

Developing Fungal Induced Calcium Carbonate Precipitation for Geotechnical Applications



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Abstract

Within a geotechnical engineering context, erosion can be problematic and impact on geotechnical and civil infrastructure. Erosion processes can trigger slope instabilities and soil mobilised as a result of slope erosion and slope failures can accumulate on neighbouring infrastructure, typically roads and railway tracks leading to major disruption with social and economic impacts.

Prior research has demonstrated that treatment of granular soils with saprotrophic fungus can improve soil resistance to erosion due to the extensive filamentous network, mycelium, which forms as the fungus grows. This thesis extends fungal treatment from not only growth, but also to precipitation of carbonate minerals and thus, focuses on developing a fungal-induced carbonate precipitation (FICP) process which could be applied to reduce soil erosion.

While recent studies have indicated that FICP can strengthen sands, these have relied upon the use of Ascomycetes, prolific spore formers. This thesis explores an alternative approach via the use of Basidiomycetes to trigger FICP. Five Basidiomycetes (*Sistotrema brinkmannii*, *Bjerkandera adusta*, *Pleurotus cornucopiae*, *Pleurotus ostreatus*, and *Pleurotus pulmonarius*) were screened for their growth behaviour and ureolytic activity. Batch experiments were conducted to understand the influence of cementing solution concentration and ratio, biomass and pH on carbonate precipitation. Finally, FICP treatment was applied to sand columns and varying treatment strategies were investigated.

Of the five fungal species investigated *Pleurotus cornucopiae* was found to be the most ureolytically active. Batch experiment results show that higher biomasses result in higher carbonate precipitation at elevated cementing solution concentrations. For FICP, here a ratio of 1:4 (calcium chloride to urea) was found to be optimal for carbonate precipitation. Treatment of sand columns indicated that increasing the number of treatment cycles, resulted in progressive increases in carbonate content, achieving carbonate contents up to 2.22%.

This research has developed the proof-of-concept that *Pleurotus cornucopiae* and other Basidiomycetes could be used to trigger FICP for ground engineering applications via a two-step: growth and treat strategy.

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Chapter 1: Introduction

1.1 Soil Erosion

Soil Erosion is the process by which soil particles become detached and transported as a result of action by water, wind or ice. Within a geotechnical engineering context, erosion can be problematic and impact on geotechnical and civil infrastructure. Erosion processes can trigger larger slope instabilities. For example, gully erosion promotes preferential flow, contributing to erosion at the toe of the slope, eventually leading to undercutting and slope failure (Chang et al., 2021). Soil mass mobilised as a result of slope erosion and/or slope failures can accumulate on neighbouring infrastructure, typically roads and railway tracks leading to major disruption with direct and indirect social and economic impacts (Stirling et al., 2021). Erosion or scouring can also lead to the removal of supporting soil around bridge abutments and piers. Soil detached during erosion processes can enter local waterways and become deposited in streams and rivers, resulting in pollution of water systems as well as reduce the channel capacity of waterways due to sedimentation, therefore increasing flooding risk (Issaka & Ashraf, 2017).

1.2 Conventional Erosion Control Methods

Traditional methods of erosion mitigation in geotechnical engineering typically come in three categories: (i) physical intervention including for example compaction, reprofiling of slopes (e.g. terracing), installation of drainage channels/layers, (ii) mechanical intervention including for example installation of geotextiles, erosion control mats, or protective facing (e.g. stainless steel mesh) and (iii) chemical intervention via the addition of lime, cementitious or chemical grouts. A combination of these measures is often employed at a single site. However, each of these methods can have various drawbacks, including the high carbon footprint of traditional construction materials (e.g. cement), and negative impacts on the environment (e.g. high pH of cementitious grouts). In particular, these measures are typically used for localised intervention at a particular point/section, and are not feasible for application over large areas, or over long stretches of linear infrastructure e.g. flood embankments (Das et al., 2018).

1.3 Nature-based methods for erosion control

To overcome some of the drawbacks associated with traditional construction materials and processes listed above, there is an increasing shift towards adopting more sustainable, lower carbon materials and construction processes. As a result, there has been increasing interest in the potential role of natural biological processes and products within geotechnical engineering (Assadi-Langroudi et al., 2022; Capobianco et al., 2025; El Mountassir et al., 2018). Some biologically induced soil modifications have been proposed in the literature for enhancing erosion resistance. These are briefly discussed below.

1.3.1 Biopolymers

A novel alternative to chemical additives or grout in unstable soil is the addition of biopolymer products such as xanthan gum, chitosan, and alginate which have been found to increase mechanical strength, improve resistance to erosion, and reduce permeability in treated soils (Chang et al., 2015, 2016; Kannan et al., 2023; Khatami & O'Kelly, 2013; Kwon et al., 2023; Maghchiche et al., 2010; Soldo et al., 2020). These materials are also considered less hazardous to the environment than traditional chemical additives as they induce less environmental toxicity and they are less likely to induce a pH change in treated soils.

1.3.2 Vegetation

The presence of plants on a slope has major effects on both the mechanical and hydrological properties of the soil due to their root systems. Roots can bestow improved mechanical properties upon a soil due to their high tensile strength and distribution in the soil (Nilaweera & Nutalaya, 1999). This results in increased shear strength (Kamchoom, Boldrin, et al., 2022; Kamchoom, Leung, et al., 2022; Mickovski et al., 2009). Additionally, the action of evapotranspiration removes moisture from the soil and increases soil suction (Garg et al., 2015; Indraratna et al., 2006; Leung, 2016; Leung et al., 2015; Leung & Ng, 2013; Ng et al., 2018). Together, these two mechanisms provided by plants cause vegetated soils to resist erosion more

effectively than unvegetated soils (Angers & Caron Jean, 1998; Boldrin et al., 2021; Fraccica, 2019; Ghestem et al., 2011; Greenwood et al., 2004; Ni et al., 2018).

Soil stabilisation by plants can occur both naturally and artificially. Artificial planting on slopes for soil stabilisation often focuses on the planting of trees with some planting of smaller species later in the process. However, natural recovery of plant communities on a slope often begins with herbaceous plants, followed by shrubs and then finally trees (Cammeraat et al., 2005; Osman & Barakbah, 2011). Chen et al. (2023) found that artificial planting had greater benefits in the short term and similar benefits in the long-term when compared to natural recovery. In either method, greater biodiversity of plant life on the slope, in addition to the presence of trees, has been shown to increase soil aggregate stability (Genet et al., 2010; Pohl et al., 2009, 2012). Note, soil aggregate stability is an indicator of the susceptibility of a soil to erosion, with lower aggregate stability linked to higher susceptibility to erosion susceptibility (Le Bissonnais, 2016).

1.3.3 Fungi

An often overlooked participant in the soil microbial community are fungi, which form filamentous network within the soil. Research has shown that the inoculation of soils with saprotrophic fungi increases soil aggregate stability (Tisdall et al., 2012) and reduces soil erosion (El Mountassir et al., 2020; Salifu, 2018; Zhang et al., 2023) in laboratory-scale experiments. Vogelsang (2010) also showed that inoculation of soil slopes with mycorrhizal fungi contributed to reduced soil erosion in field experiments. Fungi in nature are also known to contribute to biomineralisation processes in soil (Gadd, 2017). This thesis explores fungal biomineralisation as an approach to offer enhanced durability and resistance to soil erosion than that offered by saprotrophic fungal growth alone.

1.4 Aims and Objectives

The overall aim of this research is to develop proof-of-concept for fungal-induced carbonate precipitation process using a saprotrophic fungal species.

The specific objectives of this research were to:

1. Identify a saprotrophic, urease active, fast-growing fungal species suitable for inducing carbonate precipitation for use in ground engineering applications.
2. Develop methods for determining calcium carbonate content of FICP treated soils and determine the optimum growth period for fungal biomass in columns of sand and beechwood.
3. Characterise and optimise the chemistry of the fungal-induced carbonate precipitation process by investigating the influence of fungal biomass, and cementing solution chemistry (total concentration, Ca:Urea ratio and initial pH).
4. Investigate the influence of treatment strategy variables (treatment duration, number of treatment cycles, growth amendments and soil medium composition) on fungal-induced carbonate precipitation in sand.

1.5 Outline of Thesis

This thesis consists of eight chapters. Chapter 2 presents a review of the literature on fungal influence on soil behaviour, and fungal induced biomineralisation processes for civil and environmental applications. Chapter 3 presents a study targeted at identifying a suitable saprotrophic fungal strain for biomineralisation of calcium carbonate. Chapter 4 presents the development of new methodologies for assessing fungal growth, determining calcium carbonate in fungal soils, and measuring fungal biomass in soil. Chapter 5 presents a study investigating factors influencing the fungal biomineralisation reaction. Chapter 6 presents a study investigating the influence of treatment strategy on calcium carbonate precipitation in soil columns. Chapter 7 outlines how the findings of this research might be applied to improving the erosion resistance of soil. Chapter 8 concludes the main findings of this research and areas for potential future research.

Chapter 2: A Review of Literature on Fungal Induced Calcium Carbonate Precipitation

2.1 Fungal classification, growth and role in nature

2.1.1 Fungal classification

Fungi are chemoheterotrophic eukaryotes. Eukaryotes are a class of life which includes animals, plants, and fungi, in particular chemoheterotrophs consume extracellular organic compounds for energy. A eukaryote typically differs from the other two classes of life (bacteria and archaea) in that they contain membrane-bound organelles such as the mitochondria or chloroplasts. Fungi form their own kingdom separate from animals and plants because they typically have cell walls (which animal cells lack) and the cell walls are primarily composed of chitin (instead of cellulose like plants). Estimates indicate that there are ~ 2.5 million fungal species on Earth, with only 155,000 fungal species yet formally identified (Niskanen et al., 2025). Of those identified, the majority (> 97 %) are contained within the Ascomycota and Basidiomycota phyla (Hibbett et al., 2025). Ascomycetes or “sac fungi” are fungi with the defining feature of producing microscopic spore forming structures called “ascus”. Different species within Ascomycota can be asexual or sexual reproducers but are generally rapid and uncontrollable spore producers (Bennet & Turgeon, 2016; Pöggeler et al., 2006). This phylum is incredibly diverse, covering single-celled yeasts, to *Penicillium* spp., to cup fungi such as *Morchella* sp.. Within the Basidiomycota phyla, the defining characteristic is that the organisms form basidia, reproductive structures which produce spores. Many (but not all) species in this phylum produce fruiting bodies commonly known as mushrooms, the production of which is triggered by environmental stimulation (Coelho et al., 2017; Kues & Liu, 2000; Pelkmans et al., 2016).

Fungi can be further classified in terms of their mode of nutrition: (i) Saprotrophic, (ii) Parasitic and (iii) Symbiotic. Saprotrophic fungi obtain nutrients from decaying or dead organic matter such as leaf litter, soil, and wood. They do this by secreting enzymes into their surrounding environment which can break down complex organic matter, into simpler organic molecules that can be absorbed through their cell walls. Examples from the Basidiomycota phylum and Agaricomycota class

include the common button mushroom and oyster mushrooms (e.g. *Agaricus bisporus*, *Pleurotus ostreatus*). On the other hand, fungi can be parasitic and derive their nutrients from living hosts, often causing disease. An example of this includes *Armillaria* sp., which forms root-like cords (rhizomorphs), and lives on trees and woody plants, with one 1500 year old organism (genet) in Michigan estimated to have spread over an area > 15 hectares with an estimated biomass over 10 tonnes, making it perhaps one of the largest and oldest single organisms in the world (Smith et al., 1992). The third main mode of nutrition is symbiotic, where the fungus forms a mutually beneficial relationship with other organisms. It is well documented that plants and fungi can exist in symbiotic relationships. Fungi that evolved to capitalise on this symbiosis are called mycorrhizal fungi. Mycorrhizal fungi can colonise between root cells (ectomycorrhizal fungi) or penetrate into plant root cells (arbuscular mycorrhizal fungi, AMF). The fungal hyphae act to extend the plant root system, increasing the effective surface area of nutrient and water uptake for the plant, and excrete enzymes to mobilise nutrients which results in a healthier, larger plant (Ortas, 2010; Wilson & Hartnett, 1997). In exchange, the plants provide carbohydrates to the fungi. This strategy is not limited to a specific family of fungus, and likely convergently evolved multiple times (Tedersoo & Smith, 2013). Indeed, AMF belong to the phylum Glomeromycota, whereas Ectomycorrhizal fungi predominantly belong to the Ascomycota and Basidiomycota phyla (Tedersoo & Smith, 2013).

2.1.2 Fungal growth

While fungi can be both unicellular (e.g. single celled yeasts) and multi-cellular, this thesis focuses on multi-cellular filamentous fungi, which grow through the formation of hyphae. Hyphae are multi-cellular tube-like structures, consisting mainly of chitin (a nitrogen containing polysaccharide). Filamentous hyphae grow radially from a central nutrient source and can split at the hyphal tip into multiple hyphae as they grow (Boswell et al., 2007; Gervais et al., 1999; Harris, 2008). The hyphae can also anastomose, i.e. fuse back together, creating a complex three-dimensional interconnected network, known as the mycelium. Fungal hyphae can detect nutrients, grow from the tip to bridge air gaps, and even tunnel through substrates such as soil and stone to reach nutrients (Fricker et al., 2008). The resulting

mycelium network can then be used to transport nutrients and water within the organism (Heaton et al., 2010). In some species, the mycelium can extend for large distances (Smith et al., 1992).

2.1.3 Fungal Stabilisation of Soils in Nature

Biological soil crusts are well known in the Soil Science discipline to withstand erosion due to water or wind action (Eldridge & Greene, 1994). Fungi alongside bacteria, algae and other small organisms can contribute to these biological crusts with fungal hyphae contributing to the binding of soil particles and aggregates (Tisdall et al., 2012). Researchers in soil science and mycology have identified potential biophysical, biochemical, and biological mechanisms by which fungal hyphae can contribute to the stabilisation of soil aggregates (Rillig & Mummey, 2006). The biophysical process is similar to the action of plant roots (although at a smaller scale) where the fungal hyphae act to enmesh and entangle soil particles. Fungal hyphae can secrete biochemical products into their surroundings which have been previously associated with soil aggregate stability e.g. glomalin produced by AMF (Wright et al., 1998). Fungi can also influence existing microbial populations in the soil, which may also influence hydraulic and/or mechanical behaviour.

2.2 Engineering Fungi for Soil Stabilisation

2.2.1 Mycorrhizal fungal inoculation

It has long been recognised the potential beneficial effects of vegetation on soil stability (Section 1.3). However, in steep slopes susceptible to erosion, often hostile conditions persist which can inhibit plant colonisation (Graf et al., 2019). To support plant colonisation and growth, previous studies have investigated the influence of mycorrhizal fungi on plant growth and the subsequent effect on soil stability. Past literature has shown that inoculation of plants with mycorrhizal fungal inoculants results in increased plant growth, both in terms of above and below ground biomass (i.e. shoot biomass and root biomass respectively) (Graf et al., 2019; Graf & Frei, 2013). Mycorrhizal inoculation has also been shown to result in increased soil aggregate stability, due to the increased root length density of finer roots and fibres

and also due to biochemicals produced which can contribute to enhanced soil binding (Graf et al., 2018; Graf & Frei, 2013; Kimura & Scotti, 2016). The physical enmeshment of the soil by an increasingly complex and dense 3D network has also been hypothesised to increase shear strength of the soil (Fan et al., 2022). Enhanced plant growth above ground also contributes to increased rate of evapotranspiration and increased root biomass can contribute to lowering soil saturation (Roberts-Self & Tarantino, 2023), both of which can be beneficial for slopes where failures are triggered by the infiltration of rainwater and the transition towards a high degree of soil saturation. Vogelsang (2010) demonstrated that mycorrhizal inoculation of plots along a road embankment in a 3 year field trial resulted in reduced soil erosion, with the native mycorrhizal inoculum outperforming a commercial inoculum.

2.2.2 Non-mycorrhizal fungal inoculant

Given that non-mycorrhizal fungi also have similar binding properties, El Mountassir et al. (2018) proposed that saprotrophic fungi could be harnessed for ground engineering applications, within the context of biogeotechnics. Recent studies in biogeotechnics have explored the influence of the growth of non-mycorrhizal species (Ascomycetes and Basidiomycetes), on soil behaviour.

2.2.3 Soil hydraulic behaviour

Salifu & El Mountassir (2021) grew *Pleurotus ostreatus* (Division: Basidiomycota, Subdivision: Agaricomycota) in sand, before conducting water droplet tests and contact angle tests. They found that the growth of *P. ostreatus* induced water repellency in the sand (changing the sand from being hydrophilic to extremely hydrophobic). This was observed for up to 12 weeks after inoculation. They also found that water repellency was observed even when fungal growth occurred in initially saturated sands and, when the hyphal network was disrupted by mixing, water repellency was regained within 24 hours.

The induced water repellency can be explained by the fact that *P. ostreatus*, (as is the case for many other Basidiomycetes) secretes hydrophobins. Hydrophobins are amphiphilic proteins with a hydrophobic and hydrophilic part of the molecule. They can assemble into a film of hydrophobins at the water-air interface, which alters the

properties of that interface. Likewise, they can coat solid surfaces. When hydrophobins collect at an interface or surface, they can self-assemble at that surface. On a solid interface, the film self assembles and reverses the water repellency of a surface e.g. a hydrophilic surface becomes hydrophobic. It has been found that fungal hyphae use hydrophobins to lower water surface tension to allow them to breach the water-air interface and thus grow aerial structures such as aerial hyphae, and form fruiting bodies (Wösten et al., 1999). Smits et al. (2003) explored the contact angles of water droplets placed onto mycelium mats grown on filter paper with a water exposed side and an air exposed side. They found that aerial growing hyphae were hydrophobic, whilst submerged growing hyphae could sometimes be hydrophilic instead. By placing droplets at increasing distance from the central inoculant point on an agar plate, the effect of age of the hyphae on hydrophobicity was also investigated. It was found that older hyphae at the centre of the plate were more hydrophobic than younger hyphae at the edge. Chau et al. (2009) found that different fungal species had various degrees of hydrophobicity, with some inducing increasing hydrophobicity with age.

Salifu et al. (2022) investigated the infiltration behaviour of sand treated with *P. ostreatus* in a constant head infiltration test. They found that granular sand treated with *P. ostreatus* for 12 weeks exhibited reduced and delayed water infiltration and a lower saturated hydraulic conductivity than the corresponding untreated control. They also demonstrated that fungal growth resulted in a modified soil water retention curve. These effects are due to the induced water repellency making it more difficult for water to pass through the soil, as well as partial infilling of the pore space by the fungal biomass. Similar results in terms of lowering of saturated hydraulic conductivity and changes to the soil water retention curve have also been reported by Park et al. (2023) for sand treated with *Trichoderma virens*.

2.2.4 Mechanical Strength Improvement

Lim et al. (2020) and Lim et al. (2023) investigated the unconfined compressive strength (UCS) of *Pleurotus ostreatus* and *Rhizopus oligosporus* treated sand. Both studies investigated the effect of increasing mass of fungal inoculum on mechanical strength improvement. Inoculums were purchased from the commercial market, with the *R. oligosporus* inoculum containing inoculated rice flour in both

studies, and the *P. ostreatus* inoculum containing inoculated dry corn kernels. The optimum unconfined compressive strength for sand treated with *R. oligosporus* was achieved with ~ 4 - 5 % inoculum with values of UCS of 63 kPa – 100 kPa after only 4 days incubation (Lim et al., 2020; 2023), whereas *P. ostreatus* treated sand required higher inoculum dosage (16.7 %) and longer time (14 days compared to 4 days) to achieve similar UCS values (73 kPa).

Lim et al. (2020) found that while the optimum UCS achieved by *P. ostreatus* was lower and took longer to achieve (14 days compared to the 4 days by *R. oligosporus*), the UCS values were sustained for a longer duration with *P. ostreatus* than *R. oligosporus*. They suggested the reason for the greater UCS values achieved in *R. oligosporus* was that *R. oligosporus* hyphae were thicker than *P. ostreatus* hyphae. They also suggested that the reason for the greater retention of improved UCS by *P. ostreatus* was because it created a denser mycelium mesh rather than a sparser corded web as was formed with *R. oligosporus*. The optimum growth time was determined to be 14 days for *P. ostreatus* and 4 days for *R. oligosporus*, after which unconfined compressive strength of the sand column decreased. This study attributed this decline to the gradual death or inactivity of the organisms. They stated that the *R. oligosporus* could survive for 10 days whereas *P. ostreatus* could survive for 28 days.

2.2.5 Soil Erodibility

Salifu (2018) investigated the effects of treating sand mixed with lignocellulose (10 %) with *Pleurotus ostreatus* on soil erosion behaviour using a jet erosion test (JET) apparatus developed at Strathclyde. In the JET test, specimens were fully submerged and subjected to a submerged vertical jet of water impinging onto the initially horizontal surface of the soil specimen. The scour depth (i.e. the eroded depth directly under the jet) and the volume eroded (determined via laser scanning) was then plotted over time against increasing hydraulic head (i.e. jets of increasing energy). They showed that sands treated with *Pleurotus ostreatus* exhibited delayed initiation of erosion, overall smaller scour depths and lower eroded volume than control sand specimens (with no fungal inoculation). By interpreting the scour evolution against depth, it was shown that the fungal growth increased the critical shear stress required to initiate soil erosion and lowered the soil erodibility coefficient which governs the

rate of erosion. Similarly, Zhang et al. (2023) found that the critical flow velocity (the flow velocity at which erosion began) was greatly increased for sands treated with *Pleurotus ostreatus*. El Mountassir et al (2021) also showed that treatment with *Trichoderma harzianum* also considerably reduced soil erosion. In each of these papers, these results were attributed to the physical enmeshment of the sand particles by the fungal hyphae. Zhang et al. (2023) also carried out computational fluid dynamics modelling to show that the induced hydrophobicity also contributes to enhanced erosion resistance. In their specimens, they also introduced calcium chloride in the inoculation medium and correspondingly showed that after fungal treatment there was evidence of crystal precipitates along the hyphae imaged via Scanning Electron Microscopy. This observation raises the possibility that fungal induced mineral precipitation could be engineered to enhance erosion resistance in fungal-treated soils.

2.3 Fungal biomineralisation in nature

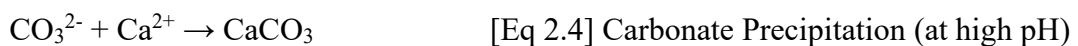
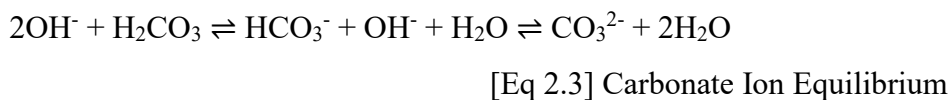
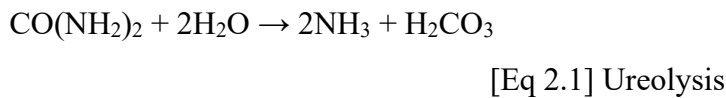
As discussed, saprotrophic fungi degrade complex organic material to feed upon the smaller simpler organic compounds. Thus, they play a key role in the carbon and nitrogen cycles, as most other organisms struggle to metabolise such complex compounds. To do this, fungi can induce high pressures at the hyphal tips and secrete specialised extracellular enzymes to burrow into or attach to plant/organic material and release useful elements and compounds contained within (Abdulla, 2009; Mendes et al., 2021; Money et al., 2004; Wild et al., 2021). These attributes also allow some fungi to grow and thrive in some of the harshest conditions, such as the surfaces of rocks. This action of attaching to and degrading rock to release vital elements is referred to as bioweathering (Burford et al., 2003). This process can also mobilise heavier metal elements which can then react with other compounds released during bioweathering of rocks and subsequently result in bioprecipitation of by-product minerals. This is known as biologically induced mineralisation and is not directly controlled by the organism, rather it is an unintended consequence of bioweathering of rocks. This is one of three categories of biomineralisation, the others being biologically controlled mineralisation (for example, the production of a mollusc's shell) and biologically influenced mineralisation (passive and spontaneous

precipitation of minerals on nucleation sites on the surface of a cell). Common examples of biominerals are oxalates, oxides, phosphates, silicates and carbonates, which can react with elements newly mobilised by fungal bioweathering (Gadd, 2017).

2.3.1 Microbially-induced carbonate precipitation

A biomineralisation reaction pathway that has been extensively studied for bacteria is that of microbially-induced carbonate precipitation (MICP) via ureolytic bacteria. This bacterially-induced process has received extensive attention due to the short times required for precipitation and the large masses of calcium that can be precipitated (van Paassen et al., 2010). MICP has been studied for a wide range of applications over the last 20 years including soil stabilisation, erosion mitigation, rock fracture grouting, enhanced oil recovery, CO₂ sequestration, concrete and stone repair as well as co-precipitation of heavy metals (Dejong JT et al., 2013; El Mountassir et al., 2018; Phillips et al., 2013).

The MICP reaction utilises the bacterially produced urease enzyme with a solution of urea and a calcium source (typically calcium chloride) to precipitate calcium carbonate onto surfaces within the treatment site. This reaction pathway is as follows (Ferris et al., 1996; Mitchell & Grant Ferris, 2006; Stocks-Fischer et al., 1999):



Ureolysis via the urease enzyme produces ammonia and carbonic acid [Eq 2.1]. Ammonia dissolves in water forming an equilibrium between the NH₃ unionised form and NH₄⁺ and the production of hydroxide ions raises the pH of the solution [Eq 2.2].

Carbonate ions also exist in equilibrium with carbonic acid, bicarbonate ions, and hydroxide ions [Eq 2.3]. At high pH (i.e. at a high concentration of hydroxide ions), the equilibrium pushes to the right. Carbonic acid is reduced to bicarbonate and then reduced to carbonate ions to attempt to balance the equilibrium. In the presence of calcium, these carbonate ions react to form calcium carbonate [Eq 2.4]. The precipitation of calcium carbonate is controlled by the saturation state, which is pH dependant. Increasing pH leads to saturation or even supersaturation of calcium carbonate in the aqueous solution, causing precipitation to become a favourable process. The high pH environment induced by hydroxide ions produced in [Eq 2.2] therefore enables precipitation of calcium carbonate crystals, usually nucleating onto surfaces in contact with the solution. The final reaction of carbonate and calcium [Eq 2.4] helps drive the reaction forward by keeping [Eq 2.2] and [Eq 2.3] unbalanced. The consumption of carbonate ions by calcium [Eq 2.4], induces the favourable consumption of hydroxide ions to produce more carbonate ions and [Eq 2.3] shifts to the right, which in turn induces the favourable reaction of ammonia into ammonium to produce more hydroxide ions and [Eq 2.2] shifts to the right. This maintains the high pH of the solution and keeps calcium carbonate precipitation favourable.

2.3.2 Fungal induced carbonate precipitation

The following section highlights studies in which the pathway outlined in [Eq 2.1] to [Eq 2.4] was induced by urease produced by fungi, (rather than bacterial urease as has been more commonly studied). Google Scholar was used to search for these studies using phrases such as “fungal biomineralisation calcite” with variations including “fungus”, “biomineralization”, and “calcium carbonate”. A limited number of studies have explored fungal induced carbonate precipitation for applications, including heavy metal precipitation, self-healing concrete, concrete protection, and soil stabilisation. Studies in each of these applications are explored in more detail here.

2.3.2.1 Heavy Metal Remediation

Fungal precipitation of calcium carbonates has the potential to be used as a treatment technology for heavy metal remediation not only because fungi can uptake heavy metals during growth (Hassan et al., 2020; Joshi et al., 2011; Talukdar et al.,

2020), but also because the heavy metals can co-precipitate out of solution and become immobilised within calcium carbonate. Studies have demonstrated heavy metal co-precipitation both for experiments carried out with heavy metal contaminated solutions but also for heavy metal contaminated soil.

Table 2.1 shows a summary of literature on fungal bioremediation of heavy metals. In this and subsequent tables in this thesis, fungal species from the Ascomycota family are labelled [A] and Mucoromycota as [M]. The displayed studies have demonstrated the potential to remove strontium (up to 68.72 % removal by J. Zhao, Csetenyi, et al. (2022)), chromium (up to 65.2 % removal by Qian et al. (2017)), lead (up to 98.8 % removal by Qian et al. (2017)) and cadmium (up to 51.2 % removal by Li et al. (2014)). The table also displays the proportion of calcium carbonate to have precipitated during the experiment (up to 93.2 % precipitated by Li et al. (2014)). These studies differ in terms of the medium within which the FICP was carried out. Most studies investigated removal of heavy metals from aqueous solutions (Dhami, Quirin, et al., 2017; Li et al., 2014, 2015; J. Zhao, Csetenyi, et al., 2022), and only one study was found to have investigated FICP for remediating contaminated soil (Qian et al., 2017).

Table 2.1 Heavy Metal Remediation Using Fungal Biomining

Reference	Media Type	Species	Heavy Metal	Treatment Duration (days)	Solution Concentrations (mM)	% Ca Precipitated	% Heavy metal removed
Dhami et al. (2017)	Aqueous	<i>Aspergillus sp.</i> [A]	Lead Strontium	7	100ml, 200 Urea + [1] 100 CaCl ₂ ,	[1] 78	[2] 34 [3] 48
		<i>F. oxysporum</i> [A]			[2] 50 CaCl ₂ + 50 PbNO ₃ , or [3] 50 CaCl ₂ + 50 SrCl ₂	[1] 67	[2] 54 [3] 31
Li et al. (2014)	Aqueous	<i>N. Crassa</i> [A]	Cadmium	12	[1] 330 Urea, 50 CaCl ₂ or [2] 200 ml 40 urea + 0.2 CaCl ₂ , then add 50 ml 500 Cd at end.	[1] 93.2	[2] 51.2
Li et al. (2015)	Aqueous	<i>Pestalotiopsis sp.</i> [A]	Strontium	14	40 Urea + [1] 50 CaCl ₂ ,	[1] 35.0 [3] 31.4	[2] 36.3 [3] 10.8
		<i>M. gramineum</i> [A]		23	[2] 50 SrCl ₂ , or [3] 25 CaCl ₂ + 25 SrCl ₂	[1] 44.2 [3] 56.0	[2] 48.9 [3] 28.0
Qian et al. (2017)	Aqueous	<i>P. chrysogenum</i> [A]	Chromium (IV) Lead	12	330 Urea, 40 CaCl ₂ + [1] 50 mg/L Cr, [2] 100 mg/L Cr, [3] 100 mg/L Pb, or [4] 200 mg/L Pb	Not Stated	[1] 65.2 [2] 39.4 [3] 98.7 [4] 98.8
	Soil				330 Urea, 40 CaCl ₂ + [1] 100 mg/kg Cr, or [2] 200 mg/kg Pb	Not Stated	Carbonate Bound: [1] ~35 [2] ~40
Zhao, Csetenyi, et al. (2022)	Aqueous	<i>P. herbarum</i> [A]	Strontium	30	40 Urea + [1] 50 CaCl ₂ ,	[1] 62.49 [3] 44.20	[2] 45.09 [3] 16.07
		<i>M. hiemalis</i> [M]			[2] 50 SrCl ₂ , or [3] 25 CaCl ₂ + 25 SrCl ₂	[1] 48.88 [3] 28.02	[2] 37.98 [3] 3.40
		<i>F. cerealis</i> [A]			[1] 53.90 [3] 49.80	[2] 60.72 [3] 37.61	

Qian et al. (2017) investigated calcium carbonate precipitation using *Penicillium chrysogenum* in farmland soil to bioremediate chromate and lead. To do this they took 4 kg farmland soil in columns, added fungal pellets with Modified Martin media amended with 2 % urea, 40 mM calcium chloride, and 100 ppm chromium or 200 ppm lead. Additional Modified Martin media amended with 2 % urea and 40 mM calcium chloride was injected every 6 days, with the experiment lasting 30 days. Soil was sampled from different depth levels for analysis of fractionation at different depths. Of relevance to this literature review, using their method, they found that the exchangeable fraction of each heavy metal was reduced during treatment. At the top of the chromium contaminated soil, there was a reduction from 41.6 % to 1.95 % in the exchangeable chromium fraction, and an increase from 10.86 % to 38.68 % in the carbonate bound chromium fraction when compared to a control which had not been treated with solution to induce precipitation. A similar result was achieved at each measured depth. At the top of the lead contaminated soil, there was a reduction from 41.27 % to 2.19 % in the exchangeable lead fraction, and an increase from 4.71 % to 28.77 % in the carbonate bound lead fraction when compared to a control which had not been treated with fungus, urea, and calcium chloride to induce precipitation. A similar result was achieved at each measured depth. Since the other studies discussed in this section only investigated treatment of aqueous solutions, this was the only study which could measure the fractionation of heavy metals in the soil. In this study, the change in exchangeable heavy metal fraction was found to be due to carbonate binding. Most of the remaining change was due to increased binding with organic matter during precipitation. Although they successfully demonstrated fungal induced calcium carbonate precipitation in soil, the study did not measure the quantity of calcium carbonate precipitated.

2.3.2.2 Self-healing Concrete

There has been interest in using fungal biomineralizing properties to create self-healing concrete. Table 2.2 displays a brief summary of research into fungal-based self-healing concrete. The Ascomycota family are labelled [A], Mucoromycota as [M], and Basidiomycota as [B]. Similar to bacterial-based self-healing concrete, the concept is that fungal hyphae could be encapsulated within the concrete mix and, when

microfractures occur, the fungus would become exposed to oxygen and the resulting aerobic respiration produces carbon dioxide, leading to production of carbonate ions via aerobic respiration rather than ureolysis. The cement is high in calcium, so when the concrete becomes wet, the resulting high pH runoff solution contains carbonate ions and calcium ions from the concrete which follow [Eq 2.4] thus producing calcium carbonate precipitate which could potentially repair the fracture (Menon et al., 2019).

This strategy presents a significant challenge in that the fungal strain encapsulated must be resistant to, and even thrive in, the high pH and extreme environment within and on the surface of concrete. Screening papers have identified fungi which innately possess this ability (Luo et al., 2018), but also investigated genetically altered fungi to resist these conditions (Menon et al., 2019).

Table 2.2 Brief Summary of Papers on Fungal Self-Healing Concrete

Reference	Species	Treated Media	Outcome
Khushnood et al. (2022)	<i>Rhizopus oryzae</i> [M] <i>Trichoderma longibrachiatum</i> [A]	Ordinary Portland Cement Columns	Successful fracture repair up to 1.3mm
Khan et al. (2023)	<i>Fusarium oxysporum</i> [A] <i>Trichoderma longibrachiatum</i> [A]	Agar plates and cement	Successful fracture repair up to 1.34mm
Luo et al. (2018)	<i>Trichoderma reesei</i> [A] <i>Aspergillus nidulans</i> [A] <i>Cadophora interclivum</i> [A] <i>Umbelopsis dimorpha</i> [M] <i>Acidomelania panicicola</i> [A] <i>Pseudophialophora magnispora</i> [A]	Plates with growth media and cement	T. reesei optimal species for precipitation
Menon et al. (2019)	<i>Aspergillus nidulans</i> [A] <i>Aspergillus terreus</i> [A] <i>Rhizopus oryzae</i> [M] <i>Phanerochaete chrysosporium</i> [B] <i>Saccharomyces cerevisiae</i> [A]	Plates with growth media and cement	A. nidulans optimal species for precipitation
Van Wylick et al. (2023)	<i>Trichoderma reesei</i> [A]	Cementing Solution in Flasks	Trichoderma reesei struggles to survive in cement paste, even after encapsulation with alginate.
Van Wylick et al. (2024)	<i>Trichoderma reesei</i> [A]	Cementing Solution in Flasks	Optimal calcium source for treatment with Trichoderma reesei is calcium lactate

From these papers investigating fungal self-healing concrete, it appears that identification of extremophilic fungal strains, and methods to encapsulate fungi within cement are high priority to ensure the effectiveness and longevity of self-healing concrete. Both Menon et al. (2019) and Luo et al. (2018) conducted screening for high pH tolerant strains of fungi. The former study identified *Trichoderma reesei* and the latter identified *Aspergillus nidulans* as the most optimal strains of those that they investigated. Menon et al. (2019) used a gene replacement procedure to attempt to enhance resistance to the high alkalinity environment. Both studies achieved calcium carbonate precipitation using the identified strains.

Khushnood et al. (2022) and Khan et al. (2023) both showed fracture healing could be achieved by fungal self-healing concrete for fractures up to 1.3 and 1.34 mm respectively. Self-healing was evaluated in both papers via ultrasonic pulse velocity (density test), compressive strength test and crack width analysis using optical microscopy. Each study followed an initial curing stage of 7 or 28 days with an initial round of the tests mentioned, then a second “curing” stage of 28 days, and a second round of testing.

Khushnood et al. (2022) found that the fungus was only viable for a few months, and that once the fungus died and degraded within the concrete, it could weaken the mechanical strength of the concrete. Hence, there has also been research into encapsulating the fungi in alginate before addition to the cement, to enhance its longevity (Khushnood et al., 2022; Van Wylick et al., 2023).

Van Wylick et al. (2024) investigated calcium chloride and calcium lactate as different sources of calcium for self-healing concrete. The study identified calcium lactate as more optimal than calcium chloride for the growth of *T. reesei* and precipitation of calcium carbonate. This contrasts with other studies into ureolytic biomineralisation of calcium carbonate outside of the self-healing concrete context. Another reason for using a calcium source other than calcium chloride when making self-healing concrete, is that chloride ions have a corrosive effect on concrete rebar reinforcement (Bajić et al., 2025; Hurley & Scully, 2006; Nguyen et al., 2012). As displayed in Table 2.1, the calcium source used for ureolytic fungal biomineralisation is most commonly calcium chloride.

2.3.2.3 Bioprotection of Concrete

Self-healing concrete requires the mixing of fungal inoculum into cement and is only relevant in new concrete production. However, another strategy being considered is to coat concrete in a layer of fungal mycelium, either as a protective or restorative method, which could then induce carbonate precipitation. This would not require the concrete to be brand new and could be used to protect or repair older concrete surfaces.

A search of literature only found a single study using this strategy (J. Zhao, Dyer, et al., 2022). In this study, mortar cylinders were made and briefly immersed in growth media. *Neurospora crassa* was harvested from separate growth media by centrifugation and applied to the surface of the cylinder to a thickness of up to 1 mm. The cylinders were then submerged and incubated for 30 days in growth media amended with cementing solution of 40 mM urea and 50 mM calcium chloride. This study found that treatment caused calcium carbonate precipitation upon the surface of the concrete, resulting in reduced rate of water infiltration to roughly 17 % the original value. It is theorised that this is due to the physical barrier posed by the hydrophobic layer of mycelium on the concrete surface, as well as clogging of surface pores by biominerals. However, the layer of mycelium on the surface of the mortar was easily disturbed by water treatment. Due to this, the study attributed a majority of the reduced water infiltration rate to the clogging of surface pores by biominerals.

2.4 Engineering Fungi for Mechanical Strength Improvement in soils by Bioprecipitation

There has been little research into use of fungal biomineralisation to stabilise soils and induce mechanical strength improvement. This literature review shall cover the few papers that were found during the search of the literature.

2.4.1 Fang et al. (2018)

This study used *Penicillium chrysogenum*, an Ascomycete, which was isolated from “cement sludge”. The fungus was grown on malt extract agar before transferral to modified martin broth amended with 2 % urea and 40 mM CaCl₂ and incubated at

25°C for 5 days whilst shaking. The mycelium was macerated with a tissue homogenizer. This biomass was mixed with “river” sand and packed into a column 14 cm long and 4 cm diameter (176 ml volume). The columns were fed continuously with modified martin broth containing 2 % urea and 40 mM CaCl₂ with flow from top to bottom. The paper states this was done at an interval of 48 hours, which this review infers to mean that fresh solution was injected every 48 hours. The experiment ran for 2 weeks. The FICP-treated sand column was subsequently air dried for 3 days and unconfined compression tests were conducted with a loading rate of 1 mm/min. SEM and XRD analysis were also performed.

One resulting FICP-treated sand column was photographed and visibly showed that the column was free standing and bio-cemented. The study noted that the fungal strain grew well in the high pH environment induced by ureolysis. This study states that compressive strength was improved in treated sand columns and achieved a value up to 1800 kPa (note: untreated clean sand has an unconfined compressive strength equal to zero). SEM analysis showed calcium carbonate crystals precipitated onto fungal hyphae. It was noted that the calcium carbonate crystals were small and scattered rather than large singular crystals. The study hypothesises that this was likely due to the many organic molecules secreted by the high surface area fungi which altered the morphologies of the crystals and prevented large crystal formation. The XRD analysis showed that the calcium carbonate present in the columns was in the form of calcite.

2.4.2 Martuscelli, Soares, Lima, et al. (2020)

This study investigated *Penicillium chrysogenum* and *Neurospora crassa*, both Ascomycetes. This paper sought to qualitatively determine urease activity, image bioprecipitated calcium carbonate, and treated sand columns via FICP using these fungal species.

To determine urease activity, 5 mm plugs of fungi were grown on Christensen’s agar amended with 20 g/L urea and 12 mg/L phenol red and incubated at 25°C in the dark. The phenol red indicator would change colour from yellow (pH 6.5) to red (pH > 8.3) with a pH increase which would be associated with ureolysis (i.e. production of hydroxide ions in [Eq 2.2] of the ureolysis reaction). Sample colours

were recorded on days 3 and 6 to determine intensity and rate of colour change. Both fungal species showed an obvious and fast colour change, but no details are given on the rate of change nor whether one species caused a faster colour change than the other.

To investigate calcium carbonate precipitation, 5mm fungal plugs of potato dextrose agar were used to inoculate modified martin broth agar amended with 2 % urea and 40 mM CaCl_2 and incubated for 7 days at 25 °C in the dark. 5mm plugs of this mycelium was then prepared for SEM imaging. The SEM images show similar crystal sizes between the fungal species, and precipitation was closely associated with the presence of fungal hyphae.

To investigate biocementation of sand, fungal biomass grown on potato dextrose agar were harvested and homogenized with modified martin broth amended with 2 % urea and 40 mM CaCl_2 . This suspension was then transferred into 70 ml of modified martin broth amended with 2 % urea and 40 mM CaCl_2 and incubated for 3 days at 25 °C whilst shaking. The fungal suspension was then mixed with sand and packed into polymeric tubes estimated to be 6 cm long and 2.2 cm diameter (22.8 ml volume) with a bulk non-compacted density of 1.58 g/cm³. The sand columns were incubated at 25 °C and treated with 10ml modified martin broth amended with 2 % urea and 40 mM CaCl_2 every 48 hours for 14 days. After treatment, the columns were dried at 50 °C for 5 days and samples imaged using SEM/EDS and unconfined compressive strength determined. The study found that sand columns treated for 14 days were cemented upon removal from casings. The study states that achieved compressive strength was 66.5 kPa and density 2.05 g/cm³ for *P. chrysogenum* and 50 kPa and 1.75 g/cm³ for *N. crassa*, compared to the original bulk non-compacted density of 1.58 g/cm³. It is not stated whether this was an average, maximum, or singular result. The higher density of *P. chrysogenum* column also coincided with a greater final compressive strength than the *N. crassa* column. *P. chrysogenum* was also suggested to have grown more biomass by Scanning Electron Microscopy images, resulting in greater calcium carbonate precipitation thought to be due to increased fungal surface area available for nucleation of calcium carbonate.

2.4.3 Ahmad et al. (2024)

This study investigated the use of *Aspergillus niger*, an Ascomycete, to precipitate calcium carbonate. The fungus was harvested from Potato Dextrose Agar by adding distilled water to the surface and scraping the fungus with a sterile loop. The suspension was used to inoculate a broth of malt extract (3 g/L), glucose (10 g/L), yeast extract (3 g/L), and peptone (5 g/L) and incubated for 4 days at 25 °C while shaken at 200 rpm. Growth rate of the fungus was determined by using filter paper to harvest fungus from the broth before oven drying at 100 °C for 24 hours and weighing to determine fungal biomass. “Poorly graded coarse-grained sand” was used as the media to be treated.

The sand was mixed with 200 ml of fungal suspension and poured and compacted into PVC tubes 500 mm long and 38 mm wide (567 ml volume). Lubricant was used to coat the interior surface of the pipe to allow for easy removal of solids from the pipe after treatment. 2 hours was given for the fungus to attached to the sand grains before cementing solution of 1 M urea and CaCl_2 was injected from the bottom of the tube. 0.5 pore volume (120 ml) was injected this way under a constant hydraulic head supplied from a tank at 2.5 meters. This took 5 mins on the first injection (0.4 ml/s). The solution was then held in the tube for 24 hours which constituted one treatment cycle. Columns were treated for 7 or 28 cycles. Samples of waste solution were taken every 7 days to monitor bioprecipitation, permeability was monitored for the duration of the experiment, and the growth media was injected after 7 days to encourage continued fungal growth. After treatment the column was removed and cut into 6 sections 76mm in height. Sections were dried in an oven at 105 °C and unconfined compression tests (UCS) and calcium carbonate content analysis performed.

UCS increased steadily up to 21 cycles when the value reached 3.87 MPa with a very small standard deviation but showed little difference between 21 and 28 cycles (3.87 - 3.93 MPa). Calcium carbonate content also increased linearly for up to 21 cycles to 14 % but remained relatively constant between 21 and 28 cycles (14 - 15 %). Distribution of calcium carbonate and UCS was similar in each section of the same column, demonstrating that these properties are similar at all depths within the column. SEM and EDS show images and elemental analysis consistent with results from Fang

et al. (2018) and Martuscelli, Soares, Lima, et al. (2020) with calcium carbonate precipitated onto fungal hyphae in many small crystals.

2.4.4 Tuckett et al. (2025)

This study investigated the use of *Penicillium chrysogenum* to reduce fugitive dust with FICP. This paper sought to optimise fungal growth duration and the concentration of cementing solution required to improve mechanical strength of soil in petri dishes using FICP.

The fungus was grown on Potato Dextrose Agar. The growing edge of the fungal mycelium was scraped off to inoculate Potato Dextrose Broth. The culture was incubated for 2 weeks whilst shaken at 200 rpm and then a sterile blender used to create a “fungal slurry” which was used to inoculate the soil. The soil was obtained from agricultural land and sieved with a 4.75 mm sieve to remove large particles.

100 mm diameter petri dishes were filled with the soil to a depth of 25 mm, and a density of 1.5 g/cm³. They were inoculated by pouring a combined 15 ml of the “fungal slurry” with Potato Dextrose Broth over the top of the soil. The amount of fungal slurry only that was used in inoculation was not stated.

The inoculated soil plates were then allowed to grow for 0, 1, 2, 3, 5, 7, or 10 days before treatment with 15 ml of cementing solution of CaCl₂ : urea. The concentration of the cementing solution was varied between 0.05 : 0.25, 0.05 : 0.5, 0.05 : 1, 0.5 : 1, 1 : 1, and 1.5 : 1.

Mechanical strength improvement was determined via a penetrometer at a constant rate of 1.3 mm/min, which gives a measure of strength of the biomineralized crust formed. Calcium carbonate was determined using an Eijkelkamp Calcimeter. Hydrochloric acid was reacted with the sample to produce carbon dioxide. The volume of carbon dioxide produced was collected and used to calculate the mass of calcium carbonate within the sample.

The study found that there was little difference in calcium carbonate precipitation between samples with different fungal growth times and treated with 0.05 M CaCl₂ and 1 M urea. However, the study also found that allowing *P. chrysogenum* to grow in the soil sample for 24 hours prior to treatment caused optimal mechanical strength improvement of 2.7 MPa. They suggest this was because in the first few days

the fungus had time to grow a scaffold within the sample and provide more effective nucleation sites but not too long to starve and dehydrate. When samples were treated with various cementing solutions after seven days of growth, treatment with 0.5 M CaCl_2 and 1 M urea, or 1 M CaCl_2 and 1 M urea, resulted in the most calcium carbonate precipitation. Samples treated with 1.5 M CaCl_2 and 1 M Urea cementing solution resulted in an optimal mechanical strength improvement of 1.8 MPa.

This study has some key results:

- A period of fungal growth in the sample before treatment with cementing solution can be beneficial for mechanical strength improvement. In this case, 24 hours.
- Increasing cementing solution concentration can improve calcium carbonate precipitation and mechanical strength.
- Mass of calcium carbonate precipitated does not necessarily directly translate to improved mechanical strength.

2.4.5 Fungal Mechanical Strength Improvement Summary

The three studies (Ahmad et al., 2024; Fang et al., 2018; Martuscelli, Soares, Lima, et al., 2020) which investigated mechanical strength improvement in sand columns by fungal bioprecipitation used similar methods. The fungus was mixed into the sand and packed into a column. The scale of the experiments was quite different with 19 ml, 176 ml, and 567 ml volume. There was little time for fungal growth before treatment with cementing solution. The column was injected with cementing solution from the bottom to remove as much air as possible and avoid preferential pathways. The cementing solution contained growth media so that the fungus would grow whilst also precipitating calcium carbonate. Columns were treated with static or continuous flow. The column was treated for at least one week, with Ahmad et al. (2024) finding that mechanical strength improvement continued for up to 28 days. Each study oven or air dried the columns for 2 to 5 days before various calcium carbonate determination, unconfined compression testing, SEM/EDS, and/or XRD analysis.

Tuckett et al. (2025) was different from the three previous studies, using petri dishes of soil rather than sand columns. The fungus was also not mixed into the soil; it was poured over the top and the cementing solution was also poured over the top

rather than injected. It was also the only study which provided an in-situ growth period for the fungus before treatment with cementing solution.

Between the highlighted studies, achieved unconfined compressive strength varied greatly, see Table 2.3.

Table 2.3 Unconfined Compressive Strength after FICP

Reference	Fungal Species	Fungal Inoculum Volume (ml)	Cementing Solution (M)		Flow Strategy	# of cycles	Treatment Time (weeks)	Volume of soil (ml)	Pore Volume (ml)	Injected solution per cycle		UCS (kPa)	
			CaCl ₂	Urea						ml	Pore Volume		
Fang et al. (2018)	<i>P. Chrysogenum</i> [A]	50	0.04	0.33	Continuous	7	2	176	Unknown	Unknown	Unknown	1800	
Martuscelli et al. (2020)	<i>P. Chrysogenum</i> [A]	70	0.04	0.33	Static	7	2	22.8	9	10	1.1	66.5	
	<i>N. Crassa</i> [A]											50	
Ahmad et al. (2024)	<i>A. Niger</i> [A]	200	1	1	Static	7	1	567	240	120	0.5	745	
						28	4					3930	
Tuckett et al. (2025)	<i>P. Chrysogenum</i> [A]	15	CaCl ₂	Urea	Static	1	Fungal Growth (days)	-	-	15	-	Solution	Growth
			0.05	0.25			0					Least	Least
			0.05	0.5			1					-	2700 (Most)
			0.05	1			2					-	1500
			0.5	1			3					1600	1600
			1	1			5					-	-
			1.5	1			7					1800 (Most)	-
			-	-			10					-	-

Fang et al. (2018) was the pioneer study investigating the compressive strength achieved in sand columns due to bioprecipitation of calcium carbonate with fungi. Compared to the other two studies, they achieved a very impressive result within 7 cycles of treatment (1800 kPa). Perhaps this was because they used continuous flow to mix cementing solution through the sand at all times, allowing more efficient calcium carbonate precipitation. They only state this one maximum result however; it was not stated what their results were below the maximum result.

Martuscelli, Soares, Lima, et al. (2020), who also tested treatment using the same species of fungus as Fang et al. (2018), *P. chrysogenum*, achieved a much low unconfined compressive strength when compared to Fang et al. (2018) (66.5 kPa and 50 kPa). This could have been due to the different treatment strategy applied, as they incorporated static periods between injection of treatment solutions, rather than the continuous flow strategy used by Fang et al. (2018). However, Fang et al. (2018) did not provide the number of pore volumes of cementing solution that they injected and thus, this could also be a major contributing factor for the much higher mechanical strength. As Martuscelli, Soares, Lima, et al. (2020) only injected 1.1 pore volumes per cycle. Martuscelli, Soares, Lima, et al. (2020) did show that mechanical strength improvement can vary between fungal species. They suggested that fungi which can grow more biomass in the allotted time resulted in greater mechanical strength improvement due to the higher availability of nucleation sites. However, they determined biomass growth visually through SEM, which does not allow a quantitative whole sample comparison of fungal biomass between columns.

Ahmad et al. (2024) showed that bioprecipitation could continue for up to 28 cycles and that between 21 and 28 cycles, the rate of bioprecipitation slowed down. They also found that UCS increased with treatment time. After splitting some columns into sections corresponding to increasing column depth, they found that UCS and calcium carbonate precipitation was similar at different depths within the column.

Tuckett et al. (2025) showed increasing cementing solution concentration improved calcium carbonate precipitation and mechanical strength, and a fungal growth period improved mechanical strength, if not the amount of calcium carbonate precipitation.

2.5 Discussion

After reviewing the scope of the literature available for engineering applications of fungal biomineralisation of calcium carbonate, some trends can be summarised and gaps in knowledge identified.

2.5.1 Fungal Species and Family

The vast majority of literature shown in this review focussed on the use of Ascomycete fungi, with a few utilising Mucoromycota, and only one Basidiomycete investigated. The papers most relevant to soil stabilisation applications used *P. chrysogenum*, *N. crassa*, or *A. niger*, which are all Ascomycetes and were also used in some of the other studies discussed here. Moreover, *Aspergillus niger* is recognised as a Category 2 Human Pathogen (Health and Safety Executive, 2023) and *Penicillium chrysogenum* is also recognised as a human pathogen (Egbuta et al., 2017). As such the deployment of these fungal strains in civil and environmental applications as cultivated inoculum is likely to be restricted due to biosafety risks. In general, Ascomycetes frequently release spores and in large quantities, and can pose a risk to respiratory health due to chronic exposure to high concentrations of spores (Alam et al., 2022; Eduard, 2009; Palmas & Meloni, 1997). Although, Menon et al. (2019) investigated a Basidiomycete (*Phanerochaete chrysosporium*) it did not grow well in agar plates containing concrete, and thus was not studied further for inducing precipitation. As Basidiomycota only fruit in specific circumstances, their sporing is generally much less frequent than for Ascomycetes (Kues & Liu, 2000; Pelkmans et al., 2016). Although, it is noted that some Basidiomycete spores (specifically *Pleurotus cornucopiae*) have been noted as potential allergen (Michils et al., 1991). A potential allergy could trigger an asthmatic response in vulnerable individuals. Allergen risks could be mitigated by avoiding fruiting behaviours and limiting exposure to spores.

