, 2015; Wijewardana *et al.*, 2015; Chan and Lourenço, 2016; Zheng *et al.*, 2017). DMDCS is a colourless liquid which undergoes hydrolysis when in contact with residual water in the soil to form polydimethylsiloxane (PDMS) and hydrogen chloride gas as shown in equation 1 below (Goebel *et al.*, 2007). WR surfaces are formed on soil grains by the outwardly oriented methyl (-CH_3) group obtained when the oxygen atoms in PDMS are bound to the polar groups of the soil particle via electrostatic forces (Goebel *et al.*, 2007; Ju, Ren and Horton, 2008).

$$n[Si(CH_3)_nCl_2] + n[H_2O] \rightarrow [Si(CH_3)_2O]_n + 2nHCl$$

Where *n* represents the number of repeating monomer units.

Chan and Lourenço, (2016) reported that DMDCS generated extreme water repellency (CAs >123° and WDPT > 5hrs) in soil grains with the addition of about 0.004% by soil mass of silane. They observed that even a marginal increase in residual soil water content led to a decrease in CAs, meaning that the amount of residual moisture in the soil poses a significant constraint to the attainment and durability of water repellency

in soils treated with DMDCS (Ng and Lourenço, 2015). The National Centre for Biotechnical Information describes DMDCS as a toxic and highly flammable chemical. The hydrolytic reaction (eq. 1) occurs vigorously and the HCl gas produced is hazardous to humans and the environment. Although soils hydrophobised by DMDCS become inert and safe to handle (Goebel *et al.*, 2007), this process might be unsafe for *insitu* soil treatment as it could lead to an increase in soil acidity and cause harm to soil organisms. The fate of DMDCS in the ground over time and under changing environmental conditions is unknown.

SA is a saturated fatty acid with a long-chain chemical structure. It is found in natural soils and is able to induce WR in soil particles due to the interaction of its hydrocarbon chain, containing a methyl group, with soil mineral surfaces (Cheng *et al.*, 2010). Several studies have used SA to achieve strong water repellency in soils (Piccolo and Mbagwu, 1999; Leelamanie and Karube, 2007; Leelamanie, Karube and Yoshida, 2008a; González-Peñaloza *et al.*, 2013; Subedi *et al.*, 2013). For instance, using 6 g SA kg^{-1} soil, (Leelamanie, Karube and Yoshida, 2008a) obtained CA of 110° and WDPT >3600s. Due to its insolubility in water, SA is often dissolved in diethyl ether before mixing with soil. Compared to DMDCS, SA is less hazardous, though reportedly harmful to aquatic life with long lasting effects (Cayman Chemical, 2014; according to Regulation (EC) No. 1907/2006 (REACH), amended by 2015/830/EU).

Natural and chemically induced WR in soils are influenced by soil water contents (de Jonge, Jacobsen and Moldrup, 1999). While there are contradictions on the nature of the influence, there seem to be general agreement that at a certain water content known as the critical water content (CWC), soils transition from being WR to being wettable

(Dekker and Ritsema, 1994; Lichner *et al.*, 2006; Leelamanie and Karube, 2007; Chau *et al.*, 2014). A clear comparison of the relationship between soil WR achieved and soil water content reported in the literature is difficult due to varied ranges of soil types, desired objectives and the test approaches/methodologies adopted. In most cases, already hydrophobic/hydrophobised soils were used (Leelamanie, Karube and Yoshida, 2008; González-Peñaloza *et al.*, 2013; Chau *et al.*, 2014) and variation of water content was achieved by using water vapour or controlling the relative humidity (Doerr *et al.*, 2002; Goebel *et al.*, 2004; Leelamanie and Karube, 2007; Leelamanie, Karube and Yoshida, 2008) or by drying soils through air-dried to oven dried water contents (King, 1981; Leelamanie and Karube, 2007; González-Peñaloza *et al.*, 2013). In one of the cases where soils were wetted prior to hydrophobisation, the chemical agent (DMDCS) reacted with the residual moisture to induce variable levels of WR (Chan and Lourenço, 2016). In general, the soil water contents considered were often < 20% with critical water contents attained at ≤5% for soils with higher fractions of sand; above the critical water content, water repellency could no longer be induced via chemical means.

The presence of organic matter is also known to affects the extent of WR induced. While the presence of organic matter was found to constrain WR induced by DMDCS (Ng and Lourenço, 2015), an increase in the persistence of WR was observed with treatment using SA (Leelamanie and Karube, 2007), however, this effects may not be consistent, as organic matter can be hydrophobic or hydrophilic depending on the type of organic compound they are composed of (Piccolo and Mbagwu, 1999; Leelamanie and Karube, 2007).

There are concerns that chemically induced water repellency in soils leads to increased surface overflow and soil erosion (Zheng *et al.*, 2017), which could mean loss of nutrient-rich top soils and hence negative effects on the soil ecology, resulting in less vegetation, loss of soil microbes/biodiversity and soil organic matter. Based on these characteristics of the hydrophobising agents highlighted so far, it is clear that they may not be suitable for deployment in field conditions within the context of environmental sustainability. There is therefore a need to seek out other less hazardous and ecologically friendly treatment alternatives, which are not constrained by existing soil moisture and are capable of inducing similar levels of water repellency in soils as the chemical agents, and do not contribute to increased soil erodibility. It is hypothesised that engineering of the growth of the hyphae of fungi in soils may be able to fulfil these requirements for sustainably inducing WR in soils.

4.1.2 Water repellent characteristics of fungi

Filamentous or mycelia-forming fungi, mostly those of the Ascomycota and Basidiomycota family, grow via the formation of thin thread-like strands (hyphae) which also serve as distribution channels for the unique protein compounds they secrete. These exudates, called hydrophobins, bestow amphipathic behaviour to fungi, making them capable of hydrophobising hitherto wetting surfaces and vice-versa (Wessels *et al.*, 1991; Wessels, 1996, 2000; Wösten, 2001; Kallio, Linder and Rouvinen, 2007; Chau *et al.*, 2012).

Fungi are ubiquitous. They typically grow by forming wide-spreading, massive networks of mycelia within the top 20 cm depth of soil surfaces, with the presence of

fungal biomass reported at 2 m depths in some forestlands (Churchland and Grayston, 2014). The mycelia networks and the hydrophobins secreted by fungi together enhance physical enmeshment of soil particles and formation/maintenance of micro/macro-aggregates which are known to influence the hydro-mechanical behaviour and erodibility of soils where they are naturally present (Tisdall and Oades, 1982; Hallett *et al.*, 2004; Rillig and Mummey, 2006; Tisdall *et al.*, 2012). Previous studies that have investigated the influence of fungi on soil WR have been aimed at (i) exploring the amphipathic behaviour of selected fungal strains (*Alternaria sp., Trichoderma harzianum,* and *Fusarium proliferatum*) with the aim of manipulating wetting properties of fungi to obtain desired soil infiltration characteristics (Chau *et al.*, 2012) and (ii) providing better understanding of the effect of WR induced by two white-rot fungi (*Phanerochaete chrysosporium* and *Coriolus versicolor*) that are of significance within the context of mobility and bioavailability of soil pollutants, for the purpose of bioremediation and restoration of remediated soils (White *et al.*, 2000).

It is believed that these fungal attributes and characteristics would have potential significance in geotechnical engineering applications as well. Exploration of the potential benefits of the incorporation of fungi in ground improvement technologies needs to begin with the specific characterisation and holistic understanding of the extent of changes that fungal species can impart in soils from a geotechnical engineering viewpoint (El Mountassir *et al.*, 2018). This is of interest to understand if short term growth of fungal mycelia can induce appreciable levels of WR in soil; and what happens to fungal induced WR over time under depleting resource conditions/environments, such as may be the case for deployment in arid areas? Does

limited and/or excess soil moisture conditions affect the ability of fungus to induce WR? What happens to the fungal induced WR in cases of external physical interference, by humans or animals, in ways that are capable of disrupting fungal growth and mycelial networks?

This chapter aims to quantitatively describe, for the first time, (within a geotechnical engineering context and using standard methods), the extent of water repellency induced in soils treated using the mycelia of *Pleurotus ostreatus*, a non-pathogenic, non-parasitic, saprotrophic, filamentous fungus of the Basidiomycota division, whose fruiting body (mushroom) is widely consumed globally. Specifically, the objectives were to: (1) determine WR induced by *P. ostreatus* in natural and sterile soils after a growth period of 1 week, (2) assess the degree and persistence of WR in sterile sands over 12 weeks of fungal growth in depleting moisture and nutrient conditions, (3) investigate the influence of varied ranges of initial water content on WR induced in sterile sands by treatment with *P. ostreatus* and (4) investigate the influence of soil mixing or disruption of the mycelial network on the persistence of WR. Based on the findings of this study, comparison was made between the WR achieved through treatment with *P. ostreatus* and that achieved via chemical hydrophobising agents as reported in the literature. The implication of the level of water repellency induced in soils by the fungus is also discussed with respect to its potential for application in ground engineering.

4.2 Materials and Methods

4.2.1 Experimental design

Details of the respective types of treatments, treatment conditions, specimen incubation periods and test intervals as well as test methods used for each experiment conducted to achieve test objectives are presented in Table 4-1 below.

4.2.2 Materials

a) Soils

Three soil samples including a fine silica sand and two natural soils were tested in this study; sterilised fine sand (FS), pyroclastic silt (PS) and a very silty sand (SS). The FS was amended with lignocellulose (described below) as a carbon source for fungal growth and sterilised by autoclaving at 121°C for 21 minutes. The FS particle sizes ranged from 0.075 – 0.425 mm, D_{60} = 0.33, D_{10} = 0.2. PS and SS were collected from Mount Faito in Campania region of Italy and 'Rest and be Thankful' in Argyll Scotland, respectively. Particle size distribution curve for PS is given in Papa *et al.*, (2012) while the SS is described in Rayner and Nicoll, (2012). These soils were selected as both sites have a history of recurrent landslides and sloped failures triggered by rainfall. Samples were collected from 2 – 20 cm depths, air dried in the laboratory at 22°C and sieved through 2 mm mesh size before the tests. The natural soils, PS and SS, were not sterilised prior to inoculation with fungi, nor was any lignocel or other nutrients added to these soils.

Table 4-1: Experimental design details showing treatment conditions and testing methods for respective specimens (wi = initial moisture content)

Experiment no.	Soil Specimen	Treatment	Test objective	Treatment conditions	Incubation period/test intervals	WR test methods
Experiment 1	FS, PS, SS	Spore suspension of *P. ostreatus* for FS; grains of fungal spawn for PS and SS	Short term WR in sterile sand and natural soils	w_i=11.1% Constant RH of 100% during growth period in dark sealed plastic boxes incubated at 25°C	1 week only	WDPT; MEDT; Modified SDM; Goniometer - for PS, SS only
Experiment 2	FS	Spore suspension of *P. ostreatus*	Effect of depleting growth resources on WR	w_i=11.1% RH of 40% during growth period in incubator at 25°C	1, 2, 4, 8 and 12 weeks	
Experiment 3	FS	Mycelia-colonised beech wood inoculants	Effect of varied initial water contents on WR	w_i = 3.1, 5.3, 11.1, 17.7, 25 & 100. RH = 100%. Incubated at 25°C	1 week only	WDPT
Experiment 4	FS	Grains of fungal spawn	Effect of soil mixing and disruption of fungal growth on WR	w_i=11.1% Constant RH of 100% during growth period in dark sealed plastic boxes incubated at 25°C	3 weeks in total; On the 7th day, specimens were tested, mixed thoroughly and incubated again. The mixing process was repeated every 48 hrs up to the 12th & 21st days when tests were performed	WDPT; MEDT & Modified SDM were also done on the 12th and 21st days

b) Lignocel

These are particles of natural wood fibres (size 0.5mm – 1mm; Grade HB 500 – 1000) obtained from J. RETTENMAIER & SÖHNE GmbH containing lignin, cellulose and hemicellulose served as carbon source for the growth of the mycelia and was added to FS for all tests.

c) Inoculants

Fungi grow through the propagation of its spores. In this study, three methods were used for inoculation of spores of *P. ostreatus* on soils for respective tests; they are: (i) spores/hypal suspension derived from spawn; used for inoculating FS for assessment of WR from 1 to 12 weeks (ii) grains of fungal spawn, that is cereal grains colonised by mycelia of *P. ostreatus* obtained from GroCycle® UK; these were used for inoculation on the natural soils - PS & SS, and on FS for investigation of the influence of disruption of hyphal growth on WR; (iii) cubes of beech wood colonised by mycelia; used as inoculant in FS to investigate influence of moisture content on WR.

4.2.3 Preparation of specimens

a) Treatment of FS using spore/hyphal suspension:

Spores of *P. ostreatus:* strain M 2191 were harvested from spawn as follows. A fixed mass of spawn was collected and placed in conical flask containing deionised water with a mass ratio of spawn of 0.1 g spawn per mL of DI water. The flask was covered with aluminium foil and vigorously shaken continuously for 10 minutes then placed on a shaker at 150 rpm for 30 minutes followed by another 5 minutes of vigorous

shaking to facilitate detachment of mycelial spores from the grains into the water. Using a 2 mm mesh sized sieve, the grains were filtered out to obtain the filtrate. The filtrate obtained is a suspension containing both harvested spores and hyphae. This was the liquid treatment added to the fungal-treated FS specimens in Experiments 1, 2 and 4 (Table 4-1). All procedures were carried out aseptically.

Respective masses of FS, lignocel and spore/hyphal suspension (or water for untreated specimens) totalling 90g were mixed by hand in the ratio 85%: 5%: 10% to obtain a homogeneous specimen and compacted in petri dishes to a target bulk density of 1.1g cm^{-3}. The initial liquid content (defined as mass of suspension/mass of dry solids) was thus 11.1%. Specimen surfaces were levelled using a spatula to limit surface variation. All procedures were carried out aseptically to minimise risk of contamination.

b) Inoculation of soils using grains of fungal spawn

Treatment of PS and SS in Experiment 1 was achieved by inoculation with 4 single grains of spawn of approximately same masses. PS and SS had initial water contents of 12.3% and 14.1% at the time of inoculation. 45 g of respective soil was lightly compacted into a 9 cm diameter petri dish and grains of spawn were placed at cardinal points on the surface of specimens.

For the determination of effect of disruption of hyphal growth on WR (Experiment 4), 8 grains of spawn were placed randomly on the surface of the specimen. The specimen was composed of 40 g of FS mixed with 2.4 g of lignocel and 4.7 mL of deionised water. Controls were prepared with similar composition but not inoculated with fungi. All specimens were incubated in the dark at 25°C.

c) Preparation of specimens for determination of influence of varied soil water contents on WR

Mycelia-colonised beech wood inocula were prepared according to the method described in Chapter 3. Briefly, 0.5 x 0.5 x 0.5 cm^3 sized cubes of beech wood were added to a 7-day old culture of *P. ostreatus* growing on malt extract agar in a conical flask. This was incubated for 14 days in order to allow for colonisation of the wood cubes by fungal mycelia. Excess mycelium were scrapped off each inoculum before being introduced into the soil. 20 g of FS was mixed with 1 g of lignocel in 6 different petri-dishes. These were each made up to pre-determined water contents of 3.1, 5.3, 11.1, 17.7, 25 and 100% (using: *w*% = (mass of water/mass of solids) x 100). Each specimen was inoculated by placing a cube of colonised beech wood on the soil surface in the middle of the petri-dish.

4.2.4 Assessment of water repellency

a) Water drop penetration time (WDPT) test

The persistence or time delay for a droplet of water to penetrate into the soil was assessed using the WDPT test. The time taken for a droplet of water, 10 µL in volume, released from a height of 5 mm above the soil surface to completely infiltrate into the soil specimen was recorded (After Letey, 1969; Doerr, 1998). This was repeated 5 times for each specimen. The relationship between the water penetration time, levels of water repellency and equivalent contact angles for sandy soil as summarised from Bisdom, Dekker and Schoute, (1993), Doerr *et al.*, (2006), Dekker and Ritsema, (1994) and Liu *et al.*, (2012) are presented in Table 4-2.

Table 4-2: Relationship between WDPT, level of water repellency and equivalent CAs for sands

Water drop penetration time (s)	Level of water repellency	Equivalent contact angle θ for sands (°)
<5	Hydrophilic/non-water repellent	0
6 – 60	Slight	0 – 80
61 – 600	Moderate	80 – 110
601 – 3600	Severe	
>3600	Extreme	>110

b) Molarity of Ethanol Droplet (MEDT) test

In soil science, soil water repellency is widely assessed using the MEDT which is based on the inverse relationship of ethanol surface tension to the concentration of its solution in water; as the molarity of ethanol increases, its surface tension at the liquid air boundary decreases. This method is valid for the estimation of contact angles ranging between 90 - 109° (Doerr, 1998; Roy and McGill, 2002; Moody and Schlossberg, 2010). The procedure used in this study followed the methods outlined in Leelamanie, Karube and Yoshida, (2008) and Carrillo, Letey and Yates, (1999). Solutions of ethanol at increasing concentrations from 0 – 6 mol L^{-1} (at increments of 0.2 mol L^{-1}) were prepared. Using a pipette, droplets of ethanol solution (vol. 50 µL, n = 3), beginning with the lowest concentration, were released from a height of 5 mm above the surface of the specimen. Water repellency is determined based on the lowest concentration of ethanol ('x' in Eqn. 4-1) which can fully infiltrate the soil specimen within 10 seconds. The 90° surface tension ($\gamma_{90°}$) can thus be determined (Eqn. 4-2) and subsequently the contact angle (θ) of the specimen (Carrillo, Letey and Yates, 1999; Leelamanie, Karube and Yoshida, 2008) (Eq. 4-2). The surface tension of water is considered to be γ_w = 71.96 mN m^{-1} at 25°C.

$$\gamma_{90°} = 61.35 - 15.46\, ln(x + 0.5) \quad (4\text{-}1)$$

$$cos\theta = (\gamma_{90°}/\gamma_w)^{0.5} - 1 \quad (4\text{-}2)$$

c) Modified sessile drop method (SDM) with CA determination via image analysis using the Low Bond Axisymmetric Drop Shape Analysis (LBADSA)

The well-known sessile drop method (SDM) (Bachmann, Ellies and Hartge, 2000; Bachmann *et al.*, 2003) involves estimation of the apparent contact angle from images of the released water droplet on a monolayer of soil particles spread onto a double-sided adhesive tape attached to one side of a microscope (glass) slide. This method has been used, with slight modification, to determine surface hydrophobicity (or contact angles) of undisturbed fungal plugs sampled from actively growing cultures (Chau *et al.*, 2009). In this present study, given that the fungal growth occurs directly on the specimen surface (rather than as a grain coating) it was considered that sampling of the grains using double-sided adhesive would be too invasive creating too much disturbance of the interaction between the fungal growth and the grains. Therefore, a modified method of the sessile drop method was conducted, where timelapse images of the water droplets released on to the bulk specimen surfaces in the petri-dishes were taken at 1s intervals using a 1000X-USB-Microscope-Camera. Care was taken to ensure a levelled specimen surface prior to fungal growth, water droplets were of vol. 10 µL, (n =3 per specimen). The contact angle was estimated from the images using the LBADSA plugin (Stalder *et al.*, 2010) in open source image processing software, *ImageJ*.

The Low Bond Axisymmetric Drop Shape Analysis (LBADSA) tool analytically estimates contact angles for images of axisymmetric sessile drops (Fig. 4-1(a)) by solving the Young-Laplace equation with the help of a first order perturbation technique (see: Stalder *et al.*, 2010). An optimised image-energy parameter together with an image interpolation model makes this technique efficient for estimating CA

from various image types and qualities. Instead of estimating CAs with reference to a baseline as obtained in the goniometer, the LBADSA considers the entire droplet profile thus being versatile for use even when image views are unclear as a result of roughness of surfaces.

d) Modified sessile drop method (SDM) with CA determination using a Goniometer

Similar modification to the SDM whereby water droplets were placed directly on the bulk specimen surface to ensure minimal disturbance was repeated for the natural soil specimens while a goniometer (KRÜSS® GmbH, DSA20 Easydrop^{TM}) along with the KRÜSS 'Advance' software was used to capture the sessile drop images and directly obtain the respective contact angles (Fig. 4-1(b)) in real time. A similar method was used by Smits *et al.*, (2003) for obtaining CAs of mycelia growing on agar blocks. Here, this technique was used to check the consistency of the data obtained from the image analysis method described in sub-section 4.2.4(c) above which is a cheaper option in the absence of a goniometer.

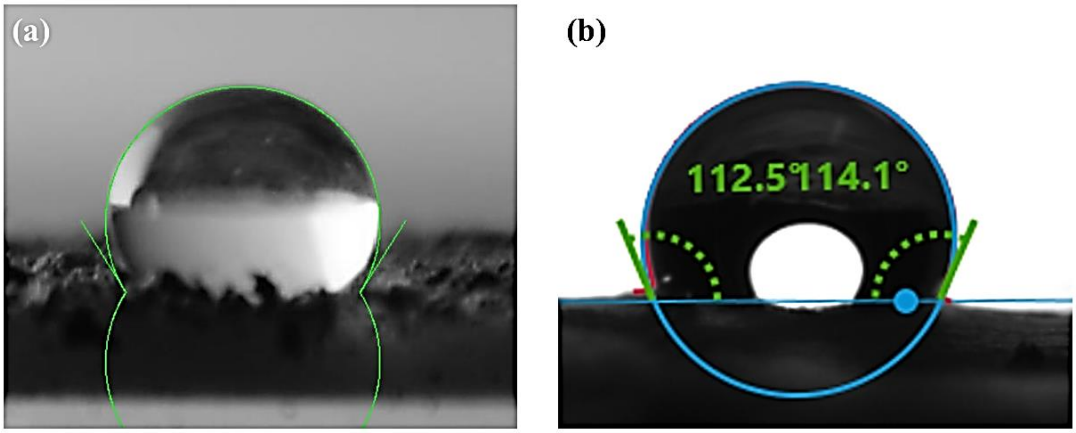


Figure 4-1: (a) An axisymmetric water droplet profile fitted with the drop shape analysis tool based on an approximation of the Young-Laplace equation (Stalder *et al.*, 2010) *for estimation of CAs. (b) Typical estimation of CAs for natural soil specimens using the ellipse fitting method in the Goniometer at University of Strathclyde*

4.3 Results and Discussions

4.3.1 Experiment 1: WR induced by *P. ostreatus* in sterile and natural soils after 1 week

Table 4-3 presents the summary of the WR determined using the various methods for sterile fine sand (FS) and natural soils - pyroclastic silt (PS) and very silty sand (SS). For all of the untreated control specimens of these three soil types, water droplets infiltrated within ≤ 2s; whereas for all the treated specimens in both sterilised and natural soils the water droplets remained on the surface of the treated specimens beyond 24hrs (Fig. 4-2) for each of the 5 repeats conducted per specimen. Observations were ceased after 24hrs as evaporation contribute to reduction of the water droplet volume.

In terms of contact angles (CAs), the mean contact angle (of 3 tests per specimen) is reported in Table 4-3. For all three soil types CAs of 109° were obtained for the MEDT, which is the maximum for this test, as it is dependent on ethanol concentration. For the natural soils the CAs determined using the goniometer and those determined by the modified sessile drop method via image analysis were in good agreement, validating the image analysis technique used here. It should be noted here that the natural soils were not sterilised nor were they amended with any additional carbon or nitrogen sources to stimulate fungal growth, beyond that present in the inoculating medium (grains of fungal spawn). Despite this, extreme water repellency (defined as >110° in Table 1, or 'severe WR' for silts (Beckett, Fourie and Toll, 2016)) was observed in both sterile and natural soils treated with *P. ostreatus* after only 1 week of growth. These results suggest that deployment and growth of single fungal strains in

natural soils is possible to create WR soils in a short time frame, even where presumably mixed microbial communities already exist. Further work is required to investigate the extent of growth possible with no further amendment of carbon and nitrogen and the potential for fungal species to be out-competed in the natural environment.

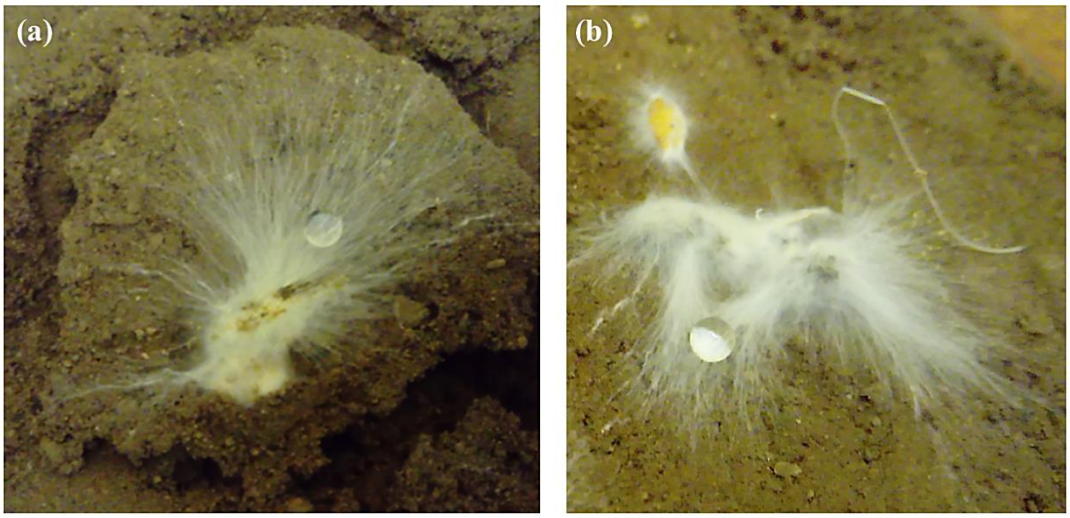


Figure 4-2: Water droplets on mycelia of P. ostreatus (t = 1 week) growing on (a) PS and (b) SS

Table 4-3: WDPT and CAs for fungal treated specimens after 1-week growth period.

Soil type	WDPT (hrs)	Contact Angle (°)		
		MEDT	Modified SDM	Goniometer
FS	>24	109	113 ± 0.7	NA
PS	>24	109	114 ± 1.2	113 ± 0.9
SS	>24	109	112 ± 0.1	111 ± 1.4

4.3.2 Experiment 2: Assess the degree and persistence of WR induced on FS by *P. ostreatus* over 12 weeks of growth

Figure 4-3 shows water droplets 2 hrs after placing them on the surface of a treated FS specimen which had been incubated for 12 weeks. The WDPT and CAs plotted against incubation period are shown in Fig. 4-4. WR was extreme and persistent 8 weeks after incubation of specimens, with WDPT > 3600s. This reduced by the 12th week as WDPT decreased markedly to 605s. The CAs determined via MEDT decreased

slightly from 109° at 2 weeks after treatment to ~107° by the 12th week, while the CA determined using the modified SDM decreased from 118° at the end of the first week to 105° after 12 weeks of incubation. Overall, the results show that even with no additional moisture or nutrients supplied to the sterile sand specimens after treatment, WR persisted for the entire 12 weeks investigated. However, both WDPT and CAs decreased, showing a change from 'extreme' to 'severe and moderate' hydrophobicity from the 4th week of incubation (Fig. 4-4). The conditions tested here can be considered to be harsh environmental conditions; if fungal growth was to be stimulated in the field, it is unlikely within a European context that three continuous months with no rainfall would be encountered. Furthermore, these tests were conducted in sterile sands with depleting nutrients over time. In the field, typically some organic matter would be present in natural soils, particularly near surface and could contribute to maintaining fungal activity/growth.

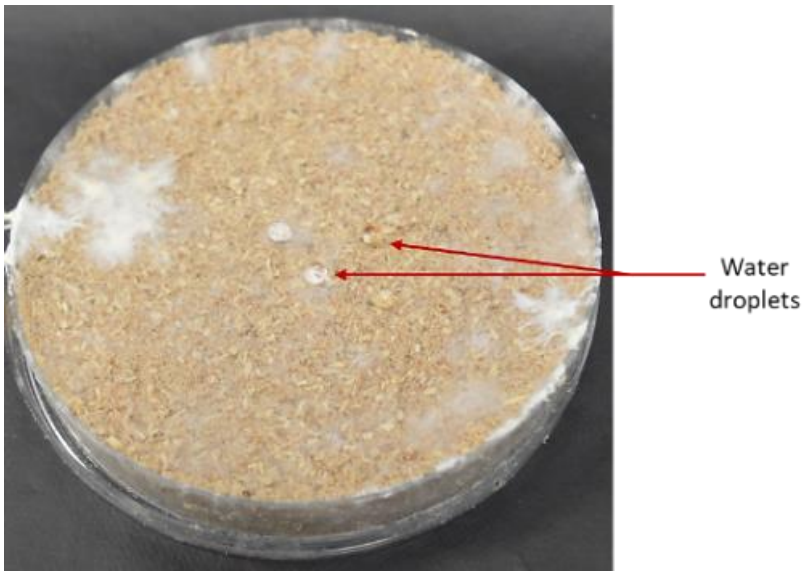


Figure 4-3: Water droplets did not infiltrate up to 2hrs after they were placed on the surface of a 12-week old treated FS specimen.

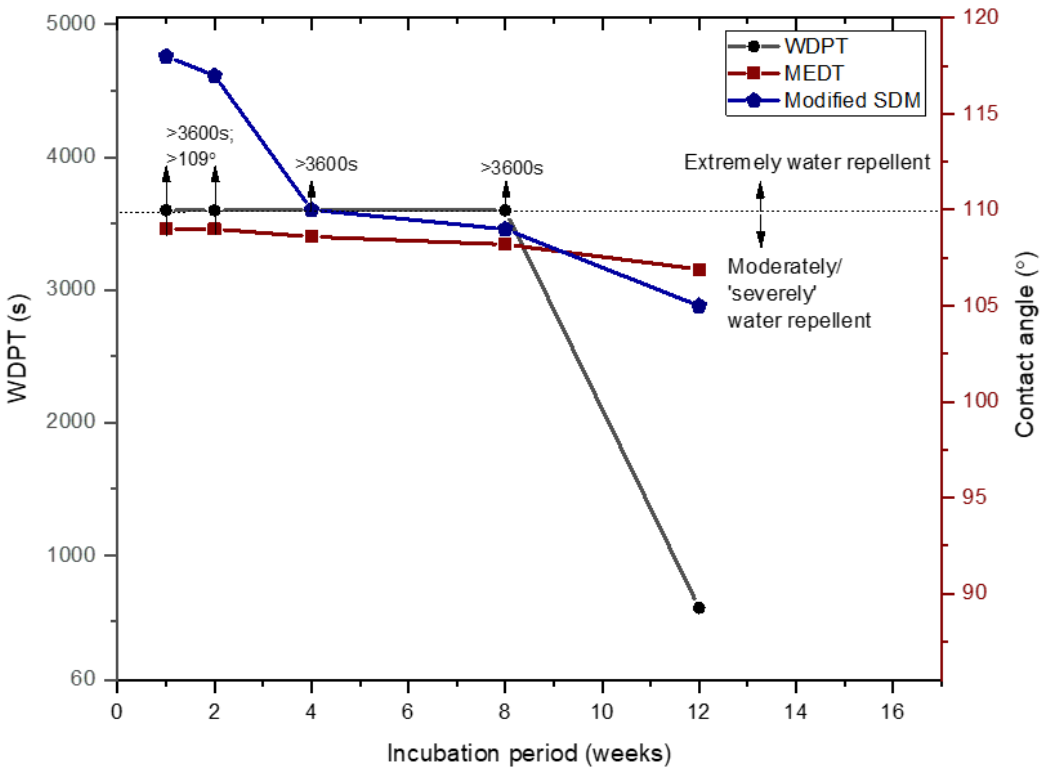


Figure 4-4: Trends in WDPT and CAs for treated specimen tested from 1 – 12 weeks after incubation

4.3.3 Experiment 3: Effect of varied initial water contents on the WR induced on FS by *P. ostreatus*

Mycelium growing radially from the colonised beech wood inoculant are visible on the surface of specimens at respective water contents shown in Fig. 4-5. The red rectangle in Fig. 4-5c show water droplets on a portion of the inundated specimen (100% moisture content).

The change in WDPT at the initial water contents of respective FS specimens investigated is shown in Fig. 4-6.

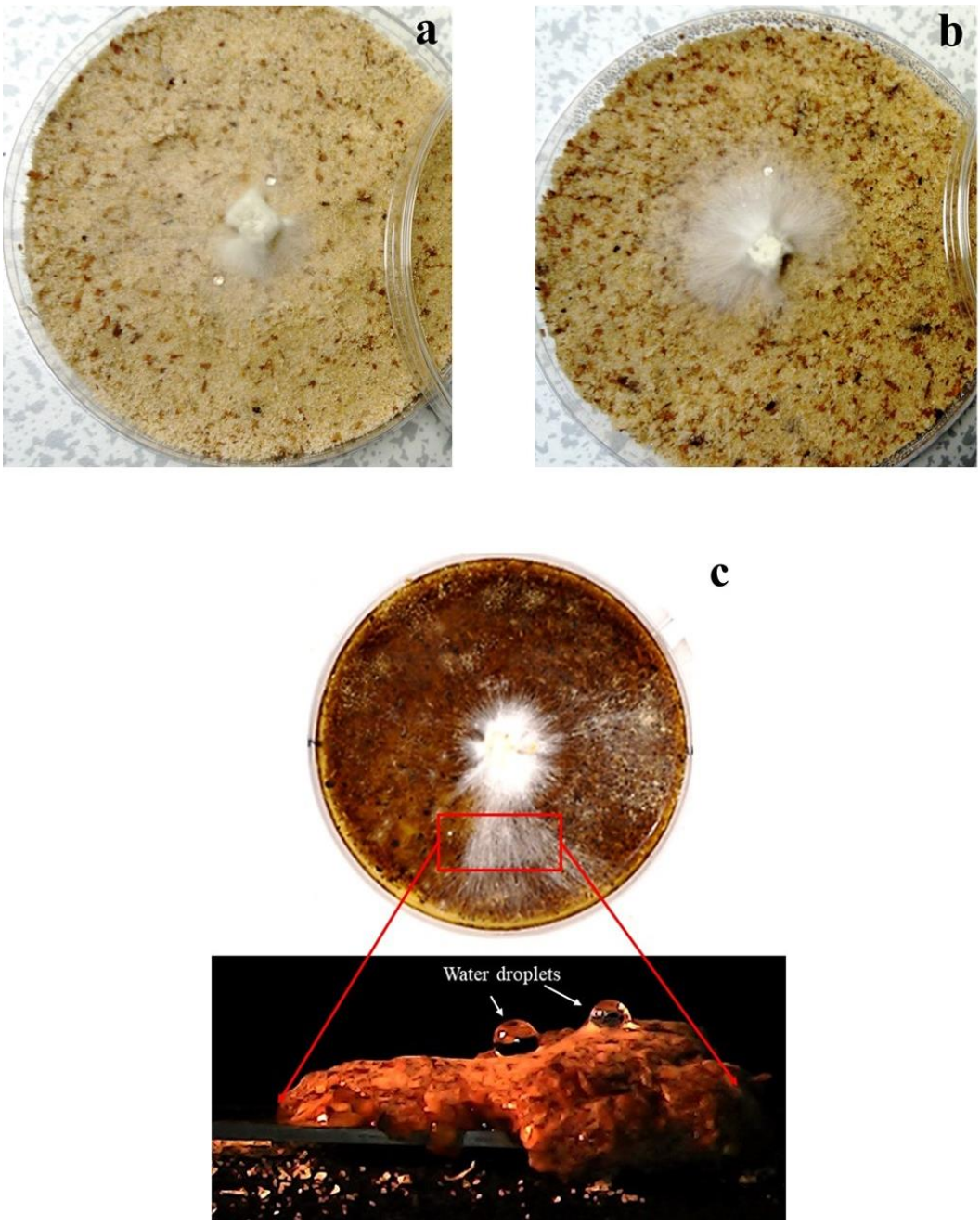


Figure 4-5: Mycelia growing radially from the beech wood inoculant in specimens at water contents of (a) 5.3% (b) 17.7% and (c) 100% on the 7th day after incubation. Water droplets are visible on the surface of specimens (a) and (b) and on a portion of the saturated specimen sampled on a slide.

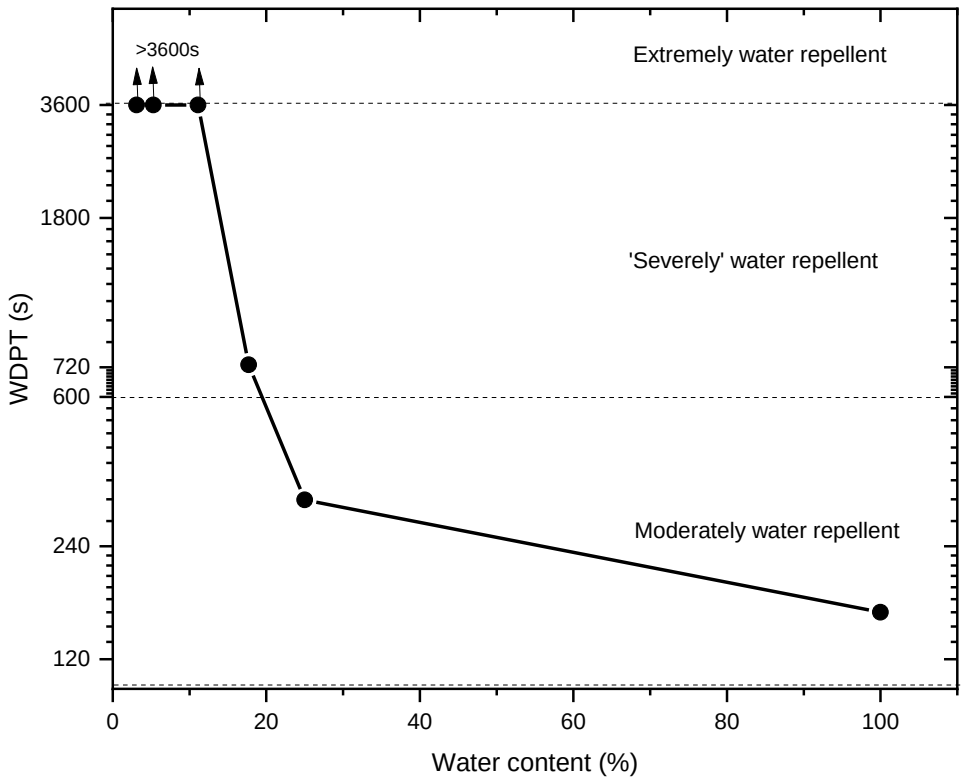


Figure 4-6: A graph of WDPTs against soil water contents. Respective levels of hydrophobicity are shown based on classification/relationships in Table 1.

WR was consistently extreme (>3600s) as water content increased from 3.1 to 11.1% but decreased to 'severe' and 'moderate' levels with further increases in water content, through 17.7 to 100%. It is worth noting that WR was not totally lost despite increasing water contents up to or beyond saturation. However, once the soil became fully saturated and subsequently inundated (at 100% moisture content), WR was not directly induced in the soil; rather, mycelia formed a hydrophobic layer on the water surface above the soil (Fig. 4-5c). It was observed that irrespective of initial soil water content, *P. ostreatus* induced WR wherever mycelia were visible. This is a significant finding, given that for most chemically induced WR in soils, hydrophobicity is completely lost when the water contents of hitherto hydrophobised soils increased up to the critical water contents which were typically <10% for different soil types hydrophobised using DMDCS or stearic acid (Leelamanie and Karube, 2007;

Leelamanie, Karube and Yoshida, 2008b; Chan and Lourenço, 2016). Whereas in fungal treated soil, induced WR persists so long as the mycelia of *P. ostreatus* forms a hydrophobic layer in or above soil surface, even once the soil is fully saturated.

4.3.4 Experiment 4: Effect of soil mixing (disruption) of fungal growth on WR induced on FS by *P. ostreatus*

To determine if fungal induced soil WR persists when mycelium growth is disrupted, which may occur in the field due to human or animal interference, FS specimens were treated with grains of spawn on the specimen surface. On the 6th day after incubation and thereafter, 48 hourly until the 21st day, the soil was thoroughly mixed using a spatula. Fig. 4-7 shows visible mycelia growth on the surface of a typical FS specimen 6 days after incubation. Each of the triplicate specimens prepared were divided into 4 parts (Fig. 4-8) and each quadrant was transferred into a fresh petri dish. This provided a total of 12 specimens such that the WDPT tests were performed on triplicate specimens for each of the 6th, 7th, 12th and 21st days. WDPT test was performed on undisturbed quadrants on the 6th day before any mixing was done (Fig. 4-9a) and immediately after mixing. Thereafter, the remaining specimens were mixed and lightly compacted to the shape of a quadrant and returned to the incubator. WDPT test was again carried out 24 hours later, that is, on the 7th day. Specimens were mixed every 48 hours and tested for WR approximately 24 hrs after last mixing/disruption, coinciding with the 12th and 21st days, following the experimental programme described previously. Fig. 4-9b shows a typical specimen during the WDPT test on the 21st day.

Figure 4-7: A typical specimen for the fungal growth disruption test 6 days after incubation. Mycelia can be seen growing around the spawn grains used as inoculants.

Figure 4-8: Specimens were each divided into four sections disrupting the already formed total surface mycelia layer. These images were taken in the process of transferring each quadrant into a fresh petri dish which also provided sufficient test samples for subsequent tests

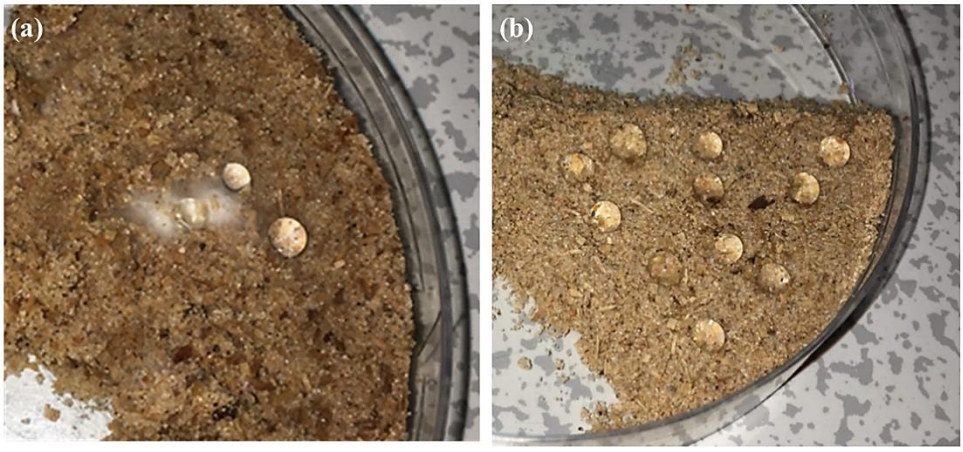


Figure 4-9: (a) WDPT test on an undisturbed quadrant on the 6th day after incubation; (b) Several water droplets on a disturbed specimen after 21 days. This specimen had been subjected to mixing every 48 hrs

For the 6-day undisturbed samples, the water droplets did not infiltrate even beyond 3600s; however, immediately after mixing the soil, WR disappeared as WDPT was < 5s. This seem to imply that an interconnected hyphal networks is required to ensure WR in soil; if this barrier is disturbed, the induced WR may be lost. The WDPT and CAs of specimens tested throughout the period of this experiment are presented in Table 4-4.

Table 4-4: WDPT, CAs and levels of WR for treated soil samples subjected to 48-hourly mixing to disrupt fungal growth up to 21 days

Day	WDPT (s)	CA (°)		Water repellency
		MEDT	Modified SDM	
*6	>86400	109	113	Extreme
**6	< 5	0	0	Hydrophilic
7	134	-	-	Slight-moderate
12	1565	103	100	Severe
21	3400	109	115	Severe - extreme

**Specimen was tested prior to mixing on the 6th day; then **tested again immediately after mixing/disruption on the same 6th day. For the 7th, 12th and 21st days, specimens were tested 24 hrs after last mixing/disruption*

It can be seen that the WR of the soils increased from slight hydrophobicity (WDPT = 134s) on the 7th day to severe hydrophobicity on the 12th day and finally to extreme hydrophobicity (WDPT 3400s; CA 115°) by the 21st day. It should be noted that the latest mixing procedure for each sample tested after the 7th day had been done ~24 hrs before testing. It seemed as if mycelia had re-mobilised and re-established hyphal networks sufficient enough to restore some level of hydrophobic behaviour to soils barely 24 hours after being disturbed. Also, hyphal strands or mycelia were not as obvious during these periods as observed in the 6-day old specimen (Fig. 4-9b), yet the extent of WR seemed to increase with additional mixing and growth duration. Two reasons are suggested for this development: (i) after mixing or growth disruption, damaged hyphae may have initiated rapid remedial measures typical of filamentous fungal hyphae (described in detail in Chapter 2), by using the Woronin bodies or septa

pore cap to plug septal pores adjacent to damaged hyphae and prevent loss of cytoplasmic fluids, thus ensuring continuous growth and/or formation of new hyphal branches (Jedd and Pieuchot, 2012). The surviving and new hyphae continue to branch out and link up with adjacent soil grains and organic matter thereby furthering inter porous networks which restore WR. (ii) Changes to grain surfaces, most likely due to hydrophobins, could have occurred with soil grains becoming gradually coated by thin films of fungal exudates/hydrophobins over time, such that without an obvious mycelia barrier, water droplets could be repelled by relatively hydrophobised grains. This is plausible because a disruption of mycelia via the action of mixing the soil could have facilitated the redistribution of residual hydrophobins. This may explain why for the Day 21 specimen the mycelial network was not clearly visible yet, sever-extreme hydrophobicity was induced. However, there is limited information from this study to support these propositions. Microscale studies or microstructural imaging could be used to further investigate the role of hyphae versus exudates and the evolution of soil grains and interactions between all components of the sample under this mixing procedure.

4.4 Brief comparison of fungal-induced versus chemically-induced soil WR

The results of this study show that once the growth of *P. ostreatus* is established in soils, it is capable of inducing WR on the soil grains/surface. And with continuous supply of required resources, including water, for continuous fungal growth, the degree and persistence of WR is expected to be sustained over time. For the 1 week old specimens, CAs ranged between 111 and 114°, while WDPT was > 24 hrs. Despite disruption of fungal growth over a 3-week period of depleting resources, WR was

often re-established within 24 hrs, showing higher CAs and WDPTs per time, up to 21 days of test period. Chemically treated sands are hydrophobised almost instantaneously although the lifespan of their hydrophobic effects is unknown, Wang and McCarthy, (2016) suggested that it could last up to 1 year depending on the composition of chemicals used. Some of the chemicals used for soils include DMDCS and organic acids like SA, oleic acid and octadecane. Table 4-5 provides a summary of the characteristics of fungal induced WR as obtained in this study, compared with chemically hydrophobised soils based on some previous studies where DMDCS and SA were used at varying soil moisture conditions. While chemically induced WR is most efficient when used on dry soils, fungi induced WR requires residual soil moisture for fungal growth and may not be effective in completely dry soil conditions. However, higher amounts of residual (or antecedent) soil moisture contents have less effect on fungal-induced WR compared to chemically-induced WR, and can even be used to create WR layers in saturated conditions.

Table 4-5: Comparison of some characteristics of fungal induced WR versus chemically induced WR

Treatment type	Test duration	Description of treatment additives	Characterisation of WR				Environmental concerns	References
			For (dry) sand		Moisture effects			
			Persistence WDPT (s)	Degree CA (°)	Varied water contents pre-hydrophobisation	Varying moisture content post-hydrophobisation of initially dry soil		
Fungal hyphae (*P. ostreatus*) on sand	1 – 12 weeks	See section 4.2.3	134 to >86400	100 - 115	WR persists in soils between water contents of 3 – 25% (WDPT reduced from >3600 to 319s); a hydrophobic hyphal layer was observed on the surface of inundated soil; (WDPT 160s)	(Not determined)	Ecological implications of introducing an alien species into a community; (however, biostimulation of suitable native species may be considered)	This study
SA on sand	-	Organic acid; Requires special solvent for dissolution (e.g diethyl ether); Soil treatment suitable conc range between 1 to 5g SA kg^{-1} sand	>3600	90 - 110	(Not available)	WR increases with increasing RH or water content up to a threshold critical water content (~2%), then decreases until it completely disappears (3 – 5%). Another study recorded increasing WR from air-dried water content (10%) to oven-dried (0%).	Similar to naturally induced WR which has been found to contribute to increased soil erodibility; SA reportedly harmful to aquatic organisms with long term effects	(Doerr *et al.*, 2002; Leelamanie and Karube, 2007; Leelamanie, Karube and Yoshida, 2008; González-Peñaloza *et al.*, 2013; Subedi *et al.*, 2013)
DMDCS on sand	-	Toxic and Highly flammable; Treatment conc. ranges between 0.04 to 10g silane kg^{-1} sand	>18000	101 - 140	Residual soil moisture reacts completely with DMDCS to induce WR. Range of moisture content tested was 0.004 to 0.16% CA increased from 124 to 137°	(Not available)	Though treatment process is hazardous and could potential increase soil acidity, treated soil is inert and relatively safe but results in increased soil erodibility	(Lourenço, Wang and Kamai, 2015) (Chan and Lourenço, 2016)

4.5 Considerations for ground engineering applications

For potential field applications within a geotechnical engineering context, the following observations and concerns for each of the treatment methods discussed in this study are noteworthy.

DMDCS will induce a more durable and strongly hydrophobic effect on soils, however, there are environmental concerns related to a potential increase in soil acidity and a potential increase in soil erodibility. WR induced by SA has also been associated with increased soil erodibility. It is also sensitive to soil moisture content changes, and may be harmful to the aquatic environment with long-term effects. In the field, soils are subjected continuously to drying-wetting cycles and a resilient hydrophobising agent is required. Attempts to study the hydro-mechanical response of DMDCS treated soils are still ongoing (Beckett, Fourie and Toll, 2016; Beckett *et al.*, 2018), the findings of such studies will provide more information on the suitability of DMDCS and SA for deployment in the field.

