Measuring DNA of soil-borne plant pathogens and biocontrol agents in soils in relation to disease and response to organic amendments

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Abstract

Soilborne pathogens pose a significant threat to agricultural productivity, necessitating sustainable alternatives to chemical control methods. This study addresses the critical need to replace or complement chemical products with biologicals and organic amendments to mitigate soilborne pathogen pressure. Understanding the dynamics of soilborne pathogens and their interactions with biological controls is essential for enhancing food security and sustainable agriculture. This research employed various methodologies, integrating soil DNA extraction techniques, qPCR quantification, and metabarcoding analysis to investigate the effects of organic amendments on soilborne pathogens and beneficial organisms. Field trials conducted in raspberry, onion, daffodil, and asparagus crops evaluated the efficacy of various organic amendments, including manure, arbuscular mycorrhizal fungi (AMF), composts, digestates, and cover crops. The study revealed substantial variability in soil DNA extraction methods, influencing the comparability of results across studies. Although the selected methods correlated with quantified DNA in inoculated soils, validation in field conditions proved challenging. Field trials failed to establish inoculum density-disease incidence relationships or relate population changes to organic amendments. Site-specific variations dominated bacterial and fungal soil communities, suggesting the influence of unmeasured variables on soil microbiomes. Despite efforts to standardize molecular methods and control environmental variables, their impact persisted throughout the study. Future trials may require extended durations to assess long-term effects on soil health and pathogen populations with repeated application of organic amendments. Increased sampling frequency throughout the growing season is recommended to capture the dynamic nature of soil microbial communities. Molecular techniques, such as qPCR and metabarcoding, offer valuable insights into soil microbial interactions, guiding future testing options and informing sustainable agricultural practices. This thesis underscores the importance of considering soil characteristics, environmental factors, and field history in long-term management strategies.

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Chapter 1- Introduction

Food security and demand have become prominent issues in today's world due to the threat of climate change and population increase. The Food and Agriculture Organisation (FAO) estimates that 2.4 billion people, out of the 8.1 billion world population, do not have access to safe and sufficient food (FAO, 2023). This population is projected to increase to 9 billion by 2050 (Hanjra and Qureshi, 2010), resulting in a rise in global food demand by 100-110% from 2005-2050, equivalent to an extra 200,000 billion calories per year (Tilman *et al.*, 2011).

Population growth is one of the major challenges facing food security (Rivas and Nonhebel, 2016). As the global population expands, there is a greater strain on available resources, including arable land and water (Satterthwaite *et al*., 2010; Popp *et al*., 2013; Rivas and Nonhebel, 2016; van Vliet *et al*., 2017). Climate change is also a significant factor impacting food security. Changes in temperature, precipitation, and extreme weather events affect crop yields and the suitability of land for agriculture (Parry *et al.*, 1999; Schmidhuber and Tubiello, 2007). As climate change alters the behaviour of pathogens, diseases can become more virulent, further threatening crops (Kettles and Luna, 2019).

Due to these challenges it is key to develop sustainable farming methods that prevent crop losses and improve crop yield and vigour. Improving plant disease management can significantly enhance global food security and pest and disease management has contributed to doubling food production in the last 40 years (Chakraborty and Newton, 2011). This study aims to explore the effects of sustainable soil management practices on crop loss due to disease by monitoring the soil DNA.

i) Soil Health

Soil has come to the forefront of public and policy agendas in terms of the environment and agriculture. This has been supported by upcoming policy and governmental incentives, as well as public perception. Defra has emphasised the importance of ‘bold new measures to protect and restore soil health’ (Soil Association, 2017) as reflected in its 25-year Environmental Plan (DEFRA, 2018), which aims to improve overall soil health as well as establishing the current state of the UK’s soil. Land and soil degradation affects over 25% of the global land area,

resulting in reduced soil quality through erosion, chemical changes, declining economic productivity, and biological activity (Webb *et al.*, 2014). Impacts of land and soil degradation have been observed in Australia where it has led to reduced cereal grain yields and hidden yield plateaus despite ongoing areal expansion (Webb *et al.*, 2014). There are many factors that are contributing to global soil decline. These include the effects of climate change on soil erosion, evapotranspiration, drought, and changes in biodiversity, increased pests and diseases (Webb *et al.*, 2014), and the use of unsustainable farming practices (Doran, 2002). For instance, soil cultivation has been found to disrupt fungal hyphae, reducing colonization by mycorrhizal and other beneficial fungi (Celestina *et al.*, 2019) and overapplication of crop management products, like pesticides and nitrogen can also lead to soil quality degradation and pose potential environmental threats (Ullah *et al.*, 2019; Turley, *et al*., 2020).

Soil health is crucial for disease control, as soilborne diseases are most damaging under poor soil conditions due to inadequate drainage, poor soil structure, low organic matter, low fertility, and/or high compaction (Abawi and Widmer, 2000). Plant-microbe interactions play a critical role in enhancing soil health (Madigan *et al.*, 2019), they improve nutrient uptake, promote plant growth, resist abiotic stress, and suppress soilborne diseases (Celestina *et al.*, 2019). Therefore, it is believed that increasing soil microbial species richness (the number of different species) leads to better plant health, productivity, diversity, and nutrient acquisition, emphasizing the importance of promoting soil microbial diversity (Chaparro *et al.*, 2012). As part of this body of work, the impact of soil microbial diversity and structure on soilborne disease is explored, to further understand the validity of this belief.

In attempts to improve soil health, studies have assessed the effects of organic amendments like poultry litter and fertilizers on soil microbial communities. For instance, Tian *et al.* (2015) found that manure compost increased microbial activity of bacteria, archaea and ammonia oxidising bacteria over 3 years and Celestina *et al.* (2019) reported that fungal community structure varied with amendment type.

To date, chemical and physical components of soil have been widely used to assess soil condition and fertilizer recommendations (McDaniel, 2017) and although they give a strong indication of soil quality, they do not provide the full picture of soil health. Soil biology has often been underestimated in its importance: soil biota has large effects on crop yield, diseases and nutrition, as biological interactions influence

available water, nutrients and fauna surrounding the underground portions of plants (Brussaard *et al.,* 2007). Therefore, the understanding of soil biology has become paramount for providing a sustainable future for agricultural soils.

Soil decline is a pressing global issue with severe consequences for agriculture and the environment. Understanding the factors contributing to soil degradation and implementing measures to improve soil health, including promoting microbial diversity and sustainable agricultural practices, are crucial steps toward ensuring the long-term viability of agricultural systems and ecosystems. This thesis aims to assess the value of analysing soil DNA as a measure of biological soil health, through the monitoring of microbial communities in response to organic amendments (See chapters 5, 6 & 7).

ii) Plant disease in Agriculture

The intensification and globalization of agriculture have led to crops with narrow genetic bases being grown away from their centre of origin, primarily in monoculture (Strange and Scott, 2005; Thrall et al., 2015). Growing these susceptible crops continuously in monocultures results in plants all in the same physiological and phenological state, increasing their vulnerability to diseases (Savary *et al.*, 2019). Moreover, this practice facilitates the accumulation of plant pathogens (Abawi and Widmer, 2000). The increasing trade and transport of agricultural products have further accelerated the global spread of pathogens (Bebber and Gurr, 2015).

Pathogen control presents a significant challenge due to their high populations and short generation times (Fisher *et al.*, 2012). Additionally, pathogens are rapidly developing resistance to conventional control methods, often outpacing the development of viable alternatives, such as the plant pathogen *Phytophthora infestans*, which causes late blight in potatoes and tomatoes, has developed resistance to several fungicides, including metalaxyl. The process of developing new controls for pathogens is time-consuming and subject to strict legislation (NFU, 2012; Newbery *et al.,* 2016; Kettles and Luna, 2019). The ability of pathogens to undergo genetic recombination, hybridization, and horizontal gene transfer adds to their capacity to generate resistant pathotypes (Fisher *et al.*, 2012). Coupled with high inputs of fertilizers and agrochemicals, these factors contribute to increased plant disease epidemics and fast pathogen evolution (Thrall *et al.*, 2015). Irrigation

practices, while beneficial for crop growth, have also been shown to aid the development and spread of soil-borne diseases, including *Verticillium* (Jefferson and Gossen, 2007).

Fungal Pathogens

Understanding the behaviour of fungal pathogens is key in understanding their significant risk to global food security. Fungal pathogens are widely dispersed worldwide and have emerged as major threats to agriculture and crop production (Gurr *et al.,* 2011). Fungi are responsible for causing many soil-borne crop diseases, including wilts and rots, leading to considerable challenges in developing effective control methods (Brussaard *et al.,* 2007). Fungal pathogens display a high degree of adaptability to their environment, enabling rapid colonization of new areas once introduced (Kettles and Luna, 2019) and often remain in the soil for decades (Metcalf *et al.,* 2007; Clarkson *et al.,* 2016). They spread through various means of transport, such as rain-splash, wind, and soilborne dissemination (Strange and Scott, 2005). Studies spanning 50 years have indicated that the number of diseases caused by fungi and oomycetes has increased over time and that these pathogens are moving polewards at a rate of approximately 8 kilometres per annum, posing new challenges in the northern hemisphere (Bebber *et al.,* 2014).