This thesis investigates Basidiomycetes as an alternative option for FICP than Ascomycetes.

2.5.2 Fungal Screening

Screening of fungi for suitability for bioprecipitation of calcium carbonate in all studies involved either (i) isolating a fungus from a concrete paste or surface, where

it has been able to survive the high pH environment, or (ii) subjecting a sample to high pH conditions and determining growth rate. This is because during ureolysis, hydroxide ions are produced which cause a high pH environment. This was especially relevant as in these studies, the fungus was provided with nutrients with the intention for it to grow whilst simultaneously inducing ureolysis to precipitate calcium carbonate, resulting in a high pH. However, this restricts the scope of fungi which are suitable for treatment to high pH tolerant species. As Basidiomycetes generally induce an acidic environment by secreting enzymes and organic acid to digest and release nutrients from lignin, they are generally accustomed to acidic environments (Ejechi, 1998). Tuckett et al. (2025) demonstrated that pre-growth of the fungus could be beneficial prior to treatment with cementing solutions. If fungal growth could be achieved in a pre-stage, this could decrease the importance of pH resistance, widening the scope of viable fungal species which could potentially be deployed in fungal induced carbonate precipitation processes to include Basidiomycetes, which has not been attempted.

2.5.3 Chemistry of FICP solutions

As well as investigating heavy metal removal, Li et al. (2014) also investigated the effect of chemical ratios and induced pH on calcium carbonate precipitation during their research. They investigated calcium carbonate precipitation at three different concentrations of treatment solution (Table 2.4). In the context of this thesis, treatment or cementing solution is the solution with which ureolysis and precipitation is induced, typically containing urea and calcium ions.

Table 2.4 pH and removal of Ca during FICP by Li et al. (2014)

Solution	Urea (mM)	CaCl ₂ (mM)	Ratio of Calcium to Urea Ions	pH	Ca removed after 12 days (mM)	Ca removed after 12 days (%)
1.	40	0.2	1:200	9.22	0.03	16.4
2.	40	50	1:0.8	7.29	26.35	52.7
3.	330	50	1:6.6	8.68	46.62	93.2

The pH of the uninoculated control for each concentration was 5.5.

Solution 1 was a pseudo-control, with low concentration of calcium. There was little visible mineral precipitation around the hyphae (observed via SEM) despite the high pH being favourable for calcium carbonate precipitation due to the lack of calcium available. Comparing Solutions 2 and 3, which both contained initially 50 mM CaCl_2 it is evident that when a higher concentration of urea was used (Solution 3, 330 mM) a higher percentage of Calcium was removed within 12 days: 93.2 % compared to 52.7 % when only 40 mM of urea was provided.

This study shows that the relative concentrations of urea and calcium chloride are important for fungal induced carbonate precipitation.

2.5.4 Bioprecipitation of Calcite

Many of the studies discussed in this review found that precipitates were closely associated with fungal hyphae, often precipitating directly onto them. Some noted that crystals were calcite and small but numerous, sometimes forming a coating across the hyphae. They also noted this was likely due to the influence of organic acids and macronutrients secreted by the fungi during growth in-situ and compounds found on and within the surface of the cell wall upon which the crystals precipitated. The morphology of crystals also varied greatly between fungal species and even within treatment sites due to the variable influence of organic contaminants originating from the fungus.

2.6 Conclusions

This chapter reviewed literature investigating the influence of fungal inoculation on soil behaviour including two approaches: (i) inoculation with mycorrhizal fungi to indirectly support plant growth and hence enhance plant reinforcement mechanisms and (ii) inoculation of soil with non-mycorrhizal, saprotrophic fungi. Both approaches have demonstrated that soil erodibility can be reduced via fungal inoculation. This thesis builds upon this literature by exploring mechanisms to further enhance the performance and durability of fungal-treated soils via fungal induced calcium carbonate precipitation (FICP). This chapter reviewed the literature on FICP for heavy metal removal, self-healing concrete, repair and protection of concrete and soil stabilisation applications. There are few studies to date

on using FICP for mechanical strength improvement of soils. The key gaps identified from this review are:

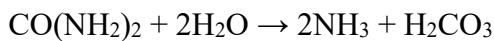
- Previous literature has focussed on using Ascomycetes, and in some studies even fungal strains which are known human pathogens have been investigated. This thesis will instead focus on screening for urease active Basidiomycetes, which have to date been mostly overlooked, and result in much less frequent spore release, generally making them safer candidates. Five Basidiomycetes are screened in Chapter 3, for their growth behaviour and ureolytic activity.
- The scope of fungi suitable for deployment with FICP treatment is currently limited by their tolerance to high pH environments. As part of the screening process, Chapter 3 explores the growth behaviour of various Basidiomycetes in high urea/high pH environments.
- The results by Li et al. (2014) indicate that the ratio of Urea to Cementing solutions plays an important role in fungal-induced carbonate precipitation. More research is needed to further explore the influence of cementing solution concentrations and ratios and their influence on FICP. Chapter 5 explores the influence of cementing solution chemistry (total concentration, ratio, pH) on the amount of calcium carbonate precipitated and the percentage conversion.
- Limited observations by Martuscelli, Soares, Lima, et al. (2020) indicate that more fungal biomass results in more calcium carbonate precipitation. In this thesis the influence of the amount of fungal biomass on FICP is explored in Chapter 5.
- With the exception of Tuckett et al., (2025), the reported literature on fungal biomineralisation of soil relied on growing fungus in the soil at the same time as treating with cementing solution. This strategy requires the fungus to be resistant to a high pH environment. Chapters 6 for the first time investigates applying a two stage treatment strategy to the treatment of sand columns using a Basidiomycete.
- Ahmad et al. (2024) found that increasing the number of cycles improved mechanical strength gain but did not explore the effect of treatment cycle duration. Chapter 6 investigates both the effect of treatment cycle duration and

number of treatment cycles on calcium carbonate precipitation in fungal-inoculated soils.

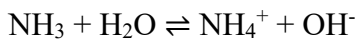
Chapter 3: Screening Urease Activity of Agaricomycetes for Fungal Induced Calcium Carbonate Precipitation

3.1 Introduction

Microbially induced carbonate precipitation (MICP) has been the focus of intensive research over the last twenty years. MICP has been investigated at laboratory- and field- scale for a wide range of civil, geotechnical and geo-environmental engineering applications including soil stabilisation, liquefaction mitigation, erosion mitigation, dust control, soil permeability reduction, stone and concrete repair, rock fracture grouting and well sealing, as well as for contaminant immobilisation (De Jong et al., 2013; El Mountassir et al., 2018; He et al., 2019; Phillips et al., 2013; Terzis & Laloui, 2019; Warren et al., 2001; Whiffin et al., 2007). The metabolic pathway which has received the most research attention is microbially induced carbonate precipitation via ureolysis, driven by ureolytic bacteria. The reaction pathway for MICP via ureolysis is shown in [Eq 3.1] to [Eq 3.4] (Ferris et al., 1996; Mitchell et al., 2010). The majority of MICP-based engineering studies utilise the laboratory-cultured bacterium *Sporosarcina pasteurii* as the source of urease enzyme due to its high urea hydrolysis rates (Terzis and Laloui, 2019). However, researchers have shown that a wide range of indigenous bacteria can produce sufficient ureolytic activity for use in engineering applications (Cheng et al., 2017; Gomez et al., 2018; Yang et al., 2016).



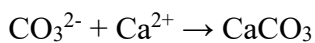
[Eq 3.1] Ureolysis



[Eq 3.2] Ammonia – Ammonium Equilibrium



[Eq 3.3] Carbonate Ion Equilibrium



[Eq 3.4] Carbonate Precipitation (at high pH)

Fungi are also known to support biomineralization processes in the natural environment either by actively contributing via enzyme activity or by passively providing a site for surface nucleation of minerals (Bindschedler et al., 2016; Gadd, 2007, 2017). However, to date fungal-induced mineralisation for engineering applications has been much less studied compared to bacterially driven systems. A limited number of studies have investigated the potential of fungal biomineralisation for the bioremediation of contaminants, i.e. co-precipitation of heavy metals (Li et al., 2014; Qian et al., 2017; J. Zhao, Csetenyi, et al., 2022), to improve the compressive strength of sands (Fang et al., 2018; Martuscelli, Soares, Camões, et al., 2020), to provide a protective coating for concrete (J. Zhao, Dyer, et al., 2022) and for use in self-healing concrete (Menon et al., 2019). All of these studies demonstrated fungal induced precipitation via urea hydrolysis using fungal species from the Ascomycota fungal division with only one species from the Mucoromycota division demonstrating precipitation (J. Zhao, Csetenyi, et al., 2022).

Recent geotechnical engineering studies have demonstrated that the treatment of soils with species from the Basidiomycota division and in particular the Agaricomycota class (e.g. *Pleurotus ostreatus*) can increase soil cohesion and improve resistance to soil erosion due to the development of three-dimensional hyphal networks that act to bind and enmesh soil particles (Salifu, 2019; Salifu et al., 2024; Salifu & El Mountassir, 2019, 2021). We propose that fungal biomineralisation could be induced to further enhance cohesion, reduce soil erodibility and ultimately improve the durability of these soil modifications.

This study seeks to identify ureolytically active Agaricomycetes which could be used to induce carbonate precipitation for deployment in geotechnical engineering applications including soil stabilisation and erosion control of slopes. To be usefully deployed, such a fungal species would need to be fast-growing and exhibit high urease activity to reduce the time required for treatment. Furthermore, given that fungal-induced mineralisation via ureolysis would induce a high pH (up to 9.5) due to the production of hydroxide ions during ammonia equilibrium [Eq 3.2], the selected species should be tolerant to high pH. Research by Ejechi (1998) shows that wood decay by Agaricomycetes is inhibited at $\text{pH} > 7$, indicating a reduction in fungal growth and metabolism. As such, the viability of Agaricomycetes at the higher pHs

induced during ureolysis requires investigation. Finally, any fungal species proposed as part of a bioaugmentation strategy for soil stabilisation should be non-toxic.

The overall aim of this study is to identify a fungal species for inducing fungal carbonate precipitation via ureolysis for soil stabilisation and improving erosion resistance. Species selection is based on the following screening criteria, that the fungal species is: (1) non-toxic, (2) fast growing, (3) has high urease activity, and (4) is tolerant to high pH.

3.2 Methods

Two main experiments were conducted to identify a suitable fungal species according to the screening criteria presented above. Experiment 1 investigates the growth behaviour and urease activity of the fungal species for cultures grown on agar (with and without urea) and in Experiment 2, urease activity and calcium precipitation was investigated for fungal cultures in liquid media. The methods for each experiment and the experimental design are presented below and summarised in Table 3.1.

3.2.1 Experiment 1 – Growth rate and urease activity of fungal species grown on agar

3.2.1.1 Fungal species

Five saprotrophic Basidiomycota (Division), Agaricomycota (Class), fungi were selected for study: *Sistotrema brinkmannii* (*S. brinkmannii*), *Bjerkandera adusta* (*B. adusta*), *Pleurotus cornucopiae* (*P. cornucopiae*), *Pleurotus ostreatus* (*P. ostreatus*), and *Pleurotus pulmonarius* (*P. pulmonarius*). These fungal species are all considered Hazard Group 1 organisms (i.e. not considered a risk to human health) and are not animal pathogens (Health and Safety Executive, 2023; The Specified Animal Pathogens Order (SAPO), 2008). Some of these are commercially sold as edible mushrooms (*Pleurotus* spp.) and all species listed have been previously investigated for bioremediation applications (Jiang et al., 2015; Lamar et al., 1999; Rodríguez et al., 2004). These five fungal species are native to the UK and fungal cultures were provided by Richard Wright from Cardiff University, who sampled them from within the UK.

3.2.1.2 Fungal inoculation and incubation

Beechwood cubes 1 cm³ in dimension (1 cm on each side) were initially sterilised by autoclaving. After cooling, they were colonised with fungus by placing them on top of fungal cultures grown for 7 days on malt extract agar. These were then incubated in the dark for a period of 10 days at 25 °C.

3.2.1.3 Preparation of specimens

Malt extract agar amended with 12 mg/L phenol red indicator was sterilised via autoclaving. The malt extract agar was then also amended with varying urea concentrations (sterilised via filtering through a 0.45 µm filter). After pouring agar into 90 mm petri dishes and allowing to cool, a single colonised beechwood cube, was placed centrally on the top of each petri dish. These were then incubated in the dark at 25 °C for up to 15 days (see Table 3.1).

3.2.1.4 Assessment of fungal growth

Fungal growth was assessed via time lapse photography. Photographs were imported into ImageJ Fiji software (Schindelin et al., 2012) to determine the area of fungal growth on the surface of the agar plates. Each image was converted to an 8-bit grey scale image, with a grey scale range between 0 (Black) and 255 (White). Image segmentation was then carried out using thresholding to obtain a binary image where the area of fungal growth could be easily distinguished from the rest of the agar plate. The extent of this growth was limited to the area of the petri dish (7088 mm²). This method did not consider three-dimensional growth, i.e. where hyphae may have penetrated into the agar or grown into the air, nor did it consider the density of the fungal growth. Note the area calculated includes the area of the beechwood cube inoculum.

3.2.1.5 Evaluation of urease activity

In Experiment 1, urease activity was qualitatively assessed using a phenol red assay. Malt extract agar was amended with 12 mg/L of phenol red indicator, following the methods used by (Martuscelli, Soares, Camões, et al., 2020; Shingha Barua et al.,

2012). Table 3.1 which plates were amended with phenol red. Phenol red provides an indirect colourimetric indication of urease activity. The colour of the agar changes from yellow at $\text{pH} < 6.8$ to red ($6.8 \leq \text{pH} \leq 8.2$) and finally to bright pink at $\text{pH} > 8.2$, in this study the pH change is due to the production of hydroxide ions during ureolysis [Eq 3.2].

3.2.1.6 Experiment 1.1

In Experiment 1.1 the growth rate and urease activity of fungal species grown on agar with varying initial urea concentration (0, 0.03, 0.1) was investigated. Specimens were incubated with growth and urease activity monitored for up to 14 days. Specimens were prepared in triplicate.

3.2.1.7 Experiment 1.2

Experiment 1.2 was conducted to understand how the growth behaviour and fungal activity of a fungal organism that is only partially exposed to a urea/ammonium rich environment, compares to an organism that is entirely exposed to a urea/ammonium rich environment. This experiment more realistically simulates the exposure conditions of an engineering application, where the fungal network may extend beyond the zone where treatment fluids are injected. In this experiment colonised beechwood cubes were sandwiched between two semi-circular discs of agar placed in petri dishes. In order to restrict diffusion of ammonium and hydroxide ions between the agar sections, the agar sections were not directly in contact. Both agar sections were amended with 12 mg/L phenol red, but one section was amended with 0.1 M of urea and the other contained no urea (0 M). The specimens were prepared in triplicate and were incubated for 7 days before observation.

3.2.1.8 Experiment 1.3

In Experiment 1.3, inoculated cubes from plates of the two fungal species which exhibited high urease activity from the first stage (grown in 0.1 M urea concentration condition) were subsequently transplanted onto fresh malt extract agar plates with and without urea present (0 and 0.1 M) and incubated for a further 14 days. This was to understand if exposure to high urea (and thus high ammonium and pH)

concentrations could (a) permanently inhibit growth and/or (b) alter urease activity. Specimens were prepared singularly.

3.2.1.9 Experiment 1.4

For the most ureolytically active fungal species observed in Experiment 1.1, an experiment was conducted to determine the concentration of urea at which growth was prevented. Agar was amended with varying urea concentration (0.03, 0.04, 0.05, 0.06, 0.07, 0.08, 0.09, and 0.1 M) and inoculated with the desired fungal species. Specimens were incubated for 7 days before growth was assessed. Specimens were prepared singularly.

Table 3.1 Experimental Design

Experiment ID	Description	Growth medium	Fungal Species	Urea Conc. (M)	Phenol Red Indicator	Measurements
Exp. 1.1	Growth behaviour and urease activity	Malt extract agar	<i>S. brinkmannii</i> <i>B. adusta</i> <i>P. cornucopiae</i> <i>P. ostreatus</i> <i>P. pulmonarius</i>	0 0.03 0.1	Yes	Growth assessed at 0, 1, 2, 3, 4, 7, 14 days
Exp. 1.2	Partial exposure to urea	Malt extract agar	<i>S. brinkmannii</i> <i>B. adusta</i> <i>P. cornucopiae</i> <i>P. ostreatus</i> <i>P. pulmonarius</i>	Half: 0 Half: 0.1	Yes	Growth assessed at 7 days
Exp. 1.3	Growth behaviour and urease activity after 2 weeks exposure to 0.1 M Urea	Malt extract agar	<i>S. brinkmannii</i> <i>P. cornucopiae</i>	0 0.1	Yes	Growth assessed at 14 days
Exp. 1.4	Maximum urea concentration	Malt extract agar	<i>P. cornucopiae</i>	0.03 0.04 0.05 0.06 0.07 0.08 0.09 0.1	No	Growth assessed at 7 days
Exp. 2	Ureolysis and calcium carbonate precipitation	Malt extract broth	<i>S. brinkmannii</i> <i>B. adusta</i> <i>P. cornucopiae</i> <i>P. ostreatus</i> <i>P. pulmonarius</i>	0.333	No	pH, NH_4^+ , Ca^{2+} measured up to 2 weeks

3.2.2 Experiment 2 - Fungal induced calcium carbonate precipitation in liquid media

3.2.2.1 Preparation of fungal inoculum

The ability of the five Agaricomycetes listed in Section 3.2.1 to precipitate calcium carbonate was investigated in Experiment 2. Fungal cultures were grown in 200 ml of malt extract broth in 250 ml conical flasks. Four replicate cultures were prepared for each fungus. Cultures were incubated at 25 °C for 14 days in the dark and shaken via a table shaker. After 14 days, each culture was individually centrifuged at 10000 rpm for 10 minutes and the pellet harvested and returned to each flask. As a result, a minimal volume of broth was present during the experiment.

3.2.2.2 Preparation of cementing solution

The cementing solution concentration comprised 50 mM CaCl_2 and 333 mM urea based on Li et al. (2014). CaCl_2 was sterilised via autoclaving, and urea was sterilised with a 0.45 μm syringe filter. 200 ml of cementing solution was added to each flask.

3.2.2.3 Control Samples

Controls were prepared with no fungal inoculum. Instead, the control flask was first rinsed with uninoculated sterilised malt extract broth, and drained off, before the addition of cementing solution. This was used to represent the small amount of malt extract broth which would have been retained within the harvested fungal pellet.

3.2.2.4 Chemical analyses

Chemical analyses were undertaken to evaluate pH, NH_4^+ and Ca^{2+} concentration at the following time points $t=0$, prior to the addition of the fungal inoculum and then after addition of the fungal inoculum at $t= 1, 2, 3, 4, 5, 6, 12, 24, 36, 48, 60, 72, 84, 96$ hrs and after 1 and 2 weeks. 3 ml of solution was sampled at each timepoint for chemical analyses. pH was measured using the Thermo Scientific Orion Star A215 pH and Conductivity Meter. NH_4^+ was measured using the Konelab Aqua30 with reagents Amml and Amm2. Ca^{2+} was measured via the Thermo Scientific iCAP 6200 Series ICP Spectrometer.

3.2.2.5 Determining fungal biomass

During the batch experiment calcium carbonate precipitation occurred. To determine the mass of the fungal biomass alone at the end of the experiment, the temperature at which all the fungal biomass was removed, without the simultaneous decomposition of calcium carbonate, was required to be identified. To identify a suitable temperature, the thermal decomposition of fungal specimens (for all five species) and chemical powdered CaCO_3 (purity 98 %) was analysed using the Agilent Technologies 7890A Gas Chromatography (GC) System following the procedure laid out in Stillings et al., (2023). Samples of fungal biomass were harvested by

centrifugation at 10000 rpm for 10 minutes and freeze-dried in a SCANVAC CoolSafe at -112°C in a low vacuum. GC analysis was intended to identify a temperature where mass loss of fungal biomass and mass loss of calcium carbonate did not overlap.

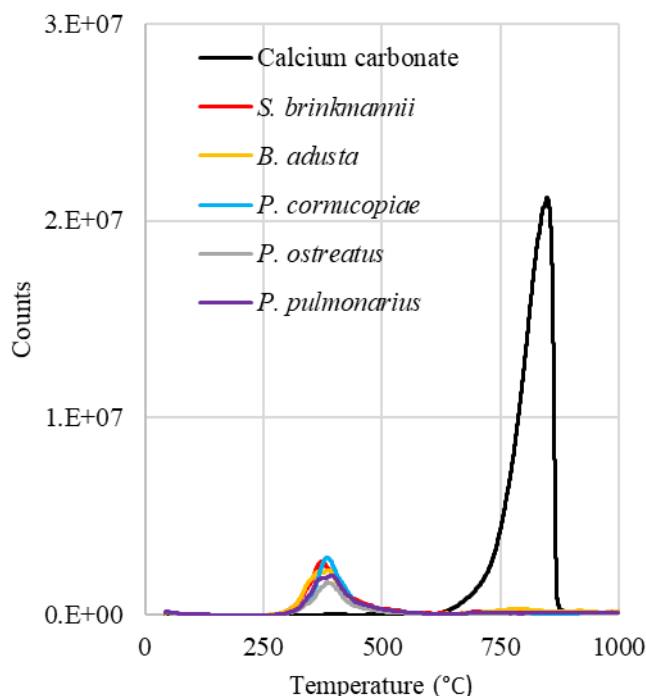


Figure 3.1 Thermal Decomposition of Different Fungal Cultures at m/z 44

Figure 3.1 presents the thermal decomposition data, shown as counts of molecules with a mass:charge ratio of 44 (i.e. CO₂), thus illustrating at which temperatures CO₂ was being produced for each specimen. The thermal decomposition data for biomass samples of each fungal species is displayed, sampled after growing in broth and then centrifuging. The fungal biomass decomposition curves are compared with abiotic powdered calcium carbonate shown in black. For the calcium carbonate specimen, CO₂ was produced between 600°C and 900°C. For all fungal biomass samples, CO₂ was produced between 250°C and 600°C with peaks within the range of 370 - 410°C corresponding to the breakdown of biomass (Figure 3.1). Based on these results it was determined that Loss on Ignition tests should be performed at 600°C to determine the dry fungal biomass present in samples. The total solid mass recovered at the end of Experiment 2 was heated in an oven initially to 105°C for 24 hrs to remove water. Subsequently, specimens were heated to 600°C for at least 8

hours, and until there was no more change in mass, and the mass lost between 105°C and 600°C is reported as the dry fungal biomass.

3.2.2.6 Calculation of urease activity

Urease activity was determined as the change in urea concentration, C_{urea} (mM) over time t , (determined from the NH_4^+ concentration results) between each successive time point determined up to $t = 12$ hrs, with the average rate determined, and normalised by the fungal biomass, $M_{biomass}$ (g). See [Eq 3.6].

$$Urease\ activity = \frac{\frac{1}{n} \sum \frac{\Delta C_{urea}}{Dt}}{M_{biomass}} \quad [Eq\ 3.6]$$

3.2.2.7 X-ray diffraction (XRD)

Solid samples were harvested from Experiment 2 by centrifugation at 10000 rpm for 10 minutes and prepared for XRD by freeze drying and mixing them with a glass rod, producing a granular powder. The powder was poured into the sample holders and levelled to the height of the rim. A Bruker D8 Advance Diffractometer was used, with a 20 - 90 θ range, 0.025 θ step size, and 1 s step time.

3.2.2.8 Scanning Electron Microscopy (SEM)

Samples from Experiment 2 were prepared for SEM by gently air drying them overnight on the SEM stubs before applying a gold coating using an Agar Sputter Coater. A Tungsten filament Hitachi S3700-N SEM was used to image samples, and elemental analysis was also performed on specific points on SEM images using an Oxford Instruments X-Max 80 mm² EDS.

3.3 Results

3.3.1 Experiment 1

3.3.1.1 Experiment 1.1 Growth behaviour and urease activity on agar plates

Experiment 1.1 was carried out to investigate the influence of urea on growth behaviour and to qualitatively assess urease activity of the five different fungal species. Figure 3.2a shows the surface area of fungal growth achieved over the initial 7-day growth period for each of the 5 fungal species grown on malt extract agar without urea amendment (total growth period was 14 days). The markers are the median value measured, and the bar is the range of growth determined between replicates. In this way, all three of the replicates for each species are displayed. It can be seen that the slowest growing fungus was *B. adusta* (Figure 3.2a square symbols), followed by *Sistotrema brinkmannii* (Figure 3.1a circle symbols). All of the *Pleurotus* species filled the extents of their plates by day 7, although *Pleurotus cornucopiae* was the slowest growing of the three, as seen on day 4 (Figure 3.2a diamond symbols).

Figures 3.2b - f show the change in growth behaviour with increasing urea concentration (0 M, 0.03 M and 0.1 M) for each fungal species. Although overall growth was slower for *B. adusta* than for the other species, this species showed only a slight reduction in growth when exposed to urea (Figure 3.2c). The fastest growing species without urea were the three *Pleurotus* species. No reduction in growth was observed for *P. cornucopiae*, *P. ostreatus* and *P. pulmonarius* at 0.03 M urea. In fact, *P. cornucopiae* appears to show a slight improvement in growth (Figure 3.2d). However, at 0.1 M urea growth of *P. ostreatus* and *P. pulmonarius* was slightly reduced (Figure 3.2e, f), whereas *P. cornucopiae* showed complete growth inhibition (Figure 3.2d). *S. brinkmannii* showed growth inhibition at both 0.03 M urea concentration and growth inhibition at 0.1 M (Figure 3.2b).

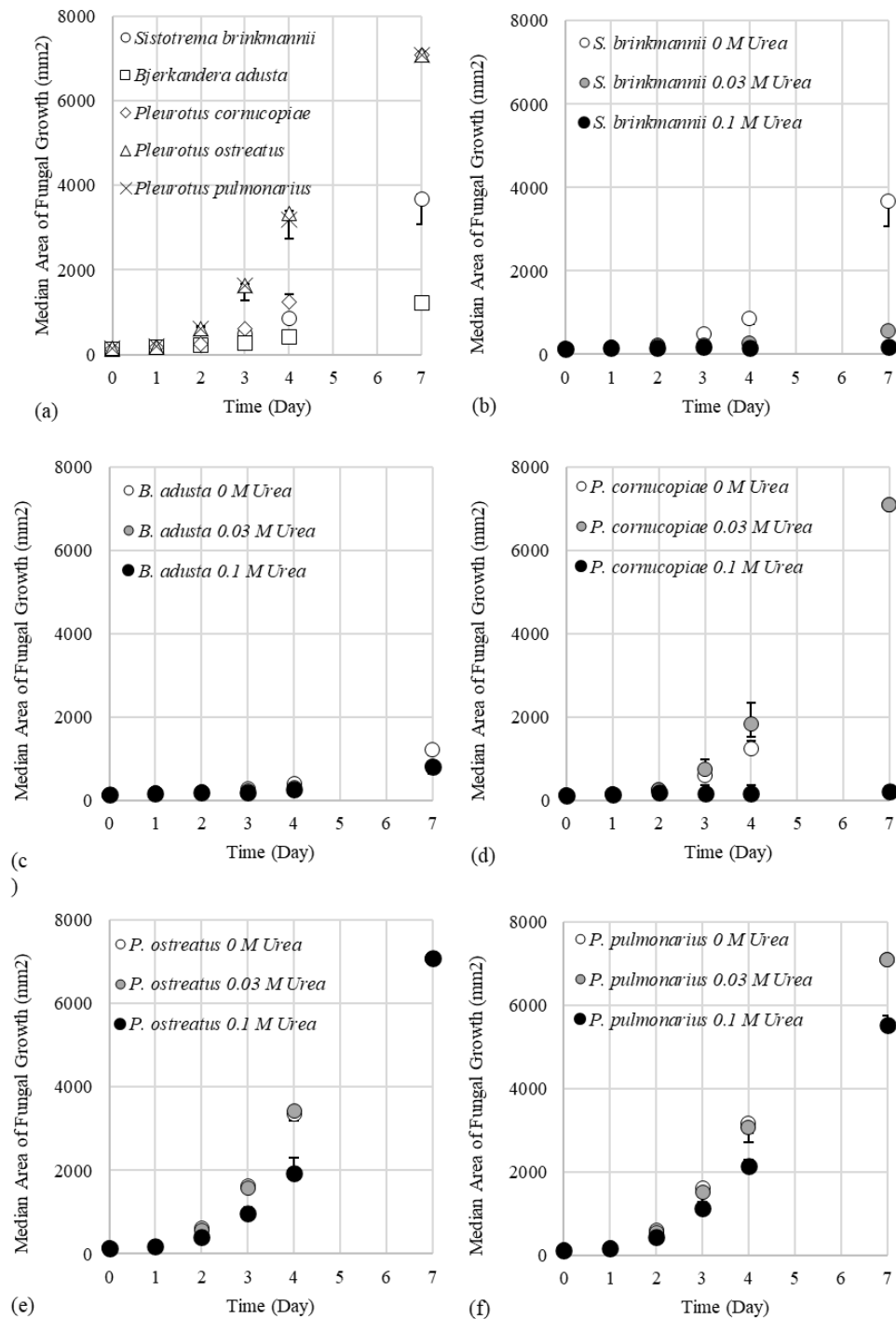


Figure 3.2 Surface area of fungal growth over 7 days (a) Comparison of all fungal species grown on agar with no urea, (b - f) Surface area of growth over 7 days on agar with 0 M, 0.03 M and 0.1M urea: (b) *Sistotrema brinkmannii*, (c) *Bjerkandera adusta*, (d) *Pleurotus cornucopiae*, (e) *Pleurotus ostreatus*, and (f) *Pleurotus pulmonarius*. Marker presents median value, bars indicate range of growth determined in 3 replicates.

From the photographs presented in Figure 3.3, it is evident that a change in colour of the agar from yellow to red occurred only in the urea amended agar plates with growth of the following fungal species: *S. brinkmannii* and *P. cornucopiae*. This indicates that *S. brinkmannii* and *P. cornucopiae* were the most urease active fungi of those screened, as they were the only fungi which caused a full plate of colour change. At 0.1 M Urea *P. cornucopiae* showed a full plate of colour change by day 4, whilst *S. brinkmannii* showed a full plate of colour change by day 7. Hence, *P. cornucopiae* likely has a higher urease activity than *S. brinkmannii* since it achieved a full plate of colour change quickest.

Both *S. brinkmannii* and *P. cornucopiae*, the fungi with the highest urease activity, exhibited completely inhibited growth in 0.1 M urea. This is likely a direct result of their high urease activity. As ureolysis proceeded, ammonium was produced as well as an increase in the local pH environment which inhibited growth. All other fungi showed much less growth inhibition than *S. brinkmannii* and *P. cornucopiae* in 0.1 M of urea.

3.3.1.2 Experiment 1.2 Influence of partial exposure to urea-rich environments on growth behaviour

Experiment 1.2 was carried out to determine if each fungal species would be able to grow in a high urea/high pH environment if the organism simultaneously had access to a low urea/low alkalinity environment. In Figure 3.3, the results from Exp 1.2 are presented in the far-right column, where each fungal species was grown in an environment with half of the agar containing 0 M urea (left side) and half containing 0.1 M urea (right side). As anticipated from Exp 1.1, *P. ostreatus*, *P. pulmonarius* and *B. adusta* were all able to grow on the high urea half of the agar plate (right side) at similar rates to those shown in the previous experiment. *P. cornucopiae* and *S. brinkmannii*, however, show different behaviour to that in Exp 1.1; growth occurs in the high urea environment (right side) when it was previously entirely inhibited by 0.1 M Urea, albeit at a reduced rate when compared to growth in the urea-free environment (left side).

It should also be noted that in this experiment, the side with agar free of urea and thus ammonium would not have remained so indefinitely. The slices of agar were separated by an air gap to limit diffusion of these chemicals as much as possible. However, urea and ammonium, and therefore also pH conditions, would have eventually diffused through the beechwood cube from the amended agar into the pure malt extract agar. This may have been the cause of the partial inhibition of growth. The fungi may also have transported the compounds between the two agar sections via their hyphal network.

These results indicate that in the case where some part of the fungal organism has access to a low urea/ammonium environment, they may still be able to grow in a high urea/ammonium/pH environment. The implications of this for civil engineering applications is further considered in the discussion section.

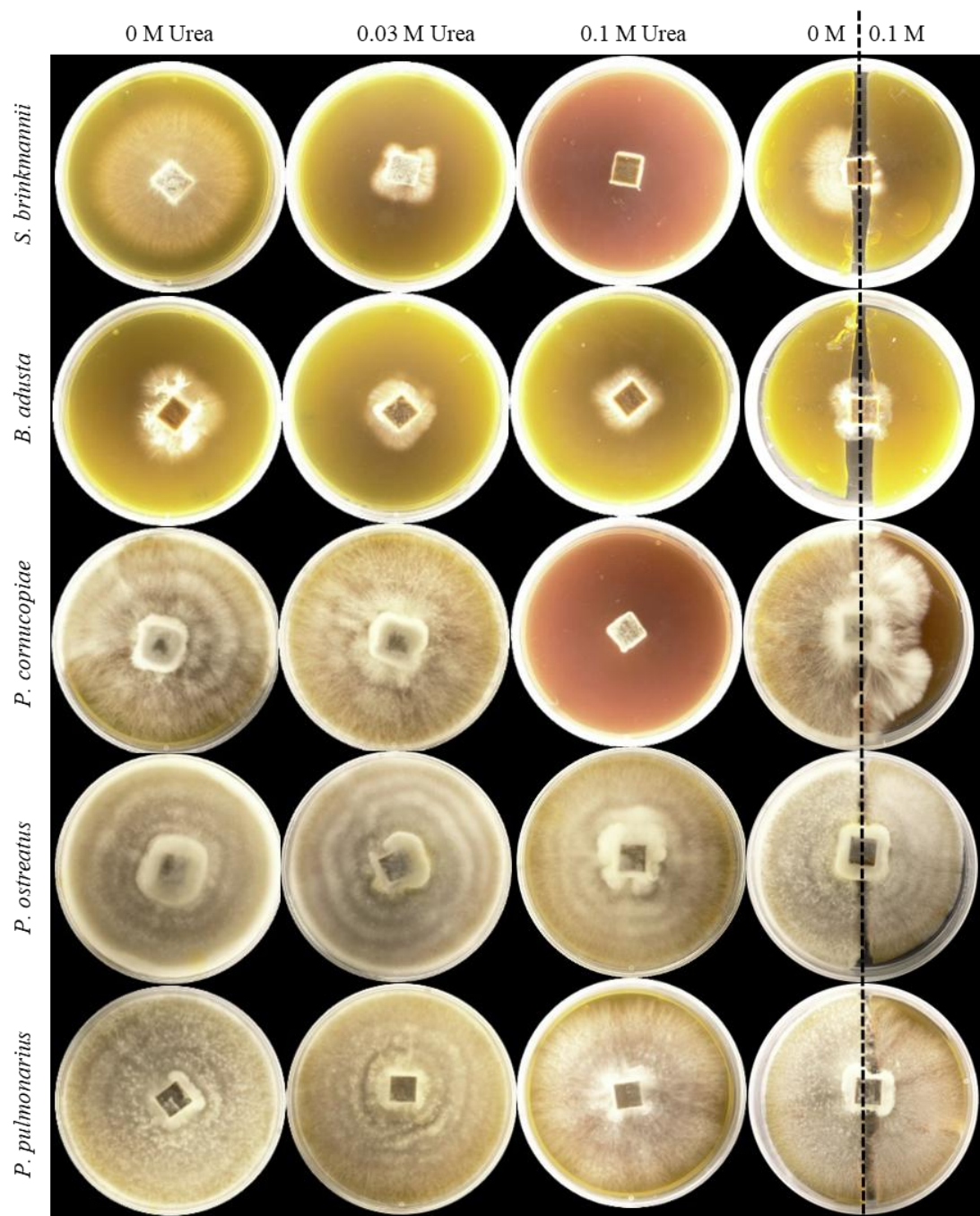


Figure 3.3 Growth after 7 days for *S. brinkmannii*, *B. adusta*, *P. cornucopiae*, *P. ostreatus*, and *P. pulmonarius* grown on agar amended with phenol red and 0 M, 0.03 M or 0.1 M urea. The farthest right hand column displays growth after 7 days when organisms have access both to agar with 0 M urea (on the left) and 0.1 M urea (on the right)

3.3.1.3 Experiment 1.3 Viability and urease activity after 2 weeks exposure to 0.1M Urea

Experiment 1.3 determined if, for the two species for which growth was inhibited by the presence of 0.1 M Urea (*S. brinkmannii* and *P. cornucopiae* Exp 1.1), they were still urease active and/or able to grow after an initial exposure to 0.1 M urea for 14 days. *S. brinkmannii* and *P. cornucopiae* grown in 0.1 M Urea amended agar for 14 days were transplanted onto new agar plates containing 0 M or 0.1 M urea and grown for a further 14 days. For both organisms, no further growth was observed (Figure 3.4) after replating on either 0 M or 0.1 M Urea, suggesting that exposure to a high concentration of ammonium and high pH for an extended period killed the fungi. Interestingly, a small colour change was still observed for *S. brinkmannii* replated in the 0.1 M urea amended agar (Figure 3.4). This colour change (which did not extend across the whole agar plate after 14 days) indicates that despite the lack of growth, in the case of *S. brinkmannii*, some urease activity was retained, but at a reduced level when compared to Experiment 1.1 (Section 3.3.1.1). This is consistent with findings from other studies where dead biomass can still perform ureolysis, although to a lesser degree (Burford et al., 2006).

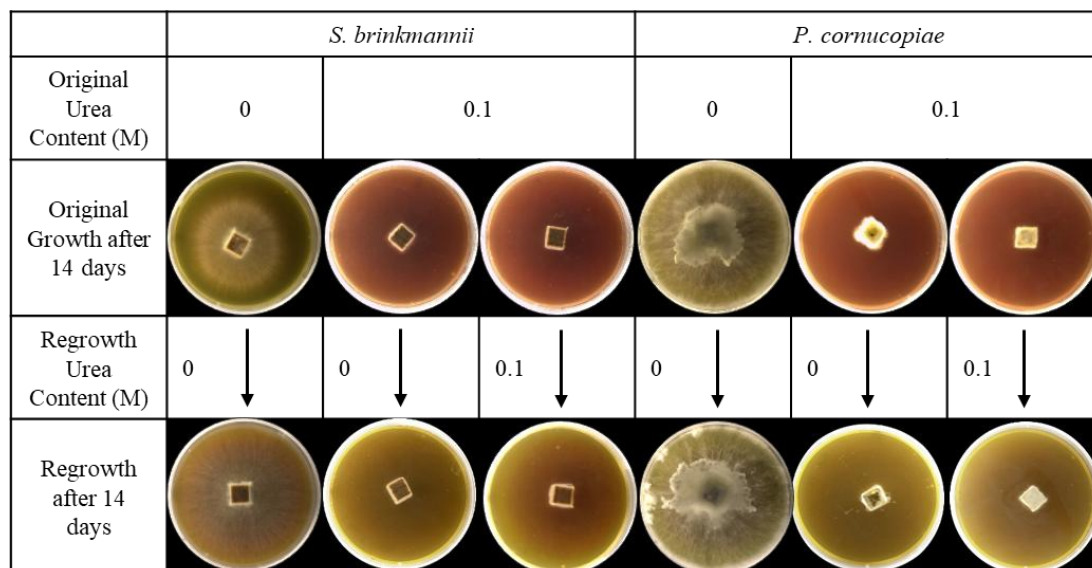


Figure 3.4 Experiment 1.3 Transplanting and regrowth of plates from Experiment 1.1.

3.3.1.4 Experiment 1.4 Urea concentration at which fungal growth is inhibited

Experiment 1.4 was used to determine the concentration of urea that was toxic to the fastest growing urease active fungi, *P. cornucopiae*. Given that *P. cornucopiae* showed improved growth behaviour at 0.03 M, yet growth was entirely inhibited at 0.1 M Urea, Exp 1.1 investigated the growth of *P. cornucopiae* at 7 days in agar with increasing urea concentrations between 0.03 M and 0.1 M. Full-plate growth occurred within 7 days at all urea concentrations up to and including 0.05 M. No growth was observed at 7 days for urea concentrations of 0.06 M and above (Figure 3.5). Hence, the concentration of urea that inhibits growth of *P. cornucopiae* lies between 0.05 M and 0.06 M.

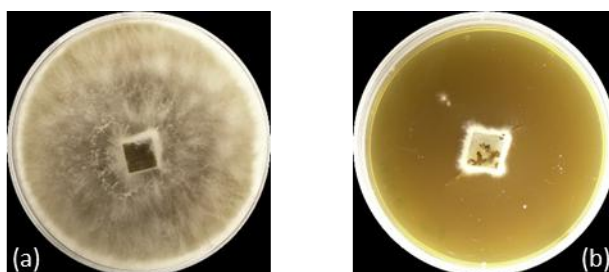


Figure 3.5 Growth of *P. cornucopiae* on agar amended with (a) 0.05 M Urea, (b) 0.06 M Urea

3.3.2 Experiment 2. Ureolysis and calcium carbonate precipitation in liquid media

Experiment 2 was carried out to quantify urease activity and calcium removal from the liquid media for each fungal species, and to analyse the mineralogy of the precipitates. Figure 3.6 presents ammonium concentration (Figures 3.6a, d, g, j, m), pH (Figures 3.6b, e, h, k, n) and calcium concentration (Figures 3.6c, f, i, l, o) over time with four replicates for each fungal species. Data for all replicates is presented. For all fungal species the general trend over time of increasing ammonium concentration, alongside an increase in pH and a decrease in the calcium concentration was observed, indicating ureolytic activity in all specimens, with the exception of Replicates 3 & 4 (red and brown in Figures 3.6d, e, f) of *B. adusta*.

From Figures 3.6a, d, g, j, and m, replicates 1 and 2 of *P. cornucopiae* produced the most ammonium via ureolysis reaching ~0.25 M by day 7 (~37.5 % conversion of available urea). Replicates 1 & 2 (blue and yellow symbols respectively) of *P. ostreatus* converted ~ 20 % of available urea, whereas for all other species and replicates < 12 % was converted. Accordingly, replicates 1 and 2 of *P. cornucopiae* also exhibited the highest pH, as can be seen in Figure 3.6h. Figures 3.6c, f, i, l, and o shows the change in calcium concentration over time and is an indicator of calcium carbonate precipitation. *P. cornucopiae* replicates 1 and 2 precipitated the most calcium out of solution and in the fastest time, removing 91.3 % and 89.0 % of calcium from the solution within 24 hrs, respectively.

Variability was observed in the behaviour between replicates of the same fungal species. During growth, despite being grown in identical conditions, there were differences in the growth behaviour observed visually. In some flasks the fungal mycelium formed in many smaller sized pellets and in others fewer larger sized pellets formed. This may be the primary cause of the variability observed in Figure 3.6 between replicates 1 to 4 for each fungal species. To investigate the relationship between the nature of fungal growth and the ureolysis behaviour, we assigned the replicates that had the smallest sized pellets as Replicates 1 and 2, and the largest sized pellets as Replicates 3 and 4.

Replicates 2 (shown in yellow in Figure 3.6) and 3 (shown in red in Figure 3.6) were selected for fungal biomass determination, to investigate the effect of the two different growth behaviours on fungal biomass production and associated urease activity. Replicate 1 was taken for SEM imaging and Replicate 4 was used for XRD analysis.

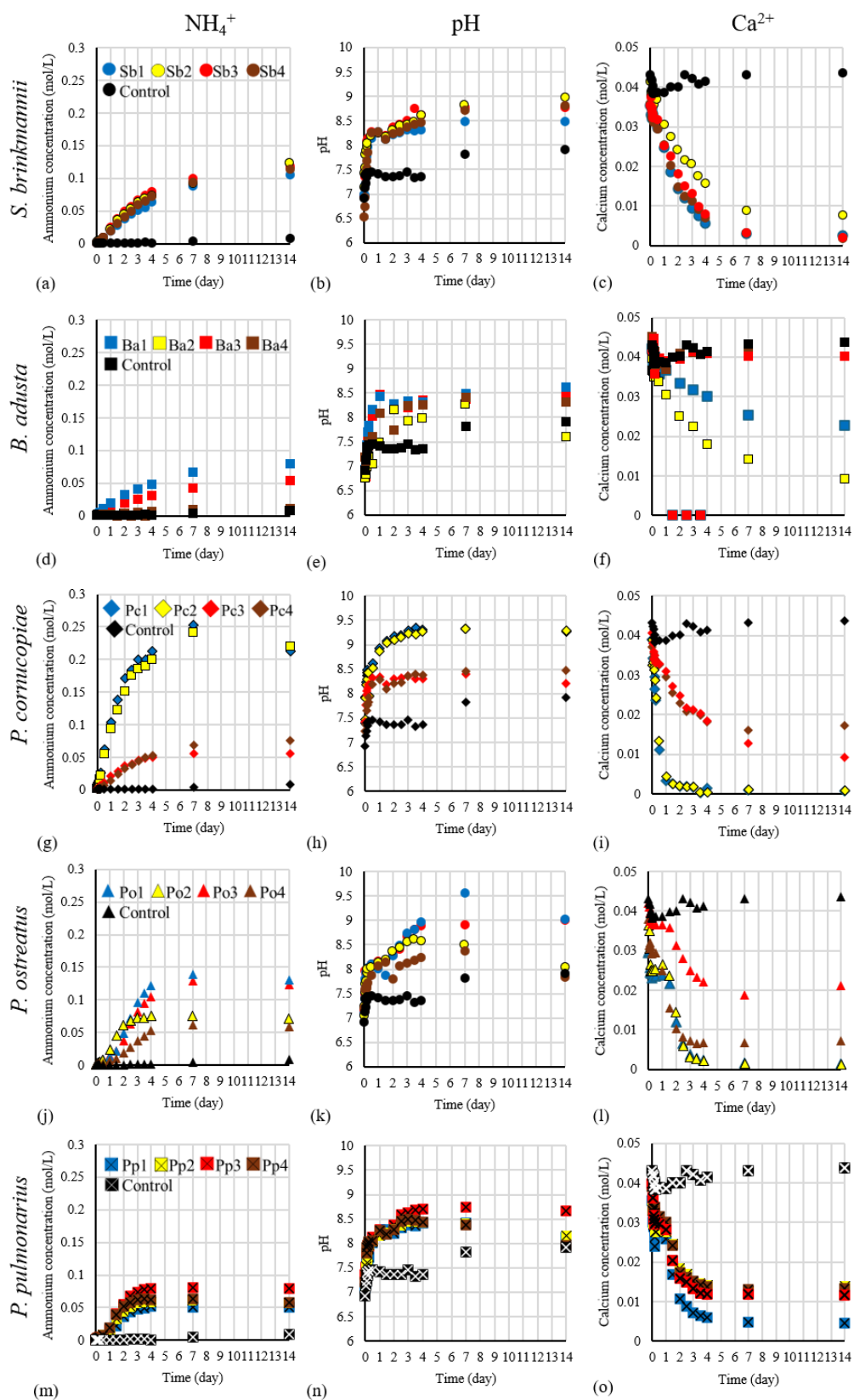


Figure 3.6 Evolution of ammonium, pH, and calcium over time for *S. brinkmannii*, *B. adusta*, *P. cornucopiae*, *P. ostreatus*, and *P. pulmonarius*

3.3.2.1 Fungal biomass and urease activity

Table 3.2 presents the fungal biomass and urease activity determined for replicates 2 and 3 from Experiment 2. *P. cornucopiae* has the highest urease activity of the five fungal species investigated, with an activity between 1.24 – 2.55 mM urea/hr/g, 2.3 to 4.9 times higher than the next highest urease activities exhibited by *B. adusta* (0.55 mM urea/hr/g) and subsequently *S. brinkmannii* (0.52 mM urea/hr/g). This is consistent with the findings in Experiment 1 (Section 3.3.1.1) where *P. cornucopiae* and *S. brinkmannii* exhibited high urease activity via the phenol red assay (Figure 3.3).

Table 3.2 also compares urease activity for each fungal species between Replicates 2 and 3 that comprised the smaller and larger sized pellets, respectively. For ease of viewing, cells shaded in orange show which replicate had the smallest values of biomass and urease activity, cells shaded in green show which replicate had the largest values of biomass and urease activity. For those species where there was a big difference in biomass and urease activity between replicates (*S. brinkmannii*, *B. adusta*, and *P. cornucopiae*), higher urease activity was associated with the smaller sized pellets (Replicate 2). This may be explained by considering that solution transport into the interior of fungal pellets is controlled by diffusion and is dependent on fungal density within the pellet (Schmideder et al., 2021). Due to this process, fungal hyphae located at the surface would have greater access to the cementing solution for ureolysis and for nucleation of calcium carbonate compared to hyphae located within the interior of the pellet. Smaller pellets (Replicate 2) would have a higher surface area for a given fungal biomass compared to larger sized pellets. Interestingly, even when the total biomass of the larger sized pellets replicate was higher than the smaller sized pellets replicate (*S. brinkmannii* in Replicate 3 versus Replicate 2) the surface area effect appears to be the dominating factor, i.e. smaller sized pellets with less total biomass exhibited higher urease activity than the larger sized pellets. *P. ostreatus* and *P. pulmonarius* did not exhibit a large difference in biomass between replicates and the replicates had similar values of urease activity.

Additionally, in four of the five investigated species, growth in smaller pellets resulted in higher biomass when compared to growth as larger pellets. This was likely because fungi mostly grow from the active edge of the hyphal mesh which in this case

was the surface of the pellet. As the smaller pellets had larger surface area, the smaller pellets had more active growing surfaces than the bigger pellets, resulting in overall higher amount of biomass.

Table 3.2 Fungal biomass and urease activity of specimens from Experiment 2

Fungal strain	Fungal biomass (g)		Urease Activity (mM urea/hr/g)	
	Replicate 2*	Replicate 3^	Replicate 2*	Replicate 3^
<i>S. brinkmannii</i>	0.163	0.246	0.52	0.35
<i>B. adusta</i>	0.181	0.018	0.55	0.26
<i>P. cornucopiae</i>	0.217	0.114	2.55	1.24
<i>P. ostreatus</i>	0.320	0.287	0.33	0.38
<i>P. pulmonarius</i>	0.307	0.258	0.20	0.32
* Many Smaller Pellets			^ Fewer Larger Pellets	

3.3.2.2 XRD Analysis

Figure 3.7 shows the results of XRD carried out on a sample from Replicate 4 for each of the fungal species. Calcite (peaks indicated in red) was present in all of the samples tested. No aragonite or vaterite (polymorphs of calcium carbonate) crystals were detected in any of the analysed samples. Additionally, calcium chlorite and urea were detected in the *Pleurotus pulmonarius* sample and calcium chlorite was also detected in the *Sistotrema brinkmannii* and *Bjerkandera adusta* samples.

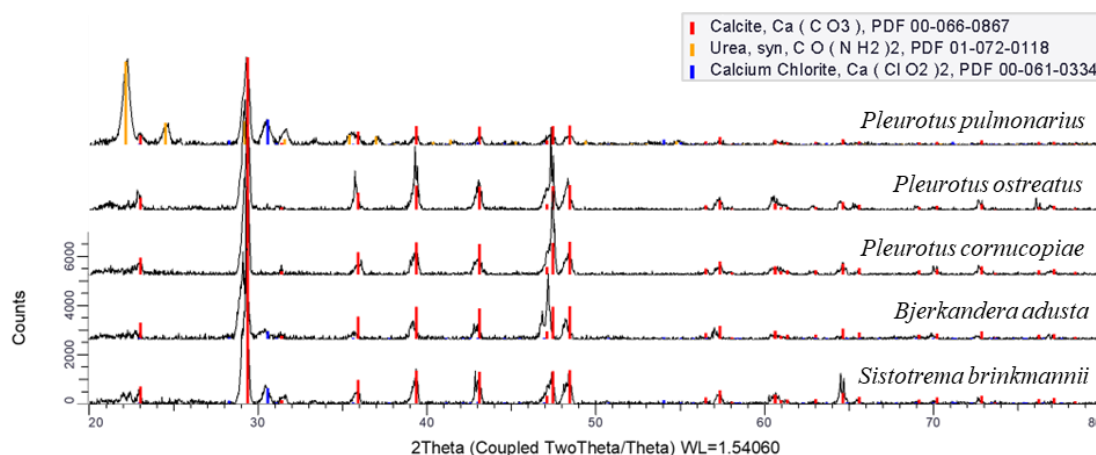


Figure 3.7 XRD analysis of Replicate 4 samples for each fungal species from Experiment 2.

3.3.2.3 SEM

Figure 3.8 shows SEM images of samples from Replicate 1 of *Pleurotus cornucopiae* from Experiment 2. Figure 3.8a shows a piece of large solid mass from Replicate 1 with crystals distributed throughout. Figures 3.8b – e show magnified areas of the specimen in 3.8a. Figure 3.8b shows large calcium carbonate crystals [1], identified based on elemental composition (Ca, C, O), which are covered by a layer of fungal biomass [2] (C, N, O); individual hyphae are evident extending from the layer of biomass. Smaller calcium carbonate crystals are also located on the thin biomass layer [3, 4]. Figures 3.8c and 3.8d show the same large calcium carbonate crystal, with 3.8d at higher magnification; imprints of fungal hyphae are clearly visible on the crystal. Figure 3.8e shows carbonate crystals [1, 4] embedded in biomass [2, 3] both with [4] and without distinguishable hyphae imprints [1]. Figure 3.8f shows another smaller piece of solid mass from Replicate 1 of *Pleurotus cornucopiae*. Figures 3.8g and 8h show two locations from Figure 3.8f at higher magnification. Figure 3.8g shows a series of calcium carbonate crystals which appear to be connected to each other, these may have formed around an individual hypha. Figure 3.8h shows a side profile of a thin layer of biomass [2] which shows calcium carbonate crystals distributed [1, 3, 4] within it.