Fungal-hyphal networks are known to contribute to soil aggregation and hence improvement of soil susceptibility to erosion. This is a major weakness in the chemical based treatment techniques. Furthermore, fungi have the tendency to grow massively across large land areas and in some cases, they continue to survive even under sub-optimal environmental conditions, being oligotrophic (Sterflinger, 2000; Gorbushina, 2007; Cantrell *et al.*, 2011). The findings of this study coupled with these fungal attributes suggest that engineered fungal hyphae could be a promising alternative for creating durable water repellency in soils with the potential of improving overtime as mycelia networks continue to grow. This technique could be deployed in areas of

ground engineering where chemically hydrophobised soils have been considered for application.

4.6 Conclusion

This study reports the extent of WR induced by bio-treatment of soils using fungi. WR induced by the fungus: *P. ostreatus* in sterile sands and natural soils was investigated and quantified for 1 week (short term) and up to 12 weeks (under depleting resource conditions); the effects of initial soil water contents and the disruption of fungal growth on WR were also investigated. The findings of this study indicate that:

1. Treatment of sterile sand and natural soils with *P. ostreatus* over a 1week growth period, induces extreme hydrophobicity (CA >110°, WDPT > 86400s)
2. WR persists in sterile sands treated with *P. ostreatus* up to 12 weeks, though with a slight reduction in CA from 109° (extreme WR) at 8th week, to 105° (severe WR) in the 12th week.
3. WR can be induced by mycelia networks of *P. ostreatus* in soils with a high initial water content and a water repellent layer can be created even on saturated or inundated sands. This is in contrast to what can be obtainable using chemical hydrophobising agents e.g. stearic acid and DCMS as reported in the literature
4. Disruption of fungal growth or the mycelial network in soils may result in an initial loss of WR but this has been shown to be re-establishing within 24 hours after physical interference.

This study is the first step in the investigation of WR induced by fungal treatment of soils. Compared to chemically-induced soil WR, fungal-induced WR has the potential of providing a safer, more resilient, durable and environmentally friendly option for deployment in geotechnical engineering applications. In order to deploy fungal treated water repellent soils in ground engineering, further studies are required to understand the microstructural interactions involved between fungal hyphae, exudates, soil grains and pore water, as well as investigation of the infiltration characteristics of these bulk soils and the impact existing soil microorganisms may have on fungal growth and on induced WR.

Chapter 5

Influence of *P. ostreatus* (fungus) on the hydraulic behaviour of sand

Abstract

The current understanding of hyphal interactions in soil in the context of biogeotechincal engineering is at its nascent stages. This study aims to provide some understanding on the change in soil hydraulic behaviour due to fungal growth. Laboratory experiments were conducted to determine the soil water retention behaviour, infiltration rate and saturated hydraulic conductivity of sand treated by growing hyphae of *P. ostreatus* for 12 weeks. A negative water column set-up was used for determining the soil water retention curve, instrumented with a tensiometer and a thetaprobe. A 1-dimensional soil column was used to determine soil infiltration characteristics by constant head ponding and also saturated hydraulic conductivity was determined. The change in hydraulic properties of treated soils was assessed by comparison with untreated controls. The growth of the hyphae of *P. ostreatus* in sand resulted in (i) an increase in the air entry value of sand (from ~0.6kPa in untreated specimens to ~6kPa), (ii) slower infiltration of water and (iii) a reduction in the saturated hydraulic conductivity of soil from 1.52×10^{-4} m s^{-1} for untreated sand to 1.3×10^{-5} m s^{-1} after growth of *P. ostreatus*. These results provide for the first time understanding of the influence of hyphae of *P. ostreatus* on the hydraulic properties of soil.

5.1 Introduction

In response to climate change, the UK and indeed countries across the globe are imposing targets to reduce carbon dioxide emissions. Cement production alone is estimated to contribute up to 8% of total global CO_2 emissions (Andrew, 2017). This presents a challenge for the construction industry and in particular ground engineering in which the use of cementitious products is pervasive. This thesis is focused on the development of using fungal hyphae as a low cost, low carbon soil improvement technique. This is premised on the unique characteristics of fungal hyphae such as their ability to grow as large 3-D networks of mycelia across massive areas of land (Smith, Bruhn and Anderson, 1992), secretion of biochemical exudates capable of altering soil wettability, making soils hydrophobic or hydrophilic, depending on fungal species and prevailing environmental conditions (Wösten *et al.*, 1999; Wessels, 2000), as well as their role in the formation and stability of soil aggregates (Rillig and Mummey, 2006). These characteristics are expected to have implications on soil hydraulic and mechanical properties and are thus considered significant for the deployment of fungi for soil improvement as proposed in this thesis.

5.1.1 Influence of fungi on soil hydraulic behaviour

Fungi are known to play an important role in the formation of biological soil crusts in arid environments, which consist also of bacteria, algae, lichens and other organisms. Biological soil crusts are well known in the soil science community to withstand erosion due to water or wind action (e.g. Eldridge and Greene, 1994). However, the influence of biological soil crusts on hydraulic properties is less clear. Many studies have reported that these crusts result in increased infiltration, with others reporting no

effect, while others still report a reduction in infiltration (review presented in Belnap, 2006).

Hallet *et al.*, (2009) and Crawford *et al.*, (2012) have used soils containing diverse microbial communities to show that the role of fungi in regulating the pore-scale arrangement within the soil matrix overtime and in enhancing soil water repellency is more significant compared to other soil organisms considered. Several studies have investigated the influence of arbuscular mycorrhizal fungi (AMF) on soil water retention (Thomas *et al.*, 1986; Augé *et al.*, 2001; Hallet *et al.*, 2009; Ruth, Khalvati and Schmidhalter, 2011). Thomas *et al.*, (1986) used a potted soil experiment to show that mycorrhizal fungi significantly increased soil aggregation and porosity, resulting in higher saturated hydraulic conductivity relative to non-mycorrhizal soil studied. However, as is the case with similar studies on mycorrhizal studies, it is difficult to disentangle the relative contributions of mycorrhizal fungi and plant roots to the hydraulic and structural changes observed, as mycorrhizal effects may be direct or indirect - i.e. via promotion of root development and plant productivity (Querejeta, 2016). A water uptake experiment performed by Ruth, Khalvati and Schmidhalter, (2011) with *Rhizoctonia intraradices* grown in an irrigated silt loam soil showed that AMF enhanced soil water retention properties even in the absence of plant roots. Other studies on the direct contributions of fungi to soil hydraulic properties have also reported similar results, that fungi increase soil porosity and connectivity of pores (Crawford *et al.*, 2012) by creating higher numbers of larger pores which result in higher hydraulic conductivity (Bearden and Petersen, 2000; Augé *et al.*, 2001). For instance, Crawford *et al.*, (2012) used the lattice Boltzman approach for predicting

soil hydraulic conductivity from images of soil structure to determine the mean simulated saturated hydraulic conductivity of ~1.76 x 10^{-7} m s^{-1} for a sandy clay loam soil inoculated with *Rhizoctonia solani* compared to ~3.13 x 10^{-8} m s^{-1} for uninoculated sterile soil.

Complete or holistic description of soil hydraulic behaviour (especially considering biological effects such as the growth of fungal hyphae) is expected to encompasses the investigation of soil infiltration characteristics, soil water retention behaviour and soil hydraulic conductivity for respective species in soil.

5.1.2 Brief background on soil hydraulic characteristics

The relationship between degree of saturation and negative water pressure found experimentally by subjecting soils to subsequent drying stages is commonly referred to as the soil water retention curve, and may be plotted in terms of gravimetric moisture content (w), volumetric moisture content (θw) or degree of saturation (Sr) against negative water pressure (-uw) or the excess of pore air pressure over pore water pressure (u_a-u_w), i.e. soil suction. Figure 5-1 illustrates the main features of a typical water retention curve for a fine grained soil (Vanapalli *et al.*, 1996; Vanapalli, Fredlund and Pufahl, 1999; Tarantino, 2010). As a soil is dried from a fully saturated condition, initially only water is removed as the curvature of the menisci increase, but the soil remains in a saturated state, albeit at water pressures below atmospheric. On continued drying some of the larger pores will empty of water, but the air phase will remain discontinuous (quasi-saturated state). Further drying will cause the menisci to recede and air to enter into the soil. Both the air and water phases are continuous in the partially saturated state. Finally, continued drying will result in only meniscus

water remaining at inter-particle contacts, the water phase is no longer continuous (residual state).

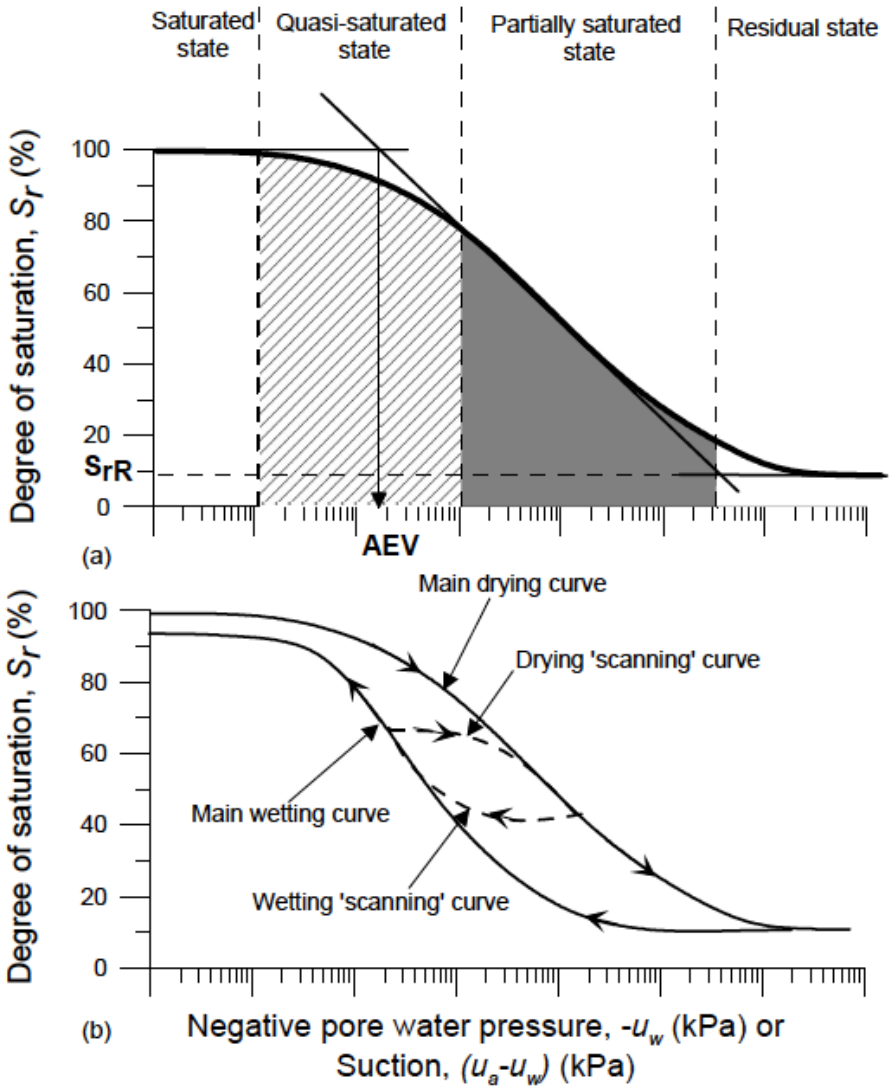


Figure 5-1: Typical features of a soil water retention curve showing (a) various saturation states along a main drying curve and (b) hysteresis between drying and wetting curves (Tarantino, 2010; El Mountassir, 2011)

In coarse-grained soils, the desaturation occurs over a much smaller range of suction than for fine-grained soils. The air-entry value (AEV) denoted in Fig. 5-1 identifies the value of negative pressure at which drainage of the largest soil pores begins, beyond this value, soils exhibit a rapid decrease in moisture content (Vanapalli *et al.*,

1996). This value is typically determined by intersecting the horizontal line drawn at Sr = 1 with the tangent to the inflection point of the drying curve (Vanapalli *et al.*, 1996). The residual degree of saturation (S_{rR}) is the saturation level at which increasing negative water pressure or suction no longer significantly causes moisture content loss and where further moisture loss must be transferred through vapour transfer (Fig.5-1).

Several curve fitting models have been proposed to describe the water retention behaviour of a soil. The equations of Van Genuchten, (1980) and Fredlund and Xing, (1994) which are based on pore size distribution function and capillary theory are the two most widely used models. The Van Genuchten, (1980) relationship between volumetric water content and suction is expressed as follows:

$$\theta = \theta_r + \frac{(\theta_s - \theta_r)}{[1 + (as)^n]^m} \tag{5-1}$$

Where: θ is the volumetric water content; θ_r = the residual water content; θ_s = the saturated water content; a, n and m are curve related parameters with *a* related to the AEV, *n* related to the rate of change of the slope of the curve and *m* is related to the shape of the curve and expressed in terms of *n* as $m = 1 - \left(\frac{1}{n}\right)$

The Fredlund and Xing (1994) equation is similar to the Van Genuchten, (1980) model and expressed as follows:

$$\theta = \theta_s + \left[\frac{1}{ln(e + (s/a))^n}\right]^m \tag{5-2}$$

where e is the base of natural logarithms while all other parameters retain the same definition as in Eq. 5-1

The mechanical behaviour of soils is coupled with the hydraulic behaviour; changes in degree of saturation and soil suction alter the mechanical response. For example, an increase in soil suction (i) increases the pre-consolidation stress (yield stress) exhibited upon externally applied stresses (Cui and Delage, 1996; Futai and Almeida, 2005) (ii) increases shear strength (Fredlund, Morgenstern and Widger, 1978; Escario and Sáez, 1986, 1987) and (iii) increases resistance to soil erosion (Nguyen *et al.*, 2017). Changes in soil suction also influence volumetric behaviour, e.g. wetting-induced collapse of soils (e.g. Booth, 1975), irreversible volumetric changes in fine-grained soils during wetting (swelling) paths, or as a result of drying-wetting cycles (Escario and Sáez, 1986). It is thus imperative to understand how fungal treatment and growth, together with its capability to induce soil water repellency, influences hydraulic behaviour of soils.

The development of soil water repellency (e.g. in wildfires or artificially induced hydrophobicity) is often associated with a reduction in infiltration, an increase in surface runoff and thus an increase in soil erodibility (Debano, 1975; Lourenço, Wang and Kamai, 2015). However, few studies have investigated the soil water retention behaviour of soils with induced water repellency (i.e. hydrophobised). Beckett, Fourie and Toll, (2016) summarises the findings of these studies indicating that induced hydrophobicity results in a shift of the water retention curve, such that water repellent soils retain less moisture for the same soil suction than the same soil in a corresponding hydrophilic condition. Similarly, available studies on the influence of fungal growth on soil water retention curves showed that fungal treatment resulted in only slight (Augé *et al.*, 2001) or negligible (Pajor, 2012) effects compared to untreated soils.

Augé *et al.*, (2001) determined the soil water retention curve of a fine sandy loamy soil colonised by a mycorrhizal species (*Rhizophagus irregularis* formerly called *Glomus intraradices*) after 7 months growth period. They found that the relative change in suction for a corresponding change in soil water content were smaller in the mycorrhizal soil compared to the non-mycorrhizal soils tested, with an excess of 0.003 g g^{-1} water content available in treated soil at higher suctions (> 4 MPa), compared to untreated soil. Overall, there seemed to be only slight or marginal difference between the influence of AMF soils and non-mycorrhizal soils on the respective water retention curves for the range of suction investigated, i.e. 0 to ~15 MPa. On the other hand, Pajor, (2012) investigated the influence of the growth of a filamentous parasitic soil fungus - *Rhizoctonia solani* on the soil water retention curve for soils inoculated with the fungus in a microcosm and grown for only 5 days. The author reported that fungal growth had no significant effect on the soil water retention curve. The short growth duration was identified as possible reason for the results obtained. The species studied by both Augé *et al.*, (2001) and Pajor, (2012) are both dependent on plants hosts for growth and development; they are filamentous and have been reported to exhibit hydrophobicity (Rillig *et al.*, 2010; Lin *et al.*, 2018; Sandoval and Cumagun, 2019). *R. irregularis* has been shown to improve soil aggregate stability, soil water repellency and significantly reduce soil erosion (Rillig *et al.*, 2010; Mardhiah *et al.*, 2016a; Zhang *et al.*, 2016). No study has yet considered the influence of a saproptrophic and non-mycorrhizal, non-parasitic/pathogen fungi species on the soil water retention curve.

In Chapter 4 of this thesis, it was shown that *P. ostreatus*, a saprotrophic, non-parasitic, non-pathogenic, filamentous fungal species is capable of inducing extreme

hydrophobicity in sands and has potential to be used as an alternative and environmentally-friendly hydrophobising agent, in place of hazardous chemicals (e.g. silane compounds) in geotechnical engineering contexts. It is hypothesized that *P. ostreatus* could significantly influence soil hydraulic behaviour since the spatial growth of its hyphae is not limited by any host (such as plant roots in the case of mycorrhizal fungi) and also due to its proven capability to induce extreme water repellent behaviour in soils, from growth periods of 1 week up to 12 weeks as shown in Chapter 4. Aside from studies on wettability, to the author's best knowledge there has been no studies on the influence of the growth of an environmentally-friendly saprotrophic fungal species like *P. ostreatus* on the hydraulic behaviour of soils, encompassing its influence on the soil water retention curve, infiltration and hydraulic conductivity.

The specific objectives of this chapter were to evaluate the (i) soil water retention behaviour, (ii) infiltration characteristics and (iii) saturated hydraulic conductivity of sand which has been treated (i.e. inoculation and growth) with *P. ostreatus.* The changes observed in the hydraulic behaviour of treated soils were assessed by comparison with untreated controls. The negative water column technique was used to determine the soil water retention curve. Soil infiltration rate was determined for one-dimensional flow while applying a constant head. The same specimen was then fully saturated and the saturated hydraulic conductivity determined.

5.2 Materials and methods

5.2.1 Materials

a) Soil

The soil used in this study composed of well-graded sand: with coefficient of uniformity, C_u defined as D_{60}/D_{10} = 3.6 and the coefficient of curvature, C_c defined as $(D_{30})^2/(D_{60} \times D_{10})$ = 1.1, mixed with lignocel (described below). A well-graded sand was selected for study in order to allow for a gradual saturation and desaturation of the soil. The particle size distribution of the sand (Fig. 5-2) was obtained by using the modified Fuller equation (Eqn. 5-3) to calculate target masses of sand corresponding to respective diameters of fine, medium and coarse-grained sand particles.

$$P = \frac{\sqrt{\frac{D}{D_{max}}} - \sqrt{\frac{D_{min}}{D_{max}}}}{1 - \sqrt{\frac{D_{min}}{D_{max}}}} \times 100 \quad (5\text{-}3)$$

Where: P = percentage passing, D = target grain/sieve size, D_{max} and D_{min} = maximum and minimum grain/sieve sizes, chosen to be 0.425 mm and 0.063 mm, respectively.

b) Lignocel

Lignocel® (HB 500 – 1000) is a commercially packaged natural softwood fibre processed and marketed by J. RETTENMAIER & SÖHNE GmbH. It is used in this study to serve as the organic substrate (i.e. nutrient source) for fungal growth as it contains basic carbon requirements, such as lignin, hemicellulose and cellulose, essential for the growth and development of saprophytic fungi.

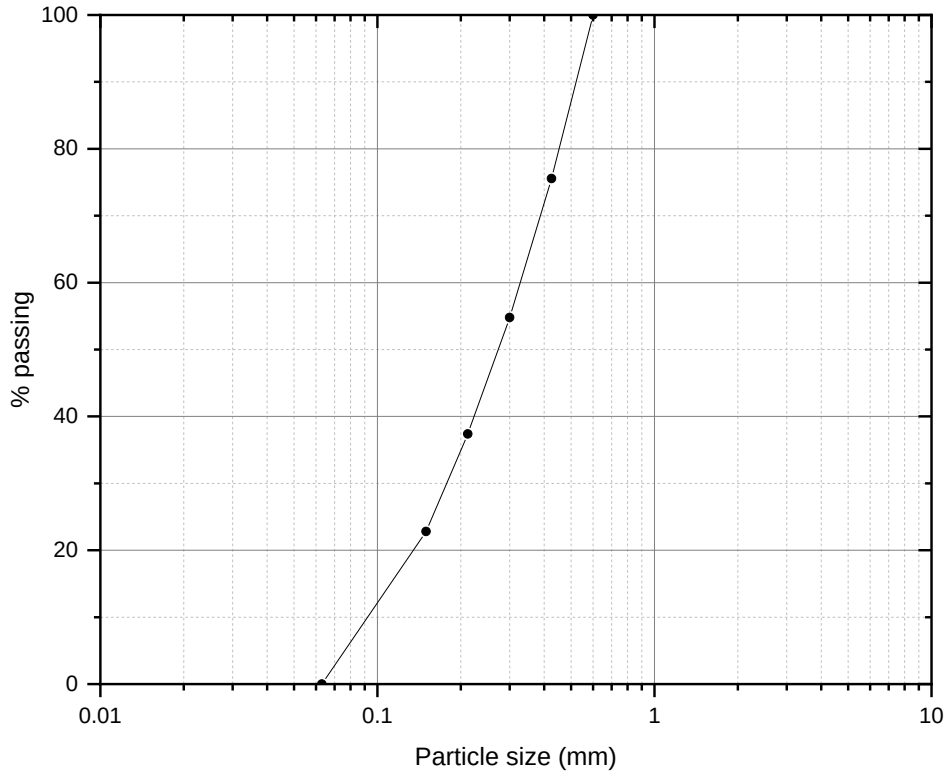


Figure 5-2: Well-graded particle size distribution of the sand

c) Fungus

Pleurotus ostreatus (strain: M 2191) was used in this study and was supplied by GroCycle UK in the form of a 500 g bag of active fungal spawn. It was stored at 4°C and used within 10 days after delivery.

d) Preparation of fungal inoculant

Inoculation of the soil was carried out using a fungal spore/hyphal suspension of *P. ostreatus*. This was prepared following the method detailed in Chapter 4. Briefly: according to the ratio in Table 5-1, a fixed mass of fungal spawn was placed in a 500 mL conical flask containing de-ionised water and subjected to vigorous shaking for 30mins in order to release spores and hyphae into the water. This was filtered through a 2 mm sieve to remove any large solid mycelium fragments and washed spawn grains

from the final spore/hyphal suspension. The suspension was always freshly prepared and used to inoculate *P. ostreatus* into the soil immediately after preparation (within 1-2 hours).

Table 5-1: Composition and characteristics of specimens

Specimen	Spore/hyphal suspension	Specimen composition & characteristics			
	Spawn:DI water	Mass of sand (g)	Mass of lignocel (g)	Liquid additive (volume cm^3)	Dry Density of specimen in column pre-incubation
Treated	1 g : 10 mL	2403	141	Spore/hyphal suspension (283)	1.08 g cm^{-3}
Untreated	-	2403	141	DI water (283)	1.08 g cm^{-3}

e) Preparation of specimens

The well-graded sand was thoroughly mixed with lignocel in the proportions shown in Table 5-1. Prior to usage, the sand-lignocel mixture was autoclaved at 121°C for 20mins and then oven-dried at 30°C overnight to ensure the material was initially sterile. Under aseptic conditions, 283 cm^3 of spore/hyphal suspension was added to the soil material and manually mixed in a bowl. For untreated specimens the same volume of DI water was used instead of the spore/hyphal suspension. A 50 mm thick layer of gravel was placed at the bottom of the column to serve as filter material. A thin sheet of non-woven geotextile was placed on the gravel filter before introduction of the soil into the column. The purpose of the geotextile was to prevent soil ingress into the pores of the filter material. The soil-liquid mixture was compacted in 9 equal layers in the column (100 mm inner diameter), with each layer tamped to form a final specimen of 300 mm length with an initial dry density of 1.08 g cm^{-3} (Fig. 5-3a). Pre-drilled slots for insertion of sensors (Fig. 5-3b & c) on the column wall were sealed using foam plugs and cellotape prior to emplacement of the specimen. After

compaction the top of the column was loosely covered with a sheet of aluminium foil. The specimen was stored in an incubator at 25°C, in the dark, for 12 weeks prior to testing.

In total, 8 specimens were prepared, comprising of 4 pairs of treated and untreated specimens. Two sets were to trial the experimental procedures used (these results are not reported in this chapter), while the results of the other two sets (4 specimens) are presented here. One pair of treated and untreated specimens was used for the soil water retention tests, while the second pair was used for the combined infiltration and hydraulic conductivity tests.

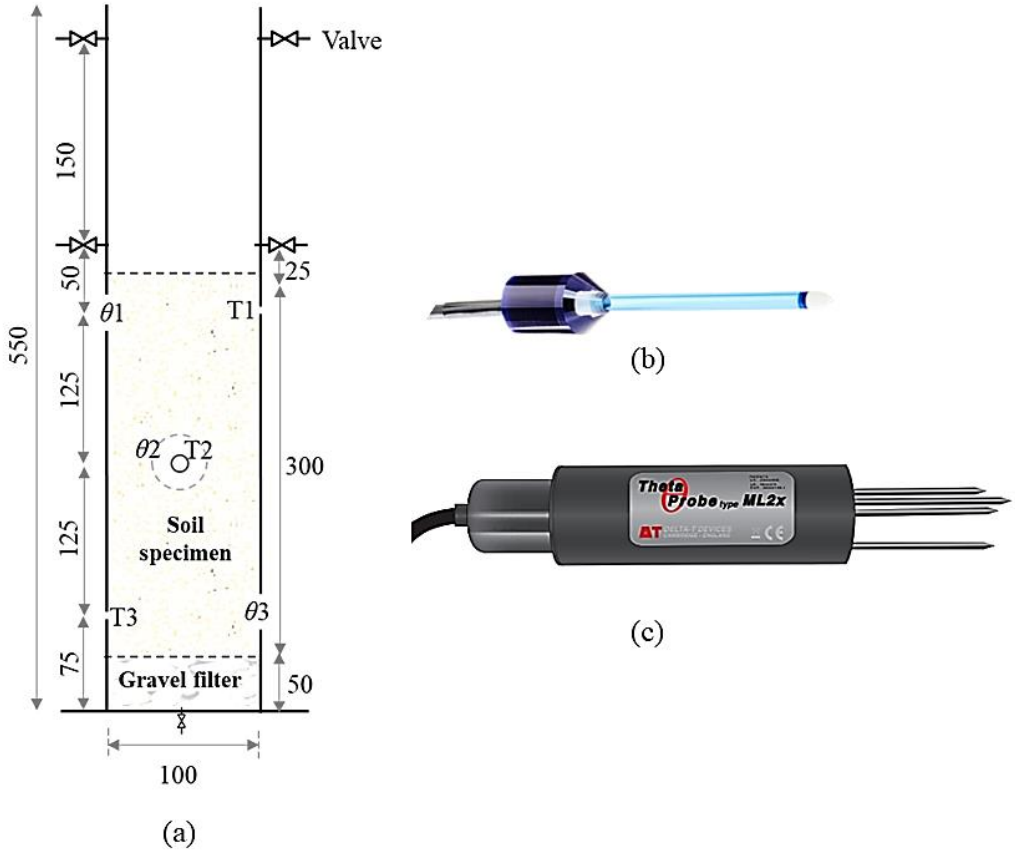


Figure 5-3: (a) schematic diagram of the 1-dimensional infiltration column showing sensor positions with 300 mm length of specimen compacted in 9-layers to make a dry density of 1.08 g cm^{-3} on a gravel filter pre-incubation. (c) T5x-5 tensiometer used for pore-water pressure measurements (d) ML2x-type Theta probe for monitoring volumetric water contents. (All dimensions are in mm; not drawn to scale)

5.2.2 Set-up, instrumentation and data acquisition

For all tests conducted, the set ups were instrumented with sensors for monitoring pore water pressure and volumetric water contents. The T5x-5 pressure transducer tensiometers (5cm shaft length) from UMS Ltd. were used for pore water pressure measurement (positive and negative) while ML2x-type Theta probes from Delta-T devices were used for determination of volumetric water content (see images in Fig. 5-3b & c). For the infiltration and hydraulic conductivity tests, three pair of sensors, i.e. tensiometers: T1, T2, T3 and Theta probes: θ1, θ2, θ3, were installed at three heights within the soil (see Fig 5-3a); while only a single tensiometer and Theta probe were used for the soil water retention curve test as shown in Fig. 5-4. An advanced data logger and controller (GP2 from Delta-T devices) with *Deltalink 3.6.2* software was used for acquisition and logging of all data from the sensors on a PC. Each set up was placed on an electronic balance and mass changes were continuously recorded using *Tera Term*, an open source programme, through a serial port connection from the balance to the PC.

5.2.3 Testing methods

a) Soil water retention curve

i) Experimental set-up

The soil water retention curve for fungal treated and untreated soils during wetting and drying cycles was determined using a negative (hanging) water column set up as shown in Fig. 5-4. The procedure adopted for this test is based on the procedure outlined by Pagano, Tarantino and Magnanimo (2018). The specimen was placed in a

cylindrical mould made of clear acrylic (diameter 100 mm, height 90 mm). The specimen sits on a silt filter (10 mm height).

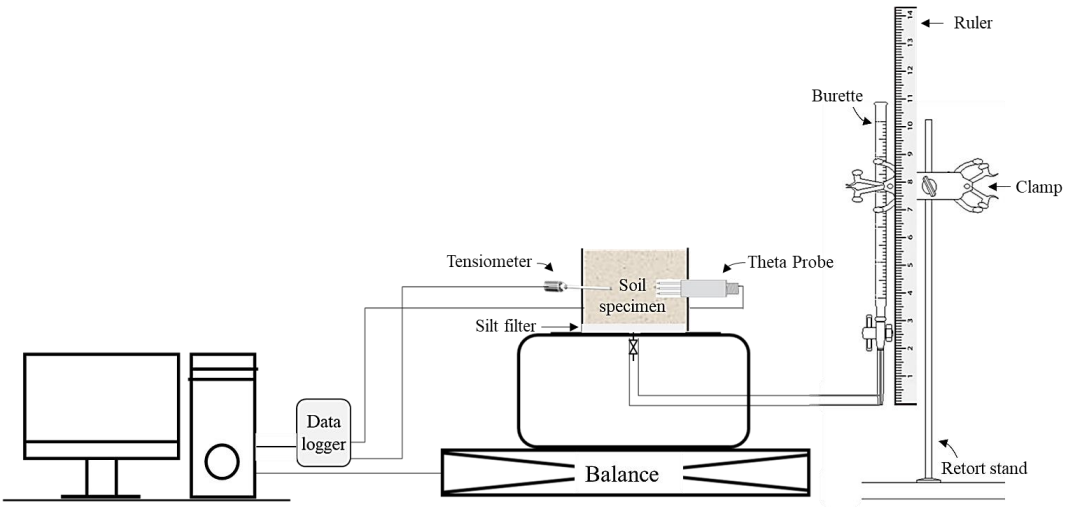


Figure 5-4: Experimental set up for determination of the soil water retention curve for fungal treated and untreated sand

The negative water column method typically relies on the use of a porous stone (e.g. ceramic) to control the matric suction at the base of the soil specimen. A silt filter was used in these experiments to control the pore-water pressure instead of the conventional porous stone because (i) it provides a high(er) air-entry interface which ensures the negative pore water pressure applied is transferred to the specimen, while ensuring the drainage system remains fully saturated; and (ii) it prevents the build-up of larger pores at the interface between the specimen and the filter, a phenomenon known as the *wall effect* which occurs at interfacial boundaries of sands or coarse grained materials (Pagano, Tarantino and Magnanimo, 2018). The negative water column technique was first used to consolidate the silt filter and later used for obtaining the wetting and drying paths of the soil water retention curve.

ii) Preparation of the silt filter

Silt, with a mean particle size of 25.2 µm produced from crushed quartz stone and commercially available as 'silica' was used for making the silt filter. 98 g of silt was mixed with 98 mL of DI water to form a slurry of 100% water content. A drainage pipe linked to a burette (negative water column) was fixed to the bottom of the empty cylindrical mould (100 mm x 100 mm), with the valve closed. A 100 mm diameter filter paper was placed inside the mould before gently pouring in the silt slurry. This was to prevent flow of the slurry into the drainage pipe. The slurry was left to settle for 48 hours forming a 10 mm thick filter. The drainage valve was opened and the height of water in the burette adjusted so that it was just several millimetres above the height of the silt filter, this enabled drainage of excess water (Pagano, Tarantino and Magnanimo, 2018).

Consolidation of the filter was achieved by applying suction up to ~6 kPa using the negative water column method. The burette was lowered in discrete steps with its water level below the bottom of the filter. Hydraulic equilibrium was targeted for each suction step, indicating consolidation of the filter. This was observed by recording the change in water level of the burette and the mass of the silt filter determined from the balance. The filter paper ensured no loss of silt particles during this stage. At the end of the consolidation process, the burette was raised in steps and topped up with water as required, until its water level was just above the surface of the silt filter, to impose zero suction on the soil specimen.

iii) Emplacement of specimen

The need to have a consolidated silt filter which remained fully saturated throughout the experiment made it difficult to have the inoculated specimen prepared and incubated in the mould from the onset, as the filter would have lost moisture during the fungal growth period in the incubator. Specimen prepared in the column in Fig. 5-3a was therefore adopted for this test. After preparing the filter, the bottom of the 550 mm column bearing the specimen was removed and the gravel filter taken out. Using a pusher that fits tightly within the column, the specimen was carefully pushed until it was flush with the base of the column. The column was then placed on to the mould containing the consolidated silt filter, perfectly aligned. With the pusher, and guided by linear scale and reference marks attached to the column, a 90 mm thick specimen was pushed into the mould and the top neatly cut using a wire hand saw. One tensiometer and one theta probe were then installed at 50 mm from the base of the mould for determination of the soil water retention curve.

iv) Wetting and drying procedure

Immediately after specimen preparation and installation of sensors, the water level in the burette was topped up (by adding water or raising the burette higher) gradually to the same level as the top of the specimen in order to impose zero suction. Using the balance and sensors, the amount of water required to reach equilibrium at zero suction was recorded. Suction and volumetric water contents measurements were logged every second. After saturation (wetting), the burette was lowered in stages, to apply the matric suction to achieve the drying path. The amount of water entering or draining from the specimen as well as the height of water in the burette during wetting and

drying were used to determine the degree of saturation and matric suction as a quality check in comparison with the sensor recordings. A drying and wetting cycle was performed twice for each of the treated and untreated specimens.

b) Infiltration and hydraulic conductivity tests

Fig. 5-3a presents a schematic diagram of the 1-dimensional soil column used for the respective combined infiltration and hydraulic conductivity tests. The column consisted of a clear acrylic tube, 5 mm thick, with inner diameter of 100 mm and a height of 550 mm. The column was set up with the aim of performing a saturated hydraulic conductivity test immediately after infiltration test on the same specimen, hence the provision of valves to maintain a constant water head at respective heights above the soil specimen.

i) Constant head infiltration test

After 12 weeks growth period, the specimen was taken out of the incubator and set up as shown in Fig. 5-5. The sensors were inserted through the pre-drilled slots into the specimen, and silicon sealant was used to fix them, ensuring a water-tight installation to the column. The column was left overnight to allow the silicon sealant to dry. The infiltration test was carried out by applying a constant head of water (25 mm) in the column above the specimen surface. A sheet of non-woven geotextile material was placed on the surface of the specimen at the soil-water boundary in order to minimise soil disturbance during column loading (i.e. setting the constant head). The wetting front and corresponding transient pore-water pressures were monitored using the moisture sensors ($\theta 1$, $\theta 2$, $\theta 3$) and tensiometers (T1, T2, T3), a pair of each were

installed opposite each other at depths of 25, 150 and 275 mm below the surface of the soil in the column. Pore air during infiltration was vented through the outlet at the base of the column. The time of commencement of water infiltration as well the time when outflow started at the bottom of the column were both recorded. The same procedure was carried out for both the treated and untreated specimens. Shortly after steady state flow conditions were achieved (water inflow = water outflow), inflow was stopped and the specimen was allowed to drain by gravity prior to conducting the saturated hydraulic conductivity test.

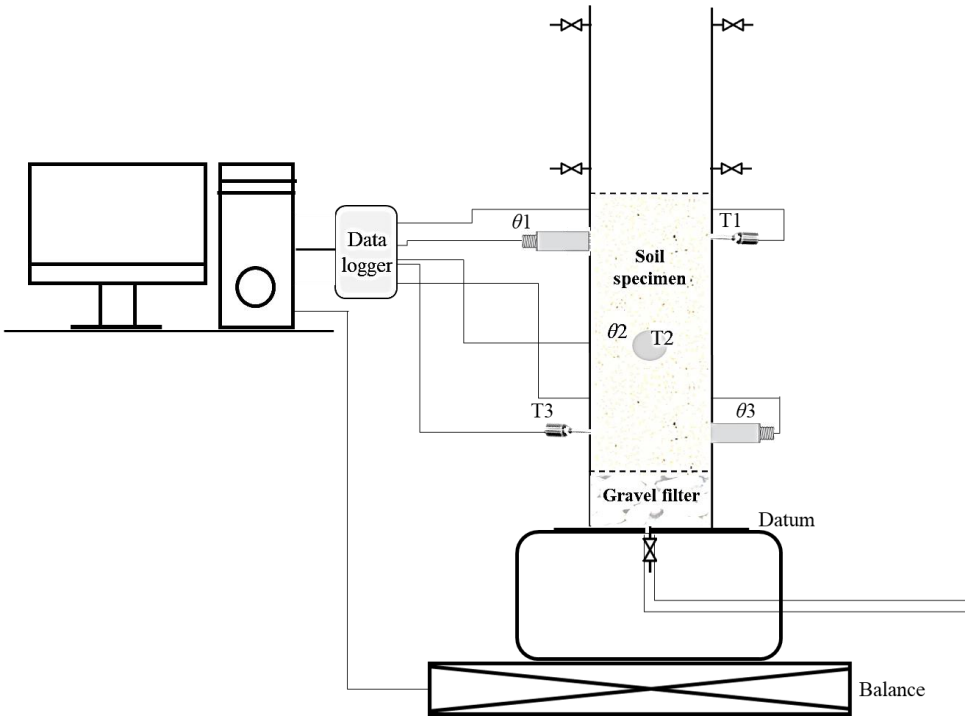


Figure 5-5: Set-up of column instrumented with tensiometers (T1, T2, T3) and water content sensors (θ1, θ2, θ 3) all connected to a data logger for continuous monitoring of infiltration front and moisture dynamics. The column sits on an electronic balance for monitoring of mass changes

ii) Determination of saturated hydraulic conductivity

Immediately after the infiltration test, the same specimen remaining in the column (Fig. 5-5) was then used for determination of the saturated hydraulic conductivity of the soil by conducting a constant head permeability test (ASTM D5084-10), in a soil

column of constant cross-sectional area (A). As described in the schematic illustration in Fig. 5-6, a reservoir was attached downstream and the specimen was initially saturated from the bottom to the top by lifting the reservoir upwards in four levels, L1 – L4, 100 mm apart. At each level, the water level in the reservoir was maintained at a constant head until hydrostatic conditions were achieved in the specimen, before proceeding to the next level. Sensor readings captured the volumetric water contents and pressure head distributions during the transient and steady-state flow conditions. After bringing the specimen to saturation, different hydraulic gradients (where i= ΔH/L, the head difference across the specimen length) were applied and the flow rate at the outlet (Q) measured over a given period of time. The saturated hydraulic conductivity (k_{sat}) was determined for upward and downward laminar flows through the specimen based on Darcy's law as described in Eqn. 5-4.

$$Q\ (m^3/s)\ =\ Ak_{sat}i \tag{5-4}$$

For both specimens, the hydraulic gradients (i) at which k_{sat} were determined are presented in Table 5-2. The water supply into the reservoir was via a peristaltic pump set at a flow rate of 1.4 $cm^3\,s^{-1}$. It was set such that there was always an excess of water above that required to maintain the constant head, such that water drained out of the outlet in the reservoir at all times.

Table 5-2: Hydraulic gradients for determination of k_{sat} via downward and upward flows for respective specimens

Specimen	Downward flow (DF)			Upward flow (UF)	
	DF-i_1	*DF-i_2*	*DF-i_3*	*UF-i_1*	*UF-i_2*
Untreated	0.33	0.72	0.91	0.67	0.75
Treated	0.35	0.69	0.83	0.53	-

A pictorial image of the treated specimen showing the typical set up during determination of k_{sat} with upward flow with hydraulic head difference of 150 mm is presented in Fig. 5-7.

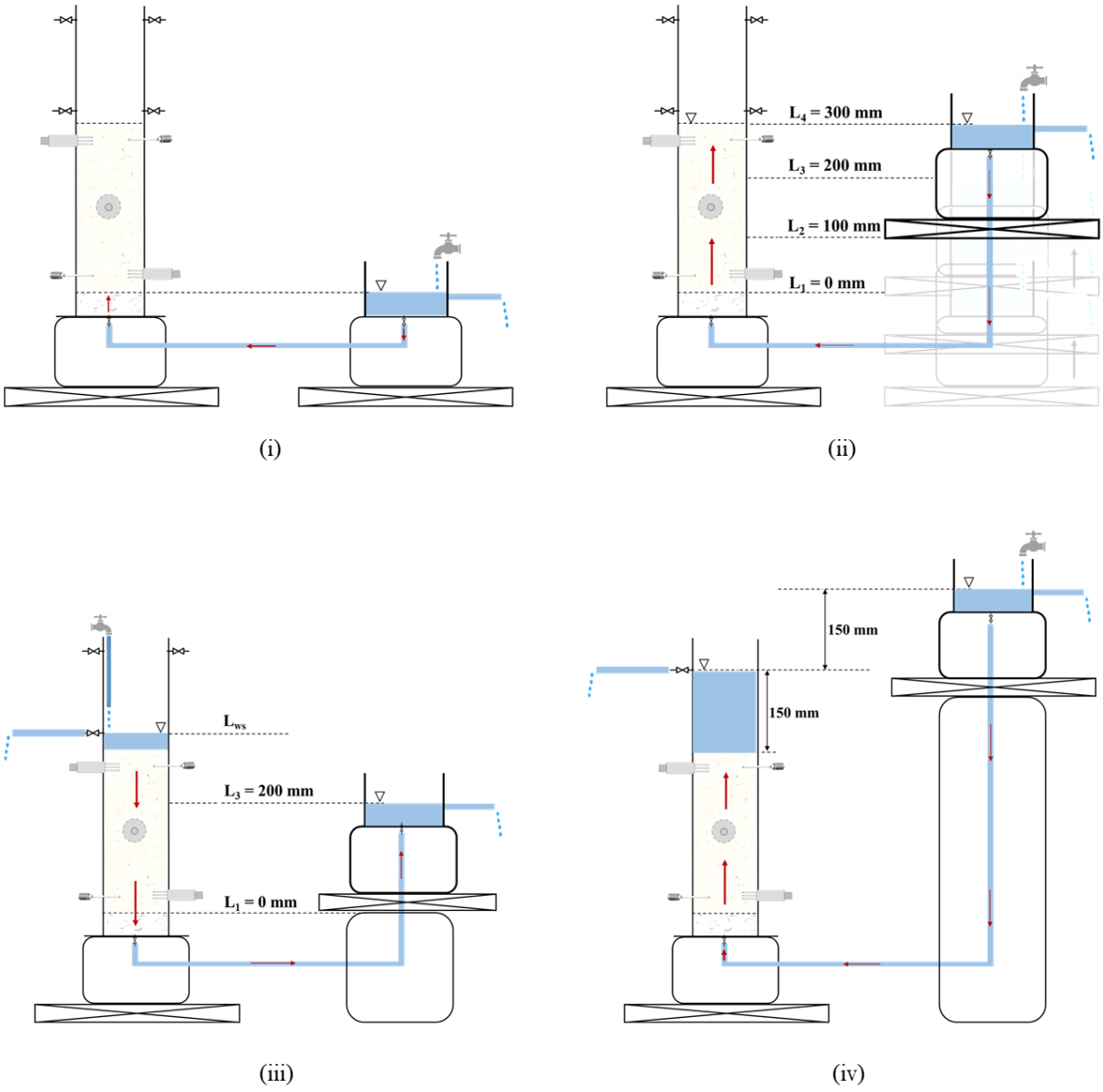


Figure 5-6: Stages followed in the determination of saturated hydraulic conductivity (k_{sat}). (i) Base of specimen connected to reservoir and constant head applied such that the gravel filter was saturated; (ii) the reservoir was then moved upwards in levels L1 - L4 to ensure saturation of specimen. At level L4 position, the specimen was expected to be fully saturated; (iii) Constant head applied with L_{ws} = 25 mm above specimen surface and the reservoir lowered to L3 for determination of k_{sat} via downward flow (iv) Reservoir raised for determination for k_{sat} via upward flow

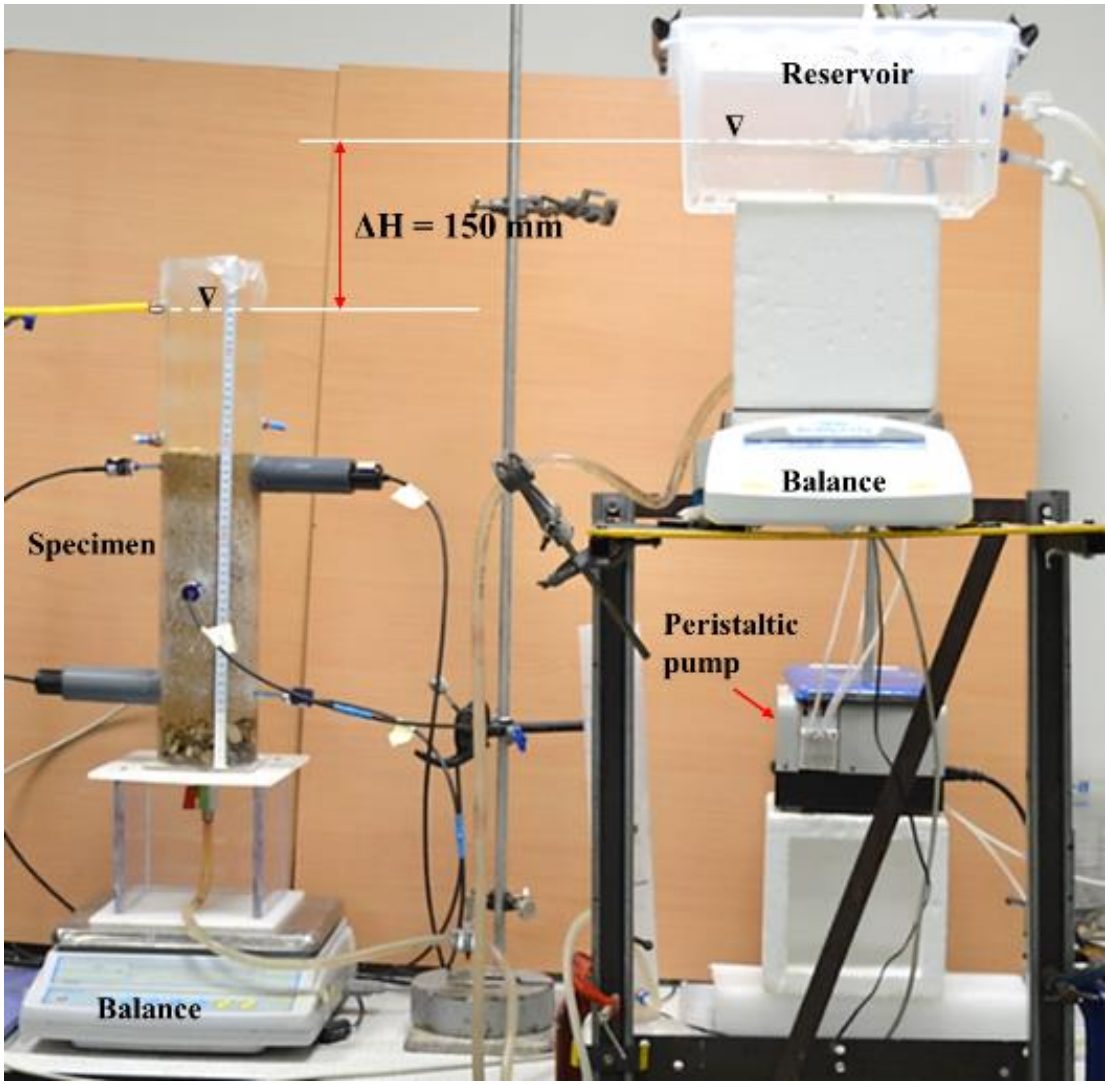


Figure 5-7: Typical set-up during the determination of k_{sat} at hydraulic head difference of 150 mm. (Pictured here is the treated specimen).