Overcoming plant resistance mechanisms is one of the key traits of fungal pathogens, facilitated by genetic changes and short generation times (Strange and Scott, 2005; Thrall *et al.*, 2015). Their multi-host nature and varied reproductive stages make it difficult to target specific control mechanisms due to their inherent variability (Kettles and Luna, 2019). Once established in a location, these pathogens can quickly dominate the microbiome. They produce substantial amounts of inoculum through sporulation and maintain large population sizes (Strange and Scott, 2005; Kettles and Luna, 2019). Moreover, they can remain dormant for extended periods by forming resting structures like sclerotia, capable of surviving up to 20 years in soils (Paplomatas *et al.*, 1992; Thrall *et al.*, 2015).

The implications of fungal pathogens extend beyond damaging crops, as some produce toxic compounds like mycotoxins, which pose risks to both crop quality and human health (Savary *et al.*, 2012). The FAO estimates that 25% of global agricultural commodities are contaminated with mycotoxins, these are often in field

pathogens that are transported into storage and stay with the edible portions of the crop (Sarmast *et al.*, 2021).

A comprehensive model analysing data over 50 years revealed an increase in diseases caused by fungal and oomycete pathogens in many countries (Bebber and Gurr, 2015). Crop production and diversity are reliable predictors of pathogen threats, and it is noteworthy that islands, like the UK, harbour a greater number of pathogens compared to land-locked or coastal countries (Bebber *et al.,* 2014).

Disease Management

At present, the primary control method for pests and pathogens in agriculture is the application of synthetic pesticides. Over the last four decades, there has been a 15-20-fold increase in pesticide use to support food production (Chakraborty and Newton, 2011) and in the last decade alone an increase in 11% pesticide use (FAO, 2021). However, despite this increase, the proportion of crop losses due to disease has risen, necessitating further exploration of alternative strategies. The global market for pesticides amounts to around $40 billion annually, making it a valuable sector in the agricultural industry (Popp *et al.,* 2013). Nonetheless, concerns regarding environmental and safety issues have led to the withdrawal of many pesticides from the market. Legislation may lead to the removal of up to a quarter of active ingredients in the UK, and potentially up to 50% if the precautionary principle is applied (NFU, 2012). The narrowing of available active substances may exacerbate the development of pesticide resistance among pathogens, as the same actives are used repeatedly, further complicating disease control and potentially costing the UK farming industry £1.73 billion in profits (NFU, 2012).

Organic amendments, such as cover crops, manures, and compost, are also demonstrating potential in reducing disease incidence and improving soil health. Crop rotations and mixed cropping systems have been effective in suppressing diseases by reducing pathogen populations and promoting beneficial microbial activity (Abawi and Widmer, 2000; Ampt *et al.*, 2018). Higher plant diversities have been associated with reduced foliar disease severity and incidence (Rottstock *et al.*, 2014). Additionally, the time of planting crops can significantly influence disease incidence, as observed in the case of onion seed planting in Tasmania when they were testing the control ability of biocontrols. They found that (Metcalf *et al.,* 2007).

While the use of disease-resistant crop varieties has proven effective, their development through classical breeding and advanced techniques like GM and CRISPR-Cas9 is a time-consuming process (Kettles and Luna, 2019). The EU's legislation against GM technologies, including CRISPR, has created challenges in the regulation and acceptance of genetically modified crops [Court of justice of the European union, 2018] (Kettles and Luna, 2019)

In response to these challenges, the search for alternative disease control methods has gained momentum. Emphasis is being placed on bio-alternatives, such as the fungal biocontrol agent *Clonostachys rosea* (formerly *Gliocladium catenulatum*), which offer potential disease control without causing environmental harm. Biocontrols in 2010 constituted approximately 2% of the global market (Moser *et al.*, 2008; Bailey *et al.,* 2010) and was estimated to be worth $1.8B in 2021 with growth to $2.3B in 2027. However, their uptake is hindered by scaling issues, perceived lower efficacy, limited promotion, lack of residual activity and infancy in research and development (Moser *et al.*, 2008). Nonetheless, biocontrols may present lower risks of resistance due to their multi-mode of action and incorporation into Integrated Pest Management (IPM) schemes (Bailey *et al.,* 2010). Studies have assessed growers' attitudes towards biological control agents, revealing a generally positive perception of their efficacy and use (Moser *et al.*, 2008). Media coverage, positive characteristics of biological control agents, and personal experience influenced growers' confidence in adopting these strategies (Moser *et al.*, 2008). Biological control methods for disease management have shown promising results. Such as *Trichoderma koningii* producing chitinolytic enzymes that dissolve sclerotia and hyphae, effectively suppressing onion white rot (Metcalf *et al.,* 2007). Crop rotation and mechanical tillage practices have also been linked to disease reduction in beans by influencing soil properties and promoting beneficial microorganisms (Abawi and Widmer, 2000). Mixed cropping systems with higher plant diversity create diverse niches for soil microbes and increase soil suppressiveness against pathogens (Ampt *et al.*, 2018). Lack of experience, belief in the superiority of chemical pesticides, and limited promotion by research centres and companies have hindered widespread biological control agent adoption (Rahman *et al.*, 2021). Therefore, further studies, as performed in this thesis (Chapters 5, 6 and 7), are valuable in demonstrating the capabilities and establishing the expectations/limitations of biological control approaches.

In conclusion, understanding the impact of fungal pathogens on global food security is of paramount importance. The globalization and intensification of agriculture have posed significant challenges to disease control in crop production. Developing effective control measures and implementing strategies to mitigate their adverse effects on crop production and human well-being is critical for ensuring a sustainable and secure food supply in the face of these formidable challenges. This may come from a balanced approach integrating biocontrols, organic amendments, disease-resistant crop varieties, and improved cultural practices. The utilization of biological control agents and other biological strategies have the potential to enhance crop productivity and reduce disease while minimizing the environmental impact associated with conventional practices. By prioritizing research and development in alternative strategies, the agricultural industry can mitigate the impact of disease and safeguard global food security, human health, and the environment. Improved disease surveillance is also essential for proactive disease control (Bebber and Gurr, 2015), and guiding research efforts into these alternative strategies. However, for these changes to occur, there is a critical need for additional research to enhance our comprehension of pathogen and biocontrol agent survival in soil, particularly concerning disease occurrence and the potential influence of soil management practices, notably the utilization of organic soil amendments.

iii) Approaches to monitoring soil microbiology

Understanding the complex dynamics of soil microbiology has become of high interest, this led to a wide array of molecular methods available for soil microbial monitoring leaving researchers with a multitude of options, each with its own set of advantages and limitations. These methods are significantly influenced by diverse variables, including soil type, environmental conditions, and sample preparation techniques. In this project, my objective is to monitor shifts in soil microbial communities and to quantify specific organisms of interest, such as pathogens and beneficial organisms. To achieve this goal, a combination of quantitative polymerase chain reaction (qPCR) and metabarcoding techniques were utilised.

Soil Sampling and Storage

Strategies for collecting and storing soil samples can play a crucial role in obtaining accurate microbial data from various analyses. Various strategies have been applied

to soil collection, from random sampling, blocking and transects (Parker *et al.,*1997; Degens and Vojvodic-Vukovic, 1999; Webster and Lark, 2018), each with their own internal variations (size, frequency and sub-sampling). Random sampling is often favoured by famers and agronomists due to its ease to perform; it has been suggested that this method is beneficial when dealing with random disease patterns, but accuracy is dependent on the number of sampling units. (Parker *et al.,* 1997). Blocking, whereby the area of interest is divided into homogenous sections, has been suggested to increase precision (Webster and Lark, 2018), however it is often quite labour intensive and not feasible for some studies as the more samples taken the more expensive the analysis becomes (Pennock *et al.,*2006). Transects are commonly used in both natural and agricultural studies; this method has proved adequate for comparing broad land use effects on biological functional diversity (Degens and Vojvodic-Vukovic, 1999). However, representative soil samples are now often collected in a zig zag or 'W' pattern for field sampling, instead of the traditional straight line transects (Pennock *et al.,* 2006). This 'W' or zig zag pattern aids in creating a picture of pathogen dissemination. It is important to select an appropriate sampling method for specific disease patterns and spatiotemporal variation, to ultimately satisfy the experimental goals. For example, It has been reported that some crop pathogens concentrate around production zones (i.e. rows) where previous susceptible crops were grown (Abawi and Widmer, 2000; McKay *et al.*, 2009). It is now general practice that a sample will be made up of a composite mixture of smaller sub-samples in soil field studies (Pennock *et al.,* 2006). It Is key that these composite samples are thoroughly mixed to ensure bias is not introduced and the microbial community is evenly distributed. An additional challenge occurs as soil is typically moist making thorough amalgamation difficult (Schulze *et al.*, 2016).