Figure 3.9 shows SEM images of *S. brinkmannii* and *B. adusta*. For both species there is evidence of calcite crystals coated in layers of biomass with hyphae

distributed throughout the biomass. In the *B. adusta* images (Figures 3.9e - h) the biomass layer appears thicker and more continuous compared to that of *S. brinkmannii*. Similarly, Figure 3.10 presents SEM images of *P. ostreatus* and *P. pulmonarius*. These images also show layers of biomass coating crystals of calcium carbonate, with individual hyphae visible where the biomass has been torn by disturbance of the sample (Figure 3.10b).

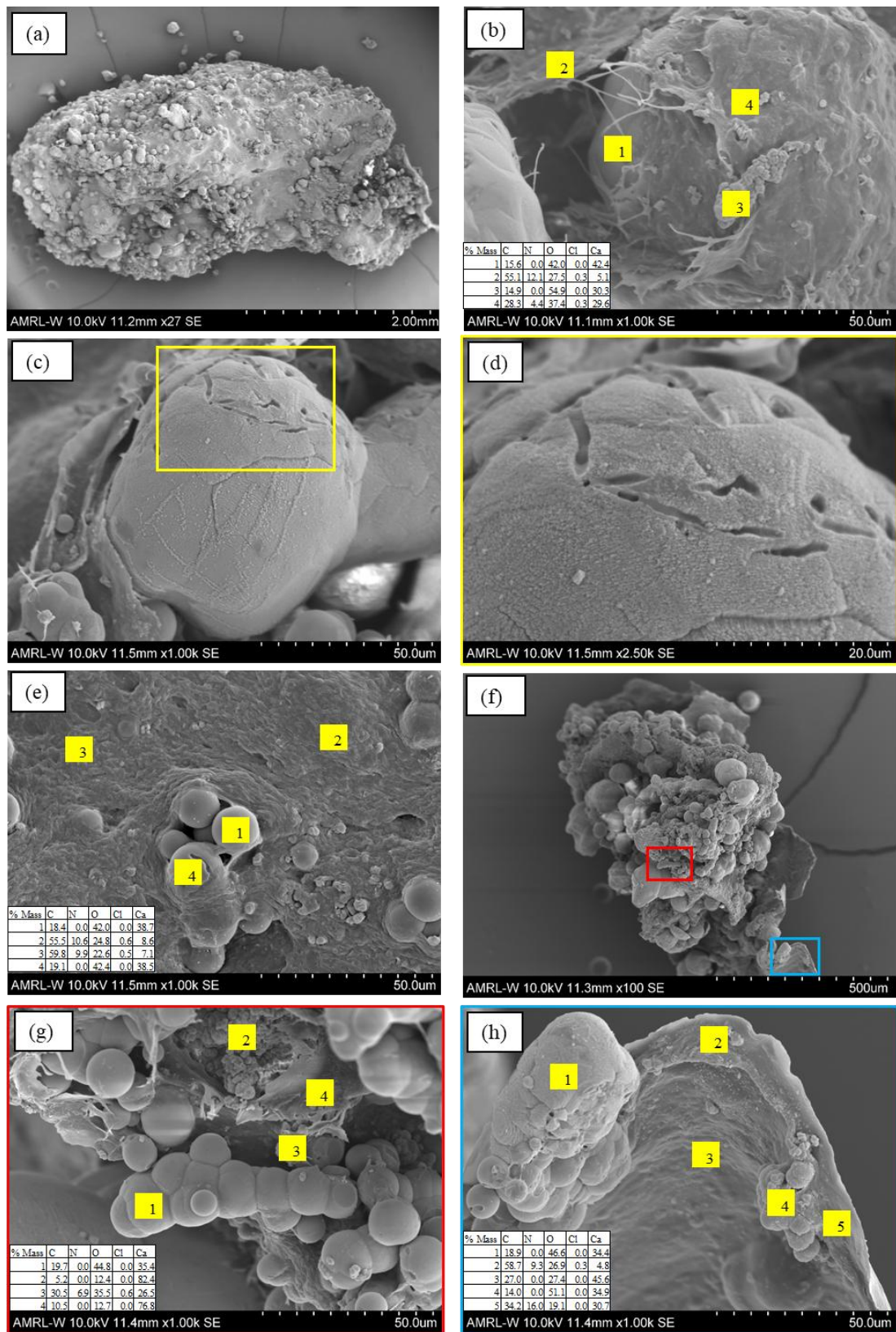


Figure 3.8a – h. SEM Images of *P. cornucopiae* Replicate 1 sampled at end of Experiment 2.

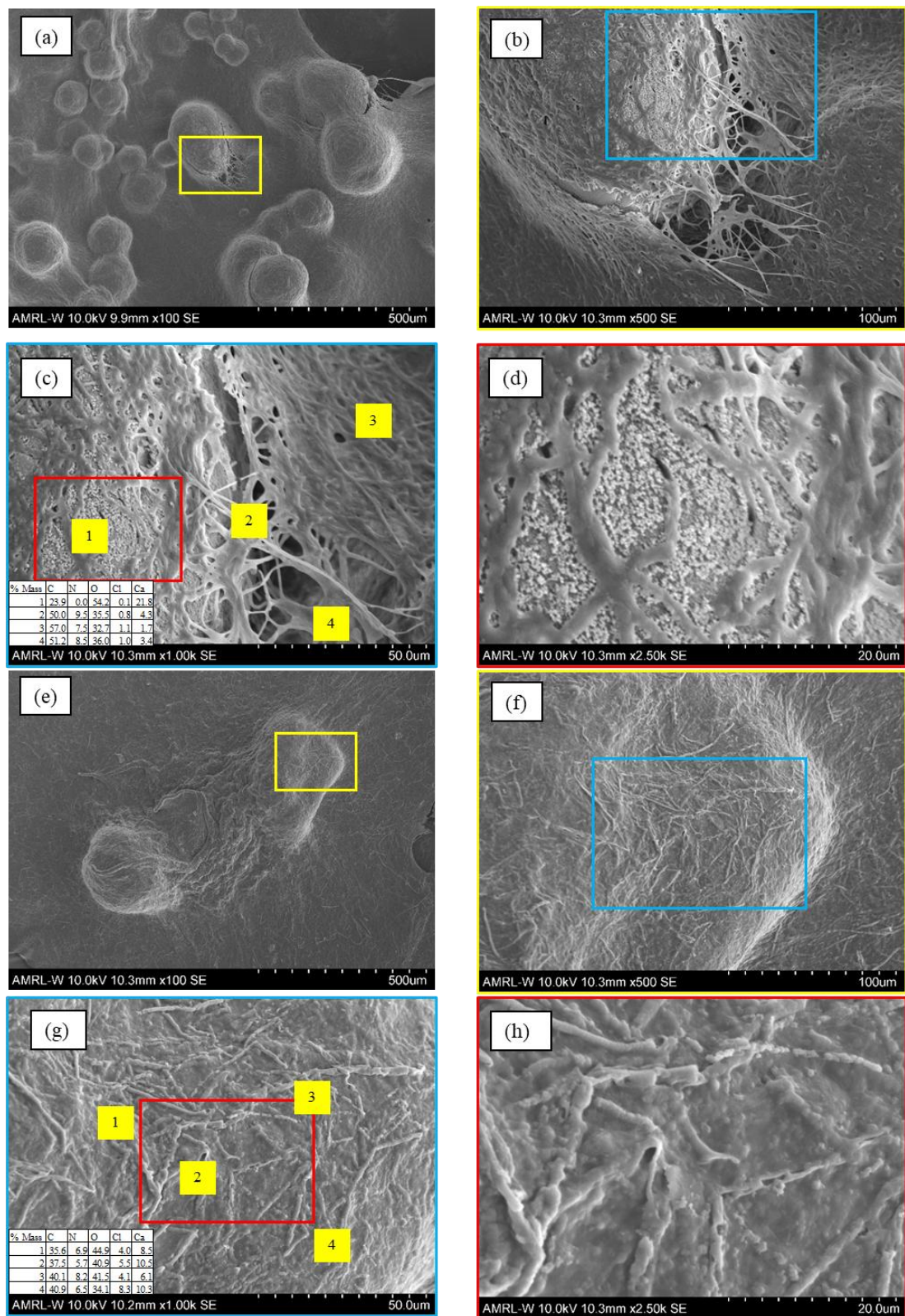


Figure 3.9a – d. SEM images of *S. brinkmannii* Replicate 1 sampled at end of Experiment 2, (e - h) SEM images of *B. adusta* Replicate 1 sampled at end of Experiment 2.

3.4 Discussion

This study investigated the urease activity and growth behaviours of five Agaricomycetes, namely *S. brinkmannii*, *B. adusta*, *P. cornucopiae*, *P. ostreatus* and *P. pulmonarius* and their ability to induce calcium carbonate precipitation. These five species were selected by convenience, due to their ready availability from a culture collection (Cardiff University). A wider screening would likely highlight further useful organisms for the studied process. The three *Pleurotus* species showed the highest growth rates, filling the 90 mm diameter agar plates within 1 week of growth. *P. cornucopiae* was observed to be the most urease active of the fungal strains investigated, assessed both qualitatively in Experiment 1 (Section 3.3.1.1) via phenol red assay, and determined via quantitative chemical analysis in Experiment 2 (Section 3.3.2) (urease activity = 1.24 - 2.55 mM Urea/hr/g of fungal biomass).

High urease activity, resulting in the production of ammonium and an increase in pH has an inhibiting effect on fungal growth. The more urease active species (*P. cornucopiae* and *S. brinkmannii*), experienced greater growth inhibition. For both *P. cornucopiae* and *S. brinkmannii*, 2 weeks exposure to 0.1 M urea concentration resulted in complete inhibition of growth and no further growth when transplanted into fresh media. However, *P. cornucopiae* could grow when exposed to urea concentrations up to 0.05 M Urea; at 0.06 M and above, growth was inhibited. Despite this finding, giving *P. cornucopiae* simultaneous access to a urea-free environment, allowed it to grow on agar with a urea concentration of 0.1 M, albeit the growth was slower than in the urea-free region.

In terms of calcium carbonate precipitation, *P. cornucopiae* exhibited the fastest consumption of Ca^{2+} ions, with between 23.7 % and 91.3 % removed from solution (i.e. precipitated) within 24 hrs.

In Experiment 1.4 (Section 3.3.1.4), it was observed that fungal growth was inhibited at high urea concentrations (0.05 - 0.06 M urea). However, in Experiment 2 (Section 3.3.2) it was demonstrated that the fungal urease enzyme was active in the high urea concentration of 0.33 M used in the cementing solution. This shows that ureolysis could still proceed despite fungal growth being inhibited by the cementing solution. This could have been because the fungal enzyme was produced during the fungal growth stage, prior to exposure to cementing solution. The enzyme remained

present after harvesting of the fungus, either extracellularly in the remnants of the malt extract broth, or intracellularly. During the cementation stage, the fungal cells may have been acting more as passive nucleation sites, rather than ongoing producers of new and fresh urease enzyme.

It should be noted that not only is fungal ureolysis and calcium precipitation dependent on the species, but ureolysis appears to also be influenced by growth behaviour i.e. the size of the fungal pellets formed. Experiments conducted in quadruplicate, with the fungal biomass in submerged conditions, showed different growth behaviours between replicates, with consistent differences in their urease activity – smaller sized pellets resulted in higher urease activity than larger sized pellets for an equivalent total biomass. Although further work should investigate the repeatability of this observation regarding the influence of the size of pellets on ureolytic activity.

Calcium carbonate precipitation was confirmed in all fungal species via XRD and SEM elemental analyses. XRD analysis indicated that calcite was formed, with no aragonite or vaterite detected. SEM images and analyses show that hyphae can become encapsulated by crystals after treatment and there is evidence of hyphal tracks on larger calcite crystals.

3.4.1 Implications for fungal-induced calcite precipitation treatment strategies

Geotechnical applications for fungal induced carbonate precipitation lie in soil improvement, with the overall goals of increasing soil strength, or improving erosion resistance. Given the preference for growth in high oxygen availability environments to maintain aerobic activity (Salifu et al., 2024), it is anticipated that fungal-induced biomineralisation using *Pleurotus* species will be most suited towards surface applications at the soil-air atmosphere, for example surface treatment of slopes to reduce wind and water-driven erosion (Salifu et al., 2024). The combination of rapid growth and high urease activity exhibited by *P. cornucopiae* make it an ideal candidate for geotechnical applications. Although, its growth was inhibited in the presence of high urea concentrations (≥ 0.06 M) as needed for calcium carbonate precipitation via ureolysis (shown in Experiment 1.1, Section 3.3.1.1), the results from Experiment 1.2

(Section 3.3.1.2) indicate that in a high urea environment, if part of the fungal network can still access an area of low urea concentration, growth can continue to occur, enabling the organism to survive the harsh conditions. Thus, if this species is to be deployed for fungal induced calcite precipitation, a two-stage treatment strategy would be recommended: Stage 1 would promote growth of the fungal hyphal network, via nutrient injection or emplacement, to act as a scaffold for calcite precipitation; Stage 2 would involve precipitation of calcium carbonate via injection of cementing solution into the treatment site. Furthermore, stepwise treatment of small regions in Stage 2 could allow for continuous access of the organism to a urea-free environment and ensure its continued survival.

3.5 Conclusions

This study investigates the urease activity and growth behaviours of five agaricomycete fungi, namely *S. brinkmannii*, *B. adusta*, *P. cornucopiae*, *P. ostreatus* and *P. pulmonarius* and their ability to induce calcium carbonate precipitation. The three *Pleurotus* species were the fastest growing species. *P. cornucopiae* was observed to be the most urease active of the fungal strains investigated. The most urease active species (*P. cornucopiae*) exhibited inhibited growth when exposed to urea concentrations of 0.06 M and above. However, growth in high urea concentration environments could occur when part of the fungal network had access to a urea/ammonium free environment. Finally, all species displayed calcium carbonate precipitation after exposure to urea and calcium chloride; this was confirmed via XRD and SEM EDS analyses. These results indicate for the first time that Agaricomycetes could be used to trigger fungal-induced carbonate precipitation for civil and environmental engineering applications.

Chapter 4: Development of Methods for Fungal Induced Calcium Carbonate Precipitation Treatment of Sand Columns

4.1 Introduction

In the previous chapter, *Pleurotus cornucopiae* was identified as a promising candidate for fungal-induced carbonate precipitation (FICP). This fungal species was selected from five agaricomycete fungi native to the UK and non-hazardous to humans. *P. cornucopiae* was found to be the most urease active fungi and had a relatively high growth rate amongst the fungi investigated. This chapter develops various methodologies to enable application of FICP for the treatment of sand columns. Firstly, the method of preparing the fungal inoculant was investigated. Subsequently, preliminary sand columns were prepared and treated via FICP to investigate several variables: (i) influence of beechwood chip size within the sand column composition, (ii) strategies for introducing treatment solutions, and (iii) cementing solution concentrations for FICP treatment. Furthermore, a methodology to determine calcium carbonate precipitation within columns containing both wood chips and fungal biomass was developed. Finally, this chapter also investigates a possible relationship between ergosterol content of columns and fungal respiration, to establish if fungal respiration could be used as a method to non-invasively determine fungal biomass within a sand column.

4.2 Preparation of fungal inoculant suspension

In order to treat sand via FICP, here a liquid fungal inoculant suspension was used as means of adding the fungus in to the sand. The liquid fungal inoculant suspension was prepared using the following methodology. Initially, *P. cornucopiae* was grown on malt extract agar for 4 - 7 days. After which 5mm diameter plugs from the fungal culture (on agar) was added to 1 L of malt extract broth (MEB) (1 plug was added per 100 mL of MEB) in a 2 L conical flask. A wide-bottomed vessel was used to increase the surface area of the broth, and a magnetic stirrer was introduced to oxygenate and increase the homogeneity of the fungal suspension. The inclusion of

the magnetic stirrer was intended to prevent the large ball-like fungal pellets that were observed during the experiments in Chapter 3. The MEB broth was amended with 0.03 M of urea, as the results in Chapter 3 showed that this may increase the growth rate of *P. cornucopiae*. The broth was incubated in the dark, at 20 °C, room temperature. After incubation the fungal suspension was centrifuged at 8000 rpm for 5 minutes and the supernatant poured off. After centrifuging a dense “sludge” of fungal suspension formed at the bottom of the tube. Note that the fungal biomass appeared to more effectively hold onto water during the centrifuging process (compared to *Sporosarcina pasteurii* bacteria). After harvesting, the fungal biomass was resuspended in sterile DI water amended with 0.5 mM NaCl to 10 % of the original broth volume (i.e. for fungal cultures grown in 1 L broth, the pellet was resuspended in 100 mL).

To understand the amount of fungal biomass that was produced during the culturing stage, the fungal biomass collected after centrifuging was freeze-dried and weighed. Table 4.1 presents the dry biomass harvested from 1 L of MEB after incubation for 1, 2, 3, 4, and 5 weeks. Table 4.1 Also indicates the location from which the agar plugs were sampled in order to inoculate the MEB broth, either from the centre of the agar plate (i.e. older mycelium), or from the edge of the fungal growth on the agar plate (i.e. newer mycelium).

Table 4.1 Measured Dry Fungal Biomass

Incubation Time (weeks)	Replicate Dry Biomass (g)		
	1	2	3
1	1.8026 	1.2226 	
2	0.5913 	1.1714 	
3	0.5800 	0.6540 	0.7941 
4	0.5868 	0.5919 	
5	0.6927 		

Inoculated with older mycelium 

Inoculated with newer mycelium 

From Table 4.1, it is evident that lower biomass (< 0.8 g) was harvested from 1 L of broth when the broth had been inoculated with plugs taken from the centre of agar plates (i.e. using older mycelium). In these “older” plates the fungal mycelium had been allowed to grow to fill the full extent of the agar plate (90 mm diameter), and a dense matt of mycelium had formed (growth of > 7 days). In contrast, biomass >

1.2g was harvested from 1 L of broth when the broth had been inoculated with plugs taken from the growing edge of fresh plates before the hyphae had reached the edge of the agar plate (i.e. between 4 - 7 days of growth) and ensured that the inoculant plugs contained highly active hyphal tips. After this trend was identified, the experiment was concluded as it showed that it only took one week for fungus from the edge of fresh plates to result in > 1g of fungal biomass and it appears this higher amount of biomass was sustained between 1 - 2 weeks (central column) suggesting the stationary growth phase had been reached after 1 week.

Experiments 1 and 2 in this Chapter used the fungal inoculant suspension prepared using fungal cultures sampled from the centre of plates (i.e. older mycelium) after 1 week of growth, whereas Experiment 3 used the fungal inoculant suspension inoculated with fungal culture from the growing edge of agar plates after 1 week of growth. All experiments in the following chapters (Chapters 5 and 6), used the method of sampling from the growing edge for inoculating the MEB.

4.3 Preliminary experiments investigating FICP treatment of sand columns

Preliminary experiments were conducted to (i) select the media composition for sand columns, (ii) to identify suitable methods for introducing treatment solutions and (iii) to test cementing solution concentrations.

4.3.1 Methods

4.3.1.1 Material

A uniform coarse sand was selected for treatment; the corresponding particle size distribution is shown in Figure 4.1 (in blue). Beechwood was added to the column to provide a carbon source for the fungus. Three different beechwood particle sizes were investigated: (1) Beechwood chips (orange), (2) Beechwood dust (in grey) and (3) Beechwood coarse dust (yellow). The particle size range and distribution are shown in Figure 4.1. Figure 4.1 Particle Size Distribution of Column Components and Table 4.2 describes the different beechwood particle sizes. The different particle sizes were

investigated to understand if they influenced fungal growth behaviour and carbonate precipitation.

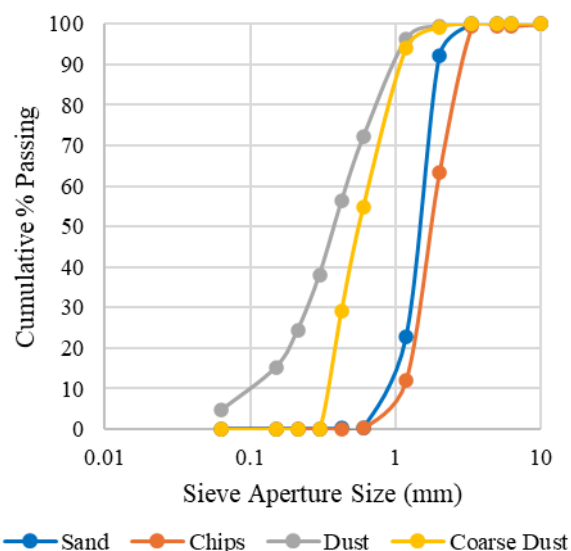


Figure 4.1 Particle Size Distribution of Column Components

Table 4.2 Beechwood Particle Sizes

Beechwood	Particle Size (mm)	Description
Chips	0.6-3.35	Commercial Animal Bedding
Dust	<0.063-2	Commercial Meat Smoking Dust
Coarse Dust	0.3-2	Beechwood Dust with particles smaller than 0.3mm removed (38.15% of mass removed).

4.3.1.2 Specimen Preparation

Each specimen comprised 50 g of solid media (95 % sand, 5 % Beechwood). To this 5 ml of fungal inoculant suspension (prepared as described in Section 4.2) was added and mixed manually throughout the media, before lightly compacting the media into the columns. The columns were made from acrylic with an internal diameter of 38 mm. A fine plastic mesh was attached to the bottom of the column with super glue to retain sand particles within the column (and avoid loss of particles during treatment).

In these preliminary experiments presented in this Chapter, (Experiments 1 and 2) relied upon visible growth to determine the required duration for fungal growth in the columns. Columns were incubated for 3 - 4 weeks at 25 °C in the dark until similar visible levels of white mycelium were observed.

4.3.1.3 Cementing Solution

After incubation, cementing solution was introduced into the columns. Here, cementing solution refers to a solution comprising both calcium chloride and urea. In Experiment 1, a low concentration of 0.05 M calcium chloride and 0.33 M urea was used as the cementing solution (following the concentration investigated by Li et al. 2014, and the concentration investigated in Section 3.2.2). Experiment 2 (Section 4.3.2.2) used an equimolar solution of 0.33 M calcium chloride and urea to test if higher concentrations of calcium chloride would lead to a greater mass of calcium carbonate precipitates.

4.3.1.4 Treatment Strategy

Experiments 1 and 2, presented here in Sections 4.3.2.1 and 4.3.2.2 respectively, used gravity-controlled flow to introduce cementing solution into the columns of fungal colonised sand. For Experiments 1 and 2, the columns were placed in beakers, and cementing solution poured down the side of the beaker, until the soil within the column was submerged and the level of cementing solution within the column became equal to that in the external beaker (see Figure 4.2). Here, the introduction of the cementing solution via the base of the column was intended to reduce the presence of air bubbles and minimise preferential flow pathways.

Previous research at the University of Strathclyde (Salifu et al., 2022; Salifu & El Mountassir, 2021) has shown that soil colonised by *Pleurotus ostreatus* exhibits strong hydrophobic properties. *Pleurotus cornucopiae* is closely related to *P. ostreatus* and also exhibits strong hydrophobic properties. Hydrophobic soils resist wetting, it becomes more difficult for wetting fluids (water, cementing solution) to displace air from the pores, meaning that air pockets can become trapped within the matrix and wetting fluids are likely to form preferential pathways. Introducing the cementing solution via the base of the column (across the entire cross-section rather than at a single injection point) was intended to reduce the formation of preferential flow pathways.

In each treatment cycle, the column was submerged in the cementing solution for 24 hours. After each treatment cycle was complete (i.e. immersion in cementing

solution), the column was removed from the cementing solution and the fluid allowed to drain by gravity. In subsequent cycles cementing solution was also introduced via the base of the column.

The effect of multiple treatment cycles was investigated to see whether introducing multiple cycles resulted in increased calcium carbonate precipitation beyond the first cycle. This was to understand if there was still sufficient active urease enzyme present, and nucleation sites available for carbonate precipitation.

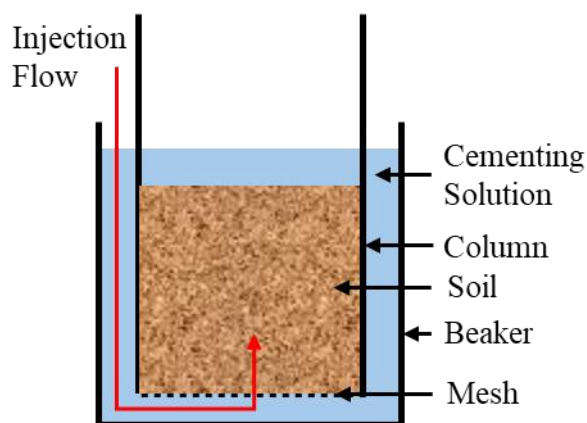


Figure 4.2 Sand Column Construction and Treatment Strategy

4.3.1.5 Media Disruption

After fungal colonisation, the sand columns exhibited strong hydrophobicity, especially close to the ends of the columns at the top and bottom where the fungus was exposed to the air. As a result, the cementing solution had difficulty penetrating through the soil pores due to gravity alone (under the small hydraulic head applied), unless the fungus had less growth time and therefore fungal mycelium growth was less dense. To counter this, some columns were *Hole-punched*. A 10 mm diameter hole was punched all the way down the centre of the column using a glass stirring rod, as shown in Figure 4.3. This broke the accumulated hydrophobicity at either end of the column, whilst maintaining most of the fungal structure. However, there was still hydrophobicity within the rest of the sample which meant that the cementing solution was not always able to visually saturate the column, as there were air bubbles visually present within. In a second set, columns were *Mixed* after the fungal incubation period, whereby the sand columns were manually stirred with a glass stirring rod to break the

hyphal network and weaken the hydrophobicity in the sample, with the intention being to enhance distribution of the cementing solution throughout the column.

These methods of media disruption are not likely to be relevant for future field applications and were used for expedience sake in the lab.

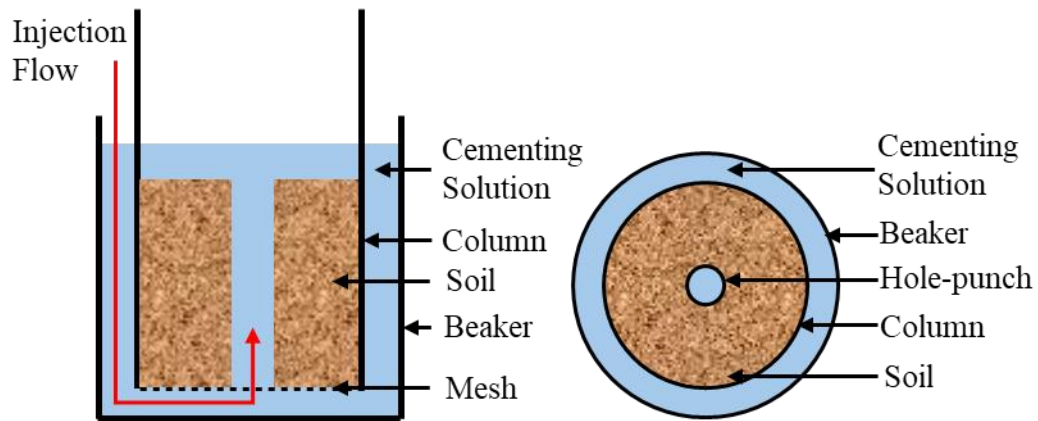


Figure 4.3 Hole-punched Column Construction

4.3.1.6 Experimental Campaign

Table 4.3 summarises the variables tested in Experiment 1 and 2. Experiment 1 tested the three different particle sizes of the beechwood (chips, dust and coarse dust), and two methods of disrupting the media: *Mixed* and *Hole-punched* columns. These different methods of media disruption were tested due to the hydrophobicity of the fungal soil. The solution had difficulty penetrating the soil without either disruption to the hydrophobicity or pressurised injection. In Experiment 1, all columns received a total of 6 cycles of cementing solution, with the first 3 cycles receiving 10 ml of cementing solution, and the second 3 cycles receiving 15 ml of cementing solution per cycle. In Experiment 2, again the particle size of the beechwood additives was investigated as well as the number of treatment cycles that each specimen received (either 5 or 10 treatment cycles of 15 ml cementing solution per cycle).

Table 4.3 Experiments 1 and 2 Summary

Exp. #	Broth/ Column Incubation Time (days)	Beechwood Particle Size	Mixed or Hole-Punched	Cement Solution (M)	Treatment Cycles	Maximum Potential Calcium Carbonate Precipitated (g)
1	21/24	Chips	Mixed	0.33 Urea, 0.05 CaCl ₂	3 x 10 ml cycles then 3 x 15 ml cycles	0.375
		Chips	Hole-Punched			
		Coarse Dust	Mixed			
		Coarse Dust	Hole-Punched			
		Dust	Mixed			
		Dust	Hole-Punched			
2	21/31	Chips	Hole-Punched	0.33 Urea, 0.33 CaCl ₂	5 x 15 ml	2.5
		Coarse Dust	Hole-Punched		10 x 15 ml	5
		Dust	Hole-Punched			
		Chips	Hole-Punched			
		Coarse Dust	Hole-Punched			
		Dust	Hole-Punched			

4.3.1.7 Calcium Carbonate Determination by Mass Lost

Calcium carbonate precipitation was determined in Experiment 1 and 2 by mass loss after digestion with 1 M HCl. After FICP treatment, the contents of each column were submerged in an acid bath for at least 15 minutes and until no further fizzing was observed before being washed and dried. The contents of the columns were dried and weighed before and after acid digestion to determine the mass of calcium carbonate precipitated.

4.3.2 Results

4.3.2.1 Experiment 1

The purpose of Experiment 1 was to explore the influence of beechwood particle size and the disruption technique (*Mixed* vs *Hole-punched*) on the mass of calcium carbonate precipitated. Table 4.4 shows the results for Experiment 1. This table shows the mass of calcium carbonate precipitated in grams and as a percentage of the maximum theoretical calcium carbonate that could have been precipitated based on the volume and concentration of calcium chloride introduced during the experiment (0.05 M of CaCl_2).

Table 4.4 Experiment 1 Results

Particle Size	Mixed Soil		Hole-Punched Soil	
	Calcium Carbonate Precipitated (g)	Calcium Carbonate Precipitated (% of theoretical maximum)	Calcium Carbonate Precipitated (g)	Calcium Carbonate Precipitated (% of theoretical maximum)
Chips	0.3791	101.1	0.3155	84.4
Coarse Dust	0.3873	103.3	0.3124	83.3
Dust	0.3633	96.9	0.3791	101.2

In the *Mixed* samples, the amount of calcium carbonate precipitated was close to the maximum possible mass > 96 % for all beechwood particle sizes. This indicates that the limiting factor for calcium carbonate precipitation was calcium chloride concentration. For two of the cases (Chips and Coarse Dust) the mass of precipitation exceeded the maximum possible; this is likely due to partial acid digestion of the fungal biomass and beechwood present and is further discussed in Section 4.4. In this experiment, for every 10 ml of cementing solution injected, a maximum of 0.05 g of calcium carbonate would be expected to precipitate. For every 15 ml, a maximum of 0.075 g would be expected. Since masses of greater than 0.3 g were achieved after treatment in all *Mixed* samples, this indicates that calcium carbonate precipitation remains achievable beyond the first treatment cycle. This indicates that multiple cycles of treatment are effective at precipitating more calcium carbonate than would be

possible with just one cycle of treatment, even when exposing *P. cornucopiae* to a high urea concentration (0.33 M), indicating that ureolytic activity is being maintained.

In general, the *hole-punched* columns showed slightly lower calcium carbonate precipitation (although note these are single specimens, without any replicates in these preliminary experiments). This is likely due to the hydrophobicity of the hyphal mesh inhibiting ingress of the cementing solution throughout the whole column. In the *Mixed* columns, the stirring action likely disrupted the fungal mycelium such that, in the *hole-punched* columns, the cementing solution was able to access a greater volume of the overall specimen (and thus a greater amount of fungal biomass). It was visibly observed that the cementing solution infiltrated more easily into the *Mixed* specimens. Although the mixing scenario appeared to result in slightly higher masses of carbonate precipitation, mixing of the soil would require additional energy input (and cost) for field-scale ground improvement applications and as such as a treatment strategy, *Mixing* is not further studied in this thesis.

From Experiment 1, the influence of the size of the beechwood additive is inconclusive due to the small masses of carbonate precipitated and the error in the calcium carbonate mass precipitated due to likely digestion of the organic matter.

4.3.2.2 Experiment 2

The purpose of this experiment was to further investigate if multiple treatment cycles would result in increased calcium carbonate precipitation, by comparing the mass of calcium carbonate precipitated after 5 treatment cycles and after 10 cycles. The effect of different beechwood particle sizes on calcium carbonate precipitation was again explored. This experiment used a higher concentration equimolar cementing solution of 0.33 M urea and CaCl_2 in an attempt to precipitate higher masses of calcium carbonate than in Experiment 1. Table 4.5 shows the results for Experiment 2.

Table 4.5 Experiment 2 Results

Particle Size	5 cycles		10 cycles	
	Calcium Carbonate Precipitated (g)	Calcium Carbonate Precipitated (% of theoretical maximum)	Calcium Carbonate Precipitated (g)	Calcium Carbonate Precipitated (% of theoretical maximum)
Chips	1.6075	64.3	2.3261	46.5
Coarse Dust	0.5092	20.4	0.6492	13
Dust	0.3848	15.4	0.9668	19.3

In all three cases, there was a greater increase in the mass of calcium carbonate precipitated after 10 treatment cycles, compared to after 5 treatment cycles, although the efficiency of the calcium carbonate precipitation was lower after 10 cycles for Chips and Coarse Dust. One cycle of treatment in Experiment 2 (15 ml cementing solution per cycle) with 100 % efficiency would result in precipitation of 0.5 g of calcium carbonate. Any column with more than 0.5 g of calcium carbonate can therefore be said to have precipitated calcium carbonate over multiple treatment cycles. This can be seen most clearly in the Beechwood Chips column. After 5 cycles, 1.6 g of calcium carbonate had precipitated, and after 10 cycles, 2.3 g of calcium carbonate had precipitated. The Coarse Dust and Dust columns exhibited less calcium carbonate precipitation than the Chip columns and showed a much smaller increase in calcium carbonate precipitation with increasing treatment cycles (comparing 5 and 10 cycles).

In Experiment 2, columns with the beechwood chips showed the highest mass of calcium carbonate precipitation. This may be due to multiple reasons: (i) the size of the beechwood source may have influenced fungal growth (e.g. by modifying aeration within the column), with potentially less growth occurring in the columns containing beechwood dust and (ii) the size of the beechwood source may have influenced the hydraulic behaviour of the column, e.g. the inclusion of the dust may have contributed to lower hydraulic conductivity (pore clogging) and potentially more preferential flow after fungal growth in these specimens, resulting in the cementing solution accessing a lower soil volume. For the same reason, draining of the columns would be less efficient in columns containing beechwood dust as there would be a higher retention

of fluids between cycles, and therefore potentially less new cementing solution introduced. Based on the results of Experiment 2, beechwood chips was selected as the carbon source for inclusion in all future column studies in this thesis.

4.3.3 Comparison of Experiment 1 and 2 Results

The *Hole-punched* columns from Experiment 1 received in total 75 ml of cementing solution. The mass of calcium carbonate precipitated in these columns is compared in Table 4.6 with that precipitated within the *Hole-punched* columns from Experiment 2 which were also treated with a total of 75ml of cementing solution. They received the same total cementing solution volume but with varying calcium chloride concentration in the cementing solution which was 0.05 M (Experiment 1) and 0.33 M (Experiment 2) respectively.

Table 4.6 Comparison of Experiment 1 and 2 results. Mass of calcium carbonate precipitated in grams and as a percentage of maximum theoretically precipitated

Particle Size	From Experiment 1		From Experiment 2	
	0.05 M CaCl ₂ , 0.33 M Urea 1:7, Ca:Urea		0.33 M CaCl ₂ , 0.33 M Urea 1:1, Ca:Urea	
	Calcium Carbonate Precipitated (g)	Calcium Carbonate Precipitated (% of theoretical maximum)	Calcium Carbonate Precipitated (g)	Calcium Carbonate Precipitated (% of theoretical maximum)
Chips	0.3791	101.1	1.6075	64.3
Coarse Dust	0.3873	103.3	0.5092	20.4
Dust	0.3633	96.9	0.3848	15.4

Considering the columns with beechwood chips, this comparison shows that a higher concentration of calcium relative to urea can result in more mass of calcium carbonate precipitated (1.6 g compared to 0.38 g) however the efficiency of precipitation is reduced (64.3 % compared to 101.1 %).

Given that Experiment 1 resulted in calcium conversion efficiencies > 100 %, it was thought likely that mass loss of the beechwood and fungal biomass was also occurring. To test this, the mass loss during acid digestion of the various media

components were tested: Beechwood chips, beechwood coarse dust, dust and fungal biomass. Each of the components were initially dried at 105°C overnight to remove moisture and then the dried mass measured before and after acid digestion. Table 4.7 presents the results.

Table 4.7 Acid Digestion Mass Loss

Material	Initial Mass (g)	Mass Lost (g)	Mass Lost (%)
Chips	9.8008	0.3197	3.26
Coarse Dust	13.9197	0.3535	2.54
Dust	11.8329	0.3484	2.94
Fungus	0.0210	0.0089	42.38

Table 4.7 indicates that an average of ~3 % of beechwood biomass (whether in chips, coarse dust or dust form) may be lost during acid digestion. Considering the initial beechwood mass of 2.5g that was used in Experiments 1 and 2, this could result in ~0.075g of mass lost from beechwood during acid digestion in each column. Combined with the low mass of calcium carbonate precipitation determined (as little as ~0.3g), the error associated with the mass loss could have been as high as 25 %. This error was without considering the mass also lost due to fungal biomass during digestion but the more significant error was likely provided by the beechwood, as the fungal mycelium did not visibly look dense enough to have a major contribution to the error on digestion. This up to 25% error was large, so a method had to be devised to remove the organic fraction from the columns before acid digestion. The development of this new method is presented in the following section.

4.4 Improved Method for Calcium Carbonate Determination in soils containing an organic fraction

Following ASTM D3437-21, the pressure change from the production of carbon dioxide during acid digestion of calcium carbonate can be used to indirectly measure calcium carbonate content of sands. However, as shown in Table 4.7, fungal biomass and beechwood can also be partially digested using 1 M HCl and, as such, the organic fraction needs to be removed prior to acid digestion.

4.4.1 Removal of Organic Fraction

In Section 3.2.2, thermal desorption using pyrolysis-gas chromatography-mass spectrometry (py-GCMS) following the procedures laid out in Stillings et al. (2023) was performed on various fungal species and on calcium carbonate (98 % purity). The results are presented again in Figure 4.4 Thermal Desorption (a) of Fungal biomass from five fungal species and of 98 % pure calcium carbonate and (b) of 98 % pure calcium carbonate and of biomineralized fungal biomass sampled from Section 3.3.2a. Furthermore, Figure 4.4b presents the thermal desorption via py-GCMS of biomineralised fungal biomass, sampled from the batch experiments in Section 3.3.2. It is evident that a temperature of 600 °C delineates the mass loss due to removal of fungal biomass and mass loss due to calcium carbonate removal.

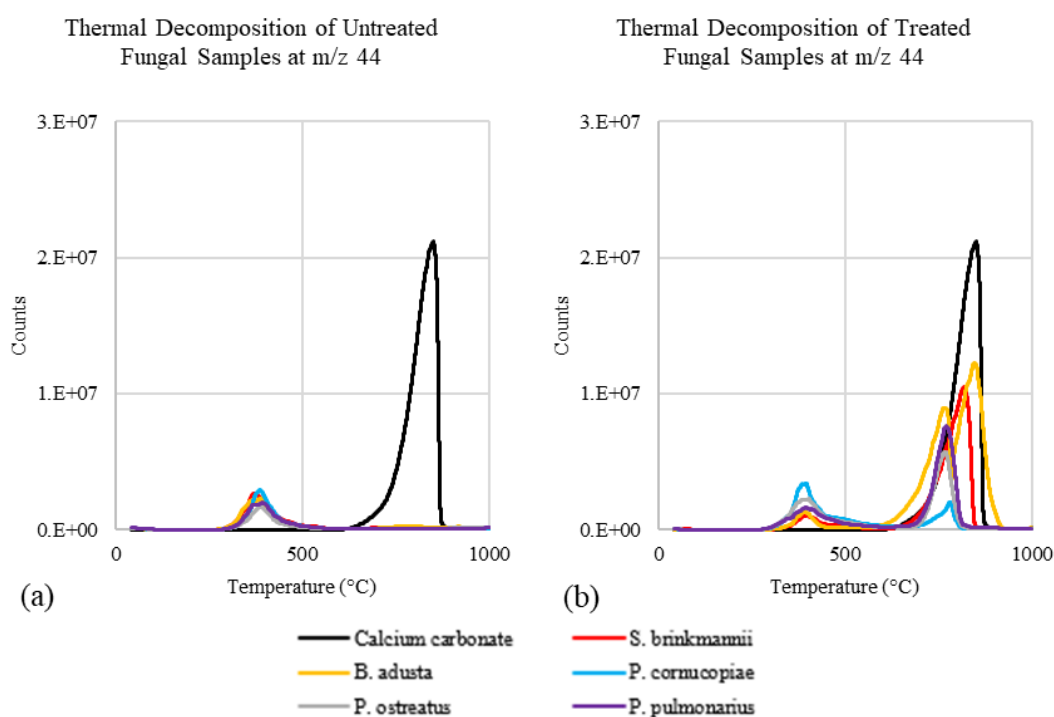


Figure 4.4 Thermal Desorption (a) of Fungal biomass from five fungal species and of 98 % pure calcium carbonate and (b) of 98 % pure calcium carbonate and of biomineralized fungal biomass sampled from Section 3.3.2

In order to investigate the temperatures required for beechwood removal thermogravimetric analysis (TGA) was also carried out using a NETZSH STA 449 F1

Jupiter and was used to analyse all the various components of the columns tested in the experiments from Sections 4.3.2.1 and 4.3.2.2: Beechwood chips, Beechwood dust, *Pleurotus cornucopiae* biomass harvested from culturing on agar, *Pleurotus cornucopiae* biomass harvested from growing in Malt extract broth, sand and 98 % pure calcium carbonate. Heating was carried out in a nitrogen atmosphere at a rate of 5 °C/min. Figure 4.5 presents the rate of mass change per minute determined via TGA analysis. Although there is overlap regarding the temperatures at which fungal biomass is removed and the temperatures at which beechwood chip mass loss occurs, there is a clear separation between removal of fungal biomass or beechwood and the mass loss due to calcium carbonate decomposition. Again, the results indicate that 600 °C would be a suitable temperature to remove fungal biomass and beechwood. Also of note is that when comparing the peaks of pure calcium carbonate and biogenically produced calcium carbonate associated with each of the fungal species, it can be seen that biogenically produced calcium carbonate can display some variation in width and position of desorption peak for different fungal species.

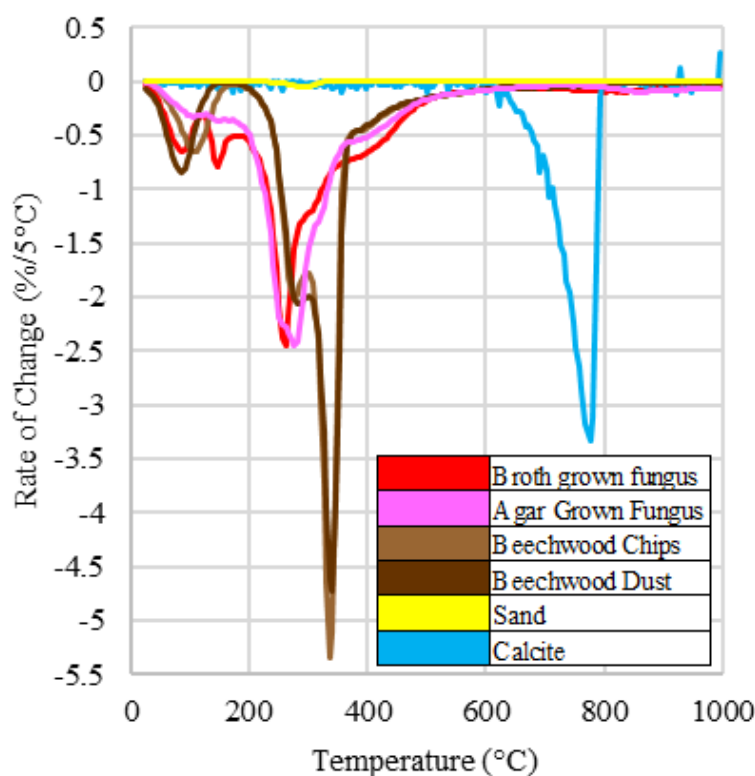


Figure 4.5 Thermal Gravimetric Analysis: Mass Rate of Change

It should be noted here, that py-GCMS analysis uses a helium atmosphere (results in Figure 4.4), and TGA (results in Figure 4.5) uses a nitrogen atmosphere. Both of these atmospheres are inert and do not support combustion. Also, both techniques required very small sample sizes (5 mg for py-GCMS, and up to 5 g for TGA) and would not be suitable for removal of the organic fraction prior to acid digestion of bulk samples from column experiments. For large samples, the organic fraction would need to be removed via heating in an oven and would involve an oxygen rich atmosphere. In practice, this would cause complete combustion which may lower the temperature at which these materials would be removed. To account for the difference in the combusting environment, the optimal temperature for removal of biomass from the soils was determined directly in the oven to be used. BS EN ISO 18122-2022 was used as a guide for using a muffle furnace to maximise removal of the organic material while minimising removal of the calcium carbonate. Following this standard, the soils should be furnace at 550 °C to remove the organic fraction. Temperatures ranging from 490 - 560 °C were investigated for their effects on the column materials including: sand, beechwood chips, calcium carbonate (purity 98 %), biomineralised sand (sand in which calcium carbonate precipitation was induced by *S. pasteurii*), *Pleurotus cornucopiae*, and beechwood chips colonised with *Pleurotus cornucopiae*.

Following BS EN ISO 18122-2022, samples and crucibles were dried separately overnight at 105 °C. Crucibles and samples were then weighed separately, and samples placed into crucibles and weighed again. The crucibles with their samples were then placed into the muffle furnace and the temperature ramped to 250 °C over one hour. This temperature was maintained for another hour to remove any volatiles. The temperature was then ramped to 490 °C at 10 °C/min and the temperature maintained for six hours. The temperature was maintained to ensure that all samples were suitably ignited, as the beechwood was resistant to ignition. The samples were then cooled in a desiccator and weighed before being placed back in the oven at 490 °C, maintaining the temperature for another 6 hours. The samples were cooled and reweighed, checking if the mass change was lower than 0.05 % of the initial mass. If it was not, then the process was repeated until the mass change was lower than 0.05 % of the initial mass.

Once a mass change below this threshold was achieved, samples would be placed back in the oven at a temperature 10 °C higher than previously, and the process repeated until the sample mass change was lower than 0.05 % once again. The temperatures used were 490 - 560 °C at 10 °C increments.

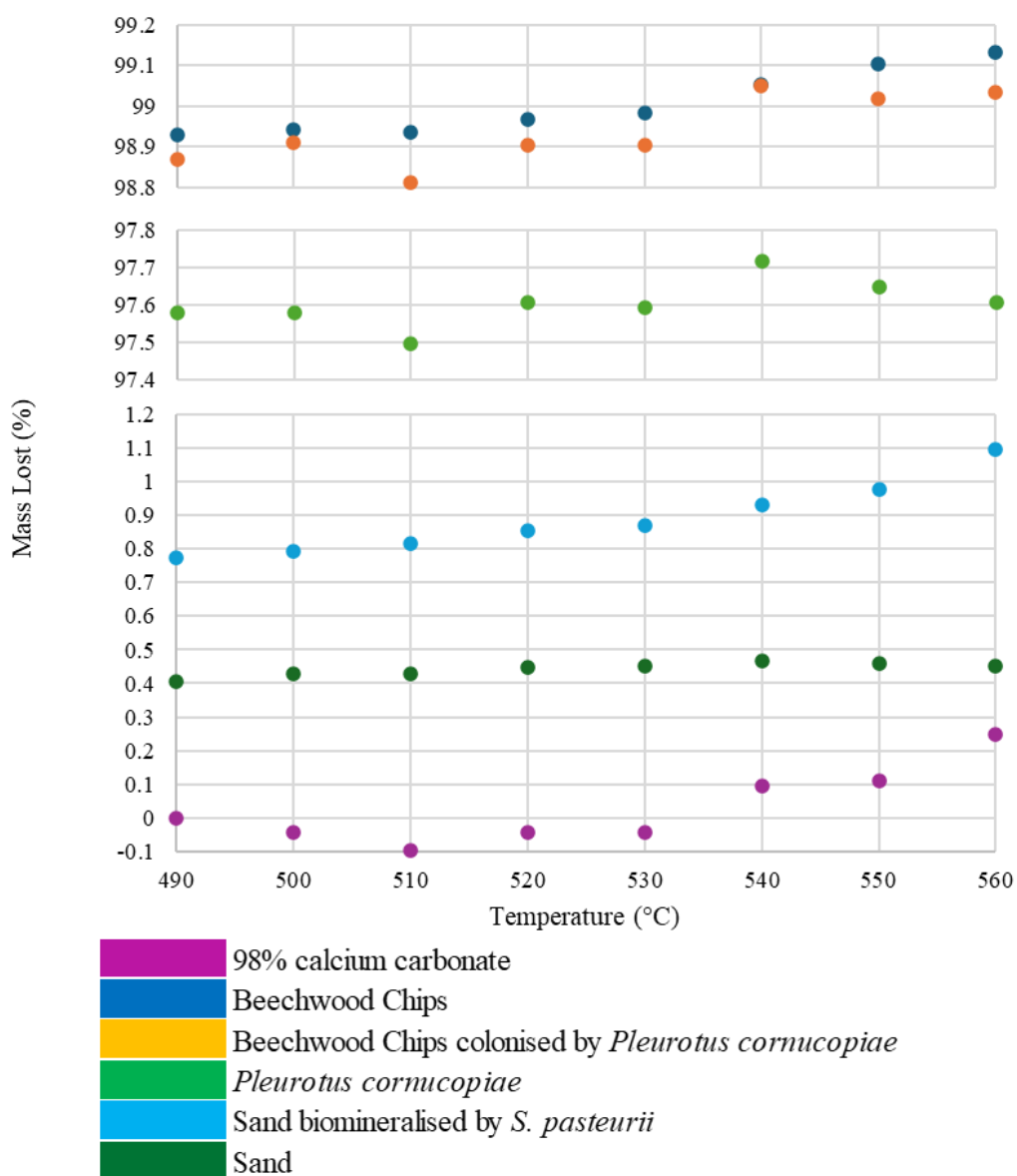


Figure 4.6 Percentage mass lost by each material after ignition at various temperatures

Figure 4.6 Figure 4.6 Percentage mass lost by each material after ignition at various temperatures shows that ignition at 530 °C removed 99 % of Beechwood Chips, 98.9 % of colonised Beechwood Chips, 97.6 % of dried fungal biomass, 0.9 %

of biomineralized sand, 0.45 % of Sand, and 0 % calcium carbonate. Ignition at 540 °C removed only slightly more beechwood and fungal biomass, whilst also removing 0.1 % calcium carbonate. There was a steady increase in the % mass loss of calcium carbonate and biomineralized sand (or sand containing microbially induced calcium carbonate precipitate) as the temperature increased above 530 °C. For this reason, it was determined that samples from FICP treated sand columns would be ignited at 530 °C to remove the organic fraction prior to acid digestion.

4.4.2 Acid Digestion

The method for acid digestion for determination of calcium carbonate followed ASTM D4373-21. The mass of calcium carbonate can be determined by considering the moles of dissolved and gaseous CO₂ generated as a result of reacting calcium carbonate with HCl:

$$m = M \times n \quad [\text{Eq 4.1}]$$

$$m_{\text{CaCO}_3} = M_{\text{CaCO}_3} \times (n_{\text{CO}_2(\text{aq})} + n_{\text{CO}_2(\text{g})}) \quad [\text{Eq 4.2}]$$

$$n = C \times V \quad [\text{Eq 4.3}]$$

$$n_{\text{CO}_2(\text{aq})} = C_{\text{CO}_2(\text{aq})} \times V_A \quad [\text{Eq 4.4}]$$

$$n_{\text{CO}_2(\text{aq})} = k_1 \times P_{\text{CO}_2(\text{g})} \times V_A \quad [\text{Eq 4.5}]$$

$$n = \rho \times V \quad [\text{Eq 4.6}]$$

$$n_{\text{CO}_2(\text{g})} = k_2 \times P_{\text{CO}_2(\text{g})} \times (V_T - V_A) \quad [\text{Eq 4.7}]$$

where:

m_{CaCO_3} = mass of calcium carbonate (g)

M_{CaCO_3} = Molar Mass of calcium carbonate (g/mol)

$n_{\text{CO}_2(\text{aq})}$ = dissolved carbon dioxide (moles)

$n_{\text{CO}_2(\text{g})}$ = undissolved carbon dioxide (moles)

$C_{\text{CO}_2(\text{aq})}$ = concentration of dissolved carbon dioxide (mol/L)

V_A = Volume of Acid (L)

V_T = Total Internal Volume (L)

k_1 = Henry's Law Constant for carbon dioxide = 0.034 mol/L/atm =
0.00033555382 mol/L/KPa

k_2 = Constant for relationship between density and pressure of CO_2 at 20°C
= 0.002839 mol/L/psi = 0.000411762882 mol/L/KPa

(NIST Standard Reference Database 69: NIST Chemistry WebBook,
<https://doi.org/10.18434/T4D303>)

$P_{\text{CO}_2(\text{g})}$ = change in pressure (kPa)

= change in partial pressure of carbon dioxide (kPa)

For this research a carbonate analyser was used which had previously been used by other researchers at The University of Strathclyde for investigating microbially induced carbonate precipitation via *S. pasteurii* (Sang et al., 2023, 2024). This was a 297 ml bottle with 40 ml of 5M HCl was used, and pressure change was measured in kPa. The bottle was chosen to be as small as possible, as the pressure change would be larger at smaller volumes, and therefore smaller masses of calcium carbonate could still be measured accurately. 40 ml of 5 M HCl was used to provide enough solution volume to submerge the sample.