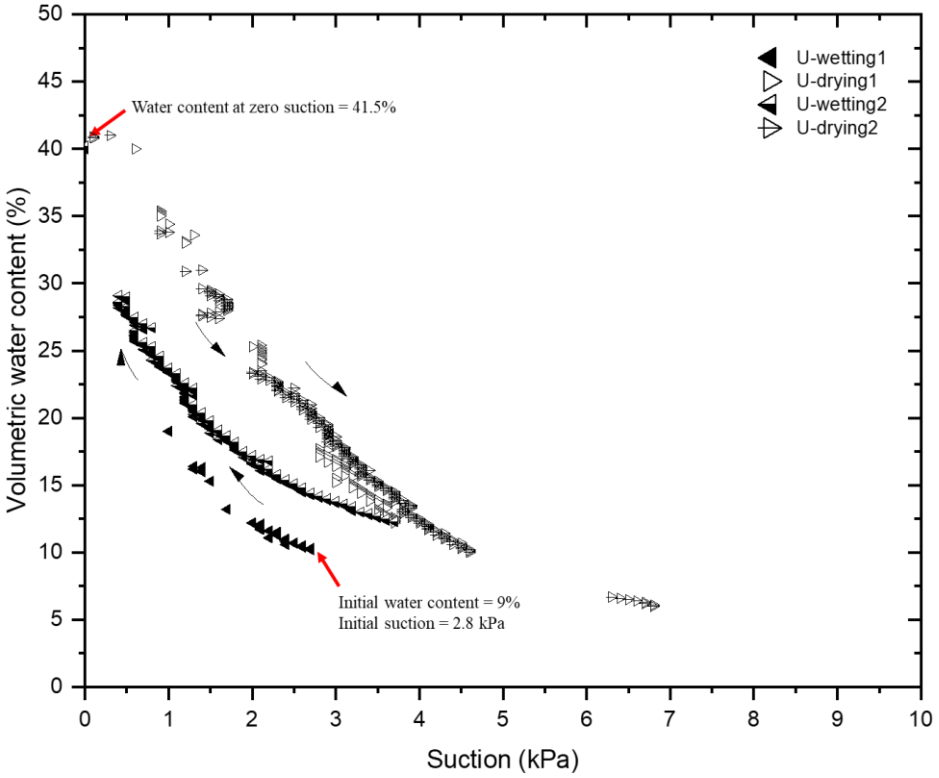
At the end of all tests, samples were collected from every 50 mm layer of each specimen to determine gravimetric and volumetric water contents as well as dry densities. These measurements were done as a quality check for the performance of the sensors and also to determine changes in soil density.

5.3 Results

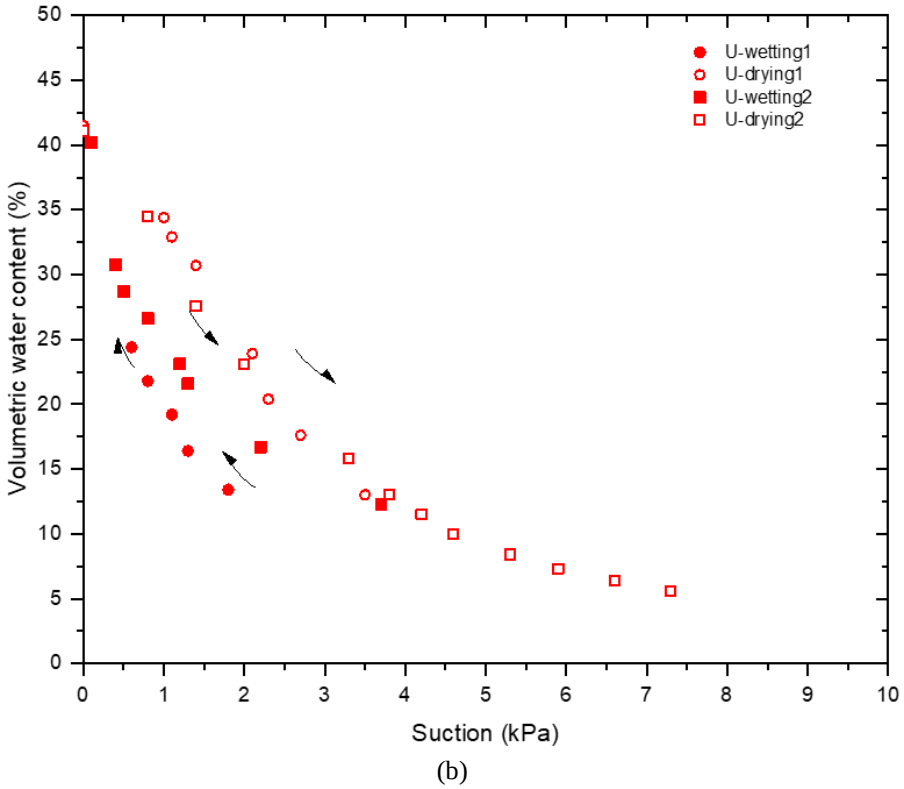
5.3.1 Effect of hyphal growth on the soil water retention curve

Fig. 5-8a presents the raw data from the tensiometer and thetaprobe sensors, i.e. volumetric water content against suction for the untreated specimen during the wetting and drying paths followed; while Fig 5-8b presents the soil water retention curve (plotted using the points when equilibrium water contents were reached at each applied suction levels, i.e. when the reservoir height was maintained constant). The equivalent of both plots for the treated specimen is presented in Fig. 5-9a &b.

The initial volumetric water content of the untreated specimen after emplacement on the silt filter was 10.2% and the initial suction measured was 2.7 kPa. Desaturation of the sand specimen occurs within a small suction range (0 - 7 kPa) as expected for sands. The water retention curve for repeated drying paths fall approximately along the same curve, typical of coarse-grained soils. It took a total of 19 days to complete the two wetting-drying cycles shown in Fig. 5-8a for the untreated specimen. From Fig. 5-8a the air entry value of the untreated specimen is ~0.6kPa.

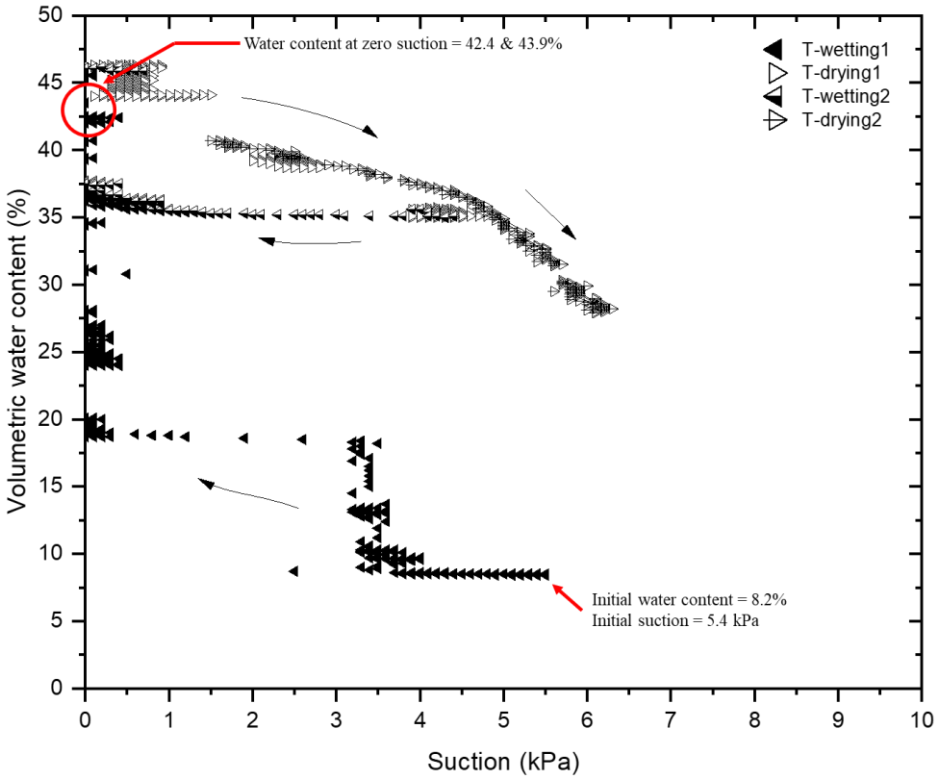


(a)

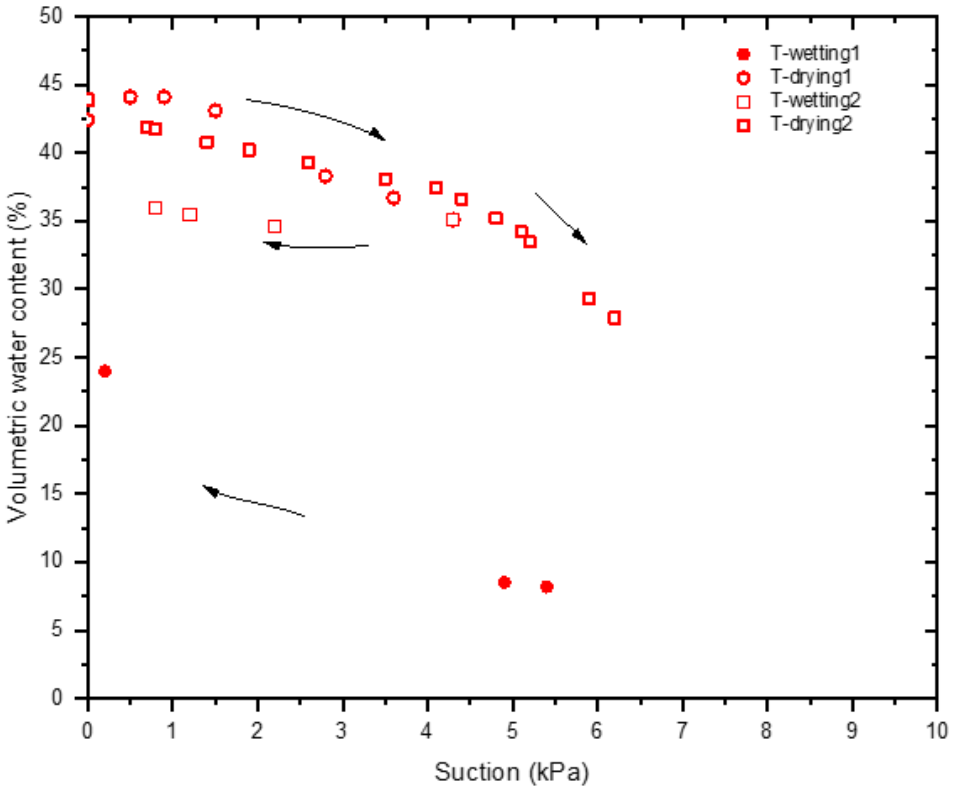


(b)

Figure 5-8: (a) Plot of the volumetric water content against suction from the experimental data for the untreated specimen (b)Soil water retention curve for the untreated specimen



(a)



(b)

Figure 5-9: (a) Plot of the volumetric water content against suction from the experimental data for the treated specimen (b) Soil water retention curve for the treated specimen

Initial wetting of the treated specimen via the base of the column was delayed for 3 days, i.e. little/no water inflow into the specimen was recorded during this time, while the water level in the burette remained level with the top of the specimen. This is likely due to a combination of factors (i) water repellency, (ii) possible entrapped gases (due to microbiological activity) at the interface between the silt filter and the specimen and (iii) alteration of the soil microstructure (i.e. pore sizes and pore architecture) due to fungal growth. A dense white mass of mycelium was visible at the base of the soil specimen and will have affected the hydraulic conductivity of the specimen (investigated in following sections). In order to induce wetting, the burette level was raised 80 mm above the top of the specimen, i.e. a higher hydraulic gradient was required to initiate water flow in the treated specimen compared to the untreated specimen. Water breakthrough (i.e. wetting) did not occur until a suction of 3.4 kPa was reached in the centre of the specimen, at this suction level there was a rapid increase in volumetric water content from 7-18%. Thereafter, the suction fell to 0 kPa as the VWC increased.

Wetting of the treated specimen was considered complete once a film of water appeared on the specimen surface and the value of VWC from the sensor corresponds to ~43%. The first drying cycle then followed. This initial drying cycle and later wetting/drying cycles lasted for a total of 48 days for treated specimen (compared to 19 for the untreated specimen), which was required in order to ensure that hydrostatic conditions and equilibrium water contents were reached at each of the respective steps. In the course of determining the soil water retention behaviour, the hyphae on the specimen surface developed into mushrooms. This was inevitable as the specimen was

exposed to light – a key requirement for mushroom growth. Fig. 5-10 shows the evolution of mushroom growth throughout the period of the drying and wetting cycles. Parafilm was used to cover the specimen surface, including the mushrooms, throughout the experiment, to prevent evaporation. The emergence of mushrooms indicates that fungal activity is continuing throughout the experiment and as such the specimen itself and its pore architecture is continuing to evolve during the determination of the water retention behaviour

In order to extract as much information as possible for comparison of the SWRCs, two different fitting models (Van Genuchten, 1980; Fredlund and Xing, 1994) were applied to the experimental data obtained during the first drying path for each specimen (Fig. 5-11a & b). The model parameters and their respective predictions for the hydraulic characteristics of treated and untreated specimens based on the SWRCs are presented in Table 5-3.

Comparison of the model parameters indicates that the treated specimen has a higher air entry value and the slope of the drying curve beyond the air entry value is steeper for the treated specimen compared to the untreated specimen. A higher air entry value indicates that the treated specimen requires a higher suction (several kPas) in order for desaturation of the pores to begin.

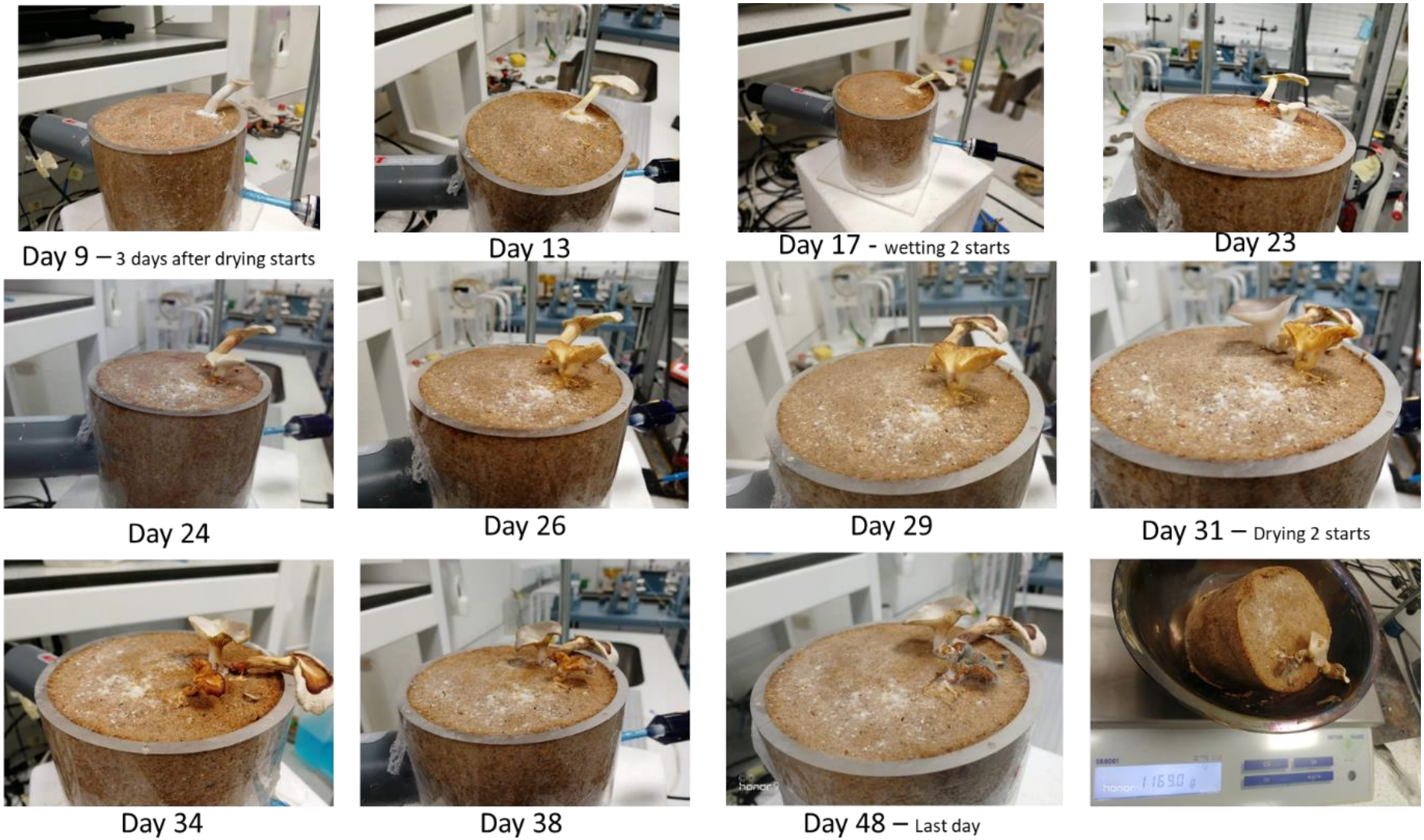
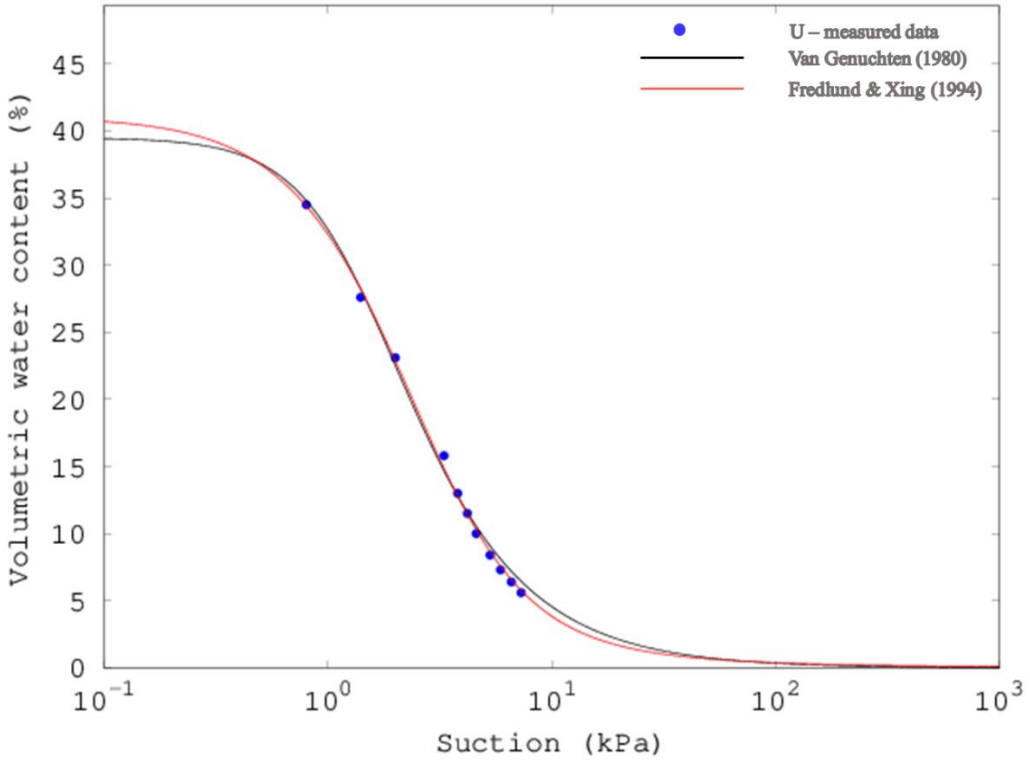
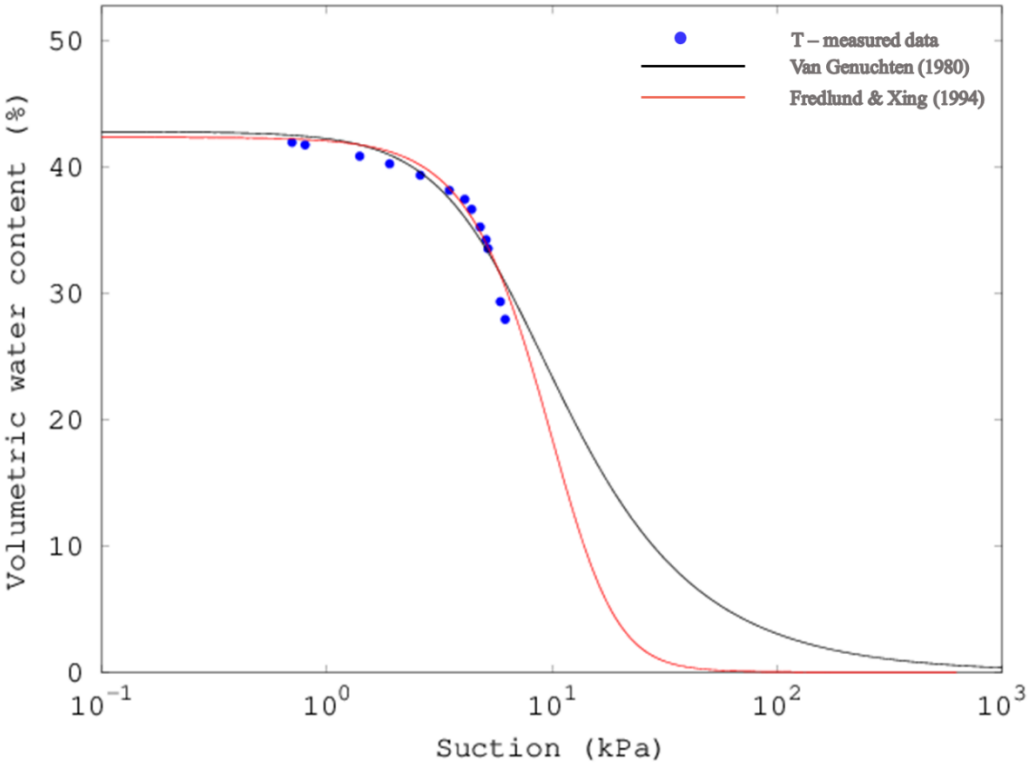


Figure 5-10: Image logging of the growth of mushrooms in the treated specimen during the wetting and drying cycles across 40 days



(a)



(b)

Figure 5-11: SWRC models fitted for (a) U- untreated and (b) T-untreated specimens

Table 5-3: SWRC model parameters for untreated and treated specimens

Model	Parameters	Specimen		R^2	
		Untreated	Treated	Untreated	Treated
Van Genuchten, (1980) (Eq. 1)	θ_s	39.49	42.73	0.99	0.92
	θ_r	$8.51x10^{-5}$	$2.8x10^{-2}$		
	a	0.67	0.16		
	n	2.13	1.96		
Fredlund & Xing, (1994) (Eq. 2)	θ_s	41.04	42.30	0.99	0.94
	θ_r	$3.25x10^{-6}$	$1.62\ x10^{-3}$		
	a	2.47	13.06		
	m	2.74	4.79		
	n	1.49	2.16		

5.3.2 Distribution of Pore-water pressure (PWP) and volumetric water contents (VWC) in specimens during infiltration

Fig. 5-12 a, b, d & e show the distribution of PWP and VWC with soil depth as recorded by the sensors before and during the infiltration period for the treated and untreated specimens. Figs. 5-12c & f are images of each specimen after 1 hr of infiltration. The ranges of time (t) presented in each case in Fig. 5-12 were selected to give a clearer indication of the advancing infiltration fronts and to cover the entire duration of the infiltration tests for each specimen. Appendix G presents the plotted raw data for the evolution of the PWP and VWC for the early periods of the infiltration: 20 minutes for untreated and 60 minutes for the treated specimens.

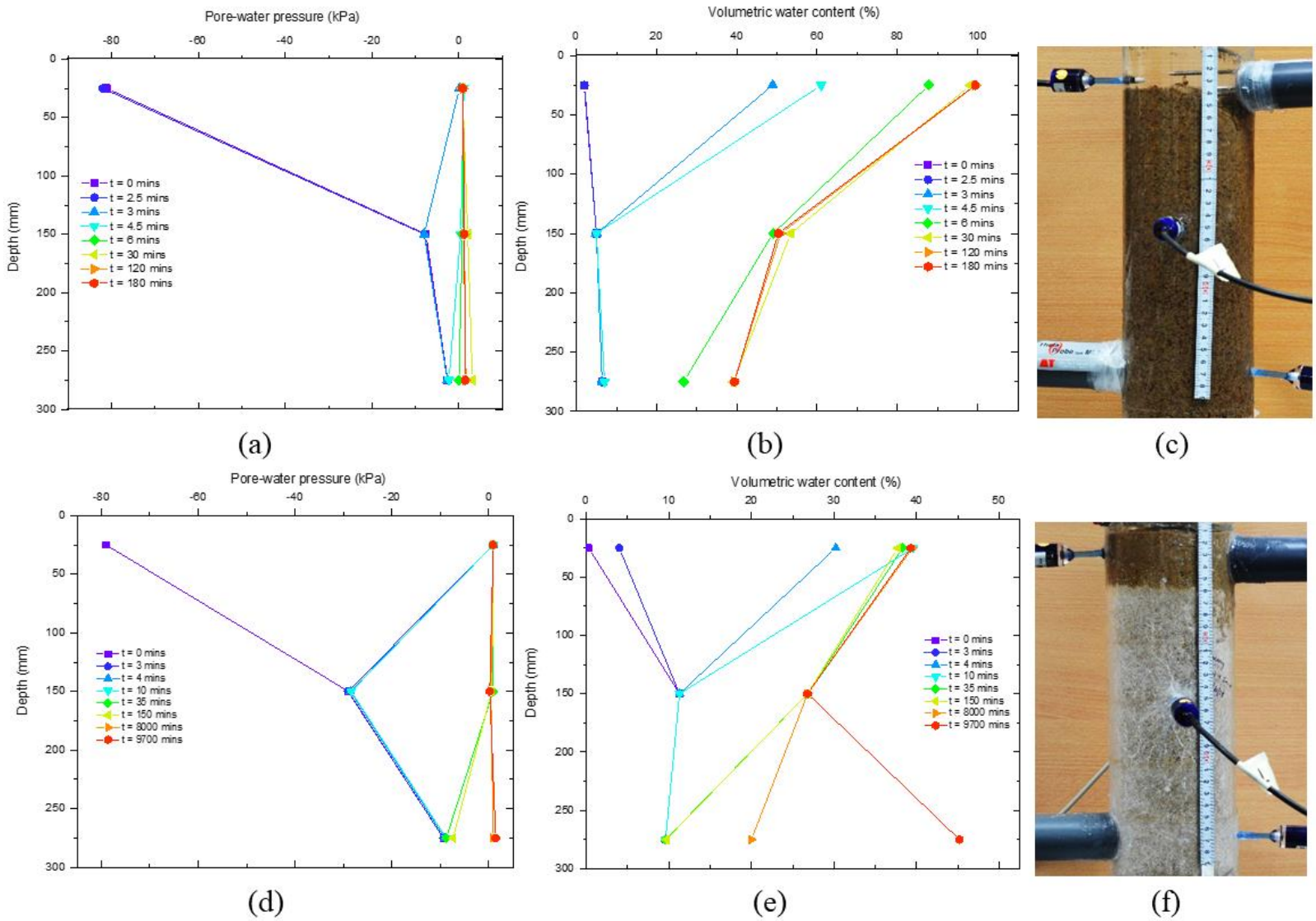


Figure 5-12: Distribution profiles of pore-water pressure and volumetric water contents with depth in the: (a-c) untreated and (d-f) treated specimens.

a) Pre-infiltration conditions

Shortly before commencement of infiltration, some variation in suction distribution was observed in the specimens (Fig. 5-12). At t = 0, the suction in the top of the specimens measured at 25 mm depth was ~80 kPa with water contents of about 1%. The initial values of PWP and VWC at the top of the specimen, prior to infiltration (t = 0), were similar for both the untreated and treated specimens. However, the middle and bottom regions of the specimens showed differences in suction and VWC as recorded by the sensors at 150 and 275 mm depths, prior to infiltration. The treated specimen recorded moisture contents of 11.3 and 9.6% while the untreated specimen had 5.0 and 6.4% at the middle and bottom respectively. It should be noted that specimens were prepared and incubated at a starting volumetric water content of 13.3%. This means that over the 12 weeks of incubation period, the growth of fungal hyphae in the treated specimen contributed to an alteration of the moisture dynamics in the soil, retaining more moisture in the bottom half of the soil, especially in the mid-region, compared to the untreated specimen.

The untreated specimen recorded lower suction values (negative pore water pressure) of 9 and 6 kPa while the treated specimen had suction values of 30 and 10 kPa at the middle and bottom respectively. As seen in the untreated specimen (at t = 0), VWC increases, and suction decreases with depth, indicating the influence of the soil-atmosphere boundary at the top of the specimen through which drying may have occurred and also the possibility that water may have percolated by gravity during the incubation period. However, the treated specimen shows a deviation from this, with higher suction and VWC at the middle compared to the bottom region. It is unclear

why these inconsistent values were recorded at this stage, as all sensors were calibrated before the experiments and the responses recorded during all the other experiments reported in this chapter appeared to be consistent; for example, tensiometer readings tending to zero as volumetric water contents reached ~40% indicating saturated conditions, occurred at similar time for each respective pair of sensors during the infiltration test and saturated hydraulic conductivity tests described in later sections. Also, checks of volumetric water contents carried out at the end of the experiment were consistent with the final volumetric water contents determined by the theta probes. Nevertheless, there is a possibility that the hyphal growth distribution may have caused a localised influence on the soil behaviour within the proximity of either of the two sensors installed at the mid region of the specimen. For instance, if there is a formation of sclerotia close to the location of the porous ceramic cup of the tensiometer (T2), it could affect the value of suction pre-infiltration. Once infiltration began, this effect may have ceased.

b) Advancing infiltration water fronts

By the 3rd and 4th minutes of infiltration into the untreated and treated specimens respectively, the advancing water fronts had reached the topmost sensors and nearly saturated the soil at a depth of 25 mm below specimen surface (see Fig. 5-12 a, b, d & e). For the untreated specimen, evidence of this can be seen in the sharp change in VWC and PWP in Fig. 5-12 a & b, where the saturated volumetric water content = 43%. By the time this infiltration front reached a depth of 150 mm (~5th minute) and water started flowing out from the bottom of the untreated column (6th minute), the soil had densified (volumetric collapse on wetting) and the topmost sensors were now

fully immersed in water instead of being emplaced within the soil, hence the 'anomalous' values of volumetric water content greater than the saturated volumetric water content (>43%) recorded by $\theta 1$ (Fig. 5-12b). This densification/collapse occurred because air in the pore spaces of hitherto loose specimen (at a dry density of 1.08 g cm^{-3}) were progressively displaced by advancing water as infiltration progressed, as such there was a loss of water menisci at grain contacts, which were providing a stabilising effect in this loose specimen. The low density of the specimens was selected in order to ensure optimum aeration for fungal growth.

On the other hand, in the treated specimen the water front advanced in a uniform manner through the top 30 – 35 mm (see Fig. 5-13, t = 0, t = 3mins). Thereafter no further advancement of the infiltration was observed for over 30mins (Fig. 5-13, t = 40mins) before a reduction in suction and increase in volumetric water content was recorded at the middle sensors located at 150 mm depth at t = 35mins (Fig 5-12e and Fig. 5-13). Although suction was lost i.e. the PWP at this point became 0 kPa, the VWC was recorded as ~25% and remained at this value for the entire duration of the infiltration test. This is probably due to be massive hyphal activity in this middle region, such that hyphal exudates and biomass may have clogged soil pore spaces and together with the hydrophobic characteristics led to a reduced quantity of water per unit volume of soil in this region of the specimen. Wetting of the middle section occurred very slowly compared to the top and bottom parts of the specimen, indicating that a semi-permeable barrier was formed here, sealing the soil sub-layer after about 35 mm depth and putting up significant resistance to wetting over such a long time. When the infiltration front broke through this layer, it was diffuse and fingering of

flow occurred via preferential flow pathways, probably through the weakest links in the semi-permeable hyphal barrier as can be seen in Fig. 5-13.

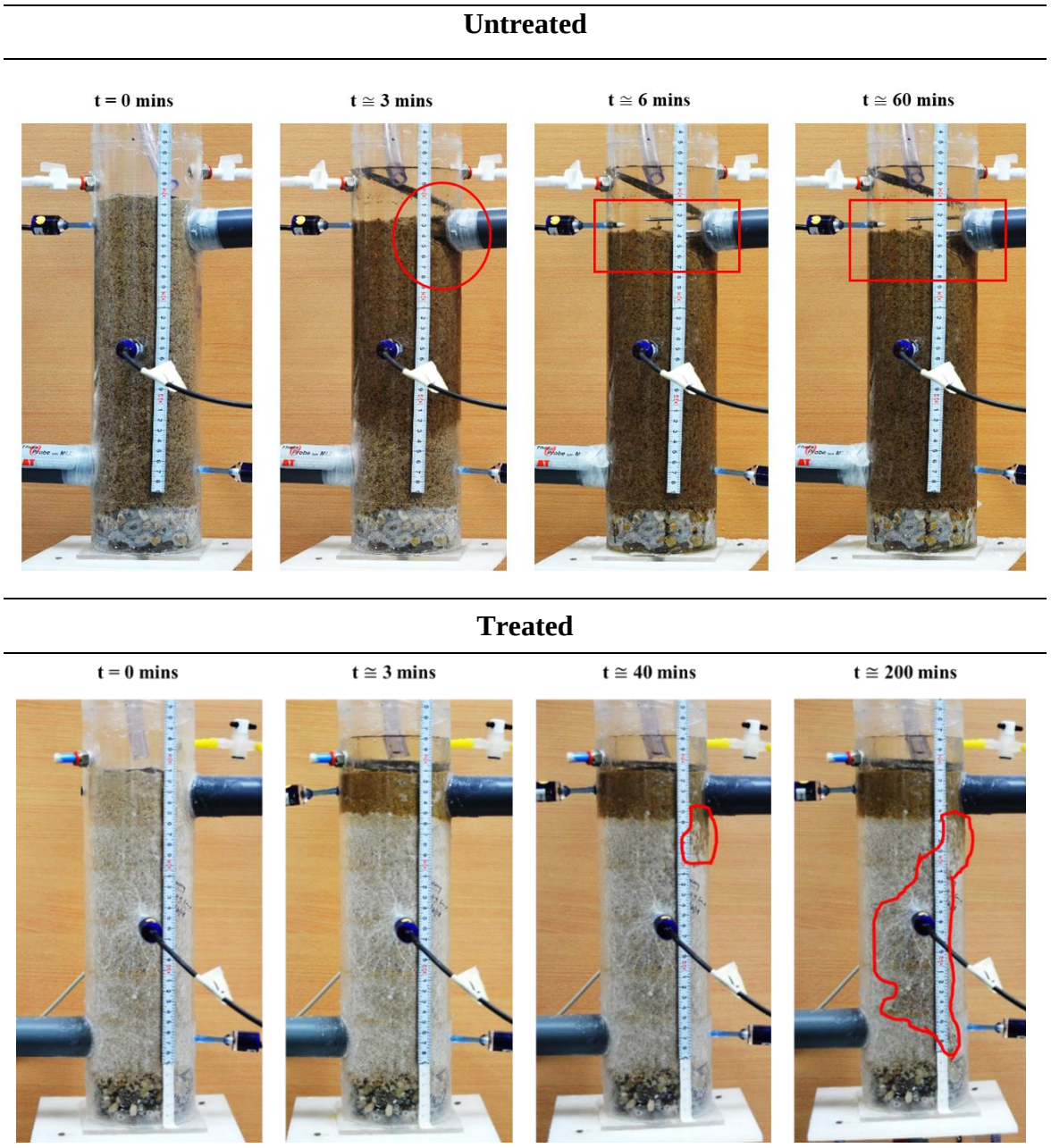


Figure 5-13: The advancing water front in the untreated and treated specimens over time. Within 3 minutes after infiltration started, the untreated specimen (above) began to collapse as seen in the red circle around the moisture sensor, θ1. By the 6th minute, both sensors T1 and θ1 were no longer in soil as soil height has reduced by ~ 30 mm. This status was maintained for the rest of the infiltration test run. Infiltration front in the treated specimen (below) advanced to ~35 mm depth within 3 minutes and was repelled from further percolation for almost 30 mins. About 10 mins after this lag time, visible signs of fingered flow through supposedly preferential pathways were noticed. This diffused flow continued gradually until it reached the position of sensors at 275 mm almost 3 hrs later.

In the treated specimen, there followed a slow and gradual wetting of the lower half of the specimen as it took another 2hrs (120mins) before the sensors at the depth of 275 mm began to show slight changes, evidencing a change in moisture conditions and indicative of water flow approaching that level. However, it was not until the 8000^{th} minute (5.7days) before a significant change in VWC was recorded at the depth of 275 mm. At this time, the VWC increased to ~20% and the PWP increased to 0 kPa. This is in contrast with the untreated specimen, which recorded maximum volumetric water contents of ~40% at a depth of 275 mm after 21mins. By the time the test was stopped, after over 9700 minutes (~6.5days), the VWC at the bottom of the treated specimen was approaching the saturated moisture content. Nonetheless, despite maintaining the constant head of 25 mm, there was no continuous outflow from the drain at the bottom of the treated column up to the end of the test, although some discontinuous trickles were spotted at the bottom of the column base, i.e. steady state flow conditions had not yet been reached under this low constant head.

These experimental outcomes are similar to observations made by Wang et al (2000) in their study on infiltration into *Ouddorp* repellent sands of the Netherlands prepared at dry densities of 1.5 – 1.6 g cm^{-3}. They reported initial infiltration lag times of 30 mins, corresponding to the water drop penetration time of their repellent sands and observed very slow and fingered infiltration into the repellent sands compared to higher infiltration rates and a uniform advancing front in wettable sand.

5.3.3 Cumulative infiltration into treated and untreated soil

Fig. 5-14 present the cumulative volume of water infiltrated into the specimen for the untreated and treated specimens. In the untreated specimen, the cumulative volume

of infiltrated water (determined from the change in mass of the specimen, recorded by the balance) increased rapidly within the first 6 minutes of inflow and subsequently became relatively constant as the advancing water front approached the bottom of the specimen under the constant ponding head. After 6 minutes the specimen became fully saturated and there was thus no further change in the volume of water retained within the specimen pores, i.e. steady state flow conditions were reached in the untreated specimen. This is expected because the untreated specimen was made of sand and lignocel, and was therefore expected to have a high hydraulic conductivity. The treated specimen also showed an initial increase in the cumulative volume of infiltrated water within the first 3 minutes of inflow. However, much higher volumes of water infiltrated into the untreated specimen at the beginning of infiltration compared to the treated specimen. Though the amount of infiltration water into the treated specimen continued to increase steadily under constant head conditions, even after 5 days it was evident that the treated specimen was not saturated and steady-state flow conditions were not reached. This implies that for any given time period, infiltration is expected to proceed at a much slower rate in the fungal treated specimen compared to the untreated specimen.

The procedure for determination of saturated hydraulic conductivity involved bringing the specimens to saturation via upward flow, which followed after drainage of the specimen after the infiltration stage discussed above. The change in soil characteristics prior to the start of infiltration and at saturation (before the saturated hydraulic conductivity test) for both untreated and treated specimens are presented in Table 4.

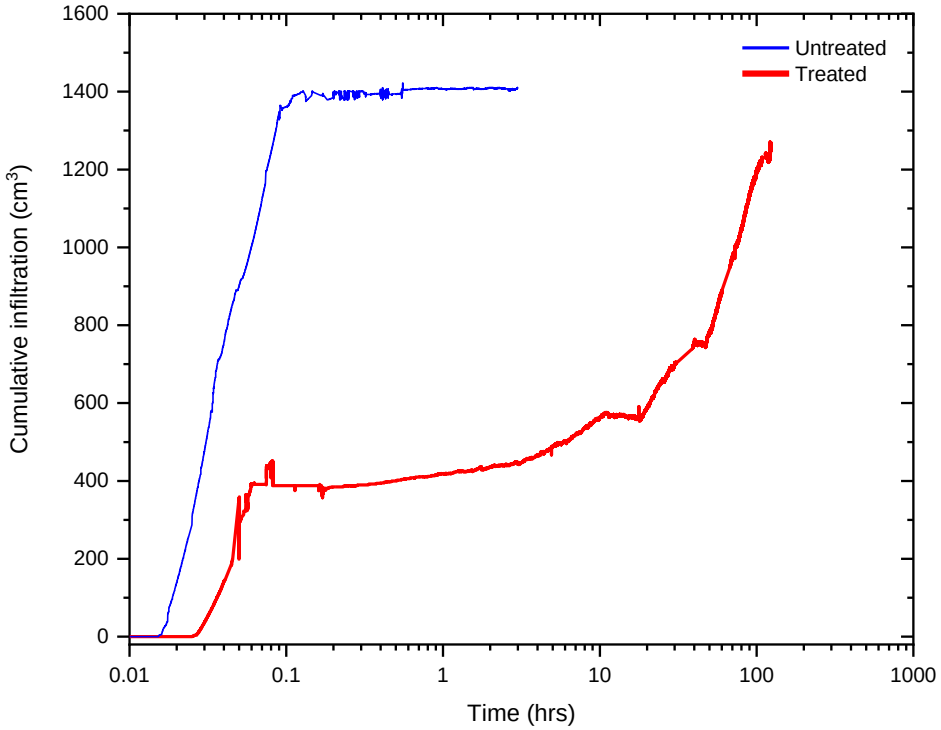


Figure 5-14: Cumulative volume of water infiltrated with time for the untreated (thin blue line) and treated (thicker red line) specimens

Table 5-4: A comparison of soil properties immediately after specimen preparation and after saturation

Characteristics	Untreated		Treated	
	Initial	Saturated	Initial	Saturated
Dry density, g cm^{-3}	1.08	1.19	1.08	1.09
Porosity *(S.G = 2.034)	0.47	0.41	0.47	0.46
Voids ratio	0.88	0.69	0.88	0.86

**Specific gravity of the soil (sand & lignocel) was determined using the water pycnometer method* (ASTM D854-14, 2014)

The change in dry density of the untreated specimen before and after saturation was due to the volumetric collapse observed (Fig 5-13). The untreated specimen exhibited volumetric collapse as a result of the suction loss during infiltration. In the treated specimen with the same initial conditions (i.e. low dry density) no volumetric collapse was observed on wetting. The fungal growth (clearly visible in Figs 5-13) contributed to stability of the treated soil in the column and as such it had a more porous arrangement of sand grains at the beginning of the saturated hydraulic conductivity test.

5.3.4 Saturated hydraulic conductivity

Saturated hydraulic conductivity was determined for both specimens by downward and upward flows. The plot of flux (Q/A) vs hydraulic gradients for untreated and treated specimen are presented in Fig. 5-15.

Linear regression line was fitted to the data points to obtain the respective saturated hydraulic conductivities k_{sat}. At 20°C, the average k_{sat} for untreated specimen from both downward and upward flows was found to be ~ 1.34 x 10^{-4} m s^{-1} while for treated specimens it ranged between 3.1 x 10^{-5} to 1.52 x 10^{-5} m s^{-1}. In all cases, the results consistently showed that treated specimens were an order of magnitude less permeable than untreated specimen, even though the untreated specimen had a slightly higher dry density than the treated specimen during this stage of testing. The growth of hyphal networks of *P. ostreatus* reduced the hydraulic conductivity of a well-graded sand.

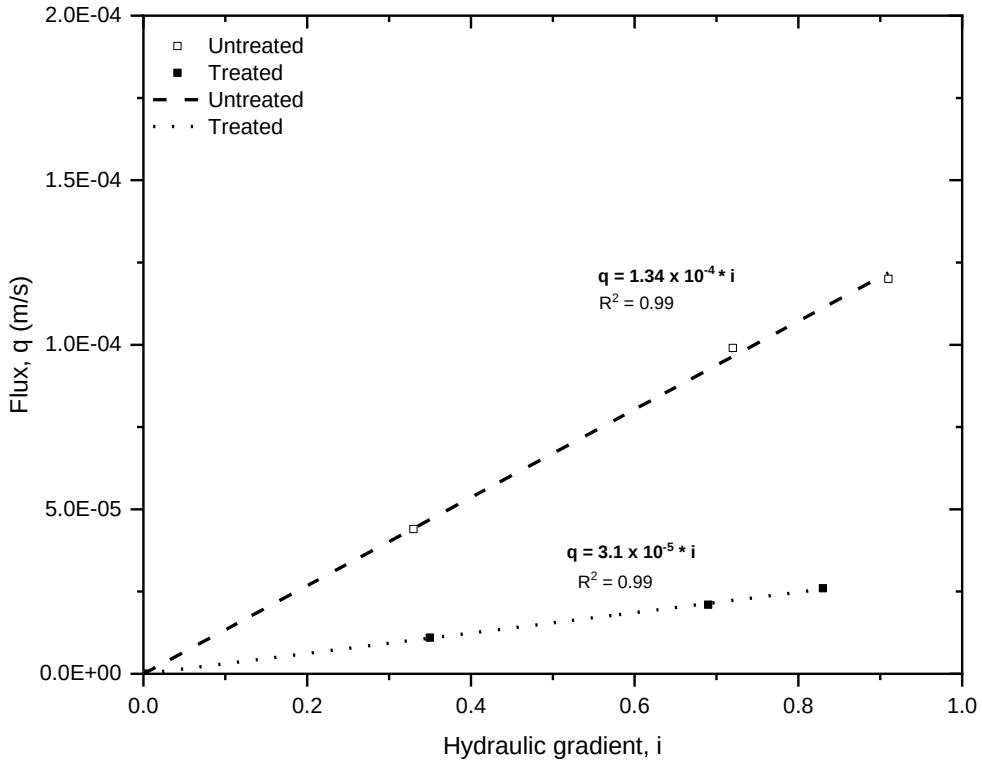


Figure 5-15: Plot of flow per area vs hydraulic gradient for treated and untreated specimen

5.4 Discussion

The findings from this study indicate that growing fungal hyphae in sand can modify soil hydraulic behaviour by (i) altering its water retention behaviour, (ii) reducing its saturated hydraulic conductivity and (iii) reducing the rate of infiltration. The proposed mechanism(s) for these fungal-induced hydraulic effects are discussed in the following sub-sections.

5.4.1 Effects on water retention curve

Water repellency alone in hydrophobised soils has been shown to shift water retention curves to lower air entry values and lower water contents for a given suction (Beckett, Fourie and Toll, 2016). The opposite has been shown here for sand treated with *P. ostreatus*. Growth of *P. ostreatus* shifted the air entry values slightly, such that the treated specimens retain more water at a given suction. For example, in the treated specimen at a suction of 5 kPa, the specimen retains 34% moisture, whereas at this suction level the untreated specimen has a moisture content of ~8%. This implies that factors aside from simply hydrophobicity are influencing the retention behaviour. The fungal hyphal mass grown within the pore space is likely reducing the effective pore entrance diameter, thereby requiring higher suctions to drain water from pores.

However, other studies have reported little or no significant effect of the growth of filamentous fungal species (a mycorrhizal species: *R. irregularis* and a parasitic Basidiomycete: *R. solani*), on the water retention curves developed (Augé *et al.*, 2001; Pajor, 2012), despite the fact that the mycelia of both species also exhibit hydrophobicity (Rillig *et al.*, 2010; Lin *et al.*, 2018; Sandoval and Cumagun, 2019)

and tests were carried out after a short (5 days) and relatively longer (7 months) growth periods. It must be noted that these studies were conducted on arable sandy-loam soils. Fungi is diverse and exhibit variable characteristics depending on species and strain. Both species reported in the studies by Augé *et al.*, (2001) and Pajor, (2012) depend on plant hosts for their growth and development; this means that the extent of hyphal colonisation of soil would be largely determined by root characteristics. This could be a limiting factor compared to saprotrophic species, like *P. ostreatus*, which can grow extensively and increase in biomass within available space as long as the environmental conditions are suitable (Chapter 3). In this study, *P. ostreatus* was inoculated into soil with sufficient carbon source and water content and incubated at 25°C based on optimal growth conditions determined in Chapter 3. After 12 weeks growth period, fungal induced water repellency still persists in soil (Chapter 4) and there was evidence of massive hyphal colonisation of soils (Fig. 5-12 & Fig. 5-13). In effect, it is proposed that this massive hyphal growth along with corresponding large amount of exudates may have resulted in the influence observed on the soil water retention curve in this study. Though hyphal growth and amount of exudates were not quantified in this study, evidence of continued fungal activity was observed during the 48-day period of the drying-wetting cycles.

Furthermore, the emergence of mushrooms and continuous mycelia activity observed on the surface of the specimen lends support to the argument that a soil improvement strategy based on fungal hyphae has the potential to, require little or no intervention, provided that suitable environmental conditions exist. These conditions may simply be seasons of precipitation and sunshine/daylight. This implies that once effectively

engineered and controlled to suit desired applications, this technique could have potentially low maintenance costs.

5.4.2 Effect on infiltration and hydraulic conductivity

In this study, it was shown that the rate of infiltration of water into treated sand was slower compared to the untreated sand. Also, the saturated hydraulic conductivity of treated sand was an order of magnitude less than the untreated sand, despite having a lower dry density compared to the untreated. These findings are in contrast with results from other studies. The presence of fungi in soils have been widely reported to cause faster drainage and higher hydraulic conductivity (Crawford *et al.*, 2012; Pajor, 2012) compared to soils with no fungal growth. The main difference is that those studies were all carried out in silty/clayey soils where fungal growth enhanced formation of aggregates and subsequent creation of more macropores with increased porosity and pore connectivity (Crawford *et al.*, 2012), whereas in sand it appears that fungal growth also contributes to pore filling.

This study assesses for the first time, the influence of the growth of the hyphae of a saprotrophic fungus (*P. ostreatus*) on the infiltration characteristics and saturated hydraulic conductivity of sand. Fig. 15-16 presents a conceptual description of the proposed mechanisms by which the growth of the hyphae of *P. ostreatus* led to relatively lower infiltration rate and lower saturated hydraulic conductivity compared to untreated sand.