After soil sampling, there is often a delay before extraction due to laboratory capabilities and travel, leading to a requirement for storage prior to analysis. In 2007, Lee *et al.* compared different storage methods for soil samples: field moist (no storage), air drying for 2 weeks followed by rewetting, field moist for 4 weeks, and freezing at -20°C and -80°C for 4 weeks. The drying and rewetting method had the most significant impact on microbial activity, affecting biomass, respiration, enzyme activity, Fatty acid methyl ester analysis (FAME), and total DNA analyses compared to field moist (no storage) samples. Freezing at -80°C was best for preserving total DNA, but community structure may be altered. Then in 2008, Clark and Hirsch,

studied soil storage from soil stored in 1843 to 2003. They found microbiological analyses could be done on dry soils, but drying caused osmotic stress affecting microbiological viability. Freezing at -80°C also induced osmotic stress, but it could be used as a mechanical lysis method to expose DNA for analysis. Černohlávková *et al.* (2009) also expressed concerns around freezing and osmotic stress in their study, where they compared storage at 4°C, -20°C and air dried at 2,4,8,16 and 32 weeks. The different soil types reacted differently to the effect of storage conditions on biological analysis, highlighting an effect of soil type on DNA extraction. Additionally, they found that soil microbial activity was not significantly impacted during the first 4 weeks of storage. Bainard *et al.,* (2010) explored additional storage options and found storage at -80°C, -20°C, under ethanol, or after silica gel drying had minimal impact on DNA concentrations. Freeze drying caused significant DNA loss, while heated drying affected fungal arbuscular mycorrhizal DNA concentrations and introduced organism bias. Wallenius *et al.* (2010) concluded that freezing was the best option, limiting changes in microbial activity. Soil characteristics again influenced the effects of storage. Peoples and Koide (2012) found freezing at -20°C better than drying, and (Lee *et al.*, 2007) supported freezing's favourable effect on preserving microbial community composition over drying and rewetting methodologies. Castaño *et al.* (2016) recommended freeze-drying, though drying methods had no effect on fungal community composition in their studies. (Weißbecker *et al.,* 2017) found significant DNA yield decrease with prolonged storage, supporting the idea that extraction should be done as soon as possible after sampling. For the purposes of the current work, it was concluded that analysis prior to storage would be most reliable, but when unavoidable, freezing at -80°C is preferable (Lee *et al.*, 2007; Bainard *et al.,* 2010), although short-term refrigeration can also prevent DNA damage from freezing.

Effective soil sampling and storage techniques are crucial for obtaining reliable microbial data. The transect sampling strategy, combined with cold or frozen storage, is recommended for accurate microbial analysis, and was utilised in this thesis. Researchers must select appropriate sampling designs to ensure representative and precise results with considerations such as crop husbandry and downstream processing feasibility. It is essential to choose suitable storage methods based on the objectives and timeframes of the study.

DNA Extraction

DNA extraction is a critical step in molecular environmental microbial studies, as it directly impacts the quality and quantity of DNA obtained from environmental samples, affecting downstream processes. Two main approaches for DNA extraction exist: direct extraction and indirect extraction. It is debatable which method is better. Direct DNA extraction involves lysing cells within the environmental sample matrix (Williamson *et al.*, 2011) (eg. soil or water) and avoids the need for cell separation before DNA extraction offering advantages such as higher DNA yield, reduced processing time, and increased representation of the microbial community (Miller *et al.*, 1999; Tien *et al.,* 1999; Carrigg *et al.*, 2007). This method can still introduce biases such as co-precipitation of humic substances (Miller *et al.*, 1999), binding of DNA to clay and organic matter leading to DNA loss and fragmentation (Anderson, 2016). In contrast, indirect DNA extraction methods initially separate prokaryotic cells from the soil matrix before lysing them outside the sample (Williamson *et al.*, 2011). However, indirect methods may suffer from lower DNA yields and may not represent the full microbial diversity present in the sample (LaMontagne *et al.*, 2002). The choice of DNA extraction method should be based on the specific research objectives and sample characteristics (Carrigg *et al.*, 2007). For the research described in this thesis, direct DNA extraction was preferred, but even within this method, many variables can impact downstream processes and the analytical results, these are further discussed in depth in Chapter 2.

In addition to the initial DNA extraction method, additional DNA purification techniques are often required to remove contaminants, especially humic substances, which can inhibit downstream applications such as PCR. Commonly used purification methods include the use of polyvinylpolypyrrolidone (PVPP), sephadex columns, gel filtration resins, caesium chloride density gradient ultracentrifugation, and activated charcoal to bind/remove unwanted inhibitory compounds and DNA-degrading enzymes (Tien *et al.,* 1999). However, additional purification steps risk introducing further bias such as loss of DNA. Various potential purification steps are further investigated within this thesis and are described in Chapter 4.

Molecular analyses of extracted DNA

Molecular analyses and genomics have minimized errors regarding the identification of microbial organisms. Technological advances in sequencing and ‘omic’

technologies have allowed for the analyses of plants and pathogens at the genome-wide scale (Kettles and Luna, 2019). The genome varies across kingdoms, genus, species, and individuals. This variation can be utilized to identify organisms at each level of classification. These differences can range from insertions, deletions and copy number, down to single nucleotide changes (De La Vega *et al.*, 2005). Such differences can be exploited in the development of molecular assays that target specific regions of a genome and can therefore be used to identify and quantify specific organisms or taxonomic groups of organisms.

Quantification of organisms from extracted DNA

For quantification of organism-specific DNA, qPCR is currently the method of choice. Although qPCR can be costly, due to extraction costs and reagents, it is relatively quick and permits multiple samples to be analysed simultaneously in the laboratory (Strange and Scott, 2005). There are now many specific qPCR assays available for accurate detection, identification, and quantification of individual fungal pathogens and biocontrol agents. Such assays need to be fully validated to make sure they do not recognize similar target DNA sequences from other organisms (Broeders *et al.*, 2014). Highly sensitive qPCR assays allow detection of small amounts of target organisms, as little as one gene copy per microliter (Saleh-Lakha et al., 2005), leading to early diagnosis and fast implementation of control methods (Atkins and Clark, 2004). qPCR has been successfully used by Dangi *et al.* (2017) and Zhu *et al.* (2014) to establish Inoculum Density-Disease Incidence (ID-DI) relationships, as well as to set thresholds for infection risk. Previously, inoculum density was quantified using methods such as wet sieving and the number of spores counted; this was a laborious task that would take a long time and errors could be made.

qPCR also offers insight into the whole microbial community through the use of genes such as 18S and 16S rDNA. These allow the quantification of all bacterial or fungal DNA, to establish ratios of communities and quantification of abundances (the relative number of individuals of each species) when combined with sequencing data. Some regions of the 18S RNA gene sequences share high similarity with other Eukaryotes (Anderson *et al.,* 2003), potentially leading to misidentification; however, this region was utilized in the research conducted within this thesis (Chapters 4 & 7) to evaluate this approach for the quantification of whole fungal communities within soil samples.

Molecular assays for qPCR

The assays used in this project are based around TaqMan technologies. They, like many PCR assays, consist of two primers (synthetic oligonucleotides) that anneal to the targeted DNA region on opposite strands of the template DNA, defining the region to be amplified and functioning as the attachment point for the DNA polymerase (Marmiroli and Maestri, 2007). Set inside these two primers is a probe. Probes are synthetic oligonucleotides with fluorescent dyes attached, they are designed to have a higher annealing temperature than the primers so it will be hybridized when cleaving begins (Butler, 2012). TaqMan probes have a fluorescent reporter dye at the 5' end and a quencher at 3' end (Atkins and Clark, 2004; Butler, 2012). When in close proximity, the quencher limits the fluorescence from the reporter dye, via the forster-type energy transfer, the closer the two dyes the stronger the effect of the quencher (De La Vega *et al.,* 2005; Marmiroli and Maestri, 2007; Butler, 2012). By limiting the fluorescence of the reporter dye background is minimised, increasing the signal to noise ratio (Marmiroli and Maestri, 2007). As the DNA polymerase cleaves the targeted region, the two dyes are separated and a fluorescent signal is generated (Atkins and Clark, 2004; De La Vega *et al.,* 2005), in each cycle more and more probes are cleaved, exponentially increasing the fluorescent signal (De La Vega *et al*., 2005). One of the most popular combinations of fluorophores and quenchers is FAM-TAMRA, which is a combination used regularly in this project. These work well in combination due to spectral overlap between the FAM fluorescence and TAMRA absorption curves.

Alternative options include non-fluorescent quenchers paired with minor groove binders (NFQ MGB) (Marmiroli and Maestri, 2007). MGB enhances probe stability by binding within the minor groove of dsDNA, while NFQ serves as a chromophore rather than a fluorophore like TAMRA (De La Vega *et al*., 2005; Marmiroli and Maestri, 2007). MGBs are often preferred when dealing with shorter-than-usual probes or unexpectedly high annealing temperatures (Butler, 2012), as seen in Chapters 4, 5, 6 & 7 of this thesis.