To calibrate the apparatus, a known quantity of calcium carbonate of 98 % purity from Sigma Aldrich was placed in the bottle, along with a small centrifuge tube with 40 ml of 5 M HCl. The bottle was then sealed and the pressure gauge, and the reading zeroed. The bottle was then gently tipped to pour the acid out of the centrifuge tube to allow it to react with the calcium carbonate contained within the bottle, then the bottle was placed securely on a shaking table at 120 rpm. The mixture fizzed as carbon dioxide was released by the reaction of calcium carbonate with the acid, and the pressure change was measured after at least 10 mins, and once the reading stabilised. The pressure change was then used to calculate the mass of calcium carbonate using Equations 4.1 to 4.7. The calculated calcium carbonate mass was then compared with the known calcium carbonate mass to assess the accuracy. Table 4.8 and Figure 4.7 show a comparison of the known and calculated calcium carbonate

masses for the calibration. Overall, the calibration was very accurate, with Figure 4.7 displaying a gradient of 0.991 (a gradient of 1 being a perfect calibration) and an R^2 value of 1. It is clear from Table 4.8 that accuracy improves with increasing mass. In this case, 95 % was selected as the minimum acceptable accuracy and as such using the results from this calibration show that, using this set-up, calculated calcium carbonate masses of 0.3 g were the minimum limit that could be measured. If the mass of calcium carbonate in a sample was determined to be below 0.3 g, then it was not considered a quantitative measurement.

Table 4.8 Calcium Carbonate Determination Calibration

Known Mass (g)	Determined Mass (g)	Accuracy (%)
0.0096	0.0072	74.6
0.0196	0.0060	30.4
0.0299	0.0203	67.9
0.0407	0.0322	79.2
0.0501	0.0358	71.5
0.0602	0.05251	87.2
0.0697	0.0633	90.8
0.0807	0.0704	87.3
0.09	0.0835	92.8
0.1	0.0931	93.1
0.2003	0.1898	94.7
0.2503	0.2351	93.9
0.2997	0.2876	96.0
0.4001	0.3879	96.9
0.4996	0.4917	98.4
0.75	0.7328	97.7
1.0002	0.9882	98.8
2.4999	2.4526	98.1
5.0006	4.9136	98.3
7.5025	7.4509	99.3

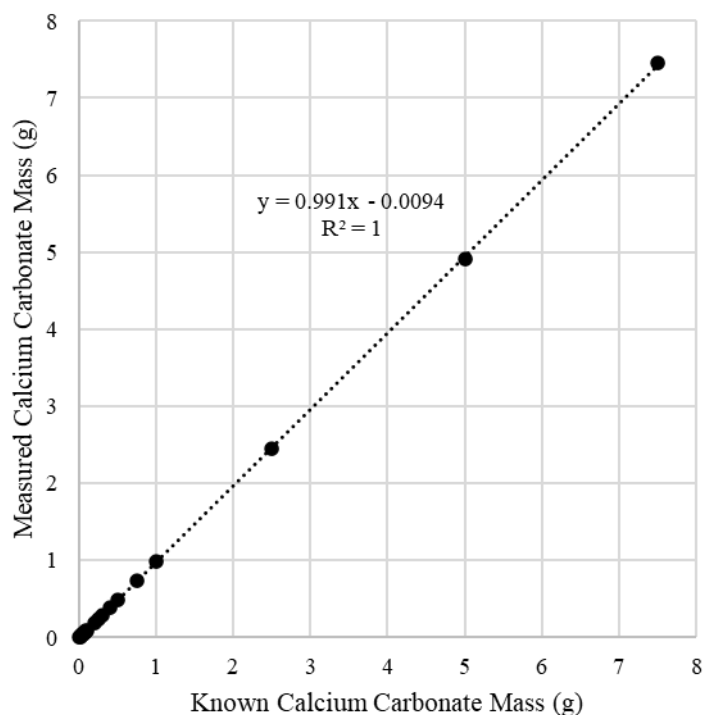


Figure 4.7 Graph of Calcium Carbonate Determination Calibration

After performing the calibration, controls of materials used in the column experiments (Sections 4.3.2.1 and 4.3.2.2) were tested. Controls of 47.5 g sand, 47.5 g sand + 2.5 g beechwood chips, and 47.5 g sand + 2.5 g *P. cornucopiae* colonised beechwood chips were first heated in the muffle furnace at 530 °C to remove the organic fraction and were then subject to acid digestion and the mass of calcium carbonate determined. These results are displayed in Table 4.9. When running these controls, the total volume of the bottle was adjusted to 279.6 ml in the calculations to account for the volume taken up by the sand (considered to be incompressible at these pressures).

Table 4.9 Calcium Carbonate Determination Controls

Materials furnaced at 530 °C	Calcium carbonate (g)
47.5 g sand	0.0079
47.5 g sand and 2.5 g beechwood chips	0.0303
47.5 g sand and 2.5 g <i>Pleurotus cornucopiae</i> colonised woodchips	0.0045

These results showed that there was little false measurement of calcium carbonate after pretreatment by ashing, well below the minimum quantitative limit for calcium carbonate content (0.3 g). This method was then used for all future calcium carbonate measurements.

4.5 Experiment 3, Relationship between Fungal Respiration and Ergosterol Concentration

Following the preliminary column experiments (Experiment 1 and 2, this chapter), it was determined that a method for biomass determination would be beneficial in order to assess the amount of fungal biomass present within the columns. The available biomass likely controls the quantity of urease available for ureolysis and the number of nucleation sites available for carbonate precipitation.

In Experiment 3 the relationship between fungal respiration and fungal ergosterol was investigated. Ergosterol is a compound unique to fungi and has been shown to strongly correlate with fungal biomass. However, measuring ergosterol content requires extraction and destruction of the sample (Davis & Lamar, 1992; Gong et al., 2001; Montgomery et al., 2000). The goal of this experiment was to determine if there was a relationship between fungal respiration evolution during growth and the amount of ergosterol present within a sample. with the overall aim that a measurement of the respiration of *P. cornucopiae* within the sample could be used to obtain an estimate of ergosterol content within the sample, and therefore a non-destructive technique of estimating fungal biomass within the sample prior to FICP treatment.

4.5.1 Methodology

4.5.1.1 Column preparation

The specimen was prepared as described in Section 4.3.2, and contained 50g of media (95 % sand, 5 % beechwood chips, an additional 10 % by weight fungal suspension) but the column was constructed slightly differently, as seen in Figure 4.8. Each column was stoppered on the bottom by a bung with a nozzle. The bottom bung was covered with a 1cm thick layer of 10mm diameter glass beads, a fine mesh, and the sand/beechwood/fungal inoculant mixture was then placed on top. This left a small headspace between the column media and the parafilm covering the top. The parafilm was left on during the growth phase to minimise water loss but was removed when measuring respiration.

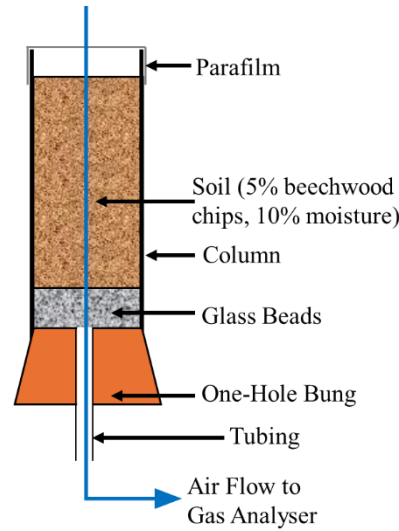


Figure 4.8 Column Respiration Design

4.5.1.2 Respiration

Respiration rate was determined by measuring the change in CO₂ concentration in air, prior to entering a column (c_i) inoculated with *P. cornucopiae* and on exiting the column (c_o). An open-measurement system was selected over a closed-loop system as closed-loop systems have been shown to underestimate CO₂ efflux by up to 10 % (Rayment, 2000). To calculate the CO₂ concentration flowing out of an open chamber, the following equation was used (Rayment, 2000):

$$c_o(t) = c_i + \frac{R}{f} - \frac{R}{f} e^{-\frac{f}{V}t} \quad [\text{Eq. 4.8}]$$

Assuming that R does not change during a single measurement, then as $t \rightarrow \infty$,

$$c_o(\infty) = c_i + \frac{R}{f} - 0 \quad [\text{Eq. 4.9}]$$

$$R = f(c_o - c_i) \quad [\text{Eq. 4.10}]$$

Where,

t = time (s)

c_o = CO₂ concentration at outlet (μmol/mol)

c_i = CO₂ concentration at inlet (μmol/mol)

f = flow rate = 1.5ml/s = 0.0000625mol/s (according to the ideal gas law)

R = respiration rate (μmol/s)

V = effective chamber volume (mol)

This calculation required a stable reading of carbon dioxide at the inlet, and a stable reading of carbon dioxide at the outlet after the airflow had been exposed to *P. cornucopiae* biomass within the column.

CO₂ concentrations of the open system were measured with an Ultra-Portable GLA 132 Gas Analyser in a fume hood. The gas analyser had an internal pump which drew in air through the intake port. The respiration measurement was conducted within a fume hood to limit interference from human respiration and to quickly remove waste respired air from the area so as not to contaminate the next samples. The intake gas was measured after inserting each column into the fume hood. Initially, the intake gas used was the compressed air line provided by the laboratory. However, the CO₂ content of this air was found to fluctuate slowly throughout the day. The intake gas was switched to the ambient atmosphere within the fume cupboard, which was found to fluctuate less than the compressed air. This alteration to the method happened at the day 14 sample point. From this point respiration measurements were less variable.

The parafilm was removed from the top of the column to allow air to easily flow through the column. To facilitate speedy measurements of the carbon dioxide produced over time by the fungus in each sand column, the column was first purged of accumulated carbon dioxide with compressed air. This removed any carbon dioxide which had accumulated within the pores and avoided a large carbon dioxide spike on connecting the gas analyser which would result in a long delay before a stable reading could be reached. The gas analyser was then attached to the bottom of the column, air was pulled through the column and out of the bottom and the carbon dioxide concentration measured in ppm, shown in Figure 4.9. It took up to 30 mins for a stable CO₂ reading to be reached. The outlet for analysed air (i.e. gas exiting the gas analyser) was placed at the back of the fume hood at the exhaust to avoid contaminating the hood with the respired carbon dioxide.

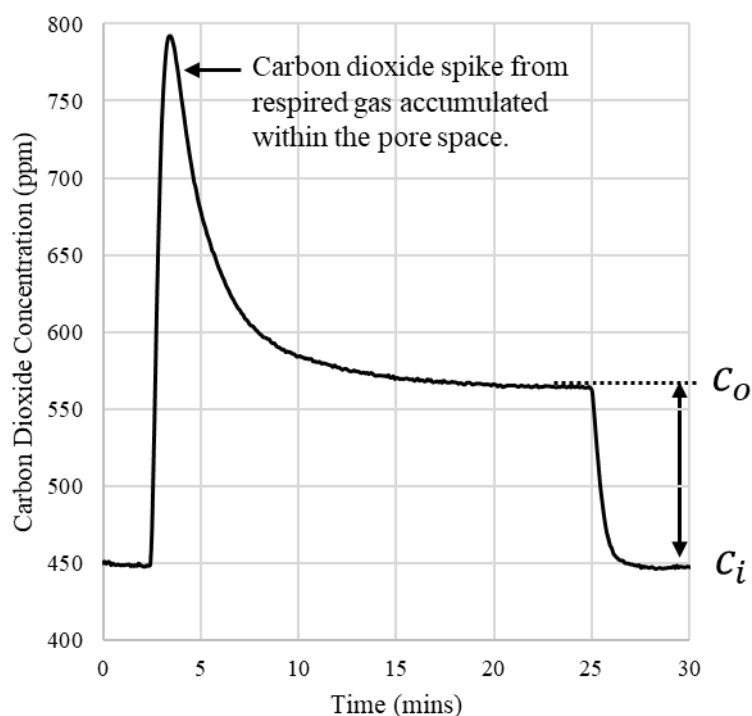


Figure 4.9 Example Carbon Dioxide Measurement from Column 12 Day 15

4.5.1.3 Ergosterol Extraction

To measure ergosterol content of the columns, the ergosterol had to first be extracted. Ergosterol extraction was carried out based on the method by Šnajdr et al. (2008). Although typically ergosterol extraction is carried out on small samples (0.5 – 1 g), here extraction was carried out on the entire soil column as follows. Briefly, the method involves removing all of the soil media from the column and adding it to a mixture of 300 ml of 10 % (or 3 M) NaOH in methanol, and 100 ml of cyclohexane in a 1 litre bottle. The mixture was then swirled for 30 seconds before sonication at 70 °C for 90 minutes. 100 ml of deionised water and 200 ml of cyclohexane was added to the mixture and swirled for 30 seconds. The mixture was allowed to settle before collection of the top phase. The top phase was concentrated in a Buchi flask using a Buchi Syncore. The bottom and top heaters were set to 40 °C. The shaking table was set to 210 rpm, and the chiller for the condensing column set to 6 °C. The vacuum was set to 90 mbarr and then 50 mbarr whilst watching for any aggressive boiling. The remaining solution in the bottle was washed twice more with 200 ml cyclohexane, with the top fraction removed and concentrated in the same Buchi flask for each sample, using the Buchi Syncore each time. The last of the super-concentrated liquid in the

Buchi flask was evaporated to dryness using Nitrogen blown across the surface. 25 ml of methanol was added to the dry sample in the Buchi flask and redissolved by heating at 40 °C for 20 mins and 210 rpm at atmospheric pressure. This 25 ml of methanol was transferred to a 100 ml volumetric flask. This was repeated twice more and the methanol solution made up to 100 ml in the volumetric flask. The solution was then taken for High Performance Liquid Chromatography (HPLC) to analyse for ergosterol content.

4.5.1.4 HPLC analysis

The solution was injected into a Dionex UltiMate 3000 standard HPLC system coupled with a UV detector. A C18 column (4.6 × 150 mm, 5 µm) was used to separate ergosterol from other compounds in the extracted samples. The mobile phase was 100 % methanol, and the flow rate was set at 1 mL/min. The lower and higher limit of the column pressure was set at 660 and 700 psi, respectively. Ergosterol was detected using a photodiode array detector at a wavelength of 292 nm.

4.5.1.5 Determining fungal biomass

A mean value of 5mg of ergosterol per gram of fungal mycelium has been used as a conversion factor when converting ergosterol content to fungal biomass in soils (Baath, 2001; Eiland et al., 2001; Ruzicka et al., 2000), indicating 200 mg of fungal biomass per mg of ergosterol.

$$B = E \times F \quad [\text{Eq 4.11}]$$

Where,

B = Column Biomass (mg)

E = Ergosterol (mg)

F = Conversion Factor (200 mg (biomass)/mg (ergosterol))

4.5.1.6 Sampling

As respiration measurements were non-invasive, the intention was that respiration measurements would be taken regularly throughout the experiment. However, ergosterol extraction requires complete destruction of the samples, as such

five sets of columns were prepared, one set for each time point of ergosterol extraction. Each set was prepared in quintuplicate, for a total of 25 inoculated columns. Sets were prepared in quintuplicate to better account for any variation in fungal growth and respiration between individual specimens. 5 uninoculated columns, where 10 % water was added instead of 10 % fungal suspension, were also prepared to act as controls. Sample Measurements are summarised in Table 4.10.

Respiration measurements were taken at the following timepoints: 1, 2, 3, 4, 7, 8, 9, 10, 11, 14, 15, 16, 17, and 98 days. Unfortunately, the gas analyser encountered a mechanical failure on the 18th day of analysis and had to be sent away for repair. It returned to operation two months later, hence the big gap in respiration measurements. Due to the practicalities of the lab, not every column could have their respiration measured every day. Sets 1, 3, and 5 were measured every odd day, and sets 2 and 4 were measured every even day. Uninoculated columns were all measured on day 2, and then individual columns were measured when possible, on the day that they were analysed for ergosterol.

For each column set, respiration measurements were taken up until they were disassembled for ergosterol extraction and measurement, therefore destroying the samples. Ergosterol content was measured in Set 1 after 7 days, in Set 2 after 14 days, in Set 3 after 21 days, in Set 4 after 35 days, and Set 5 after 98 days (after the return of the gas analyser). Ergosterol was also extracted for an uninoculated column along with each fungal inoculated set. Before extraction of each column, a last measurement of respiration was taken (when the gas analyser was available).

Table 4.10 Sample Measurement Summary

Column Set	Respiration Measurement Time Points (days)	Ergosterol Extraction and Measurement Time Point (days)
Set 1	1, 3, 7	7
Set 2	2, 4, 8, 10, 14	14
Set 3	1, 3, 7, 9, 11, 15, 17	21
Set 4	2, 4, 8, 10, 14, 16	35
Set 5	1, 3, 7, 9, 11, 15, 17, 98	98
Uninoculated	2, 7, 14, 98	7, 14, 21, 35, 98

4.5.2 Results

Figure 4.10a shows the change in respiration rate over all columns over 14 weeks. This is displayed as the mean, with error bars displaying the standard deviation of the data. Figure 4.10b shows the respiration of individual columns 21 - 25 in Set 5, which were measured for respiration rate over the entire range of measurement points. Figure 4.10a shows that fungally treated columns had a higher respiration rate after one week than the uninoculated controls. Combining these two figures, it can be seen that the fungus within the columns had high variability respiration for the first day, before settling and slowly increasing in respiration rate until between day 8 and 10. The respiration rate then began to decrease, until week 14 when in some columns respiration was negligible.

Figure 4.10c shows the mean estimated fungal biomass of each quintuplicate set of columns, with the error bars showing the standard deviation of the data. The graph also shows the estimated biomasses in the uninoculated control columns, which remained very low in all columns other than the control column tested after 3 weeks of incubation. This one measured a small amount of fungal biomass (14.58 mg) and had visible patches of slight fungal growth. Figure 4.10c shows that the first week of growth is quite rapid, with the average estimated biomass increasing from 6.8 mg in the fungal inoculant, to 94.1 mg. Between weeks 1 and 5, fungal biomass grew to 129.8 mg. Fungal growth slowed considerably after 5 weeks, only increasing from 129.8 mg at week 5 to 145.0 mg at week 14.

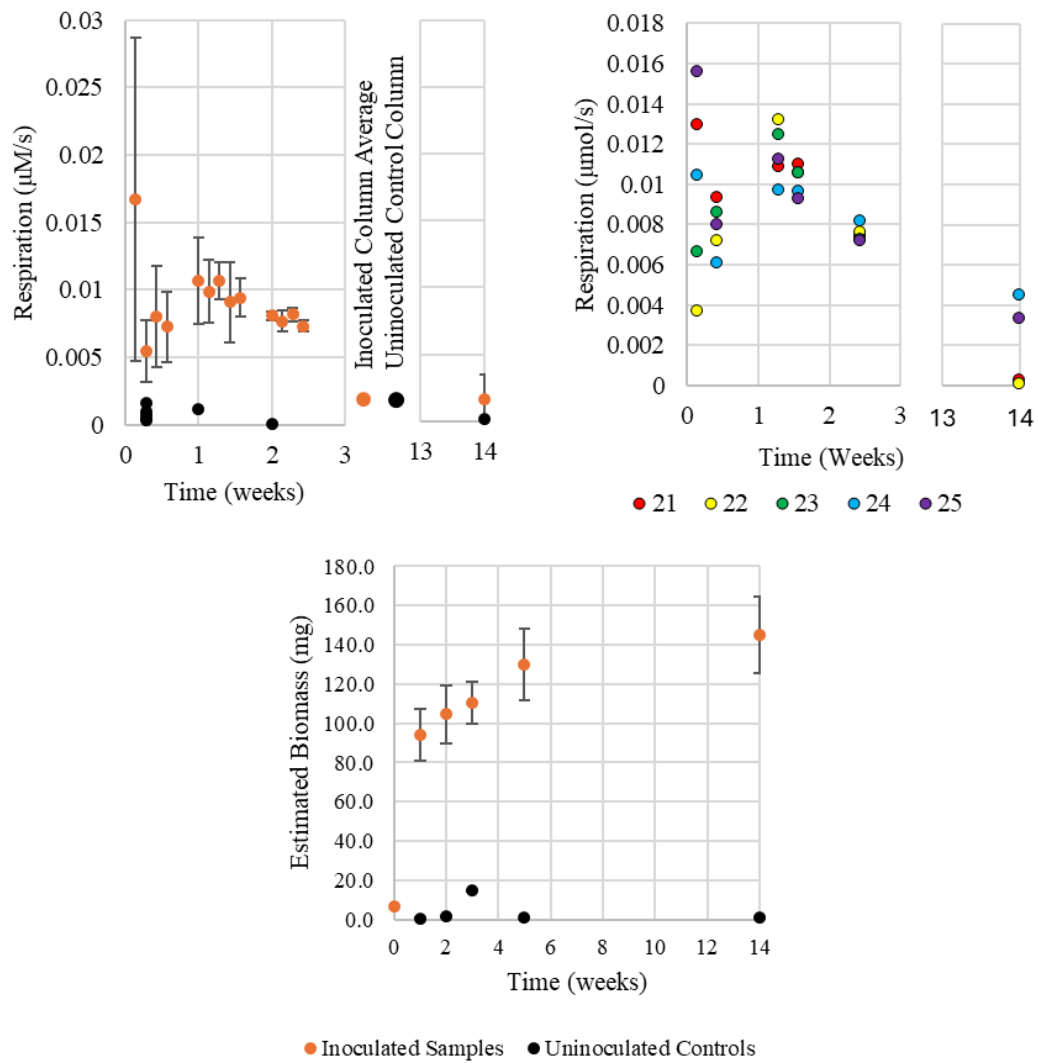


Figure 4.10: (a) Mean Respiration and Standard Deviation for all Columns Over 14 Weeks; (b) Respiration measurements for Columns 21 - 25 which were monitored over the entire 14 week period; (c) Mean Biomass and Standard Deviation in each set of columns

4.5.3 Discussion

Previous work to determine respiration rates of saprotrophic fungi (*Paxillus obscurusporus*, *Pleurotus ostreatus*, and *Ganoderma lucidum*) have found that respiration rates peaked after 16 or 20 days (Pavlík et al., 2020; Wilkinson et al., 2010). Wilkinson et al. (2010) used melin nokrans agar and Pavlík et al. (2020) compared respiration rates after growing fungus on potato dextrose agar, oakwood chips, beechwood chips, or rye straw. In contrast, after the initial peak in respiration rate

found on Day 1, respiration rates were found to peak less extremely for a second time at around 9 to 11 days in this study. This could be because the scale of experiments in this study was much smaller (50 g) than in that literature (1 kg) which may have reduced the time to reach peak respiration. It could also be due to the substrate being different, which has been shown to alter growth rate (Nashiruddin et al., 2022; Schoder et al., 2024). Nashiruddin et al. (2022) compared rice husk, sawdust, or sugarcane bagasse and Schoder et al. (2024) investigated different ratios of cotton fiber and pine bark. They are much more nutrient rich than the environment provided by the soil columns in this thesis, as they contained no sand. Nevertheless, fungal biomass of the columns showed initially fungal growth is rapid and then slows down over time. The limit on growth that the samples were tending towards could be due to several factors: Oxygen availability, moisture content, nutrient supply, or available pore space (Nashiruddin et al., 2022; Salifu et al., 2024; Schoder et al., 2024).

In Section 3.3.1.1, it could be seen that on agar plates, *Pleurotus cornucopiae* growth experienced a lag phase of 2 days, with growth being observed only after 3 days. It is likely that a similar lag phase occurred during column growth. Literature shows that this “lag time” has been found in other species of agaricomycetes (Nashiruddin et al., 2022; Schoder et al., 2024) and is likely due to the organisms acclimitising to its new environment and new nutrient sources. From the data presented in Figure 4.10, there may be a relationship between respiration and the three stages of fungal growth: lag, exponential, and stationary phases (Kavanagh, 2005; Seto & Tazaki, 1975) as follows: Lag Phase (Before Day 3): the fungus acclimitases to the new substrate, there is likely little growth, and the respiration rate is highly variable. Exponential Phase (Day 3 until Day 11): the fungus has acclimatised to the new environment and nutrient conditions and biomass increases rapidly, as does respiration rate. Stationary Phase (Day 11 to Week 14): Biomass growth slows and respiration rate decreases during this time. By Week 14, some columns had respiration rates close to zero.

In *Aspergillus oryzae* (ascomycete), it has been found that aerial hyphae are the major contributors to respiration rate (Rahardjo et al., 2002). These hyphae then transport oxygen to areas of the fungus which are not exposed to oxygen, eg wet hyphae. Seto, et al. (1975) found that a decrease in fungal respiration rate was

accompanied by the appearance of vacuolated hyphae and chlamydospores. These features appear as environmental conditions become unfavourable, allowing the organism to survive harsh conditions such as heat, dessication, or starvation (Hwang & Ko, 1978; Lin & Heitman, 2005; Sitton, 1981). They studied an ascomycete, but a similar effect may occur in agaricomycetes, with respiration decreasing as resources become scarce.

In Experiment 3, there was no clear relationship established between ergosterol content and fungal respiration. During growth, the mass of ergosterol determined within the columns steadily increased, but respiration increased and decreased at various stages. This experiment also showed that respiration peaked after 1.5 weeks of growth in the column but the biomass continued to increase. The data suggests that after 5 weeks the accumulation of biomass slowed down, as such, for column experiments in Chapter 6 the fungal column incubation period was set at 5 weeks prior to treatment with cementing solution.

4.6 Conclusions

In this chapter, a method for growing fungal culture in broth was refined, namely ensuring that cultures sampled from agar to inoculate broth were sampled from the growing edge. A revised method for determining calcium carbonate content was developed to remove fungal biomass and any beechwood present by heating in the muffle furnace to 530 °C, prior to conducting acid digestion for calcium carbonate determination in accordance with ASTM D3437-21.

Preliminary column experiments were also carried out to trial treatment strategies, select the nutrient substrate, and investigate the influence of cementing solution concentrations on fungal-induced carbonate precipitation. The key findings are summarised here:

Experiment 1 explored the effect of mixing or hole-punching fungal colonised soil on calcium carbonate precipitation. *Mixed* samples, whereby the fungal mycelium was disrupted by manually mixing prior to treatment with cementing solutions (calcium chloride and urea) displayed higher calcium carbonate precipitation, however such a strategy would be impractical for applications in the field. *Hole-punched*

samples, whereby a small hole was formed at the upper surface, exhibited slightly reduced calcium carbonate precipitation compared to *Mixed* soil specimens.

Experiment 2 demonstrated that columns using a finer fraction of beechwood (dust > 0.3mm) and dust beechwood, precipitated less calcium carbonate than columns containing only beechwood chips. This may be due to differences in fungal growth as a result of differences in aeration, or due to changes in the hydraulic behaviour, i.e. the samples containing dust sized beechwood being more likely to retain fluids during drainage, and thus have less access to fresh cementing solution. Based on these results, the sand column experiments presented in Chapter 6 were composed of sand and beechwood chips.

Experiments 1 and 2 showed that it is possible to increase the total mass of calcium carbonate by (i) increasing the number of treatment cycles applied and (ii) increasing the concentration of CaCl_2 in the treatment solution used. However, comparison between Experiments 1 and 2 indicates that as the ratio of Urea : Calcium Chloride changes, the efficiency of the process is greatly affected. Using a ratio of 1:7 (Calcium Chloride: Urea), 100 % of Calcium available was precipitated as CaCO_3 (for beechwood chips samples). Whereas at a ratio of 1:1 the efficiency reduced to 64 % for the same total treatment solution volume applied (75mL) and the same concentration of Urea (0.33 M).

Experiment 3 attempted to find a relationship between fungal biomass present within a column, (determined via ergosterol extraction and quantification via HPLC analysis) and fungal respiration as a means to non-invasively measure the fungal biomass of a column. Unfortunately, no correlation was observed but the different growth phases of fungus within the column were identified. Based on the results of Experiment 3, a column growth period of 5 weeks in the dark at 25 °C was selected for the fungal incubation period for the column experiments in Chapter 6.

Chapter 5 investigates further the influence of fungal biomass, Calcium chloride:Urea ratios, total concentration of CaCl_2 and Urea, and pH on ureolysis and calcium carbonate precipitation in a series of batch experiments.

Chapter 5: Investigating the Influence of Biomass, Cementing Solution Concentrations, and pH on Fungal Induced Calcium Carbonate Precipitation

5.1 Introduction

To date, only a limited number of studies have investigated the potential of fungal biomineralisation for the bioremediation of contaminants, i.e. co-precipitation of heavy metals (Li et al., 2014; Qian et al., 2017; J. Zhao, Csetenyi, et al., 2022), to improve the compressive strength of sands (Fang et al., 2018; Martuscelli, Soares, Camões, et al., 2020), to provide a protective coating for concrete (J. Zhao, Dyer, et al., 2022) and for use in self-healing concrete (Menon et al., 2019). Moreover, although these studies demonstrate fungal induced carbonate precipitation (FICP), the variables which may influence the process have not yet been systematically studied.

Given the limited work in this area, information on possible variables which could affect fungal biomineralisation of calcium carbonate might be gleaned from microbially-induced calcium carbonate precipitation via ureolysis using *S. pasteurii* which has been much more widely studied. Previous studies on microbially induced carbonate precipitation (MICP) indicate that higher bacterial cell concentrations induce faster ureolysis and (Okwadha & Li, 2010; Tobler et al., 2011; Q. Zhao et al., 2014). Here, it is hypothesised that similar may be true for fungal-induced carbonate precipitation, and that biomass may be a key limiting factor in the treatment process. Higher amounts of biomass present in the system, should result in larger quantities of available urease enzyme for ureolysis and may also provide more nucleation sites available for nucleation of precipitating calcium carbonate. This should contribute to a faster rate of calcium carbonate precipitation.

As outlined in Section 2.3.2.1, only one study Li et al. (2014) has to date investigated the influence of the treatment solution concentrations in the cementing solution in FICP. Li et al. (2014) investigated in a limited experimental study the influence of the ratio of calcium concentration to urea concentration on calcium carbonate precipitated after exposing the Ascomycete *Neurospora crassa* to cementing solution for 12 days. Three cementing solution ratios were investigated as shown in

Section 2.5.3, Table 2.4. In Table 2.4, for a ratio 1:200 (Calcium: Urea), a high pH was reached, but low conversion of calcium to calcium carbonate (16.4%) was achieved. This was intended as a control given the very low CaCl_2 concentration supplied. For the ratio 1:0.8, more calcium carbonate was precipitated due to the higher availability of calcium ions, however only 53 % of the available calcium was precipitated as calcium carbonate. As the ratio changed to 1:6.6, and the urea concentration was in excess of the CaCl_2 , a greater amount of calcium carbonate precipitation occurred, and higher calcium conversion was observed (93.2%). These results show the influence that the ratio between CaCl_2 and Urea, however only three ratios were tested. Additionally, most studies investigating FICP have used similar cementing solution concentrations of 40 mM CaCl_2 and 330 mM urea (Fang et al., 2018; Martuscelli, Soares, Camões, et al., 2020; Qian et al., 2017). A similar cementing solution concentration (50mM CaCl_2 and 333mM) was also used in Section 3.2.2, with calcite precipitation induced in *Pleurotus cornucopiae* specimens (confirmed via XRD). There is scope to investigate in more depth the influence of different cementing solution concentration ratios to understand its influence on the rate and quantity of calcium carbonate precipitation, in order to optimise the FICP process for *Pleurotus cornucopiae*.

In this Chapter, the Agaricomycete, *Pleurotus cornucopiae*, was selected to study the influence of different variables upon fungal-induced carbonate precipitation. *P. cornucopiae* was selected due to its native status in the UK, non-hazardous nature, high urease activity and growth rate as determined in Chapter 3. This chapter explores variables which may influence ureolysis and carbonate precipitation in order to optimise the fungal induced carbonate precipitation process, namely: (1) Fungal biomass, (2) Overall concentration of the cementing solution, (3) Ratio of calcium concentration to urea concentration in the cementing solution and (4) initial pH of reagents. This research seeks to optimise carbonate precipitation via ureolysis using the species *P. cornucopiae*, and to identify any inhibitory conditions that should be avoided.

5.2 Materials and Methods

5.2.1 Fungal suspension

Pleurotus cornucopiae was grown on malt extract agar at 25 °C in the dark for 7 days. 5 mm diameter plugs were subsequently sampled using a sterilised stainless steel borer and used to inoculate sterilised (via autoclaving) malt extract broth – using one 5 mm diameter inoculated agar plug per 100 ml of broth. 5 L vessels were used to incubate 3 L of broth each. The inoculated broth was incubated in the dark at 20 °C for 7 days and stirred using a magnetic stirrer. After 7 days, the broth was centrifuged, and the harvested biomass collected and resuspended in deionised water at one tenth of the original volume of the broth. To vary the fungal biomass added to each flask, 30, 35 or 40 mL of the resuspended fungal suspension was added to each 250 ml conical flask.

5.2.2 Preparation of batch experiments

The fungal suspension was distributed into 250 ml flasks by volume (30 mL, 35 mL or 40 mL). Calcium chloride and urea solutions were separately prepared as 5 M stock solutions using ultra-pure deionised water. Calcium chloride stock solution, ultra-pure deionised water, and deionised water were all sterilised by autoclaving, and urea stock solution was syringe filtered through 0.45 µm syringe filters. Cementing solutions were then prepared from the stock solutions with sterile ultra-pure deionised water to concentrations such that, upon addition to the fungal suspension and reaching a total solution volume of 100 ml per flask, the concentration of the cementing solution would be as desired. The final CaCl₂ and urea concentrations tested in each experiment can be found in Table 5.2. All experimental flasks were prepared in triplicate. After addition of the cementing solution to the fungal suspension (i.e. treatment), flasks were stored at 20 °C, (not in the dark), and all flasks were kept static with manual shaking carried out immediately before sampling. In control flasks, the flasks were prepared singularly in the same manner, but the fungal suspension was replaced with deionised water.

5.2.3 Chemical analyses

Samples were taken at times ranging from 0 – 31 days, sample t_0 was taken immediately after mixing the fungal suspension and cementing solution together. Samples were analysed for pH, ammonium, and calcium. pH was measured using a SevenExcellence Mettler Toledo pH Meter, ammonium was measured using the Konelab Aqua30 with reagents Amm1 and Amm2 following ISO 15923-1:2013, and calcium concentration were measured using Thermo Scientific iCAP 6200 Series ICP Spectrometer following ISO 11885:2009.

As well as the measured data, this chapter also presents data calculated from the measured data. Percentage calcium remaining was calculated using the following equation:

$$Ca_{Remain}(\%) = \frac{C_{Ca}}{C_{Ca_i}} \times 100 \quad [\text{Eq 5.1}]$$

where, Ca_{Remain} is the percentage of calcium remaining in solution (%), C_{Ca} is the calcium concentration (M), C_{Ca_i} is the initial calcium concentration (M).

The mass of calcium carbonate precipitated was calculated using the following equation:

$$m_{CaCO_3} = (C_{Ca_i} - C_{Ca}) \times M_{CaCO_3} \times V \quad [\text{Eq 5.2}]$$

where, m_{CaCO_3} is the mass of calcium carbonate precipitated (g), C_{Ca} is the calcium concentration (M), C_{Ca_i} is the initial calcium concentration (M), M_{CaCO_3} is the molar mass of calcium carbonate (100.09 g/mol), V is the initial volume of treated solution = 0.1 L. It is assumed here that all calcium removed from solution was precipitated as calcium carbonate.

The estimated mass of calcium carbonate precipitated per gram of biomass was calculated using the following equation:

$$m_b = \frac{m_{CaCO_3}}{m_f} \quad [\text{Eq 5.3}]$$

where, m_b is the mass of calcium carbonate per gram of biomass (g/g), m_{CaCO_3} is the mass of calcium carbonate precipitated (g), m_f is the mass of fungal biomass (g).

5.2.4 Determination of fungal biomass

The fungal biomass in each flask was determined after treatment. The remaining solution in each flask was first centrifuged at 8000 rpm for 5 mins. This produced a biomass “sludge” rather than a pellet, due to the fungus’ ability to hold onto water. The supernatant was then removed and the sludge mixture of fungus and calcium carbonate placed into a dried and pre-weighed crucible. The sludge was dried at 105 °C and weighed. The sample was then ramped to 530 °C at 10 °C/min and maintained for 6 hours (temperature selected based on Section 4.4) to determine the mass of biomass without decomposition of calcium carbonate. To estimate the initial biomass in each flask, the determined biomass was linearly extrapolated to account for any biomass that may have been removed during sampling.

5.2.5 Experimental Design

5.2.5.1 Experiment 1 Influence of biomass and overall cementing solution concentration

To investigate the influence of fungal biomass on ureolysis and carbonate precipitation, the amount of fungal suspension added to each flask was varied to achieve a low, medium, and high relative fungal biomass. The volumes of fungal suspension added to each flask were 30ml, 35ml, and 40ml of fungal suspension. The actual biomass in each flask was determined (as in Section 4.4) after the experiment and each individual flask labelled with high, medium, or low biomass according to that measurement. In Experiment 1, the overall concentration of the solution was also varied but the ratio of calcium to urea concentration was held constant at 1:7. The concentrations used were: (1) 0.11 M urea and 0.0165 M $CaCl_2$, (2) 0.33 M urea and 0.05 M $CaCl_2$, and (3) 0.5 M urea and 0.075 M $CaCl_2$. Table 5.1 shows the intended distribution of conditions across flask replicates. The combination of these variables led to an intended nine possible different flask conditions, with twenty-seven individual experimental flasks, and three control flasks (i.e. containing no fungus) representing each of the different cementing solution concentrations.

Table 5.1 Experiment 1: Intended distribution of flask replicates in terms of fungal suspension inoculant volume and cementing solution concentrations.

# of flasks	0.11 M Urea, 0.0165 M CaCl ₂	0.33 M Urea, 0.05 M CaCl ₂	0.5 M Urea, 0.075 M CaCl ₂
40 ml inoculant	3	3	3
35 ml inoculant	3	3	3
30 ml inoculant	3	3	3
No Inoculant	1	1	1

5.2.5.2 Experiment 2 Influence of Calcium to Urea Concentration Ratio

In Experiment 2, the urea concentration was held constant at 0.5 M and the CaCl₂ concentration was varied. The fungal suspension inoculant volume was held constant at 35 ml. Calcium to urea ratios of 1:7 and 1:0.8 were previously tested in Li et al., (2014) for FICP with *Neurospora crassa*. Here, ratios of 1:10, 1:7, 1:4, and 1:1 were investigated for *Pleurotus cornucopiae*. Each ratio was tested in triplicate.

5.2.5.3 Experiment 3 Influence of pH Adjustment

Five different pH conditions were investigated in this experiment by adjusting the initial pH of the cementing solution. The cementing solution was prepared either with sterile tap water to give an initial pH before fungal inoculation of 10.11 or sterile ultra-pure deionised water (UPDW), giving an initial pH before fungal inoculation of

8.94, to investigate any potential effect on calcium carbonate precipitation. Furthermore, additional pH adjustments were made by amending UPDW with increasing concentrations of NaOH to determine if higher pH starting solutions resulted in faster rates of calcium precipitation. To amend the cementing solution, 1ml of either 0.1M, 0.5M, or 1M NaOH was added to the 65 ml of cementing solution (requiring the fungal inoculum to be reduced by 1 ml to 34 ml), resulting in a final NaOH concentration in each flask of 1mM, 5mM, or 10mM. The amended cementing solution was then mixed with the 34 ml fungal suspension to make a total solution volume of 100 ml. The final concentration of the cementing solution was fixed at 0.125 M CaCl₂ and 0.5 M Urea.

Table 5.2 Experimental Design Summary

Experiment #	Description	Volume of Fungal Inoculum (ml)	CaCl ₂ Concentration (mM)	Urea Concentration (mM)	Ca:Urea Ratio	1 ml of initial pH Adjustment (mM NaOH)	Solvent						
1	Influence of fungal biomass and overall solution concentration	30	16.5	110	1:7	-	UPDW						
			50	330									
			75	500									
		35	16.5	110				1:7	-	UPDW			
			50	330									
			75	500									
		40	16.5	110							1:7	-	UPDW
			50	330									
			75	500									
2	Influence of Ca:Urea Ratio	35	50	500	1:10	-	UPDW						
			75		1:7								
			125		1:4								
			500		1:1								
3	Influence of initial pH	35	125	500	1:4	-	Tap Water						
		34				-	UPDW						
						1							
						5							
						10							

5.3 Results

5.3.1 Experiment 1 Influence of biomass and overall cementing solution concentration

Figure 5.1 presents each flask no. in experiment 1 and its corresponding estimated initial fungal biomass. Based on this each flask was assigned as having a low (diamond symbols), medium (square symbols), or high (triangle symbols) amount of biomass. Flasks containing greater than 0.14 g were considered to have a high biomass, flasks containing between 0.09 and 0.14 g were categorised as having medium biomass, and flasks with below 0.09 g were categorised as having low biomass. This is indicated in Figure 5.1. Originally the experimental design was such that the intention was to have an even split of flasks between the 9 different test conditions as seen in Table 5.1. Instead, due to the distribution of biomasses in the flasks, only 7 of the 9 conditions were represented (there was no low concentration (red), high biomass scenario, nor high concentration (black) low biomass scenario)., and only 5 of the conditions were at least triplicated. Table 5.3 shows the actual distribution of flask conditions. Flasks 10, 20, and 30 are not displayed because they were the control flasks and contained no biomass.

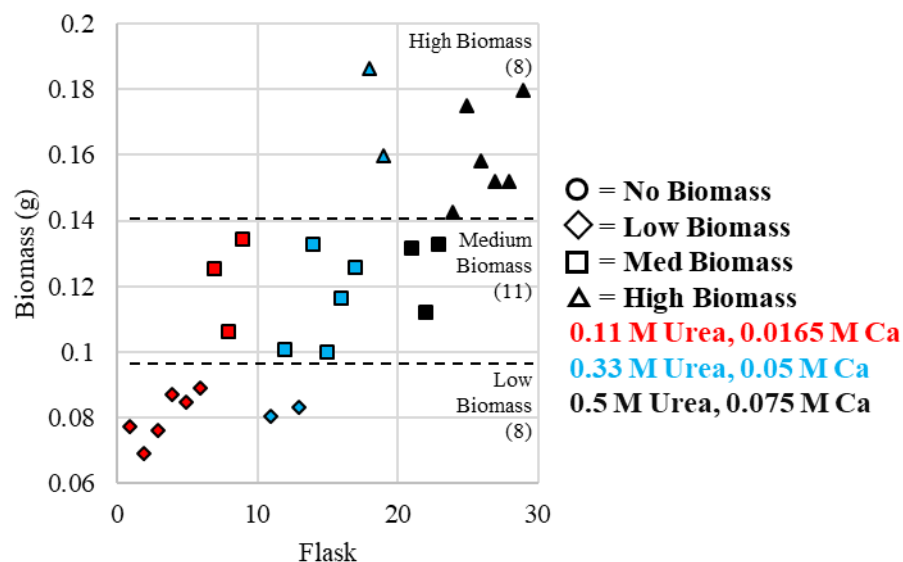


Figure 5.1 Experiment 1: Estimated biomass in each flask

Table 5.3 Experiment 1: Actual distribution of flask replicates in terms of fungal suspension inoculant volume and cementing solution concentrations.

# of flasks	0.11 M Urea, 0.0165 M CaCl₂	0.33 M Urea, 0.05 M CaCl₂	0.5 M Urea, 0.075 M CaCl₂
High Biomass	0	2	6
Medium Biomass	3	5	3
Low Biomass	6	2	0
No Biomass	1	1	1

The results for this experiment are separated into individual graphs for each cementing solution concentration (red: 0.11 M Urea, 0.0165 M CaCl₂, blue: 0.33 M Urea, 0.05 M CaCl₂; black: 0.5 M Urea, 0.075 M CaCl₂) allowing for comparison of behaviour between different biomasses. The results of each direct measurement (ammonium concentration, pH and calcium concentration) are displayed in Figure 5.2, and the results of the processed data (percentage calcium remaining in solution, the estimated mass of calcium carbonate precipitated, and calcium carbonate precipitated per gram of fungal biomass) are displayed in Figure 5.3. The data points in the figures are the mean of the results of that flask condition, and the error bars represent one standard deviation. Control data points (circle symbols) were single specimens so those points have no associated error bars.

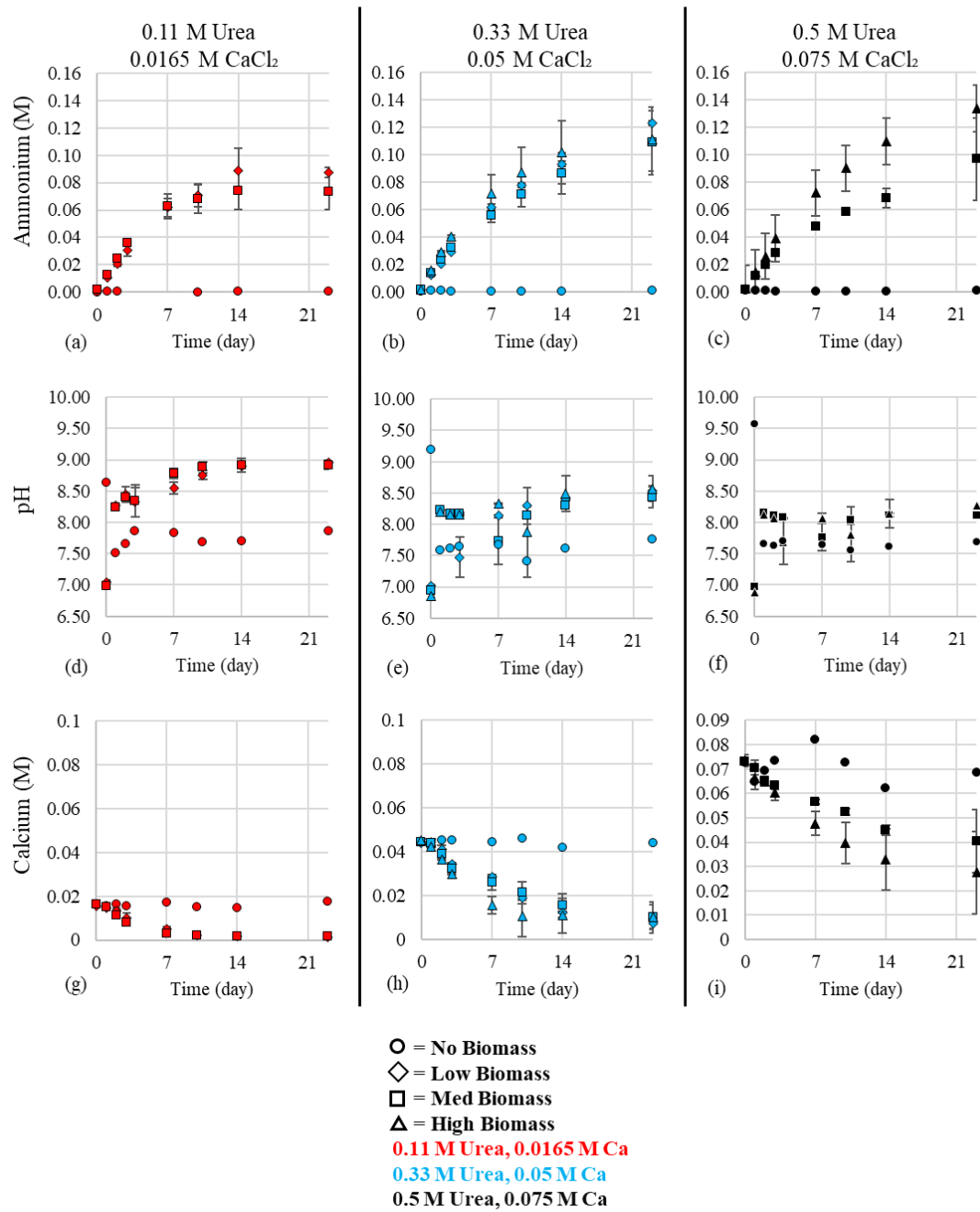


Figure 5.2 Experiment 1. Evolution of ammonium concentration: (a - c), pH: (d - f), calcium concentration: (g - i) over time. Three cementing solution concentrations were tested - 0.11 M Urea and 0.0165 M CaCl₂: (a), (d), (g) - 0.33 M Urea and 0.05 M CaCl₂: (b), (e), (h) - 0.5 M Urea and 0.075 M CaCl₂: (c) (f), (i)

Figure 5.2a - c show the ammonium evolution with time. Control samples (circle symbols) show that there was no ammonium produced in flasks where no fungal suspension was added (i.e. no fungal biomass present).

In Figure 5.2a the rate of ammonium production for 0.11 M urea and 0.0165 M CaCl_2 cementing solution concentration is very similar between the low (diamond symbol) and medium (square symbol) biomasses and reaches a plateau at ~ 0.08 M after 14 days. In Figure 5.2b rate of ammonium production for 0.33 M Urea and 0.05 M CaCl_2 cementing solution concentration was also very similar for all three biomasses categories and did not reach a plateau within the experiment timeframe. In Figure 5.2c the rate of ammonium production with 0.5 M Urea and 0.075M CaCl_2 solution concentration did vary with biomass, with higher biomass (triangle) resulting in a higher rate of ammonium production. There was no plateau in ammonium production within the experiment timeframe. Comparing Figure 5.2a - c, the rate of ammonium evolution up to 7 days did not vary with increasing cementing solution concentration, however at the lowest concentrations tested (i.e. 0.11 M Urea, 0.0165 M CaCl_2) ammonium production plateaued, whereas at higher concentrations ammonium production was still continuing at the end of the experiment (i.e. 23 days).

Figure 5.2d - f show the pH evolution with time. The control samples (circles) show that the pH of the flasks with no fungal biomass were higher at t_0 than in the specimen flasks which contained fungal biomass. Initial pH in the control samples increased with increasing concentration of the cementing solution (pH 8.63, 9.19, and 9.57). After one day the pH in each control sample had decreased to a value between 7.5 and 8 and subsequently remained in that range for the remainder of the experiment. The flasks containing fungal biomass all had lower initial pH at t_0 than the controls, as the fungal biomass counteracted the high pH of the cementing solution, bringing the initial pH to between 6.89 – 7.05.

In Figure 5.2d pH in the 0.11M urea and 0.0165M CaCl_2 solution concentration increased in samples from pH 7 to greater than pH 8.5 after 7 days. The rate of pH change was similar for both the low and medium biomass flasks, and the pH reached a plateau by 14 days. Figure 5.2e shows pH evolution in 0.33M Urea and 0.05M CaCl_2 solution concentration which increased over the first day from pH 7 to pH > 8 . After this, pH generally remained higher than 8 but with some variability in the data. After

14 days, the pH stabilised around pH 8.5. Figure 5.2f shows pH for the 0.5 M Urea and 0.075 M CaCl_2 solution concentration flasks increased from pH 7 to greater than pH 8 after the first day but also showed great variability until day 14 when the pH stabilised at values slightly above pH 8. Comparing Figure 5.2d - f, the final pH reached between different cementing solution concentrations was different, despite the ratio being maintained constant. For 0.11M Urea and 0.0165M CaCl_2 , the final pH was ~ 8.9 ; for 0.33M Urea and 0.05M CaCl_2 , the final pH was ~ 8.5 ; and for 0.5M Urea and 0.075M CaCl_2 , the final pH was ~ 8.2 . In all flasks containing fungal biomass the final pH was higher than that in the control flasks.

Figure 5.2g - i show the concentration of calcium ions in the solution over time. Control samples (circle symbols) show that without fungal biomass, there was no calcium removal from solution. Concentrations remained approximately the same as the initial concentration (red and blue circles), however in the control flask with the highest initial calcium concentration (Figure 5.2i) there were large fluctuations in calcium concentration (black circles).

Figure 5.2g shows little difference in the rate of calcium depletion for the 0.11 M urea and 0.0165 M CaCl_2 solution concentration scenario between the low and medium biomass flasks but does show that little calcium remained in solution by the end of treatment with only 1.3 mM and 1.6 mM, remaining respectively. Figure 5.2h shows little difference in the rate of calcium depletion in the 0.33 M Urea and 0.05 M CaCl_2 solution concentration between low and medium biomass but appears to show a faster rate of calcium depletion in the higher biomass flasks. At this concentration by the end of treatment, 7.1 - 10.2 mM of calcium remained in solution. Figure 5.2i also shows a clear difference in the rate of calcium depletion for the 0.5 M Urea and 0.075 M CaCl_2 cementing solution concentration between the medium and high biomass flasks. At medium biomass, 40.4 mM calcium remained and, at high biomass only 27.4 mM calcium remained in solution. Higher fungal biomass resulted in more calcium ions removed from solution. Comparing Figure 5.2g - i, as expected the initial calcium concentration between the different solution concentrations is different. As the initial calcium chloride concentration increases (moving from Figure 5.2g to i), more calcium is removed from solution. However, at low concentrations (Figure 5.2g and h) the

calcium ions were depleted, whereas at the highest CaCl_2 concentration tested, after 23 days there remains a high concentration of calcium ions in solution (Figure 5.2i).