It is proposed that as hyphae of *P. ostreatus* grow in sand, they release gummy exudates, fill soil pores, and physically enmesh particles, thereby causing binding of

sand grains. The filling of soil pores acts to reduce pore entrance diameters, which contributes to the increase in the air entry value observed in the water retention curves of the treated specimen. At the same time, water repellence of the sand grains is induced due to the hyphal growth, since there are air pockets which provide room for the development of amphipathic membranes of hydrophobins (Chapter 4). The induced water repellency and reduction in pore size both contribute to a lag in water breakthrough, i.e. a delay in infiltration, which will be governed by the hydraulic loading (in this case infiltration under one constant head value was investigated), the degree of water repellency mobilised by the hyphal network and the alteration of the pore size distribution induced by fungal growth. The latter two depending on the uniformity of hyphal growth. Where water breakthrough later occurs, infiltration through this 'semi-permeable' hydrophobic barrier may occur in a diffuse manner, slowly wetting adjacent soil micro-aggregates and advancing in a fingering pattern. Infiltrating water is retained and protected within residual hydrophobic pore-pockets and within the cell walls of massive pore-filling and grain-enmeshing hyphal networks.

The delay to infiltration, the lower infiltration rates and differential wetting due to discrete flow paths provide an opportunity for suction levels to remain higher than in the same untreated soil, thus enabling the soil to benefit from the enhanced mechanical behaviour associated with higher suction/unsaturated conditions. For application in the field (or in clay soils for instance), where differential wetting may result in undesirable effects like swelling, further studies are necessary for effective engineering of fungal growth to create a totally uniform hydrophobic barrier suitable for such conditions

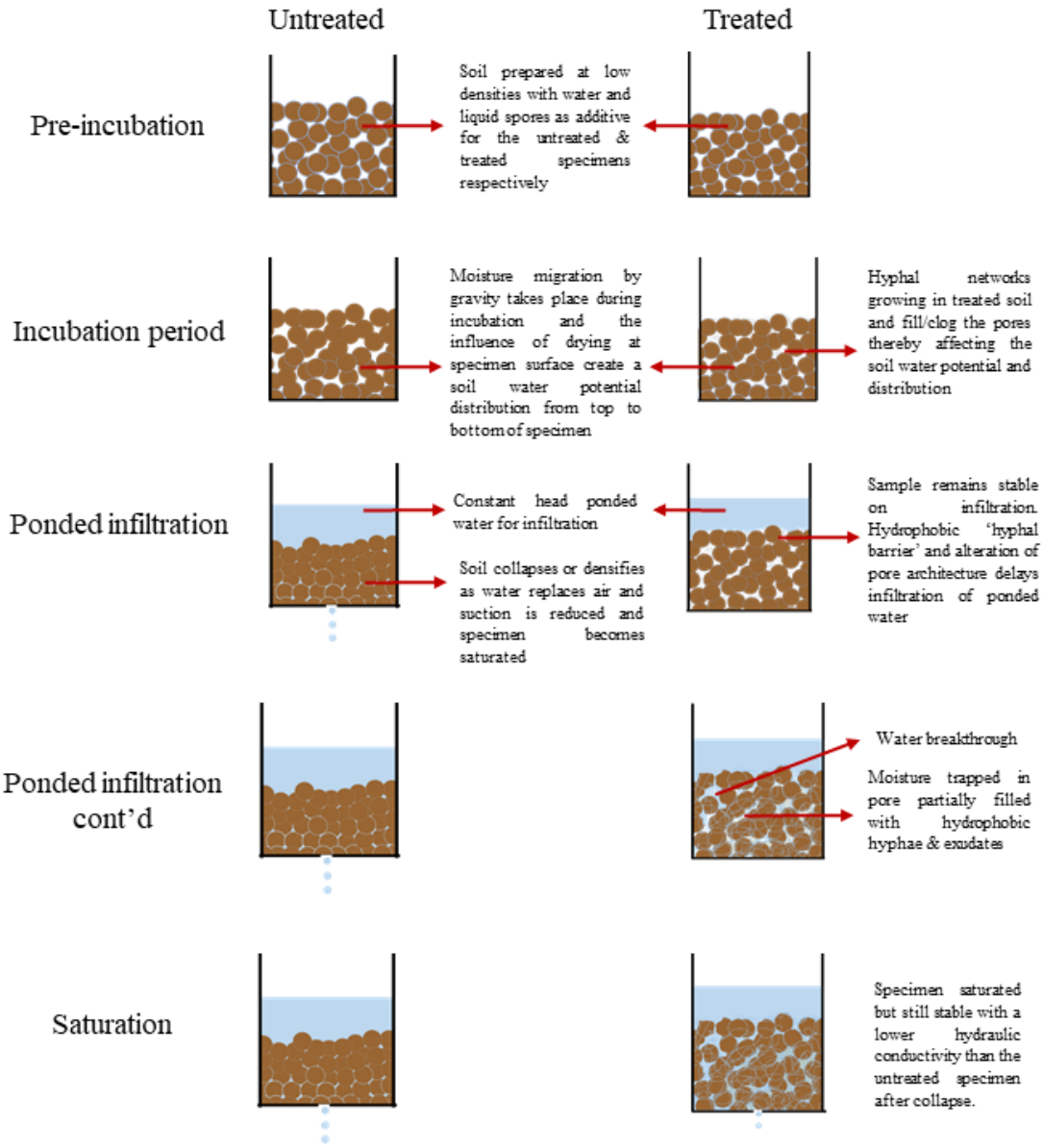


Figure 5-16:Conceptual description of effect of hyphae growth on soil based on findings from this study

Pre-infiltration moisture retention and localised variations were seen in the lower part of the treated column in this study. These may be attributed to the action of fungal hyphal networks during growth and development, as follows: (i) that significant amount of the initial moisture in the specimen may have been absorbed by hyphae for their growth and development. Hyphae have been found to contribute to moisture retention in dry arid soils as they absorb local resource (in solution) into their cells and

limit rapid loss of protoplasmic fluids due to their strong cell walls (Schimel, Balser and Wallenstein, 2007). This characteristic, coupled with the formation of protective hydrophobic layers by species like *P. ostreatus* keeps moisture intact within the hyphal walls and within pore pockets with hyphal growth, thereby contributing to the soil net moisture content overtime. (ii) Unlike bacteria, hyphal growth involves extension and branching of hyphae which form massive 3-dimensional networks called mycelia. There is also the localised release of biochemical exudates by hyphae as they grow (Rillig and Mummey, 2006). In these processes, there is huge mobility and spatial distribution of hyphae within the soil matrix. This would expectedly result in translocation or redistribution of moisture within the soil profile (Guhr *et al.*, 2015). (iii) Hyphae-induced soil moisture dynamics may be influenced by gradients in antecedent soil suction and hyphal tropisms or response to variable environmental stimuli, as is typical of filamentous fungi (Brand and Gow, 2009). There is the likelihood that after an initial period of percolation during incubation, the eventual moisture content at the mid-region of the specimen was optimal for fungal growth compared to less moisture at the top and more at the bottom, leading to increased hyphal activity in the middle region. It was shown in Chapter 3 that a moisture content of 11% was optimal for growth of the hyphae of *P. ostreatus*. Even though the specimen here was prepared with a starting gravimetric moisture content of 11%, there would be different moisture contents across the specimen overtime as percolation would lead to a moisture gradient across the 300 mm high specimen. The higher moisture content recorded at the middle region with higher fungal activity is also plausible based on the results of the soil water retention curve where the growth of *P. ostreatus* led to higher moisture retention and the explanation in (i) above.

For the top 50 mm of treated specimen where the influence of fungal growth seemed negligible, it is likely that the disturbance of specimen during experimental set up as well as the period of leaving the set up overnight for the silicone sealant to dry may have affected the continuity of hyphal growth barrier within that area. In Chapter 4 it was shown that destruction of established hyphal networks by any interference, e.g. soil mixing would disrupt surface hydrophobicity; although this could be regained within 48 hours if suitable growth conditions exist.

5.5 Conclusion

This study was undertaken to determine the influence of the growth of hyphae of *P. ostreatus* on the soil-water retention behaviour, infiltration characteristics and saturated hydraulic conductivity of a well-graded sandy soil amended with organic matter (lignocel). An untreated control was set-up and compared with the treated specimen to determine the extent of modification on the soil hydraulic properties due to fungal growth. Findings from this work are as follows:

1. From the soil water retention curve obtained, it was observed that growth of hyphae of *P. ostreatus* increased the air entry value of sand, improving the water retention behaviour of sand, such that at an applied suction, the treated specimen retained more water than their untreated counterpart.
2. Due to the very low initial dry density (1.08g cm^{-3}) of these specimens, the untreated specimen exhibited volumetric collapse upon infiltration as it tended toward saturation and suction was reduced. In contrast the treated specimen exhibited no collapse and remained stable throughout infiltration and saturation with no volume changes observed. The presence of hyphae, their

enmeshment of particles and possible exudates are thought to contribute to binding of particles and were responsible for keeping the soil stable during infiltration.

3. Fungal treated soil resulted in significantly lower infiltration rates compared to that obtained in untreated soil. This is attributed to clogging of soil pores by hyphal biomass which are relatively non-wetting as a result of hydrophobic biochemical secretions by hyphae. These combined characteristics result in reduced overall pore spaces, significant delay (up to 5 days' difference) in advancement of the wetting front, diffuse flow through tortuous paths and ultimately, slowed down infiltration rate in treated sand.
4. The saturated hydraulic conductivity (k_{sat}) for the fungal treated soil was found to be an order of magnitude lower than k_{sat} for the untreated soil, for specimens after 12 weeks of incubation.

The results presented in this chapter illustrate the potential for the deployment of fungal treated soils as a low cost, low carbon technique for creating semi-permeable layers deployable in the design of slow draining water holding layers in capillary barriers or as surface layers in geo-infrastructures utilised for water diversion purposes. In such cases, a mechanism for moderating the growth distribution across the soil profile/layers may be required. Also, the results suggest that hyphae growth could potentially enhance soil water retention at higher suction values, implying that the stabilising effects of suction in sands could be further explored for variable moisture contents by engineering fungal growth to obtain suitable outcomes.

Furthermore, the method of hyphal inoculation for the 300 mm high specimen in this study did not provide uniform distribution of growth networks as seen in the negligible fungal effect in the top 50 mm of the treated specimen and later preferential flow paths and variations in the middle and bottom regions due to different concentrations of hyphal growth. It would be worthwhile to investigate different treatment methods coupled with microscale analysis of hyphal distribution per method, as this is crucial for obtaining required outcomes in terms of growth uniformity. Microscale imaging of a section through the specimens from preparation through incubation to infiltration and saturation stages could help to provide a clearer picture of the overall interaction between hyphae, hyphal exudates, soil mineral particles, lignocel (or organic matter), water and air. Micro-scale investigation of hyphal biomass distribution and evolution under variable moisture conditions could provide more evidence to understand the key mechanisms involved. This will also aid any future plans of upscaling to field site conditions and will be necessary for any predictive modelling of bulk soil behaviour response to fungal growth.

Based on the findings in this chapter, the influence of the growth of *P. ostreatus* on soil erodibility was investigated; this is presented in Chapter 7. This was carried out as a first attempt towards application of the proposed fungal-based soil improvement technique to a real problem.

Chapter 6

Observations of the Shear Behaviour of Fungal Treated Soil[1]

Abstract

This Chapter presents results of an investigation into an entirely novel technique for ground improvement involving the use of fungal hyphae. Fungal hyphae (long filamentous branches) are known to contribute to soil aggregation and soil hydrophobicity, and are hypothesised to also influence the hydro-mechanical behaviour of soil. We present here preliminary observations of the mechanical behaviour of sands treated with the fungal species *Pleurotus ostreatus.* Direct shear tests were carried out on sand containing different percentages of organic substrate and treated with *P. ostreatus*. The stress-strain behaviour of fungal treated and untreated soil was investigated. Results show that irrespective of the percentage of organic matter, treatment type and duration, fungal treated specimens tended to show a loss in the peak behaviour characteristic of the untreated control specimens and an associated transition towards a more contractive volumetric response. The results show similarities in behaviour to both hydrophobic sands and artificially reinforced sands reported in the literature. Further investigation is required to fully elucidate the mechanisms influencing the mechanical behaviour of fungal-treated soils.

[1] *This chapter has been accepted for publication in the EPJ web of conferences as part of proceedings of the 7th International Symposium on Deformation Characteristics of Geomaterials to be held at the University of Strathclyde, Glasgow – UK 26-28th June, 2019. Authors: Salifu, Emmanuel & El Mountassir, Gráinne. Title: Preliminary Observations of the Shear Behaviour of Fungal Treated Soils. (Some changes/edits have been made to parts of the manuscript such as the discussion section, for consistency and to fit within this thesis*

6.1 Introduction

Within the emerging sub-discipline of bio-geotechnical engineering, El Mountassir *et al.*, (2018) proposed a novel technique involving the engineering of fungal hyphae, with a view to deploying it as a potential low-carbon technique for soil modification and ground improvement. Filamentous fungi (such as *Pleurotus ostreatus*) grow within near-surface soil layers as a network of hyphae; this network is commonly referred to as the mycelium. These vegetative parts of the fungus develop from fungal spores or cells. Protein compounds in the cells are mobilised to one end, from where the cell extends structurally, forming a tubular elongated, thread-like shape (the hypha), which can fuse with adjacent hyphae or produce 'branches' capable of spreading over large areas and distances in environments where suitable conditions exist (Smith, Bruhn and Anderson, 1992).

From the field of soil science, it is widely known that fungi present naturally in the environment contribute to soil architecture and soil aggregation (e.g. Rillig, 2004). It is hypothesised that the growth of fungi can be engineered to bring about changes of geotechnical significance. The fungal characteristics of interest include their physical form. For filamentous fungi, that is the form of the hyphae or rhizomorphs which are typically 1-30 μm in diameter and range from several microns up to metres in length, and can form extensive 3D hyphal networks. The physical action of the mycelium as it grows through the soil tends to enmesh or entangle soil grains, thereby contributing to the formation and maintenance of soil aggregates (Caesar-TonThat and Cochran, 2000; Rillig, 2004).

The biochemical exudates released during the life-cycle of the fungus have also been found to influence soil wettability (Liu, Ma and Bomke, 2005) and in an ecological community, tends to serve as a binding agent that contributes significantly to the enhancement of soil aggregate stability (Tang *et al.*, 2011; Rashid *et al.*, 2016; Baumert *et al.*, 2018). Soil aggregation and soil hydrophobicity are expected to affect soil water retention characteristics and hydro-mechanical behaviour. Fungal growth in soils requires the presence of organic matter as a nutrient source. As such the engineered growth of fungi in the field may require the addition of organic matter/nutrients.

This chapter investigates for the first time, the shear behaviour of sands treated with fungi. The specific objectives of the study are: (i) to investigate the stress-strain behaviour of fungal-treated sands subjected to direct shear tests and compare with the untreated control specimens; (ii) to investigate the influence of the percentage of amended organic matter and (iii) to investigate the influence of growth duration.

6.2 Materials and methods

6.2.1 Materials

a) Soil and lignocel

Silica sand mixed with lignocellulose (Lignocel®) formed the composition of soil used in this study. Its characteristics are shown in Table 6-1. Lignocel (Grade HB 500 – 1000), a processed form of fibres derived from natural softwoods, is a commercially available organic material obtained from J. RETTENMAIER & SÖHNE GmbH and was used as the nutrient source (substrate) for fungal growth in this study. The

chemical constituents of lignocel typifies that of decaying organic matter in natural soils (Imran *et al.*, 2016) and was selected for study in this chapter based on results presented in Chapter 3. In all tests performed, sand and lignocel were sterilised prior to preparation of the specimens and inoculation with *P. ostreatus* by autoclaving at 121°C for 20mins. The composition in terms of the percentage of sand and lignocel content (of the total mass of solids) for each specimen is given in Table 6-2.

Table 6-1: Characteristics of sand and lignocel

Properties	Sand	Sand & lignocel	Lignocel
D_{60} (mm)	0.33		Particle size range:
D_{30} (mm)	0.28		0.5 – 1mm; with
D_{10} (mm)	0.2		2% >1.25mm;
C_c	1.19		65% >0.63m;
C_u	1.65		90% >0.5mm.
ASTM classification	Uniformly graded fine sand	-	(from supplier's data sheet in Appendix F)
Specific gravity	2.65	2.03	

b) Fungus

P. ostreatus strain: M 2191 was used in this study and was obtained from GroCycle UK as active spawn (mycelia grown on a wheat substrate). The fungal spawn was stored in a cold room at 4°C and used within 30 days.

6.2.2 Experimental procedures

a) Preparation of fungal treatments

In this study two different types of additive were used (i) A spore/hyphal suspension and (ii) fungal homogenate. The methods for preparation for each are as follows. A spore/hyphal suspension of *P. ostreatus* was prepared from spawn as was carried out for experiments presented in Chapters 4 and 5. Fresh spawn of a known mass was

collected, weighed and placed in a conical flask containing de-ionised water. This was then shaken vigorously by hand to ensure that spores and hyphae were released into the water. The mass ratio of spawn:water was 1g:10mL. The mixture was then further shaken at 320 rpm for 30mins and then filtered through a 2 mm sieve to remove larger spawn residues. The filtrate obtained is a suspension containing both harvested spores and hyphae. This was the liquid treatment added to the fungal-treated (T) specimens.

Fungal homogenate (FH) was also prepared for a set of experiments (described in section 3.3, see Table 6-2) because in comparison to the hyphal/spore suspension described above the homogenate contains a higher concentration of fungal biomass and exudates. FH was prepared following a similar method to that described in Caesar-TonThat and Cochran (2000). A fixed mass of sclerotia (thick spongy mass formed by older mycelia) extracted from the exterior of a 25 - 30day old spawn was obtained, aseptically crushed by hand and blended to a pulp. This pulp was then added to DI water (mix ratio = 2g sclerotia pulp:1mL DI water) and homogenised using a sonicator for 20mins. The mixture was filtered through a 2 mm sieve to remove any larger solid pieces leaving a cloudy-watery-suspension or filtrate, the homogenate.

Table 6-2: Test specimens: composition and test conditions

Specimen	Normal stress (kPa)	Sand (%)	Lignocel (%)	Liquid content (%)
T-25kPa	25	94	6	11
U-25kPa	25	94	6	11
T-50kPa	50	94	6	11
U-50kPa	50	94	6	11
T-100kPa	100	94	6	11
U-100kPa	100	94	6	11
T_94S:6LIG	25	94	6	11
U-25kPa	25	94	6	11
T_70S:30LIG	25	70	30	11
U_70S:30LIG	25	70	30	11
T_50S:50LIG	25	50	50	11
U_50S:50LIG	25	50	50	11
U-25kPa	25	94	6	11
TFH_94:6LIG (Day 0)	25	94	6	11
TFH_94:6LIG (1 month)	25	94	6	11

T (fungal-treated using hyphal/spore suspension as additive), U (untreated), TFH (treated with fungal homogenate as additive)

b) Specimen preparation

Each treated specimen was composed of sand, lignocel and fungal treatment (either hyphal/spore suspension or fungal homogenate). For the untreated (control) specimens the volume of spore/hyphal suspension was replaced with deionised water. The liquid content (spore/hyphal suspension or deionised water) is presented in Table 6-2 for each test specimen and is determined as the mass of liquid/mass of solids.

After mixing, the soil-lignocel-liquid mixture was lightly compacted into plastic moulds (Fig. 6-1). Multiple plastic moulds were manufactured using an 'Ultimaker^2 Extended' 3D printer. Multiple random pin-holes were perforated on the sides of the mould to allow passage of oxygen into the specimen. These moulds were required to enable growth in multiple test specimens over 3 weeks under controlled environmental conditions. The hollow square-shaped moulds had outer dimensions of 58.97 x 58.97

x 40 mm and wall thickness of 0.5 mm to facilitate minimal disturbance of the specimen when transferring to the shear box for testing.

Specimens were prepared with dry densities in the range of 0.9 – 1.1 g cm^{-3} to ensure adequate porosity and thus aeration. All specimens treated with the hyphal/spore suspension were incubated at 25°C in the dark and taken out for shear testing after a growth period of 3 weeks.

After assembling the specimens in the shear box, the specimens were inundated with water and left for 2 hours prior to testing. For the specimens treated with fungal homogenate, (TFH, Table 6-2) two specimens were prepared, one which was tested directly after mixing of the homogenate with the soil specimen and subsequent saturation, and a second which was incubated at 25°C in the dark for a growth period of 4 weeks.

c) Direct shear test

A digital direct shear test apparatus (ELE International, UK) with a load cell of 5 kN capacity was used in this study. It has a 60 mm square shear box and is equipped with two displacement transducers for monitoring both horizontal and vertical displacements. The apparatus was connected to a computer for data acquisition via a LabVIEW® programme interface. Specimens were tested at normal stresses of 25kPa, 50kPa and 100kPa, Specific test conditions of each specimen are listed in Table 6-2. All specimens were sheared at a shearing rate of 1 mm min^{-1}.

Figure 6-1: Specimen lightly compacted into plastic mould before incubation

d) Determination of fungal presence after shear tests

To investigate the presence of fungi in the specimens after shearing, the sheared surfaces of selected specimens were stained using fluorescein diacetate (FDA) and acridine orange (AO). FDA glows green under UV light when viable (i.e. living cells) are present, indicating the presence of live fungi. AO on the other hand stains and fluoresces varied intensities of green-yellow-red depending on stages of fungal colonisation indicating the presence of live or dead fungal cells (Houtman *et al.*, 2016).

6.3 Results and discussion

6.3.1 Visual observations

It was visibly evident for the fungal treated specimens, that fungal growth resulted in binding of the test specimen components. As such for the treated specimens it was possible to transfer the specimens directly into the shear box from the plastic mould using a pushing tool. In this procedure the fungal treated specimens remained intact

(as a whole specimen) with minimal disturbance. On the other hand, during transfer of the untreated control specimens (which were also initially placed in the plastic moulds and in the controlled environment for 3 weeks) via the same procedure, it was clear that the control specimens remained composed of cohesionless loose sand/lignocel. As such these specimens were recompacted into the shear box with the same procedure as during initial preparation with the aim of achieving the same initial target dry density (0.9-1.1gcm^{-3}).

Fig. 6-2b shows the shearing plane of typical treated samples stained with FDA (on the right) and AO (on the left). The FDA-stained samples (on the right) did not give off a green glow however the samples stained with AO showed a clear fluorescence, implying that fungal cells and (what appear to be) hyphae are present but are no longer alive. It is possible that as these samples were taken from the shear plane, the mechanical action may have ruptured hyphae and cells.

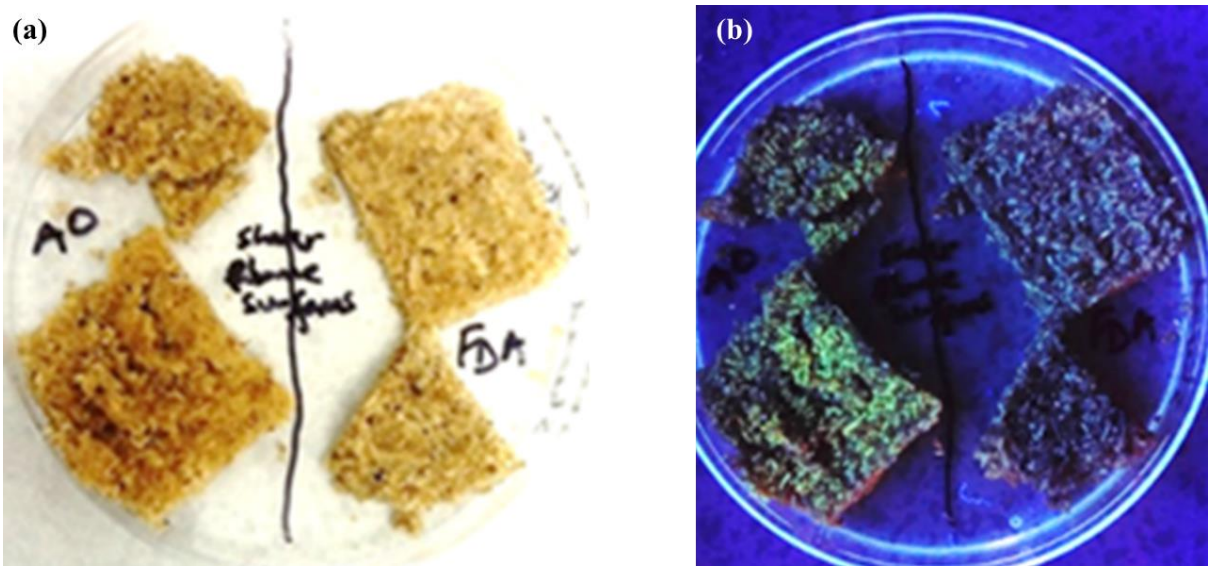


Figure 6-2: : Samples of sheared surfaces (a) before and (b) after staining with FDA (to right) and AO (to left).

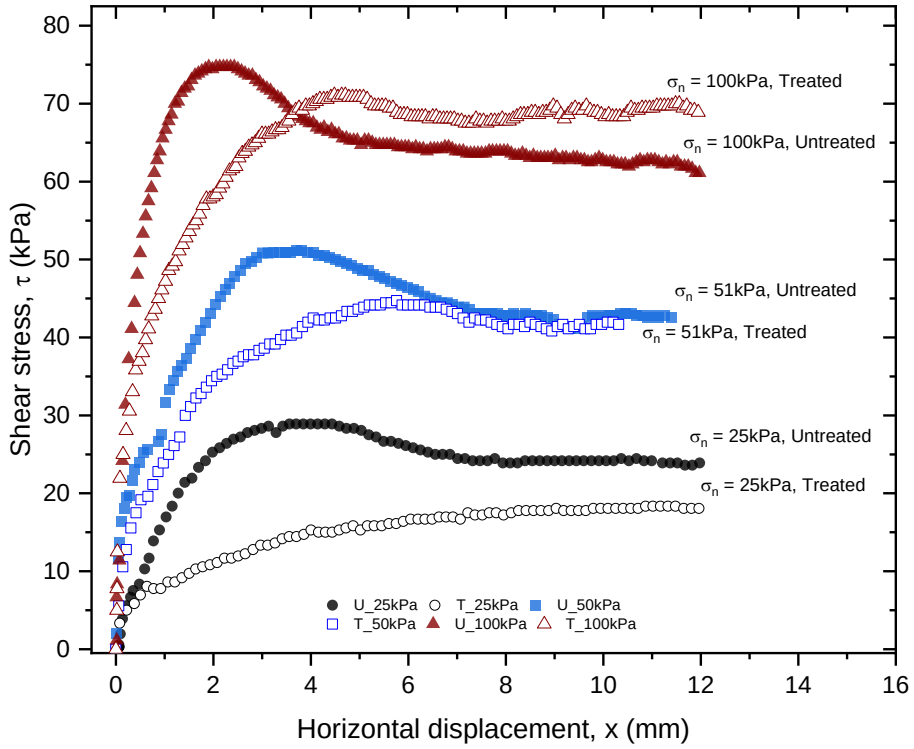
6.3.2 Effect of fungal treatment on shear behaviour

Figure 6-3 present plots of (a) shear stress and (b) vertical displacement against horizontal displacement as well as (c) the peak and ultimate failure envelopes for treated and untreated specimens under applied normal stresses of 25, 51 and 100kPa. It is evident in Figure 6-3a that the fungal treated specimens exhibit a lower peak shear strength than the untreated specimens and have reduced initial stiffness, i.e. a greater strain is required to reach failure. Fig. 6-3b highlights that for all the normal stresses tested dilatancy is inhibited in the fungal treated specimens compared to the untreated specimens and this effect is more pronounced at lower normal stresses.

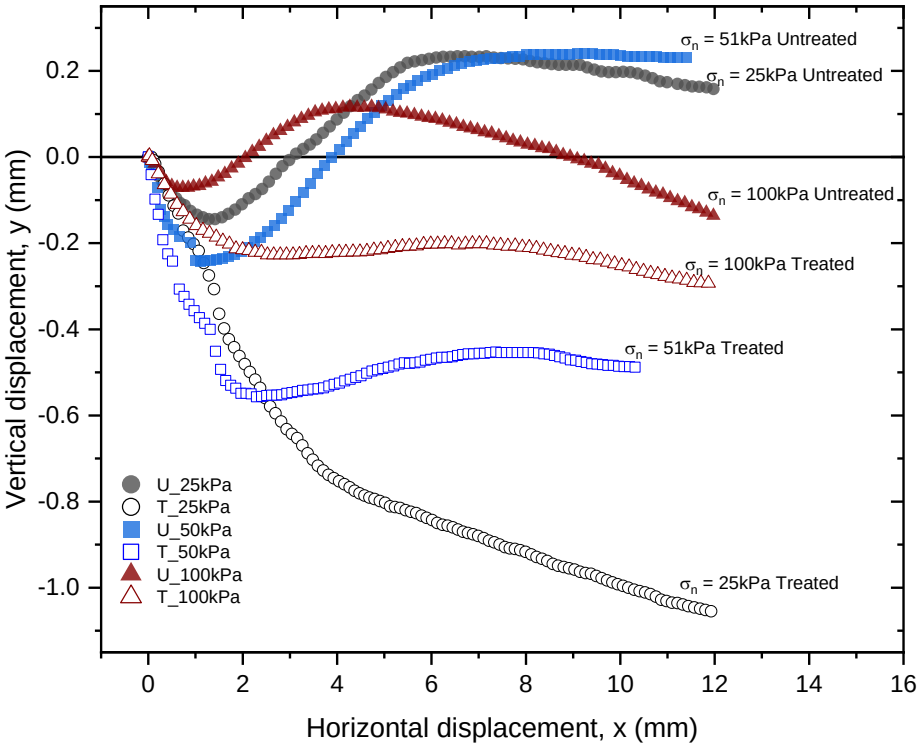
As seen in Fig. 3c, the peak friction angle (ϕ'_{peak}) decreased from 39° for untreated specimens to 35^o for treated specimens, while the ultimate $\phi'_{ultimate}$ was approx. 34^o for both treated and untreated specimens. Cohesion (c') of the treated specimens was determined as c'_{peak} = 3kPa and $c'_{ultimate}$ = 4kPa for the peak and ultimate failure envelopes respectively.

Experimental results of direct shear tests on glass beads and sands chemically hydrophobised using silanisation techniques show similar behaviour, with a reduction in peak shear strength, a suppression of dilatant behaviour and a corresponding reduction in internal friction angles (Karim, Tucker-Kulesza and Derby, no date; Banitz *et al.*, 2011; Byun and Lee, 2012; Byun *et al.*, 2012; Kim, Kato and Park, 2013). This has been shown for hydrophobic media at different degrees of saturation and for different particle shape (perfectly round beads and crushed sands). Byun *et al.*, (2012) propose that modification of the silica surfaces via silanisation results in a reduction of interparticle friction. The hydrophobins secreted by the fungal hyphae to induce

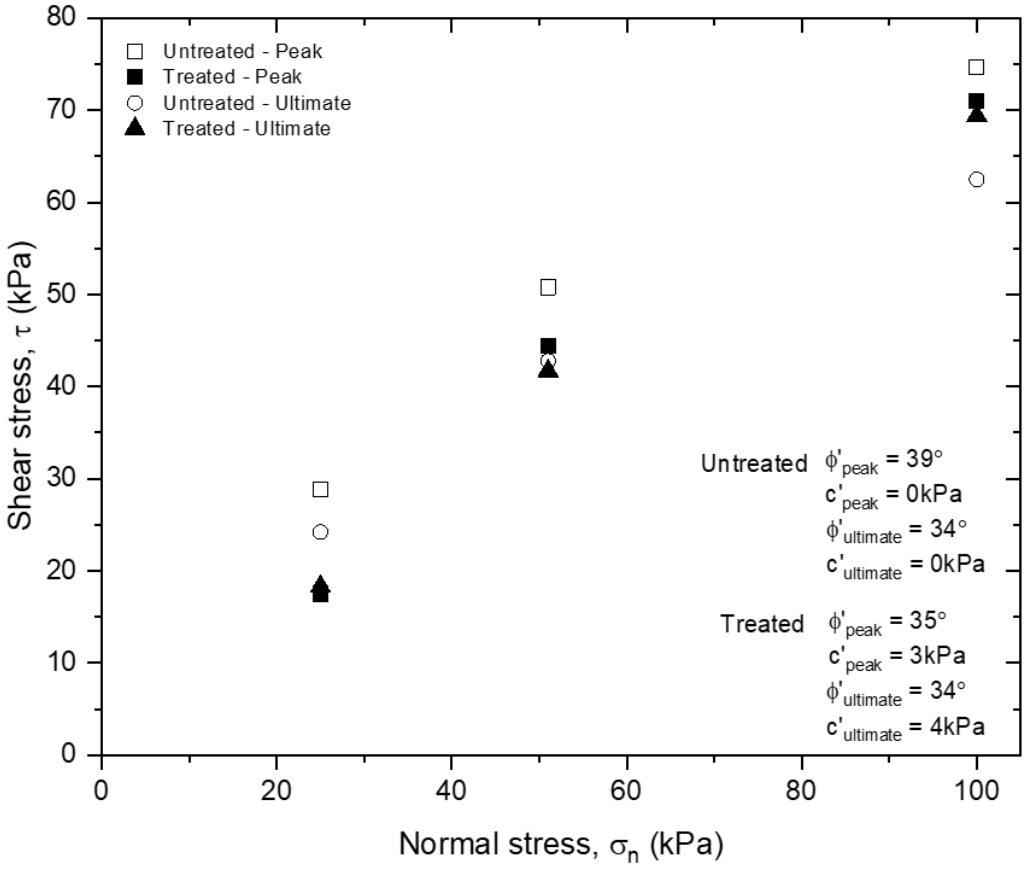
water repellency in the fungal treated soils (and other exudates) may similarly be contributing to a lowering of the interparticle friction at grain contacts.



(a)



(b)



(c)

Figure 6-3: (a) Shear stress - horizontal displacement and (b) volumetric behaviour of treated and untreated soils under applied normal stresses of 25, 51 and 100kPa; (c) failure envelope of treated and untreated soil

Experimental studies (triaxial and direct shear test) on artificial fibre reinforcement (e.g. steel or polyamide) in sands also present similarities to the behaviour presented in Figure 4-3. Although fibre reinforcement in sands tends to lead to a greater ultimate shear strength or deviatoric stress; the inclusion of fibres is also typically associated with inhibition of the development of dilatancy, and with increasing fibre content a lower initial stiffness (Michalowski and Zhao, 1996; Michalowski and Čermák, 2003; Gray and Ohashi, 2008).

As well as the influence of the induced hydrophobicity of the sand grains and the hyphal fibres, there are a number of other factors which may also have contributed to the reduction/ of the peak shear strength observed in the fungal treated sands: (i) The enmeshment of the sand and lignocel grains by the fungal hyphae created a stiffer specimen, with the consequence that on the application of the vertical stress the presence of the hyphal network prevented the sand/lignocel grains from rearranging to the same extent as in the untreated specimens. Thus despite having the same initial dry density, after applying the vertical stress, the treated specimens likely maintained a lower dry density and more open structure compared to the untreated specimens. In early trials larger vertical displacements were recorded for treated specimens compared to untreated specimens during loading. Unfortunately, this data is not available for the test specimens presented in this chapter. (ii). As the fungus grows, lignocel is digested as the nutrient source, thus it is possible that this is also partly responsible for a lower dry density in the fungal treated specimens (compared to the untreated) immediately prior to shearing.

6.3.3 Effect of amount of organic matter on shear behaviour of treated soil

Treated and untreated soil made up of variable proportions of lignocel and sand (see Table 6-2) were investigated. This was based on the hypothesis that increasing the amount of organic matter added to the soil would affect fungal growth and by extension may influence the soil shear behaviour. Fig. 6-4 shows the plots for shear stress and vertical displacement against horizontal displacement for both treated and untreated specimens with the amount of lignocel (LIG) ranging from 6 – 50%.

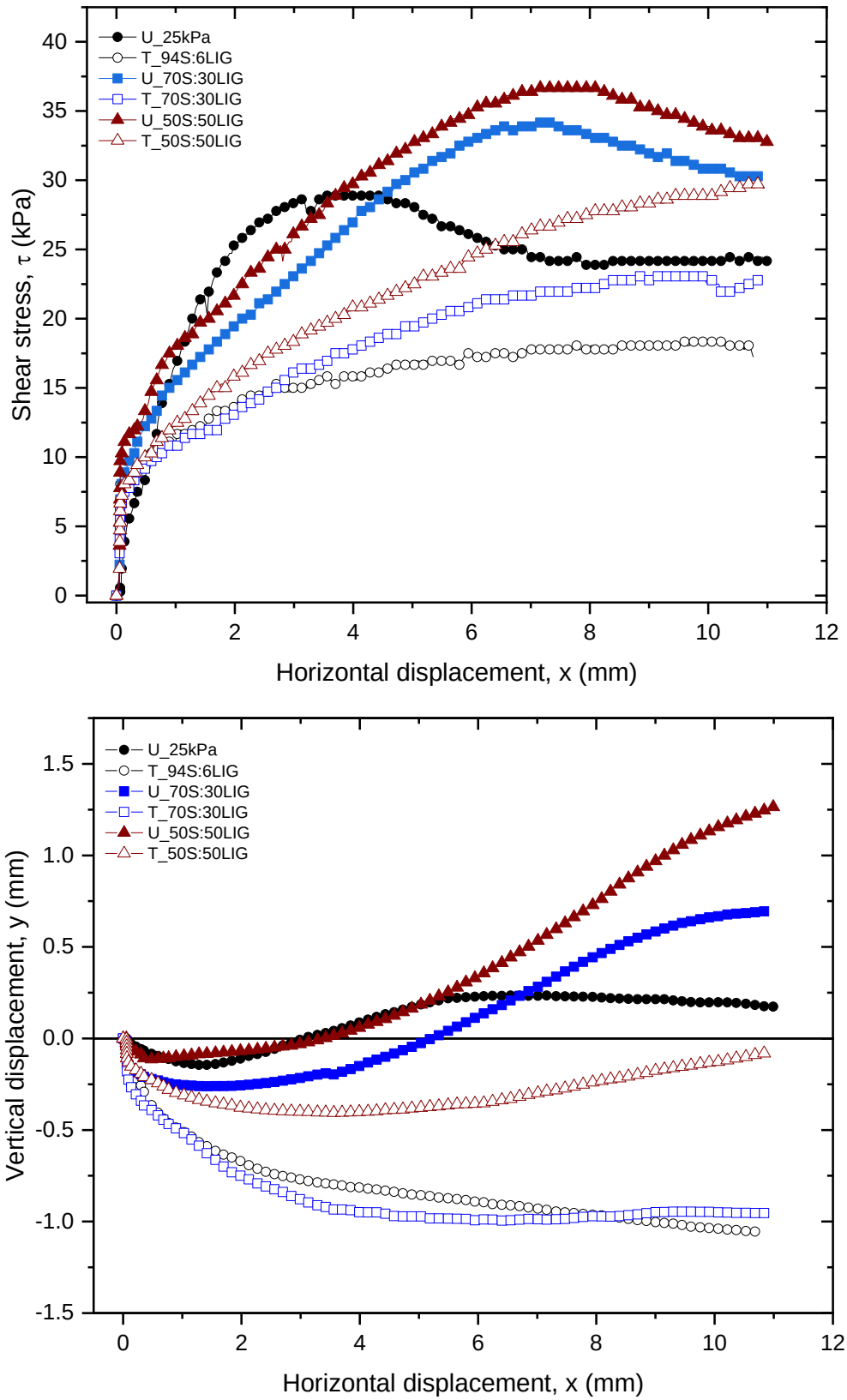


Figure 6-4: Shear stress and volumetric change for treated and untreated specimens with varied amounts of LIG

The results show a similar reduction/loss of the peak shear strength behaviour for treated specimens as seen in section 6.3.2 above. For all untreated specimens, dilative behaviour was exhibited and, for all treated specimens, dilatancy was inhibited. As the percentage of LIG increased from 6-50% shear resistance increased in both the treated and untreated specimens. Given that the 'particle' size of lignocel is larger than that of the sand grains (see Table 6-1) and that the shape of the lignocel is cuboid in nature (rather than spherical), this is likely to be due to the increased opportunity for interlocking with an increasing percentage of lignocel.

6.3.4 Influence of growth duration on shear behaviour

In order to investigate the possible factors that are likely responsible for the difference in behaviour observed between untreated and treated specimens in 6.3.2 and 6.3.3, a series of experiments were conducted on specimens prepared with fungal homogenate. Fungal homogenate was selected for these experiments as it contains a higher concentration of fungal biomass and exudates compared to the hyphal/spore suspension. A specimen prepared with fungal homogenate was tested in the shear box immediately after mixing and assembling, i.e. there was no opportunity for hyphal growth permitted. A second specimen was left for a growth duration of one month (i.e. opportunity for hyphal growth and enmeshment). Both are compared to an untreated control specimen in Figure 6-5.

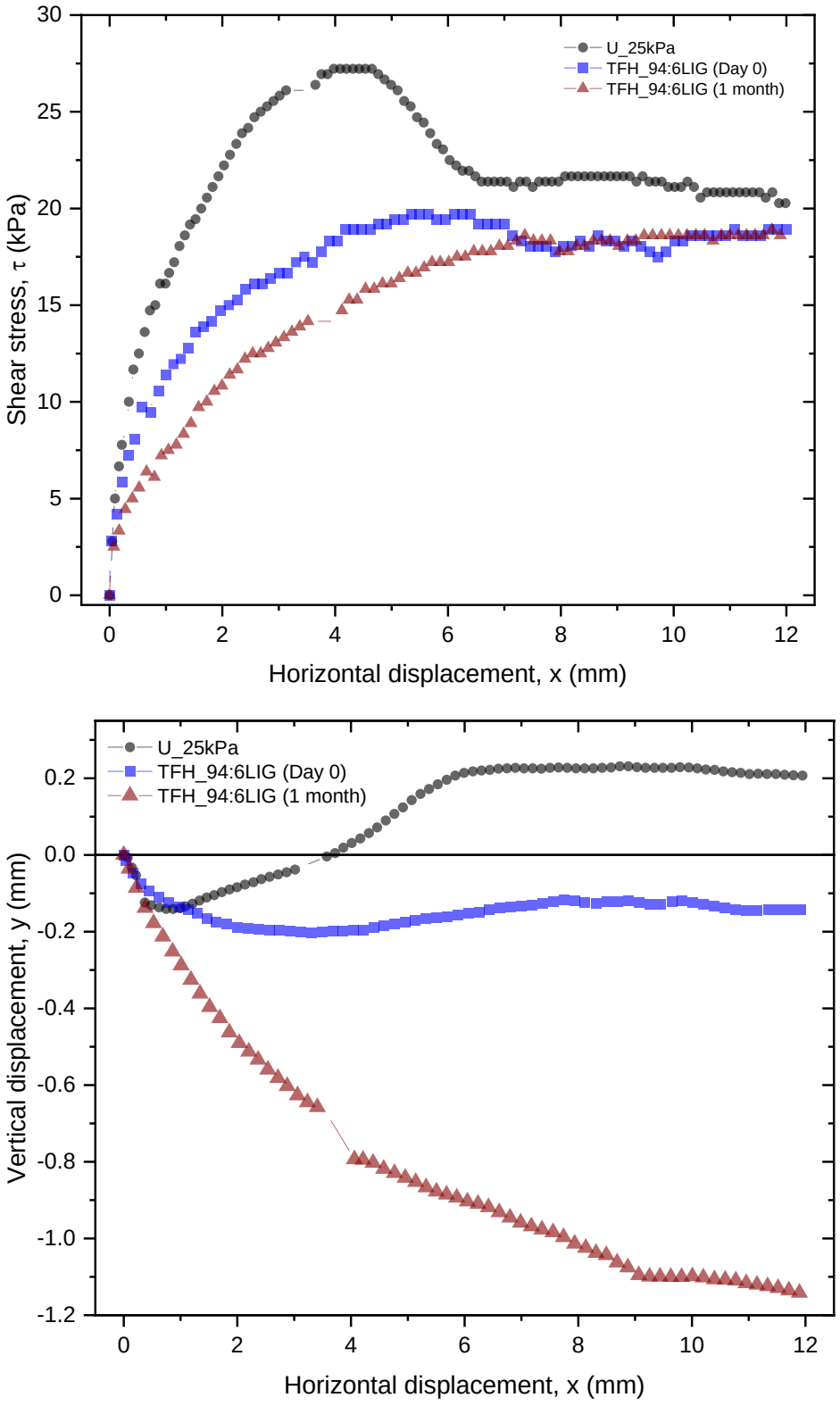


Figure 6-5: Shear stress-horizontal displacement and volumetric change for untreated specimen and specimens treated with homogenate & tested immediately after mixing (day 0) and 1 month after, sheared at a normal stress of 25 kPa..

It is evident that a large reduction in the peak shear strength occurred even on Day 0, in the specimen prepared simply by mixing with the fungal homogenate. This suggests that this loss in shear strength is not due to hyphal growth enmeshing grains and creating a stiffer overall specimen which might be more resistant to densification on loading. Furthermore, for the specimen tested on Day 0, it is proposed that no/minimal fungal growth would have occurred in-situ and therefore lignocel would not have been digested in such a time frame, as to be responsible for the change in behaviour observed. Fungal hyphae, although not binding soil particles on Day 0, may be behaving similar to the presence of artificial fibres, and restricting the development of dilation and thus the development of peak shear strength.

Furthermore, the materials composing the fungal homogenate (hyphal chitin, hydrophobins and exudates) may all be contributing to lower interparticle friction on Day 0, similar to the behaviour observed for hydrophobic soils. The peak shear strength and dilatancy is further reduced after 1 month suggesting that fungal hyphal growth, i.e. increasing aspect ratio of the hyphae, binding of grains and further production of fungal products also contributes to this behaviour. Based on these limited results however, it is not possible to elucidate the extent to which each of these mechanisms influences the shearing response observed.

6.4 Conclusion

This chapter presented the first investigation into the stress-strain behaviour of fungal treated soils tested under direct shear conditions. It is evident from the experiments conducted that a loss in peak shear strength was observed and an associated reduction in dilative behaviour. The experimental results presented show similarities in

behaviour to both hydrophobic sands and artificially reinforced sands reported in the literature. Further investigation is required to fully elucidate the mechanisms influencing the mechanical behaviour of fungal-treated soils.

It is acknowledged that this study is the first attempt to characterise this type of bio-geotechnical sample and as such a number of modifications in the experimental method are suggested and recommendations for further studies are given here: (i) Inoculate and allow for fungal growth directly in specimens mounted in the shear box and already loaded to the desired vertical stress. This would eliminate any disturbance of specimens due to transferring from the mould. Furthermore, it is possible that any effect of hyphal enmeshment has been minimised in this study due to loading of the specimen after hyphal growth, such that the hyphae became slack on loading and thus individual hyphae were not mobilised to the point of failure during shearing. (ii) Use organic matter in powder/liquid form instead of large fibres like lignocel, whose physical form also influenced the resulting stress-strain behaviour, (iii) Conduct tests at lower normal stresses which may be more applicable to the contexts of interest, e.g. interface applications; and (iv) investigate the influence of increasing treatment duration.

Chapter 7

Influence of fungal growth (*Pleurotus ostreatus)* on soil erodibility

Abstract

Damages caused by both on-site and off-site effects of soil erosion affect agriculture, geo-infrastructure and the environment. Strategies employed for erosion mitigation and control have evolved overtime; however, with recent global challenge of climate change, more attention is now focussed on low carbon erosion mitigation strategies that are technically effective, socio-economically viable, eco-friendly and adaptive to climate change. This study investigates, for the first time, the erodibility characteristics of sands treated with a spore/hyphal suspension of the filamentous and saprophytic basidiomycetes, *P. ostreatus* as a potential low carbon alternative for mitigation of water-induced soil erosion. A laboratory JET test apparatus was used to induce soil erosion and analytical methods were used to assess the erodibility characteristics (erosion rates and pattern, erodibility coefficient: k_d and critical shear stress: τ_c) of fungal treated and untreated sands. The effect of inoculation methods and growth duration on the erodibility of fungal treated sand were also investigated.

Findings show that fungal treatment significantly increases the critical shear stress and reduces the erodibility coefficient for specimens with growth duration $\geq$ 3 weeks, compared to untreated sands. Also, the growth duration has a significant effect on the resistance of fungal treated sands to erosion as a one week growth period was shown to be insufficient for improving soil resistance to erosion.

7.1 Introduction

It is estimated that 130 billion metric tonnes of soil are translocated annually across half of the global land area due to water-induced soil erosion (Reich, Eswaran and Beinroth, 2001). This is comparatively higher than soil movement from landslides. Furthermore, soil erosion is critical to food security since arable lands alone account for about half of the total soil eroded by water (Stokes *et al.*, 2013). Soil can be considered as a non-renewable natural resource. The formation of about 2.5 cm of top soil takes between 200 and 1000 years and the damages caused by wind- and water-induced soil erosion (both onsite and offsite) amount to over $44 billion annually in the United States alone (Pimentel *et al.*, 1995). Some off-site consequences of soil erosion include siltation of water bodies, nutrient enrichment of downstream waters resulting in cascading effects such as algal blooms, loss of aquatic lives and ecosystem imbalance. For agriculture soil erosion control and mitigation is critical for conserving nutrient-rich top soil. In a geotechnical context water-induced erosion can trigger failures of embankment dams, flood embankments and natural riverbanks. Foster, Fell and Spannagle, (2000) in a study analysing over 11,000 dams attributes 46% of all dam failures to piping (internal erosion of soil, in the embankment core or foundation) and 36% to overtopping/overflow. The latter can lead to breaching of the dam/embankment due to subsequent erosion of the landward slope (Powledge *et al.*, 1989). Furthermore, water-induced erosion can lead to instability of road and rail embankments (e.g. Polemio and Lollino, 2011; Raj and Sengupta, 2014) as well as instability of hillslopes via rill and gully formation (Van Beek *et al.*, 2008).