Alternatively, SYBR green serves as an alternative to TaqMan. Unlike TaqMan's dual-labelled fluorescent assay, SYBR green employs intercalating dyes (Butler, 2012). While TaqMan relies on fluorescence changes from specific sequence cleavage, SYBR green detects the presence of any PCR product (Butler, 2012).

SYBR green offers cost-effectiveness and simplicity, albeit at the expense of reduced specificity (Ponchel *et al.*, 2003).

Other probe options include molecular beacons, which self-anneal into stem-and-loop structures carrying fluorescent dyes and quenchers with the complementary sequence in the loop. Scorpion probes similarly utilize a stem-and-loop configuration (Marmiroli and Maestri, 2007).

Key advantages of TaqMan are its single enzymatic step, that assays are universal in their reactions and thermal cycling conditions, primers can be flexible around the target, they can be easily automated via the use of robots and there is no post-PCR processing (De La Vega *et al*., 2005). Because of this and the availability of published TaqMan assays, this technology was used throughout the research described in this thesis.

iv) Thesis Summary

This thesis assesses the value of using molecular techniques to quantify and characterise the DNA of soil-borne organisms, as a measure of their impact on biological soil and plant health.

To facilitate this, an in-depth assessment of methods for the extraction of microbial DNA from soils was conducted as a systematic review (Chapter 2) which aimed to investigate and select the most appropriate methodology for use in the following investigations.

Building on this review of DNA extraction methods, some additional experiments were conducted (Chapter 4) in order to test and refine the selected DNA extraction methodology for use in qPCR analysis of soils for quantification of targeted pathogens and biocontrol agents, quantification of whole bacterial and fungal communities and ultimately for characterisation of these communities using high throughput metabarcode sequencing.

The DNA extraction and qPCR methodologies were evaluated under controlled glasshouse conditions (Chapter 5) before being used in a series of field trials established to investigate the effects of soil amendments and biocontrol agents of soil borne diseases of different crops. For the initial glasshouse experiments,

strawberry plantlets were grown in composts inoculated with varying populations of *Verticillium dahliae*, some of which were also pre-treated to varying extents with either the biological control agent *C. rosea* or by amendment with anaerobic digestate. Detection and quantification of both organisms could then be assessed and related to eventual disease development. It was hypothesised that successful detection and quantification of the organisms in the compost would vary with the initial inoculum levels and could be correlated with the incidence or severity of disease observed over time. It was also hypothesised there would be a negative correlation between the populations detected of the pathogen and the biocontrol agent, indicating an effective biological control strategy. A final hypothesis was also tested that organic amendment of the compost with anaerobic digestate would affect populations of pathogen and biocontrol agent detected.

Further evaluation of these methodologies was then performed under field conditions by sampling a series of field trials under different cropping systems and attempting to quantify additional relevant soilborne pathogens (Chapter 6). These included *Fusarium oxysporum, Stromatinia cepivora, Stemphylium vesicarium* and *Verticillium dahliae*, in asparagus, daffodil, onion and raspberry. Under field conditions, the robustness of the methodology was further evaluated taking into consideration additional variables such as presence of PCR-inhibitory substances in different soils, variation amongst natural soil microbiomes, differences in soil management/farming husbandry and environmental differences between trial sites. In addition to quantifying the populations of soilborne pathogens in field soils, the effect of cover crops, organic amendments and applications of arbuscular mycorrhiza and biocontrol agents could be assessed across different trials to determine their influence on soilborne pathogen populations and any resulting crop diseases. It was hypothesised that such treatments have potential to influence soil microbiomes, in turn impacting soilborne pathogen populations and their ability to infect the crop and/or cause disease. For example, it was theorised that if organic amendments improved biological soil health through increasing organic matter and ultimately microbial diversity, then the soil environment may become suppressive to pathogens due to increased competition or antagonism, offering a natural resistance to infection. This was assessed by quantifying any changes in pathogen populations at the beginning of the season (pre-planting) and at the end of the season (pre-harvest) and relating these to the observed disease development in the crops.

To further investigate any major effects of these soil management treatments on whole soil microbial communities, a comparison of qPCR and metabarcoding approaches aimed to investigate changes in whole bacterial and fungal communities in response to soil management treatments (Chapter 7). It was theorised that soils with a higher microbial diversity may lead to a higher resistance to disease through a variety of mechanisms such as competition with soilborne pathogens and healthier root development. Furthermore, it was hypothesised that altering the soil microbiome over a season would in turn impact the development of soilborne crop diseases.

Overall, this thesis aims to assess the current state of molecular techniques and their applicability to soil microbial communities. There have been calls for these molecular techniques to advise farmers especially in light of recent efforts to protect and improve soil health. Therefore, tools such as these might help achieve these goals.

Aims and Objectives

- Validate Molecular Analysis Methods: Validate the use of qPCR approaches for comprehensive analysis of soil microbial communities, including bacterial and fungal populations.
- Investigate Soil Management Practices: Investigate the effects of various soil management practices, such as cover crops, manures and biocontrol agents, on soil microbial communities and soilborne pathogen populations.
- Assess Soil Health Indicators: Evaluate soil health indicators derived from molecular analyses, such as microbial diversity and pathogen abundance, as predictors of soilborne disease risk and overall soil health status.
- Support Soil Health Initiatives: Support ongoing efforts to protect and improve soil health by providing scientific evidence and tools, such as molecular techniques, that can aid in monitoring and managing soil microbial communities

Chapter 2 – Review of methods for extraction of DNA from soils

i) Introduction

DNA extraction/purification is a crucial step in the successful application of molecular diagnostics and is utilised to offer insights in ancestry to crime. The prominence of DNA analysis has led to the development of a wide range of extraction methods, evident in the 3306 papers already dedicated to 'DNA extraction' before May 2021. Research to date has highlighted the significance and challenges associated with the influence of extraction method on the quantity and quality of DNA retrieved from a wide range of substrates. Due to the diversity of commercial kits and laboratory-based protocols, it was necessary to include a systematic review, focusing on methods for DNA extraction on soil, in order to select appropriate procedures for use in the experimental studies on soil DNA quantification and diversity to be undertaken subsequently as part of this thesis.

Soil properties bias

Soils vary greatly across all scales, from the global scale to variation within a single field. Each soil has its own characteristics made up of multiple elements, such as pH, type/ structure, organic matter content and composition, all influenced by its history and current use. Soil types are mainly determined by sand, silt, clay and organic matter content and through the organisation of micro and macro-aggregates (Robe *et al.*, 2003). All of these elements have been found to affect the quality, yield and efficiency of DNA extracted using different extraction methods (Hu *et al.*, 2010; Dequiedt *et al.*, 2012; Young *et al.*, 2014; Schulze *et al.*, 2016).

Salts

Xie *et al*. (2018) identified soil types as the most important factor in their study assessing microbial diversity and community abundances in saline soils using different extraction methods. They tested 6 different methods which included; the method developed by Zhou *et al*. (1996) with varied lysis methods, the ISO standard method 11063 and the Powersoil™ DNA isolation kit from MoBio, on saline and non-saline soils. Individual samples could be distinguished using principal component analysis but could not be separated by method, showing that the soil characteristics, namely saline content, influenced the resulting community composition rather more

so than did the choice of extraction method. Salts are known to interact with DNA and therefore may impact on the efficiency of DNA extraction. Salts are regularly used to precipitate proteins and polysaccharides out of solution (Roose-Amsaleg *et al.*, 2001; Demeke and Jenkins, 2010), therefore salts from the environment may be performing similar roles potentially introducing a bias during DNA extraction. Vandeventer *et al.* (2012) tested different buffers with different pH, ionic compounds and salts for their ability to bind DNA, they found that more DNA was adsorbed to a silica membrane with buffers containing chaotropic salt; sodium perchlorate. This further indicated that soils with higher salts may introduce bias within downstream DNA analyses. They attributed the ionic environment, from charged ions including sodium, to poor binding of DNA to silica DNA-purification columns. Currently there has been no study considering the potential effects of salts in environmental samples on DNA extraction.

Aggregate size and porosity

Other soil characteristics that may impact DNA extraction are aggregate size and porosity. About 80% of microorganisms are found within micro-aggregates and micropores in the soil. Fungi tend to be in macropores between microaggregates, while bacteria prefer microporous areas (Robe *et al.*, 2003). Fungal structures are closely linked with soil particles (Damm and Fourie, 2005). Some studies sieve their soils prior to extraction in an attempt to break up aggregates, whereas others try to incorporate this disruption as part of the DNA extraction protocol. Ranjard *et al*. (1998) separated soil samples into aggregate size (<2um, 2-20 μm and 20-50μm) and extracted 1g of each aggregate size grouping and then quantified 16S and 18S DNA using agarose gels. They found significant differences between all the various soil microenvironments, for example DNA yields ranged from 0.7-51μg per gram of soil, dependant on aggregate size. Lysis efficiency also varied across aggregate size, ranging from 60% to 84% (20-50μm former and <2μm latter). Therefore, disruption methods need to be successful across all aggregate sizes to avoid risk of introducing bias to the quantification of those organisms that are most easily accessible.