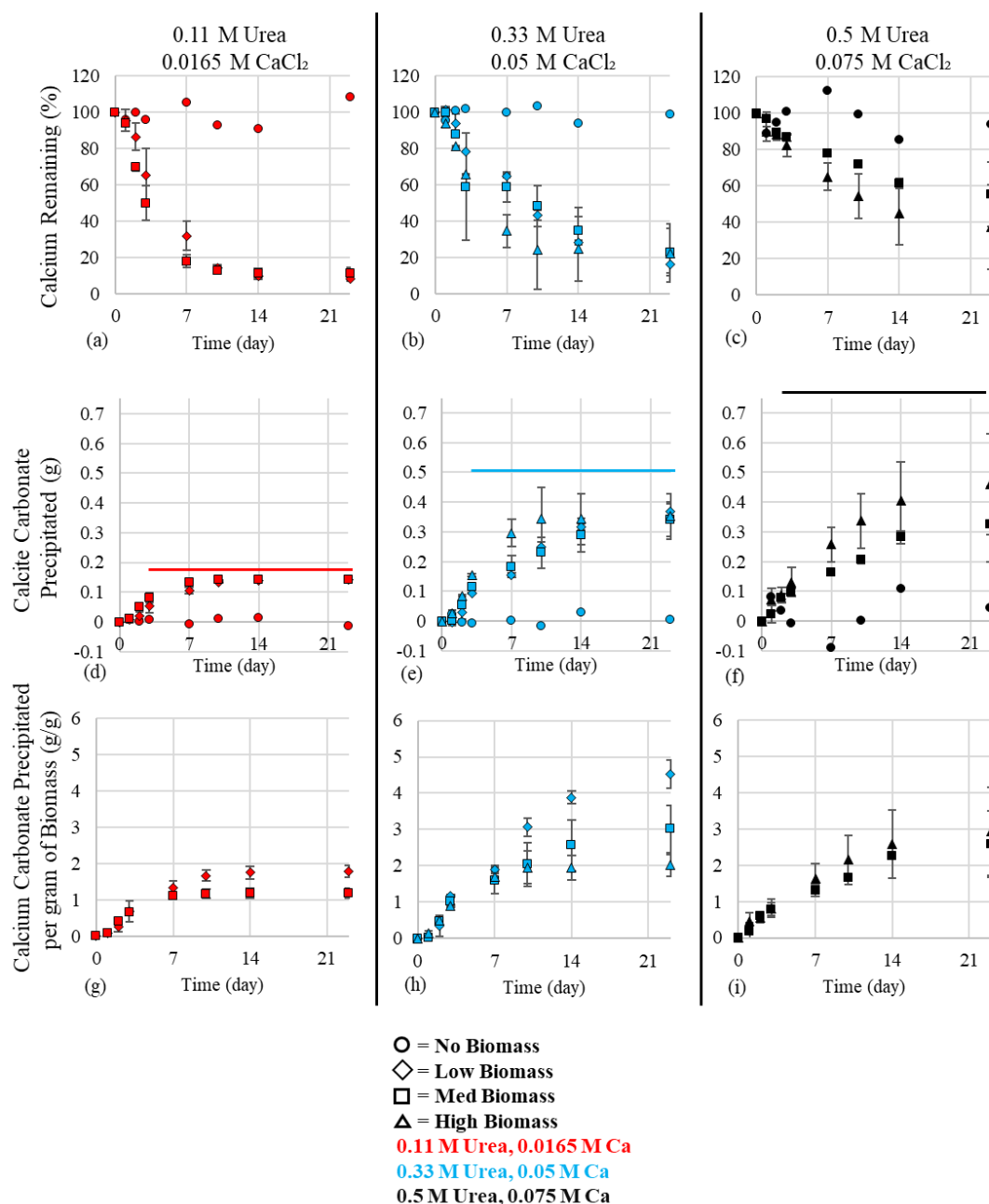


Figure 5.3 Experiment 1. Evolution of percentage calcium remaining: (a - c), mass of calcium carbonate precipitated with coloured lines displaying the maximum theoretical mass that could be precipitated : (d - f), and mass of calcium carbonate precipitated per gram of biomass: (g - i). Three concentrations were tested - 0.11 M Urea and 0.0165 M CaCl_2 solution: (a), (d), (g) - 0.33 M Urea and 0.05 M CaCl_2 solution: (b), (e), (h) - 0.5 M Urea and 0.075 M CaCl_2 solution: (c), (f), (i)

Figure 5.3a - c shows the percentage calcium remaining in solution over time. The controls (circles) show the percentage fluctuating around 100 % calcium remaining. This was expected because there was no fungal biomass present in the control flask.

Figure 5.3a shows that by the end of treatment with 0.11 M Urea and 0.0165 M CaCl_2 , there was only 8.4 % (low biomass, diamond) and 11.1 % (medium biomass, square) of calcium remaining – negligible difference in remaining calcium between different biomasses. This figure also highlights that there was a slightly faster rate of calcium precipitation with medium biomass than with lower biomass. Figure 5.3b shows that by end of treatment with 0.33 M Urea and 0.05 M CaCl_2 , 16.2 % (low biomass, diamond) 22.9 % (medium biomass, square), and 22.4 % (high biomass, triangle) of calcium remaining. This figure does not show a difference in final calcium remaining after treatment, however the rate of calcium removal from solution appeared to be faster with high biomass although these values had higher variability. Figure 5.3c shows that by end of treatment with 0.5 M Urea and 0.075 M CaCl_2 , there was 55.4% (medium biomass, square) and 37.5 % (high biomass, triangle) of calcium remaining in solution. This figure shows that at 0.5 M Urea and 0.075 M CaCl_2 solution concentration, higher biomass results in more and faster calcium removal from solution. The final results of % calcium removed after exposure to cementing solution for 23 days are displayed in Table 5.4 for ease of viewing.

Table 5.4 Experiment 1: Average % calcium removed from solution after 23 days

	0.11 M Urea 0.0165 M CaCl_2	0.33 M Urea 0.05 M CaCl_2	0.5 M Urea 0.075 M CaCl_2
Low biomass	91.6 %	83.8 %	-
Medium biomass	88.9 %	77.1 %	44.6 %
High biomass	-	77.6 %	62.5 %

Figure 5.3d - f shows the estimated mass of calcium carbonate precipitated over time. Each graph indicates the maximum theoretical calcium carbonate precipitate at each concentration with coloured line corresponding to that concentration: 0.165 g for 0.11 M Urea and 0.0165 M CaCl_2 cementing solution; 0.5 g for 0.33 M Urea and 0.05 M CaCl_2 cementing solution; and 0.75 g for 0.5 M Urea and 0.075 M CaCl_2 cementing

solution. The controls (circles) show little to no calcium carbonate precipitated without biomass.

Figure 5.3d shows that by the end of treatment with 0.11 M Urea and 0.0165 M CaCl_2 , both low and medium biomasses had precipitated a similar amount of calcium carbonate, ~ 0.14 g. It is evident that the plateauing observed was due to the limitation of calcium in the system, as the mass of calcium carbonate precipitated approaches the maximum theoretical amount. Figure 5.3e shows that by end of treatment with 0.33 M Urea and 0.05 M CaCl_2 , that similar masses of calcium carbonate were precipitated for low, medium, and high biomass, 0.34 to 0.37 g. Figure 5.3f shows that by the end of treatment with 0.5 M Urea and 0.075 M CaCl_2 , 0.33 g (medium biomass) and 0.46 g (high biomass) of calcium carbonate was precipitated, clearly a higher mass of CaCO_3 precipitated with higher fungal biomass. The final results of mass of calcium carbonate precipitated after treatment for 23 days are displayed in Table 5.5 for ease of viewing.

Table 5.5 Experiment 1: Average calcium carbonate precipitated after 23 days (g)

	0.11 M Urea 0.0165 M CaCl_2	0.33 M Urea 0.05 M CaCl_2	0.5 M Urea 0.075 M CaCl_2
Low biomass	0.142 g	0.369 g	-
Medium biomass	0.143 g	0.342 g	0.325 g
High biomass	-	0.352 g	0.459 g

Figure 5.3g - i shows the change in calcium carbonate precipitated per gram of biomass over time. Controls are not included on this graph as they had no biomass present in the flasks. In both Figure 5.3g and h, it can be seen that calcium carbonate precipitated per gram of biomass decreased with increasing biomass, with the low biomass flasks appearing to be more efficient. In Figure 5.3i, this difference is not reflected, and both biomass conditions (medium and high) exhibited similar calcium carbonate precipitation per biomass but, in these flasks, there was much higher variability. The final results of the mass of calcium carbonate precipitated per biomass after treatment for 23 days are displayed in Table 5.6 for ease of viewing.

Table 5.6 Experiment 1: Average calcium carbonate precipitated per gram of biomass after 23 days (g)

	0.11 M Urea 0.0165 M CaCl ₂	0.33 M Urea 0.05 M CaCl ₂	0.5 M Urea 0.075 M CaCl ₂
Low biomass	1.78 g/g	4.53 g/g	-
Medium biomass	1.19 g/g	3.01 g/g	2.58 g/g
High biomass	-	2.01 g/g	2.94 g/g

In summary, the most efficient system in terms of calcium conversion to calcium carbonate was the lower concentration cementing solution condition (0.11 M Urea and 0.0165 M CaCl₂) with low biomass, with conversion > 90 %. If we consider efficiency in terms of the mass of calcium carbonate precipitated per mass of fungal biomass, the most efficient system was the cementing solution concentration (0.33 M Urea and 0.05 M CaCl₂) containing the low amount of fungal biomass. However, for engineering systems it may be desirable to achieve the greatest amount of total calcium carbonate, and this was achieved by the high cementing solution concentration (0.5 M Urea and 0.125 M CaCl₂) with high biomass, precipitating 0.46 g of calcium carbonate.

5.3.2 Experiment 2 Influence of Calcium to Urea Concentration Ratio

Figure 5.4 shows the estimated biomass from each flask in Experiment 2. The estimated fungal biomass is shown on the y-axis, the flask ID number is shown on the x-axis. The colour of the biomass data point indicates the concentration of CaCl₂ that was used in each flask. Urea concentration was held constant for all flasks at 0.5 M. Although all flasks were inoculated with the same volume of fungal suspension inoculant (35 ml), the estimated fungal biomass contained within the flasks varied considerably. Flask 10 (grey triangle circled in black) was removed from consideration as it had very different biomass than its accompanying replicates (both grey triangles). The flasks with the highest CaCl₂ concentration (black triangles), Flasks 13, 14, and 15, all had high biomass relative to the other flasks but since they were all relatively similar to each other, as a set of replicates, they are still included in the discussion of the data presented here.

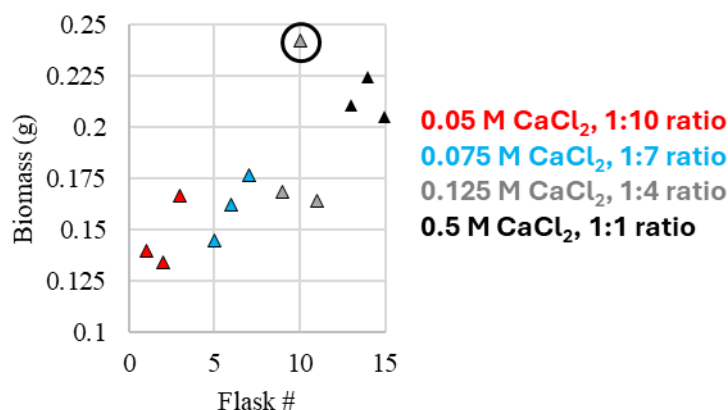


Figure 5.4 Experiment 2: Estimated biomass in each flask

Figure 5.5a - f presents the results comparing the flask experiments with varying ratios of Calcium chloride to Urea concentrations (1:10, 1:7, 1:4, 1:1). Note Urea concentration for all flasks was 0.5 M. Each figure shows the mean of the replicates of the same cementing solution ratio, and the error bars display the standard deviation of the measured replicate values.

Figure 5.5a shows evolution of ammonium over time. The cementing solution with a CaCl₂ to Urea ratio of 1:4 (grey) produced the most ammonium, with a rate (up to 11 days) that was similar to the 1:10, and 1:7 conditions. The cementing solution with a 1:1 ratio (black triangles) produced less ammonium. Initially, ammonium production was slightly slower in rate compared to the other ratios but, by day 10, ammonium production was almost completely inhibited at the 1:1 ratio. Figure 5.5b shows evolution of pH over time. The 1:10, 1:7, and 1:4 ratio specimens showed similar trends in pH, starting at pH ~7.4, exhibiting an initial increase to pH values between 8.0-8.21, thereafter there was a decrease in pH, until day 7, followed by an increase. However, for the 1:1 ratio, the initial pH was higher, and then decreased until day 3, and from day 7 onwards remained fairly constant. Final pHs were achieved of: 8.59 for 0.05 M (red), 8.29 for 0.075 M, 8.16 for 0.125 M (grey), and 7.83 for 0.5 M (black) calcium concentration. Figure 5.5c shows evolution of calcium over time. Samples show that all ratios tested showed calcium depletion over time, with the exception of the 1:1 ratio which showed fluctuation of calcium concentration around the initial concentration (and values even higher than the initial concentration at later time points), despite having the highest amount of fungal biomass present. Figure 5.5d

shows the percentage remaining calcium over time. The cementing solution ratio of 1:10 (0.05 M calcium, in red) showed the most efficient removal of calcium from solution, with efficiency reducing with increasing calcium concentration.

As there was no clear indication of calcium carbonate precipitation at the 1:1 ratio (see Figure 5.5c and d), the 1:1 ratio results are not presented in Figure 5.5e and f. Figure 5.5e shows estimated mass of calcium carbonate precipitated over time. Although at early timepoints the mass of calcium carbonate precipitates was similar, at later timepoints (days 10 and beyond) a 1:4 ratio of calcium to urea (calcium concentration of 0.125 M) consistently resulted in the most calcium carbonate precipitated (after 31 days, 0.67 g). Figure 5.5f shows estimated calcium carbonate precipitated per gram of biomass over time. Similarly, the 1:4 ratio appeared also to be the most efficient, with the most calcium carbonate precipitated per gram of biomass (~4 g/g after 31 days).

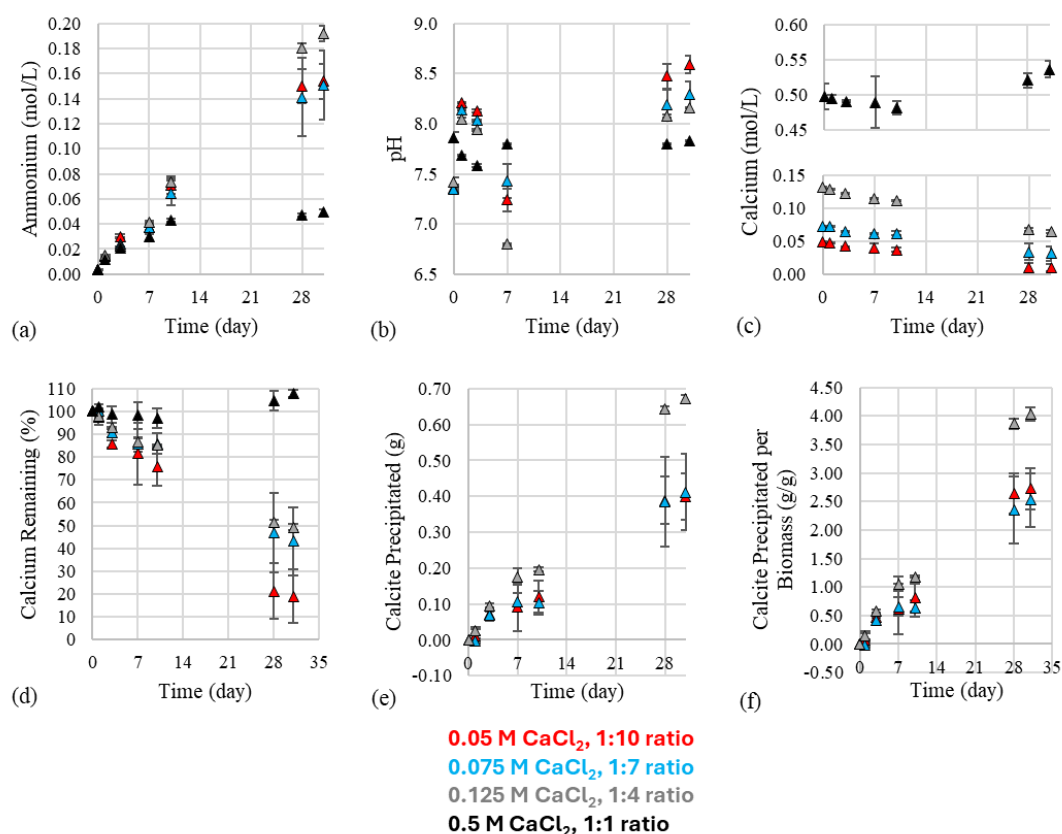


Figure 5.5 Experiment 2. Evolution of (a) ammonium concentration, (b) pH (c) calcium concentration (d) percentage calcium remaining (e) mass of calcium carbonate precipitated and (f) mass of calcium carbonate precipitated per gram of biomass

5.3.3 Experiment 3 Influence of pH Adjustment

Figure 5.7 shows the estimated biomass in each flask. The estimated biomass is shown on the y-axis, the flask ID number is shown on the x-axis. The colour of the biomass data point indicates the solvent and pH amendment that the biomass was treated with. Grey means the solvent was tap water with no pH amendment, black means UPDW was the solvent used to prepare the cementing solution with no pH amendment. Furthermore, UPDW was also amended with 1 ml of 1 mM NaOH (red), 5mM NaOH (blue), 10mM NaOH (yellow). Flask 3 and 10 (circled in Figure 5.7) were removed from consideration as their biomass was far higher than the other flasks within their replicate sets. Flasks 4, 8, 12, 16, and 20 were not included in this figure as they were controls and contained no biomass.

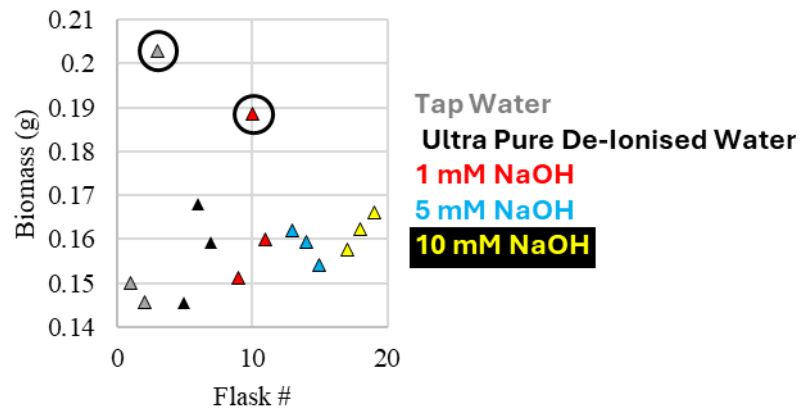


Figure 5.7 Experiment 3: Estimated biomass in each flask

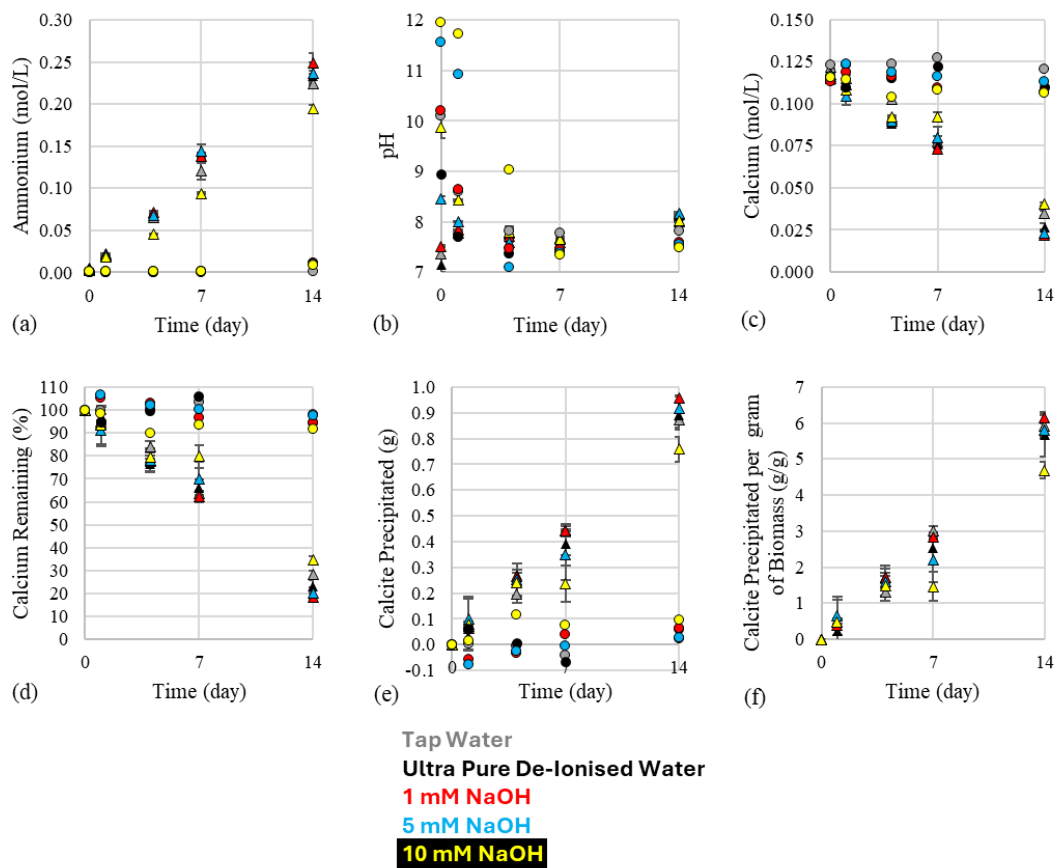


Figure 5.6 Experiment 3. Evolution of (a) ammonium concentration, (b) pH, (c) calcium concentration and (d) percentage calcium remaining (e) mass of calcium carbonate precipitated (f) mass of calcium carbonate precipitated per gram of biomass. 1:4 ratio Ca:Urea, 0.125 M CaCl_2 , 0.5 M Urea cementing solution

Figure 5.6a - f shows the influence of different pH adjusted cementing solutions on urea hydrolysis and calcium carbonate precipitation for Experiment 3. In Experiment 3, a ratio of 1:4 was tested, with concentrations of 0.125 M CaCl_2 and 0.5 M Urea cementing solution. Each figure shows data points (triangle) which are the mean of the replicate values of the same cementing solution ratio, and error bars display the standard deviation of the measured replicate values. Control data points (circle) were not replicated so those points are not averages and do not have error bars.

Figure 5.6a shows the evolution of ammonium over time. The controls (circle) show that without the addition of fungal biomass there was no production of ammonium. The samples containing fungal biomass (triangle) show that all of the different solutions produce similar ammonium concentrations during treatment. The most obvious difference was that amendment with 10 mM NaOH, which resulted in the highest initial pH (9.87, yellow), had a lower rate of ammonium production than the other pH amendments tested.

Figure 5.6b shows the evolution of pH over time. The controls (circle) show that the starting pH of the cementing solution was lowest (pH = 8.94) for the control specimen with cementing solution prepared with UPDW (black), 10.11 for cementing solution prepared with tap water (grey), 10.21 for UPDW amended with 1 mM NaOH (red), 11.55 for UPDW amended with 5 mM NaOH (blue), and highest at pH = 11.94 for UPDW amended with 10 mM NaOH (yellow). This starting pH decreased over the first week to between pH 7.34 and 7.45. Tap water solution after a week had the highest pH at 7.78. pH stabilised at these pHs (7.3 – 7.8) for the control flasks. Samples containing fungal biomass (triangles) showed a different initial pH for each different treatment solution. The pH of the flasks containing fungal biomass was always lower than its corresponding control, although the initial pH immediately after inoculation was again lowest (pH = 7.16) for the flask prepared with UPDW (black), 7.37 for cementing solution prepared with tap water (grey), 7.52 for UPDW amended with 1 mM NaOH (red), 8.46 for UPDW amended with 5 mM NaOH (blue), and 9.87 for UPDW amended with 10 mM NaOH (yellow). After 1 week, each of these treatment solutions reached pH values between 7.59 and 7.67. After another week, these treatment solutions increased to between pH 8.01 and 8.16. Whilst these treatment

solutions had different initial pHs, they all reached similar pH values towards the end of treatment.

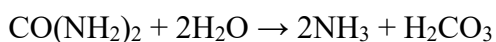
Figure 5.6c - e show the evolution of calcium in solution, percentage calcium remaining in solution, and calcium carbonate precipitated over time. The controls (circle) in each figure show that without the addition of fungal biomass, as expected, there was little change in the calcium in solution, percentage calcium remaining in solution, and calcium carbonate precipitated respectively. Samples containing fungal biomass (triangle) showed that the different pH adjustment strategies all showed similar trends and values. Nevertheless, the greatest pH adjustment (shown in yellow triangles) did appear to precipitate the least amount of calcium carbonate, this is reflected in all three figures.

Figure 5.6f shows the evolution of calcium carbonate precipitated per gram of biomass over time. Controls are not displayed on this figure as they had no biomass present. Fungal containing flasks showed little difference in calcium carbonate precipitated per biomass between the different treatment solutions, except that the solution amended with the most pH (yellow) exhibited the least amount of calcium carbonate precipitation per gram of biomass by the end of the experiment (14 days).

5.4 Discussion

This series of experiments were designed to better understand the influence of biomass, cementing solution and initial pH on ureolysis and calcium carbonate precipitation.

When considering these variables, it is useful to also consider the chemistry of the reaction being studied:



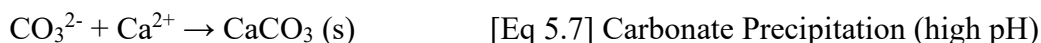
[Eq 5.4] Ureolysis (via fungal urease)



[Eq 5.5] Ammonia Equilibrium



[Eq 5.6] Carbonate Ion Equilibrium



Fungal urease hydrolyses urea into ammonia and carbonic acid in Eq 5.4. Ammonia in an aqueous solution exists in equilibrium [Eq 5.5] as ammonia (NH_3) and ammonium (NH_4^+). Critically, the production of ammonium from the balancing of this equilibrium also produces hydroxide ions, which raises the pH of the solution. Carbonic acid consumes these hydroxide ions in its own series of equilibria [Eq 5.6] to produce carbonate ions. If a calcium source is then included or introduced to the solution, then the calcium ions react with the carbonate ions to produce calcium carbonate [Eq 5.7], which can only precipitate at high pH. However, this final reaction consumes carbonate ions, forcing Eq 5.6 to shift to the right, also consuming hydroxide ions, and therefore lowering the pH. Because of this, there needs to be an excess of urea provided to the system to ensure that the pH remains high during the reaction and calcium carbonate precipitation is favourable. In the other case, if there is no calcium source, or no calcium source remaining, the pH should begin to increase, as there is no consumption of hydroxide ions due to the reaction of the calcium source (Tobler et al., 2011).

This was why pH was monitored throughout these experiments: to ensure that a high pH was induced and calcium carbonate precipitation remained favourable. This series of equations are important to consider when discussing the results of this chapter.

5.4.1 Biomass

The influence of increasing biomass contributed to faster rates of ureolysis (i.e. ammonium production) only at higher cementing solution concentrations, at the lowest concentration tested (0.11 M Urea and 0.0165 M CaCl_2) the biomass did not have an effect on ureolysis, indicating that in this condition the urease enzyme was not the limiting factor for ureolysis. Here ammonium production plateaued when the calcium became depleted in the solution (~ 7 days), even though excess urea remained in solution. The pH evolution at all concentrations tested was similar, showing no effect of different amounts of biomass. Increasing biomass did appear to increase the rate of

calcium carbonate precipitation at the higher concentrations tested when calcium was not limited (i.e. 0.33 M Urea and 0.05 M CaCl_2 ; 0.5 M Urea 0.075 M CaCl_2), possibly due to increasing the rate of availability of carbonate ions (via ureolysis) but possibly also providing more nucleation sites for calcium carbonate precipitation. The rate of calcium carbonate precipitation per gram of biomass was similar between all biomasses up to 7 days, confirming that increasing biomass would increase the rate of calcium carbonate precipitation.

5.4.2 Overall cementing solution concentration

Increasing cementing solution concentration did not appear to change the initial rate of ureolysis (up to 7 days), indicating that this was being limited by either the availability of urease or the availability of calcium ions. Although, as expected, increasing the cementing solution concentrations increased the maximum amount of ammonium produced. For the lowest cementing solution concentrations tested (i.e. 0.11 M Urea and 0.0165 M CaCl_2), shown in Figure 5.5a, it can be seen that ammonium production plateaued at 0.08 M. A possible reason for this is that the pH was too high in the low concentration flasks and the fungal urease may have become inhibited. Note on day 10, when ureolysis appeared to end, i.e. ammonium plateaued, pH reached 8.88 for medium biomass, and 8.76 for low biomass. Notably, the pH only reached these high values after most available calcium ions had been consumed in these flasks, (as expected). Higher concentration treatment solution did not reach such high pH values, as they had more available calcium, and so did not experience the same degree of calcium depletion as the low concentration flasks. Microbial urease has been found to become inhibited by pH at least partially (Fujita et al., 2017; Imran et al., 2019; Lauchnor et al., 2015; Singh & Nye, 1984), although the exact pH can vary depending on the microbe producing the enzyme, depending on the bacteria's own abilities to self-regulate and survive such extreme conditions.

Higher concentration treatment solutions did not appear to affect the rate of calcium precipitation but did increase the maximum amount of calcium carbonate precipitated as higher concentration solutions were not calcium limited.

5.4.3 Calcium to Urea Ratio

When the concentration of urea was fixed at 0.5 M, and the concentration of calcium varied, urease inhibition occurred at a calcium concentration between 0.125 M and 0.5 M, corresponding to a ratio of Calcium:Urea between 1:4 and 1:1. At a 1:1 ratio, the pH never exceeded 7.86, much lower than the other ratio conditions tested. This is likely due to the interactions of Eq 5.6 and 5.7 and overconsumption of hydroxide ions due to the high concentration of calcium ions. This low pH then did not favour calcium carbonate precipitation. Additionally, it appears that urease activity itself was inhibited after 10 days of treatment with a 1:1 ratio of Calcium:Urea with a concentration of 0.5 M CaCl_2 . This would have meant that there were very few carbonate ions free in solution to react with the calcium ions and resulting in the negligible calcium carbonate precipitation. Of the ratios and calcium concentrations tested here, a solution concentration of 0.125 M CaCl_2 and 0.5 M Urea (1:4 ratio) resulted in the highest rate of ureolysis, most total calcium carbonate precipitated, and most calcium carbonate precipitated per gram of biomass. This study found a cementing solution of 0.125 M calcium chloride and 0.5 M urea was the most effective cementing solution of the concentrations and ratios investigated.

5.4.4 pH Amendment

When designing this experiment, it was hypothesised that increasing the initial pH of the cementing solution could increase the favourability of calcium carbonate precipitation [Eq 5.7]. It could also push [Eq 5.6] to the right, causing more carbonate ions to be available for calcium carbonate precipitation.

However, this experiment found that there was no beneficial effect from increasing the initial pH. Cementing solutions prepared using sterilised tap water and sterilised UPDW showed similar ureolysis and calcium carbonate precipitation. Similarly, amending the cementing solution with sodium hydroxide did not have a positive effect, and amendment with higher NaOH concentrations resulted in slightly inhibited ureolysis and CaCO_3 precipitation.

During experimental design, it was thought that additional hydroxide ions should not affect the rate of ureolysis [Eq 5.4] unless the conditions begin to inhibit the activity of the urease enzyme. From the results, we can see that this is true, with

little effect on ureolysis due to increases in the initial pH, apart from with the addition of 1 ml of 10 mM NaOH. With this amendment, the rate of ureolysis appears to be lower than the other conditions, noticeable by considering $t = 3$ days when ammonium production is lower than for the other pH conditions tested (Figure 5.6). It has been hypothesised in the discussion of overall concentration that a high pH could inhibit urease activity and that might also be contributing to the lower ureolysis rate observed here (at $t < 3$ days). After the pH decreases, the urease activity appears to recover to a similar rate to the other flask conditions (i.e. based on slope of ammonium production between 7-14 days, Figure 5.6).

5.4.5 Batch variability

Another point to note is that some samples from Experiment 2, Section 5.3.2 should be replicates of Experiment 3, Section 5.3.3. Specifically, the grey triangles from Fig 5.5 and the black triangles from Fig 5.8. Both of these samples used cementing solutions of 0.125 M CaCl_2 and 0.5 M urea in UPDW to treat similar quantities of fungal biomass inoculant. They should have produced similar results in this similar environment. However, the results are quite different, the results obtained after 7 days are compared in Table 5.7.

Table 5.7 Comparison of average results from Experiment 2 and 3 after 7 days

Figure	Data Point Appearance	Section	Average Biomass (g)	Average Ammonium (mol/L)	Average pH	Average Precipitate (g)	Average Precipitate per Biomass (g/g)
5.5	Grey triangle	5.3.2	0.166	0.04	6.8	0.18	1.06
5.7	Black triangle	5.3.3	0.157	0.14	7.67	0.39	2.53

This table shows that despite having similar biomass on average, the samples from Figure 5.5 underperformed in all aspects, compared to the samples in Figure 5.6, their “replicate”. This suggests that the fungal biomass used to inoculate the flasks in Figure 5.5 had a very low urease activity. This is most obvious when considering the ammonia production, the first step in the reaction, which was much lower than in UPDW samples, despite having a higher fungal biomass. This highlights that urease activity of individual fungal inoculants is quite important, and a method for measuring

urease activity before growth would be beneficial. Additionally, it highlights that it can be difficult to compare results produced from different batches of fungal inoculant, as there can be a variance in urease activity. Hence, in this chapter conclusions have been drawn based on comparisons between samples sets within each individual Experiment, where the fungal inoculant used was prepared from the same batch of fungal suspension.

5.5 Conclusions

The key findings of this chapter are: (1) a 1:1 ratio of calcium chloride to urea should not be adopted for FICP as it can inhibit calcium carbonate precipitation, (2) a cementing solution ratio of 1:4, 0.125 M calcium chloride and 0.5 M urea, was found to be optimal of the investigated cementing solution concentrations in terms of ureolysis, total calcium carbonate precipitation and calcium carbonate precipitation/g of biomass. (3) Increasing the amount of fungal biomass was observed to increase the rate of ureolysis and calcium carbonate precipitation, when the systems were not calcium limited. (4) Increasing the initial pH of the cementing solution did not have a beneficial on ureolysis or calcium carbonate precipitation, indeed once the initial pH reached a value of 9.87 the pH adjustment appeared to have a negative impact on the rate of ureolysis and carbonate precipitation.

Chapter 6: Investigation of treatment strategies for FICP of sand

6.1 Introduction

To date, studies into fungal induced calcium carbonate precipitation (FICP) have mostly focussed on applications within self-healing concrete (Menon et al., 2019) and heavy metal remediation of water and soil (Dhami, Quirin, et al., 2017; Li et al., 2014, 2015; J. Zhao, Csetenyi, et al., 2022). However, another potential application for FICP is the improvement of the mechanical properties of soil and erosion resistance.

Recent research has shown that the growth of saprotrophic filamentous fungi within soils has potential benefits for ground engineering applications. This includes improving soil cohesion, modifying soil water repellency, water infiltration, hydraulic conductivity and erosion resistance (Bhurtel et al., 2024; Park et al., 2023; Salifu, 2019; Salifu et al., 2022; Salifu & El Mountassir, 2019, 2021). There is potential for further treatment using FICP to enhance further mechanical fungal-induced modifications, in particular erosion resistance, and the durability of these modifications.

6.1.1 Fungal strain

Table 6.1 displays the limited studies available at the time of writing which have investigated the treatment of soils using FICP via ureolysis. It can be seen from Table 6.1 that there were some similarities between these studies. Each of these studies utilised Ascomycetes for FICP via ureolysis, marked with [A]. Ascomycetes commonly reproduce spores asexually and have frequent release of spores. Some Ascomycetes are known to be harmful to human health, specifically the respiratory system. The asexual spores of *Penicillium chrysogenum*, (a common mold found in indoor environments) are known human allergens (Der Shen et al., 2003). *Aspergillus niger*, also previously studied for FICP via ureolysis, is known to cause lung disease (aspergillosis) and ear infection (otomycosis) (Schuster et al., 2002).

6.1.2 Treatment solutions

Each of the studies listed in Table 6.1 included inoculated growth media in the cementing solution, which was introduced into sand columns by mixing, to support fungal growth during FICP treatment. The only one which did not was Tuckett et al. (2025) who poured the “fungal slurry” over the top of the soil to inoculate and also allowed the fungus a period of growth before treatment with cementing solution. All three fungal species previously studied (*Penicillium chrysogenum*, *Aspergillus niger*, *Neurospora crassa*) induced calcium carbonate precipitation indicating resistance to the high pH environment induced during FICP treatment via ureolysis. These studies are discussed below.

6.1.3 Treatment strategies

Total treatment time (i.e. fungal growth + FICP treatment) for the three older studies listed in Table 6.1 (Ahmad et al., 2024; Fang et al., 2018; Martuscelli, Soares, Lima, et al., 2020) were in the scale of weeks, with treatment cycles of cementing solution injection lasting between 1 and 6 days. Each paper defined a treatment cycle differently. Fang et al. (2018) injected an unspecified volume of cementing solution (330 mM urea, 40 mM calcium chloride and broth media) through the sand column continuously, refreshing the cementing solution every 48 hours. Whereas Martuscelli, Soares, Lima, et al. (2020) injected 10ml of cementing solution (330 mM urea, 40 mM calcium chloride and broth media) into their columns every 48 hours. Ahmad et al. (2024) injected 120ml cementing solution (1 M urea, 1 M calcium chloride and broth) and held the cementing solution within the sand column for 24 hours before injecting the next cycle.

All of these highlighted studies treated the sand columns with cementing solution almost immediately after mixing fungal suspension into the sand. There was no time allowed for growth of the fungus in situ within the sand prior to treating.

The outlier was Tuckett et al. (2025), who planned a growth period for the fungus within the soil up to 10 days, before introducing a single cementing solution cycle. This was poured onto the top of the sand, as they treated a petri dish containing sand, instead of a sand column.

6.1.4 Post-treatment analyses

All four studies, highlighted in Table 6.1, directly measured the change in unconfined compressive strength (UCS) in sand due to FICP treatment. Note the initial UCS of untreated sand is zero. These papers found a UCS improvement of 50-66.5 kPa (Martuscelli, Soares, Lima, et al., 2020), up to 1800 kPa (Fang et al., 2018), up to 2700 kPa (Tuckett et al., 2025), and 3930 kPa (Ahmad et al., 2024). These differences are likely due to differences in the mass and distribution of calcium carbonate precipitated within these FICP columns, due to variations in cementing solution concentration, fungal strain selected, treatment strategy used, as well as variations in the material tested. However, only one of these papers quantified the calcium carbonate precipitation achieved in these FICP specimens (Tuckett et al., 2025), they found an increase of up to 0.9% calcium carbonate content by weight after treatment.

The studies in Table 6.1 were used to inform the research conducted in this chapter but with a key difference. This study used a Basidiomycete, Agaricomycete: *Pleurotus cornucopiae*, instead of a species from the Ascomycota phylum. This was to minimise hazards associated with frequent release of asexual spores from microscopic sacs associated with Ascomycetes. Generally, agaricomycetes instead spore from a temporary large visible fruiting body (commonly known as a mushroom) which requires certain environmental conditions to form (Coelho et al., 2017; Kues & Liu, 2000; Pelkmans et al., 2016).

The overall aim of this study was to develop a strategy for treating sand via FICP using *P. cornucopiae*. The objectives of this study were to:

1. Investigate the influence of the duration of the exposure of cementing solution to fungal biomass (cycle duration) and the number of treatment cycles on the mass and distribution of fungal-induced calcium carbonate precipitation.
2. Investigate the influence of inoculant amendments and particle grain size on fungal-induced calcium carbonate precipitation.
3. Systematically investigate the microstructure of FICP treated sand and the fungal-carbonate-sand interactions.

Table 6.1 Previous studies which have treated sand columns to improve mechanical strength with fungal-induced carbonate precipitation via ureolysis

Reference	Fungal Species	Cementing Solution	Treatment strategy	Cementing Time	Cemented Sample	Investigation Focus
Fang et al. (2018)	<i>Penicillium chrysogenum</i> [A]	Modified Martin Broth, 40 mM CaCl ₂ , 330 mM urea	Continuous flow. Cementing solution refreshed every 48 hours equating to one cycle.	2 weeks, 48 hour cycle, 7 cycles	Sand	Compressive strength improvement
Martuscelli, Soares, Lima, et al. (2020)	<i>Penicillium chrysogenum</i> [A] <i>Neurospora crassa</i> [A]	Modified Martin Broth, 40mM CaCl ₂ , 330 mM urea	10 ml injection of cementing solution every 48 hours, equating to one cycle	2 weeks, 48 hour cycle, 7 cycles	Sand	Compressive strength and density
Ahmad et al. (2024)	<i>Aspergillus Niger</i> [A]	1 M Urea and CaCl ₂ , MGYF growth media after 7 cycles	120 ml injection of cementing solution, hold for 24 hours, equates one cycle	7 and 28 cycles of 24 hours each	Sand	UCS improvement, permeability decrease
Tucket et al. (2025)	<i>Penicillium chrysogenum</i> [A]	Various growth times (0 – 10 days) and cementing solution concentrations (0.05 – 1 M Urea, 0.05 – 1.5 M CaCl ₂)	Pored over the top of a shallow petri dish filled with soil	1 – 2 days	Soil	Penetrometer UCS improvement, mass of calcium carbonate precipitation

6.2 Method

6.2.1 Broth Incubation

Pleurotus cornucopiae was grown on malt extract agar amended with 0.03 M urea at 25 °C in the dark for four days. Twenty 5 mm diameter plugs were taken from the growing edge of the fungal culture and used to inoculate 2 L of malt extract broth amended with 0.03 M urea in a 5 L flask. The broth was agitated with a magnetic stirrer and grown in the dark at 20 °C for 1 week. Fungus was harvested from suspension by centrifugation at 8000 rpm for 10 mins. The resulting sludge was then resuspended up to 200 ml with 0.5 mM NaCl sterile de-ionised water to make the fungal inoculant.

6.2.2 Column Construction and Incubation

Specimens were prepared by mixing 90 % sand, and 10 % beechwood chips (dry weight). For Column Sets 1-8, coarse sand was used, with 91.9 % of the particles with a grain size between 0.6 - 2 mm, whereas for Column Set 9, a medium sand with 86.8 % of the particles with a grain size between 0.3 - 0.6 mm (see Figure 6.1). The initial porosity of the columns was calculated using the following equation:

$$Porosity (\varphi) = \frac{V_{soil} - (\frac{m_{sand}}{\rho_{sand}} + \frac{m_{beechwood}}{\rho_{beechwood}})}{V_{Soil}} \quad [Eq 6.1]$$

Where,

V_{soil} is the total volume of the soil as compacted in the column,

m_{sand} is mass of sand,

$m_{beechwood}$ is mass of beechwood,

ρ_{sand} = particle density of sand = 2.65 g/cm³ (Schjønning et al., 2017),

$\rho_{beechwood}$ = particle density of beechwood 1.43 g/cm³ (Appendix 1).

The initial porosity was 0.51 for the coarse sand columns and 0.53 for the medium sand columns.

Beechwood chips were selected as the nutrient source based on the results in Section 4.3.3.2. Before column construction, dried beechwood chips were weighed out, autoclaved, and then soaked overnight in a sterile 40 ml “soaking solution” of

deionised water. Excess soaking solution was removed by simply pouring away before chips were mixed with the sand. At this point, 8 ml of the fungal suspension inoculant was added to the sand/beechnod and manually mixed. Both the soaking solution and fungal inoculant suspension included 0.5 mM NaCl and, depending on the column set, were both sometimes amended further with 0.03 M urea or was replaced with malt extract broth.

The mixture was emplaced and lightly compacted into clear split acrylic columns (re-sealed with silicon) with dimensions of 35 mm internal diameter, to form soil columns with length 76 mm. The columns had an initial moisture content of 32.5 %. For Column Set 7, the fungal suspension and water used to soak the beechwood chips also contained 0.03 M Urea. This was the same concentration of urea used to grow *P. cornucopiae* in the broth. For Column Set 8, the fungal suspension and water used to soak the beechwood chips included malt extract broth instead of NaCl.

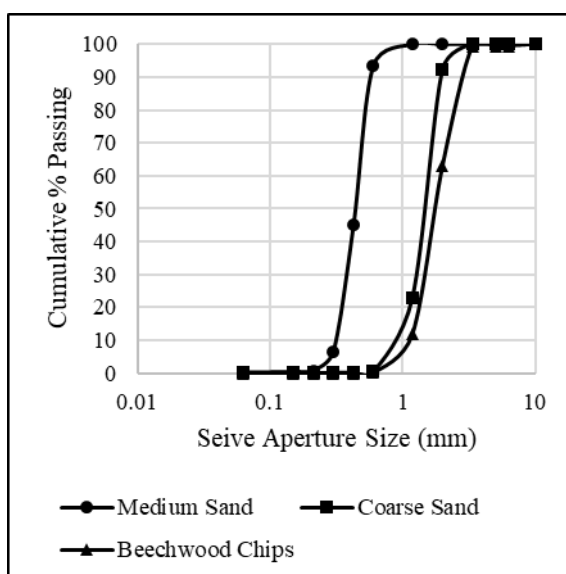


Figure 6.1 Particle Size Distribution of Soil Components

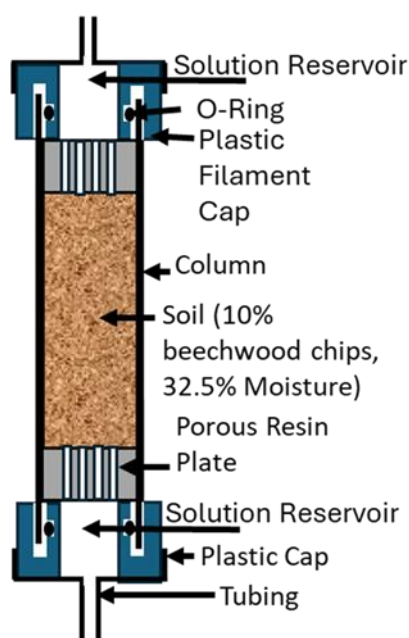


Figure 6.2 Column Construction

Columns were constructed to allow pumping of treatment solutions through the inoculated soil, as the fungal biomass induced hydrophobicity in the samples which limited infiltration via gravity-fed percolation as observed in the preliminary sand columns in Chapter 4. Each column contained a porous resin plate above and below the soil sample, and each end was sealed with a plastic cap, with an inlet/outlet port to facilitate injection and drainage of the treatment solutions. Note, each end of the column had a space of 11.5 ml referred to as the solution reservoir, see Figure 6.2.

Column sets were constructed in quintuplicate and incubated for 5 weeks in the dark (selected based on results in 4.5.2) at 25 °C with 40 % Relative Humidity.

6.2.3 Treatment strategy

Based on the results in Section 5.3.2, a cementing solution comprising 0.125 M CaCl₂ and 0.5 M urea (Ca:Urea ratio of 1:4) was selected for all treatments. The treatment solution used in this study did not include growth media, unlike the previous studies outlined in Table 6.1 using other fungal species. This was because *P. cornucopiae* is sensitive to high pH environments and so here the approach taken was to first grow the fungus in situ within the sand, before a second stage of introducing the cementing solutions for FICP. This was adopted based on the results shown in Section 3.3.1, which indicated that growth of *P. cornucopiae* can be inhibited in high ammonium/high pH conditions.

In each cycle, cementing solution was injected into the base of the column using a peristaltic pump at a rate of 0.5 ml/min for the first cycle, and 1 ml/min for all subsequent injections. During each treatment cycle cementing solution was injected until it was observed to exit the outlet tubing, i.e. both the bottom and top solution reservoirs were filled with cementing solution as well as the pore space within the sand column.

In Sections 5.3.1 - 5.3.3, it was observed that calcium carbonate precipitation occurred over weeks in solution in flask experiments. Previous studies investigating FICP treatment of sand used cycle durations of 1 to 6 days (Table 6.1). Here the cycle duration refers to the length of time that the sand column was exposed to cementing solution during a single cycle. Here, cycle durations of 1 day, 3 days, or 7 days were investigated. The number of treatment cycles chosen for investigation were: 3, 6, and 9 cycles.

For Column sets 1 - 3 and 5 - 9, after injection the cementing solution was held within the column for the cycle duration, i.e. the valve at the base of the column was closed after injection. After the desired cycle duration had passed, the cementing solution fluid was drained from the columns using a peristaltic pump at a rate of 1 ml/min. This constituted one treatment cycle for these columns, and the strategy is referred to in this chapter as “*Submerged*”. These columns were immediately injected

with fresh cementing solution for the next cycle. However, for Column Set 4, the columns were drained immediately after completing injection of the cementing solution i.e. once the cementing solution visibly exited the tube at top of the column, the fluid was drained from the base of the column using a peristaltic pump at a rate of 1 ml/min, therefore only residual cementing solution was left in the pore spaces for the cycle duration. This constituted one treatment cycle for these columns, and this strategy is referred to in this chapter as “*Drained*”.

6.2.4 Maximum calcium available

The total quantity of calcium available in each soil column for precipitation was calculated using a series of equations, assuming that each soil column received 1 pore volume of 0.125 M CaCl₂ and 0.5 M Urea (note 0.3 PV was held within the top and bottom reservoirs during a treatment cycle):

$$V_V = V_{soil} - \left(\frac{m_{sand}}{\rho_{sand}} + \frac{m_{beechwood}}{\rho_{beechwood}} \right) \quad [\text{Eq 6.2}]$$

where, V_V = Volume of voids in the soil (cm³) = 1 PV, V_{soil} = Total volume of the soil samples as compacted into the column (cm³), m_{sand} = mass of sand (g), $m_{beechwood}$ = mass of beechwood (g), ρ_{sand} = density of sand (g/cm³), $\rho_{beechwood}$ = density of beechwood (g/cm³) and

$$m_{CaCO_3} = V_V \times c_{Ca} \times M \quad [\text{Eq 6.3}]$$

where, m_{CaCO_3} = maximum theoretical mass of calcium precipitated (g), V_V = Volume of voids (L), c_{Ca} = concentration of calcium in solution (M), $M_{(Ca)}$ = molar mass of calcium carbonate (g/mol).

It was calculated that in each treatment cycle in coarse sand, 0.47 g of calcium carbonate was the theoretical maximum possible calcium carbonate precipitate. During treatment of the medium sand, the theoretical maximum possible calcium carbonate precipitate within the soil per cycle was 0.49 g.

6.2.5 Experimental Campaign

Table 6.2 displays each set of columns and outlines the variables tested. The variable that each set investigated is highlighted in green for ease of viewing.

- Column Sets 1 - 3 investigated the influence of cycle duration with durations of 1, 3 and 7 days respectively, each received 6 cycles in total.
- Column Set 4 investigated the effect of draining the soil immediately after injection and can be compared with Column Set 2. Both sets received 6 cycles with a cycle duration of 3 days.
- Column Sets 1, 5 and 6 investigated the influence of the number of treatment cycles with 3, 6, and 9 cycles. All three columns were treated with a cycle duration of 1 day.
- Column Sets 7 and 8 investigated the effect of using slightly different fungal inoculant and beechwood soaking solutions on calcium carbonate precipitation. Set 7 inoculant and soaking solution was amended with 0.03 M urea. Set 8 inoculant and soaking solution included malt extract broth rather than 0.5 mM NaCl. These sets could be compared with Set 6 which had an inoculant and soaking solution of 0.5 mM NaCl and was treated with the same number and duration of cycles (9 cycles of 1 day each).
- Column Set 9 investigated the effect of using sand with a smaller particle size, referred to here (and described previously) as “medium” sand. This set could be compared with Set 6 (Coarse sand) which was also treated with 9 cycles of 1 day each.

Table 6.2 Column Sets Explanation

Column Set	Column #	Media	Soil Mass (g)	Beechwood Mass (g)	Inoculant (ml)	Inoculant Amendment (mM)	Cycle Duration	# of Cycles	Treatment Method	Max Theoretical Calcium Carbonate (g)
1	1 – 5	Coarse	78.7	8.8	8.75	NaCl: 0.5	1	6	Submerged	2.8
2	6 – 10	Coarse	78.7	8.8	8.75	NaCl: 0.5	3	6	Submerged	
3	11 – 15	Coarse	78.7	8.8	8.75	NaCl: 0.5	7	6	Submerged	
4	16 – 20	Coarse	78.7	8.8	8.75	NaCl: 0.5	3	6	Drained	
5	21 – 25	Coarse	78.7	8.8	8.75	NaCl: 0.5	1	3	Submerged	1.4
6	26 – 30	Coarse	78.7	8.8	8.75	NaCl: 0.5	1	9	Submerged	4.2
7	31 – 35	Coarse	78.7	8.8	8.75	NaCl: 0.5 Urea: 30	1	9	Submerged	
8	36 – 40	Coarse	78.7	8.8	8.75	Malt Extract Broth	1	9	Submerged	
9	41 – 45	Medium	75	8.3	8.33	NaCl: 0.5	1	9	Submerged	4.4

The broth and columns were grown and treated in two batches: Batch 1 was used to inoculate Sets 1 - 4, Batch 2 was used to inoculate Sets 5 - 9 (see Table 6.2). Each set was prepared in quintuplicate, resulting in 45 specimens.