Soil erosion is defined as the detachment, transportation and deposition of soil particles due to the influence of rainfall, runoff/overland/stream flow, or wind action. In principle, soil detachment occurs when the erosive forces (fluid shear stress) overcome the soil's resistive forces (weight of particle/gravity, interaction with neighbouring particles: cohesion and adhesion, friction); that is to say, when the shear stress exerted by the fluid exceeds a critical shear stress the motion of soil particles is initiated. Depending on soil properties and slope/topography, erosion may occur as a 'particle level phenomenon' where individual particles are detached as a result of relatively small values of fluid shear stress imparted; or as 'mass (block) erosion' where larger values of fluid shear stress cause the detachment and/or transport of much larger soil masses (Pan *et al.*, 2015). Particle erosion is typically observed in cohesionless soils, with block erosion characteristic of cohesive (i.e. clayey) soils. As such erosion of coarse-grained flood embankments has been observed to be via a progressive mechanism of surface erosion, whereas in cohesive soils, a stepped head-cut profile is typically formed as a result of block erosion (Morris, Kortenhaus and Visser, 2009).

Erosion control and mitigation strategies are developed based on factors influencing erosion and may vary for different locations and land use. Aside from the properties of the eroding agent (wind, water, ice), Morgan (2009) identified the key factors affecting soil erosion to include soil properties, slope and nature of soil protective cover (*e.g.* vegetation) available in a location. Traditional engineering approaches for erosion control and mitigation include the installation of drainage to remove excess surface or subsurface water (Sanborn, 1962; Istok, Boersma and Kling, 1985), the

installation of geosynthetics/geotextiles/geomembranes to provide filtration, drainage or reinforcement (Carroll, Rodencal and Collin, 1992) and stabilisation of soils using soil-binding chemicals (*e.g.* cement, lime, fly ash) or organic polymers (*e.g.* xanthan gum, guar gum, polyacrylamide) (Diamond, 1975; Karol, 2003; Chen *et al.*, 2016). The production and installation processes of these techniques are energy intensive, require the use of heavy machinery which disturb the ground, and are expensive to install and maintain over large areas for a long time. More critically, some of these processes affect the environment negatively because they involve the destruction or removal of soil vegetative cover, release of greenhouse gases into the atmosphere and/or introduction of toxic by-products in the soils. With the recent global challenge of climate change, more attention is now focussed on low carbon erosion mitigation strategies that are technically effective, socio-economically viable, eco-friendly and resilient to climate change.

The use of vegetation for erosion control has been widely studied (e.g. Rickson, Clarke and Owens, 2006; Stokes *et al.*, 2007; Morgan, 2009; Ola, Dodd and Quinton, 2015) and implemented. The mechanical influence of plant roots has been well studied; they bind soil particles and aggregates together providing an additional apparent cohesion against shearing (e.g. Stokes *et al.*, 2009). The level of reinforcement provided is dependent on root tensile strength and root architecture (e.g., root diameter, root length density). Greater shearing resistance is provided by many smaller diameter roots than by a smaller number of larger diameter roots, where the fraction of the soil plane occupied by the plant roots is the same (Stokes *et al.*, 2009). Furthermore, plants also interface with the soil-water-atmosphere interaction leading to modification of soil

water content and suction. The combined hydro-mechanical effects of vegetative cover have been shown to positively contribute to erosion mitigation and soil stabilisation (Greenway, 1987). Some of the main challenges related to engineering vegetation for soil improvement and erosion include: (i) variability in plant characteristics with species - anatomy, life cycle, root diameter, architecture and (ii) adaptation of vegetation to climatic conditions in specific locations. As a means of promoting revegetation of degraded soil systems, some studies have investigated the introduction of mycorrhizal fungi in conjunction with plants (e.g., Requena *et al.*, 2001; Caravaca *et al.*, 2003). The presence of mycorrhizal fungi promotes the formation and stability of aggregates acting as stores for nutrients and water for plant growth (Tisdall and Oades, 1982), thus accelerating and aiding plant colonization (Jeffries *et al.*, 2003; Graf and Frei, 2013; Peng, Guo and Liu, 2013). Furthermore, mycorrhizal have been shown to increase root production, root length density, and for some species even enhance plant root tensile strength (Stokes *et al.*, 2009; Burri, 2011). Plant symbiosis with mycorrhizal fungi have also been explored with the aim of improving the capacity of roots to enhance the formation of soil aggregates which improves soil resistance to erosion (Burri, 2011). Peng, Guo and Liu (2013) showed that independent of the involvement of plant roots, hyphal networks of filamentous fungi had a positive impact on the formation of stable soil aggregates. As the mechanisms by which arbuscular mycorrhizal fungi may influence soil aggregations and other fungal types are expected to be similar (Rillig and Mummey, 2006) and that binding substances are known to be closely associated with hyphal surfaces for a range of fungal types (Aspiras *et al.*, 1971), it is proposed that other fungal species could by themselves also be considered for soil improvement applications.

Recently microbial-based technologies for erosion control have also been investigated. These include the use of bacterial biofilms and microbial-derived soil binding secretions including extracellular polymeric substances (Vignaga *et al.*, 2013; Van De Lageweg, McLelland and Parsons, 2018) and bio-mineralisation strategies. The most commonly investigated bio-mineralisation process by the geotechnical community to date is microbial induced calcite precipitation, MICP in which a denser layer of calcium carbonate is produced at the soil surface that is more resistant to shear stresses imposed by wind or water (Gomez et al., 2015; Salifu et al., 2016; Amin et al., 2017; Montoya, Do and Gabr, 2018). Despite the recent increase in interest in microbial-based technologies few studies have investigated the influence of fungi alone on soil erosion behaviour. The few that have are described in section 7.1.1 below.

7.1.1 Use of fungi in erosion control

The contribution of fungus to soil aggregation has been described in Piotrowski *et al.* (2004), Rillig (2004) and Rillig and Mummey (2006) using typical characteristics of arbuscular mycorrhizal fungi species. Soil aggregate formation is directly correlated to soil resistance to erosion (Wright and Upadhyaya, 1998; Tisdall *et al.*, 2012) and some studies have demonstrated that fungal hyphae can stabilise soils against water or wind-induced erosion (Chaudhary *et al.*, 2009; Tisdall *et al.*, 2012; Mardhiah *et al.*, 2016b).

Chaudhary *et al.*, (2009) performed a study to determine the individual contributions of biological surface crusts, plants and AMF to surface (top 1cm) and subsurface (15 cm deep) stability of fine sands, fine sandy loams and very gravelly loam soils sampled from semi-arid shrublands in Utah, USA. Soil stability in this sense refers to aggregate

stability determined via an infield aggregate stability tests, and is considered to have implications for erosion control. All the biological components investigated were found to contribute to surface stability but only plants and AMF contributed directly to subsurface stability. It was surprising to record some influence of AMF on surface stability since they are generally considered to be less abundant in the top few millimetres of the soil.

Mardhiah *et al.*, (2016) tested the erosion behaviour of soil samples seeded with *Achillea millefolium* (a flowering plant) and inoculated with the AMF fungi *Rhizophagus irregularis* in a hydraulic flume experiment. The specimens inoculated with AMF showed a reduction in the soil mass loss compared to the planted only specimens. Interestingly there was no correlation between the percentage of water stable aggregates and the soil mass loss, indicating that aggregate stability was not the main mechanism contributing to reduced erosion.

Tisdall *et al.* (2012) tested the abrasion resistance (as a proxy for resistance to wind) and tensile strength of soil aggregates inoculated with six different species of saprotrophic soil fungi. All species exhibited enhanced abrasion resistance and tensile strength compared to the uninoculated control samples and a positive correlation with hyphal length density was found. The improvement observed varied for different fungal species, indicating perhaps the degree to which different mechanisms play a role in aggregate stability for different species.

The studies by Chaudhary *et al.*, (2009) and Mardhiah *et al.*, (2016) showed positive contributions of AMF to soil stability and improvement of soil erodibility respectively. However, AMF require plant hosts and their influence (which may be more significant

in the subsurface) depends on plant roots characteristics in soils. This could be a limitation in areas where suitable plant hosts do not exist or are unable to thrive. Tisdall *et al.,* (2012) showed that saprotrophic species could improve soil resistance to wind erosion, although to varied degrees, depending on specific mechanisms involved. Since these mechanisms vary for different species, there is the need to investigate the effects of a single species with known characteristics on the erodibility of a sterile soil, so as to better delineate possible mechanisms involved. Also, based on existing literature, the influence of saprotrophic fungi on water-induced soil erosion is still unknown. This study seeks to investigate the influence of the growth of a selected species of saprotrophic fungi on water-induced erosion in sand assessed using an appropriate standard method.

7.1.2 Assessment of soil erodibility

The erosion of soil is often described by geotechnical and hydraulic engineers using the linear excess stress equation (Partheniades, 1965); eqn. 7-1), commonly called the erosion law. This law states that the rate of erosion for a cohesive soil is proportional to the difference between the effective hydraulic shear stress τ (Pa) imposed on the soil at the soil/water interface and the critical shear stress τ_c (Pa), which is the threshold shear stress at which erosion is initiated.

$$\varepsilon_r = k_d(\tau - \tau_c)^a \tag{7-1}$$

where ε_r (cm s^{-1}) is the erosion rate, k_d (cm^3 N^{-1} s^{-1}) is the erodibility coefficient, and a is an empirical exponent often assumed to be unity (Partheniades, 1965; Ariathurai and Arulanandan, 1978; Hanson, 1989). The quantitative description of soil erosion is

achieved using the values of τ_c and k_d, collectively known as the erodibility parameters of a soil. k_d represents the susceptibility of soil to erosion, it controls how quickly erosion proceeds once initiated; soils with higher values of k_d are said to be more erodible. While τ_c represents the threshold at which particle movement or erosion begins; higher values of τ_c implies better resistance to initiation of erosion. These two parameters are significant for understanding and modelling erosion or scour in dams and embankments, channels and spillways, bridge piers; as well as in sheet, rill and gully erosion prevalent in rural catchment areas and hilly landscapes (Hanson and Cook, 1997).

Several testing methods have been developed for quantitative estimation of the soil erodibility parameters. These include: flume-type tests which may be large (e.g. 29 m length and 1.8 m wide with side walls 2.4 m high; (Hanson and Cook, 2004)) or small like the erosion function apparatus - EFA (Briaud *et al.*, 2001), rotating erosion test apparatus – RETA (Bloomquist *et al.*, 2012), hole erosion test – HET (Wan and Fell, 2004) and the jet erosion test – JET (Hanson, 1990; Hanson and Cook, 2004). In these tests, soil erosion rates are measured or inferred and plotted as a function of applied shear stresses, thereby modelling the assumed erosion law and obtaining values of the soil erodibility parameters. By design and intent, these tests methods are preferred for cohesive soils i.e. clayey soils. However, the JET apparatus is a simple, versatile device which is conceptually suitable for determination of erodibility parameters of cohesive or non-cohesive soils (Stein, Julien and Alonso, 1993) both *in-situ* or in the laboratory (Hanson, 1990; Hanson and Cook, 1997; Hanson and Simon, 2001).

Erodibility characteristics of some bio-treated soils have been studied using the jet test apparatus. These include sandy clay loams permeated with vegetation (Mcnichol *et al.*, 2017), sand treated with microbial induced calcite precipitation (Montoya, Do and Gabr, 2018). No studies could be found in the literature which have investigated the behaviour of soil treated with fungal hyphae using the JET apparatus.

This study presents for the first time an assessment of the erodibility of sand treated with fungal hyphae using *P. ostreatus* species for a bio-geotechnical engineering context. A laboratory JET erosion testing apparatus was constructed at the University of Strathclyde. Jet erosion tests were carried out to determine the erodibility characteristics (i.e., erosion rate, erodibility coefficient and critical shear stress) of sand treated with *P. ostreatus* and control specimens. Specifically, the objectives of this study were: (i) to determine the erodibility characteristics of fungal treated and untreated sands (ii) to determine the effect of inoculation methods and (iii) growth duration on the erodibility of fungal treated sand.

7.2 Methods

7.2.1 Materials

a) Sand

Fine silica sand with particle size distribution shown in Fig. 7-1 was used in this study. The sand was supplied as kiln dried but was autoclaved to ensure sterility before use in all experiments.

b) Lignocel

Processed natural fibres derived from softwoods and commercially available as lignocel (Grade HB 500 – 1000) supplied by J. RETTENMAIER & SÖHNE GmbH was used as the nutrient substrate for fungal growth. Lignocel contains lignin, cellulose and hemicellulose (Imran *et al.*, 2016), which are representative of typical constituents of organic matter which saprophytic fungi feed on in natural soils.

The soil used in this study is composed of lignocel (6%) and fine sand (94%).

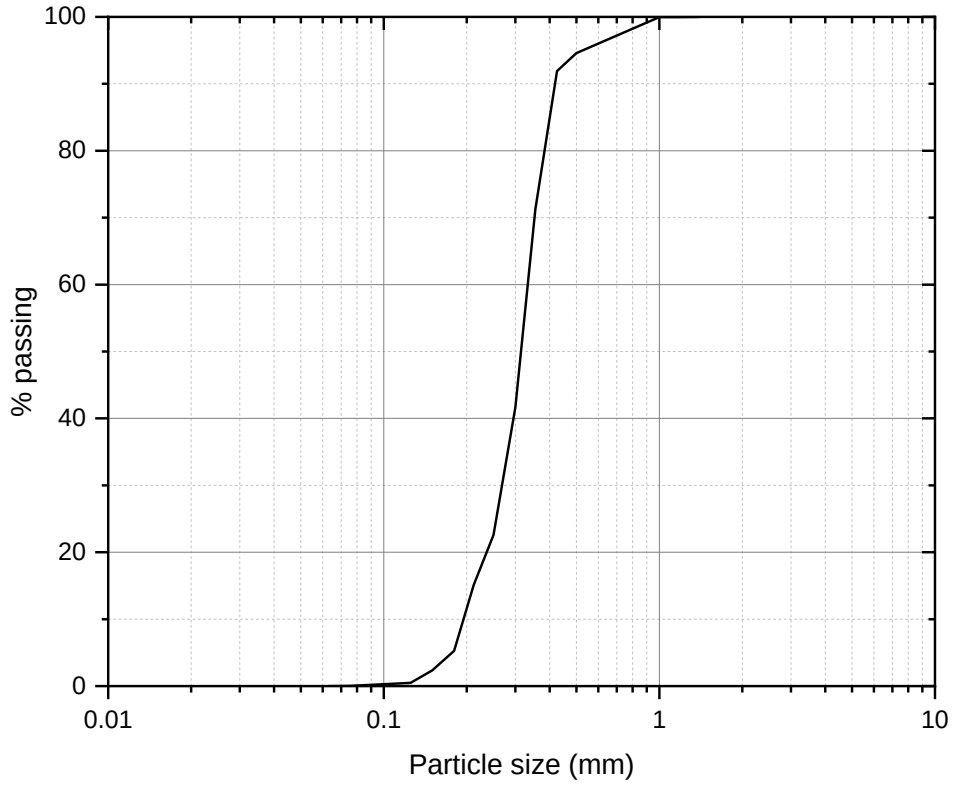


Figure 7-1: Grain size distribution for uniform fine sand

c) Fungus

The fungal species used in this study is *Pleurotus ostreatus* (strain: M 2191) obtained from GroCycle UK. It was supplied as active fungal spawn (Fig. 7-2), that is, wheat

grains colonised by mycelia of *P. ostreatus*. Prior to use, the spawn was stored at 4°C in a cold room. Only fresh fungal spawn was used, i.e. within 30 days after delivery.

d) Preparation of fungal spore/hyphal suspension

Fungal growth was initialised in the soil via inoculation with a fungal spore/hyphal suspension of *P. ostreatus* prepared from spawn according to the following method (Fig. 7-2). A fixed amount of fungal spawn was added to deionised (DI) water (ratio 1g spawn:10mL DI water) in a conical flask and shaken vigorously by hand for 5 minutes. Thereafter, the mixture was placed on a shaker set at 320 rpm for 30mins. This was to ensure effective detachment of fungal spores/hyphae from the spawn into the water, leaving the grains floating on the surface. The fungal spore/hyphal suspension was then separated from the wheat grains by filtration using a sieve with a mesh size of 2 mm.

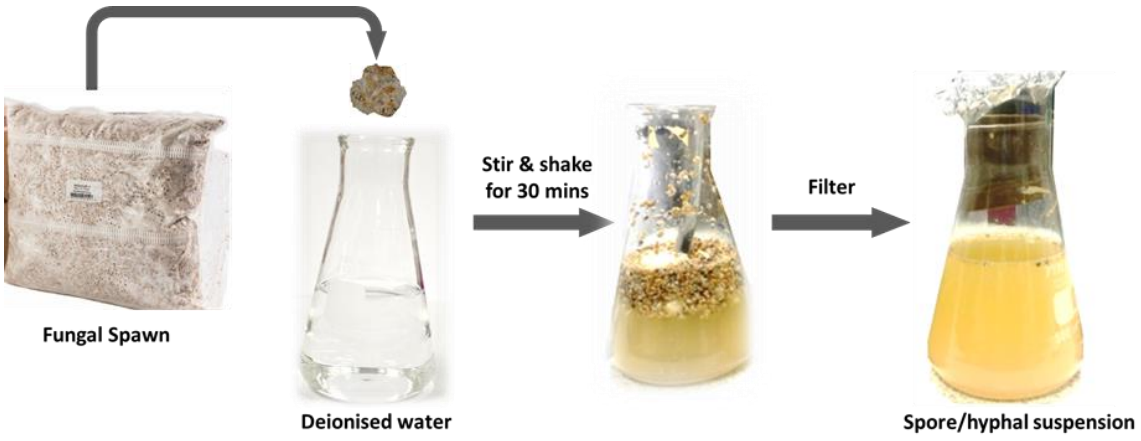


Figure 7-2: Procedure for preparation of the fungal spore/hyphal suspension

e) Specimen preparation and incubation

All specimens were prepared by mixing the soil (sand: 94% & lignocel: 6%) with liquid (spores/hyphal suspension for treated or DI water for untreated) such that the respective specimen was made up to a moisture content of 11.1% (i.e. mass of liquid/mass of sand and lignocel). The water content and lignocel content were selected based on the experimental results presented in Chapter 3. Specimen compositions are shown in Table 7-1. The specimens were compacted to a dry density of 1.1 g cm^{-3} in 3 layers in a 100 mm high x 100 mm in diameter cylindrical mould. The mould was placed upright on an acrylic base.

Table 7-1: Masses of respective specimen constituents

Specimen composition	Masses of components for respective treatment/inoculation method				
	Control (g)	Fully-treated (g)	Half-treated (g)		Surface-treated (g)
			Top half	Bottom half	
Sand	802	802	401	401	802
Lignocel	47	47	23.5	23.5	47
Spore/hyphal suspension	-	94	47	-	47
DI Water	94	-	-	47	47

Depending on the respective inoculation strategy, the amount and procedure for addition of liquid (spores/hyphal suspension and water) to the soil differed. Deionised (DI) water was added to the untreated specimens (i.e. controls) in place of the fungal treatment suspension. Fig. 7-3 provides a schematic representation of the different treatment methods for respective specimens.

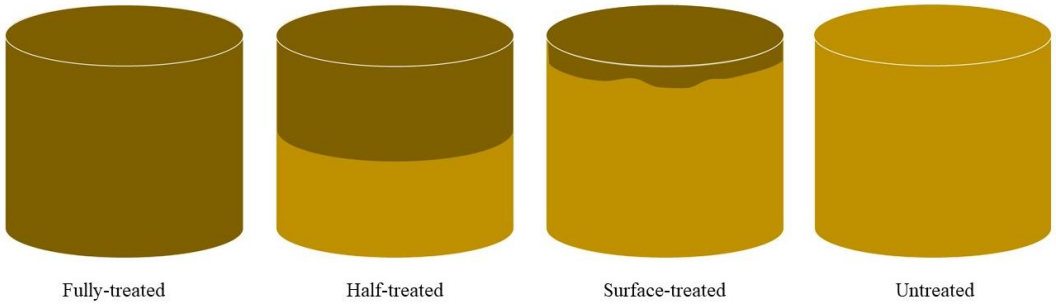


Figure 7-3: Schematic representation of soil specimens in 100mm x 100mm hollow cylindrical moulds. The darker regions represent the soil areas inoculated with spores/hyphal suspension of P. ostreatus before incubation of specimens

For *fully-treated* specimens, the total mass of treatment suspension (94g) was added to and mixed through the total mass of sand and lignocel. This was to create a well-mixed specimen, mimic a situation where soil is pre-inoculated prior to emplacement in earthworks, for example fill material for embankments. For *half-treated specimens*, the bottom half of the specimen was mixed with water while the top half was mixed with the treatment suspension; in this case, the mass of treatment suspension is half that used for the full-treatment. Half-treated specimens were prepared to investigate the transition between the fungal treated and untreated profiles in the soil. While *surface treated specimens* were prepared to simulate *in-situ* delivery of fungal inoculant using techniques like hydroseeding. Here, the entire soil mass was mixed with water (47g) while the treatment suspension (47g) was introduced to the specimen surface by spraying using a gardening spray bottle with adjustable nozzle. A method of spraying was developed such that spraying was done at random intervals and completed within 5-7 minutes to ensure minimal soil movement on the specimen surface. In this way, the treatment suspension gradually inundated the surface of the specimen and infiltrated into the specimen.

All specimens were incubated in the dark at 25°C. The temperature was selected based on results presented in Chapter 3. The decision to incubate the specimens in the dark was based on (i) additional experiments by the author, which showed enhanced mycelium growth in specimens incubated in the dark vs replicate specimens incubated in the light for specimens with the same organic and water content and incubated at the same temperature; and (ii) evidence from literature showing that exposure of overgrown mycelium to light would trigger the formation of carpophores, that is the stems of the fruiting bodies (mushrooms) of *P. ostreatus*, depending on the exposure time and light intensities (Trukhonovets, 1991; Siwulski, Ziombra and Sobieralski, 2012). After the required incubation/growth periods, each specimen was removed from the incubator and a JET erosion test was conducted on it.

7.2.2 Experimental plan

All experiments in this study involved three key phases: (i) the treatment of soil using fungal spore/hyphal suspension (ii) incubation of the specimens at 25°C in the dark, for fungal growth over specified periods of time and (iii) the performance of the Jet erosion test on the specimens.

To achieve the experimental objectives, two work plans were executed. Firstly, triplicate specimens of fully treated soil, where all the soil was thoroughly mixed with the fungal spore/hyphal suspension, were prepared (see Fig. 7-3) and incubated to grow for three weeks before performing the JET erosion test on them. Untreated specimens were also prepared and subjected to the same conditions and incubated for the same duration, but not inoculated with the fungus. This was carried out to determine the erodibility characteristics of fungal treated and untreated soils and

establish suitable analytical methods for the data obtained. The second work plan was carried out to determine the influence of inoculation methods and fungal growth/incubation periods on erodibility. This involved the preparation of specimens using three inoculation methods (i.e. initial treatment strategies): (i) fully treated - FT, (ii) half treated - HT and (iii) surface treated – ST, as shown in Fig. 7-3/described in section 7.2.1(e) and subsequently placing them in the incubator for fungal growth periods of 1, 3, 6 and 9 weeks respectively.

Table 7-2 shows the experimental conditions including respective inoculation/treatment strategy and growth duration for each specimen tested.

Table 7-2: Experimental conditions showing treatment methods and growth duration for respective specimens

Treatment type	**Duration (weeks)**	**Notation**
Untreated-1	3	U1
Untreated-2	3	U2
Untreated-3	3	U3
Fully-Treated	1	1wk-FT
Half-Treated	1	1wk-HT
Surface-Treated	1	1wk-ST
Fully-Treated-1	3	3wk-FT1
Fully-Treated-2	3	3wk-FT2
Fully-Treated-3	3	3wk-FT3
Half-Treated	3	3wk-HT
Surface-Treated	3	3wk-ST
Fully-Treated	6	6wk-FT
Half-Treated	6	6wk-HT
Surface-Treated	6	6wk-ST
Fully-Treated	9	9wk-FT
Half-Treated	9	9wk-HT
Surface-Treated	9	9wk-ST

The triplicate control specimens U1, U2 and U3 used in this study were incubated for only 3 weeks (as shown in Table 7-2). This is because preliminary trials conducted on control specimens incubated for 1, 3, and 9 weeks (not reported here) showed similar behaviour.

7.2.3 Jet erosion test (JET) apparatus

Several studies have presented a detailed description of the evolution of the jet erosion test, the testing methodologies and the analytical approaches for obtaining erodibility parameters of soils (see Hanson and Cook, 1997; Hanson and Simon, 2001; Al-Madhhachi, Hanson and Fox, 2013; Wahl, 2016). Conceptually, the submerged impinging jet system provides an experimental representation of soil erosion by mimicking jet diffusion in a pool with underlying soil. This can be considered to simulate erosion that may occur on the slopes of flood embankments, e.g. as a result of overtopping/overflow. In its standard form (Hanson and Cook, 2004), the JET apparatus consists of a jet tube with a jet nozzle of specified diameter attached to its lower end; a deflector plate for opening the flow through the nozzle or deflecting flow when required; a submergence tank which is filled with water and houses the jet tube nozzle and the inundated soil sample; an adjustable tank for supplying water to the system; and a point gauge for taking scour depth readings. Recently, the original device has been modified into a much smaller unit called the mini-JET apparatus, adapted for laboratory use (Al-Madhhachi, Hanson and Fox, 2013).

a) Theoretical background and methods of data analysis

A widely adopted analytical approach for determining τ_c and k_d in jet tests was developed by Hanson and Cook, (1997) and Hanson, Robinson and Cook, (2002) based on diffusion principles proposed by Stein and Nett, (1997) and a hyperbolic function for predicting equilibrium scour depth (Blaisdell, Hebaus and Anderson, 1981).

Fig. 7-4 (Hanson and Cook, 2004) provides a schematic diagram of an impinging jet and the shear stress distribution profile. A circular jet from a submerged nozzle (of diameter = d_o) impinges the soil surface perpendicularly, from an initial height J_i. The jet is at uniform velocity $U_o = \sqrt{2gH}$ and pressure head, H. Scour begins and continues until an equilibrium depth (J_e) is reached, where no further erosion takes place (i.e., scour rate = 0) and at this point, the hydraulic shear is equivalent to τ_c (Hanson and Cook, 1997). The applied shear stresses as a result of the impinging jet are a function of J_i and d_o (Hanson, Robinson and Temple, 1990). Directly under the jet, the shear stress applied is zero (i.e. $\tau = 0$, at the stagnation point; Fig. 7-4), then at some distance from the centreline of the jet, the shear stress applied reaches a maximum (τ_{max}), after which it decays with increasing distance from the centre of the jet. The average velocity of the jet decreases as it diffuses radially throughout the surrounding water; however, the centreline jet velocity remains constant (U_o) up to a certain distance from the nozzle known as the potential core length, J_p. The velocity, U is maximum along the centreline beyond J_p, until it decreases due to diffusion.

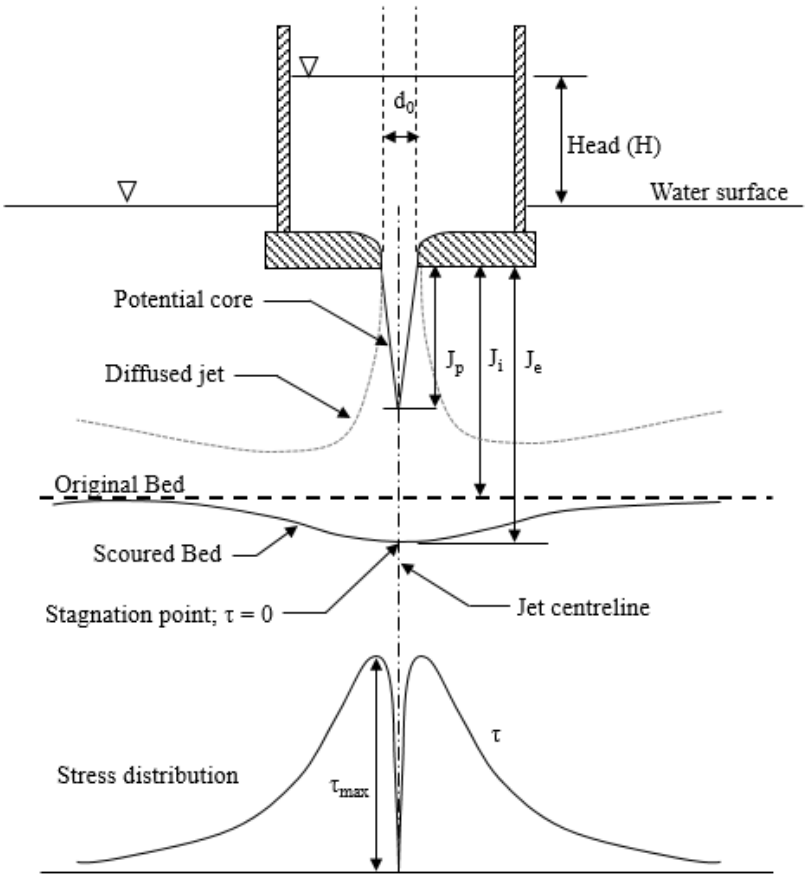


Figure 7-4: Schematic illustration of the impinging jet and shear stress distribution profile (Hanson and Cook, 2004)

Albertson *et al.*, (1950) provides a formula for the centreline velocity where $J > J_p$ as follows:

$$\frac{U}{U_o} = C_d \frac{d_o}{J} \tag{7-2}$$

Where J = distance from the jet origin along the centreline (mm); and the diffusion constant C_d is often assumed to be 6.2 or 6.3 (Beltaos and Rajaratnum, 1974).

When $U = U_o$ at $J = J_p$, Eq. 7-2 becomes:

$$J_p = C_d d_o \tag{7-3}$$

The maximum shear stress τ on the soil surface is related to U using Eq. 7-4 ((Hanson and Cook, 1997)

$$\tau = C_f \rho U^2 \tag{7-4}$$

Where C_f is a coefficient of friction taken as 0.00416, based on (Hanson, Robinson and Temple, 1990) and ρ = density of water (kg m^{-3})

From Eqs. 7-2 to 7-4, the maximum shear stress due to the jet velocity at the nozzle $\tau_{o,}$ can be obtained as follows:

$$\tau_o = C_f \rho U_o^2 \qquad J \leq J_p \tag{7-5}$$

$$\tau = C_f \rho \left(C_d U_o \frac{d_o}{J} \right)^2 \qquad J > J_p \tag{7-6}$$

In the analysis of the jet test, it is assumed that the rate of change in the scour depth, (dJ/dt) is the erosion rate and is a function of the maximum shear stress and erodibility coefficient k_d and this maximum shear stress causes the maximum scour beneath the impinging jet. Combining Eqs. 7-1, 7-3, 7-5 & 7-6, the erosion rate for jet scour (Hanson and Cook, 1997) becomes:

$$\frac{dJ}{dt} = k_d \left[\frac{\tau_o J_p^2}{J^2} - \tau_c \right] for\, J \geq J_p \tag{7-7}$$

As stated earlier, τ_c is assumed to occur when the equilibrium depth J_e is reached and $dJ/dt = 0$ (Hanson and Cook, 1997), therefore:

$$\tau_c = \tau_o \left(\frac{J_p}{J_e} \right)^2 \tag{7-8}$$

Hanson and Cook, (1997) combined Eqs. 7-7 & 7-8 into a dimensionless form to obtain:

$$\frac{dJ^*}{dT^*} = \frac{(1 - J^{*2})}{J^{*2}} \tag{7-9}$$

Where the dimensionless term $J^* = J/J_e$ and $J_p^* = J_p/J_e$. The dimensionless time T^* and the reference time T_r are given by Stein et al., (1993) and Stein and Nett, (1997) as follows:

$$T^* = t/T_r \tag{7-10}$$

$$T_r = \frac{J_e}{k_d \tau_c} \tag{7-11}$$

Where t = time of a scour depth measurement

Hanson and Cook, (1997) determined the dimensionless T^* by integrating Eq. 7-9 from the initial soil surface J_i to the scour depth J

$$T^* = 0.5\, ln\left(\frac{1 + J^*}{1 - J^*}\right) - J^* - 0.5\, ln\left(\frac{1 + J_i^*}{1 - J_i^*}\right) + J_i^* + T_i^* \tag{7-12}$$

While actual measured time t_m since scour started is given by the equation:

$$t_m = T_r \left[0.5\, ln\left(\frac{1 + J^*}{1 - J^*}\right) - J^* - 0.5\, ln\left(\frac{1 + J_i^*}{1 - J_i^*}\right) + J_i^*\right] \tag{7-13}$$

Following the complete analysis of the theory of a submerged jet, Hanson and Simon, (2001) and Hanson and Cook, (2004) provide an excel spreadsheet routine, such that using Eqs. 7-2 to 7-13 the values of τ_c and k_d can be determined. However, determination of τ_c is based on reaching the equilibrium scour depth J_e, which is

difficult to achieve because a very long testing time is required (Blaisdell, Hebaus and Anderson, 1981). A hyperbolic function (Eq. 7-14) developed by Blaisdell, Hebaus and Anderson, (1981) can be used to estimate J_e and the corresponding time from the scour data.

$$x = [(f - f_o)^2 - A^2]^{0.5} \quad (7\text{-}14)$$

Where: $x = \log[U_o t/d_o]$; $f = \log[J/d_o] - \log[U_o/d_o]$; $f_o = \log(J_o/d_o)$; t = time of data reading; d_o = nozzle diameter; and A = the value of the semi-transverse and semi-conjugate axis of the hyperbola.

In the spreadsheet routine, the scour depth data (J_i, do, Uo and T) are fitted based on a plot of f against x; thereafter, A and f_o are obtained by iteration by minimising the standard error using Microsoft Excel Solver. J_e is then calculated using $J_e = d_o 10^{f_o}$. Subsequently, k_d is estimated in the spreadsheet by iteration for best fit values using Eq. 7-12.

b) The JET apparatus at the University of Strathclyde

Figs. 7-5 and 7-6 show the pictorial representation of the apparatus constructed at the University of Strathclyde based on the mini-JET apparatus designs of Al-Madhhachi, Hanson and Fox, (2013). It consists of an acrylic jet tube (50 mm dia, 920 mm length), a nozzle plate with orifice diameter of 6.4 mm, a deflector plate with handle control and a 300 mm high x 300 mm in diameter submergence tank made of transparent acrylic plastic. The apparatus is supported by screw rods and acrylic sheets and can be easily dismantled and re-assembled (Fig. 7-5). A mould holder is provided at the bottom of the submergence tank for fitting the sample mould. The sample mould is

100 mm high x 100 mm diameter, and made of acrylic. Inlet valves at the bottom of the submergence tank are used for draining and filling the tank; and at the top of the jet tube for filling the tube and maintaining a constant hydraulic head. A graduated thin rod (63mm dia) which can pass through the jet nozzle, was used as the point gauge.

7.2.4 The JET procedure

The test procedure followed the method outlined by Al-Madhhachi, Hanson and Fox (2013) with some modifications.

Firstly, the cylindrical mould containing the specimen was carefully placed in the sample holder centrally located in the submergence tank (Fig. 7-5B) and this part of the apparatus was taken for laser scanning (see details in section 7.2.5) to record the specimen surface profile, prior to commencing the erosion tests. The complete JET test apparatus was then assembled as shown in Fig. 7-5. Using a point gauge, the initial distance from the surface of the specimen to the jet nozzle (J_i) was fixed. For these experiments, J_i was set at 40 mm. The submergence tank was slowly filled via inlet ports at the bottom of the tank until water began to flow from the openings provided at the top of the tank. This constant head of water above the specimen surface was maintained in the tank with the specimen left completely submerged for 1 hour.

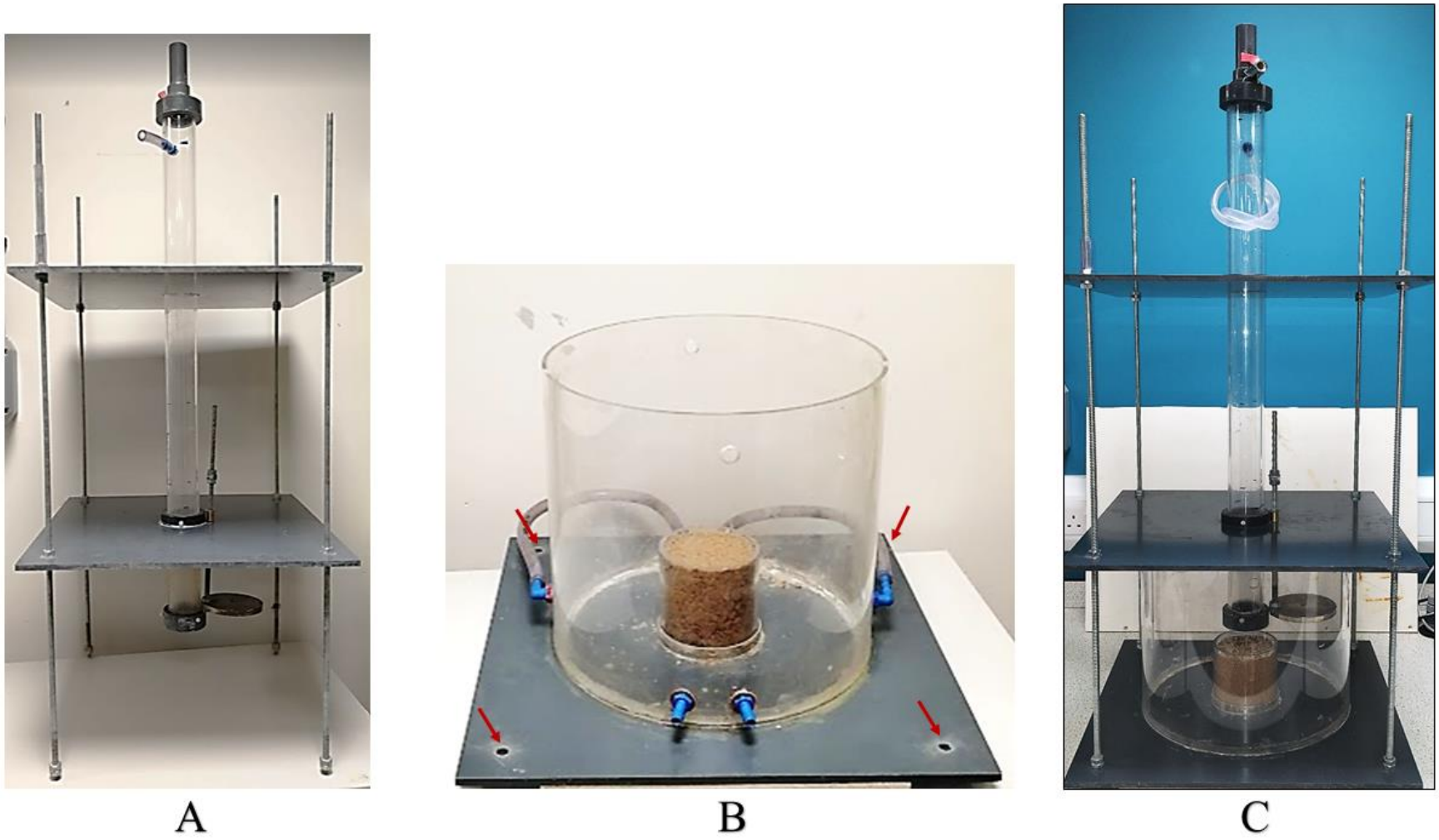


Figure 7-5: The apparatus is assembled by lifting the frame 'A' and fitting the ends of the screw rods into the respective slots (shown by the red arrows) at the base of the component part 'B'. 'C' is the fully assembled apparatus

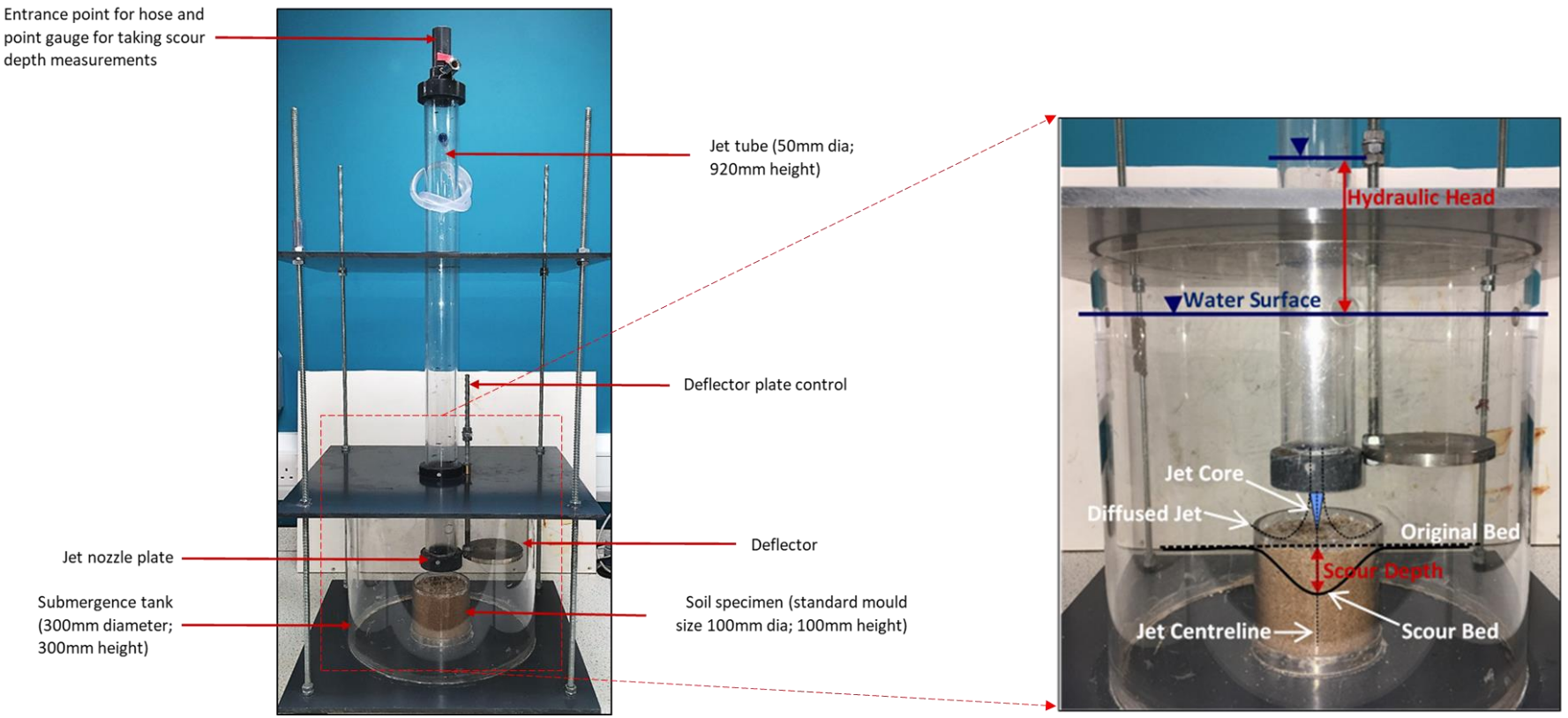


Figure 7-6: The JET erosion test apparatus at the University of Strathclyde, Glasgow – UK

The deflector plate was used to obstruct the jet nozzle in order to protect the specimen surface when the jet tube is filled, i.e. it prevents the jet from impinging on the soil surface. The jet tube was then slowly filled with water to attain initially a hydraulic head of 50 mm in the jet tube (i.e. height of water in jet tube is 50mm above the water level in the submergence tank) see Figure 7-6. The height of water in the jet tube was maintained constant by adjusting the valve from the supply tap. The deflector plate was then rotated away from the nozzle, this represented the commencement of the jet erosion test. The deflector plate was returned to stop the jet impinging the soil surface after the specified time, and the scour depth (the distance from the initial soil surface to the newly eroded soil surface) was measured and recorded using the point gauge. At this constant hydraulic head, the jet erosion process was repeated with scour depths measured and recorded at predetermined times of t = 1, 2, 3, 4, 5, 10, 15, 20, 30 ,40 and 60 minutes.

After running the test for 60 minutes, the submergence tank was drained slowly, the upper part of the apparatus was carefully lifted off and the lower part with the specimen in the tank was taken for laser scanning again to obtain more detailed information on the specimen's surface profile at this time. This represents the completion of a first stage of test for each specimen. The jet test for each specimen was performed for hydraulic heads of 50, 100 and 250 mm, (representing stages 2, 3 and 4), going up to 500 mm (stage 5) or a maximum of 750 mm (stage 6) depending on the behaviour exhibited.

The range of jet pressure values, beginning from as low as 50 mm were chosen based on preliminary tests, to enable data collection for evolution of erosion in cohesionless

sand for comparison with fungal-treated sands. Furthermore, since the presumption of linearity between rate of erosion and excess shear stress in the excess stress erosion model may only be realistic over a short stress range, it is recommended to apply shear stresses that are comparable to the magnitude of shear stresses expected in the field (Wahl, 2014). Hanson and Cook (2004) provide an equation (Eq. 7-15) for calculating the values of initial shear stress (τ_i) applied to the soil surface at the beginning of jet impingement, i.e. prior to scour. The initial shear stress, τ_i is a function of the height of the nozzle above soil surface (J_i) and the hydraulic head (H).

$$\tau_i = 0.13\left(\frac{H}{J_i^2}\right) \tag{7-15}$$

Where, τ_i = initial peak stress prior to scour (Pa); H = the differential head measurement (m); J_i = the initial height of jet nozzle from soil surface (m), taken here to range from 40 mm to 120 mm.

Using Eq. 7-15 and Eq. 7-3, the relative values of τ_i for changes in J_i with respect to the range of hydraulic heads used in this study (50 – 750 mm) were calculated and are presented as respective ratios of J_p/J_i in Fig. 7-7. To calculate J_p (using Eq. 7-3), the diffusion constant was assumed to be 6.3 and the nozzle diameter (d_o) for this study = 6.4 mm was used.

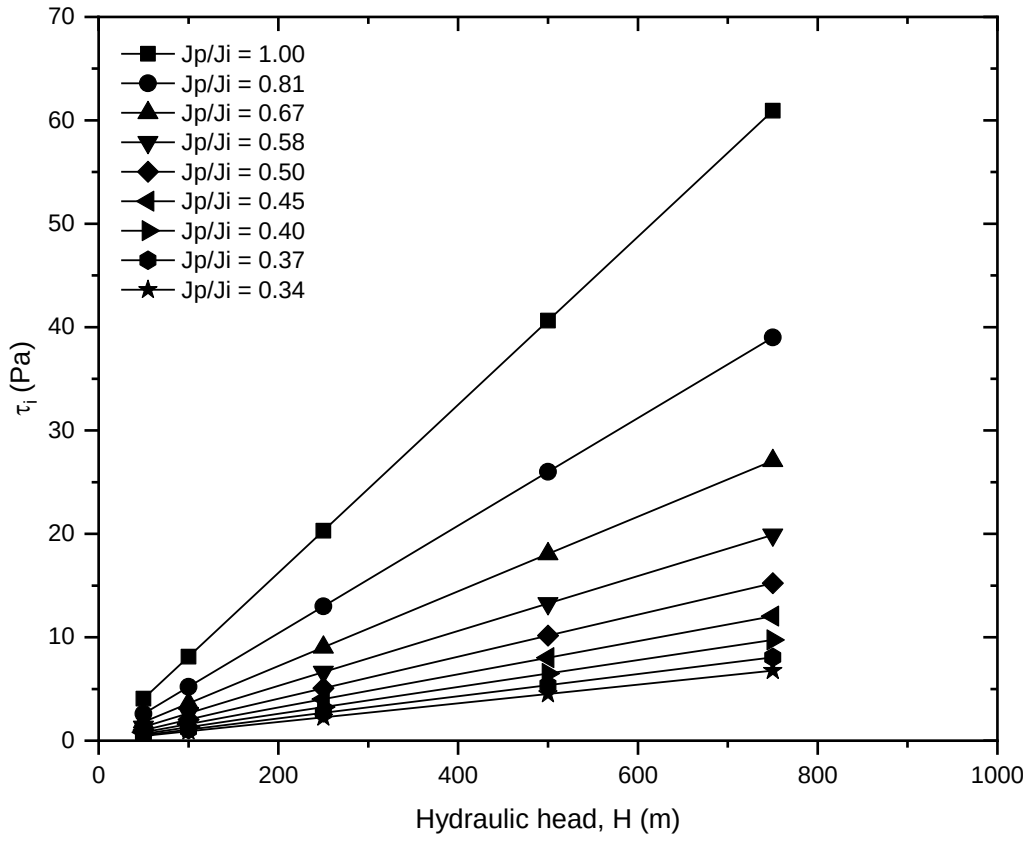


Figure 7-7: Initial peak boundary stress and respective hydraulic heads expressed as the ratio of J_p/J_i (Based on Hanson and Cook, 2004)

From Fig. 7-7, the values of τ_i for specimens in this study are expected to range between 0.45 Pa (for Jp/Ji = 0.34 and H = 50 mm) up to a maximum of 61 Pa (for Jp/Ji = 1.00 and H = 750 mm). These values of τ_i were not the actual initial shear stresses on the specimens tested at each stage, as for each stage (beyond stage 1) J_i is greater than 40mm (the initial fixed distance between soil surface and nozzle) and is dependent on the scouring achieved in the previous stage. For Stage 1 J_p/J_i will be maximum (1.00) for each specimen as there was no previous scour recorded, and so, the ratio J_p/J_i will reduce with increasing scour depths in subsequent stages of test per specimen. Nevertheless, Fig 7-7 gives an indication of the order of magnitude of the initial shear stresses applied to the specimens in the JET tests.

It should be noted that τ_i is different from τ_o which is the maximum stress due to the velocity of the jet at the nozzle. Fig. 7-8 presents a plot of the τ_o (calculated using Eq. 7-5) for respective hydraulic heads (H) used in this study. The value of τ_o = 61 Pa at the hydraulic head of 750 mm is comparable with the maximum shear stress of 50 – 60 Pa obtained for the toe region downstream of an embankment in a study on numerical simulations of overtopping by Briaud *et al.*, (2008).