Organic Matter

Adsorption studies have indicated that free DNA binds more easily to inorganic than organic components in soils (Young *et al.*, 2014). Consequently, soils with higher inorganic content exhibit greater DNA adsorption, introducing another potential

source of bias. However, this does not imply that organic soils are more suitable for DNA extractions, as higher organic matter often contains substances like humic acids that are inhibitory to downstream PCR analysis (LaMontagne *et al.*, 2002). It does however suggest that DNA released from microbial cells may be adsorbed onto the inorganic components of the soil rather than being released into solution affecting extraction/purification and creating biases during analysis.

Organic matter content and composition is regularly cited as a consideration in soil DNA extraction methods (Robe *et al.*, 2003; Lakay *et al.,* 2007; Sagova-Mareckova *et al.*, 2008). Van Elsas *et al*. (1997) investigated different DNA extraction and purification methods on a range of soils. They found that soils with higher organic matter (30% w/w) required multiple purification steps to generate PCR-amplifiable DNA, and they hypothesised that this was due to the release of PCR-inhibiting substances from the organic matter. Kuske *et al*. (1998) similarly tested soils with a range of organic matter levels. DNA yields ranged from 0.18- 21.3 µg/g despite being inoculated with the same sample size of *Pseudomonas putida,* indicating that the soil organic matter content was affecting extraction/detection of DNA. However, unlike the study by Van Elsas *et al*. (1997) which proposed that inhibitory substances were limiting the detection of DNA, Kuske *et al*. (1998) quantified the levels of humic substances released by each soil using UV spectroscopy. This ranged from 49-2200 µg/g but this was not related to organic matter levels in the soil. Knauth *et al*. (2012) proposed a correlation between organic matter and total DNA yield, they consistently got higher DNA yields from the higher organic matter soil across the DNA extraction methods used, the high organic matter soil also generated marginally higher 260:230 ratios than the other soils, indicating higher inhibitory substance release. However, the organic matter range was limited, as it only ranged from 1.0-1.5%. This relationship between organic matter and DNA yield has been indicated by other studies, Frostegård *et al*. (1999), Miller *et al*. (1999), Zhou *et al*. (1996) and Dequiedt *et al*. (2012) which also found that higher DNA yields were achieved from higher organic matter soils in the soils tested. Tien *et al*. (1999) reported that the same soil treated with organic fertiliser compared to the soil being treated with chemical fertiliser yielded more DNA, potentially due to the increase in organic matter from the organic fertiliser and the effect of this organic matter on the soil microbial communities. The relationship between total extracted DNA yield and organic matter is unsurprising, given that organic matter contains microbial organisms and plant

materials. Consequently, it is challenging to discern whether the DNA extraction method improved the release of DNA or if there was simply a greater quantity of DNA readily available.

Soil pH

Soil pH can also impact the efficiency of DNA recovery (Robe *et al.*, 2003; Lakay *et al.,* 2007; Hu *et al.*, 2010; Dequiedt *et al.*, 2012). Lower pH levels, typically below 5.5, are associated with heightened adsorption capacity for free DNA within the soil matrix, whereas higher pH levels exhibit reduced adsorption capabilities (Guerra *et al*., 2020). Sagova-mareckova *et al*. (2008) study highlighted a correlation between DNA purity, PCR performance and soil pH, postulating that lower pH levels may trigger the release of humic acids, thereby impairing PCR performance. A study by Hebda & Foran (2015) investigated DNA extraction techniques applicable to skeletal remains within soil. They linked decreased DNA recovery to shifts in soil pH, which impacted the DNA binding capacity of silica (Vandeventer *et al.*, 2012). This highlights the significance of pH as a pivotal factor to consider when optimising DNA extraction protocols.

Organism bias

As well as soil characteristics affecting DNA extraction, the characteristics of the target organisms can affect DNA extraction success. Bacterial and fungal organisms can have multiple forms as part of their life cycles, for instance, fungi often have hardy resting structures which are difficult to extract DNA from (Paplomatas *et al.*, 1992; Damm and Fourie, 2005; Thrall *et al.*, 2015; Habib *et al.*, 2017). Bacteria offer their own set of challenges, gram negative bacteria often rupture more efficiently than gram positive bacteria, in response to physical disruption, this being due to the latter's thick peptidoglycan cell wall layer (Robe *et al.*, 2003; Bakken and Frostegård, 2006). Bakken and Frostegård (2006) also found the shape and size of bacteria effects their rupturing efficiencies, for instance rods are more readily ruptured than cocci and larger cells more so than small ones. This was corroborated by the study by Berthelet *et al.,* (1996) which found that their lysis method, involving SDS and bead mill homogenisation, resulted in smaller cells being left intact. More *et al.* (1994) also showed that small bacterial cells (0.3-1.2 µm) were more difficult to lyse than larger bacteria (2-10 µm).

Kuske *et al*. (1998) compared three lysis methods (freeze thaw, hot detergent and bead beating) in different combinations at different temperatures and times, on spore suspensions and soil samples. They found that DNA from their target organism, the Gram-negative rod-shaped bacterium *Pseudomonas putida,* could be released by the majority of methods, as discussed previously (Robe *et al*., 2003; Bakken and Frostegård, 2006). However, hot detergent and freeze thaw were unsuccessful at releasing DNA from *Bacillus atrophaeus* (previously *B. globigii*) and were only moderately successful at rupturing *Fusarium moniliforme*. The difficulties surrounding *Bacillus sp.* were also experienced in the study by More *et al.* (1994) which found 94% of *Bacillus sp.* survived freezing, meaning freeze thawing was not an acceptable method for this organism. Similarly, Tien *et al.,* (1999) tested methods for their ability to extract fungal DNA from soils, they were unable to detect *Pythium aphanidermatum* and *Fusarium solani* using any of the 7 methods selected. This may be resolved by tailoring the lysis method to a specific target organism, but this is not possible when performing community studies without introducing bias.

Method bias

Risks of mechanical disruption

Efforts to effectively lyse challenging organisms often involve employing harsher lysis methods, which come with the inherent risk of DNA shearing (Robe *et al.*, 2003; Bakken and Frostegård, 2006; Sagar *et al.*, 2014). DNA shearing can result in the formation of chimeric products, alteration of DNA fragment size, and hinder PCR primer binding (Kuske *et al.*, 1998; Sagar *et al.*, 2014). The impact of DNA shearing can be limited by the downstream analysis method, for instance qPCR amplifies shorter specific regions of DNA-and is therefore less impacted by shearing due to the lower likelihood of the shearing occuring in this region. Williamson *et al.* (2011) hypothesized that this bias could explain the underrepresentation of *Proteobacteria* in their community study, given that proteobacteria consist primarily of gram-negative cells.

Among mechanical lysis methods, bead beating is one of the most commonly employed techniques, but has been frequently associated with DNA shearing across multiple studies (van Veen *et al.,* 1997; Wintzingerode *et al.,* 1997; Miller *et al.*, 1999; Roose-Amsaleg *et al.,* 2001; Delgado-baquerizo *et al.*, 2020). Carrigg *et al.* (2007) observed significantly higher shearing with the Mobio Ultraclean Soil DNA Kit

compared to other methods, which consistently produced DNA fragments of at least 23 kb. In a comparative analysis, Knauth *et al.,* (2012) found that the FastDNA spin kit for soil, utilizing a mixture of ceramic and silica particles, resulted in minimal shearing, evidenced by sharper gel bands during electrophoretic separation of the DNA. Feinstein *et al.* (2009) explored various lysis methods in combination with the Powersoil DNA isolation kit, demonstrating only slight increases in shearing with more vigorous methods like grinding and bead-beating, with all fragments ranging from 5 to 50 kb. Bakken and Frostegård (2006) and Bürgmann *et al.* (2001) noted that extending the duration of bead beating can enhance lysis efficiency but at the expense of increased DNA shearing. Bakken and Frostegård (2006) further suggested that while DNA shearing might be negligible within intact cells, it becomes significant once DNA is freed into the solution, particularly with prolonged bead beating durations. Sonication, another commonly utilized lysis method, has also been implicated in causing DNA shearing (Wintzingerode *et al.,* 1997). During sonication, samples are subjected to high-frequency sound waves to disrupt cells and release DNA. Desai and Madamwar (2006) compared sonication with other lysis methods such as freezing, bead beating, and ultrasonic processing, confirming its association with DNA shearing. Overall, it is widely acknowledged that achieving an entirely unbiased DNA extract from complex communities without some degree of shearing remains challenging across all methods (Miller *et al.*, 1999; Bakken and Frostegård, 2006).