6.2.6 Post-treatment Analyses

6.2.6.1 Calcium carbonate determination

Once the column had been treated with the desired number of cycles, the columns were injected with water and drained immediately, injected with water again and left submerged for 24 hours then drained, and injected with water and immediately drained again for a final time. This meant each column was washed with water three times. This was done to remove any residual salts from the pore space which may affect the calcium carbonate measurement.

Three columns from each quintuplicate set were taken for whole column calcium carbonate determination. The columns were heated at 530 °C to remove

beechwood chips and fungal biomass (as outlined in Section 4.4.1), before being digested with hydrochloric acid in a closed system using a pressure gauge to measure the carbon dioxide emissions. This result was then used to calculate the mass of calcium carbonate present in the whole sample in accordance with ASTM D3437-21 (as outlined in Section 4.4.2.).

The fourth column replicate from each was split into three sections of ~25 mm length to represent the top, middle, and bottom portions of the column. Each section was dried, weighed, and then analysed for calcium carbonate. This was to observe the distribution of calcium carbonate throughout the column.

6.2.6.2 Scanning Electron Microscopy

Samples from the fifth and final column replicate were used for SEM imaging and were taken from the columns at depths of 20 mm, 40 mm, and 60 mm, at a location of 8.75 mm from the centre (i.e. 0.5x radius). Samples were prepared for Scanning Electron Microscopy (SEM) by gently air drying them overnight on the SEM stubs before applying a 15 nm thick gold coating using an Agar Sputter Coater. A Tungsten filament Hitachi S3700-N SEM was used to image the samples, and elemental analysis was also performed on specific points on SEM images using an Oxford Instruments X-Max 80 mm² EDS.

XRD was not performed in this case, as mineralogy had already been confirmed in Chapter 4 via XRD. Chapter 6 on the otherhand investigates the distribution of precipitates in the samples, and the interactions between precipitates and surfaces in the sample media.

6.3 Results

6.3.1 Column Calcium Carbonate Determination

The following sections describe the results of calcium carbonate determination. Each set is presented with other sets that allow for a comparison of different variables. Comparisons of each variable are presented via a figure displaying average total calcium carbonate per column, with error bars showing standard deviation, and a dashed line displaying the maximum theoretical calcium carbonate precipitation that could be precipitated based on one soil pore volume. Comparisons for calcium

carbonate distribution throughout the columns in sections split into top, middle, and bottom are also shown. The red section shows the calcium carbonate content of the top third of the soil, the yellow section shows the calcium carbonate content for the middle third of the soil, the blue section shows the calcium carbonate content for the bottom third of the soil located next to the injection point. The number within each section shows the mass of calcium carbonate measured in that section.

A table of results for each variable is provided for ease of viewing. The greyed out cell for individual column calcium carbonate readings represents the total calcium carbonate within the columns used for the calcium carbonate distribution measurements (top, middle, bottom). The green cells highlight the variable being compared.

6.3.1.1 Influence of Cycle Duration

Figure 6.3a shows the results of the column sets which investigated the influence of varying the cycle duration (Sets 1, 2, and 3). Treatment with 6 cycles of a 1 day cycle duration precipitated 1.05 g, 3 days precipitated 1.17 g, and 7 days precipitated 1.15 g. There does not appear to be an influence of cycle duration on calcium carbonate precipitation.

Figure 6.3b shows the distribution of calcium carbonate within one of these columns. This figure shows that in each column, where the distribution was tested, there was more calcium carbonate precipitated within the bottom and middle sections

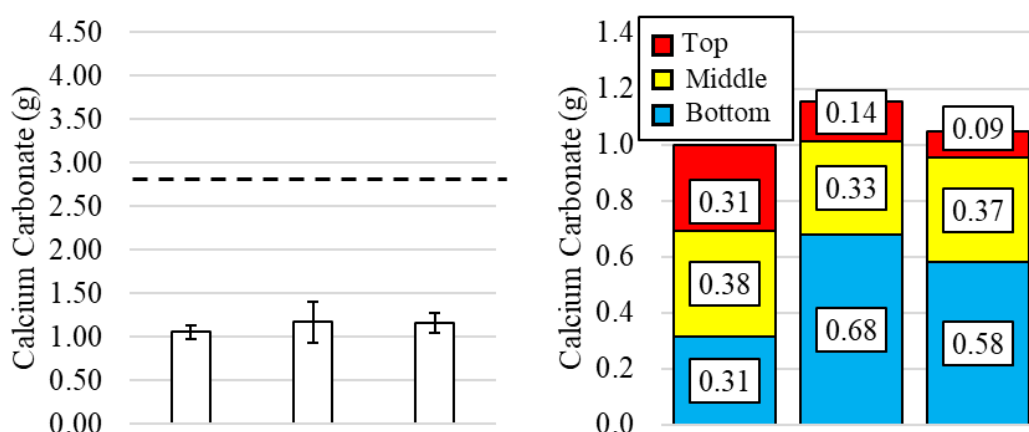


Figure 6.3 Influence of Cycle Duration on (a) total calcium carbonate, and (b) calcium carbonate distribution

of the columns, and similar to Fig 6.3a the mass of calcium carbonate precipitated does not appear to be differ with increasing cycle duration.

Table 6.3 Influence of Cycle Duration: Results Summary

Set	Cycle Duration	# of Cycles	Media	Treatment Method	Inoculant Amendment (mM)	Calcium Carbonate (g)	Average Calcium Carbonate (g)	St Dev	Max Theoretical Calcium Carbonate (g)	Calcium Carbonate Precipitated (%)
1	1	6	Coarse	Submerged	NaCl: 0.5	0.985	1.05	0.075	2.8	37.7
						1.127				
						1.110				
						0.996				
2	3	6	Coarse	Submerged	NaCl: 0.5	1.008	1.17	0.237	2.8	41.9
						1.013				
						1.513				
						1.153				
3	7	6	Coarse	Submerged	NaCl: 0.5	1.202	1.16	0.109	2.8	41.4
						1.099				
						1.291				
						1.046				

6.3.1.2 Influence of Number of Cycles

Figure 6.4a shows the results of the column sets in which the number of cycles was varied (Sets 5, 1, and 6). Treatment with 3 cycles of 1 day precipitated 0.6 g, 6 cycles precipitated 1.1 g, and 9 cycles precipitated 2.0 g. There was a clear increase in calcium precipitation with an increasing number of treatment cycles. Even up to 9 treatment cycles, the increase in calcium carbonate precipitation was proportional with the number of cycles.

Figure 6.4b displays calcium carbonate distribution measurements and showed that most precipitation occurred within the bottom or middle sections, and this remained the case with increasing numbers of treatment cycles. Consistent with Figure 6.4a, the overall mass of calcium carbonate precipitated again showed an increase with an increasing number of cycles.

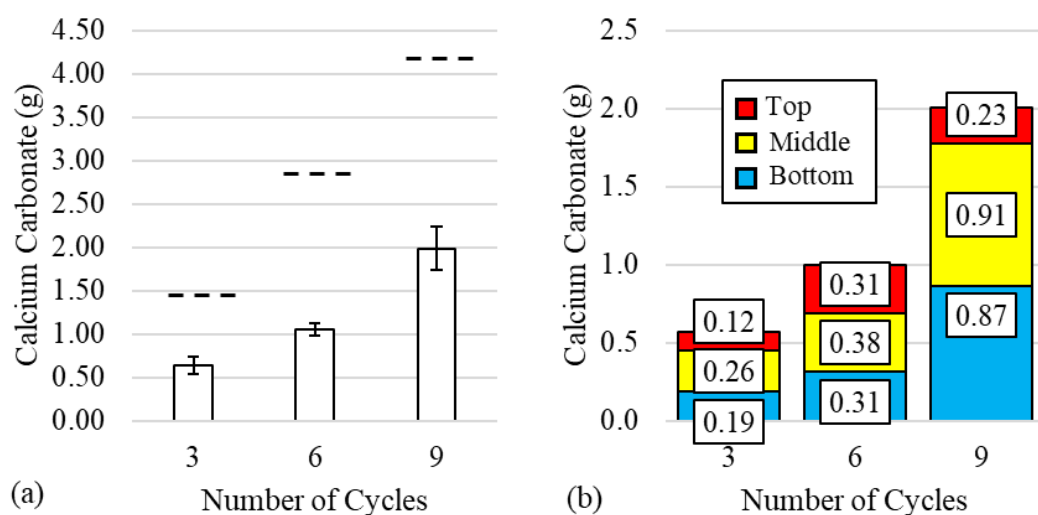


Figure 6.4 Influence of Number of Cycles on (a) total calcium carbonate, and (b) calcium carbonate distribution

Set	Cycle Duration	# of Cycles	Media	Treatment Method	Inoculant Amendment (mM)	Calcium Carbonate (g)	Average Calcium Carbonate (g)	St Dev	Max Theoretical Calcium Carbonate (g)	Calcium Carbonate Precipitated (%)
5	1	3	Coarse	Submerged	NaCl: 0.5	0.748	0.64	0.096	1.4	45.8
						0.546				
						0.693				
						0.574				
1	1	6	Coarse	Submerged	NaCl: 0.5	0.985	1.05	0.075	2.8	37.7
						1.127				
						1.110				
						0.996				
6	1	9	Coarse	Submerged	NaCl: 0.5	2.205	1.99	0.251	4.2	47.4
						2.112				
						1.632				
						2.010				

Table 6.4 Influence of Number of Cycles: Results Summary

6.3.1.3 Influence of Injection Strategy

Figure 6.5a shows column sets which investigated the influence of the injection strategy (Sets 2 and 4). Treatment using the “*submerged*” treatment strategy precipitated 1.2 g, and *drained* treatment strategy precipitated 1.1 g. There appeared to be little difference between the *Submerged* and *Drained* treatment strategies. Figure 6.5b displays distribution of calcium carbonate. Again, most calcium carbonate precipitated in the bottom and middle portions of each column, and consistent with Figure 6.5a, the distribution of precipitate was similar between the two treatment strategies.

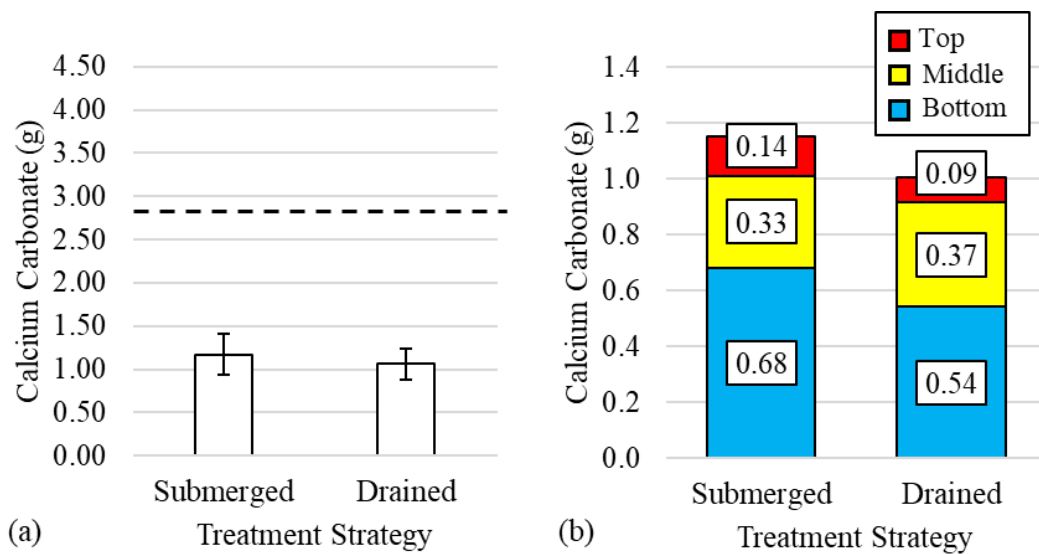


Figure 6.5 Influence of Injection Strategy on (a) total calcium carbonate, and (b) calcium carbonate distribution

Table 6.5 Influence of Injection Strategy: Results Summary

Set	Cycle Duration	# of Cycles	Media	Treatment Method	Inoculant Amendment (mM)	Calcium Carbonate (g)	Average Calcium Carbonate (g)	St Dev	Max Theoretical Calcium Carbonate (g)	Calcium Carbonate Precipitated (%)
2	3	6	Coarse	Submerged	NaCl: 0.5	1.008	1.17	0.237	2.8	41.9
						1.013				
						1.513				
						1.153				
4	3	6	Coarse	Drained	NaCl: 0.5	0.907	1.06	0.180	2.8	37.9
						1.321				
						1.008				
						1.007				

6.3.1.4 Influence of Inoculant Amendment

Figure 6.6a shows the results of the column sets which investigated the influence of inoculant amendments (Sets 6, 7, and 8). A mean mass of calcium carbonate precipitated for columns where the fungal inoculant contained only 0.5 mM NaCl precipitated 2.0 g, for columns amended with 0.5 mM NaCl and 30 mM Urea precipitated 2.0 g, and columns amended with Malt Extract Broth precipitated 2.2 g. The inoculant amendment did not appear to have an influence on calcium carbonate precipitation, although the variability was smaller for columns grown with malt extract broth.

Figure 6.6b shows the calcium carbonate distribution within columns for each inoculant amendment. The distribution did not appear to be different between the different conditions and consistently showed more precipitation in the bottom and middle sections.

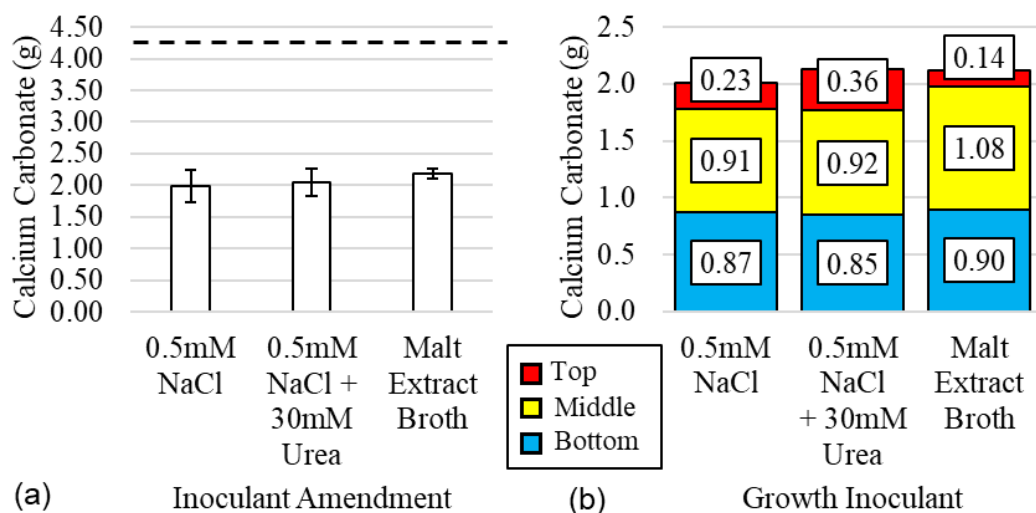


Figure 6.6 Influence of Inoculant Amendment on (a) total calcium carbonate, and (b) calcium carbonate distribution

Table 6.6 Influence of Inoculant Amendment: Results Summary

Set	Cycle Duration	# of Cycles	Media	Treatment Method	Inoculant Amendment (mM)	Calcium Carbonate (g)	Average Calcium Carbonate (g)	St Dev	Max Theoretical Calcium Carbonate (g)	Calcium Carbonate Precipitated (%)
6	1	9	Coarse	Submerged	NaCl: 0.5	2.205	1.99	0.251	4.2	47.4
						2.112				
						1.632				
						2.010				
7	1	9	Coarse	Submerged	NaCl: 0.5 Urea: 30	2.175	2.05	0.212	4.2	48.8
						2.156				
						1.732				
						2.131				
8	1	9	Coarse	Submerged	Malt Extract Broth	2.279	2.18	0.080	4.2	52.1
						2.223				
						2.114				
						2.121				

6.3.1.5 Influence of Grain Size

Figure 6.7a shows the results of the column sets which varied grain size (Sets 6 and 9). Treatment of columns with smaller particle sizes (medium sand) precipitated 1.5 g, while the coarser sand columns contained 2.0 g of CaCO_3 . Treatment in the columns containing the smaller medium grain size appeared to show less calcium

carbonate precipitation when compared to the coarse grain columns, this is despite the fact that the columns comprised of medium sand had an overall slightly higher porosity, and therefore slightly higher maximum theoretical amount of calcium carbonate that could have been precipitated.

Figure 6.7b shows the calcium carbonate distribution for each grain size. Medium grain size showed much less precipitation within the middle section than for coarse sand and accounted for most of the difference in the overall calcium carbonate precipitation.

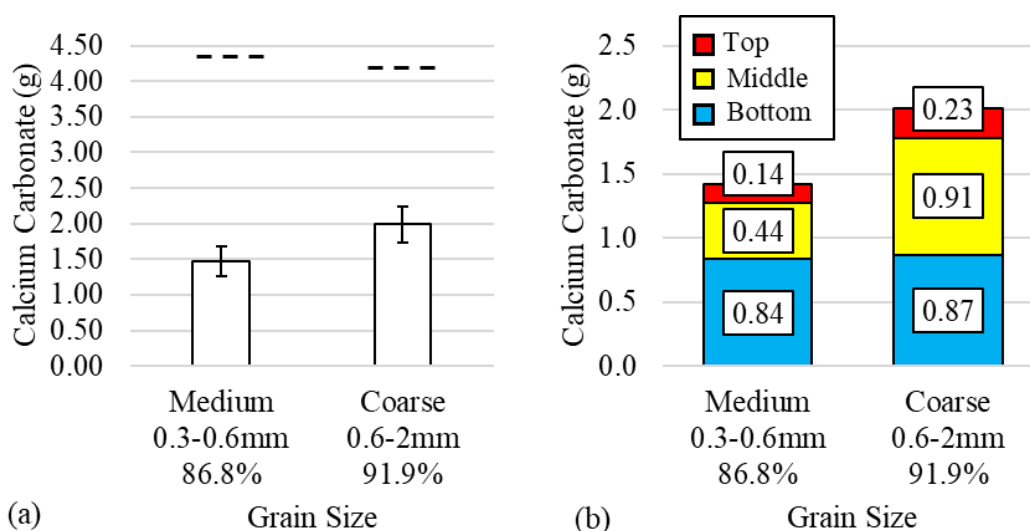


Figure 6.7 Influence of Grain Size on (a) total calcium carbonate, and (b) calcium carbonate distribution

Table 6.7 Influence of Grain Size: Results Summary

Set	Cycle Duration	# of Cycles	Media	Treatment Method	Inoculant Amendment (mM)	Calcium Carbonate (g)	Average Calcium Carbonate (g)	St Dev	Max Theoretical Calcium Carbonate (g)	Calcium Carbonate Precipitated (%)
6	1	9	Coarse	Submerged	NaCl: 0.5	2.205	1.99	0.251	4.2	47.4
						2.112				
						1.632				
						2.010				
9	1	9	Medium	Submerged	NaCl: 0.5	1.784	1.47	0.209	4.4	33.6
						1.327				
						1.372				
						1.416				

Table 6.8 is a final summary of calcium carbonate determination for all column sets.

Table 6.8 Calcium Carbonate Determination Results Summary

Set	Cycle Duration	# of Cycles	Media	Treatment Method	Inoculant Amendment (mM)	Calcium Carbonate (g)	Average Calcium Carbonate (g)	St Dev	Max Theoretical Calcium Carbonate (g)	Calcium Carbonate Precipitated (%)
1	1	6	Coarse	Submerged	NaCl: 0.5	0.985	1.05	0.075	2.8	37.7
						1.127				
						1.110				
						0.996				
2	3	6	Coarse	Submerged	NaCl: 0.5	1.008	1.17	0.237	2.8	41.9
						1.013				
						1.513				
						1.153				
3	7	6	Coarse	Submerged	NaCl: 0.5	1.202	1.16	0.109	2.8	41.4
						1.099				
						1.291				
						1.046				
4	3	6	Coarse	Drained	NaCl: 0.5	0.907	1.06	0.180	2.8	37.9
						1.321				
						1.008				
						1.007				
5	1	3	Coarse	Submerged	NaCl: 0.5	0.748	0.64	0.096	1.4	45.8
						0.546				
						0.693				
						0.574				
6	1	9	Coarse	Submerged	NaCl: 0.5	2.205	1.99	0.251	4.2	47.4
						2.112				
						1.632				
						2.010				
7	1	9	Coarse	Submerged	NaCl: 0.5 Urea: 30	2.175	2.05	0.212	4.2	48.8
						2.156				
						1.732				
						2.131				
8	1	9	Coarse	Submerged	Malt Extract Broth	2.279	2.18	0.080	4.2	52.1
						2.223				
						2.114				
						2.121				
9	1	9	Medium	Submerged	NaCl: 0.5	1.784	1.47	0.209	4.4	33.6
						1.327				
						1.372				
						1.416				

6.3.2 SEM Imaging and Elemental Analysis

Table 6.9 shows the elements analysed using EDS. Elemental Analysis included the elements carbon, nitrogen, oxygen, aluminium, silicon, chlorine, calcium, and iron. These elements were chosen to indicate the presence of specific objects.

Table 6.9 Elements for SEM Elemental Analysis

Element(s)	Object
Carbon	Background carbon reading, biological carbon, calcium carbonate, carbon stub
Nitrogen	Fungal cell wall (chitin), ammonium chloride, protein, amino acids, biofilm
Oxygen	Sand grain, calcium carbonate, background oxygen reading.
Aluminium	Sand grain
Silicon	Sand grain
Chlorine	Ammonium chloride, calcium chloride
Calcium	Calcium carbonate, calcium chloride, calcium oxalate
Iron	Sand grain
Other	Trace Elements of Titanium, Rubidium, Astatine, Manganese, Magnesium, Chromium, Copper, Niobium, Tellurium, Yttrium, Iridium, Potassium, Germanium, Sodium, or Phosphorus.

Figure 6.8 shows sample spectra with peaks labelled to the corresponding elements. Elements were determined as a percentage weight of all elements measured. Elements that were light, scarce at the sample point, and/or with a peak situated between two much heavier or more abundant elements could possibly be obscured from measurement. This was sometimes the case for nitrogen as shown in Figure 6.8a and b. The peak for nitrogen was very small and was located between the large peaks of very abundant carbon and oxygen and so was difficult to observe because it was obscured by the large peaks on either side. Because of this, the software would sometimes require manual prompting to check for a nitrogen peak instead of automatically recognising it, and it may not have recognised a peak when it would be anticipated to do so. This may explain why some sample points of fungus, which would be expected to contain nitrogen, did not show any measured nitrogen.

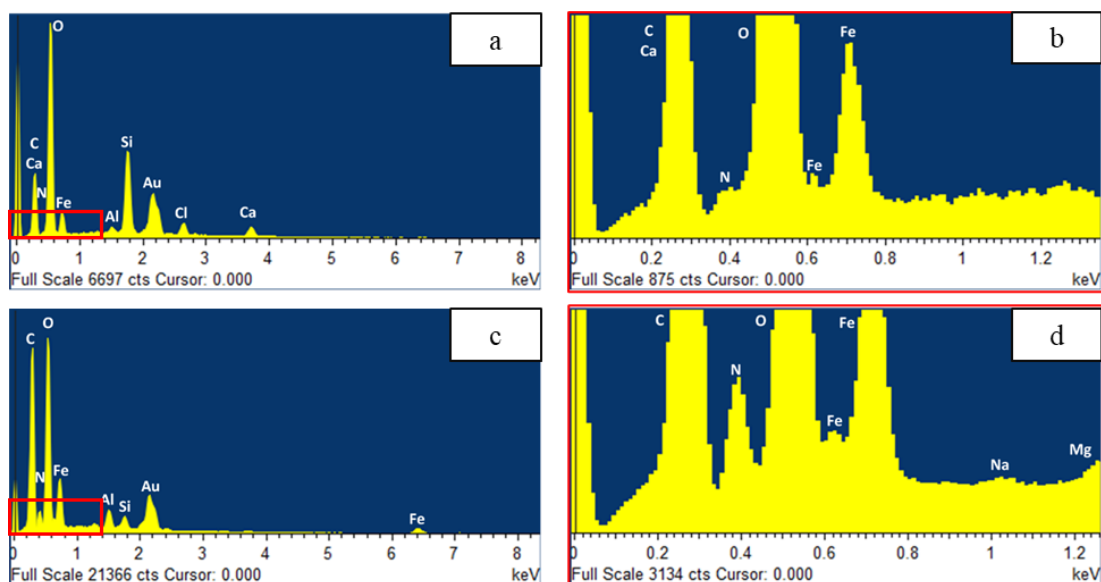


Figure 6.8 Example Elemental Spectra (a) Set 6, Mid Section Site 4 (see Figure 6.26b and d), (b) magnified view of nitrogen peak in (a), (c) Set 5, Mid Section, Site 1, (d) magnified view of nitrogen peak in (c)

6.3.2.1 Control Samples

Before performing SEM imaging and elemental analysis of samples, controls were imaged and analysed to better understand the features and elements contained within the untreated materials.

The aluminium stub and the adhesive carbon tab used to secure the sample to the aluminium stub were imaged and analysed, along with untreated samples of coarse sand, medium sand, *Pleurotus cornucopiae* grown on agar, and beechwood chips. Additionally, calcium carbonate powder (98 % purity, Sigma-Aldrich) was also imaged and analysed for elements.

Figure 6.9 displays the aluminium stub and showed a predictably large proportion of aluminium but also contained carbon and oxygen. Figure 6.10 displays the adhesive carbon tab and showed a predictably large proportion of carbon with some nitrogen and carbon. Notably, the carbon tab prevented any detection of aluminium from the aluminium stub located below it. This means that the carbon tab blocked any background interference during elemental analysis from the aluminium stub.

It was important to know how the untreated samples appeared before analysing treated samples. Untreated samples of coarse sand, medium sand, *Pleurotus cornucopiae* grown on agar, and beechwood chips were imaged and elements

analysed. Figure 6.11 and Figure 6.12 show coarse sand samples, and Figure 6.13 and Figure 6.14 show medium sand samples. There was no obvious difference in elemental compositions between each type of sand. Both coarse and medium sand showed large proportions of oxygen, but also silicon, iron, and small proportions of aluminium, and carbon. Figure 6.15 and Figure 6.16 display scans of untreated *Pleurotus cornucopiae* hyphae grown on malt extract agar and removed from the agar before imaging. The *P. cornucopiae* shows a large proportion of carbon and oxygen; nitrogen was detected at all sample locations, except for two where hyphae appeared to have become bundled together (6 out of 8 sites analysed). Figure 6.17 and Figure 6.18 displayed beechwood chip samples and showed high proportions of carbon and oxygen. Nitrogen in low quantity was present at most sites within the sample (5 out of the 6 sites analysed). Figure 6.19 shows crystals from commercial calcium carbonate. The crystals in this image exhibit a typical rhombohedral morphology as expected for the crystal structure of calcite.

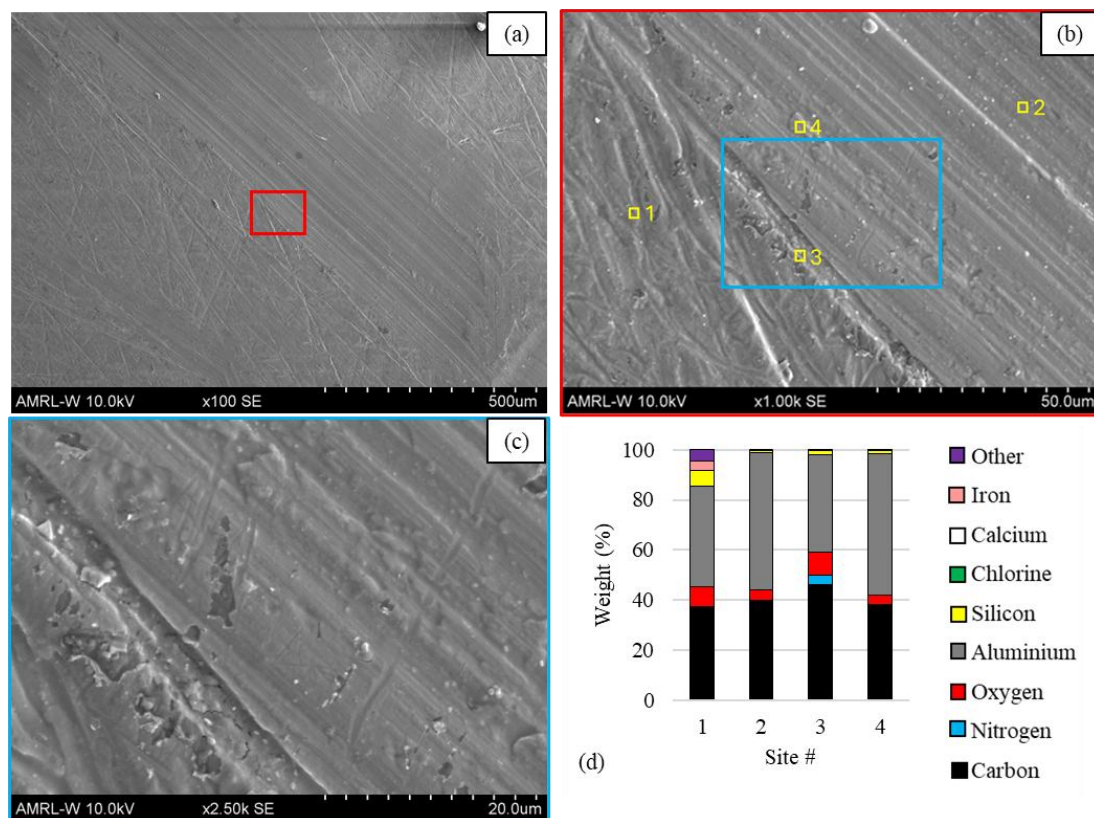


Figure 6.9 Aluminium stub at 100x, 1000x and 2500x magnification

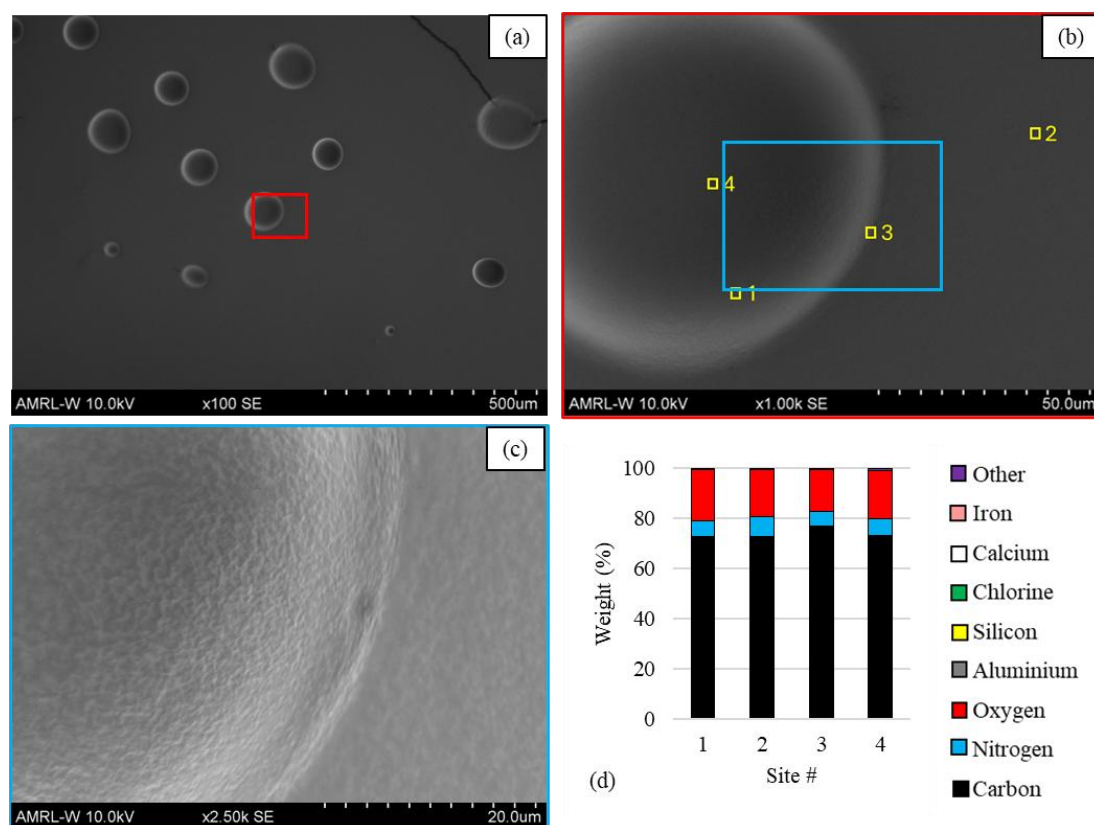


Figure 6.10 Carbon sticker at 100x, 1000x and 2500x magnification

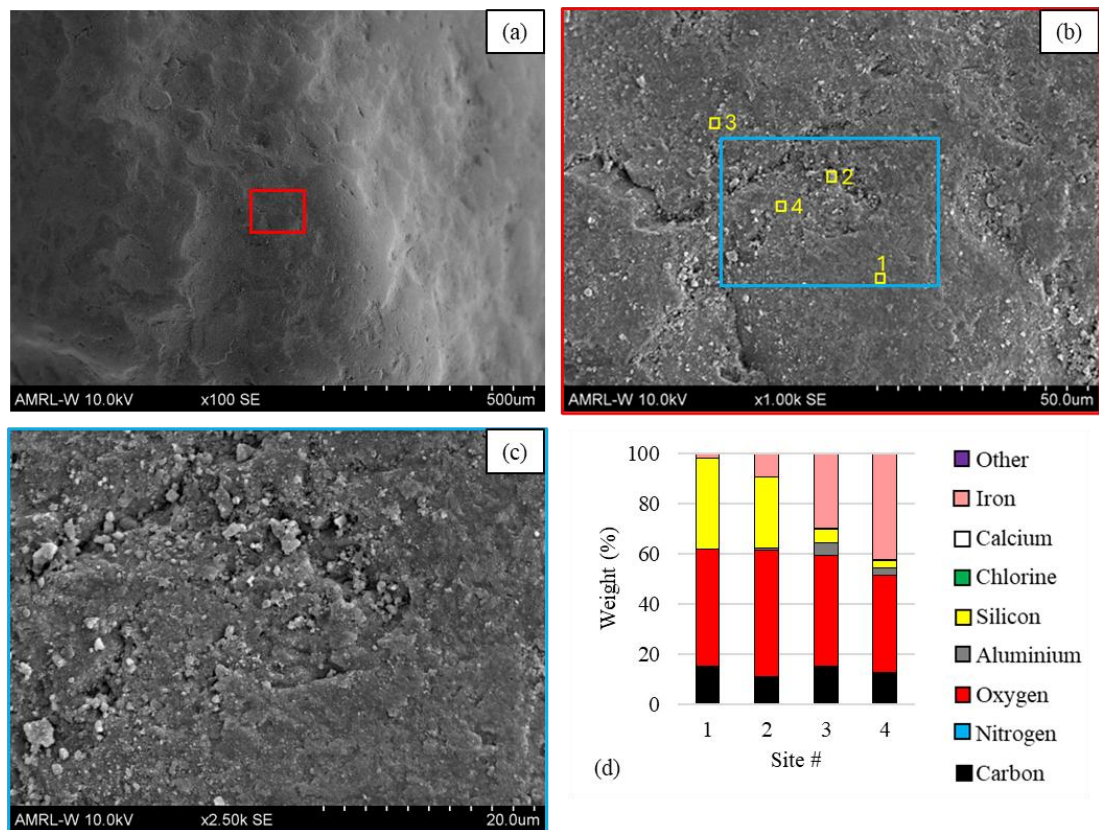


Figure 6.11 Untreated Coarse Sand Grains at 100x, 1000x and 2500x magnification

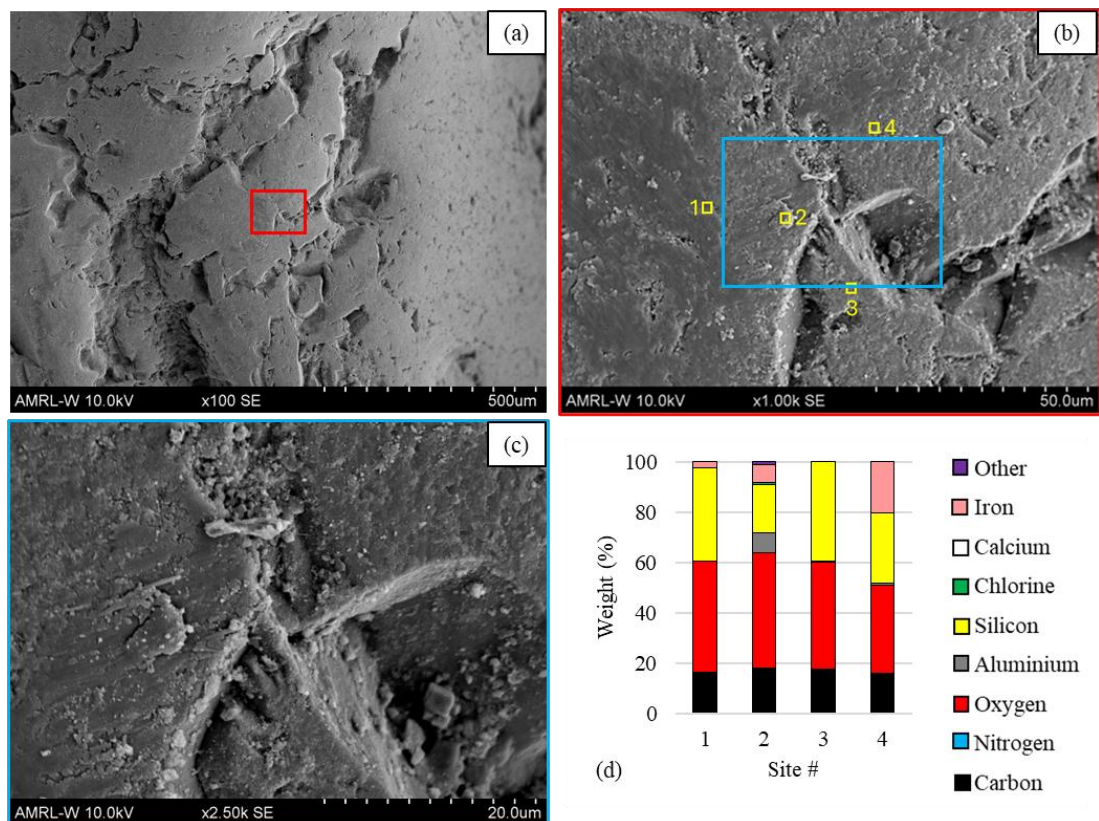


Figure 6.12 Untreated Coarse Sand Grains at 100x, 1000x and 2500x magnification

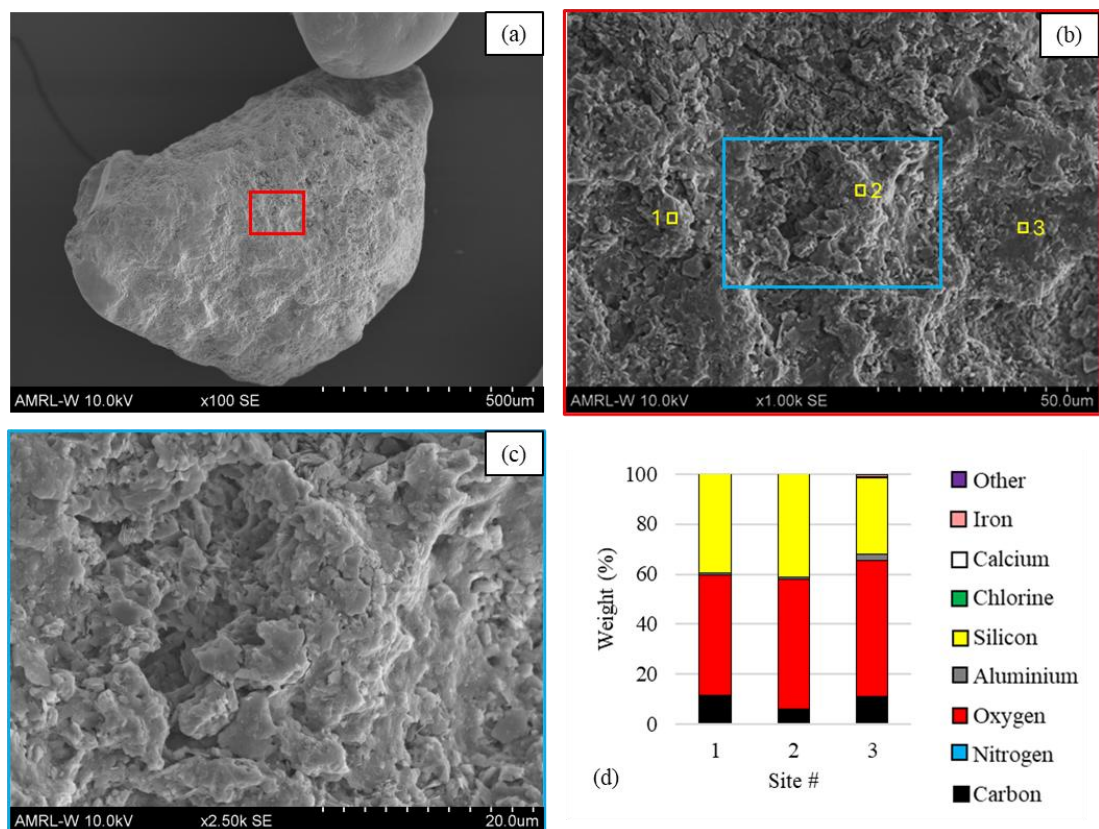


Figure 6.13 Untreated medium sand grains at 100x, 1000x and 2500x magnification

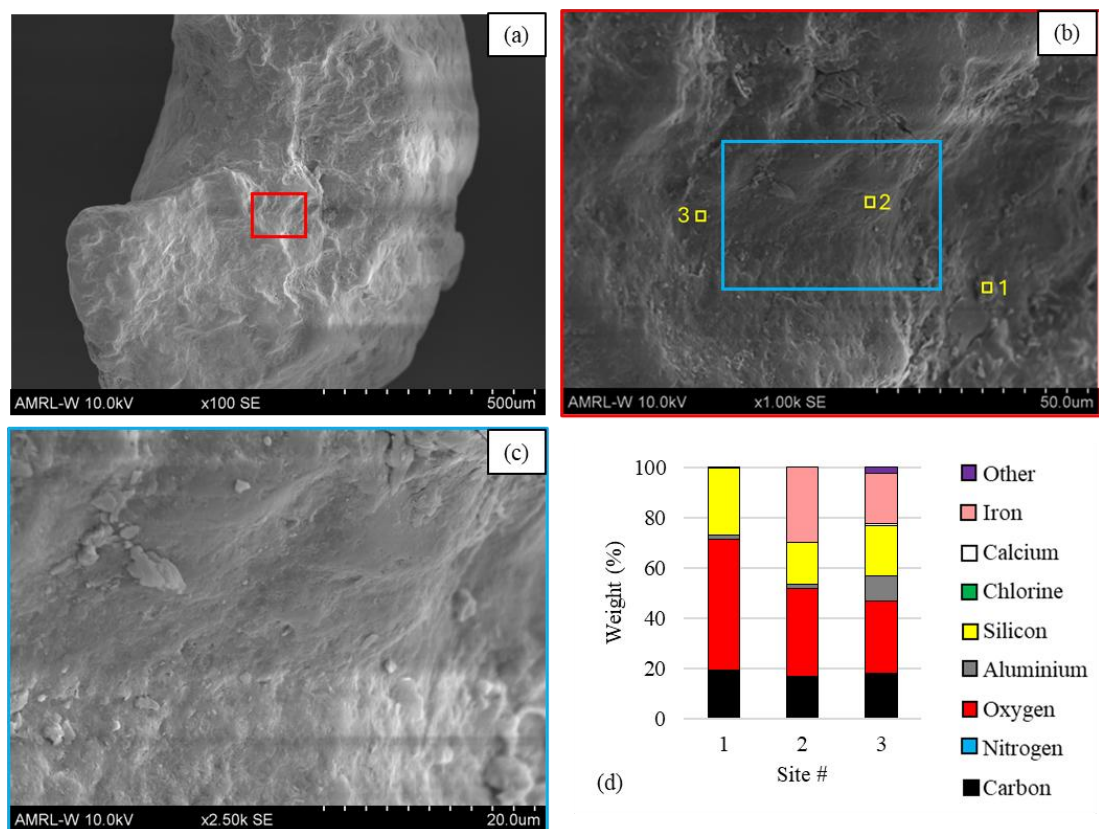


Figure 6.14 Untreated medium sand grains at 100x, 1000x and 2500x magnification

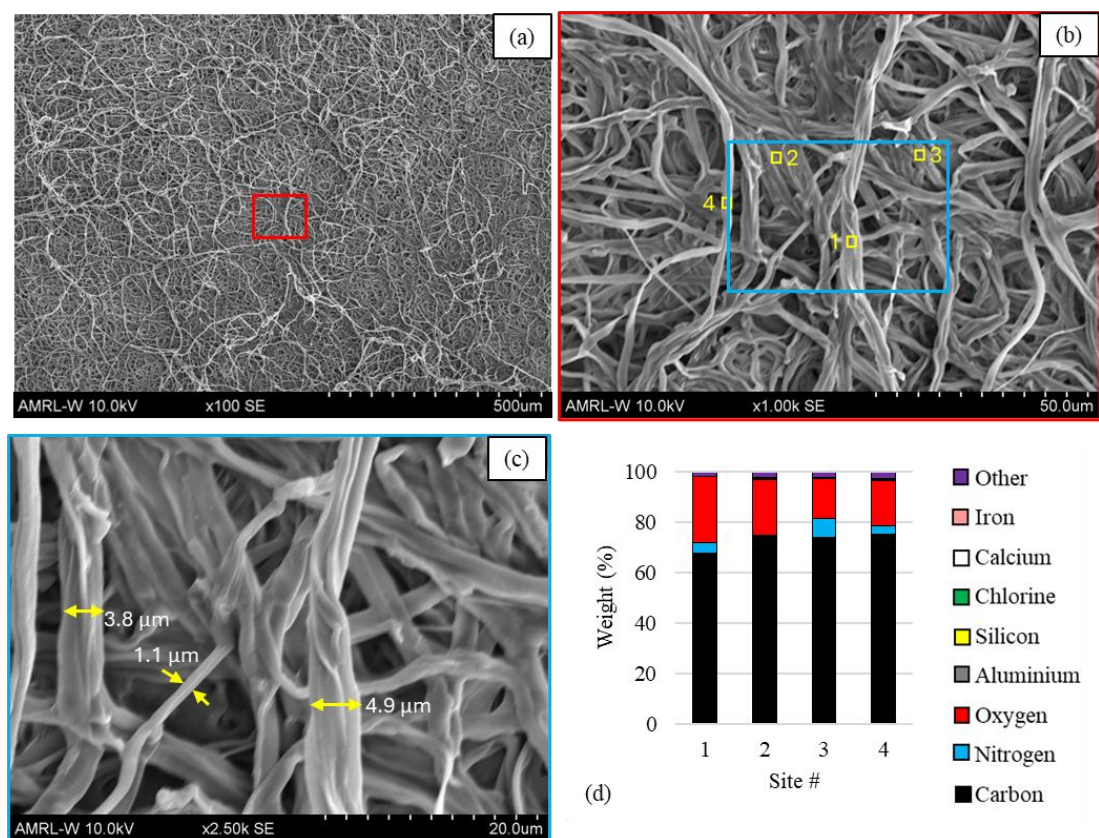


Figure 6.15 *P. cornucopiae* grown on agar at 100x, 1000x and 2500x magnification

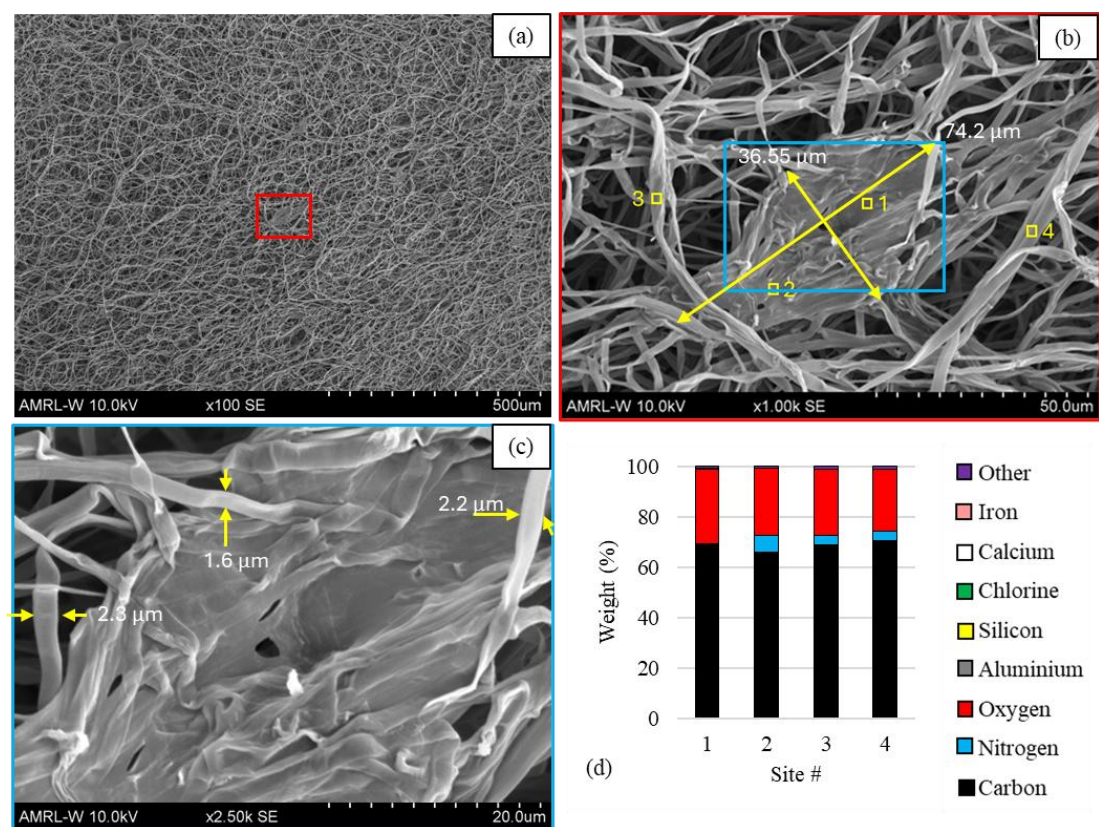


Figure 6.16 *P. cornucopiae* grown on agar at 100x, 1000x and 2500x magnification

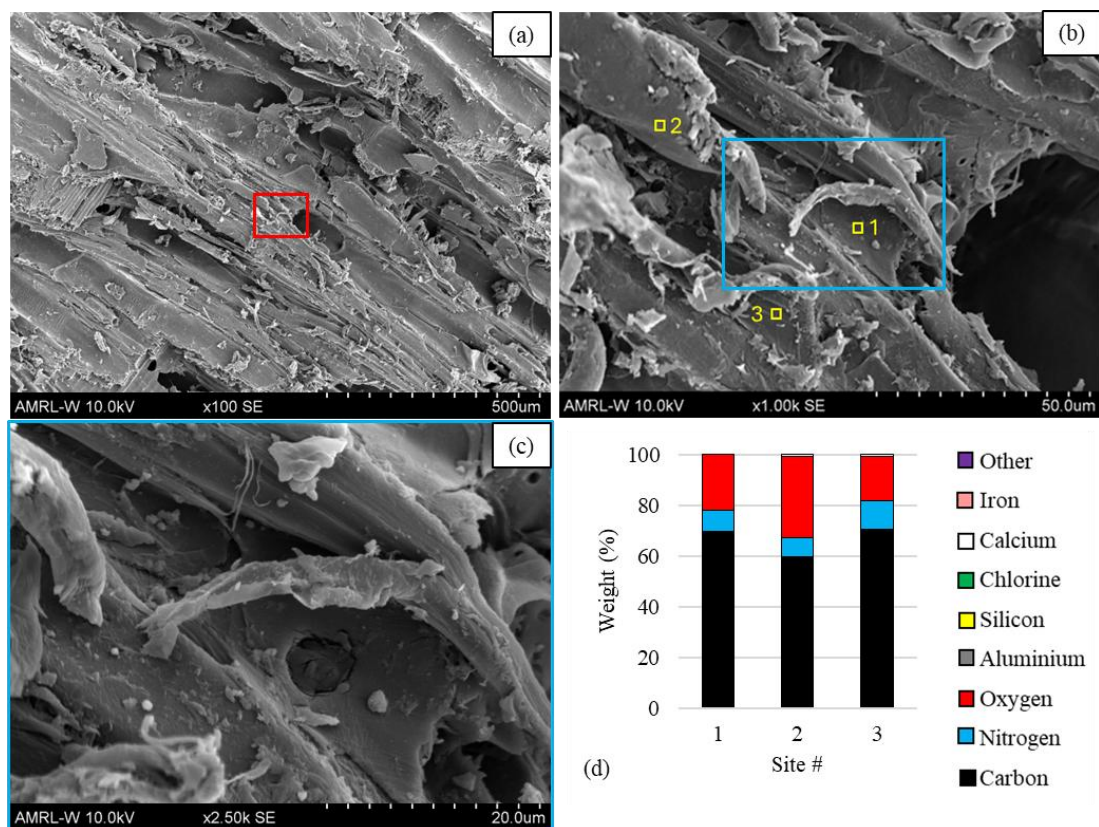


Figure 6.17 Untreated beechwood chip at 100x, 1000x and 2500x magnification

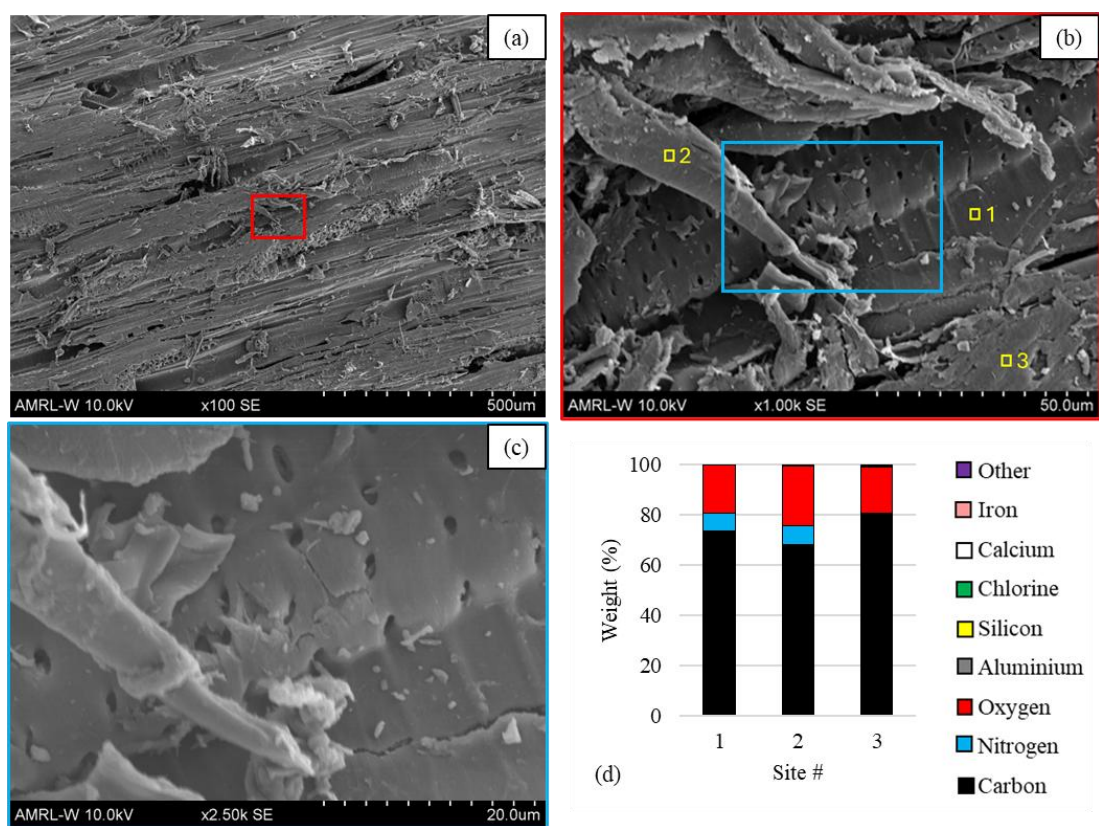


Figure 6.18 Untreated beechwood chip at 100x, 1000x and 2500x magnification

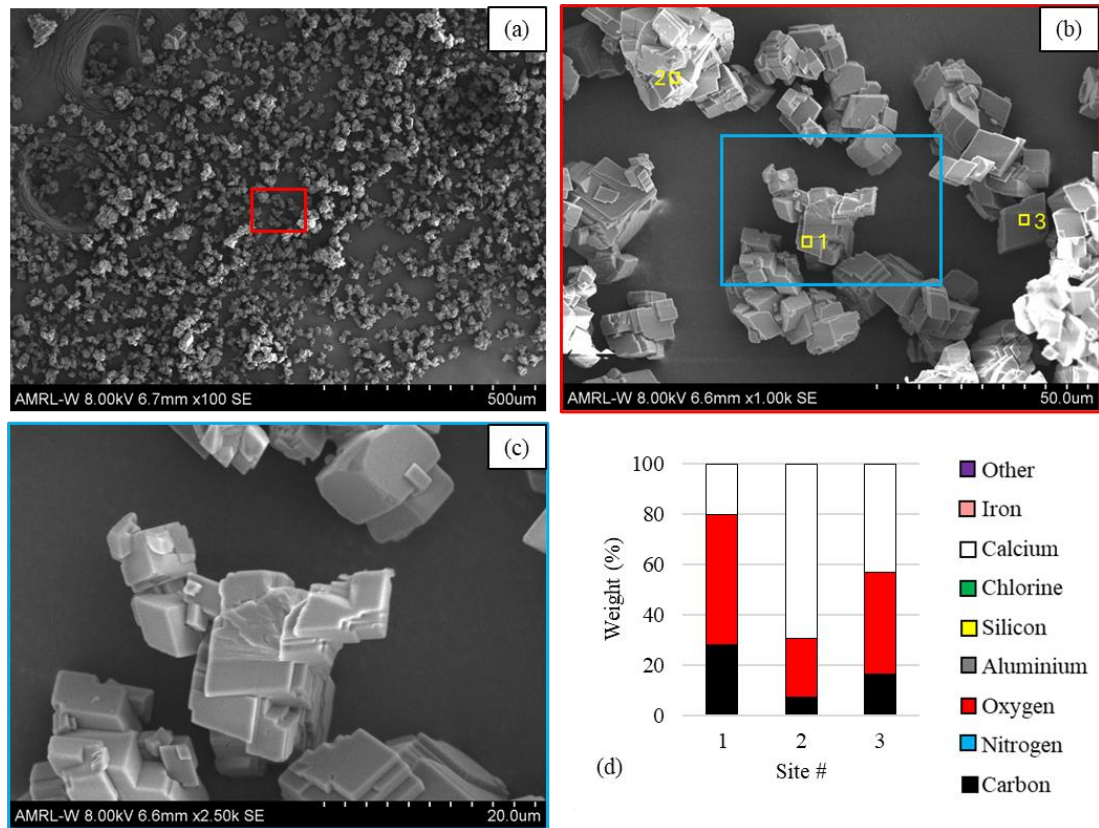


Figure 6.19 Calcium carbonate (98 %, Sigma-Aldrich) at 100x, 1000x and 2500x magnification

6.3.2.2 SEM Results for Treated Columns

Before comparing images across different sets, first the typical features found within these FICP treated sand columns are presented, using as a baseline a column from Set 2. Figure 6.20 - Figure 6.24 show SEM images and elemental analysis of a sample from a column in Set 2 (Coarse sand, NaCl amendment), which was treated with a cycle duration of 3 days during which the specimen remained submerged in the cementing solution and received 6 cycles in total.