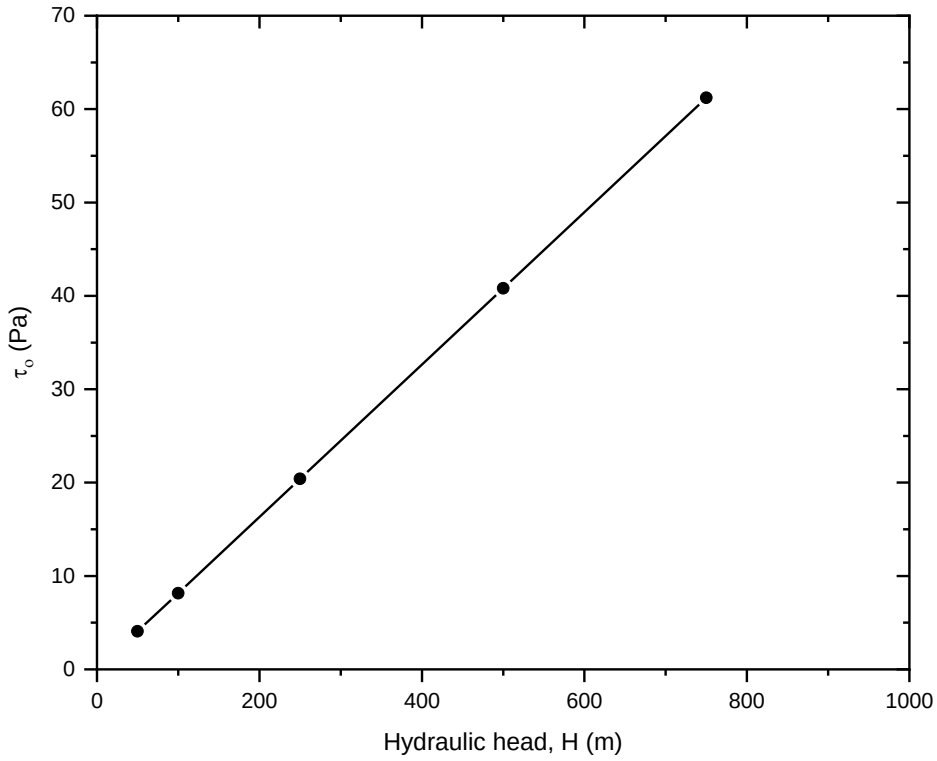


Figure 7-8: Maximum shear stress due to the velocity of the jet nozzle plotted against respective hydraulic heads

a) Determination of k_d and τ_c using the Blaisdell method

The traditional method for jet test analysis developed by Hanson and Cook (2004) adopts a series of assumptions and equations which refers to the hydrodynamic of an axisymmetric, circular impinging jet in unconfined conditions. However, there are studies (e.g. Ghaneeizad, Atkinson and Bennett, 2015), which show that the bed shear

stress values are greatly affected by the confinement condition of the JET apparatus and that the actual procedure tends to underestimate the shear stress distribution. The Blaisdell method, which is based on determining the scour depth at equilibrium, employs a projection technique involving hyperbolic function to calculate k_d and τ_c (section 7.2.3a; Eq. 7-14). This approach has been widely used by many authors (Hanson and Simon, 2001; J. Hanson, M. Robinson and R. Cook, 2002; Hanson and Cook, 2004).

Alternative approaches have been developed based on modifications of the Blaisdell method. These include the Modified Blaisdell Method (Karamigolbaghi *et al.*, 2017), the Iterative Principle (Simon, A.; Thomas, R. E.; Klimertz, 2010) and Scour depth Principle (Daly, Fox and Miller, 2013). There is continued discussion over the analysis procedures and the influence of experimental procedures on results, in particular the range of pressure heads selected and the intervals of scour depth measurement (Mahalder *et al.*, 2018). There is also the argument that a non-linear equation might provide better prediction of soil erodibility (Houwing and Van Rijn, 1998; Ghaneeizad, Atkinson and Bennett, 2015; Walder, 2015) compared to the widely adopted definition using a linear equation. A review and critique of the different procedures is given in Karamigolbaghi *et al.*, (2017) and Mahalder *et al.*, (2018).

In this study the Blaisdell method, which remains the most commonly used approach, was selected as a means of comparing between untreated sands and fungal treated sands rather than an attempt to determine the absolute values of k_d and τc which may be required for design purposes.

7.2.5 Laser scanning for estimation of volumes of soil eroded

In order to obtain more information regarding the volume of soil eroded, and a second assessment of scour depth, specimens were scanned after 60 mins at each hydraulic head using a laser scanning unit. Laser scanning systems are versatile non-contact measurement technologies that provide quick and accurate representation of surface profiles providing the opportunity for 3D surface visualisations and further post-processing of data.

For this study, the laser scan imaging unit used was the 2D/3D scanCONTROL 2700-100/BL from Micro-Epsilon. It was mounted on a moveable frame coupled with a software-timed motion controller OWIS PS-II. This scanner uses the triangulation principle and was operated dynamically, by moving the sensor unit relative to the specimen at a height of aprox. 500 mm above the surface of the fixed specimen (see Fig. 7-9).

Sanchez *et al.*, (2013) used this same laser scanning unit set-up at the University of Strathclyde and provides more details on the operating principle. The reference resolution in the vertical direction for this model is 15 μm. The imaging and control units were linked to a computer using interface cables while operational configurations or settings, data capture, visualisation and data export were achieved using the ScanCONTROL 3D-view software. Data were exported as ASCII files for further processing using CloudCompare (Girardeau-Montaut, 2015), a 3D point cloud open source processing software. In this software, the initial specimen surface was used as the reference against which the subsequent eroded volumes and scour depth were determined using the 'compute 2.5D volume' command. In cases where profiles of the

surface scans were incomplete due to deeper scour depths or shadowed from incident scan beam, four scans were taken by rotating each specimen through four cardinal points; these were combined as layers to produce the full profile before computation of scour depths or eroded volume.

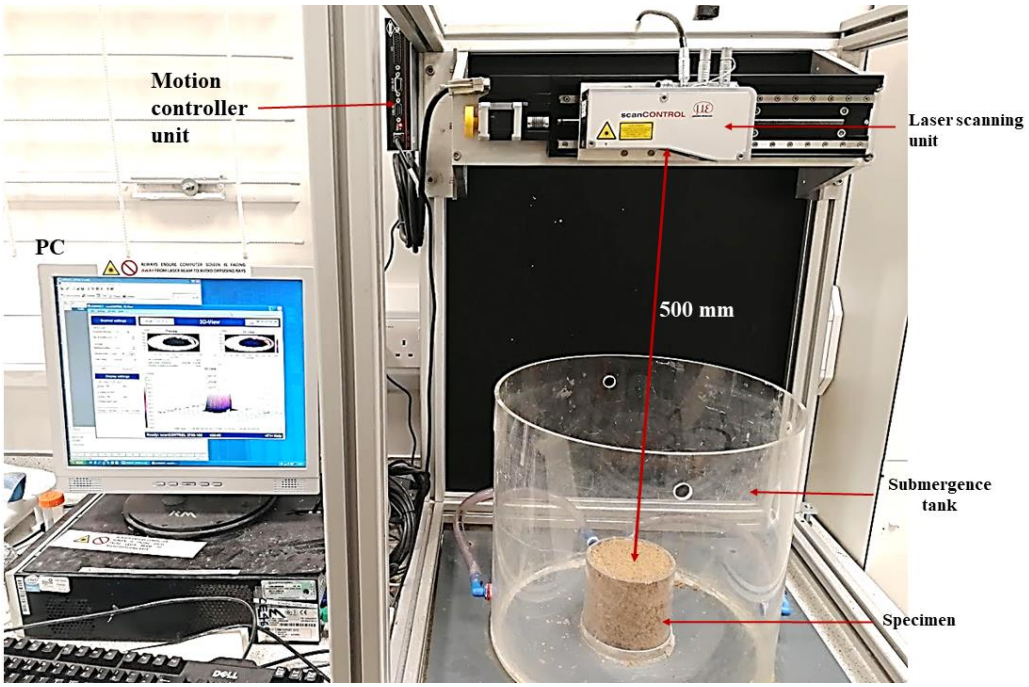


Figure 7-9: Set-up of the laser scanning unit, showing test specimen in the submergence tank and the PC unit for data acquisition .

7.3 Results

7.3.1 Erodibility characteristics of fungal treated and untreated soil

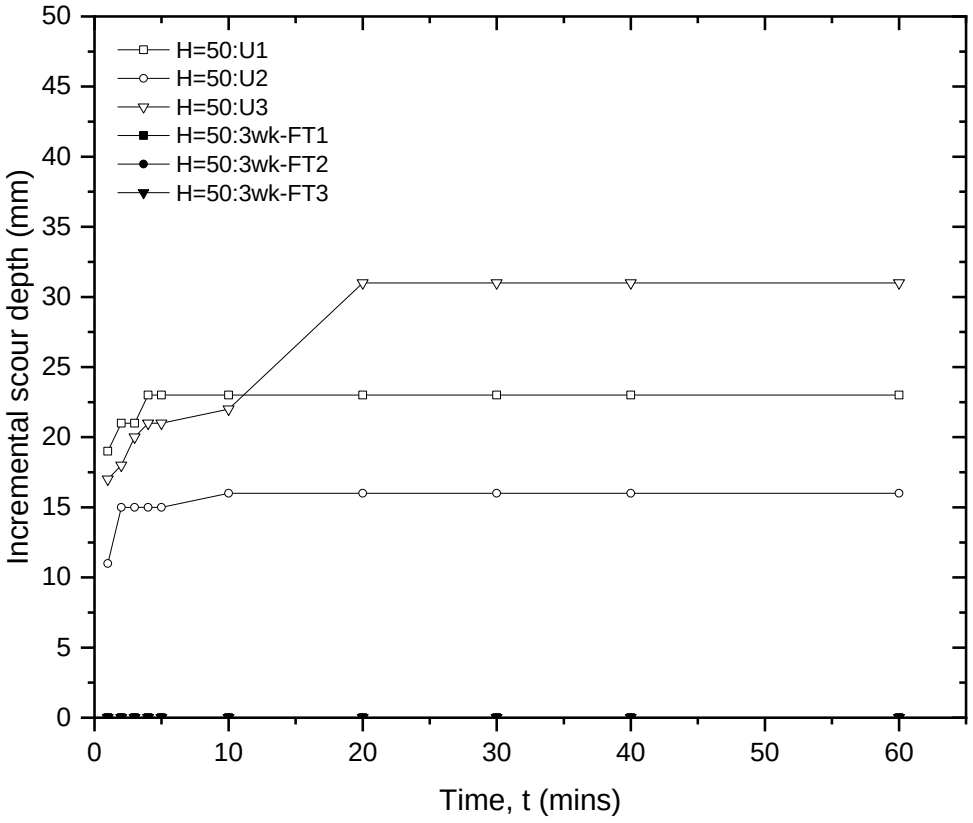
a) Scour depth evolution

Figs. 7-10a – e presents the respective scour depth evolution occurring at each specified hydraulic head (incremental scour depth). At a head of 50 mm, the untreated specimens began to scour immediately once the jet impinged on the specimen surface. After 1 minute, scour depths recorded ranged between 11 – 19 mm for the triplicate

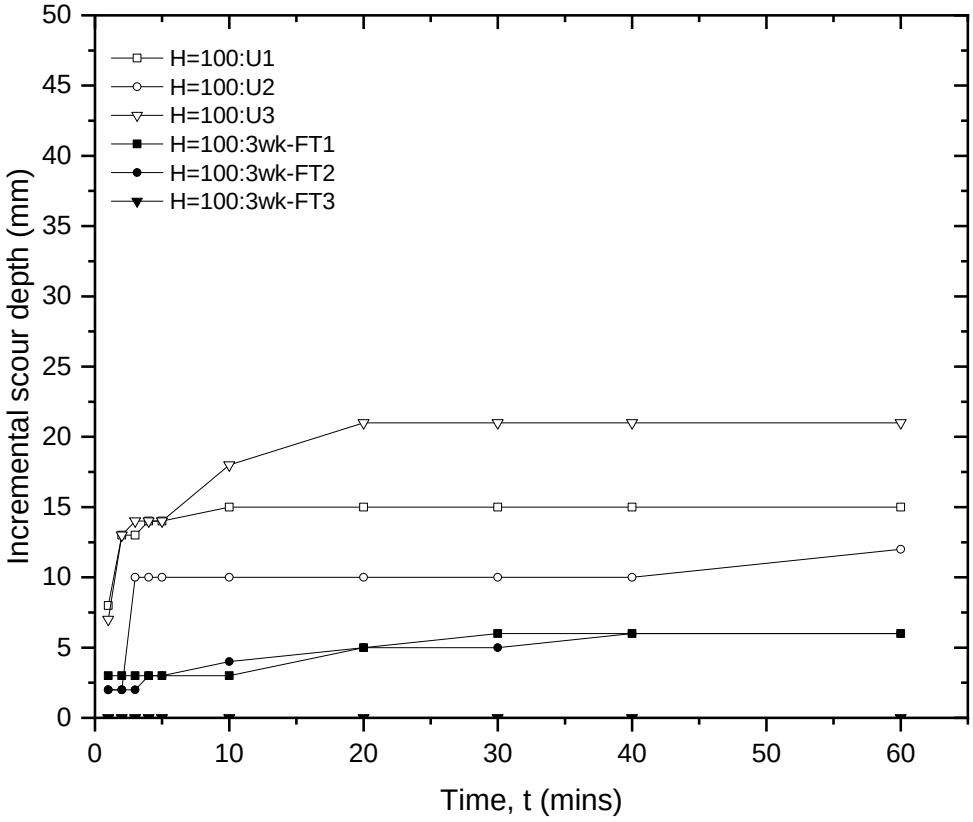
specimens, reaching maximum depths of 16 – 31 mm within 10 – 20 minutes. Negligible (or no further) increase in scour depth occurred up to the 60th minute. Similarly, when the hydraulic head was increased to 100 mm, the maximum scour depths were reached within 10 - 20 mins, and maintained until the end of the test (60th minute). The largest variation of scour depths achieved for the untreated specimens were recorded at hydraulic head of 250 mm. Initial depths ranged from 20 – 40 mm (after 1min of jet) while final depths were 25 – 47 mm at the 60th minute.

The reason for the variation in initial and final values of scour depths for the replicates of the same untreated soil specimens may be attributed to measurement errors due to the point gauge. For a cohesionless sand submerged in water, it is difficult to accurately observe the exact point when the tip of the point gauge touched the scour surface. This subjective error may have contributed to the variability observed. Furthermore, even for relatively well-mixed specimen of sand + lignocel only, variations at the micro-scale regarding soil compaction and resulting structural arrangement of sand grains and lignocel could contribute to variations in erosion behaviour for the three replicate specimens. Despite the variations, statistical ANOVA (Appendix H) showed that there was no significant difference, at $P<0.05$, in the scour rates (determined as the maximum incremental scour depth/total time tested at that hydraulic head) for all the untreated specimens at each respective hydraulic head.

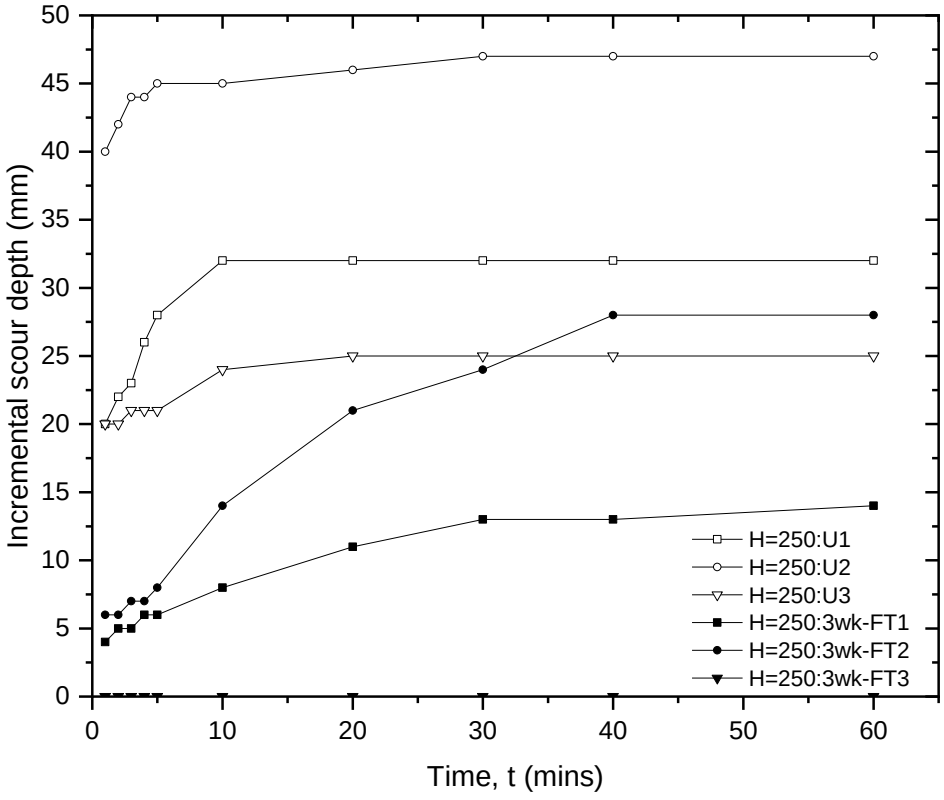
For the treated specimens, no erosion (scour) was observed at a hydraulic head of 50 mm for any of the three specimens and there was also no significant difference (at $P<0.05$) between the three replicates in the scour rates recorded at hydraulic heads of 100, 250, 500 and 750 mm (see ANOVA in Appendix I)


H=50:U1
H=50:U2
H=50:U3
H=50:3wk-FT1
H=50:3wk-FT2
H=50:3wk-FT3
Incremental scour depth (mm)
Time, t (mins)
0
5
10
15
20
25
30
35
40
45
50
0
10
20
30
40
50
60

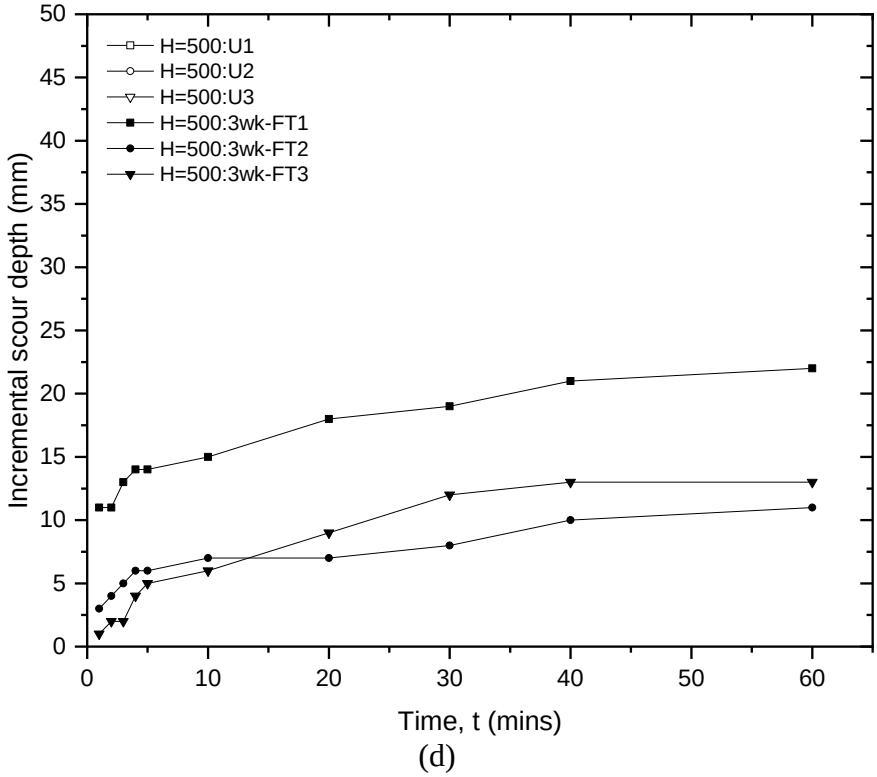
(a)


H=100:U1
H=100:U2
H=100:U3
H=100:3wk-FT1
H=100:3wk-FT2
H=100:3wk-FT3
Incremental scour depth (mm)
Time, t (mins)
0
5
10
15
20
25
30
35
40
45
50
0
10
20
30
40
50
60

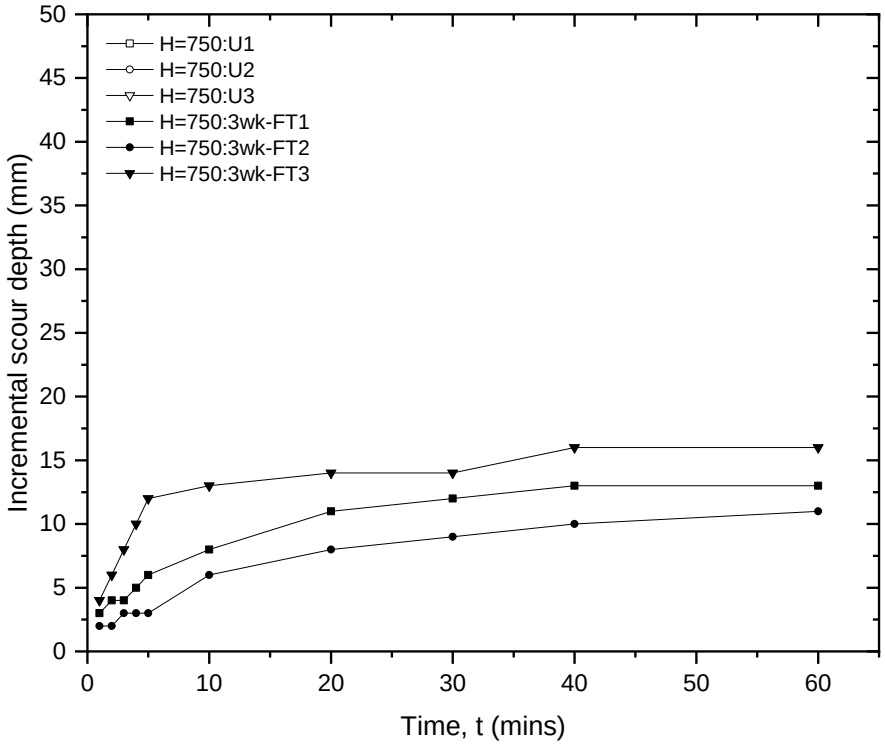
(b)


Incremental scour depth (mm)
Time, t (mins)
H=250:U1
H=250:U2
H=250:U3
H=250:3wk-FT1
H=250:3wk-FT2
H=250:3wk-FT3

(c)


Incremental scour depth (mm)
Time, t (mins)
H=500:U1
H=500:U2
H=500:U3
H=500:3wk-FT1
H=500:3wk-FT2
H=500:3wk-FT3

(d)



(e)

Figure 7-10: Plot of incremental scour depths with time, for untreated (U) and treated (T) specimens at respective hydraulic heads ranging as follows: (a) H = 50 mm (b) H = 100 mm (c) H = 250 mm (d) H = 500 mm (e) H = 750 mm.

The cumulative scour depth recorded over the entire period of the test including all values of hydraulic head tested for the untreated (U1, U2, U3) and treated (F1, F2, F3) replicate specimens are presented in Fig. 7-11. These specimens were all incubated for a period of 3 weeks. It is evident from Fig 7-11 that the performance of the fungal treated specimens subjected to the jet tests differed from the untreated control specimens in the following ways:

(i) the onset of erosion in the treated specimens was delayed, only beginning at H = 100 mm for F1 and F2 and 500 mm for F3. This is in contrast to the untreated specimens where erosion began from the first minute of testing at a hydraulic head of 50 mm.

(ii) the untreated specimens showed an initial rapid increase in scour depths for each change in hydraulic head, and quickly reached a constant scour depth (equilibrium) at each respective heads within about 10-20mins, whereas the increase in scour depth for the treated specimens on the other hand was much more gradual, levelling off after about 30-45mins, and

(iii) the scour depths for the untreated specimens were higher than those observed for the treated specimens for all the hydraulic heads tested, even at H=750mm (representing relatively high shear stresses, τ_o = 61 Pa) the maximum scour depth reached by the treated specimens was in the range of 5-16mm whereas untreated specimens were almost completely eroded at H = 250 mm.

At a hydraulic head of 250 mm, scour depths > 60 mm were recorded for the untreated sand; that is erosion of more than 60% of the total specimen height occurred. Untreated sand specimens subjected to jet erosion at a hydraulic head of 500 mm recorded a scour depth of ~90 mm in the first few minutes with the mould almost empty (mould height = 100mm). Further removal of sand particles did not occur as the grains were continuously recirculated locally within the mould. Since this stage did not last for the expected 60mins, values of scour depths for untreated specimens were not recorded for hydraulic heads above 250 mm. However, images and laser scans were obtained. On the other hand, maximum scour depths of approximately 55 mm were recorded for F1 and F2 after being subjected to a jet with a hydraulic head of 750 mm for 60 mins. Furthermore, F3 only exhibited a scour depth of 29 mm after 60mins at 750mm.

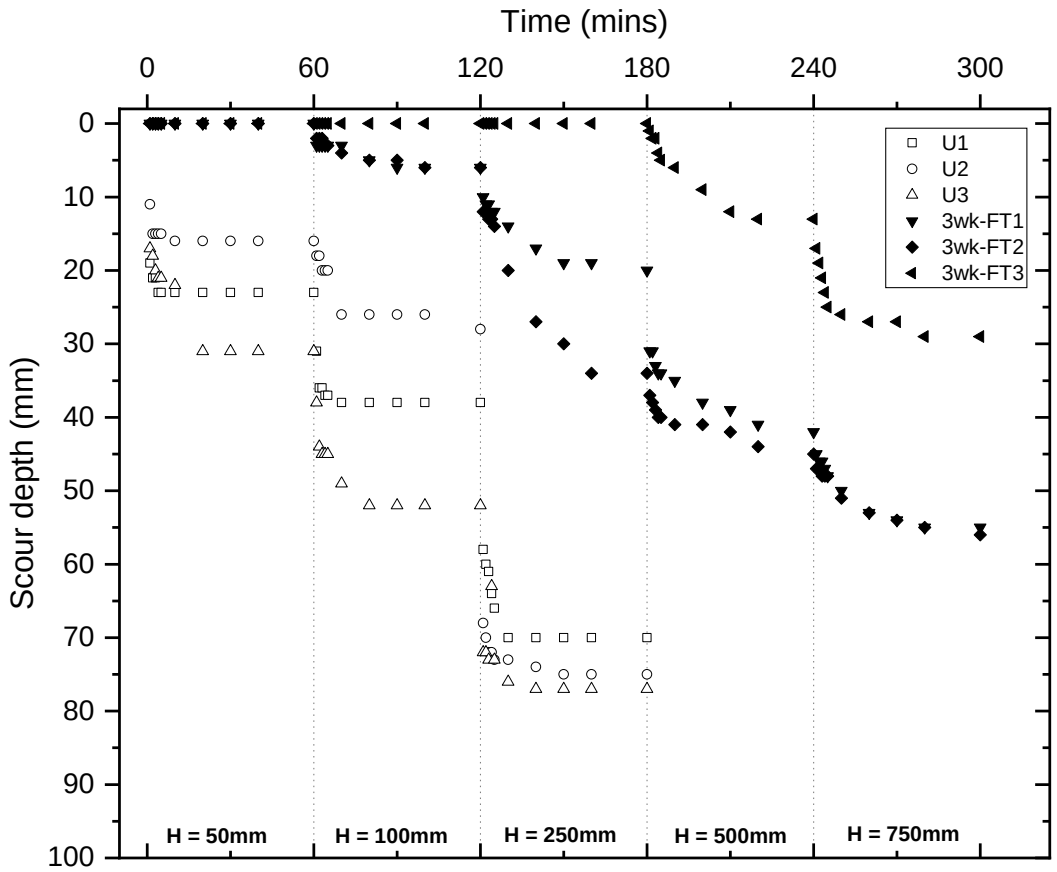


Figure 7-11: Experimental results showing evolution of scour depth at each hydraulic head (H) for untreated and treated specimens. Time is cumulative from beginning to the end of each test for each specimen and respective hydraulic heads.

Fig. 7-12 presents the cumulative scour depth at t = 60mins for each hydraulic head for all triplicates of the treated and untreated specimens. Linear regression lines were fitted to this data. It is evident that erosion proceeds more rapidly with increasing hydraulic heads i.e. applied shear stress for untreated specimens than for the fungal treated specimens.

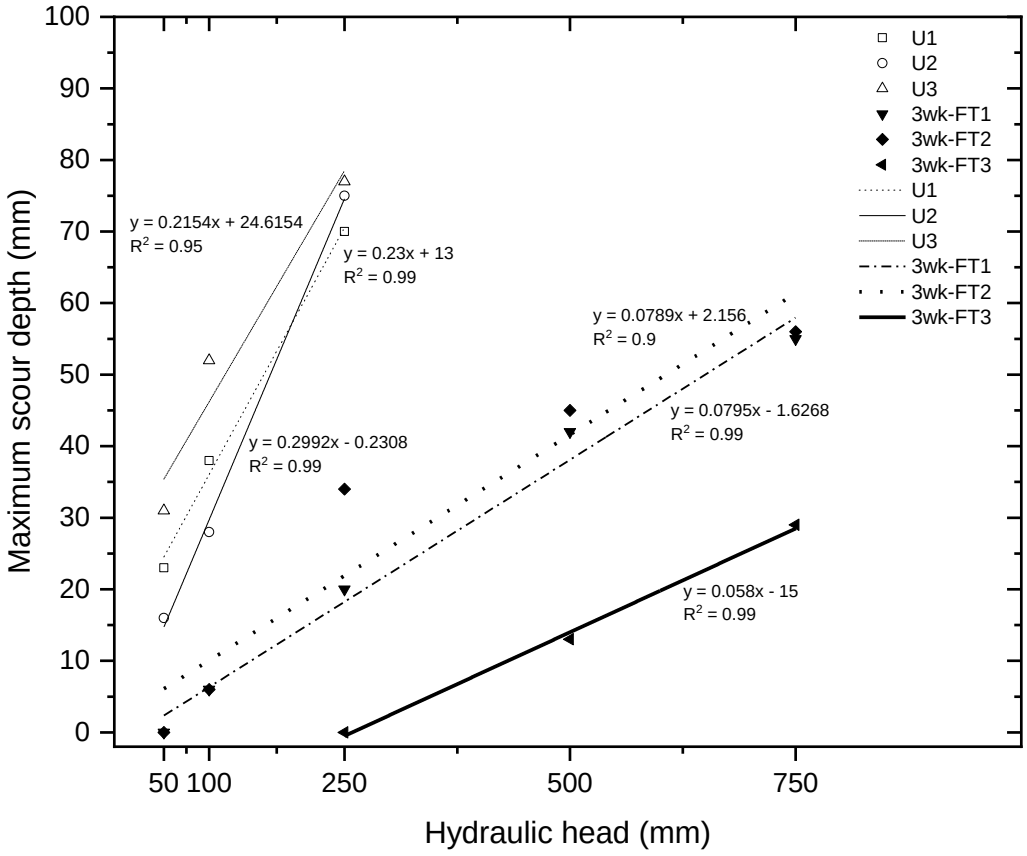


Figure 7-12: Scour depth against hydraulic head for fungal treated and untreated specimens

b) Volume of soil eroded

The scour depth only gives an indication of the depth of erosion reached within the soil specimen at a central point directly below the impinging jet. It gives no information on the volume of material eroded and how this may vary spatially across a specimen. To obtain this additional information, laser scanning profiles were obtained prior to the start of the tests and at the end of each stage (i.e. after 60mins at each hydraulic head). Fig. 7-13 presents photographic images of untreated (U1 & U2) and treated (3wk-FT1, 3wk-FT2) specimens before commencement of the jet tests and after being subjected to hydraulic heads of 100, 250 and 500 mm, while Fig. 7-14 presents the surface profiles obtained from laser scanning for untreated, U2 and

treated, 3wk-FT2 specimens. Figures 7-13 and 7-14 illustrate that as erosion progressed in the untreated specimens, the surface of the specimens were conical in shape extending with a gradual slope from the centre to the edge of the mould. This is in contrast to the treated specimens, where scour holes formed at the centre of the treated specimens directly below the impinging jet. That is to say that even where the scour depth reached may have been similar the volume of material eroded would have been considerably less for the treated specimens. The maximum diameter of the scour hole shown in Fig 7-13 and Fig 7-14 was ~33 mm; beyond this, little to no erosion occurred. The change in nature of the erosion behaviour indicates that binding of soil particles has occurred to some extent within the treated specimens as a result of fungal treatment.

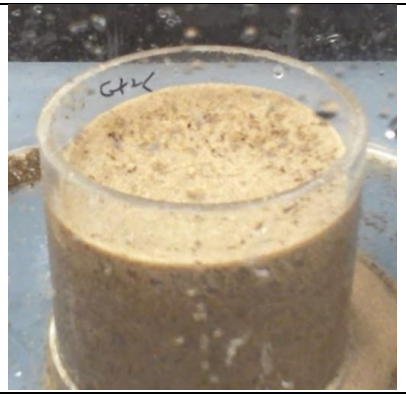
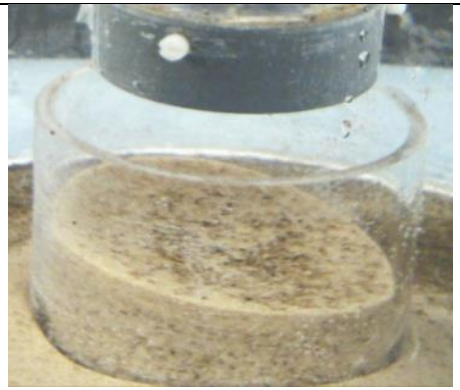
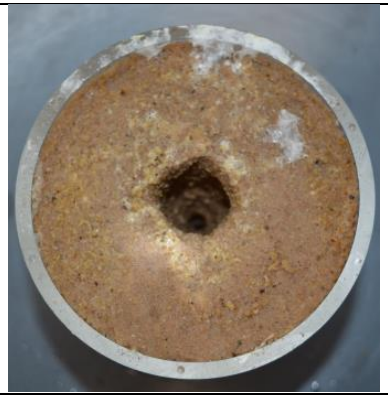
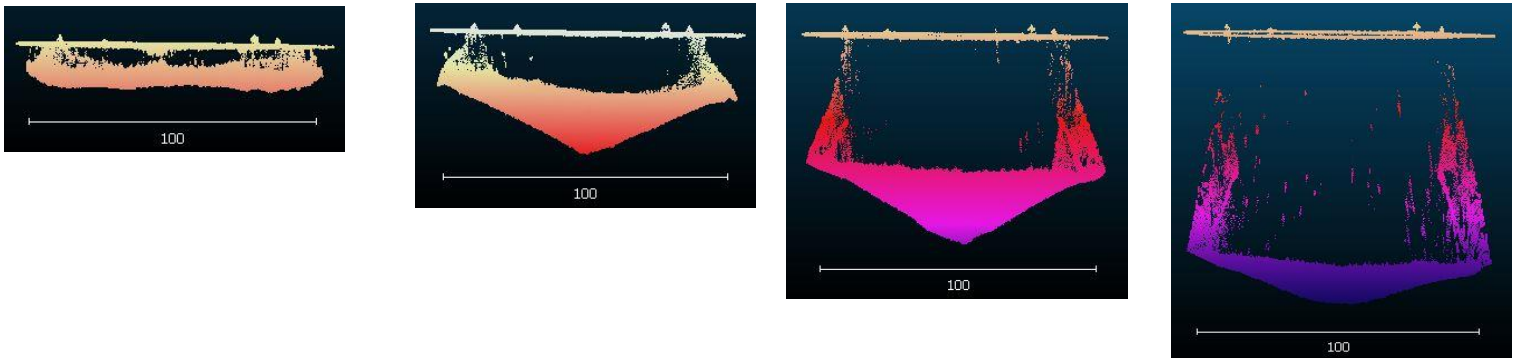
Untreated			
U2 - Before	U1 – Stage 2; H = 100 mm	U1 – Stage 3; H = 250 mm	U2 – Stage; H = 500 mm
			
Treated			
3wk-FT1_Before	3wk-FT2_Stage 2; H = 100 mm	3wk-FT2_Stage 3; H = 250 mm	3wk-FT1_Stage 4; H = 500 mm
			

Figure 7-13: Erosion patterns for fungal treated and untreated specimens.

Untreated (U2)

Before	Stage 2; H = 100mm	Stage 3; H = 250mm	Stage 4 (*t = 1min); H = 500mm



Fully-treated (3 wk-FT2)

Before	Stage 2; H = 100mm	Stage 3; H = 250mm	Stage 4; H = 500mm	Stage 5; H = 750mm

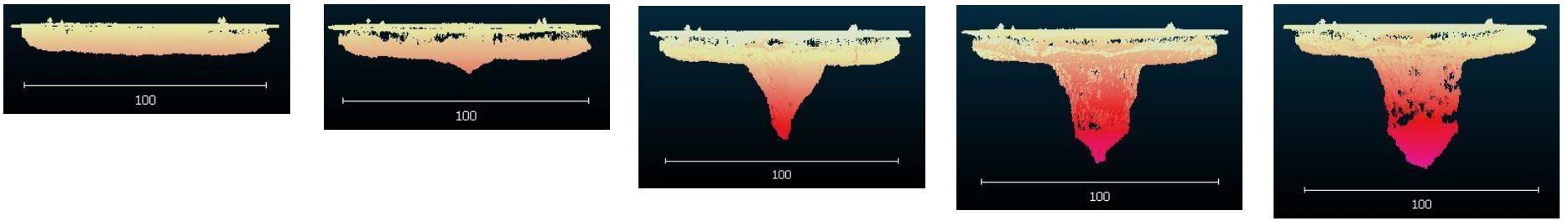


*Figure 7-14: 2D images of a 3D surface obtained from laser scanning, showing erosion profile/pattern for fungal treated and untreated specimens before commencement of test and at indicated stages. 2D images of a 3D surface obtained from laser scanning, showing erosion profile/pattern for fungal treated and untreated specimens before commencement of test and at indicated stages. The highlighted part represents either the initial soil surface, or the newly eroded soil surface. *Erosion tests for untreated specimens were stopped after 1 minute of stage 4 (at H = 500 mm).*

Results from the laser scanning also provided more information on the quantitative volumes of soil loss and provided an opportunity to validate the scour depths hitherto measured using the point gauge. Accurate measurement of scour depth using the point gauge largely depends on the pattern of scour obtained since the point gauge is passed through the nozzle and traces the jet centre line down to the bottom of the scour hole. Fig. 7-15a presents a schematic diagram showing the typical expectation for the evolution of scour depth under an impinging jet of water. However, where soils exhibit some level of cohesion, an uneven scour pattern (Fig. 7-15b) may form. This is because under the influence of the applied shear stresses, blocks of weaker aggregated soils may be eroded from locations that are offset from the jet centreline or the typical scour hole. For such occurrences (e.g. Fig. 7-15b) measurements using the point gauge may not be representative of the true scour depth. In this study, the scour depth measurements were supplemented by the use of a laser scanner to obtain more information regarding the erosion pattern in the soil.

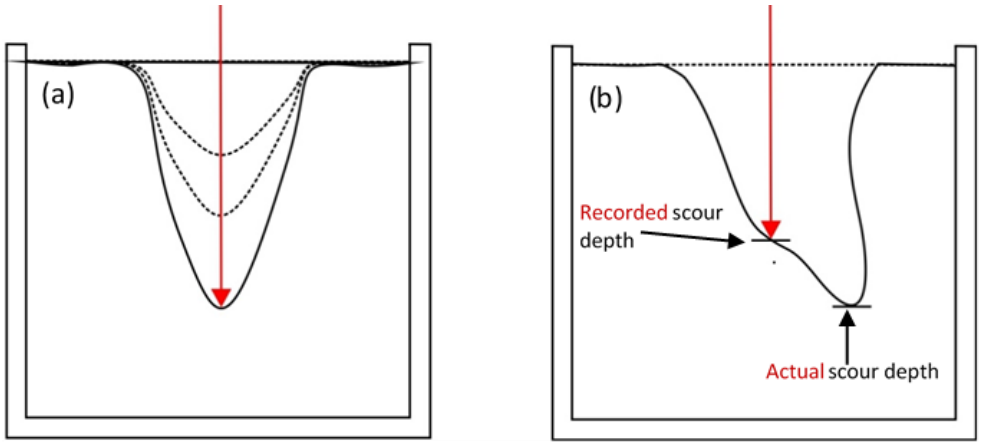


Figure 7-15: Schematic diagrams of possible scour depth evolution showing limitation in the use of a point gauge for measuring scour depth. (a) Typical scour depth pattern expected for relatively accurate measurement with point gauge (b) Possible scour pattern (in cohesive soils), where point gauge reading taken down the jet centreline may not accurately represent depth of scour erosion

Estimates of volume of soil eroded (%) (defined as the eroded soil volume/initial soil volume) were determined using the laser scanned images, by removing the newly eroded surface profile at the end of each stage from the initial specimen surface. These values were plotted against the respective hydraulic heads for each specimen. Fig. 7-16 clearly highlights that less soil is eroded in the treated specimens compared to the untreated specimens. The volume of material eroded and respective scour depths obtained using the laser scans give a more representative description of the respective erosion behaviour of soils tested opposed to relying only on the scour depth measurements determined using the point gauge. With reference to the scour depths determined from the laser scans in this study, the point gauge method underestimated the scour depth values at the end of each stage by 0.2 – 6.9 mm (Appendix J).

Fig. 7-16 shows that after subjecting to a jet with a hydraulic head of 250 mm, approximately 50% of the untreated specimens were eroded, whereas for the same hydraulic head <5% of the soil was eroded in the fungal treated specimens. Furthermore, after testing at 500 mm hydraulic head when little or no soil is left in the untreated specimen moulds, the fungal treated specimens had lost < 8% of total specimen volume. For the treated specimens the soil eroded was largely confined to that removed from central scour holes.

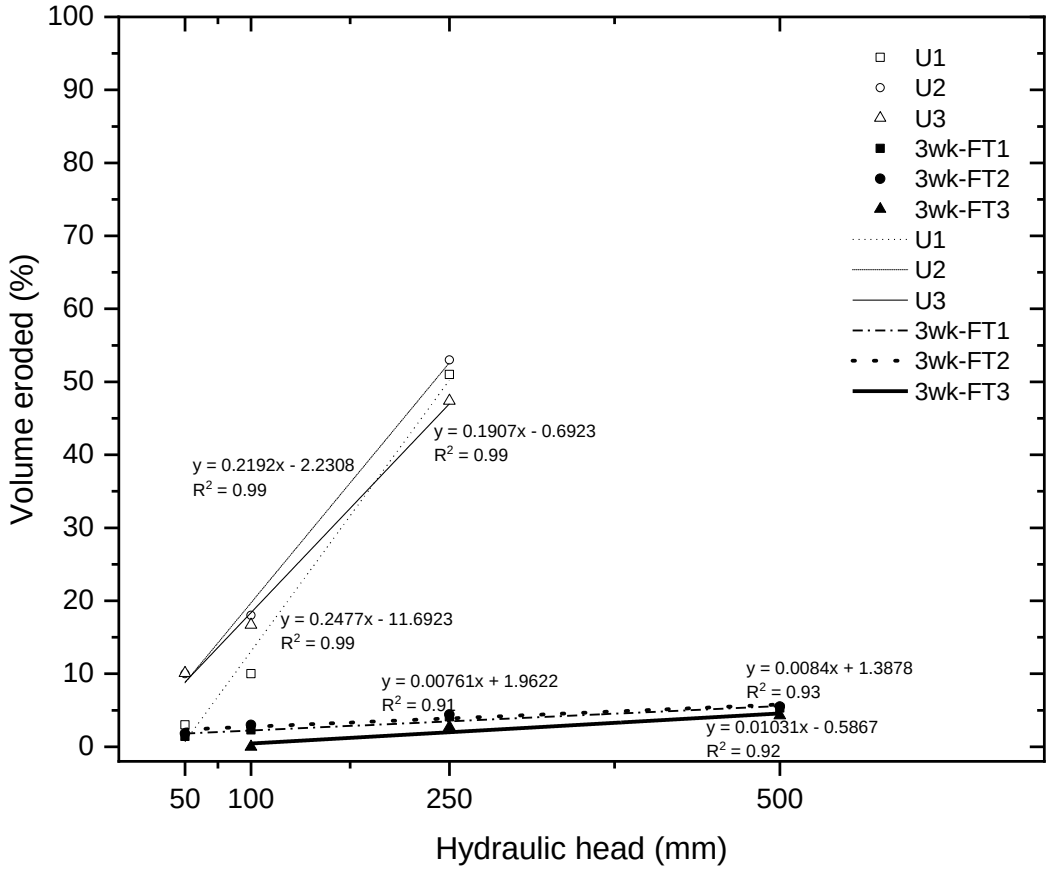


Figure 7-16: Trends % volume eroded per specimen at different hydraulic heads. Linear regression fitting lines are shown with R^2 values ranging from 0.91 to 0.99. Clearly, less volume of soil is eroded from the treated soil compared to the untreated soil.

c) Erodibility parameters, k_d and τ_c for fungal treated and untreated sands

In this study, the erodibility parameters from the JET data were determined using the common jet theory, often called the Blaisdell method (Blaisdell, Hebaus and Anderson, 1981). Data for the incremental scour depth (i.e. scour depth during each individual stage) evolution at each hydraulic head tested were analysed for each specimen, using an Excel routine of the Blaisdell method (Hanson and Simon, 2001). Each hydraulic head was treated as a different experiment with value of the input *Ji* (initial distance from jet nozzle to specimen surface) adjusted accordingly. Fig. 7-17 presents a plot of k_d versus τ_c for the untreated and treated specimens. The dashed red lines shown in the plot were taken from a categorisation of levels of soil erosion by

(Hanson and Simon, 2001) to provide a context for discussion of results. Values of k_d and τ_c have been determined for each stage tested, thus for the untreated specimens there are three points plotted for each specimen (50 mm, 100 mm, 250 mm) It is evident that all the untreated specimens plot well within the 'very erodible' category with the exception of one test stage for specimen U2 which plots at the boundary between 'very erodible' and 'erodible'. It is clear that all the treated specimens had lower values of erodibility and higher critical shear stress values compared to untreated specimens, with the exception of one test stage for specimen F2. From the values of erodibility parameters in the classification chart, it is clear that the fungal treatment has improved soil resistance to erosion, making hitherto 'very erodible/erodible' soils become 'erodible/moderately resistant' to erosion. Fungal inoculation and growth in sand specimens contributes to enhancing soil resistance to water-induced erosion.