Release of PCR inhibitory substances

In addition to the concern of DNA shearing, another challenge arises from the release of PCR-inhibitory substances during the extraction process (Frostegård *et al.*, 1999). These substances include proteins, polysaccharides, non-target nucleic acids, polyphenols, heavy metals, soil and cell debris, as well as fulvic and humic acids (Saleh-Lakha *et al.*, 2005; Bakken and Frostegård, 2006; Gibson *et al.*, 2012; Wang *et al.,* 2012; Hargreaves *et al.,* 2013; Sharma *et al.,* 2013; Sagar *et al.*, 2014; Hebda and Foran, 2015) each present in varying concentrations depending on soil type and environmental conditions (Kuske *et al.*, 1998; Deora *et al.*, 2015). Hebda and Foran (2015) delved into the impact of various inhibitors—such as collagen, calcium, and humic acids—on the extraction of bovine bone DNA from soils. They conducted experiments by introducing known amounts of each inhibitor into soil samples. Their findings suggest that DNA extraction kits tailored for soil applications are better

equipped to mitigate the inhibitory effects of humic acids compared to kits not specifically designed for soil samples. This underscores the prominent role of humic acids as a primary concern regarding PCR inhibition in soil DNA extracts.

The action of the humic acids interferes with PCR reactions (Berthelet *et al.,* 1996; Saleh-Lakha *et al.*, 2005; Carrigg *et al.*, 2007; Butler, 2012; Gibson *et al.*, 2012; Aggarwal *et al.*, 2014; Deora *et al.*, 2015), by either outcompeting the DNA for enzyme binding sites or by interfering with the action of the polymerase molecule (Hu *et al.*, 2010; Gibson *et al.*, 2012). Since humic acids resemble nucleic acids, they can compete for active sites of enzymes during PCR reactions (Lakay *et al.,* 2007). The phenolic groups in humic acids bond to amides, denaturing biological molecules (such as polymerases) or oxidise to form quinone which covalently bonds to nucleic acids (Robe *et al*., 2003). This means that there are less available binding sites for the target DNA to bind to active polymerase molecules to perform the PCR reaction.

As PCR inhibitors are a major limitation of molecular studies involving soils, there have been a multitude of studies investigating their occurrence and removal. As little as 10 ng/µl of humic acid can inhibit a PCR reaction (Tsai and Olson, 1992; Sagar *et al.*, 2014). Lamontagne *et al*. (2002) found that humic acid contamination varied significantly between extraction methods in their study testing different DNA extraction methods from soil. To monitor inhibition in samples, the use of internal controls has been used, this is where a known amount of DNA or material containing DNA is added to the sample either before extraction or before analyses. This allows the assessment of inhibitory effects when compared to testing known concentrations of uninhibited internal control in water (McKay *et al.*, 2009; Romdhane *et al.*, 2019; Delgado-baquerizo *et al.*, 2020). Another way inhibition is monitored in DNA samples is through spectrophotometric analysis of the DNA extract (Sagar *et al.*, 2014). A A260:230nm ratio less than 2.1 indicates humic acid contamination in the DNA extract (Verma and Satyanarayana, 2011; Knauth *et al.,* 2012). The widely accepted threshold for PCR reactions to perform is a ratio of 260/280 (Anderson, 2016).

There are many methods for minimising inhibition in soil DNA samples, during extraction to within the PCR reaction. Efforts are mostly focused on the lysis stage of extraction, where inhibitors are most prevalent. To counteract this challenge, additives such as CTAB (Cetyltrimethylammonium bromide) and PVPP (Polyvinylpolypyrrolidone) are incorporated into lysis buffers. These compounds form insoluble complexes with inhibitors, impeding their progression through the extraction

process (Robe *et al.*, 2003; Bakken and Frostegård, 2006; Verma and Satyanarayana, 2011). Studies by Braid *et al*. (2003) and Sharma *et al.,* (2013) explored the effectiveness of different ions—$FeCl_3$, $MgCl_2$, and $CaCl_2$—added to lysis buffers. While Braid *et al*. (2003) favoured $FeCl_3$, Sharma *et al.,* (2013) found $MgCl_2$ to be superior, noting significant DNA losses with $FeCl_3$. This variance in outcomes underscores the importance of soil composition, as inhibitors vary in type and concentration based on soil type. Additionally, ammonium ferric sulphate dodecahydrate in the lysis buffer was found by Braid *et al*. (2003) to successfully remove inhibitors without compromising DNA yield within acceptable limits. Activated charcoal, known for its efficacy in removing contaminants from water, has been adapted for DNA extraction methods due to its porous nature and large surface area, enabling the adsorption of humic acids (Verma and Satyanarayana, 2011; Barbaric *et al.,* 2015). Enzymes such as proteinase K and lysozymes have also been employed to mitigate inhibition. Proteinase K targets contaminating proteins, while lysozymes act hydrolytically against humic acids (Robe *et al.*, 2003; Bakken and Frostegård, 2006). These approaches collectively contribute to minimizing inhibition and optimizing DNA extraction from challenging soil samples.

DNA purification

Additional purification steps can be incorporated into the DNA extraction protocol to enhance the purity of the DNA extract. These steps can include techniques such as gel filtration resins, caesium chloride density centrifugation, chemical flocculation, size exclusion chromatography, ion exchanges and agarose gel electrophoresis (Braid *et al.*, 2003; Damm and Fourie, 2005; Desai and Madamwar, 2006; Sharma *et al.,* 2013; Sagar *et al.*, 2014). While these methods effectively remove inhibitors from the DNA extract, they are not without limitations. Gel filtration-based separations operate by molecular weight, yet they may fail to fully eliminate humic molecules, which can form large polymeric complexes similar in size to the target DNA, thereby remaining in the extract (Verma and Satyanarayana, 2011; Hebda and Foran, 2015). Similarly, DNA precipitation using isopropanol and ethanol may not efficiently precipitate degraded or sheared DNA, resulting in DNA losses (Hebda and Foran, 2015). Due to the structural similarities between humic acids and nucleic acids, distinguishing them during purification poses a considerable challenge (Bakken and Frostegård, 2006).

To address inhibition during PCR reactions, dilution of the sample in water is a common strategy (Damm and Fourie, 2005). However, this approach may not always sufficiently alleviate inhibition (Roose-Amsaleg *et al.,* 2001) and risks diluting the target DNA below the limit of detection. PCR enhancers such as t4 gene 32 protein, DAX-8, and Bovine Serum Albumin (BSA) can also be employed, but they may alter PCR efficiency and melting temperature (Damm and Fourie, 2005; Schriewer *et al.,* 2011). BSA is a carrier protein that has multiple modes of action to prevent inhibition, including helping to stabilize enzymes such as DNA polymerase and protect them from degradation. It binds to nonspecific DNA fragments or inhibitors, thereby preventing them from interfering with the PCR process and reduces surface tension to help improve the efficiency of PCR reactions. The t4 gene 32 protein, another protein, binds to ssDNA, thereby preventing the formation of secondary structures and enhancing the accessibility of DNA polymerase to the template DNA, and DAX-8, a chemical regent, works by selectively binding and removing inhibitors present in the DNA sample.

Additional steps are often essential to obtain a positive PCR signal from soil extracts (Berthelet *et al.,* 1996; Tien *et al.,* 1999) and sometimes multiple purification steps are required (Van Elsas *et al.,* 1997; Delgado-baquerizo *et al.*, 2020). However, extensive purification can result in DNA losses and compromise the detection of rare DNA sequences (Robe *et al.*, 2003). Thus, the decision to purify DNA involves a trade-off between achieving high-quality DNA and removing inhibitors (Kuske *et al.*, 1998; Braid *et al.*, 2003; Lakay *et al.,* 2007; Bilodeau, 2011; Sharma *et al.,* 2013).

End Use Bias

The end use of the DNA extracted must also be a consideration when choosing a DNA extraction method. Many of the papers referenced comment on the appropriateness of the extracted DNA for their chosen end use (Van Elsas *et al.,* 1997; Kuske *et al.*, 1998; Krsek and Wellington, 1999; Roose-Amsaleg *et al.,* 2001; Vandeventer *et al.*, 2012; Sagar *et al.*, 2014; Basim *et al.*, 2020; Guerra *et al.*, 2020) and some have even developed methods for specific end uses (Bollmann-giolai *et al.* 2020).

End use results can be impacted very early on in the DNA extraction method process, even simply the amount of sample taken through the process. Dopheide *et al*. (2019) found that extraction of larger samples resulted in higher estimates in

metazoan biodiversity, when compared to the smaller sample sizes. Correspondingly, Ranjard *et al*. (2003) found that sample size had no influence on the bacterial diversity using ribosomal intergenic spacer analysis (RISA), however an affect was observed in the fungal community and it was concluded that at least 1g is needed if assessing fungal communities and that sample size should be dependent on the target. Dequiedt *et al.* (2012) also found that fungal diversity patterns were affected by the DNA extraction method chosen, perhaps indicating fungi are more susceptible to bias introduced through the choice of DNA extraction method.