Figure 6.20 shows a beechwood chip with fungal hyphae evident and crystals spread across the surface of the beechwood chip, with two spherical shaped crystals measured with diameters of 53.9 and 56.1 μm . Hyphae are clearly visible extending into/from the crystals. Sites 1-4 are located on visible crystals, here there is typically a larger % of Calcium detected (shown in white), indicating that these are calcium carbonate crystals. Sites 5-6 are located on beechwood. There is trace aluminium at each analysed point from an unknown source. Nitrogen is also detected at each site other than 5, likely from the beechwood located behind or fungal biomass present within the calcium carbonate crystals.

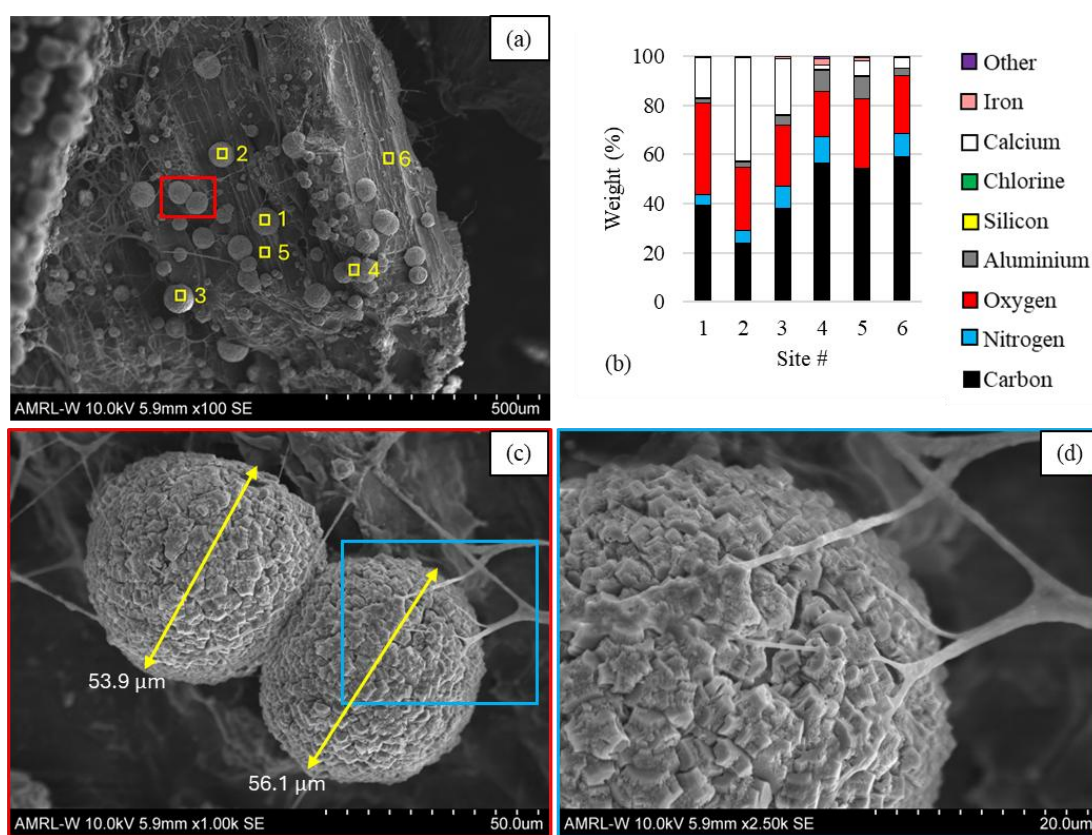


Figure 6.20 SEM Images from the top portion of a column from Set 2: Coarse sand, NaCl inoculant amendment, submerged treatment strategy, with 6 cycles of 3 days each

Figure 6.21 shows a sand crystal covered in a sheath of material that could be a biofilm. There are a few small calcium carbonate crystals distributed throughout the image, identified as such by elemental analysis of site 3 and 4. There were traces of nitrogen at all sites other than 3. This could be due to the layer of biofilm visible on the sand grain. This layer of biofilm was hard to view when it was not peeling off a sand grain, as it was practically invisible when fully attached to the sand grain.

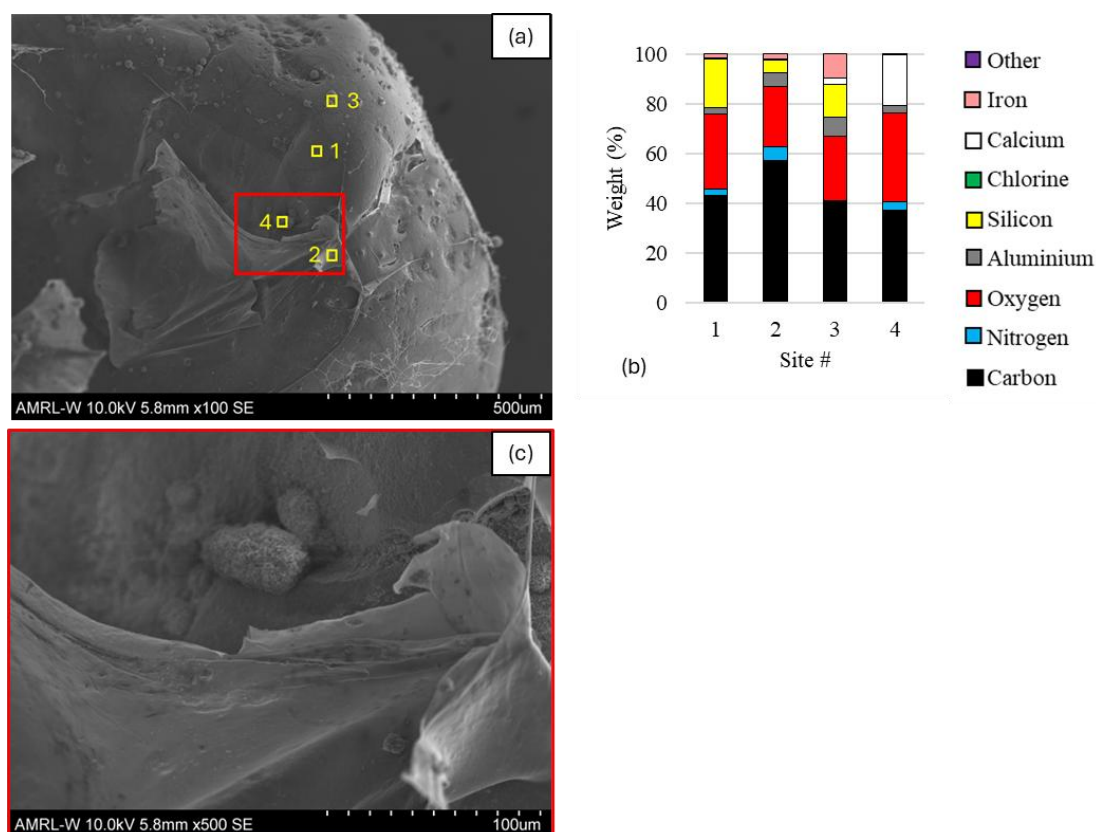


Figure 6.21 SEM images from the top portion of a column from Set 2: Coarse sand, NaCl inoculant amendment, submerged treatment strategy, with 6 cycles of 3 days each

Figure 6.22 shows a beechwood chip with crystals precipitated across the surface. Only sites 2 and 9 in Figure 6.22b show the presence of calcium. Many of the sites chosen for elemental analysis had crystals visually present but no calcium measured. It could have been that the crystals were too small to have a detectable quantity of calcium carbonate, but these crystals could also have been a secondary precipitate such as another different carbonate, or they could have been unreacted urea which had avoided the reaction within the woodchip. This latter explanation could also have contributed to the presence of nitrogen at all sites, in addition to from the woodchip. The crystals appeared to have a flat surface, as if they had been previously attached to another surface during precipitation. They were also an irregular shape. There was considerable aluminium measured at almost all sites in this sample from an unidentified source, similar to Figure 6.20 which was also of a beechwood chip.

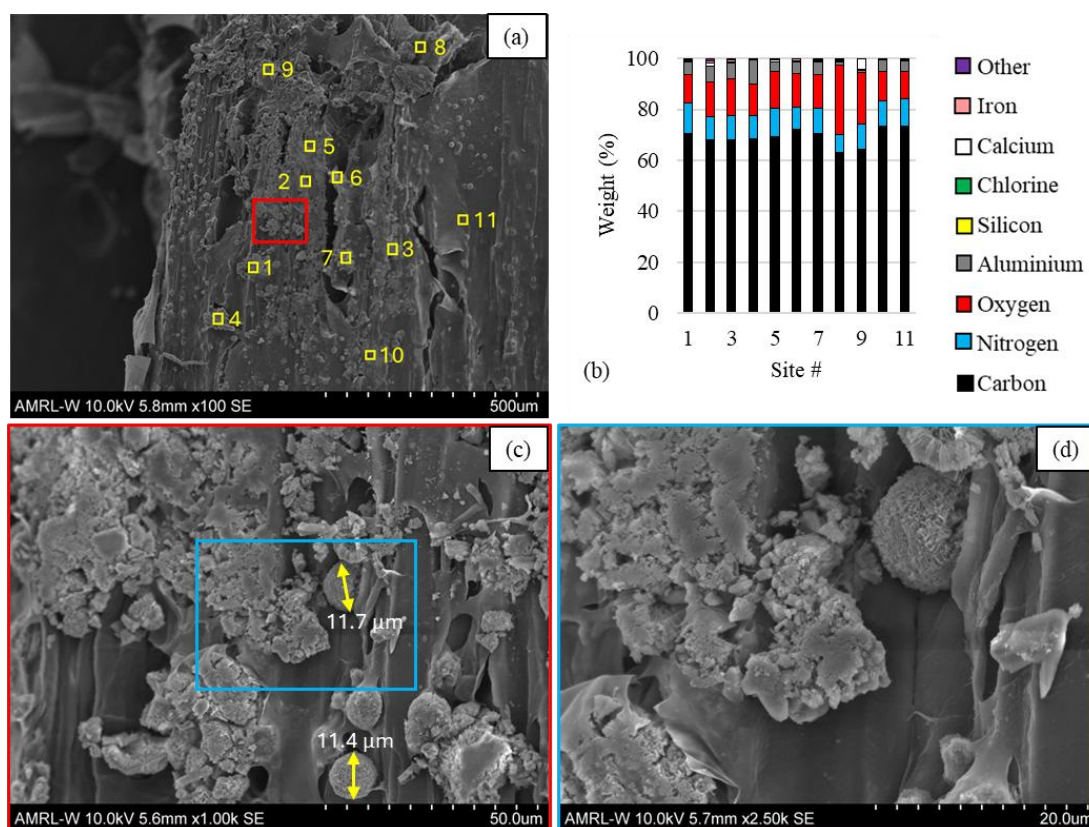


Figure 6.22 SEM images from the mid portion of a column from Set 2: Coarse sand, NaCl inoculant amendment, submerged treatment strategy, with 6 cycles of 3 days each

Figure 6.23 shows a woodchip with fungal hyphae and crystals throughout the image. The hyphae have grown around the beechwood chip, and the calcium carbonate has precipitated upon the hyphae. This suggests that the crystals are nucleating on the fungal cell surfaces. The crystals showed the presence of calcium, and the visible structure was irregular or spherical. The two measured crystals were 13.1 and 22.3 μm in diameter. Sites 3, 6, and 7 were of crystals and clearly showed calcium, and there were nitrogen and aluminium present at most analysed sites.

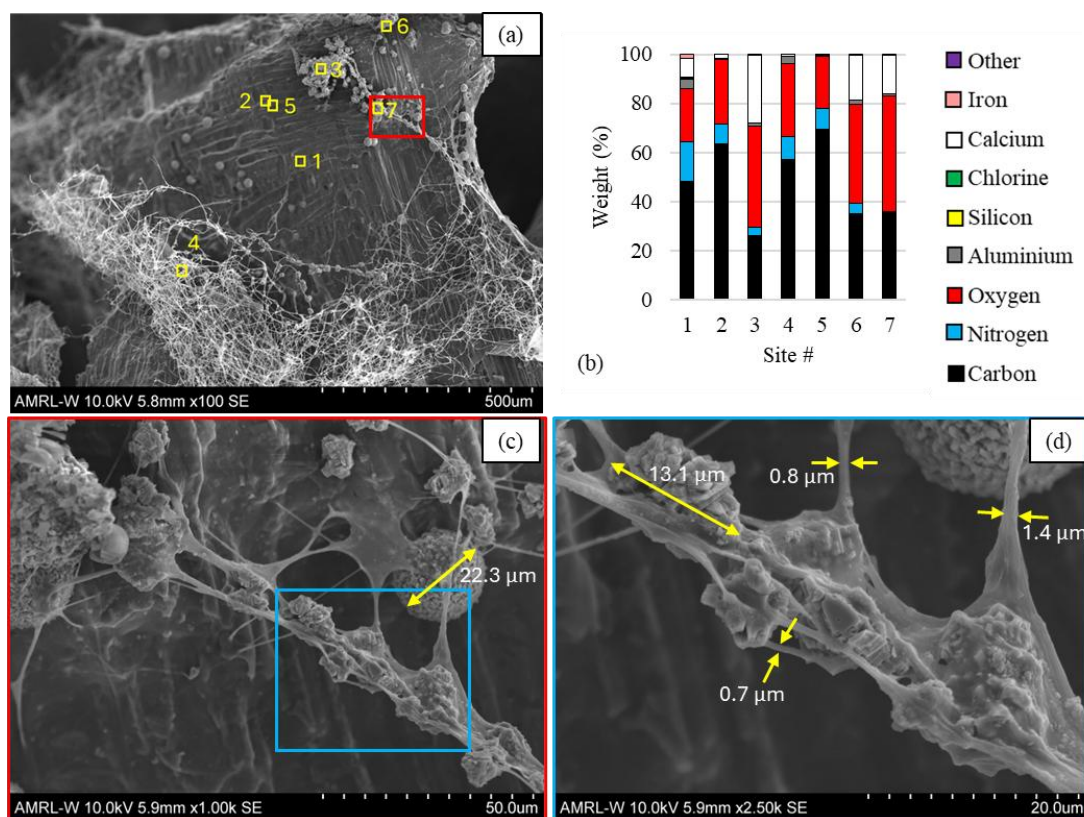


Figure 6.23 SEM images from the mid portion of a column from Set 2: Coarse sand, NaCl inoculant amendment, submerged treatment strategy, with 6 cycles of 3 days each

Figure 6.24 shows a sand grain with crystals precipitated across the grain surface. The focus of Figure 6.24c was a single large crystal 50.2 μm in diameter. This large crystal was composed of lots of regular edges, suggesting that it is calcite. Figure 6.24d focuses on the smaller crystals surrounding the large crystal. These crystals are small and spherical. Many of these crystals had a hole in them. One of these calcium carbonate crystals was 7.8 μm wide and had a hole of diameter 3.3 μm . It was hard to determine if these crystals precipitated onto fungal hyphae, biofilm, or directly onto the sand grain due to the surface coverage of the precipitated crystals. Referring to later **Error! Reference source not found.** (Loges et al., 2006), it appears that these small crystals were likely vaterite and may even have precipitated as hollow crystals rather than the hole being caused by dislodged hyphae.

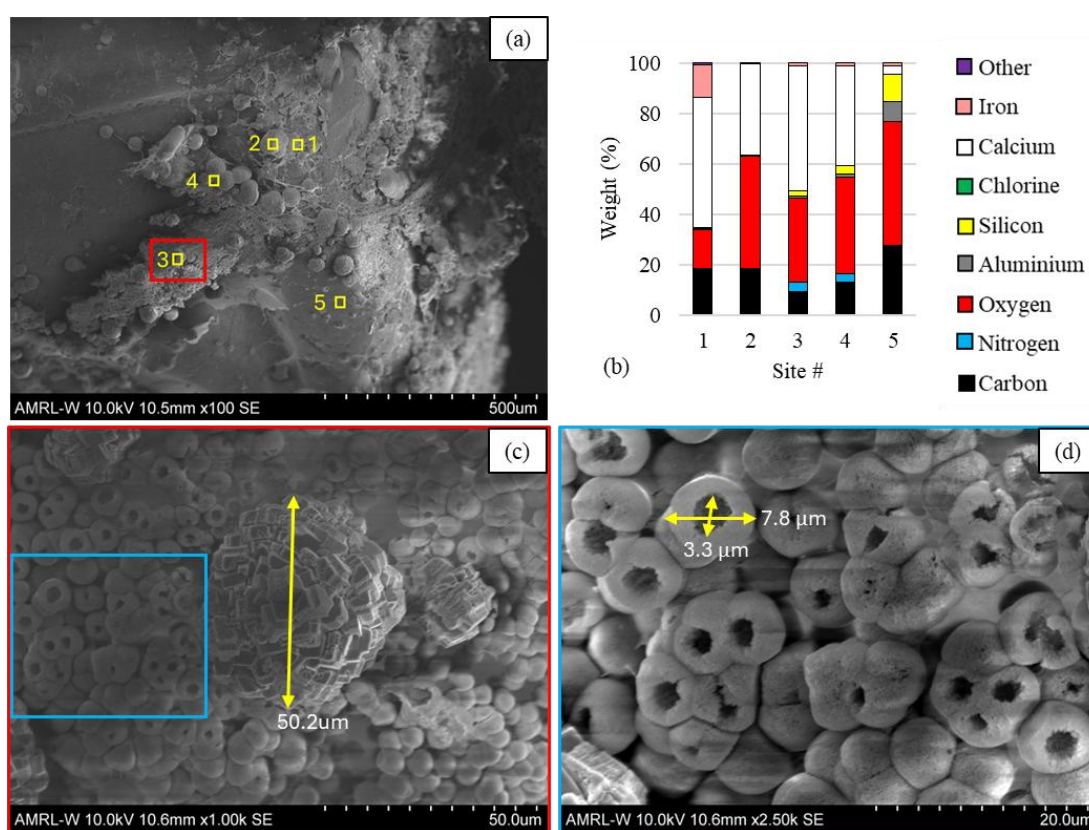


Figure 6.24 SEM images from the bottom portion of a column from Set 2: Coarse sand, NaCl inoculant amendment, submerged treatment strategy, with 6 cycles of 3 days each

In order to understand the implications of FICP treatment on soil mechanical behaviour, here SEM images and elemental analysis of column samples from Set 6, which received treatment of 9 cycles of one day each are presented (Figure 6.25 – Figure 6.27). In Set 6, coarse sand was tested with NaCl inoculant amendment and a submerged treatment strategy.

Figure 6.25 shows three sand grains touching each other. Between two of the sand grains calcium carbonate crystals and hyphae appear to bridge across between the sand grains connecting the two. The focus of the images is on a 27.9µm diameter spherical crystal which showed the presence of calcium and appeared to be attached to both sand grains. The crystal is spherical but made up of small angular segments. The crystal has a 2µm wide hole in it which corresponds to a hyphal filament which the crystal may have precipitated around before the filament was dislodged during sampling. Crystals in this image at first glance appear to be precipitated directly onto the sand grain, but on closer inspection there are hyphae distributed across the sand grain. Crystals on the sand grain are likely actually precipitated onto a fungal hypha attached to that sand grain.

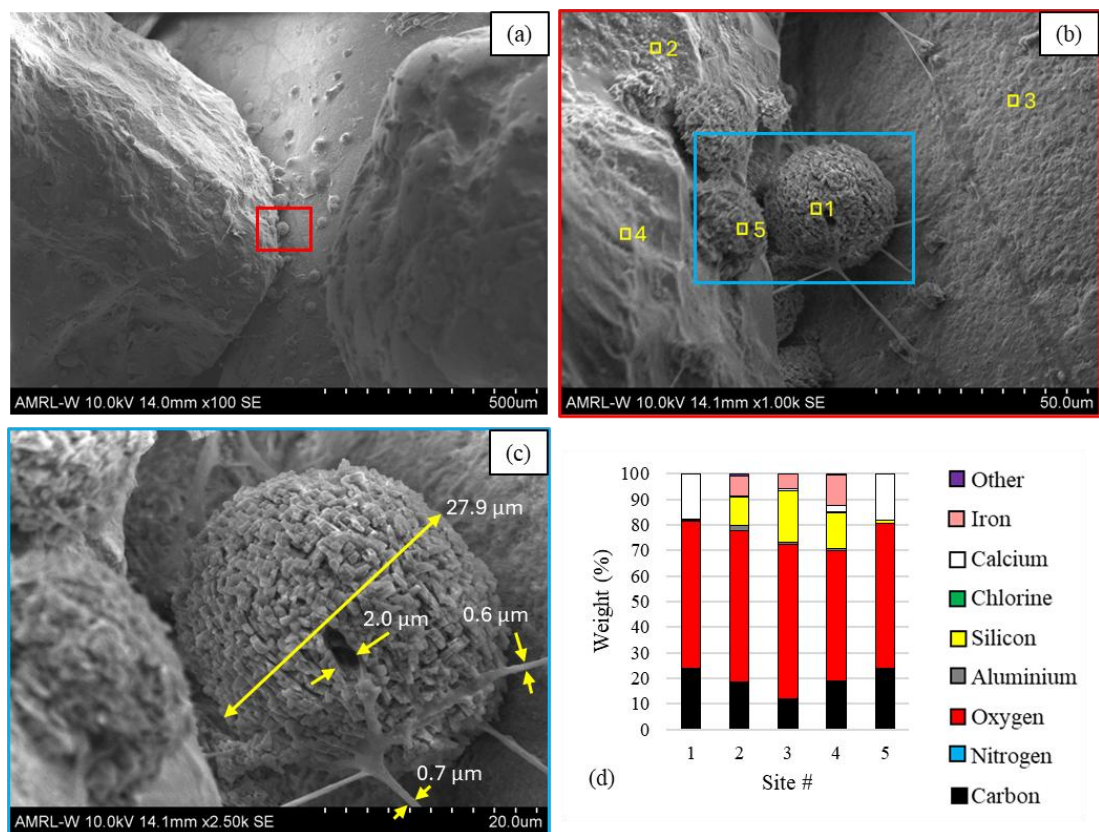


Figure 6.25 SEM images from the top portion of a column from Set 6: Coarse sand, NaCl inoculant amendment, submerged treatment strategy, with 9 cycles of 1 day each

Figure 6.26 shows a sand grain and two woodchips attached to each other. The focus of the images is on where the connecting biomass and biofilm attaches to the sand grain. Crystals are scattered across the biomass with measured sizes of 44.6, 38.7, and 47.6 μm wide. All elemental analysis of crystals showed calcium carbonate, while analysis of the biomass showed nitrogen. It was difficult to visually determine if the crystals precipitated directly onto the sand grain or onto biofilm which had coated the surface of the sand grain.

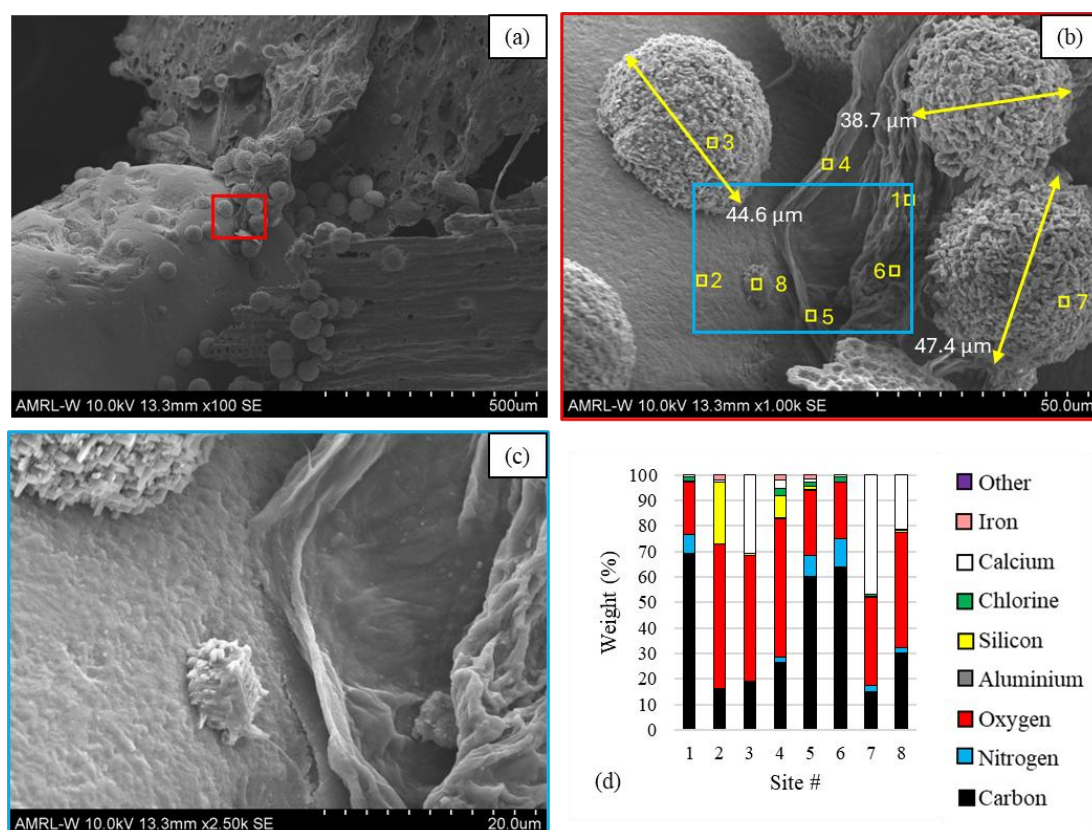


Figure 6.26 SEM images from the mid portion of a column from Set 6: Coarse sand, NaCl inoculant amendment, submerged treatment strategy, with 9 cycles of 1 day each

Figure 6.27 shows a crevice between three sand grains. Further magnified images are not shown as the quality of those images was very low due to poor gold coating inside the crevice. Inside the crevice can be seen sheets of biomass and many crystals precipitated upon them. This figure provides evidence that further carbonate precipitation could lead to binding between grains, with a potential for inducing a measurable effect on soil mechanical behaviour.



Figure 6.27 SEM images from the bottom portion of a column from Set 6: Coarse sand, NaCl inoculant amendment, submerged treatment strategy, with 9 cycles of 1 day each

6.3.2.3 Comparisons of SEM Analyses

SEM and elemental analyses are used in this section to probe the following research questions:

- (1) *Do more treatment cycles results in the precipitation of more crystals or larger crystals?*

Figure 6.28 presents SEM images from the top, middle and bottom sections of columns that received varying number of treatment cycles (3, 6, 9 cycles). In Figure 6.28, it was difficult to determine number of crystals quantitatively, as crystals were of many different shapes and sizes, some very small and hard to count or identify. Additionally, some crystals were a merging of multiple crystals together. Because of this quantitative counting of crystals was not conducted.

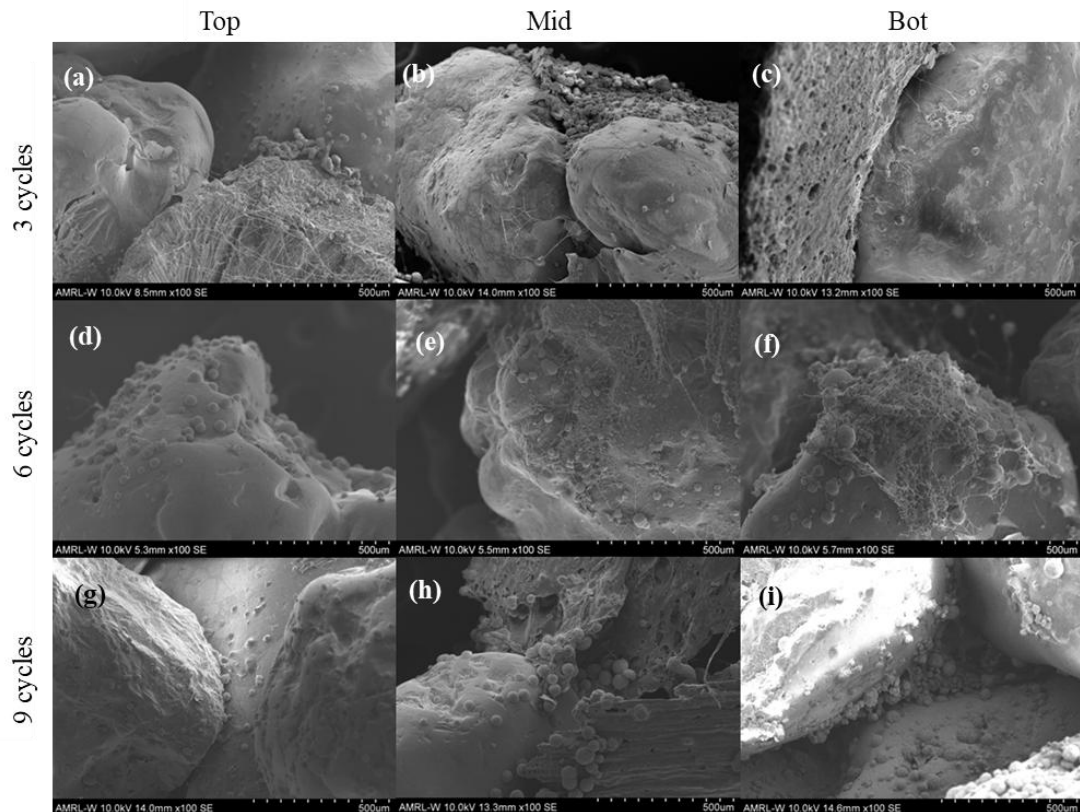


Figure 6.28 Comparison of SEM images (x100 magnification) from sets with different numbers of treatment cycles sampled from different locations within those columns.

Comparing the quantity of precipitated crystals between number of cycles and location within the column was difficult because each image only covers a narrow field of view and crystal precipitation was very heterogeneous even within the same column segment. Because of this, a single image was not always useful as a representation of crystal distribution throughout the column. However, comparing the top, middle and bottom of the columns, it appears that there are more calcium carbonate crystals present in the images of the middle and bottom sections, compared to the SEM images taken from the top of the columns. The exception to this is Figure 6.28c in which there appeared to be only a few crystals. Although, this is consistent with Figure 6.4c which indicated for the 3 cycles specimen, that more calcium carbonate precipitate was located in the middle of the column. There may also be crystals located within the woodchip present on the left side of Fig 6.28c.

(2) Do different growth inoculants cause different crystal morphologies or different crystal polymorphs?

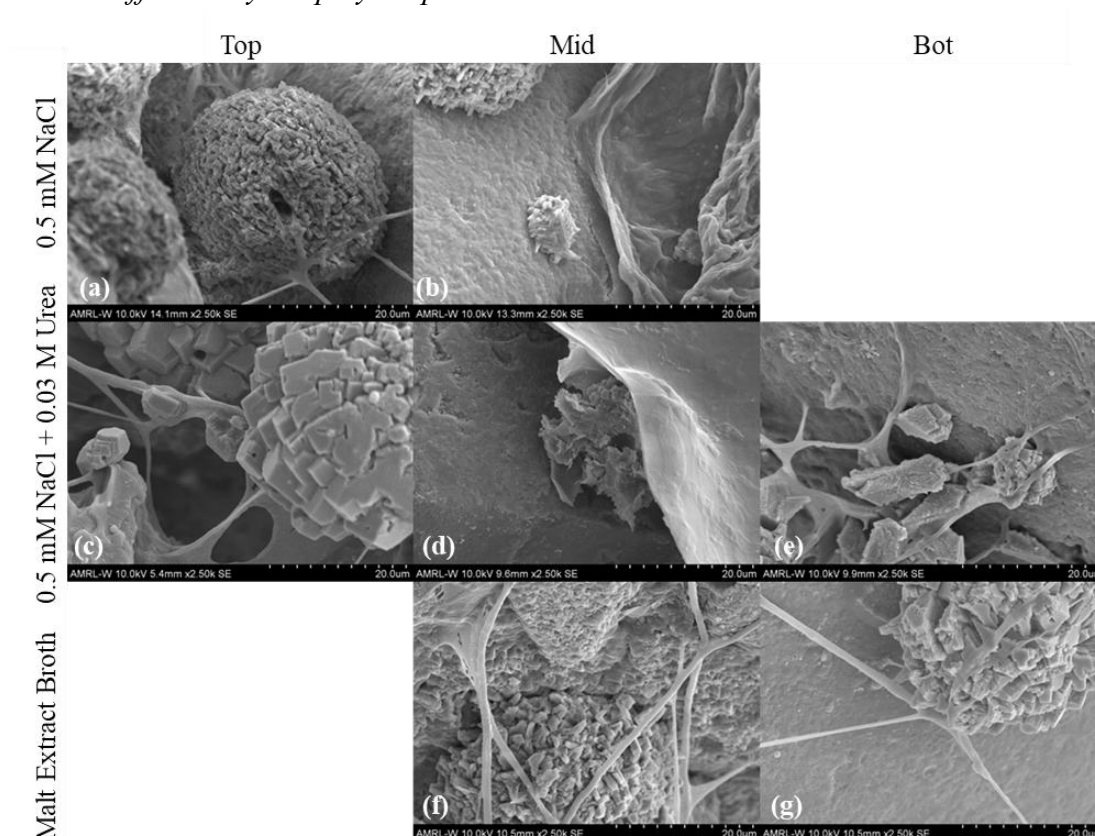


Figure 6.29 Comparison of SEM images (x2500 magnification) from sets with different growth inoculants and different sections within those columns

The polymorph or internal arrangement of molecules within a crystal determines the external appearance or morphology of the crystal. This morphology of the precipitated crystals can therefore be used to estimate the polymorph of the crystalline compound. For example, calcium carbonate has three polymorphs: calcite (blocky, angular morphology), vaterite (spherical, lumpy morphology), and aragonite (needle morphology) (Liendo et al., 2022). In order to identify the polymorphs present the SEM images are compared against images in the literature for vaterite (**Error! Reference source not found.** and **Error! Reference source not found.**), calcite (**Error! Reference source not found.** and **Error! Reference source not found.**), and calcium oxalate (**Error! Reference source not found.**). Comparing to **Error! Reference source not found.** (Shen et al., 2006), it can be seen that the larger blocky spheres (Figure 6.29c) were likely vaterite which transformed into polycrystalline calcite superstructures. Comparing to **Error! Reference source not found.** (Loges et al., 2006), the small blocky angular spheres (Figure 6.29a, b, f, g) were likely also calcite but in a more disorganised form. Comparing to **Error! Reference source not found.** (Ruiz-Agudo et al., 2013), the small sharp crystals in Figure 6.29d and e could be calcium oxalate crystals, precipitated due to oxalate acids secreted by the fungus. Comparing each of the images in Figure 6.29 to **Error! Reference source not found.**, **Error! Reference source not found.**, and **Error! Reference source not found.** (Loges et al., 2006; Shen et al., 2006; Zhou et al., 2010), no vaterite could be identified.

These comparisons may give an indication of how growth inoculant could affect crystal polymorph or morphology. Growth with only 0.5 mM of NaCl or malt extract broth appeared to result in more disorganised calcite crystals after treatment with cementing solution, whilst growth with urea appeared to result in blocky, spherical calcite crystals and also calcium oxalate crystals. These are only small images of larger samples, so are not representative but provide an indication of crystal morphology due to FICP treatment.

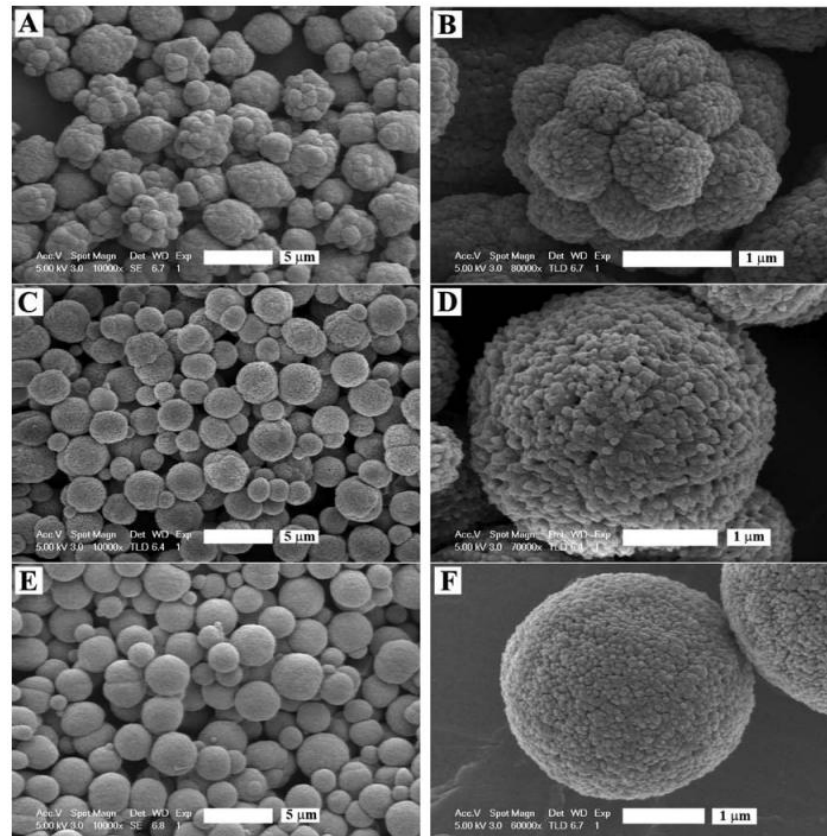


Figure 6.31 Example SEM of Vaterite crystals from Zhou et al. (2010)

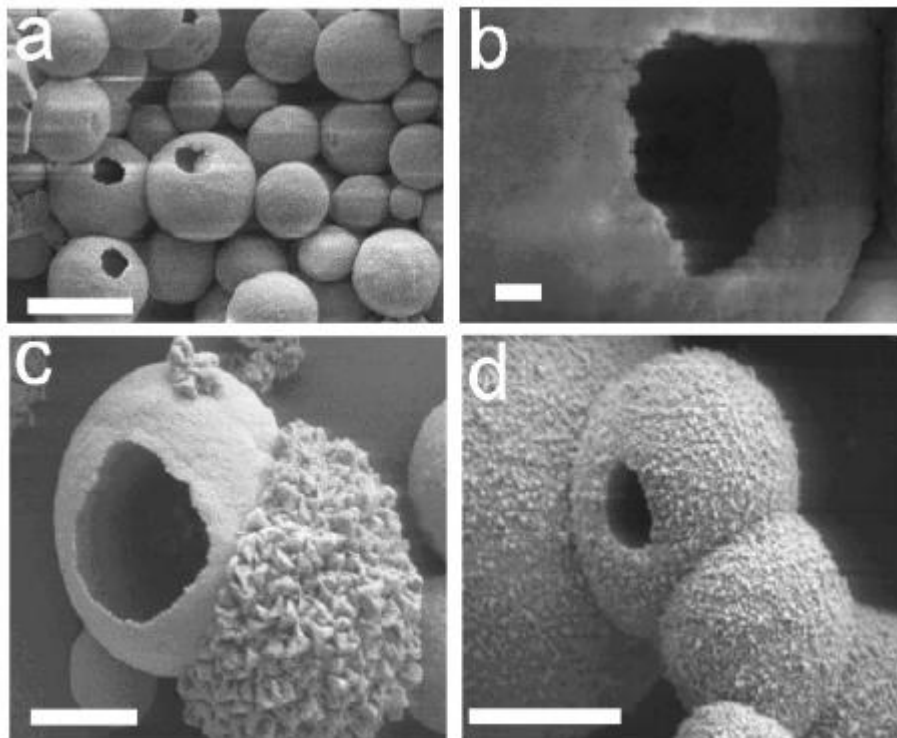


Figure 6.30 Example SEM of hollow vaterite crystals from Loges et al. (2006). Scale is (a) 5 μm , (b) 0.2 μm , (c) 5 μm , (d) 10 μm

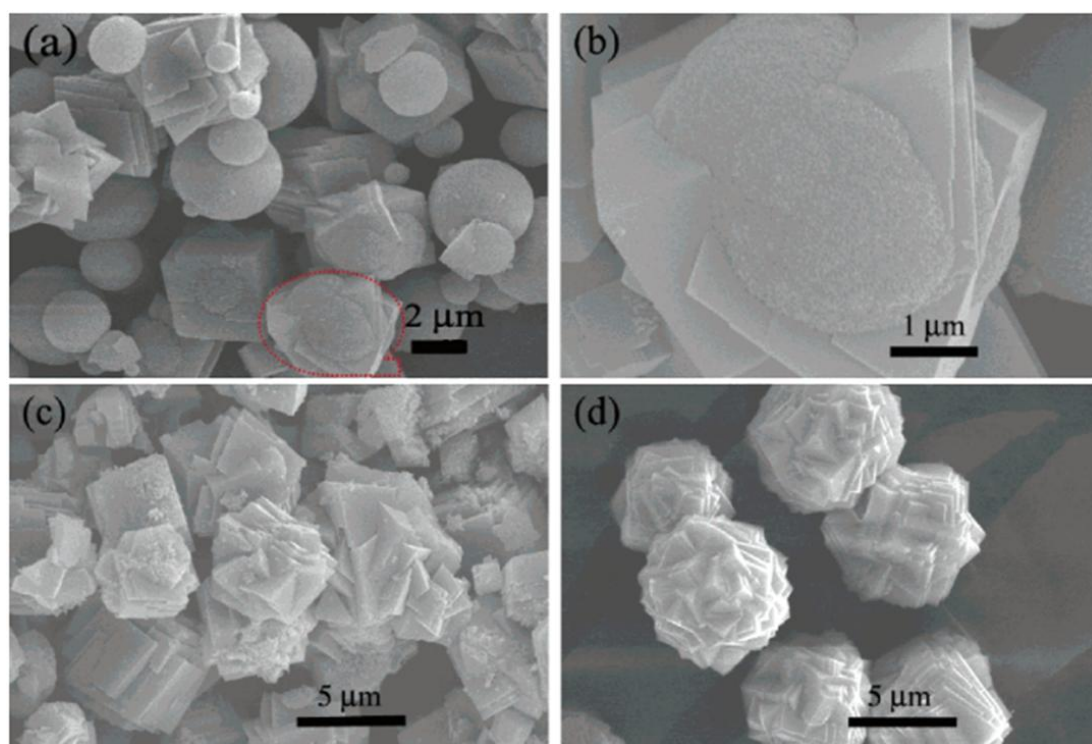


Figure 6.33 Example SEM of vaterite (a, b) transforming into polycrystalline calcite superstructures (c, d) from Q. Shen et al. (2006)

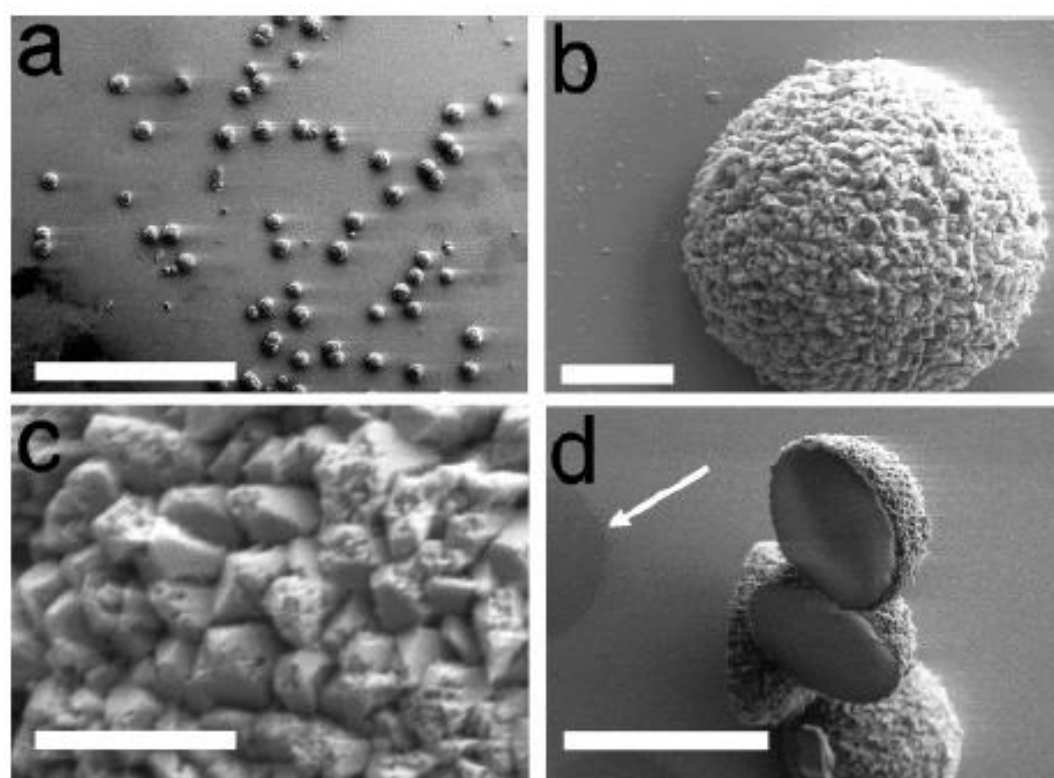


Figure 6.32 Example SEM of spherical calcite crystals from Loges et al. (2006).

Scale is (a) 0.2 μm , (b) 5 μm , (c) 2 μm , (d) 20 μm

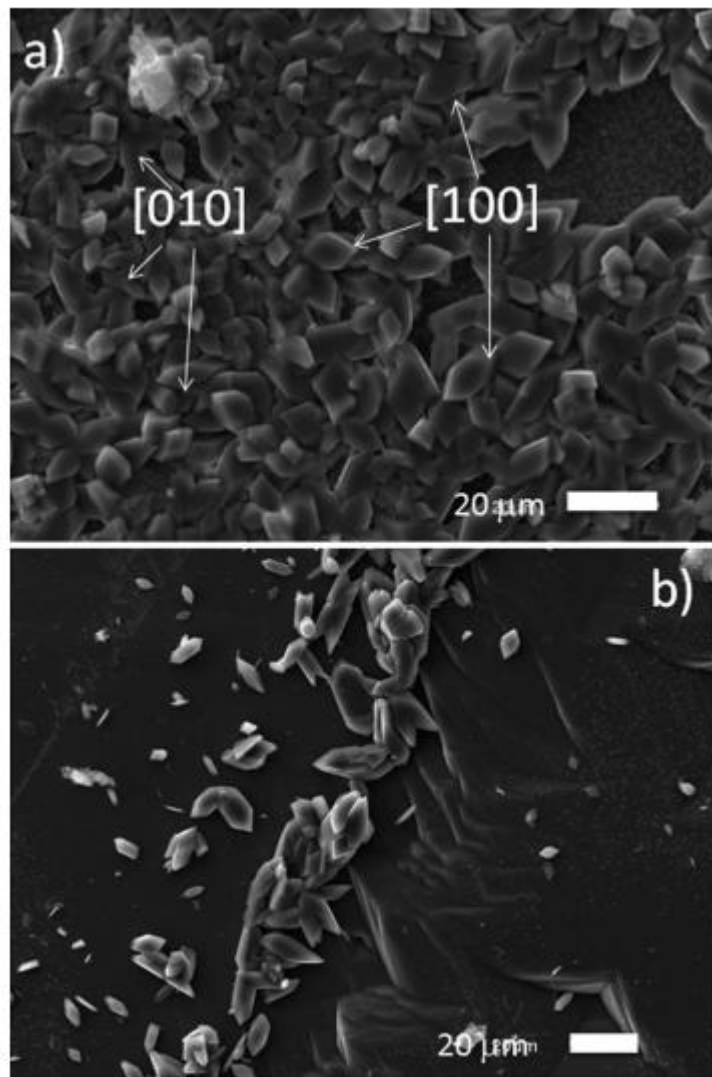


Figure 6.34 Example SEM of Calcium Oxalate precipitated onto Calcium Carbonate from Ruiz-Agudo et al. (2013)

6.3.2.4 Additional insights from SEM Images

The following figures are notable additional images from amongst the column sets which were analysed. Figure 6.35 shows two crystals which appear to have peeled off the surface of another particle with a beechwood chip in the background. These crystals were 52.3 μm and 54.9 μm in diameter. Fungal hyphae can be seen on the surface of the crystal, which would have been located between the crystal and the missing sand particle. These hyphae then continue to extend beyond the crystal edges. The thickest of these hyphae was 4.1 μm . This image is notable as it shows the underside of a removed crystal, this appears to indicate that the crystals are nucleating on the fungal hyphae.