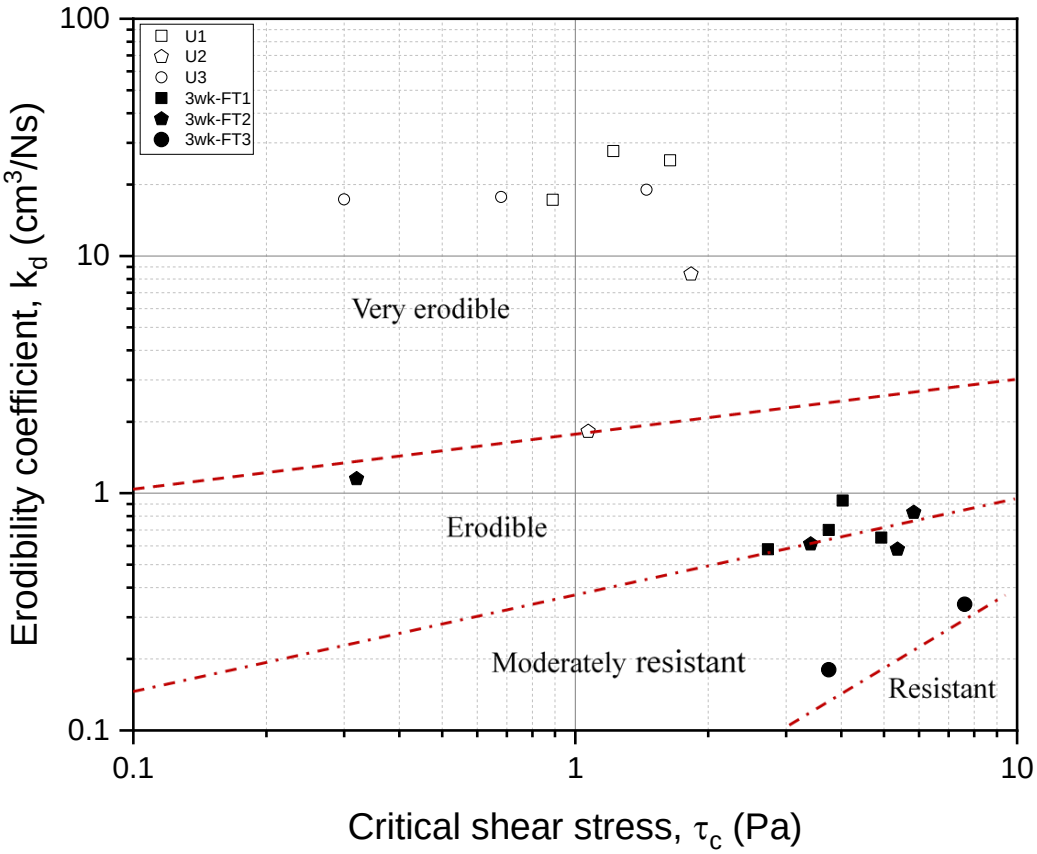
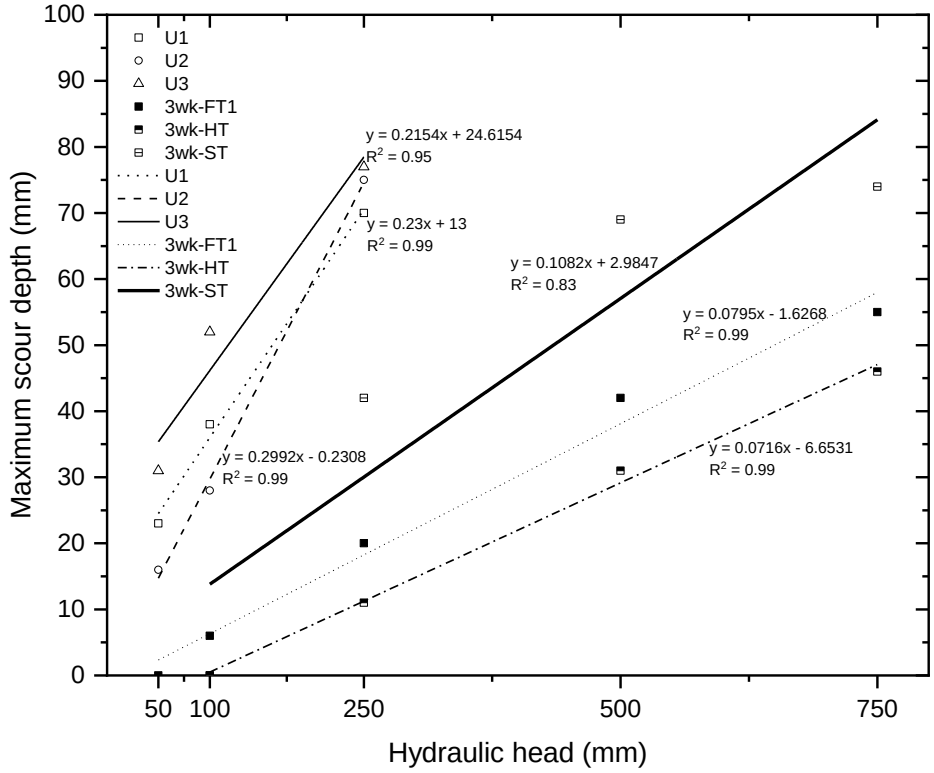


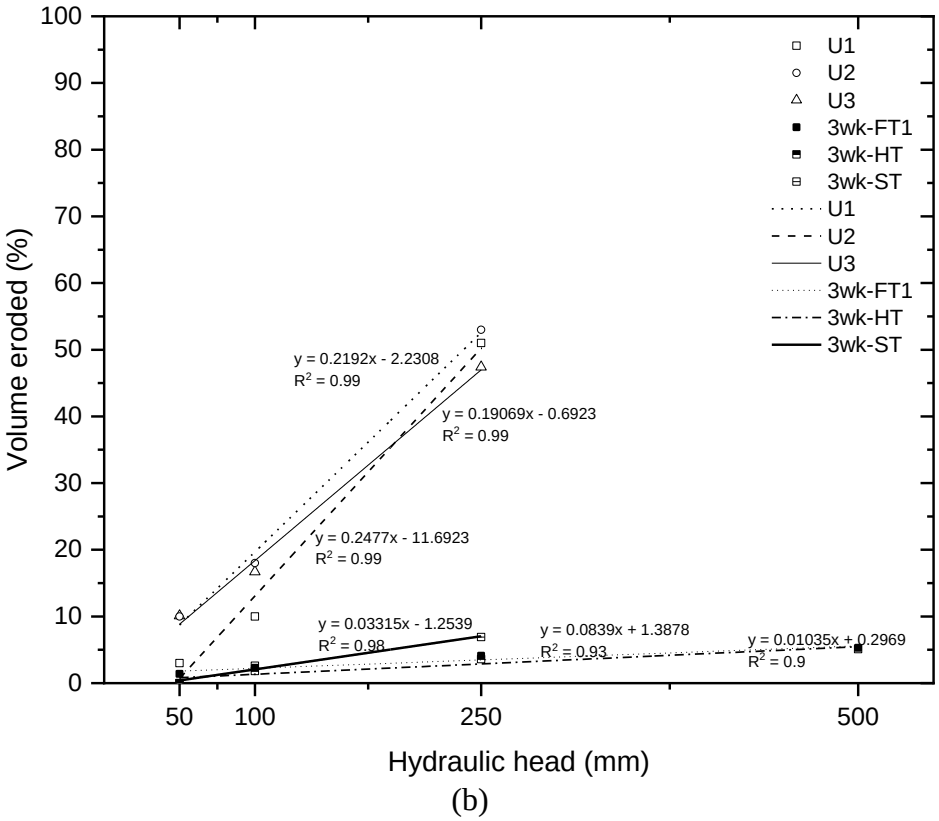
Figure 7-17: Classification of levels of resistance to erosion for fungal treated and untreated sands, based on erodibility parameters

7.3.2 Effect of method of treatment on erodibility

Figure 7-18a & b presents the scour depth and % volume eroded after 60mins at each hydraulic head tested for the 3wk-FT, 3wk-HT and 3wk-ST specimens and for comparison also presents the 3 untreated specimens (after an incubation period of 3 weeks). It appears that the surface treatment method has been less successful in reducing soil loss, reaching a greater scour depth and a greater percentage soil volume eroded compared to the other treatment methods. The fully treated and half-treated specimens appear to behave more similarly.



(a)



(b)

Figure 7-18: (a) Maximum scour depth and (b) % volume eroded versus respective hydraulic heads for the different treatment methods investigated

Fig. 7-19 presents the erodibility parameters for the 3wk-FT, 3wk-HT and 3wk-ST specimens and for comparison also presents the 3 untreated specimens (after an incubation period of 3 weeks). It is clear that all treatment methods resulted in lowering the erodibility coefficient and increasing the critical shear stress for erosion to be initiated, thus shifting the soil from being 'very erodible' to being classified as 'erodible/moderately resistant'. However, the half-treated specimen seems to show better resistance, with highest τ_c and lowest k_d values, compared to full-treated and surface-treated specimens (see Fig. 7-19).

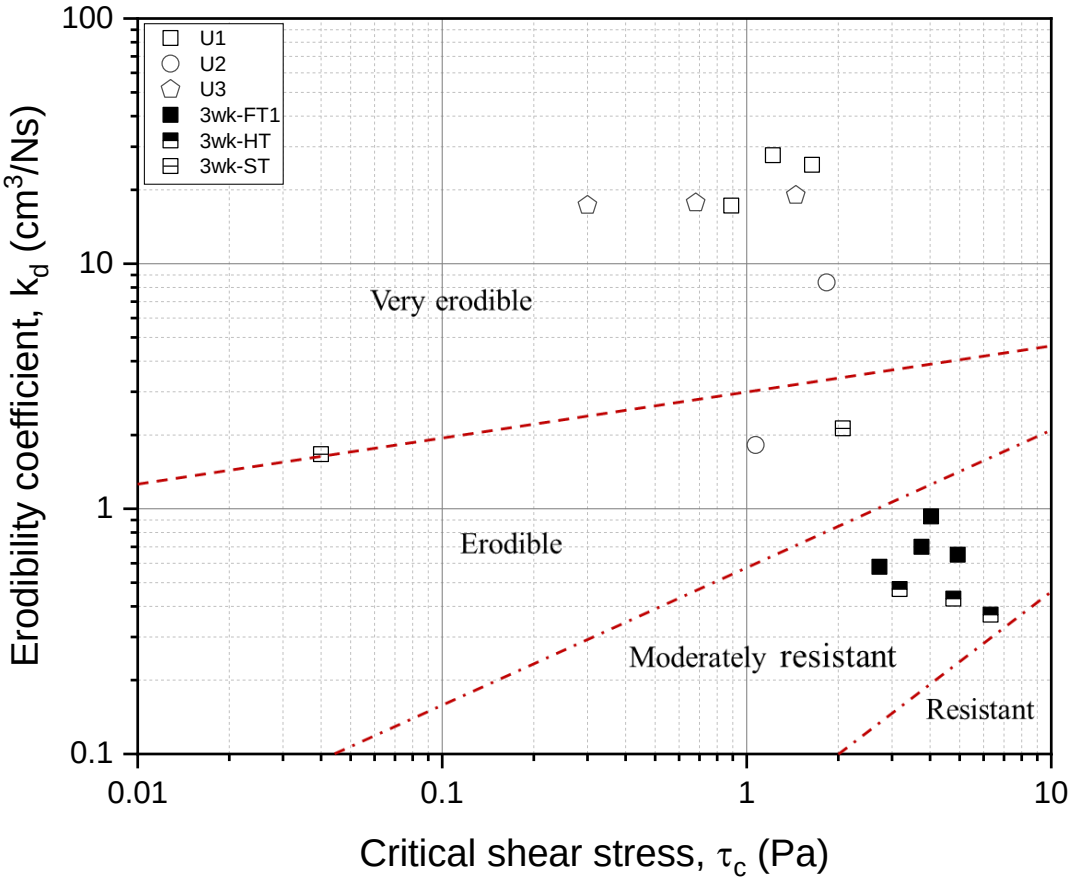


Figure 7-19: Erodibility chart for 3-wks old specimens with different treatment methods - FT, HT & ST.

7.3.3 Influence of combined inoculation method and growth duration on erodibility

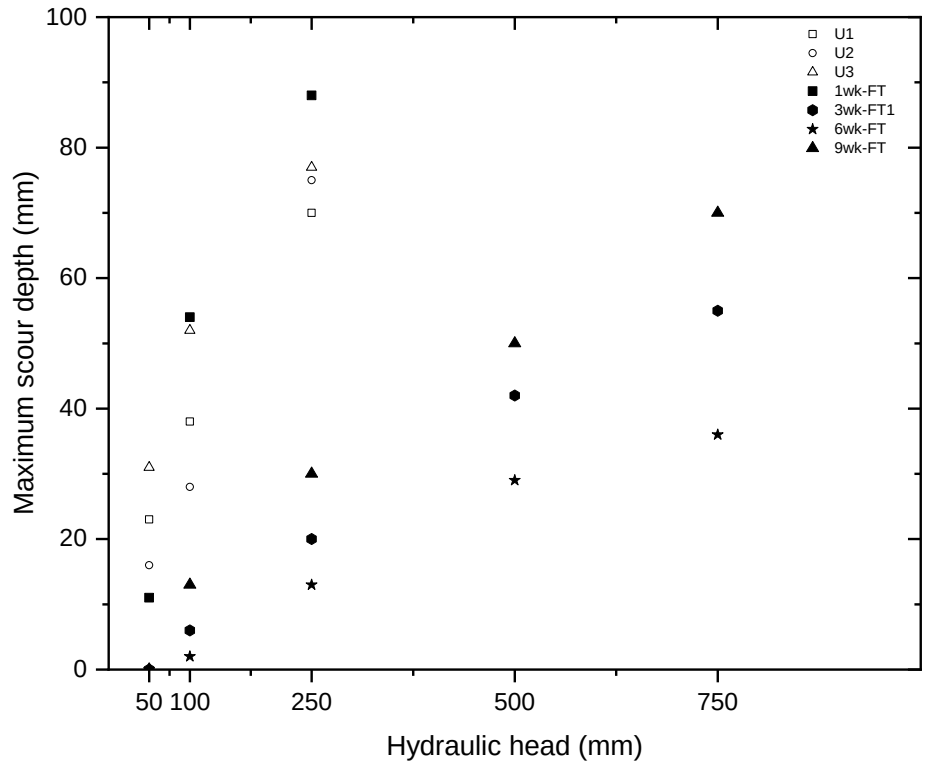
Jet erosion tests were conducted on specimens that were fully-, half-, or surface-treated and grown in the incubator for durations of 1, 3, 6 and 9 weeks respectively, to investigate the effect of method of inoculation and growth duration on erodibility characteristics of the soil.

Fig. 7-20a - f show maximum scour depths and soil volume eroded (%) plotted against the respective hydraulic heads for all the fully treated (Fig. 7-20 a & b), half treated (Fig. 7-20 c & d) and surface treated (Fig. 7-20 e & f) specimens, incubated between 1 – 9 weeks, as well as the untreated (control) specimens.

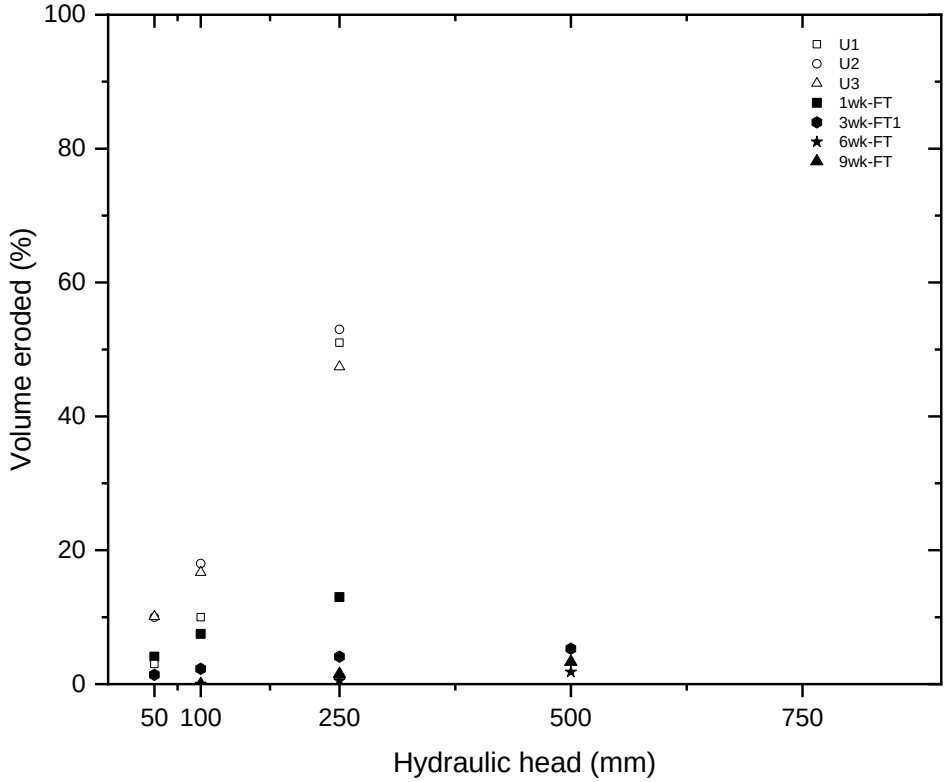
Figure 7-20 highlights that regardless of treatment method, all specimens incubated for only 1 week showed similar erosion behaviour (scour pattern) as the untreated control specimens. This was evident in both the scour depth measurements and % soil volume eroded determined from laser scanning. The % volume of surface treated specimens (Fig. 7-20f) eroded after 1week incubation period were significantly higher than soil loss in the other specimens. Whereas all fungal treated specimens with incubation/growth durations greater than 1 week exhibited enhanced resistance to erosion. The maximum scour depths (at the end of each stage) for half- and fully-treated 1-wk old specimens were not significantly different from the depths recorded for the surface-treated specimens, although the half- and fully-treated specimens were comparatively less erodible at lower hydraulic heads than the surface treated specimens. During the jet test on these 1-wk old specimens, at hydraulic head of 50 mm, blocks of aggregated soil mass were detached, resulting in mass soil loss and

deep scour holes developing within short periods. This behaviour can be attributed to early formation of weak soil aggregates by the fungi after a short growth period of 1week. This implies that a treatment period of 1 week is not sufficient for fungal-enhanced soil resistance to erosion, irrespective of inoculation method used.

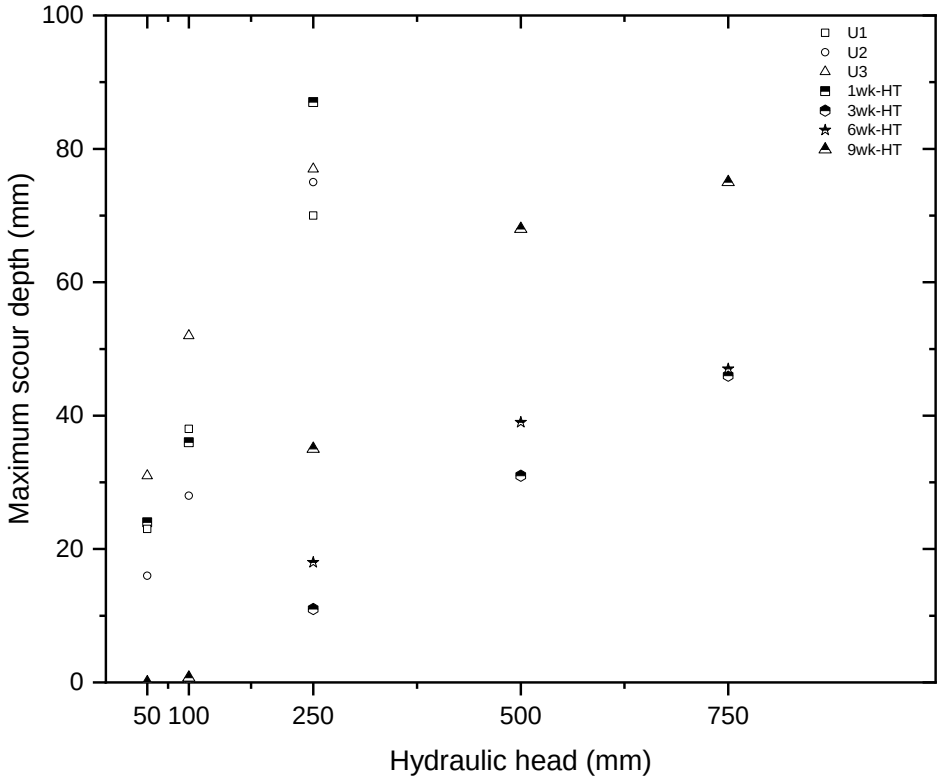
Furthermore, from the laser scans presented for the 1 week specimens (Fig. 7-21), it appears that fungal hyphal growth is more limited in the vertical extent for the surface treated specimens compared to the other treatment methods. As such, initially, erosion occurred in the form of a scour hole, but once a hydraulic head of 250mm was reached block mass erosion proceeded until the erosion behaviour reverted to that of the control untreated specimens, indicating that the growth of the fungi had not penetrated the full depth of the specimen. In the 6 and 9 week specimens, fungal mycelium was visibly observed at the bottom of surface-treated specimens and the behaviour was similar to that of the other inoculation methods.



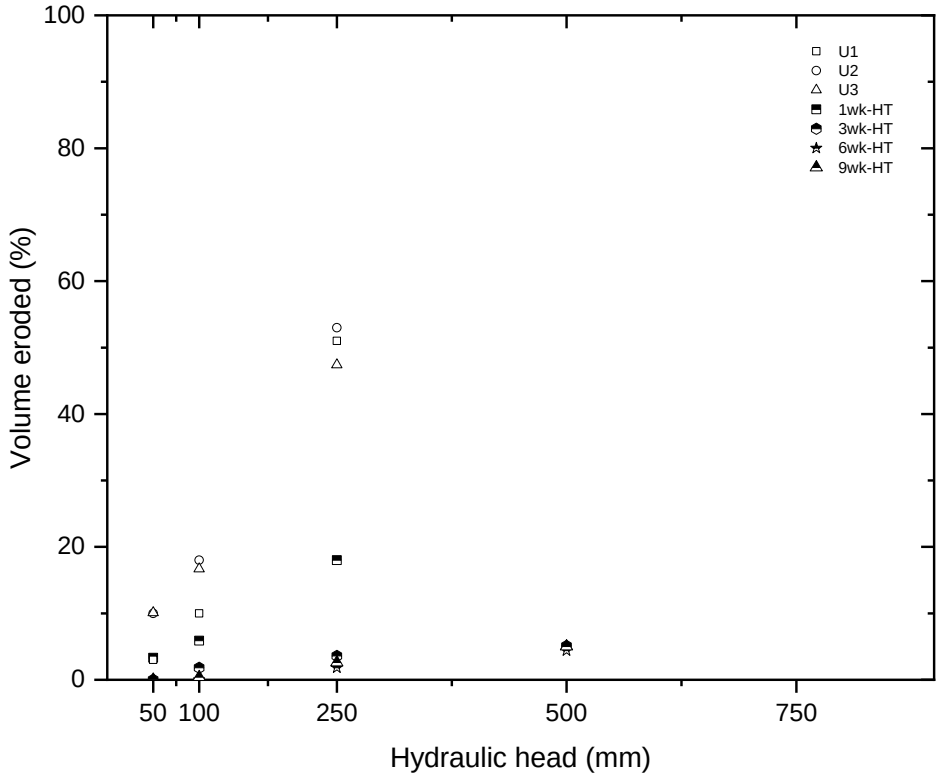
(a) Maximum scour depth vs hydraulic head for all fully-treated (FT) specimens incubated for 1 – 9 weeks



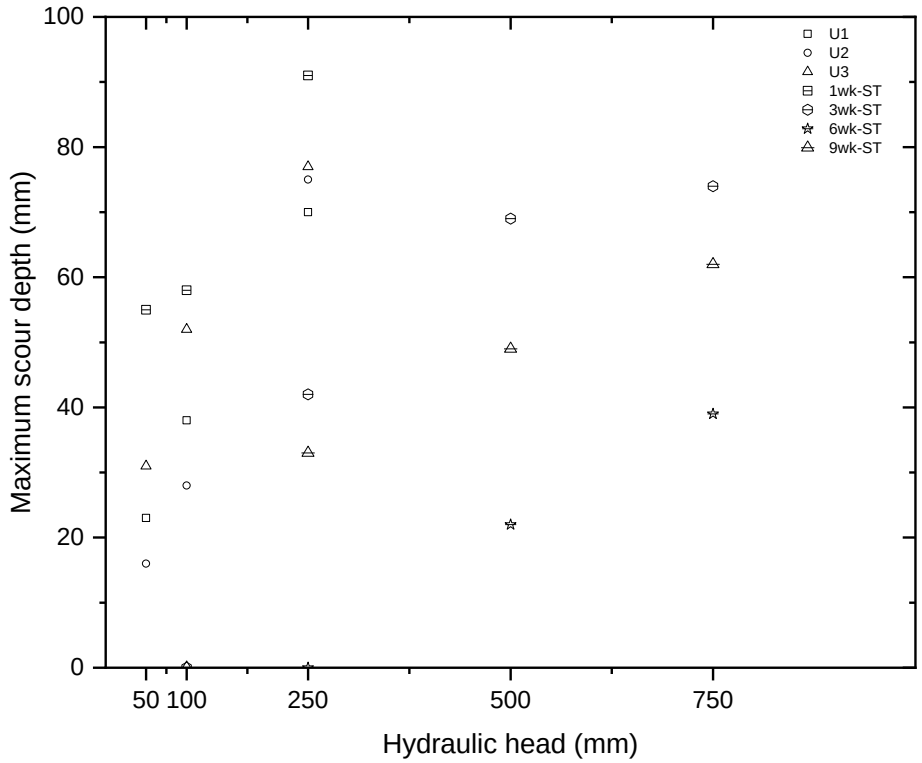
(b) % volume eroded vs hydraulic head for all fully-treated (FT) specimens incubated for 1 – 9 weeks



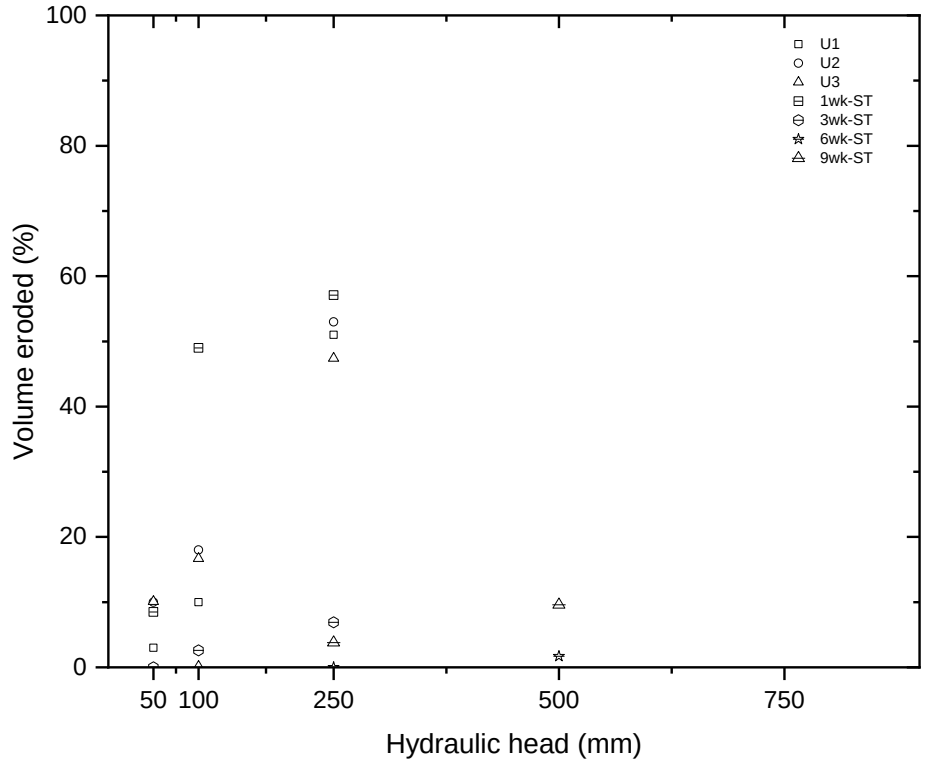
(c) Maximum scour depth vs hydraulic head for all half-treated (HT) specimens incubated for 1 – 9 weeks



(d) % volume eroded vs hydraulic head for all half –treated(HT) specimens incubated for 1 – 9 weeks



(e) Maximum scour depth vs hydraulic head for all surface-treated (ST) specimens incubated for 1 – 9 weeks



(a) % volume eroded vs hydraulic head for all surface-treated (HT) specimens incubated for 1 – 9 weeks

Figure 7-20 (a – f): Plots of maximum scour depths and volume eroded (%) against the respective hydraulic heads for all the treated specimens incubated between 1 – 9 weeks

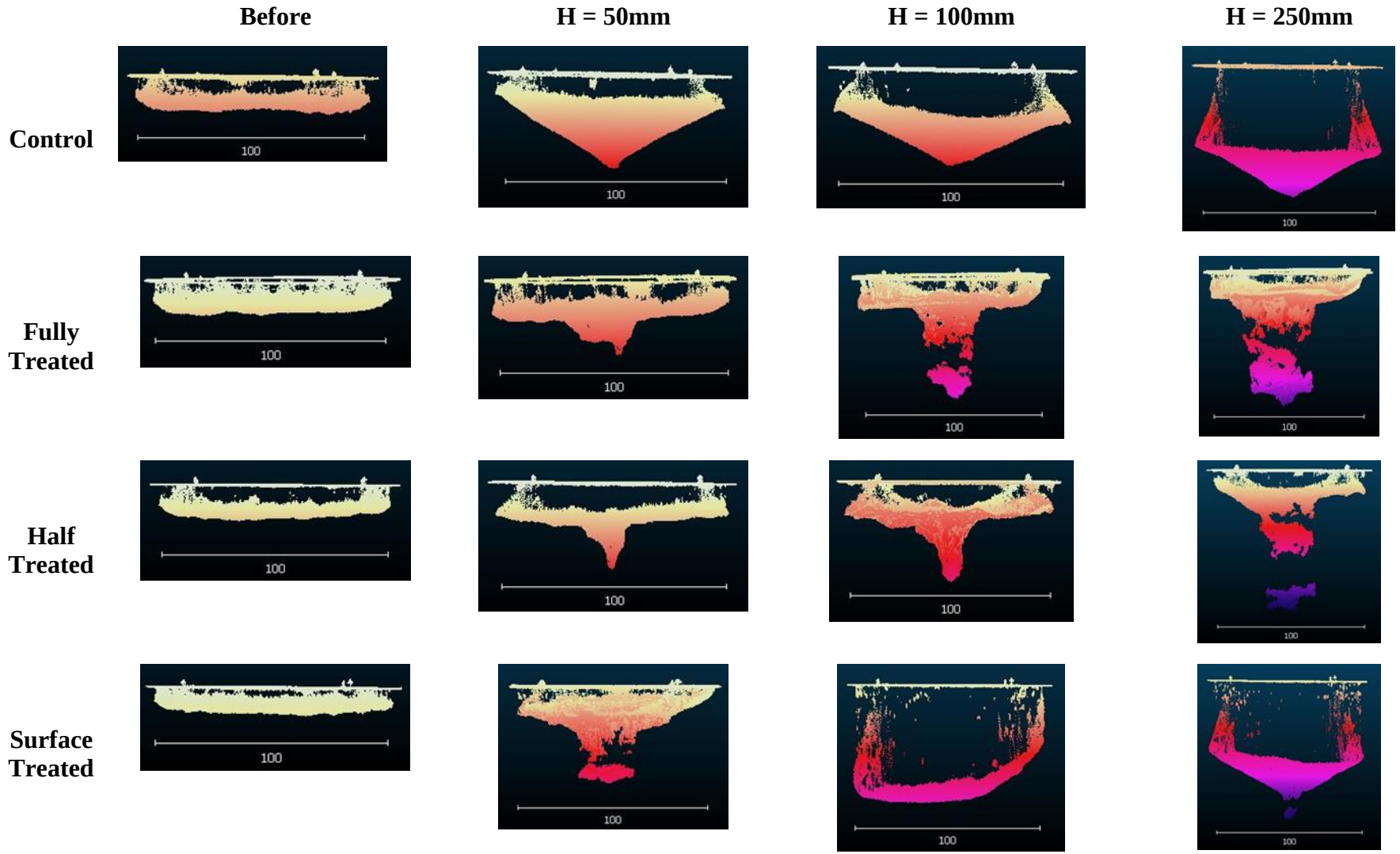


Figure 7-21: Laser scans of all the one-week treated samples and control showing different patterns of erosion with increasing hydraulic heads and varied treatment methods.

For specimens with 3 – 9 weeks' growth duration, there was statistically no significant difference (at $P < 0.05$) in the maximum scour depths recorded and % volumes eroded at each respective head (See ANOVA in Appendix K). Based on these experimental results it is clear that treatment of sand with *P. ostreatus* beyond 3 weeks of growth period will enhance the soil's resistance to erosion significantly.

The erodibility coefficient k_d and critical shear stresses τ_c obtained from the Blaisdell routine at different hydraulic heads are presented in Fig. 7-22. Again, it can be seen that surface treated 1-week specimens performed poorly with the lowest values of τ_c and highest k_d values determined. Generally, all 1-wk old specimens had a higher erodibility coefficient, meaning higher rates of erosionOn the other hand, half-treated specimens incubated for 6-9 weeks appeared to perform best. The parameters interpreted for the 3 week specimens appear to be more closely located at the boundary between the erodible/moderately resistant soil categories whereas the 9 week specimens appear to be located more distributed throughout the 'erodible' category. Possible reasons are discussed in section 1.4 below.

To determine the most significant main effect (i.e. treatment methods - FT, HT, ST or growth duration - 1, 3, 6, & 9 weeks) on soil susceptibility to erosion, a 2-factor ANOVA test was performed on the respective values of % volumes of soil eroded, maximum scour depths, k_d and τ_c for the specimens. Since the 1-wk treated specimens seemed to vary significantly from the others, the ANOVA was performed twice, one for all specimens using values at hydraulic head of 250 mm, and secondly, for all specimens with exception of the 1-wk treated specimens at hydraulic heads of 500 mm (and 750 mm where applicable).

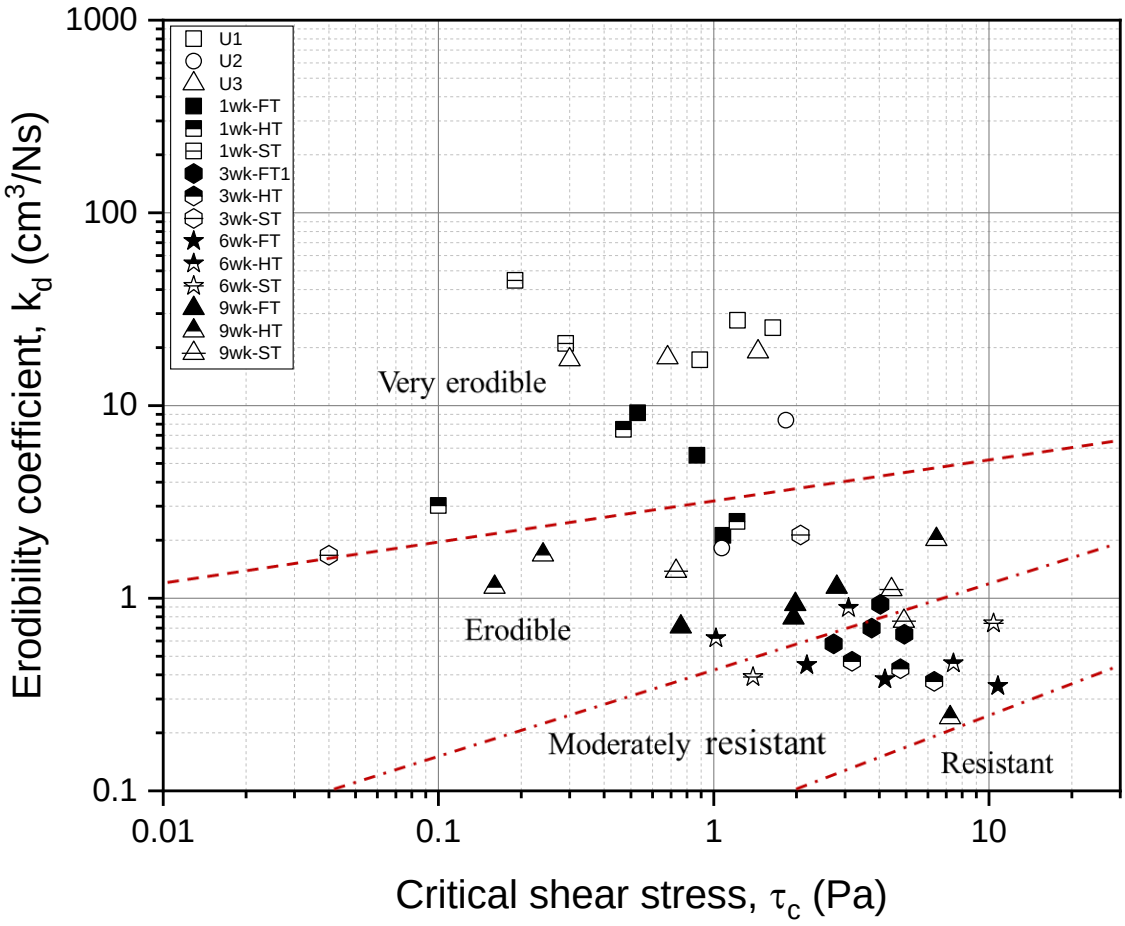


Figure 7-22: : Erodibility chart for all the treated specimens incubated between 1 – 9 weeks

Table 7-3 presents a summary of the statistical results. Analysis of the maximum scour depth and volume eroded of all the fungal treated specimens showed that the duration of fungal growth is the most significant main effect on erosion behaviour of fungal-treated soils tested at a hydraulic head of 250 mm (and up to 750 mm for maximum scour depths only). The method of treatment (full, half or surface) was not significant at $P<0.05$ and $P<0.1$. However, analysis of the % volume eroded for all treated specimens with growth durations of 3 weeks and beyond showed that there was no significant main effect due to inoculation method on the susceptibility of the soils to erosion. In conclusion a fungal growth period of 3 weeks and beyond was sufficient for reducing erosion irrespective of the method of treatment used.

Table 7-3: Statistical significance of all treated specimens (1-9 wks) and treated specimens with incubation periods > 1 week

Specimens	Hydraulic head	Maximum scour depth		% Volume eroded	
		Method	Duration	Method	Duration
All treated 1-9weeks	250	N	Y (*P<0.05*)	N	Y (*P<0.1*)
All treated > 1 week	500	-	-	N	N
	750	N	Y (*P<0.1*)	-	-

Y = significant; N = not significant

7.4 Discussion

7.4.1 Variation in fungal treated specimens

Fungal treatment is a biological based technique and relies on growth of the fungus to alter soil behaviour, as such there may be variability. Specimens F1, F2 and F3 were all treated in the same manner (fully treated) and incubated for a period of 3 weeks. Two of the specimens performed similarly in terms of erosion behaviour, with ~55 mm maximum scour depths achieved at 750 mm hydraulic head, while F3 exhibited only 29 mm scour depth at the same hydraulic head. However, all treated specimens showed a similarly low % volume of soil eroded (~5% at hydraulic head of 500 mm) whereas U1, U2 and U3 each recorded ~50% volume eroded at 250 mm hydraulic head.

7.4.2 Influence of treatment method

The author initially hypothesised that the fully treated specimens would outperform the other treatment methods due to an initial even distribution of spores throughout the specimen, it was anticipated that this would therefore translate into more hyphal biomass, also evenly distributed throughout the soil specimen. However, this was not found to be the case. Fig. 7-23 shows typical images of a fully treated and a half-

treated specimen just before the start of a JET test. These were incubated for 3 weeks. While patches of mycelia can be seen in colonies spread throughout the entire profile for a fully-treated specimen (Fig. 7-23a), more visible indication of fungal presence (*i.e.* the marked whitish mycelia) exists in the bottom half of the half-treated specimen (Fig. 7-23b) and similarly observed for surface treated specimens (no image). This mode of mycelial growth distribution was observed for half- and surface-treated specimens at 3 weeks growth period and beyond. One may have expected the reverse to occur for the partially treatment specimens, i.e. denser hyphal growth in the top half of the specimen where the spore suspension was applied to or mixed with the soil. (Sufficient information to provide better explanation for this behaviour could be obtained from a post-mortem analysis of hyphae distribution across the vertical extent of all specimens. Unfortunately, these are not available for this study). For a relatively loose sand (bulk density of 1.2 g cm^{-3}) with lignocel particles mixed through it at an initially low moisture content (6.5% for the surface-treated specimens and 11% for the half-treated specimen), permeability is expected to be high enough for quick percolation after infiltration. There is the possibility that after application, visible hyphae and spawn particles within the treatment suspension are attached to the soil particles and retained on the specimen surface (surface-treated) or within the top half of specimen (half-treated) while the treatment suspension percolates to the lower regions, resulting in higher concentration of spores at this hitherto uninoculated bottom half of the soil. Growth within the top half may have progressed, but less rapidly or extensively compared to the bottom region with more spores and moisture available for a relatively longer time. It should be noted that the amount of the liquid content at the bottom of the fully treated specimen and half-treated specimen were the

same during specimen preparation and at the start of the test, only that the former was mixed with the fungal spore suspension and the latter with water; while the surface-treated specimens had a much lower water content before inoculation with spore suspension (see section 1.2.1(e)).

During an erosion test on a half-treated and a surface-treated specimen incubated for 3 and 6 weeks respectively, cracks were observed along the boundary between compaction layers at the upper parts of the specimens. Subsequently these blocks of soil mass became separated from the rest of the specimen (Fig. 7-23c) barely a minute into the erosion test at the stage where the hydraulic head was increased to 750 mm. This could be attributed to combined effects of (i) possible build-up of pressure due to entrapped air within pores along the boundary of the compaction layer; meaning that treated specimens may not have been completely saturated during the period of submergence, and (ii) relatively weaker aggregation and hyphal growth within the top half of the half- treated specimens compared to the bottom half, due to the concentration of spores and higher moisture at the bottom region, as suggested earlier.

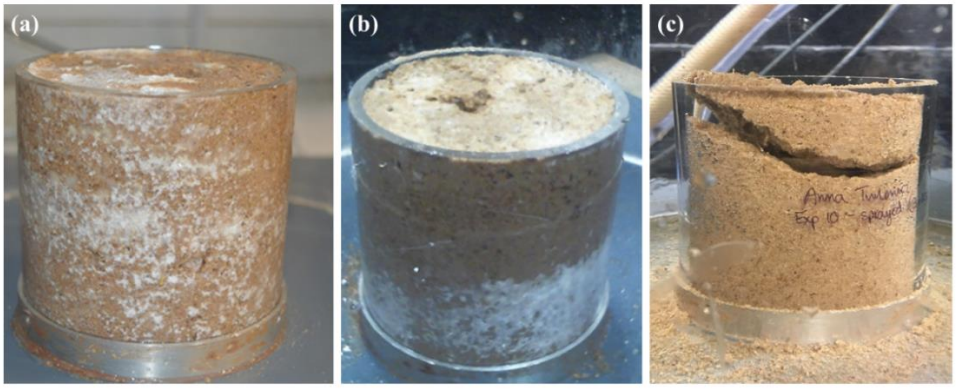


Figure 7-23: (a) A typical fully treated specimen after 9 weeks of incubation showing evidence of well distributed mycelia colonies or growth throughout the specimen and (b) a half-treated specimen after 9 weeks incubation period with relatively denser 'whitish' patches of mycelia colony observed at the bottom half and top surface of the specimen. (c) A block of soil mass completely detaches from the rest of a surface-treated specimen (incubated for 3 weeks) during erosion test after 1 minute at hydraulic head of 750 mm.

The surface treated specimens were more susceptible to erosion after 1 week incubation than the other methods. In this case erosion occurred in the form of mass blocks of variable sizes, indicating that particle aggregation after 1 week was weaker than in the fully- or half-treated specimens. This is probably because more time is required for hyphal growth to extend across and interconnect farther adjacent particles and also to extend to the bottom of the mould. Also, the deployment of hyphae to scavenge for resources may also have been limited by time, as the proximity of the inoculated soil surface still had abundant nutrients.

Previous studies have shown that variable foraging characteristics of fungal species in time and space, influences the extent of hyphal growth and fungal biomass produced (Boddy *et al.*, 2006). Extra-radical hyphae of some filamentous fungi species deploy thicker hyphal cords to scavenge for resources in nutrient-depleted areas whereas less hyphal growth extent but denser mycelium is seen in nutrient rich areas because no extra growth effort is required when resources are within easy reach (Dowson, Rayner and Boddy, 1986; Donnelly and Boddy, 1998; Boddy *et al.*, 2006, 2009). This may also have contributed to the growth gradients observed - and may influence future growth distribution - whereby mycelium grew more extensively in the non-inoculated layer of the half- and surface-treated specimens, compared to the fully-treated specimen (Fig 7-23b). Additional time (beyond 1 week) was required for the surface treated specimens to grow hyphae to such an extent as to behave similarly to the other inoculation methods.

For field applications, it is imperative that irrespective of the inoculation method used, the time required for initial hyphal establishment and growth should be considered in

the planning and design as well as implementation. Uniformity of treatment or growth distribution of mycelium across the soil profile may vary widely in fields, depending on inoculation method, soil type and composition, availability and location of nutrients/resources, influence of existing microbial communities as well as time. Further studies may be required, preferably at small and large-scale, to understand the specific foraging behaviour of the hyphal networks of *P. ostreatus* in soils under abundant and depleting nutrient conditions, as this will aid the engineering of its growth for erosion control in field conditions.

7.4.3 Influence of growth duration

The above discussion implicitly suggests that time factor can significantly modify the behaviour of the specimens inoculated in different ways. As mycelia grows, it depletes the amount of substrate, moisture and pore space and thus oxygen available. This alters the hyphal growth dynamics and distribution (Chapter 6). With no replacement of these resources, some older hyphae may senescence and begin to sporulate (i.e. form spores) (Jedd and Pieuchot, 2012) or become food for younger ones, while others may regroup into thicker cords and begin to explore farther regions for resources (Donnelly and Boddy, 2001). The conditions tested in the experiments presented herein were controlled constant temperature, fixed initial moisture content (11%), and fixed initial substrate condition. Given that below a moisture content of 3% (see Chapter 3) no growth occurred, moisture content may have limited fungal growth and viability of the longer duration specimens (6 week and 9 weeks) and contributed to the growth gradient visually observed in the partially-treated specimens. In the field, addition of moisture in the unsaturated zone may occur due to rainfall and infiltration for near-

surface soils, and from groundwater via capillary rise and is therefore less likely to be a limiting factor (depending on site location and climatic conditions).

7.4.4 Mechanisms contributing to enhanced erosion resistance

The results presented herein all indicate that fungal treatment of a cohesionless soil can reduce soil erosion. Photographic evidence and laser scanning profiles indicate that untreated specimens eroded via a particle mechanism, with the entire soil surface eroding at a similar rate (forming conical surface with a gradual slope from centre to edge), whereas the fungal treated specimens exhibited a scour hole, which developed directly below the JET but with surrounding soil bound together in a manner that is characteristic of cohesive soils. Specimen 1wk-ST illustrated a switch from the scour hole pattern typical of a treated specimen, to the pattern of an untreated specimen below a depth of 55 mm.

It is the development of the fungal hyphae which are considered responsible for the change in erosion behaviour. This may be through physical entanglement of the grains, or enhanced cohesion resulting from biochemical exudates secreted or contained in the hyphal walls. In addition, small air bubbles were visibly observed in the fungal treated specimens suggesting that the presence of the hyphae of *P. ostreatus* may have prevented complete saturation of the soil during the specimen submergence phase due to water repellent grain surfaces and a slower rate of infiltration. Considering this, soil suction could therefore have been higher in treated specimens with an associated higher resistance to soil erosion (Nguyen et al., 2017). In two out of the total number of treated specimens, it was observed that during infiltration (and displacement of air by water), air bubbles were trapped due to the lower permeability treated soil surface

and confinement of the experimental specimen mould. Air bubbles accumulated and formed larger air pockets, and when the air pressure exceeded the overburden stress, cracks and uplift of soil occurred (See Fig. 7-23c). This phenomenon has also been observed in experiments investigating soil desaturation using biogenic gas formation (van Paassen *et al.*, 2017).

7.4.5 Erosion behaviour of water repellent soils

As highlighted in Chapter 4, the development of water repellency in soils whether natural (e.g. wildfires) or induced via chemical treatments tends to be associated with an increase in soil erosion. Fungal treatment due to the ability of the hyphae and associated polysaacharides to bind soil particles can offer the ability to induce water repellency, reduce soil hydraulic conductivity while also increasing resistance to soil erosion. Chapter 8 discusses further potential applications of this technology.

7.5 Conclusion

This study has investigated, for the first time, the erodibility characteristics of sands treated with a spore/hyphal suspension of the filamentous and saprophytic basidiomycetes, *P. ostreatus*. A laboratory JET test apparatus was used to induce soil erosion to compare behaviour between fungal treated and untreated specimens. The key findings of this work are:

(i) Fungal treatment significantly increases the critical shear stress and reduces the erodibility coefficient following a growth period of 3 weeks, compared to untreated sands.

(ii) The growth duration has a significant effect on the resistance of fungal treated sands to erosion; a one week growth period in these studies was shown to be insufficient for improving soil resistance to erosion.

(iii) This study compared specimens inoculated in the following manner: fully treated, half-treated and surface-sprayed. Beyond growth duration of 3 weeks, there was no significant difference on the susceptibility of soil to erosion due to different treatment methods.

These findings are for sterile sand amended with lignocel as a single organic substrate with specimens incubated under controlled environmental conditions and with no other soil microorganisms competing with *P. ostreatus*. These findings form a reference point for further explorations into the potential applicability of fungi as a bio-treatment for improving soil susceptibility to erosion. Further studies are required to investigate the resulting behaviour following fungal inoculation in soils where a community of soil microorganisms exist. Future studies should transition from these initial sterile simple conditions to more complex conditions (ecological and soil type) relevant to field applications.

Chapter 8

Applications of Fungal Hyphal Networks in Ground Engineering

8.1 Introduction

This chapter outlines potential areas of applications for the deployment of fungal hyphal networks in ground engineering.

8.2 Potential applications for engineered fungal-hyphal networks

Based on the experimental results presented in this thesis, there are two potential main areas of application of fungal-based technology using the species *P. ostreatus:*

8.2.1 To create semi-permeable barriers

It has been suggested that hydrophobic soils are potentially suitable for use as infiltration barriers, with advantages over conventional soil surface sealing materials which include: i) water repellent soils remain gas permeable despite inhibiting infiltration, and ii) the extent of water repellency may be manipulated to produce impermeable or semi-permeable barriers (depending on the hydraulic head applied and the extent of treatment) (Lourenço *et al.*, 2018b). In this regards, several authors have investigated the potential use of chemically-hydrophobised soils (Myers and Frasier, 1969; Fink, Cooley and Frasier, 2007; Dell'Avanzi *et al.*, 2010; Lourenço, Wang and Kamai, 2015).

In this thesis, it has been shown that hyphal networks of *P. ostreatus* induces water repellency in sands. While chemically-hydrophobised soils are limited by antecedent moisture content and tend to lead to increased susceptibility to erosion (Lourenço, Wang and Kamai, 2015; Chan and Lourenço, 2016), this is not the case for fungal-hydrophobised soils. Water repellency induced by fungal treatment requires time to develop as it is dependent on in-situ hyphal growth. However, it was shown that a water repellent barrier was formed in sands with a moisture content up to 25%. Furthermore, infiltration rates into sand were considerably lowered by treatment with *P. ostreatus*, and saturated hydraulic conductivity was reduced by one order of magnitude (from 1.34×10^{-4} to 3.1×10^{-5} m s^{-1}) in specimens which had undergone 12 weeks of fungal growth. It is however envisaged that the saturated hydraulic conductivity could be further reduced beyond that demonstrated in this thesis by encouraging more mycelial/sclerotia growth to enhance pore filling. It should be noted that growth conditions were here selected on the basis of hyphal growth (density and extent of growth). Fungal growth conditions selected solely for biomass production could lead to a greater influence on hydraulic behaviour. For example this thesis only investigated nutrient supply in solid form; liquid supply of nutrients could perhaps facilitate faster growth (due to easier digestion) and more fungal biomass production. The influence of liquid supply of nutrients should also be investigated on induced water repellency.