As previously discussed PCR can be heavily disrupted by inhibitors, meaning that when extracting for PCR-based end uses (rt-PCR, qPCR, sequencing), then methods that release high amounts of inhibitory substances should be avoided, or further purification steps added (Berthelet *et al.,* 1996; Sagar *et al*., 2014). Another consideration for PCR-based end uses is the shearing of DNA and the formation of chimeric products. The shearing of DNA may prevent the binding of the primers to the target DNA (Van Elsas *et al.,* 1997; Sagar *et al*., 2014) and chimeras can cause template misrepresentation, generating artifactual amplification products and competition for amplification with genuine targets. In the study by Krsek and Wellington (1999) assessing different combinations of mechanical, chemical and enzymatic lysis as part of DNA extraction from soil, they found that different 16S rRNA primers, targeting different regions, all produced different results dependant on method used. They concluded that the primers vary in sensitivity to impurities, however this study clearly shows the impact of DNA extraction on end use and results.

The nature of the study significantly influences the selection of DNA extraction methods, whether the focus is on targeting specific organisms or conducting a comprehensive community analysis (Roose-Amsaleg, *et al.,* 2001; Sagar *et al*., 2014). As previously emphasized, certain organisms exhibit different susceptibility to lysis methods, potentially introducing biases in community studies. Thus, this consideration becomes pivotal in the choice of extraction method.

Conversely, when the aim is to specifically target easily lysed organisms for detection or quantification, opting for a less invasive extraction method becomes pertinent to minimize the release of non-target organisms. These non-target organisms might outcompete the desired target due to non-specific off target primer binding or lead to an overestimation due to the presence of extracellular DNA (Frostegård *et al*., 1999)

or inhibitory substances that could compromise the efficiency of downstream applications.

Numerous community studies have investigated the impact of DNA extraction methods on results. For instance, Dopheide *et al*. (2019) found that each of the tested methods (Powersoil RNA extraction kit, Nucleospin Soil extraction kit, Powermax DNA extraction kit) resulted in a biased assemblage of Operational Taxonomic Units (OTUs). Similarly, Xie *et al*. (2018) observed that evenness (how evenly the abundance of different species is distributed within a sample, taking into account the number of species but also their relative abundances) indices in their community study were influenced by the choice of method, although richness appeared to be less affected. Moreover, Martin-Laurent *et al*. (2001) demonstrated that the phylotype abundance and composition of bacterial communities, as assessed using ribosomal intergenic spacer analysis (RISA), were dependent on the DNA extraction method utilized.

Multi-study comparison

DNA extraction method can heavily bias end results and often studies are not comparable due to the different extraction methods used. Numerous protocols have been published (Knauth *et al.,* 2012) and Bakken and Frostegård (2006) described the number of bespoke protocols for DNA extraction as ‘daunting’. As of the 1st of June 2021 1,155, papers (according to web of science) were published with ‘DNA extraction method’ in the title, not including those that are embedded within papers and commercial kits, or the minor iterations published in many papers. For example, Xie *et al*. (2018) tested four iterations of the same method published by Zhou *et al.,* (1996). As previously discussed, DNA extraction method has an effect on the end results, meaning that it is near impossible to compare studies using different DNA extraction protocols (Martin-Laurent *et al.*, 2001).

Numerous studies have identified the need for a standard unbiased and transparent protocol (Martin-Laurent *et al.*, 2001; LaMontagne *et al.*, 2002; Carrigg *et al.*, 2007; Wang *et al.,* 2012; Anderson, 2016; Dopheide *et al.*, 2019). An ISO standard method (11063) has been created, in an attempt to fulfil this need for a standardised protocol. It was validated by 12 independent European laboratories on 12 soils, comparing DNA quantity and the abundance and structure of bacterial communities. It was designed with bacteria in mind, minimising its usefulness for all the other kingdoms.

Dequiedt *et al.* (2012) evaluated the performance of the ISO standard method (11063) and found that the standard ISO protocol achieved lowest DNA yields, had ~10 times less bacterial gene copies, ~7 times less fungal gene copies and ~5 times less archaeal gene copies than the other two methods, with GnS-GII consistently performing the best. This perhaps indicates that the lysis step in the ISO standard (11063) is not sufficient at lysing the harder organisms and therefore creates a biased extraction. Xie *et al.* (2018) also tested the ISO standard (11063) against 5 other methods, including the commercial kit; Powersoil (Mo Bio Laboratories, Carlsbad, CA, United States). The ISO standard appeared to underestimate the rRNA gene abundance, and similarly this was attributed to its lysis capability.

It has also been suggested to tailor methods in response to soil types, such as pH and calcium content (Hebda and Foran, 2015; Guerra *et al.*, 2020). Soil type is often linked to DNA extraction efficiency, supporting this claim. However, as discussed above, there are calls for a standard protocol to remove DNA extraction bias. Perhaps this goal of achieving a standard protocol for all soils with no bias is unachievable, for the many reasons aforementioned. Frostegård *et al.* (1999) proposed the use of reference soils, that do not change over time. This allows the comparison of different methods, without having to perform them all at the same time. This could perhaps offer the opportunity for all these studies and future method papers to be comparable, and possibly allow for corrections to be applied to results retrospectively. Nevertheless, the creation of a reference soil comes with its challenges. Firstly, what soil attributes do you choose to apply? Secondly, do you use a natural microbiome or create a synthetic one? And finally, many studies have investigated the issue of storing soils, and how their microbiomes change during storage. Changes have been recorded only four weeks after storage with a range of different storage methods (Clark *et al.*, 2008; Černohlávková *et al.*, 2009; Wallenius *et al.*, 2010).

Krsek and Wellington in 1999 identified the lack of a systematic survey of soil DNA extraction methods and to this date no such review has been performed. There have been many comparative studies of potential methods on a select few soils, however none have assessed the trends of DNA extraction choices across the field of study and often fail to justify the choice of methods they have chosen to review. Therefore, this review aimed to give an overview of the DNA extraction methods used for the detection of specific organisms in soil.

ii) Systematic Review method

A search criterion was developed to find papers that extracted total DNA from soil for PCR based detection of specific bacterial or fungal organisms. To identify key words for the search, five papers were chosen based on their applicability to the criteria; 1: Habib *et al*. (2017), 2: Budge *et al*. (2009), 3: Deora *et al*. (2015), 4: Huang and Kang (2010) and 5: Nunes *et al*. (2010). Common words that were used in the title, abstract and key words were identified, and their occurrence counted, as shown in Table 1. These were used to develop an initial search, this was then adapted as limitations in the results were identified, for example human or animal studies. Each time the search criteria was adapted, the results were checked for these five papers and if one of the papers was not found in the results the adaptation was removed. The search criteria were also adapted to be used in two online search databases: SCOPUS and Web of science (WOS) (

Table 2).

Table 1. Common words identified in the five control papers, and their occurrence in each paper

Word/Paper	1	2	3	4	5	Occurrence
Polymerase chain reaction	1	1	1	4	2	5/5
DNA	0	5	3	11	10	4/5
Soil	7	9	8	14	8	5/5
Real time	0	4	0	8	3	3/5
qPCR	1	0	4	5	5	4/5
PCR	0	2	0	5	2	3/5
Detection	2	1	1	7	1	5/5
Crop	2	0	0	2	0	2/5
Field	0	1	1	3	0	3/5
Inoculated	5	2	2	0	0	3/5

Molecular	1	2	0	0	0	2/5

Table 2. Search criteria adapted for use in both SCOPUS and Web of Science

Database	**Search criterion**	**Number of results (date searched)**
SCOPUS	(TITLE-ABS-KEY (*"Polymerase chain reaction"*) OR TITLE-ABS-KEY (*qpcr*) OR TITLE-ABS-KEY (*pcr*) OR TITLE-ABS-KEY (*rtpcr*) OR TITLE-ABS-KEY (*rt-pcr*) AND TITLE-ABS-KEY (*soil*) AND TITLE-ABS-KEY (*detect**) AND NOT TITLE (*"gene" OR "genes"*) AND NOT TITLE (*compar**) AND NOT TITLE (*method**) AND NOT TITLE (*extract**) AND NOT TITLE-ABS-KEY (*human*) AND NOT TITLE-ABS-KEY (*animal*) AND NOT TITLE-ABS-KEY (*vir**) AND NOT TITLE-ABS-KEY (*parasit**) AND NOT TITLE-ABS-KEY (*nematode*) AND NOT TITLE-ABS-KEY (*insect*)) AND (LIMIT-TO (PUBSTAGE , *"final"*)) AND (LIMIT-TO (DOCTYPE , *"ar"*)) AND (LIMIT-TO (LANGUAGE , *"English"*)) AND (LIMIT-TO (SRCTYPE , *"j"*))	2233 (15/5/20)
WOS	(TS=("Polymerase chain reaction" OR qpcr OR pcr OR rtpcr OR rt-pcr) AND TS= ("soil") AND TS=(detect*) NOT TI= (method* OR extract* OR "gene" OR “genes”) NOT TS= (human OR animal OR vir* OR parasit* OR nematode OR insect)) AND LANGUAGE: (English) AND DOCUMENT TYPES: (Article)	2354 (15/5/20)