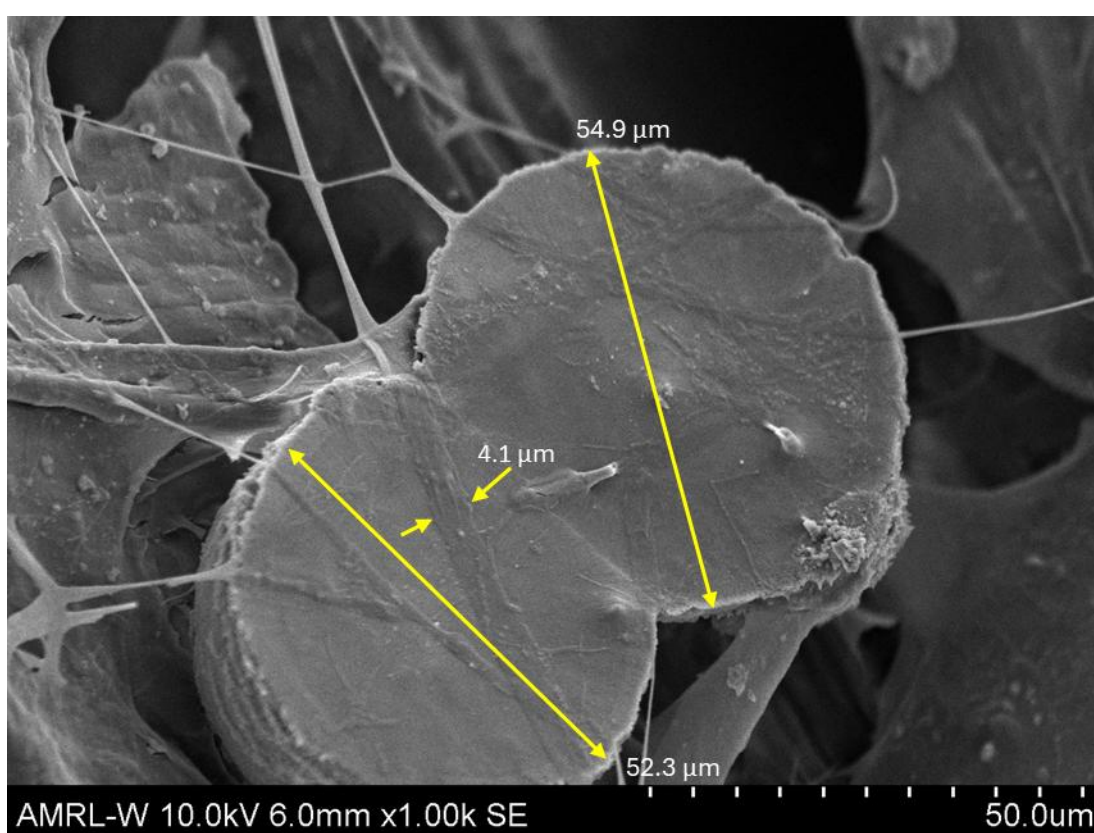


Figure 6.30 Magnified image of a Set 3 column which was treated with 6 cycles of 7 days each

Figure 6.36 shows a sand grain attached to a large sheet of biofilm/biomass. The sheet of biofilm/biomass can be seen peeling off the sand grain, suggesting that the rest of the sand grain is also coated in biofilm/biomass. Considering the elemental analysis, Site 1 in Fig 6.36b could be a calcium oxalate crystal due to its very angular and irregular appearance, sites 2 and 6 are located on the surface of uncoated sand grains, site 3 appears to be a calcium carbonate coated hypha, and site 4 fungal hyphae. Site 5 is a biofilm coated sand grain, and this is reflected in the elemental analysis which registered nitrogen in that area. From these results, it can be surmised that areas which appear to be just sand grain surface but show nitrogen in the elemental analysis may be coated in non-visible biofilm/biomass. Figure 6.36c focuses on a crystal situated underneath the biofilm. This crystal is hard to identify visually, appearing to contain shreds of what might be biomass. It is fairly sharp and angular, indicating calcium oxalate.

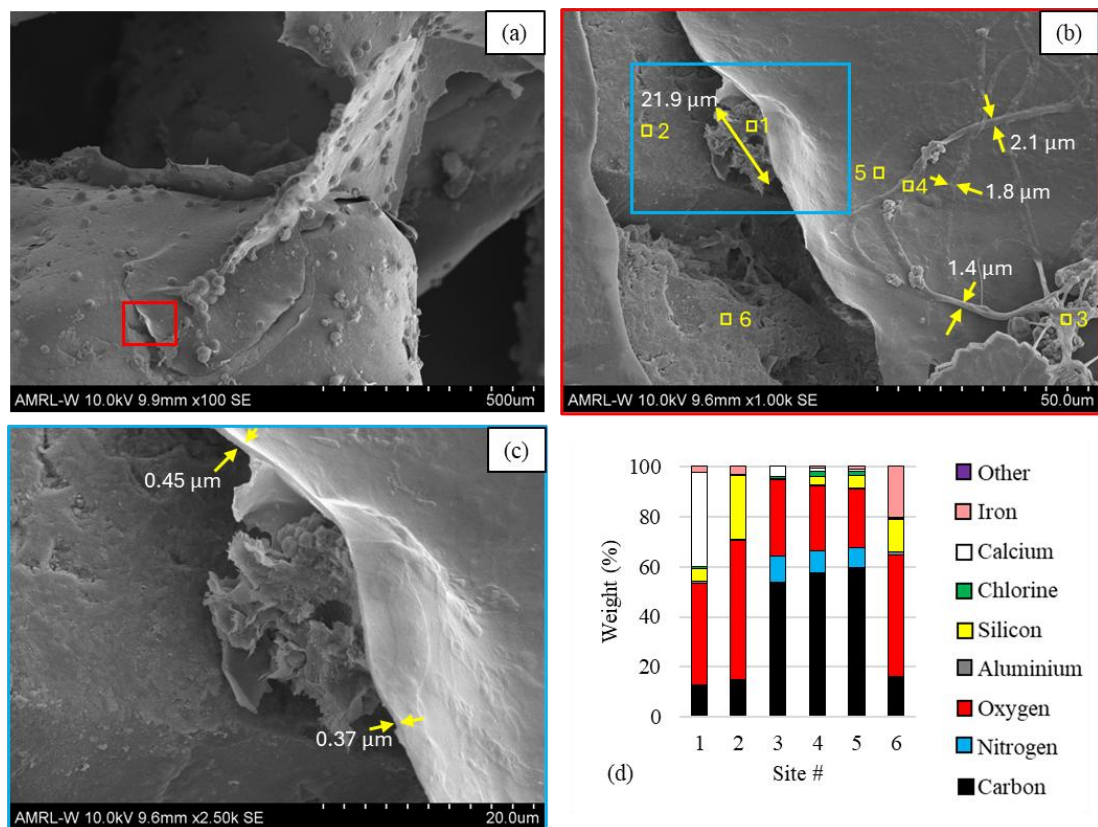


Figure 6.31 SEM image from the middle section of a column from Set 7, treated with 9 cycles of 1 day each, with NaCl and urea growth inoculant

Figure 6.37 shows a long string of fungal hyphae bridging between two sand grains. The left sand grain has sheets of biofilm/biomass peeling off. This image shows that there can be complex bridging between sand grains by the fungal hyphal mesh. It is also another example of sand grains being covered in sheets of biofilm/biomass.

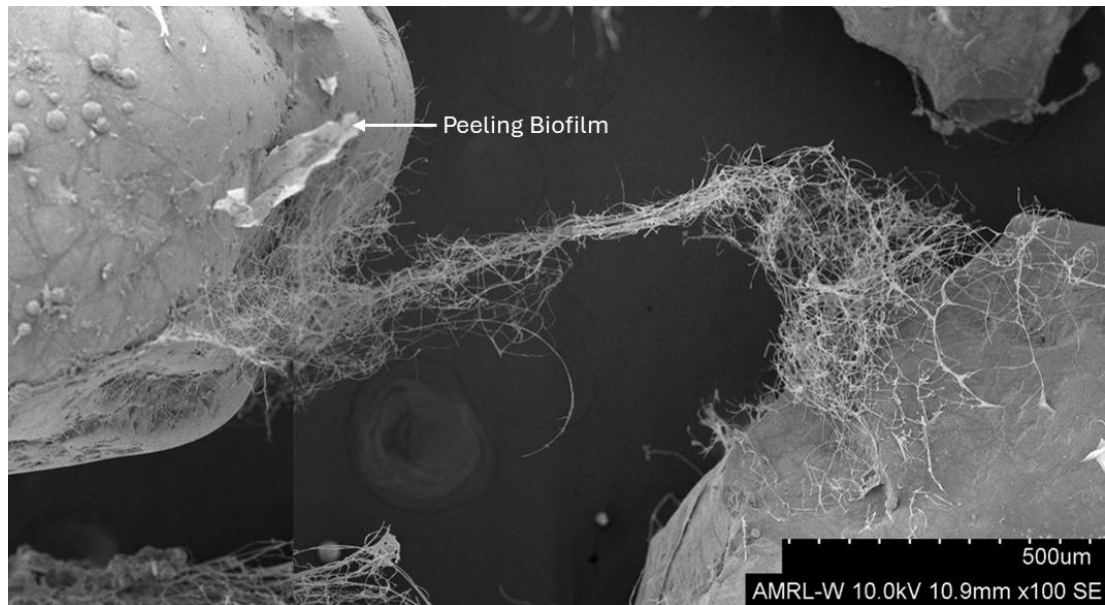


Figure 6.32 SEM image made up of combined SEM images from the bottom section of a column from Set 7, grown with NaCl and urea and treated with 9 cycles of 1 day each

Figure 6.38 shows either a fungal cord or a shard of beechwood stretching from the sample. The piece was 180.9 - 201.4 μm in diameter and had observable fungal hyphae and crystals along its length. There was high nitrogen detection at several points in the analysed area, suggesting that the piece was either a fungal cord or a beechwood shard heavily coated in fungal biomass. Comparing visual crystal morphology in Figure 6.38 with those from **Error! Reference source not found.** and **Error! Reference source not found.**, it appears that all crystals in Figure 6.38 are

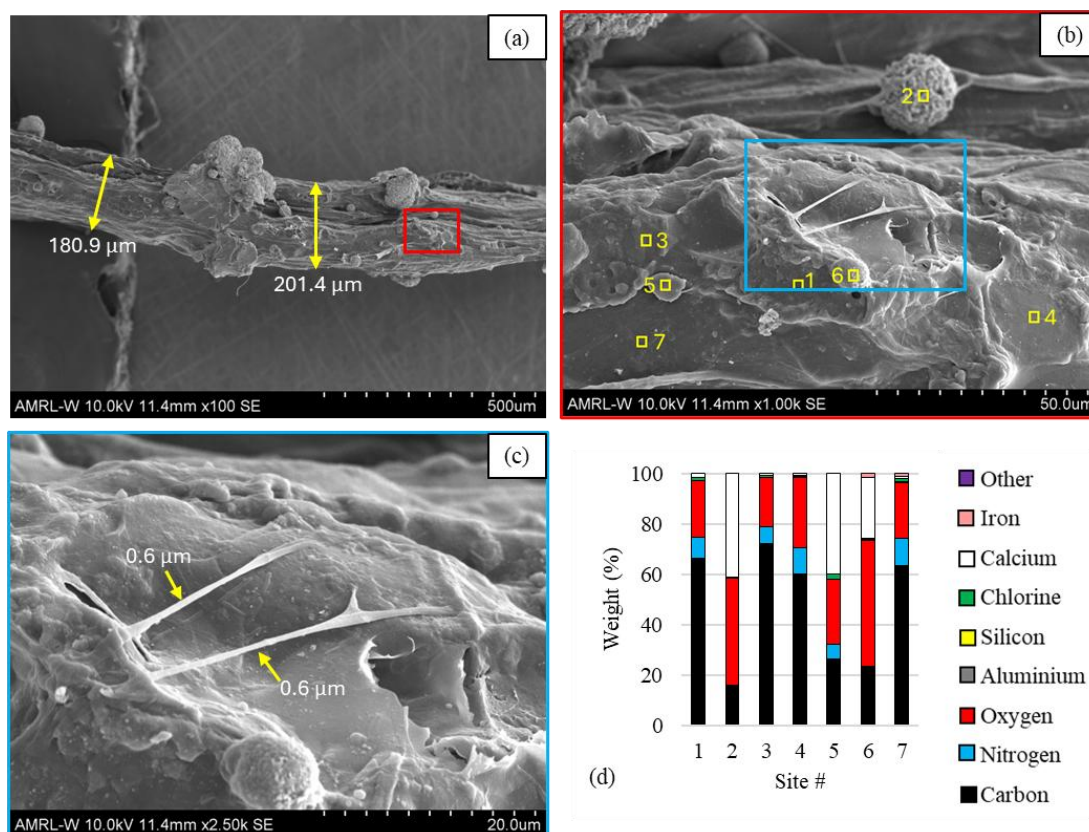


Figure 6.33 SEM images from the middle section of a column from Set 8, grown with malt extract broth and treated with 9 cycles of 1 day each calcite.

6.4 Discussion

6.4.1 Effective Pore Volume

The maximum amount of calcium carbonate that could be precipitated was calculated based on the assumption that the cementing solution fully saturated the pore space of the soil (i.e. that one pore volume of cementing solution was available). However, due to the formation of preferential flow paths caused by the heterogeneous growth of the hydrophobic fungal mycelium, it was visibly observed that the entire pore space was not accessed by the cementing solution during injection. This means that the effective pore volume (i.e. the pore volume accessed by the cementing solution) is likely to have been lower than the pore volume calculated for the sample (based on total volume – volume of solids). Future studies should ensure that the actual volume injected per cycle is recorded. Therefore, the presented maximum potential calcium carbonate precipitation is likely to be overestimated in Figure 6.3 - Figure 6.7 and would be lower if the effective pore volume was used for the calculation instead. In turn, this would also influence the percentage of calcium carbonate precipitated given in Table 6.3 - Table 6.7 and likely show that the achieved calcium conversion efficiency was higher than Figure 6.3 - Figure 6.7 and Table 6.3 - Table 6.7 suggest.

6.4.2 Influence of Cycle Duration and Number of Cycles

In columns treated with different cycle durations (1, 3, 7 days), there was very little difference in mass of calcium carbonate precipitated. This suggests that precipitation occurred quickly (within 1 day). After 1 day of exposure, increasing the exposure to the cementing solution may not have resulted in further increases in calcium carbonate precipitation due to local depletion of available calcium. It was unlikely that degradation of the urease enzyme occurred with increasing exposure time to the cementing solution.

More treatment cycles resulted in increased precipitation of calcium carbonate in a proportional manner, without further addition of a urease enzyme source, i.e. there was only one introduction of the urease enzyme, via the growth of *P. cornucopiae* prior to any treatment with cementing solution. Injection of more cementing solution via an increase in the number of treatment cycles likely displaced fluids in which the calcium was depleted and allowed for further calcium carbonate precipitation, up to at

least 9 cycles. This result corroborates the results of Ahmad et al. (2024), who found that increasing the number of treatment cycles (from 7 to 28) also increased the quantity of calcium carbonate precipitated, as evidenced by the increase in mechanical strength of the treated sand column. While (Ahmad et al., 2024) showed this for *Aspergillus niger*, a category 2 hazard group from the Ascomycota phylum, this chapter demonstrates that repeated treatment cycles can achieve increased carbonate precipitation for a Basidiomycete, category 1 hazard group. Furthermore, although (Ahmad et al., 2024) tested different numbers of cycles, they also simultaneously introduced additional growth media after 7 days, this study demonstrates that, without additional growth media, repeated injections of cementing solution can increase the amount of carbonate precipitated. Additionally, this result suggests that the urease enzyme present in the sand columns was likely intracellular, as any extracellular enzyme would be removed during injection and draining.

6.4.3 Effect of Injection Strategy

There was no difference in the total mass of calcium carbonate precipitation between the injection strategies tested, *drained* vs *submerged*. This is notable because the *drained* strategy columns only had access to the full quantity of cementing solution for ~1 hour during injection and drainage, after which only residual pore fluid would have remained within the column. This suggests that the first hour of treatment (including during injection) was when most of the calcium carbonate precipitation occurred and indicates that a continuous flow treatment where fresh cementing solution is continually being injected might be a more effective treatment strategy, similar to the method used by Fang et al. (2018).

6.4.4 Calcium Carbonate Depth Distribution

Given that calcium carbonate precipitation appears to occur within the first hour of treatment, this might explain why most calcium carbonate was precipitated in the bottom and middle sections of the column compared to the top section of the column. As the cementing solution was injected into the bottom, by the time the cementing solution reached the top portion of the column it contained less calcium and so less calcium precipitated in the top portion. Another possible cause for this

distribution might be that there was more fungal biomass in the lower portion of each the column. This was visibly obvious when comparing the lower reservoir with the upper reservoir where, in general, more fungal hyphae had grown into the lower reservoir during the incubation period, (when the reservoirs were air-filled). This lower section with higher biomass may have exhibited a higher rate of calcium precipitation than the upper section which had lower biomass, according to the results presented in (Section 5.3.1).

6.4.5 Effect of Inoculant Amendment

There was little difference in the total amount of calcium carbonate precipitated when using different inoculant amendments. Possible differences in calcium carbonate precipitation may have been observable within the first 24 hours, but no columns investigated cycle duration times shorter than 24 hours. Because most of the calcium carbonate precipitation occurred within the initial 24 hour window, this experiment would not have captured improvements in rate of calcium carbonate precipitation within that time.

6.4.6 Effect of Grain Size

There was a difference in calcium carbonate precipitation between medium grade and coarse grade sand. In the columns composed of medium grade sand less calcium carbonate was precipitated than for the columns composed of coarse grade sand. This may have been due to the smaller pore size in the medium grade sand. This smaller pore size may have allowed the hydrophobicity of the fungal mycelium to more easily counteract capillary rise within the sand columns, resulting in more clogging of individual pore throats, causing a lower effective pore volume compared to the coarse sand, resulting in less cementing solution being available in the medium grade sand columns, and therefore lower calcium carbonate precipitation achieved.

6.5 Conclusion

The key findings from this chapter are:

- Fungal induced calcium carbonate precipitation via *P. cornucopiae* inoculation was not improved by increasing the cycle duration (i.e. the time that the sand was exposed to cementing solution) beyond 24 hours.
- Calcium carbonate precipitation increased in sand columns which were treated with an increasing number of treatment cycles.
- A similar mass of calcium carbonate precipitation was observed in columns treated with the *drained* and *submerged* treatment strategies, suggesting that the cycle duration could be further reduced below 24 hours.
- Calcium carbonate precipitation was reduced in columns composed of finer grain size.
- In all sand columns, more calcium carbonate precipitation occurred in the bottom and middle portions of the column, closer to the injection point.
- Observed crystal precipitates in SEM images appear to indicate that the polymorph precipitated was predominantly calcite, although some calcium oxalate may have also been precipitated.
- Calcium carbonate precipitation observed in SEM images appeared to be closely associated with fungal hyphae and biomass.

Chapter 7: Discussion

In this chapter, the overall findings of this thesis will be discussed, and comparisons drawn between chapters and with past literature. Implications of the findings from this research for field deployment and potential engineering applications of FICP are also discussed.

7.1 FICP treatment: Comparison between Flask and Column Experiments

This PhD research comprised both FICP treatment in batch (i.e. flask) experiments, i.e. with no porous media and also column experiments involving treatment of porous media (i.e. sand grains and beechwood chips). Overall, it was observed that the rate of calcium carbonate precipitation was much higher in the sand column experiments compared to the corresponding flask experiment. Table 7.1 below compares calcium carbonate bioprecipitation and calcium conversion results obtained in Chapters 5 and 6 of this thesis.

In Section 5.3.3, fungal biomass was grown submerged in ME broth, harvested as described in Section 5.2.1, and then 35ml of the resultant inoculant submerged in a cementing solution of 0.125 M CaCl_2 and 0.5 M Urea without the presence of sand or beechwood. The average amount of calcium precipitated out of solution unamended with sodium chloride (Figure 5.7, black triangles) was 0.03 g (2.7 %) after one day of treatment, 0.27 g (23.4 %) after 4 days, and 0.39 g (34.1 %) after 7 days, indicating a clear increase in calcium precipitation with increasing treatment duration over the course of a week and beyond.

As a result of the Chapter 5 flask experiment results, the column experiments in Chapter 6 were designed to investigate treatment cycle durations of 1, 3, and 7 days. The expectation was that the 1-day treatment cycle duration would show the lowest calcium precipitation, followed by the 3-day treatment cycle duration, and that the 7-day treatment cycle duration would show the highest calcium precipitation. However, even though the sand columns in Section 6.3.1.1 were treated with the same cementing solution concentrations and ratio (0.125 M CaCl_2 and 0.5 M Urea) as described above

Table 7.1 Comparison of results from Chapters 5 and 6

Section	Experiment Type	Experiment Description	Cementing Solution (M)	Fungal Inoculant (ml)	# of Cycles	Cycle Duration (days)	Total Treatment Time (days)	Average Calcium Carbonate Precipitated (g)	Average Calcium Carbonate Precipitated (%)
5.3.3	Flask	Experiment 2. pH amendment	0.125 CaCl2, 0.5 Urea	35	1	1	1	0.03	2.7
					1	4	4	0.27	23.4
					1	7	7	0.39	34.1
6.3.1.1	Column	Cycle Duration		8	6	1	6	1.05	37.7
					6	3	18	1.17	41.9
					6	7	42	1.16	41.4

for the flasks in Section 5.3.3, the mass of calcium precipitated in the columns did not increase with increasing treatment cycle duration as in the flasks. Instead, the average calculated % of available calcium precipitated (Table 6.3) was 37.7 % for 1 day treatment cycle duration, 41.9 % for 3 day treatment cycle duration, and 41.4 % for 7 day treatment cycle duration. This indicates that most of the calcium precipitation in the sand columns was occurring within the first 1 day of exposure to cementing solution. As highlighted in Chapter 6 these calcium precipitation conversion percentages are also likely to be an underestimation, as not all of the pore space of the sand columns was accessed during injection due to the hydrophobic nature of the fungal mycelium (i.e. a reduce effective pore volume). Therefore, a lower volume of cementing solution was present within the sand column during the exposure to cementing solution than used to calculate calcium conversion.

It is important to note that the fungal biomass inoculated was different in the flask experiment and in the sand column experiment. The fungal inoculant volume added into the flasks was initially higher at 35 ml than in the columns (8 ml) but in the

flasks cementing solution (urea and calcium chloride) was added immediately after with no chance for further fungal growth. From the ergosterol measurements undertaken on total sand column contents (Chapter 4, Section 4.5.2), it was seen that after inoculation of columns with 5ml of fungal biomass (6.6 mg), the biomass grew to 129.8 mg which was an increase in biomass of 1867 %. Based on this result, it was anticipated that the growth of biomass over 5 weeks from an initial 8 ml of fungal inoculant in the Chapter 6 columns would far outstrip the fungal biomass present in the Chapter 5 flasks, with 35ml of biomass inoculated into but with no time for any further growth. Differences in fungal biomass are likely to have contributed to the difference in calcium carbonate precipitation and calcium conversion efficiency between Chapter 5 Experiment 2 and Chapter 6 (Varying cycle duration). Chapter 5, Experiment 1, (Section 5.3.1) has demonstrated that increasing fungal biomass increases calcium carbonate precipitation.

The fungal inoculants in the two chapters also experienced different growth conditions immediately before treatment. In Chapter five the inoculant was grown in aerated broth and then treated, in Chapter 6 the inoculant was grown in aerated broth before addition to sand columns and growth in the porous media. These growth conditions are very different from each other (eg oxygenation, stiffness, hydrophobicity) and may have also contributed to the different final urease activities observed.

In addition, the sand columns from Chapter 6 contained sand grains and beechwood chips, which may also have acted as nucleation sites, contributing to the greater surface area available for calcium carbonate precipitation in the sand columns compared to the flasks from Section 3.3.2.

The batch to batch variability of urease activity discussed in Section 5.4.5 may also be relevant to this comparison and to the wider research. Every batch of fungus grown for inoculation had the potential to vary in urease activity, which may have also been true for fungal growth in individual sand columns. Results in chapter 6 combine the results from two different batches of fungus (See Section 6.2.5). However, this was minimised as much as possible. The only results that compare sand columns inoculated from two different batches of fungal inoculant are the columns with varying number of treatment cycles (Section 6.3.1.2, Figure 6.4, Table 6.4). In these results the columns

treated with 6 cycles were from a different inoculant batch than the columns treated with 3 and 9 cycles. However, even discounting the columns treated with 6 cycles and only considering the columns treated with 3 and 9 cycles (from the same inoculant batch), the trend for proportionately increasing carbonate precipitation with increasing treatment cycles is still obvious. Ideally, experimental planning should prioritise using the same batch of fungal inoculant where possible. A method for measuring urease activity of the fungal inoculant before treatment with cementing solution would be beneficial to ensure fungal inoculants have a useful high urease activity. This would also enable quicker investigation of different methods of fungal growth to promote and enhance urease activity of the final fungal biomass.

7.2 The impact of changing methods for determination of carbonate

Section 4.4 investigated a new and improved method for carbonate determination in media containing beechwood chips and fungal biomass. It was found that these organic fractions should be removed by ignition at 530 °C. However, in Section 3.2.2.5, it is stated that the temperature used to determine fungal biomass shown in Section 3.3.2.1 was 600 °C based on a preliminary investigation using the inert atmosphere of a py-GCMS instead of the oxygenated atmosphere of an oven as was done in Section 4.4.1. From the findings from Section 4.4.1, Figure 4.6 Percentage mass lost by each material after ignition at various temperaturesFigure 4.6, it can be extrapolated that at 600 °C it was likely that some small quantity of calcium carbonate may have also been removed. This adds additional uncertainty to the results in Section 3.3.2.1.

7.3 Results from different cementing solution component concentrations and ratios

Different chapters of this thesis explored the use of different concentrations of the components of the cementing solution. Chapters 3 and 4 used 0.05 M CaCl₂ and 0.33 M urea (1:7 ratio of CaCl₂ to urea), with Chapter 4 also exploring an equimolar solution of 0.33 M CaCl₂ and urea (1:1) . Chapter 5 explored four different cementing

solution component ratios of 0.05 M CaCl_2 and 0.5 M urea (1:10), 0.075 M CaCl_2 and 0.5 M urea (~1:7), 0.125 M CaCl_2 and 0.5 M urea (1:4), and 0.5 M CaCl_2 and urea (1:1).

The findings of Chapter 5 (Section 5.3.2) indicate an optimal cementing solution (of those investigated) of 0.125 M CaCl_2 and 0.5 M urea. With an equimolar (1:1) solution of 0.5 M CaCl_2 and urea inhibiting carbonate precipitation. However, results from Section 4.3.2.2 showed that carbonate precipitation occurred in a sand column treated with an equimolar (1:1) solution of 0.33 M CaCl_2 and urea. There are some key differences in the methodologies of these two experiments. Chapter 4 used a lower overall concentration of equimolar cementing solution (0.33 M) than Chapter 5 (0.5 M), however the results discussed in section 5.4.2 suggest that this would not have been a major contributing factor. Further, Chapter 4 was a hole-punched sand column based experiment whilst Chapter 5 was a flask based solution experiment with no solid media and therefore likely less nucleation points. A key point is that despite there being no calcium depletion observed in the flask experiments in Chapter 5 (for an equimolar concentration), urea hydrolysis did occur, indicated by an increase in ammonium concentration. This suggests that the presence of additional nucleation sites (in the form of grain surfaces) in Chapter 4 may have supported the calcium carbonate nucleation and subsequent crystal growth. It should also be noted that the experiments in Chapter 4 were preliminary and did not include replicates, whilst the equimolar result from Chapter 5 (which was included in the experimental plan specifically to clarify whether a 1:1 ratio cementing solution was effective for treatment) was performed in triplicate and had a low standard deviation. The highlighted cementing solution of 0.125 M CaCl_2 and 0.5 M urea was then used to successfully precipitate calcium carbonate in Chapter 6.

7.4 Engineering Applications of FICP

Geotechnical applications for fungal induced carbonate precipitation lie in soil improvement, with the overall goal aim of increasing soil strength and stiffness. Given the preference for growth in high oxygen availability environments to maintain aerobic activity, it is anticipated that fungal-induced biomineralisation will be most suited towards surface applications at the soil-air atmosphere, for example surface treatment

of slopes to reduce wind and water-driven erosion (Salifu et al., 2024). Consequently, further research into submerged deployment would be required to evaluate or optimise suitability and effectiveness for any applications which would occur in saturated conditions, or below the water table. MICP driven by bacteria has already received significant attention for erosion control applications (with laboratory and field-scale studies) (Amin et al., 2017; Chek et al., 2021; Ghasemi & Montoya, 2022; Gomez et al., 2015; Salifu et al., 2016).

Fungal-based precipitation systems present a number of potential benefits over bacterially-driven systems: (i) filamentous fungi exhibit higher surface-to-volume ratios than single-celled bacteria (Menon et al., 2019). Given that fungal cell walls comprise chitin, known to act as a template for biomineralisation (Ehrlich, 2010) by acting as a substrate for binding Ca^{2+} ions, fungal-based systems would provide a greater surface area for carbonate nucleation compared to bacterially based systems for the same microbial volume; (ii) some Agaricomycete species are known to grow to vast sizes in the natural environment with individual organisms measured up to 10km^2 (Ferguson et al., 2003). As such, fungal-based systems may be suited to deployment for erosion control of slopes/hillslopes, covering large areas with fewer injection/inoculation points needed than for bacterially driven systems; (iii) fungal-induced carbonate precipitation treatment relies on growth of the organism through the porous media, this provides an opportunity to optimise and reduce the volume of the inoculant that are required to be grown ex-situ, potentially reducing costs associated with production and transport.

However, so far the FICP process suggested in this thesis requires a long growth phase of 5 weeks, much longer than required for full treatment using MICP. There is however scope for this time to be reduced in the future by selecting a different organism or changing the experimental methods, eg spraying the fungus onto the surface of sampled soils instead of mixed into inert laboratory sand.

In contrast to MICP, FICP may also require the addition of nutrients in the form of beechwood chips or nutrient broth into the treatment site. This additional organic material may affect the efficiency of precipitation of calcium carbonate by also causing precipitation of calcium oxalate and other secondary precipitates, as discussed in Section 6.3.2.3. In real soils, this would also be much more likely to occur due to

the many organic materials and native microbes present in the treatment site when compared to sands. Further research would need to be conducted to establish if these secondary precipitates would have a net positive or net negative impact on the mechanical behaviour of the final treated soil.

7.5 Influence of FICP on erosion behaviour

At the end of FICP treatment of the sand columns in Chapter 6 there was visual evidence of clumps of biocemented grains and woodchip present within the treated sand columns from Chapter 6, however the columns remained fragile to touch. From Section 6.3.1.2, Table 6.4, it can be seen that columns from set 6 (9 treatment cycles of 1 day cycle duration) contained a mean mass of calcium carbonate of 1.99 g in 89.49 g of treated soil (calcite, sand, and beechwood). This amounts to a mean bulk calcium carbonate content within the sand columns of 2.22 % by mass. Considering that a higher amount precipitated closer to the injection point, the calcium content in the bottom third of the column (set 6) was ~2.9 % by mass.

Previous research into MICP treated sands has found through mini JET erosion laboratory testing that a calcium carbonate content as low as 2.5 % could increase the critical shear stress required to initiate erosion and decrease the erodibility coefficient (Do et al., 2019, 2020). In erosion flume tests, Amin et al. (2017) showed that mean carbonate contents in the range 2.7 - 3.2 % also increased critical shear stress and reduced the erodibility coefficient of MICP treated sands. Furthermore, Gomez et al. (2015) demonstrated that calcium carbonate content as low as 2.1 % were sufficient to inhibit water induced erosion in field testing of MICP treated sands. Ghasemi & Montyoya, (2022) also showed in a field trial of MICP treated sands that a calcium carbonate content of 1 % resulted in an increase in critical shear stress, and a 2 % calcium carbonate content led to a 3 orders of magnitude reduction in erodibility coefficient. These studies indicate that FICP and the process developed here (and the carbonate content achieved) has potential to reduce soil erosion. For applications to reduce surface erosion (e.g. in slopes), direct application on the surface may be preferable to develop a biocemented crust with higher calcium carbonate content to enhance erosion resistance.

However, this thesis has not directly investigated a surface-spraying methodology and this should be further investigated for developing site treatment protocols. In addition, the research in this thesis did not involve measurement of erosion resistance improvement after FICP treatment, nor mechanical strength improvement as the FICP sand columns were not solid after treatment. Further investigation and studies are required to directly test the erosion behaviour of FICP treated soils.

7.6 Field-scale treatment strategy

Comparing this experiment with the papers discussed in (Ahmad et al., 2024; Fang et al., 2018; Martuscelli, Soares, Lima, et al., 2020; Tuckett et al., 2025), the greatest difference in treatment strategy of sand was the adoption of an initial in-situ fungal growth phase before treatment with cementing solution. Tuckett et al., (2025), did have a ten day of growth period but did not investigate multiple cycles of treatment with cementing solution. So, this thesis proposes for the first time, a new multi-step treatment strategy for FICP treatment of sand comprising: 1) inoculation of soil with fungal biomass 2) growth of fungus in situ for several weeks (based on the previous research indicating that 3 weeks of saprotrophic fungal growth can inhibit soil erosion, (Salifu, 2019), followed by 3) treatment with multiple cycles of cementing solution. As such, this research has been able to show for the first time that less alkaliphilic fungi, such as *P. cornucopiae*, can be successfully deployed in FICP treatment.

This multi-step treatment approach resulted in fungal-induced cementation and increasing mass of induced calcium carbonate with repeated treatment cycles regardless of the high urea concentrations used (500 mM Urea). This was more than the 60 mM urea concentration identified in Section 3.3.1.4 that completely inhibited *P. cornucopiae* growth after 1 week exposure. This indicates that either short term exposure (1 day) to the high urea concentration (500mM) does not kill the fungus or that enzyme activity is still being maintained even after death of the fungus due to residual enzyme activity (evidence of this was also observed in Section 3.3.1.3).

The results from the sand columns in Chapter 6, indicate that the treatment duration can be 1 day or less (with no advantage to longer treatment durations). Indeed, even shorter times may be adequate, as the “drained” columns showed similar calcium

carbonate mass to the “submerged” columns. *P. cornucopiae* may be able to survive this high urea/high ammonium exposure, if it is for much shorter durations. It was also found in Section 3.3.1.2 that, if *P. cornucopiae* was provided with access to an environment uncontaminated with urea, it could survive and even grow simultaneously in the high urea/pH environment. Within the sand columns tested in Chapter 6, this mechanism could have been facilitating the repeated cementation behaviour observed, by allowing *P. cornucopiae* access to pore spaces that did not become filled with the cementing solution (as a result of the hydrophobic nature of the mycelium). Therefore, it is possible that growth was continuing to occur during FICP treatment.

Using the multi-step approach, and the results observed on fungal network resilience, for field scale engineering deployment of FICP treatment, it is proposed that on a large scale such as an embankment *P. cornucopiae* could be grown across the site and then treated with cementing solution conducted in a staged manner within distinct treatment zones, to enable the fungus to survive by relying on the untreated portions (see Figure 7.1). The embankment could be split into a patchwork of zones, with one third of the sections treated on the first day, the next third on the second day, and the remaining sections on the third day. In one of the sections not receiving treatment of cementing solution on a given day, the section could instead be given nutrients to facilitate recovery from the high pH environment. This could be repeated until every section had achieved the desired erosion resistance, and the fungus might even still be alive to grow further for treatment again in the future.

Future studies should focus on developing knowledge and evidence base to extend beyond the controlled experiments conducted in this thesis towards the proposed deployment strategy outlined above. The experiments in this thesis were conducted in controlled, sterilised environments, whereas deployment in the field would be in uncontrolled environmental conditions. The inoculant organism would be in competition with native microbes and the soil would have a more complex soil composition, including e.g. additional grain sizes, organic matter. Further testing would be required to investigate the effects of variable conditions such as temperature, light, and different nutrient sources. As well, specific fruiting conditions for the treatment species should be confirmed so that they may be prevented/controlled to

avoid allergen-related hazards. Future research should investigate these aspects in small and large-scale laboratory experiments before field-testing.

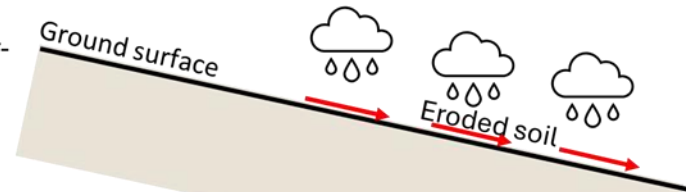
The proposed deployment strategy would also require an investigation into use of spraying solutions onto the surface of soils to be treated rather than injection. The hydrophobicity induced in the soil would be a significant hurdle to overcome. In Section 4.3, columns were mixed or hole punched specifically because injection of cementing solution was challenging due to induced hydrophobicity. In Chapter 6, cementing solution was injected into the fungally colonised sand columns using a peristaltic pump because otherwise the solution would not penetrate into the sand. A similar problem may occur in field-scale deployment onto slopes, causing some of the cementing solution to simply run off fungal-colonised soil.

In Chapter 6, it was noted that the hydrophobicity induced by the fungus within the sand columns likely induced channelling, caused uncertainty in the pore-volume calculation, and therefore uncertainty the percentage of potential calcium carbonate precipitated. This was in addition to the calculation not considering pore spaces potentially becoming progressively smaller during treatment, the volume of residual treatment solution from previous treatment cycles, and that precipitate not physically bound to a surface would be lost during draining. Together, this has implications for field treatment, suggesting that even with some adjustment in method there will likely be some fraction of a field treatment site that would receive less effective treatment.

Figure 7.1 Suggested method of field-scale deployment

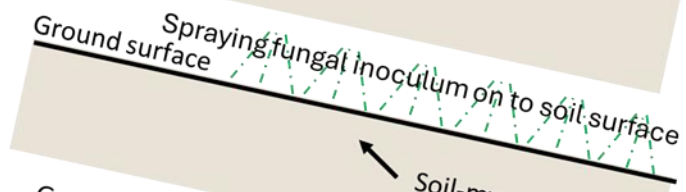
Stage 1:

Initial slope vulnerable to water-induced erosion



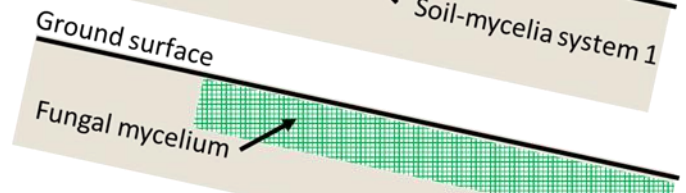
Stage 2:

Inoculate slope with fungi via surface spraying



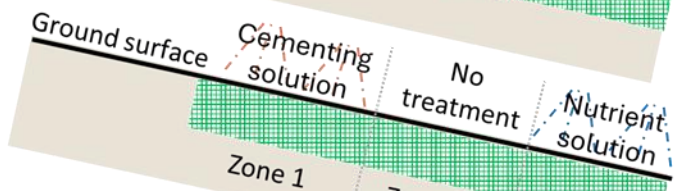
Stage 3:

Growth of fungal mycelium across slope over a period of weeks



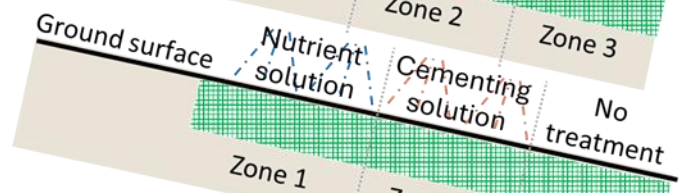
Stage 4:

Treatment of slope with cementing solution



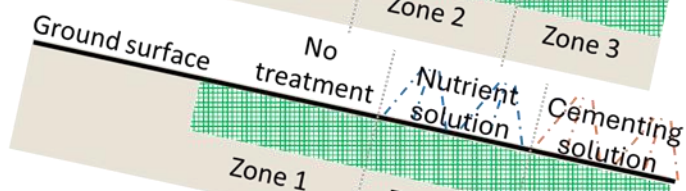
Stage 5:

Treatment of slope with cementing solution (cont.)



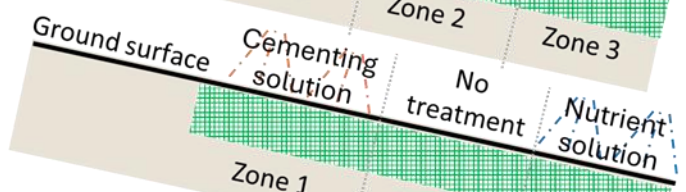
Stage 6:

Treatment of slope with cementing solution (cont.)



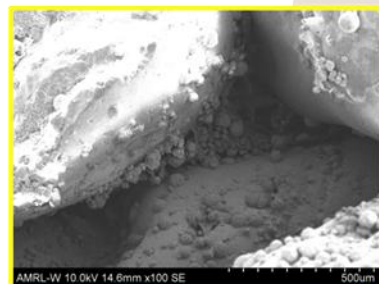
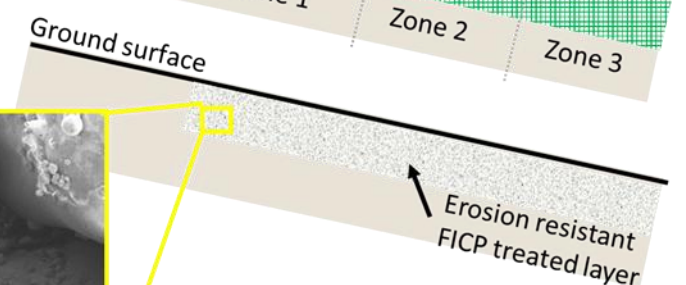
Stage 7:

Treatment of slope with cementing solution (cont.)



End Stage:

Formation of erosion resistant FICP layer



Chapter 8: Conclusions, limitations & future work

8.1 Contributions to knowledge

This thesis has focussed on developing a proof-of-concept for fungal-induced carbonate precipitation process using a saprotrophic fungal species from the Basidiomycota phylum.

The specific objectives of this research were to:

1. Identify a novel saprotrophic, urease active, fast-growing fungal species from the Basidiomycota phylum suitable for inducing carbonate precipitation for potential use in ground engineering applications.
2. Develop novel methods for determining calcium carbonate content of FICP treated soils and determine the optimum growth period for fungal biomass in columns of sand and beechwood.
3. Characterise and optimise the chemistry of the fungal-induced carbonate precipitation process by investigating the influence of fungal biomass, and cementing solution chemistry (total concentration, Ca:Urea ratio and initial pH).
4. Investigate the influence of treatment strategy variables (treatment duration, number of treatment cycles, growth amendments and soil medium composition) on fungal-induced carbonate precipitation in sand.

In each of the following sections, the key findings related to each objective are presented with novel contributions to existing knowledge highlighted.

Objective 1: Identification of suitable Basidiomycete for FICP

Pleurotus cornucopiae was identified for the first time as the fastest growing, most urease active (1.24 – 2.55 mM urea/hr/g) candidate for use in FICP. However, all of the screened species exhibited some calcium carbonate precipitation when exposed to a cementing solution of 50 mM CaCl₂ and 333 mM urea. Calcium carbonate was observed via SEM imaging and confirmed as calcite via XRD analyses. In addition, it was found that even though growth of *P. cornucopiae* was inhibited on

agar at urea concentrations above 0.06 M, it could grow on agar amended with 0.1 M urea if it also had simultaneous access to a source of nutrients unamended with urea. This is due to the networked nature of the mycelium, providing additional resilience, allowing the fungus to survive in a harsh environment.

While previous studies have investigated Ascomycetes for engineered fungal induced carbonate precipitation, this research demonstrated for the first time that Basidiomycetes and in particular Agaricomycetes, and some edible mushrooms (*P. cornucopiae*, *P. ostreatus*, and *P. pulmonarius*) are ureolytically active and can induce carbonate precipitation.

Objective 2: Development of methods

While acid digestion is a commonly used technique to determine carbonate content in MICP treated soil samples, the results in Chapter 4 demonstrated that organic matter (beechwood chips and fungal biomass) are also sensitive to mass losses during acid digestion. Therefore, in this research a new method was developed for the determination of calcium carbonate in fungal treated soils, i.e. comprising sand, fungal biomass and beechwood after igniting the sample at 530 °C. This temperature was identified as removing 99 % of beechwood and 97.6 % of fungal biomass, while removing 0 % calcium carbonate. Once the biomass and organic matter was removed, samples were acid digested with pressure change measurements to determine the mass of calcium carbonate. In order to understand how fungal biomass evolved during incubation, ergosterol was extracted from fungal inoculated sand and beechwood columns over a period of 14 weeks. An optimal growth time for fungal biomass in the columns was identified at 5 weeks of incubation in the dark at 25 °C.

Objective 3: Optimising FICP process

The optimisation of FICP treatment for fungal biomass with a cementing solution of CaCl₂ and urea in a flask had multiple novel findings. A greater fungal biomass treated at higher cementing solution concentration resulted in more calcium carbonate precipitation. A cementing solution of 0.125 M CaCl₂ and 0.5 M urea was identified as the optimal concentration and ratio (1:4) of each component, with this system being the most efficient in terms of mass of calcium carbonate precipitated per

gram of biomass. As fungal biomass lowers the initial pH of the cementing solution, Chapter 5 also explored amending the initial pH amendment of the cementing solution, intended to increase the initial pH with NaOH. However, increasing the pH was not an effective method to improve calcium carbonate precipitation. Indeed, if the initial pH was too high (observed here for initial cementing solution pH = 11.94), there was an inhibitory effect on urease activity and calcium carbonate precipitation.

While MICP research has commonly adopted an equimolar cementing solution concentration, the results from this research indicate that for FICP using *P. cornuopiae* this is not a suitable ratio, with ureolysis and calcium carbonate precipitation inhibited at a 1:1 ratio of CaCl₂ to Urea.

Objective 4: Investigation of FICP treatment strategies

This research demonstrated for the first time that despite the sensitivity of *P. cornuopiae* to high pH/high urea environments, when treating sand columns exposed to 0.5M Urea in the cementing solution, repeated treatment cycles of 1 day each resulted in progressively increased calcium carbonate precipitation. This was shown in Section 6.3.1.2 which reported calcium carbonate precipitation of 0.64 g after 3 cycles, 1.05 g after 6 cycles, and 1.99 g after 9 cycles. Increasing the cycle duration from 1 to 3 to 7 days, did not enhance calcium carbonate precipitation, indicating that most of the calcium available in the cementing solution was already depleted and that a treatment cycle duration could be reduced to less than 24 hours. This latter finding was supported by the similar result between the two treatment strategies: submerged and drained (Section 6.3.1.3), which achieved a similar mass of calcium carbonate precipitate, despite the drained scenario having much less exposure time to the cementing solution (up to 1 hour) compared to 1 day for the submerged scenario. Growth amendment with urea or malt extract broth had little effect on calcium carbonate precipitation, and smaller sand grain size reduced the amount of calcium carbonate precipitated during treatment. In most columns the majority of calcium carbonate precipitation occurred in the middle and bottom portions of the sand column, nearer to the cementing solution injection point.

These novel results provide key findings which will underpin future development of FICP using *P. cornuopiae*, namely that exposure duration can be

greatly reduced below that tested here, which would facilitate the injection of more pore volumes of cementing solution, and the potential to substantially increase the amount of calcium carbonate that can be precipitated, with corresponding implications for erosion resistance.

8.2 Limitations

8.2.1 Biomass Determination of treated columns

While ergosterol measurements (and biomass estimates) provided useful information in Chapter 4, they were not conducted on the sand columns in Chapter 6 due to restricted space, time, and materials. While ergosterol extracted columns from Chapter 4 contained no calcium carbonate, it must be noted that the high NaOH concentration introduced to facilitate extraction of the ergosterol is likely to react with calcium carbonate to produce lime and sodium carbonate (Hanein et al., 2021; Simoni et al., 2022). Another barrier for applying this method to FICP treated sand columns is the large volume of solvents required, 300 ml of methanol and 700 ml of cyclohexane of solvents required per treated 50 g sand column. This would be expensive and the evaporation of over 0.7 L of cyclo-hexane was the bottleneck in the method for this study due to laboratory capabilities. If ergosterol extraction was to be performed regularly at this scale, optimisation of the method would be required to reduce the volume of solvents, and therefore the time required.

8.2.2 Measurement of urease activity of fungal inoculant

The variability in urease activity between batches of fungal inoculant has been noted in sections throughout this thesis. A method for measurement of fungal urease activity would be beneficial both for quality assurance to ensure that a given batch of fungal inoculant has sufficient useful urease activity, as well as to ensure appropriate comparisons, when other variables are tested. This would save time in the long-run, and improve the quality of the experimental research and results.

8.3 Recommendations for future work

8.3.1 Refinement of treatment strategy

Sand column treatment in this thesis incorporated long static periods, (i.e. periods with no flow). However, the results indicate that such prolonged static period was not required in the sand columns, either because of the high biomass achieved after growth, and/or the greater availability of nucleation sites within the sand column (compared to the batch experiments). It was also visually observed that the effective pore volume (i.e. the pore volume accessed by the cementing solution) was considerably lower than the total pore volume due to the hydrophobic nature of the fungal mycelium. As such, there is scope to modify the FICP treatment strategy to incorporate either continuous flow injection or pulsed flow to enhance the efficiency of calcium carbonate precipitation by allowing the cementing solution greater access to hydrophobic pore spaces. Such treatment strategies are anticipated to enhance the calcium carbonate content achieved via FICP.

8.3.2 Testing mechanical and hydraulic behaviour of FICP treated columns

This thesis focused on identifying a suitable fungal species, on optimising biological and chemical factors controlling carbonate precipitation and efficiency during FICP treatment, as well as treatment strategy variables. In future work, with increasing calcium content achieved via FICP by refining the treatment strategy, the hydraulic and mechanical behaviour of the treated FICP soils should be tested. For erosion applications direct testing of erosion behaviour is necessary and future work could include e.g. JET erosion tests. While evaluation of the hydraulic (water retention, permeability) and shearing behaviour (direct shear, or triaxial testing) of FICP treated soils is also worthwhile to explore other potential geotechnical engineering applications of FICP-treated soils.

8.3.3 Influence of soil properties

Although this thesis conducted FICP treatment in medium and coarse-grained sands, further work should also explore FICP treatment of fine sands. Treatment of

fine sand or more well-graded sand could result in greater mechanical strength improvement due to FICP as smaller pore spaces require less mass of calcium carbonate to bridge and bind particles together than the large diameter pores in the coarse sand used in this study (Liufu et al., 2023; Nafisi et al., 2018; Wang et al., 2025). Similarly, the initial dry density is also expected to play a role on the strength gain achieved for a given amount of calcium carbonate precipitation. Future studies could also explore the influence of different mineralogy on fungal-induced carbonate precipitation,

8.3.4 Fungal species

While this thesis has focused on a bioaugmentation approach, which would rely on the introduction of *P. cornucopiae*, experience from the environmental engineering discipline, where bioaugmentation has been carried out for contaminated land remediation, shows that bioaugmentation strategies involving injection of non-native microbes can fail due to competition from the existing native community (Van Veen et al., 1997). Investigating the potential to harness native fungi for FICP is an interesting future research avenue. Such species would already be adapted to growing in the local environment and so would be much more likely to survive and grow on site during treatment. There are two main approaches to harnessing in situ native microorganisms: 1. Isolating fungi from treatment site, culture ex-situ and re-inject for treatment (Bibi et al., 2018; Maureira et al., 2024; Mwandira et al., 2023; Nayanthara et al., 2019; Safdar et al., 2020), or 2. Biostimulating the native fungal community in situ as part of the growth phase before treatment with cementing solution. Such approaches have already been successful for bacterial-based MICP (Dhami, Alsubhi, et al., 2017; Gomez et al., 2017, 2018; Graddy et al., 2021).

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Appendices

Appendix 1: Density of beechwood chips

Analysis Gas: Helium
 Sample Mass: 1.7244 g
 Temperature: 26.74 °C
 Number of Purges: 10

Equilibration rate: 0.0050 psig/min
 Expansion volume: 8.4414 cm³
 Cell Volume: 11.5350 cm³

Density and Volume Table						
Cycle #	Volume (cm ³)	Volume Deviation (cm ³)	Density (g/cm ³)	Density Deviation (g/cm ³)	Elapsed Time (mm:ss)	Temperature (°C)
1	1.2037	0.0013	1.14326	-0.0016	20:51	26.54
2	1.2018	-0.0005	1.4348	0.0006	30:21	26.61
3	1.2057	0.0033	1.4302	-0.004	39:56	26.62
4	1.1981	-0.0042	1.4393	0.0051	50:43	26.73
5	1.2111	0.0087	1.4238	-0.0104	59:58	26.76
6	1.2067	0.0044	1.429	-0.0052	67:48	26.73
7	1.2034	0.0011	1.4329	-0.0013	77:36	26.82
8	1.1959	-0.0064	1.4419	0.0077	88:52	26.85
9	1.1999	-0.0025	1.4371	0.0029	96:40	26.85
10	1.1972	-0.0051	1.4403	0.0061	107:10	26.94

Summary Data	Average	Standard Deviation
Volume	1.2024 cm ³	0.0045 cm ³
Density	1.4342 g/cm ³	0.0053g/cm ³
Temperature	26.74 °C	0.12 °C