8.2.2 To increase resistance to erosion

The experimental results presented in Chapter 7 highlight that fungal treatment of sand with *P. ostreatus* led to a significant reduction in the volume of sand eroded compared

to untreated control specimens. The erodibility characteristics were demonstrably altered in the fungal treated sand, resulting in a higher critical shear stress, i.e. the shear stress required to initiate erosion, and a lower erodibility coefficient. The growth period was a significant factor affecting erodibility as it was observed that 1-week growth duration did not have a positive effect on erodibility. Beyond 3 weeks of fungal growth period however, fungal treatment showed a 30 – 90% reduction in the volume of soil eroded compared to untreated specimens under the same test condition.

Fig. 8-1 presents a schematic illustration of specific applications in ground engineering, where the installation of infiltration barriers or enhanced resistance to erosion or a combination of both may be beneficial as discussed below.

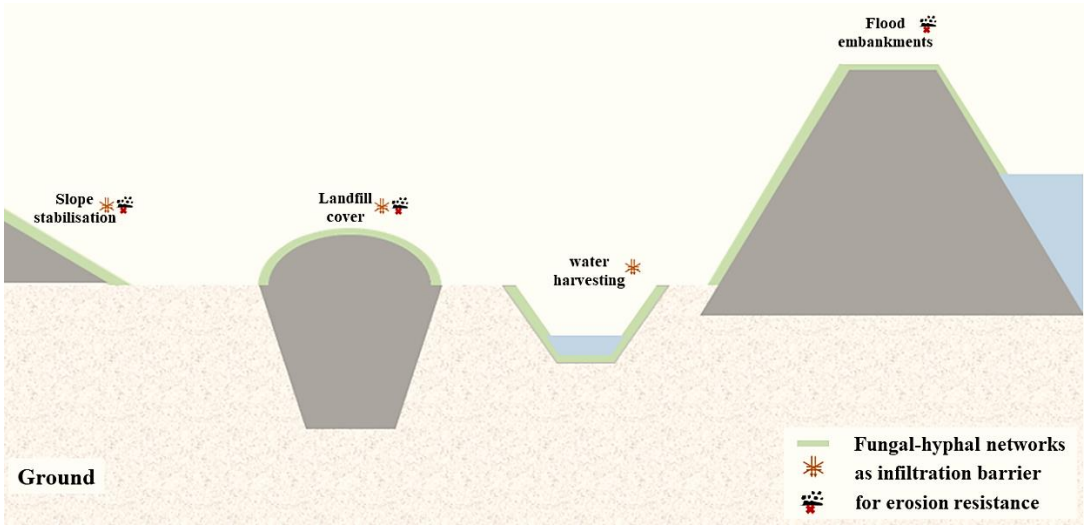


Figure 8-1: Proposed potential areas for application of engineered fungal networks on ground surfaces

a) Landfill covers

Conventionally, capillary barriers and evapotranspiration covers are deployed effectively as landfill covers in arid areas. However, Dwyer, (1998) and Dell'Avanzi *et al.*, (2010) observed that innovative solutions and alternative landfill covers, like the use of water repellent soils, are required for long-term prevention of infiltration in

locations exposed to higher rainfall events and requiring resilience to cyclic infiltration-evapotranspiration processes.

The water repellent effects of commercially available Hydrophobic Sand (HS) created using Trimethylhydroxisilane ($(CH_3)_3SiOH$) is estimated to last up to 30 years (Salem, Al-Zayadneh and Cheruth, 2010) and modifications have been suggested in the design of barriers formed with water repellent soils to include the installation of a vegetated soil layer above the water repellent soil layer in order to protect against erosion (Subedi *et al.*, 2013). Hydraulic tests conducted by Dell'Avanzi *et al.*, (2010) on poorly graded sand hydrophobised using Polytetraflourethylene (PTFE) showed decreased infiltration rates with increasing water repellency and hydraulic conductivity of water repellent soils were about three orders of magnitude lower than untreated controls (k_{sat} for untreated sand = 6.4 x 10^{-6} m s^{-1}, while k_{sat} for treated sand was 5.6 x 10^{-9} m s^{-1}). Overall, it was reported that water repellent sand performed better than conventional capillary barriers and were recommended for use as landfill cover systems in tropical climates.

Similar outcomes to Dell'Avanzi *et al.*, (2010) have been obtained in the hydraulic tests conducted for sands treated with *P. ostreatus*, although the k_{sat} obtained here was only one order of magnitude lower than untreated soil. Nonetheless, the research presented in this thesis is a first demonstration of the reduction of the hydraulic conductivity of sands using fungal treatment. It is envisaged that with further studies it may be possible to reduce further the saturated hydraulic conductivity and enhance the water repellency effects by altering growth conditions. The added advantages of fungal-hyphae in this context would include: 1) soil erodibility is also improved; 2)

continuous growth of fungal hyphae over time (provided sufficient nutrients exist) implying that the cover system should require little on-going maintenance.

b) Slope stabilisation

Prolonged rainfall duration and high intensity are common triggers for shallow landslides. The infiltration of water into the surface layers leads to a transition from an unsaturated state towards a saturated state with a corresponding a reduction in soil suction and thus shear strength (e.g. Kim *et al.*, 2004; Olivares and Picarelli, 2004; Springman, Jommi and Teysseire, 2004). Lourenço, Wang and Kamai, (2015) discuss the deployment of wettable soils to regulate water infiltration after intense rainfall, and improve the factor of safety of slopes susceptible to landslides. Water-repellent barriers (with associated lower infiltration rates) could be used to divert water into drainage gullies for example, to ensure that higher levels of soil suction are maintained, thereby reducing the risk of shallow landslides.

Fungal-hyphal networks in soil may be capable of creating infiltration barriers if effectively engineered or treated. Bio-stimulation of local species or bio-augmentation, i.e. the introduction of a non-native fungal species to landslide prone areas could be potentially used to initiate a natural process of obtaining an infiltration barrier that could maintain soil suction in upper soil layers such as to reduce the likelihood of rainfall-induced landslides. Fungal species for this application must have the ability to hydrophobise soils, like *P. ostreatus.*

c) Flood embankments

The experimental results presented in Chapter 7 clearly highlight that the treatment of sands with *P. ostreatus* can reduce its susceptibility to erosion. Although this has only been tested in sands and with a single fungus species to date, it is envisaged that with further study, fungal based technologies could be deployed to protect geotechnical earthen infrastructure against surface erosion due to flowing water, for example in flood embankments. A number of common failure mechanism of flood embankments are associated with erosion: erosion of the outward face and toe due to river current or wave impact, (which may culminate in shallow slippage), erosion of the landward face due to overtopping, erosion of slopes and crest due to heavy rainfall, as well as internal erosion (El Mountassir *et al.*, 2011).

Further study would be required to investigate the performance of fungal treated soil layers under fully submerged conditions, in order to assess feasibility for deployment on the outward face.

8.3 Limitations & recommendations for future work

In order to develop further the proof-of-concept for use of fungal-based technologies in the applications listed above, there are limitations in the research presented here and additional research questions that still need to be addressed. These are discussed below:

a) A single species, P. ostreatus, was used in this study.

There are many species of saprotrophic and non-parasitic soil fungi, safe for introduction into the environment, and probably capable of performing in a similar manner to the species used here. It would be worthwhile to explore other species as they may have useful traits that are superior to, complementary with or even very different from *P. ostreatus.* For instance, rhizomorphs (root-like mycelium) of certain fungal species could provide improvement for soil mechanical behaviour. Other species may support growth to greater depths (i.e. facultative anaerobes), or exhibit enhanced survival under extreme conditions. It may be worthwhile to combine different species to promote a range of different growth patterns simultaneously.

b) This study has been mainly conducted on sterile sands under laboratory controlled conditions

These idealised conditions have been used, as this study represents the first step towards understanding the influence of fungal-hyphal networks in soil. As knowledge is gained, it is recommended that further studies should consider gradual introduction of variables including more complex soil types (e.g. silt, clay, natural), different forms of organic matter (instead of the lignocel fibres used here). Although this study has been carried out in sterile sands, for field applications it would be necessary to investigate the survival of *P. ostreatus* when introduced as an alien species into an established ecological community. The long-term goal would be to carry out bio-stimulation of native fungal species to engineer desirable hydro-mechanical behaviour.

c) Inoculation methods

One key contribution of this study to knowledge is the method for preparation of spores/hyphal suspension for inoculation of the fungus into the soil. Going forward, it is recommended that other inoculation methods be explored, specifically targeted to field rather than laboratory deployment. For example, inoculation methods could be developed whereby a mix of spores and organic matter are sprayed on to the soil surface, or by broadcasting spores on a field in form of seeds, as used in agriculture, but ensuring minimal ground disturbance.

d) Growth duration

The alterations to soil behaviour presented in this thesis, all rely upon the growth of fungi in soils. Growth duration had a significant effect on the hyphal growth characteristics (lengths and biomass), which directly correlated with the influence it had on soil behaviour in this study. While growth duration was investigated for its influence on wettability and soil erodibility, due to time constraints, it was not possible to study the influence of growth duration on the soil water retention behaviour, infiltration and saturated hydraulic conductivity, nor the shearing behaviour. It is recommended that behaviour at different growth durations be further investigated.

e) Manage extent of fungal growth

The growth of fungal hyphae and mycelia in soil is often spatially explorative, typically in response to soil and environmental conditions. Where conditions are suitable, hyphal growth and biomass may increase across space and time, and in different forms, depending on characteristics of species. For deployment in soil

improvement works, it may be required that hyphal growth be restricted to a specific radius, or depth, or only within a particular soil layer. These restrictions may also be required in fulfilment of environmental regulations, especially for technologies involving the introduction of non-native species into the ground. It is therefore recommended that future work involve the development of safe, appropriate and environmentally-friendly method(s) for controlling or managing hyphal extent in the ground.

f) Micro-scale imaging and microstructural understanding of hyphae interaction in soil matrix is lacking in this current study

This is strongly recommended for future work as it has the potential of revealing the actual interactions between hyphal strands, exudates, soil mineral, organic matter and water or air. This would allow a better understanding of the underlying mechanisms contributing to altering hydraulic and mechanical behaviour which is critical for effective engineering of fungal-hyphal growth in soils.

g) Quantification of fungal biomass

Samples were taken at different depths from the infiltration columns (Chapter 5) and erosion specimens in (Chapter 7) for determination of ergosterol content and hence fungal biomass. However prolonged difficulties and delays with the HPLC (high performance liquid chromatography) equipment at the university of Strathclyde has meant that these results could not be obtained, hence they are not included in this thesis. Further research is recommended for the development of other less cumbersome methods of quantifying fungal biomass such as possible use of non-

invasive geophysical techniques for quantifying fungal growth pattern and biomass directly from specimens during laboratory tests.

h) Durability

In Chapter 5, the growth of mushrooms during the soil water retention tests was seen as an indication of continuous hyphal growth, which is assumed to contribute to improvement of fungal influence in the soil overtime. Further investigation is recommended to understand the durability of engineered fungal hyphae in the ground, in terms of the maintenance of desired properties/behaviour and the evolution of properties over time. Furthermore, it is imperative to understand if engineered fungal hyphae will be resilient enough to withstand the prevalent environmental cyclic actions, such as wetting-drying cycles governed by multivariate factors. For instance, how durable will engineered fungal technology be in saturated environments/conditions? It is recommended that future work should also involve the development of models to predict fungal growth and response as well as its implications on soil hydro-mechanical behaviour under various conditions and over extended periods of time.

Chapter 9

Conclusions

9.1 Introduction

As a first step in a larger systematic research campaign investigating the potential use of fungal treatment in ground engineering applications, this PhD study has focussed on gaining an in-depth understanding of the growth behaviour of *P. ostreatus* in sand and the influence of this growth on the hydro-mechanical behaviour of sand.

The specific objectives of this research were to:

1. Investigate the influence of environmental conditions (temperature, moisture content, type and organic substrate content) on growth and biomass produced by fungal mycelia (*P. ostreatus*) in soils,
2. Investigate the extent of alteration caused to the wettability (water repellency) of sand treated with *P. ostreatus*, and to investigate the persistence of these changes with time.
3. Determine the (i) soil water retention curve, (ii) infiltration behaviour and (iii) saturated hydraulic conductivity of sand treated with *P. ostreatus*.
4. Investigate the stress-strain behaviour of sand treated with *P. ostreatus* subjected to direct shear tests, and the influence of organic content.

5. Assess the erodibility characteristics of sand treated with *P. ostreatus*. Moreover, to investigate the influence of inoculation methods and growth duration on the erodibility of fungal treated sand.
6. Outline potential applications of deployment of fungal hyphal networks in ground improvement based on the growth results and hydro-mechanical behaviour evaluated for fungal-treated sand in this research programme.

In each section below, the key findings related to each of the objectives listed above are presented while the contributions to existing knowledge are briefly highlighted. This chapter concludes with a list of proposed areas for future research towards field application of this technology.

9.1.1 Environmental conditions for growth of hyphae of *P. ostreatus* in sands

A preliminary investigation was done to determine the suitable ranges of temperature (between 5 - 30°C), moisture content (from 3 – 40%) and substrate type/composition (lignocel, spent coffee grounds and guar gum ranging 1% -15%) for growth of hyphae of *P. ostreatus* in sand. The results from Chapter 3 showed that the hyphae of *P. ostreatus* can grow under extreme environmental conditions with limited resources. Average mycelium growth rates of 1.08 – 5.51 mm day^{-1} were recorded after 6 days growth period. Selected ranges of most suitable conditions were further investigated and analysed using the coupled Taguchi-Grey method to determine optimal conditions for hyphal growth.

For the parameters investigated in this study, the optimal conditions for growth of hyphae of *P. ostreatus* over a short duration (6 days) in sands were found to be 25°C,

5.3% moisture content and 3% guar gum as organic substrate. Whereas for longer growth periods, up to 24 days, the optimal conditions found were: 20°C, 11.1% moisture content and 1% spent coffee grounds.

This is the first study to investigate the environmental conditions for growth of the hyphae of *P. ostreatus* in soil for the purpose of ground improvement. Moreover, it is the first study to apply the coupled Taguchi-Grey method for optimisation of the selected growth parameters for growth of fungal hyphae in soil.

9.1.2 Influence of fungal-hyphae on the wettability of sand

The extent of water repellency (WR) induced by growth of *P. ostreatus* was investigated and quantified for sands and natural soils after growth durations of 1 week (short term) and up to 12 weeks (with no further supply of nutrients). The effects of initial soil water contents and the disruption of fungal growth on WR were also investigated.

Short-term growth of *P. ostreatus* in sterile sand and natural soils resulted in extreme hydrophobicity (CA >110°, WDPT > 86400s). This WR persisted in sterile sands for up to 12 weeks growth though with a slight reduction in CA from 109° (extreme WR) at 8th week, to 105° (severe WR) in the 12th week recorded. It was also found that the disruption of fungal growth or mycelia hydrophobic barrier in soils does not imply total loss of WR as *P. ostreatus* was capable of re-establishing growth and WR within 24 hours after physical interference.

Although the influence of hyphal growth on the wettability of soil have been investigated in previous studies, this thesis presents the first step in the investigation

of WR induced by fungal treatment of soils which potentially seeks to provide a more environmentally friendly option for harnessing the advantages of water repellent soils in ground engineering. Furthermore, this study established for the first time through a systematic experimental investigation, that fungal induced WR was not limited by initial soil water content (up to 40%) and a water repellent barrier can be created even on saturated sands. This is in contrast to what can be obtained using chemical hydrophobising agents like stearic acid and DCMS as reported in the literature. With the potential of continuous fungal growth where/when suitable conditions prevail, it is suggested that fungal induced WR has the potential of providing a safer and environmentally friendly option for deployment in geotechnical engineering applications compared to chemically induced soil WR. Further studies are required to investigate the resilience and durability of fungal-induced WR.

9.1.3 Influence of fungal-hyphae on hydraulic behaviour of sand

The growth of the hyphae of *P. ostreatus* increased the air entry value of sand, improving the water retention behaviour of sand, such that at an applied suction, the treated specimen retained more water than their untreated counterpart. Also, during infiltration, the fungal treated sand exhibited significant delay (up to 5 days' difference) in advancement of the wetting front with diffuse flow through tortuous paths and ultimately, slower infiltration rate compared to untreated sand. While the untreated specimen exhibited volumetric collapse upon infiltration due to the initial low dry density, the treated specimen exhibited no collapse and remained stable throughout infiltration and saturation. It is suggested that the presence of hyphae, their enmeshment of particles and possible exudates contributed to binding of particles and

were responsible for keeping the soil stable during infiltration. The saturated hydraulic conductivity (k_{sat}) for the fungal treated soil was found to be an order of magnitude lower than k_{sat} for the untreated soil, for specimens tested after growth duration of 12 weeks.

Prior to this study, the understanding of the influence of the growth of the hyphae of saprotrophic fungal species on the hydraulic behaviour of soils had been largely investigated for silty/loamy/clayey soils, where fungal growth typically resulted in enhancing soil aggregation, macroporisty and thus increased hydraulic conductivity. This is the first study to have investigated the influence of fungal growth on the hydraulic behaviour of sands and to demonstrate a reduction in hydraulic conductivity.

9.1.4 Influence of fungal-hyphae on the shear behaviour of sand

This thesis reported, for the first time, an investigation into the stress-strain behaviour of fungal treated soils tested under direct shear conditions. It is evident from the experiments conducted that fungal treatment resulted in a loss in peak shear strength and an associated reduction in dilative behaviour. The results obtained showed similarities in behaviour to both hydrophobic sands and artificially reinforced sands reported in the literature. Being the first attempt to characterise fungal-treated specimens several lessons were drawn from the observations made and results obtained, with a number of modifications suggested and recommendations for further studies. In particular specimens for future work should be installed in the shear box and subjected to a vertical load prior to inoculation, with this being maintained throughout the growth period.

9.1.5 Influence of fungal hyphae on the erodibility of sands

One of the main contributions of this PhD research is the investigation of the erodibility characteristics of sands treated with a spore/hyphal suspension of the filamentous and saprotrophic basidiomycetes, *P. ostreatus* assessed using a laboratory JET test apparatus constructed at the University of Strathclyde Geomechanics Laboratory. It was found that fungal treatment significantly increased the critical shear stress and reduced the erodibility coefficient following a growth period of 3 weeks, compared to untreated sands. A one week growth period in these studies was shown to be insufficient for improving soil resistance to erosion. Furthermore, based on the available data in this study there was no significant difference on the susceptibility of soil to erosion beyond a growth duration of 3 weeks and/or due to different treatment methods (fully treated, half-treated or surface-sprayed).

These findings form a reference point for further explorations into the potential applicability of fungi as a bio-treatment for improving soil susceptibility to erosion. This study also provided a slight modification to the conventional data collection process in the JET test procedure. A laser scanner was used for determination of the evolution of the volume of soil eroded and the scour depths for respective specimens. This provided useful additional information to better understand the pattern of erosion exhibited by fungal treated sand and for comparison with the untreated sand.

9.2 Proposed future research areas

This thesis has opened up a novel area of research within the biogeotechnical community. The findings of this study provide a starting point for future work on the

engineering of fungal networks for ground improvement. As such, the research presented here raises many questions which require in-depth investigation and exploration. The following is a list of suggested topics, based on lessons learnt from this research, for extension of this work in the future:

1. Investigation of the microstructure of fungal treated soil to determine the specific mechanisms responsible for the hydro-mechanical behaviour found in this study. Micromodels with suitable imaging techniques could be used to provide better understanding of the interactions and alterations in the soil matrix as a result of hyphal growth.
2. Survey and investigation of other non-pathogenic, non-parasitic fungal species suitable for use in ground improvement.
3. Investigation of the influence of the growth of fungal hyphae on the hydromechanical behaviour of different soil types, including natural soils.
4. Development of predictive models to understand the spatial evolution of hyphal growth in soils with time, for different environmental conditions, soil types and fungal species.
5. A life-cycle assessment of the use of fungal hyphae for soil improvement.
6. Development of industrially suitable and cost effective hyphal growth protocols and inoculation procedures suitable for varied soil treatment scenarios.
7. Investigation of appropriate techniques for engineering the spatial growth of fungal hyphae for effectively harnessing required soil modifications over time.
8. Field trials and deployment of fungal hyphal techniques.

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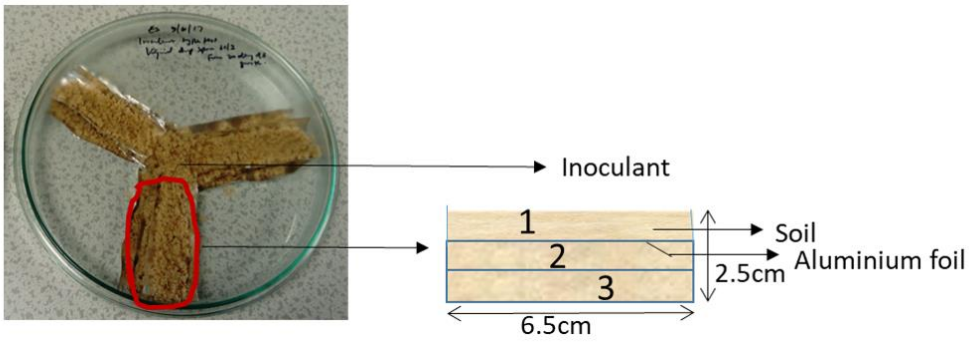
Appendices

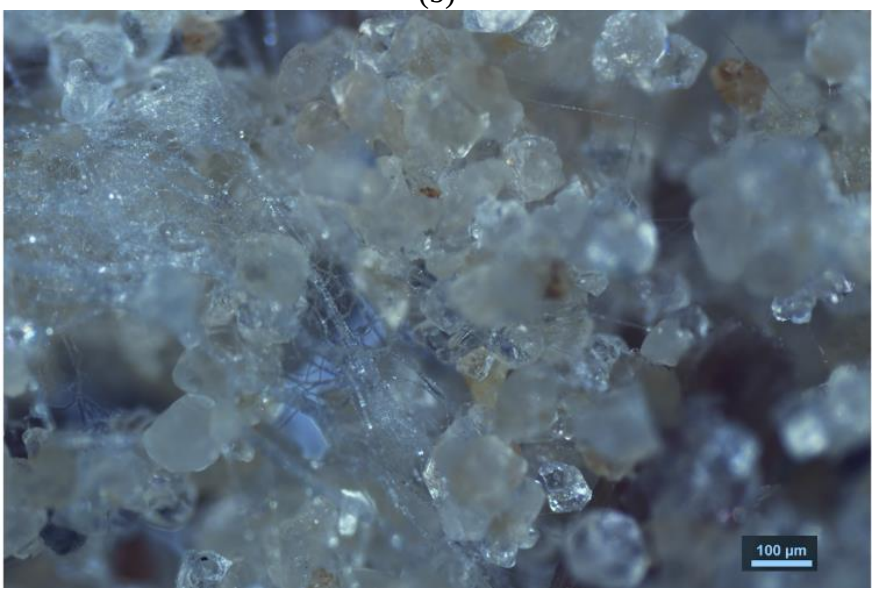
Appendix A – Development of suitable inoculant and inoculation method

In order to initiate hyphal growth in soil, it is expedient that a suitable type of spores-bearing inoculant as well as method for inoculation of spores be developed. Based on methods used in related literature, four different option of spores-bearing inocula were identified: (i) hyphae-colonised agar blocks (AB) of 5 mm diameter removed from actively growing edges in a petri dish, using sterile cork borers (Kim, Xiao and Rogers, 2005) (ii) hyphae-colonised cube of beech wood (BW) 1 mm^2 (Donnelly and Boddy, 1998) (iii) hyphae-colonised soft wood chips (WC) (Rühl, Fischer and Kües, 2008) and (iv) spores/hyphal suspension (SHS) extracted directly from hyphal culture grown on agar plates using a simpler modification to method by Montgomery *et al.*, (2000). A simple experiment was set-up to determine the growth rates, extent and biomass density of hyphae in sands inoculated using these options (Fig. xx). Visual appearance and microscopic imaging were adopted to describe hyphal growth characteristics.



(a)

(b)



(c)

(d)

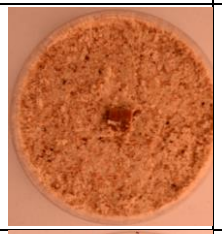
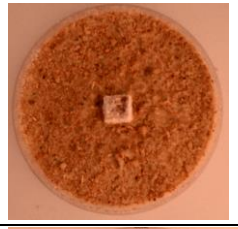
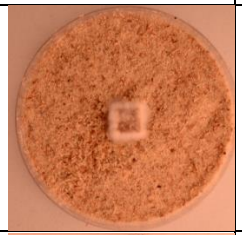
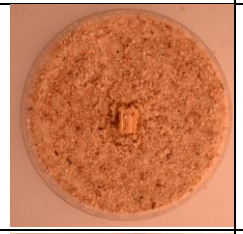
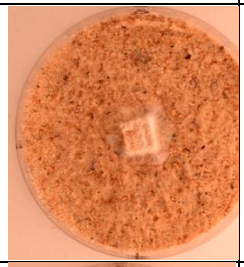
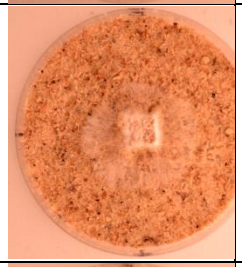
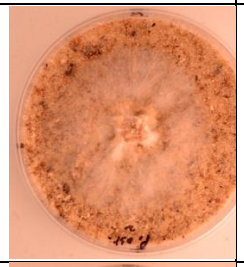
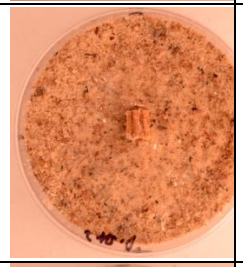
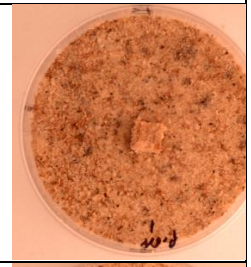
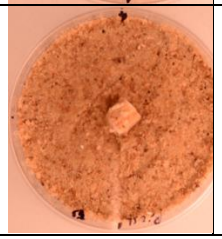
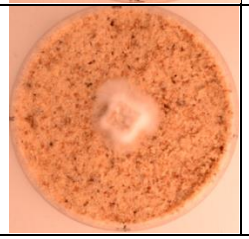
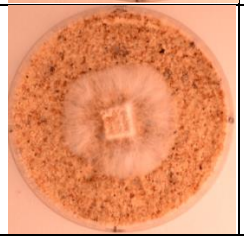
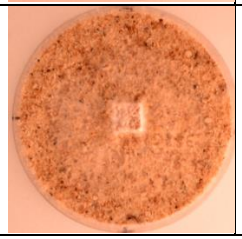
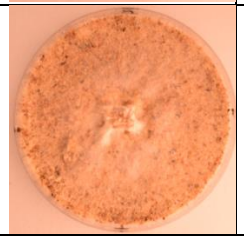
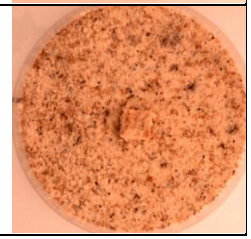
Fig. 2: (a) set-up of specimen to test growth rate of respective inoculation method. Sand was prepared with organic matter and set in layers (1 – 3) separated using aluminium foil; each unit was made in triplicates put together in form of separable 'Y-shaped' units. The inoculant was placed in the middle and the specimen was incubated to grow in the dark at 25°C. Each specimen was removed from the incubator after every 3 days' growth period (b) and one part of the three 'Y-shaped' units is removed; samples are taken across length and depths and viewed under the microscopic (c) to see the extent and density of hyphal growth. This observation was repeated on the 6th day (d) and 9th day after growth.

The level of performance of each type of inoculum was assessed, based on growth density and hyphal lengths per time, and it was found to be in the order: BW > SHS > AB > WC. Denser growths were recorded around the BW and AB inocula, but the farthest growth at comparatively lower hyphal density was seen in the specimen inoculated with SHS. BW which is 'hard-wood' was probably difficult for hyphae of *P. ostreatus* to penetrate, compared to the ease of growing into the soil and utilising the surrounding organic matter, while WC (soft wood) and AB were immediately available rich nutrients sources for the hyphae and therefore there was the 'reluctance' for hyphae to extend into surrounding soil or organic matter, hence the slower hyphal extension but relatively denser growth. The drops of SHS was the only inoculant in liquid form tested. Though hyphal growth in this specimen was not as visually dense as BW and AB, its strands were visible under the microscope at the ends of the foil, which is about 60 mm away from the inoculation point (droplets) on the 3rd day after inoculation, making it the specimen with the farthest hyphal length in a short time, compared to the others.

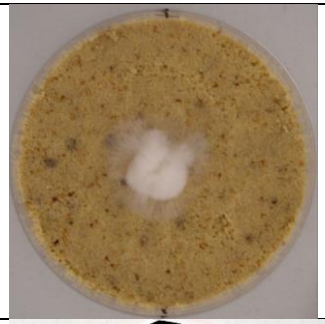
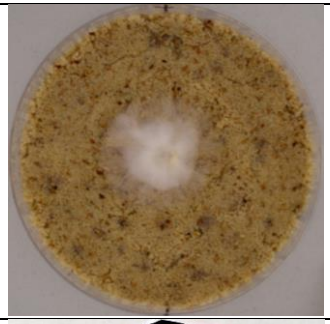
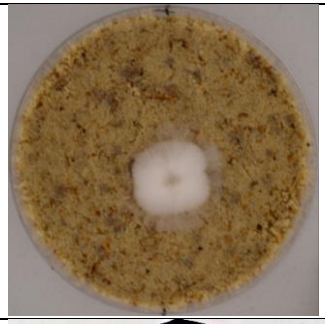
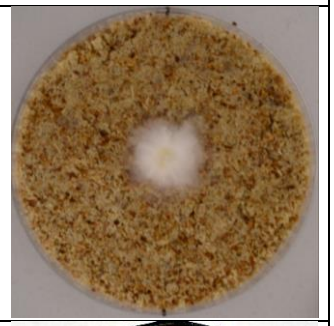
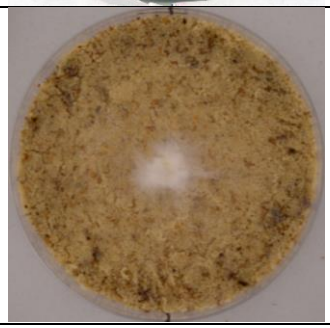
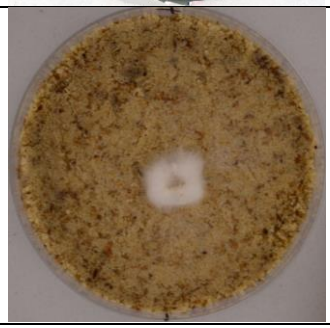
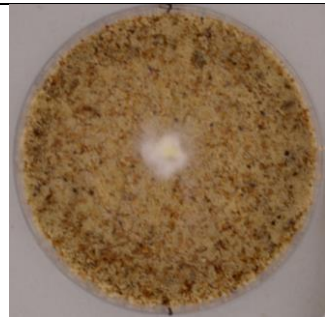
Based on the findings of this test, the BW and SHS inoculants were adopted for subsequent experiments. BW was used in the experiments conducted to optimise growth conditions for hyphae of *P. ostreatus* (Chapter 3), while a modified form of the SHS was used in all other experiments in this study (Chapters 4-7).

Appendix B – Images of mycelia growth extent captured during the preliminary tests

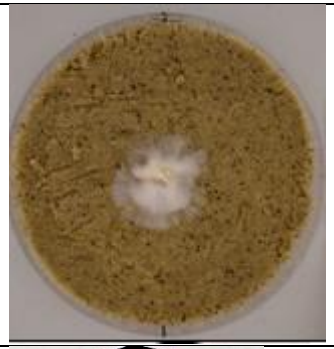
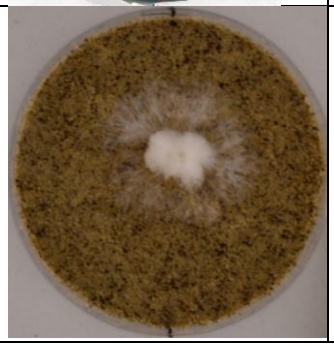
Temperature effect

	5˚C	10 ˚C	15 ˚C	20 ˚C	25 ˚C	30 ˚C	35 ˚C
Day 3							
Day 9							
Day 12							

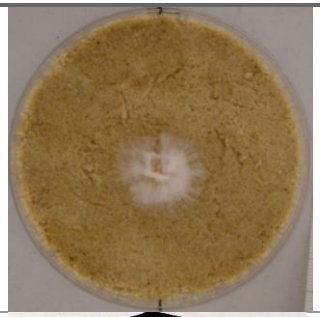
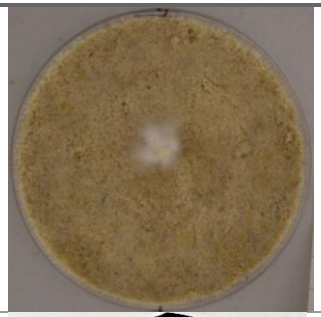
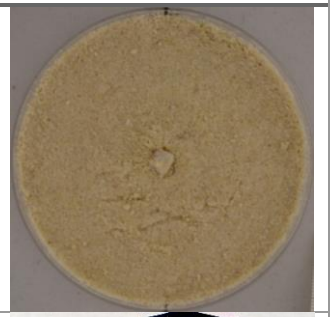
Effect of nutrients on mycelial growth: Lignocel

	1%	3%	5%	10%	15%
3rd day					
					
6th day					

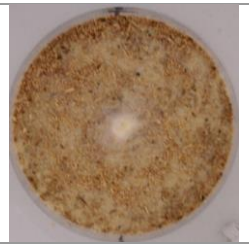
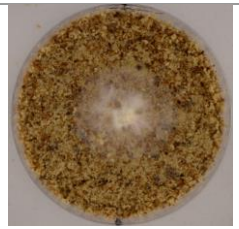
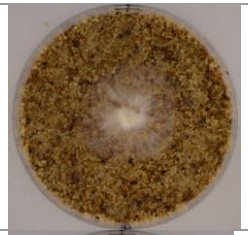
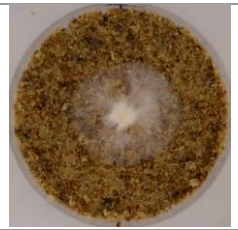
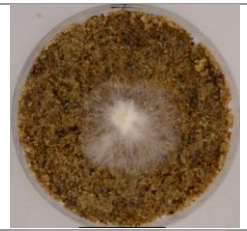
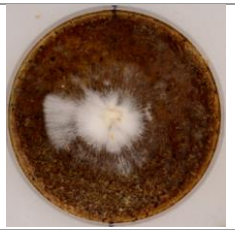
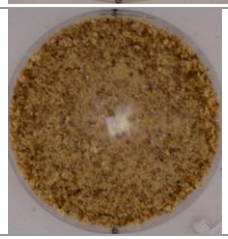
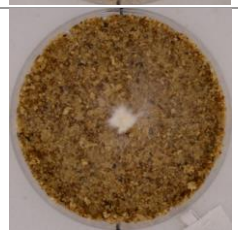
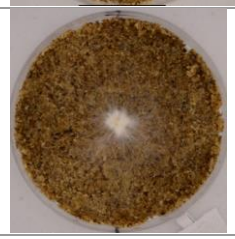
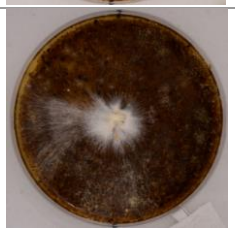
Effect of nutrients on mycelial growth: Spent Ground Coffee

	1%	3%	5%	10%	15%
3rd day					
					
6th day					

Effect of nutrients on mycelial growth: Guar gum

	1%	3%	5%	10%	15%
3rd day					
3rd day - Flip side (bottom of plate)					

Effect of Moisture content on mycelial growth

Day/Moisture content	0%	1%	3.1%	5.3%	11.1%
4th day					
12th day					
Cont'd	**17.7%**	**25.0%**	**42.9%**	**33.3%**	**100%**
4th day					
12th day					

Appendix C – ANOVA results for Taguchi dynamic analyses for optimal dry fungal biomass overtime showing factors and interactions between factors

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Regression	20	13.0930	0.65465	10.91	0.004
Incubation period (days)	2	0.3165	0.15827	2.64	0.151
Temperature	2	0.1487	0.07433	1.24	0.354
Moisture content	2	0.2059	0.10295	1.72	0.257
Organic substrate	2	0.6152	0.30762	5.13	0.050
Incubation period (days)*Temperature	4	0.4522	0.11305	1.88	0.233
Incubation period (days)*Moisture content	4	0.1383	0.03457	0.58	0.691
Incubation period (days)*Organic substrate	4	1.3866	0.34665	5.78	0.030
Error	6	0.3600	0.06000		
Total	26	13.4529			

Appendix D – Response tables for S/N ratios and Means, showing ranking of factors based on delta statistics in the Taguchi analysis of combined mycelium radius and fungal dry biomass versus Temperature, Moisture content and Organic matter

Response Table for Signal to Noise Ratios

Larger is better

Level	Temperature	Moisture content	Organic substrate
1	-8.173	-5.092	-8.443
2	-6.510	-6.963	-7.081
3	-5.203	-7.831	-4.363
Delta	2.970	2.739	4.080
Rank	2	3	1

Response Table for Means

Level	Temperature	Moisture content	Organic substrate
1	0.3910	0.6129	0.3786
2	0.4777	0.4548	0.4433
3	0.6053	0.4063	0.6521
Delta	0.2143	0.2065	0.2736
Rank	2	3	1

Appendix E – ANOVA results for GRG

Source	DF	Adj SS	Adj MS	F-Value	F-tab (0.35,2,2)	Contribution (%)	Significance
Temperature	2	0.0697	0.0349	1.19	1.86	21.73	No
Moisture content	2	0.0699	0.0345	1.2		21.82	No
Organic substrate	2	0.1226	0.0613	2.1		38.23	Yes
Error	2	0.0585	0.0292			18.22	
Total	8	0.3208					

**Significance at 65% confidence level (F-value > F-tab)*

Appendix F – Supplier's info sheet for Lignocel

Data sheet

Grade
HB 500-1000

LIGNOCEL

Natural wood fibers

Basic raw material

Selected leaf-wood

Chemical and physical properties

Colour		light brown
Structure		cubic
Average particle size / particle range		0.5 mm - 1mm
Oxide ash (850 °C, 4 h)	~	1 %
pH-value (10 % suspension)		5 - 7
Bulk density (in accordance with DIN EN ISO 60)		205 g/l - 305 g/l

Screen analysis (with RETSCH vibrating sieve) with an interior mesh aperture of:

> 1.25 mm	> 0.63 mm	> 0.5 mm
max. 2 %	max. 65 %	max. 90 %

General remarks

LIGNOCEL wood fibers are environment friendly products, gained from replenishable raw materials. Among other things, they are used as thickeners, for fiber reinforcement, as an absorbent and diluent or as a carrier and filler in most manifold application fields.

As with all natural products slight differences to the above given values may arise.

Release date: 2013-11 / 1 page

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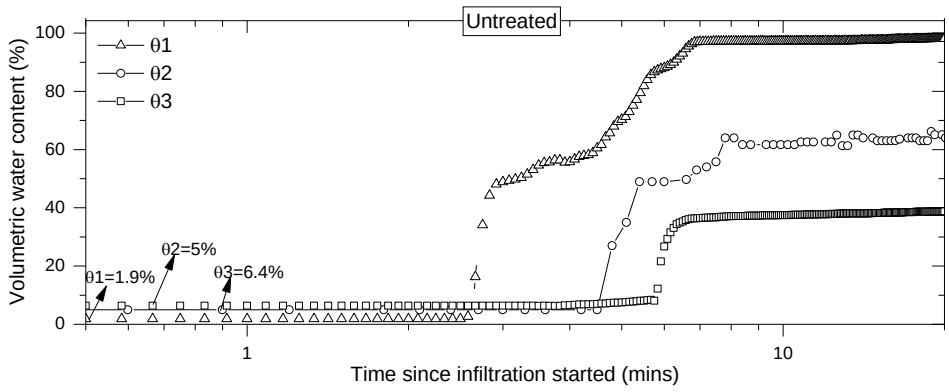
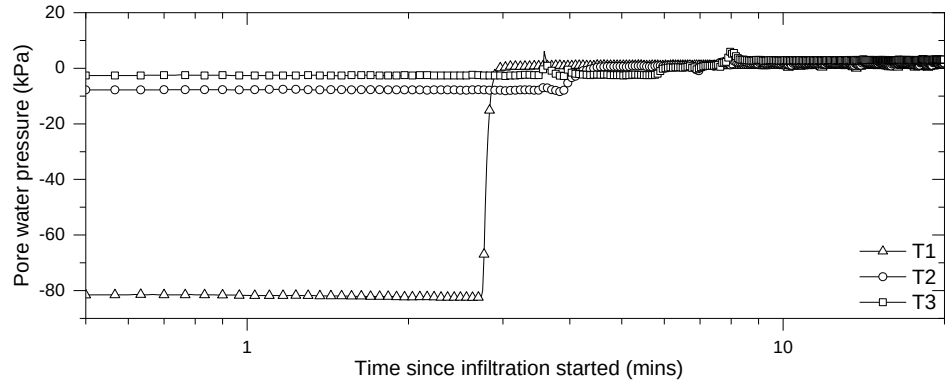
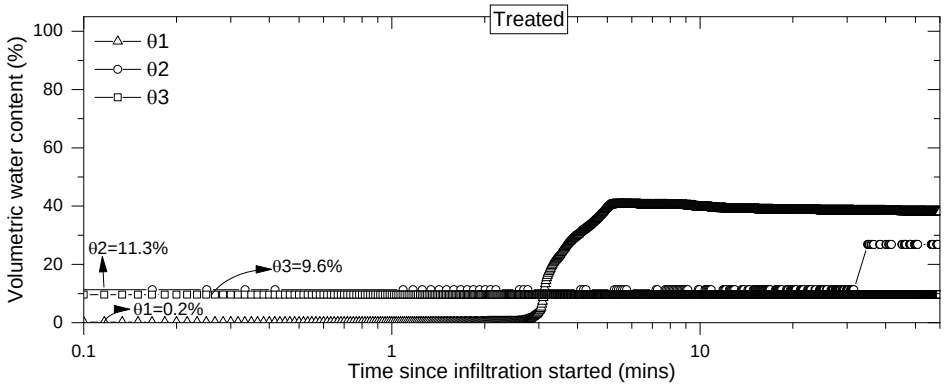
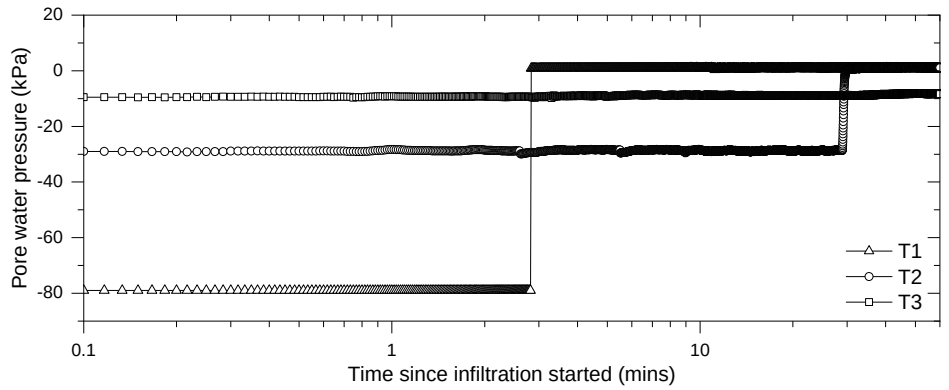
Telefax: +49 7967 152-222

Appendix G – Evolution of pore water pressures and volumetric water contents for untreated and treated specimens during the infiltration tests









Appendix H – ANOVA table for maximum scour rates in untreated specimens: U1, U2, U3

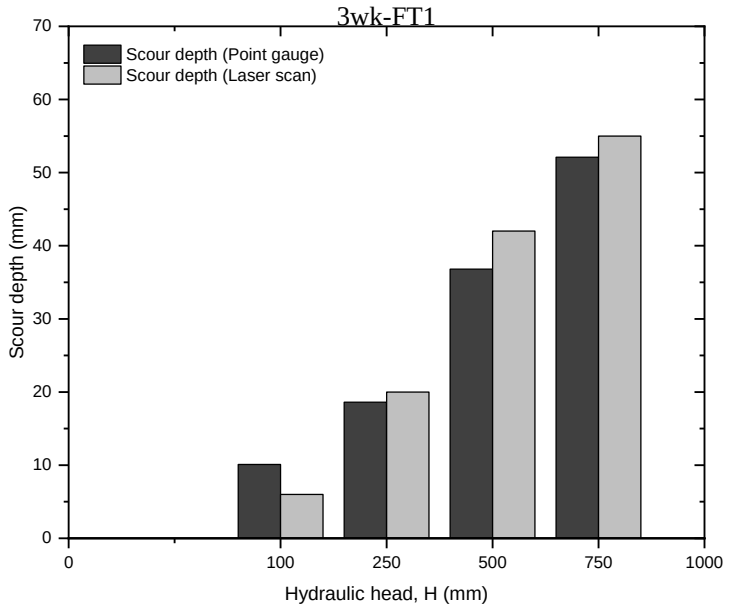
Source	DF	Adj SS	Adj MS	F-Value	P-Value
Factor	2	0.002407	0.001204	0.03	0.973
Error	6	0.258148	0.043025		
Total	8	0.260556			

Appendix I – ANOVA table for maximum scour depths in treated specimens: 3wk-FT1, 3wk-FT2, 3wk-FT3

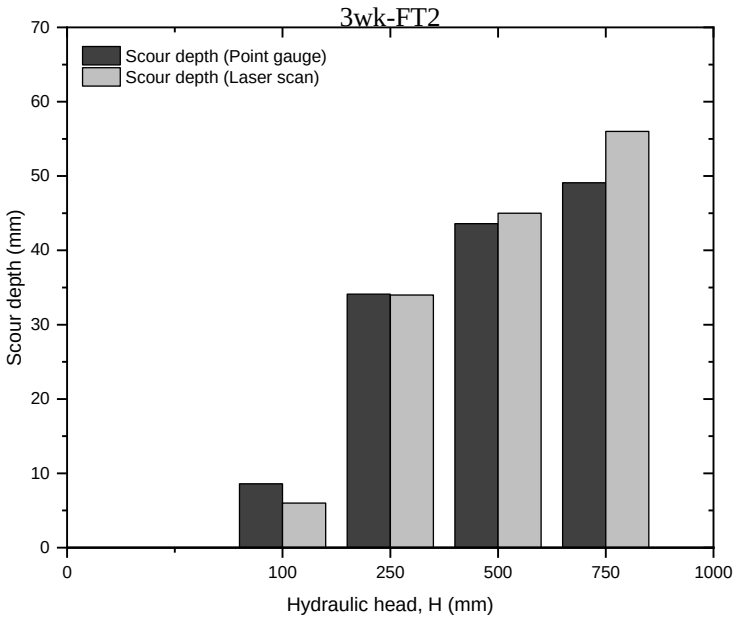
Source	DF	Adj SS	Adj MS	F-Value	P-Value
Factor	2	0.02604	0.01302	0.58	0.575
Error	12	0.26989	0.02249		
Total	14	0.29593			

Appendix J - Comparison of scour depths determined using point gauge and estimated from laser scans

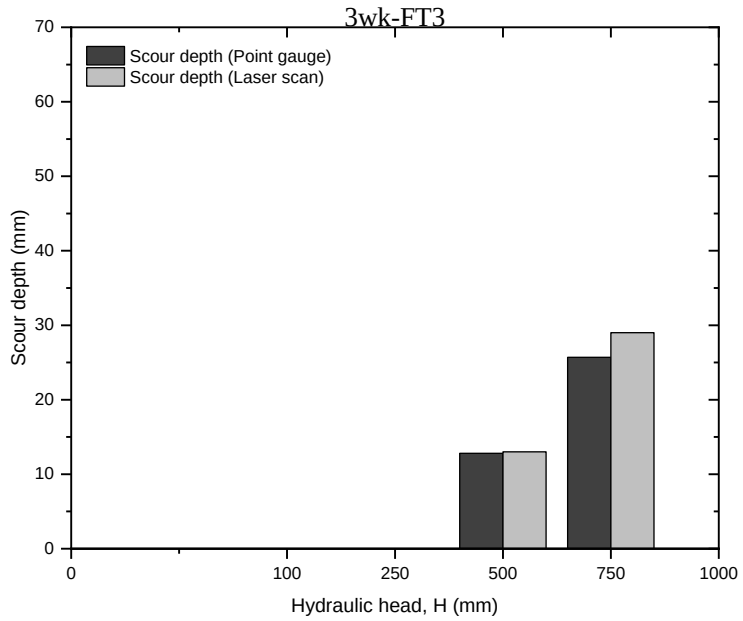
3wk-FT1



3wk-FT2



3wk-FT3


3wk-FT3
Scour depth (Point gauge)
Scour depth (Laser scan)
Scour depth (mm)
Hydraulic head, H (mm)
0
10
20
30
40
50
60
70
0
100
250
500
750
1000

Appendix K - ANOVA table for maximum scour depths of treated specimens from 3-9 weeks (3wk-FT1, 3wk-HT, 3wk-ST, 6wk-FT, 6wk-HT, 6wk_ST, 9wk-FT, 9wk-HT, 9wk-ST)

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Factor	8	3245	405.6	0.59	0.777
Error	36	24616	683.8		
Total	44	27860			

ANOVA table for % Volume eroded of treated specimens from 3-9 weeks (3wk-FT1, 3wk-HT, 3wk-ST, 6wk-FT, 6wk-HT, 6wk_ST, 9wk-FT, 9wk-HT, 9wk-ST)

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Factor	8	40.85	5.106	0.88	0.544
Error	26	150.52	5.789		
Total	34	191.36			