	Indexes=SCI-EXPANDED, SSCI, A&HCI, CPCI-S, CPCI-SSH, ESCI Timespan=All years	

All results were downloaded for screening, this gave a total of 4587 matches. Results that had missing information, i.e. abstract or authors, that could not be retrieved were removed. Then all duplicates were removed. This left 3030 results. Results were then screened for their applicability to the study based on a set criteria for removal (2593 removed). Those that were removed at this stage were categorised into six options: not plant related (412), incorrect media (361), not PCR detection (212), community analyses/sequencing/functional genes (1512) and method/review papers (96). 'Not plant related' included papers that were detecting human and animal pathogens or organisms associated with pollution degradation that were not removed by the initial search. 'Incorrect media' included papers that extracted the DNA from other media, not soil, for example water or agar. 'Not PCR detection' was for methods that did not involve PCR for the detection, for example, plating or LAMP assays. Sequencing or community studies were removed due to the study investigating the use of DNA extraction on detecting specific organisms within soils. Method/Review papers were removed as these were investigations into DNA extraction from soil rather than the choice a laboratory had made to detect a specific organism in soils. The assumption was made that all these papers were scientifically robust as they had been peer reviewed and published. Removal based on scientific robustness would create a bias which is counter intuitive to the nature of a systematic review.

For papers that passed the screening stage, DNA extraction methods were recorded by breaking content down into sample size, mechanical disruption, and lysis buffer. The lysis buffer for bespoke methods was broken down into chelating agents, detergents, salts, enzymes and 'other'. If a commercial kit was used this was recorded and the commercial kit protocol was broken down into its components such as the mechanical disruption, but the lysis buffer and its components were not recorded as many are protected and not publicly available.

Results were recorded as the number of times each method occurred, including in combination. This data was also recorded per decade to monitor how trends had changed over time.

iii) Results and discussion

After screening, 271 papers were suitable for inclusion into the review. Table 3 shows the number of papers per decade. Within the 1980's, only 1 paper was suitable for this study, indicating the beginning of this type of research. PCR was first developed in 1983 by Kary Mullis (Bartlett and Stirling, 1996), so for the first paper to utilise this technology for specific detection of an organism in such a complex medium as soil only 5 years later (1988), shows how quickly this technology developed and how valuable of a discovery it was. In the 1990's there was a steady increase in the number of studies, which carried on into the 2000's and 2010's. The increase in studies maybe linked to the release of commercial kits, due to their ease and high throughput nature, as well as the increased interest in soil DNA analyses (Erana *et al.,* 2019). The 10 papers from 2020 were only from the first half of the year, as the search was performed in May 2020.

Table 3. Number of papers from systematic review results in each decade after screening

DECADE	NO. PAPERS
1980'S	1
1990'S	18
2000'S	75
2010'S	167
2020	10

Sample size

One of the main variations across all the various protocols, both bespoke and commercial methods, is the starting sample size. The results recorded sample size as little as <0.1g up to 500g. Many studies have commented on the impact of sample size on end results (Ranjard *et al.*, 2003; Budge *et al.*, 2009; McKay *et al.*, 2009; Taberlet *et al.*, 2012; Woodhall *et al.*, 2012; Dopheide *et al.*, 2019; George *et al.*, 2019; Bollmann-giolai *et al.*, 2020; Guerra *et al.*, 2020). The highest percentage of studies used 0.1<0.25g, followed by 0.25<0.5g. Only 32% of studies used more than 1g (Figure 1). This may be attributed to the majority of commercial kits functioning under 1g, other than a select few; DNeasy PowerMax Soil Kit and UltraClean® Mega

Soil DNA Isolation Kit by MO BIO Laboratories Inc., extract DNA from up to 10g of soil.

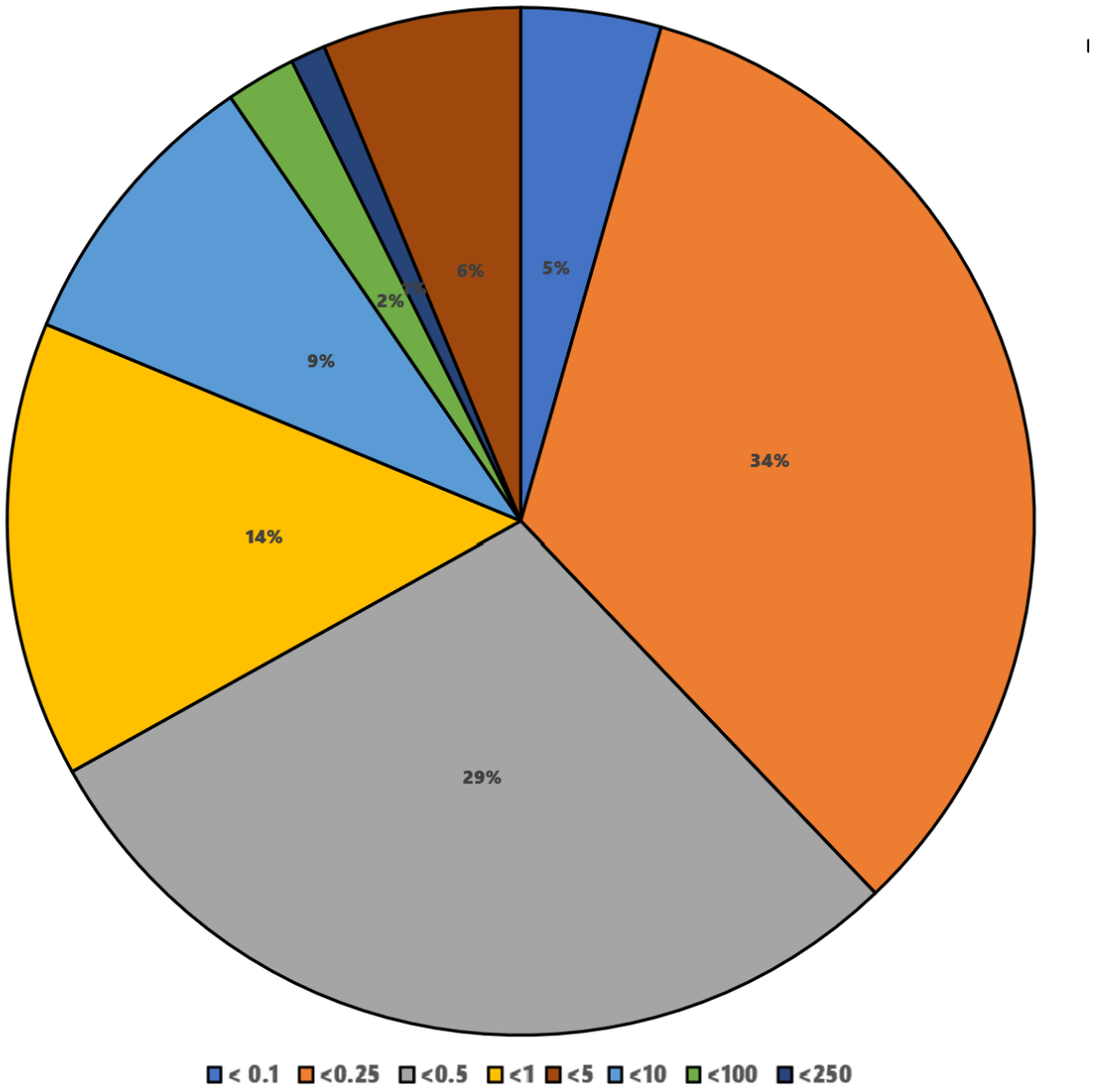

Figure 1. Percentage of sample size (g) used over all time

Breaking down the trends of sample size over time, an increase in popularity of the smaller sizes (under 0.5 g) was apparent (Figure 2), with a decline in starting sample size over 10g. Both of these are indicators of the uptake of commercial kits over time, as commercial kits operate at the lower starting sample size and do not exceed 10 g. From the choice of commercial vs bespoke methods, this trend is clearer. As expected in the commercial kit graph (Figure 3), smaller sample size dominates, however the percentage of studies using over 0.5 g remained stable, with 5-10 g increasing in use in 2020. Within the bespoke methods, a higher variation of different sample size was used. This is expected with the flexibility bespoke methods allow.

Only in the bespoke methods were sample sizes over 10 g used across both the 2000's and 2010's. There however appeared to also be a steady incline in the use of sample size under 0.5g within the bespoke methods, with a decrease in those using between 0.5-5g. No studies in the period reviewed in 2020 used bespoke protocols (Figure 4).

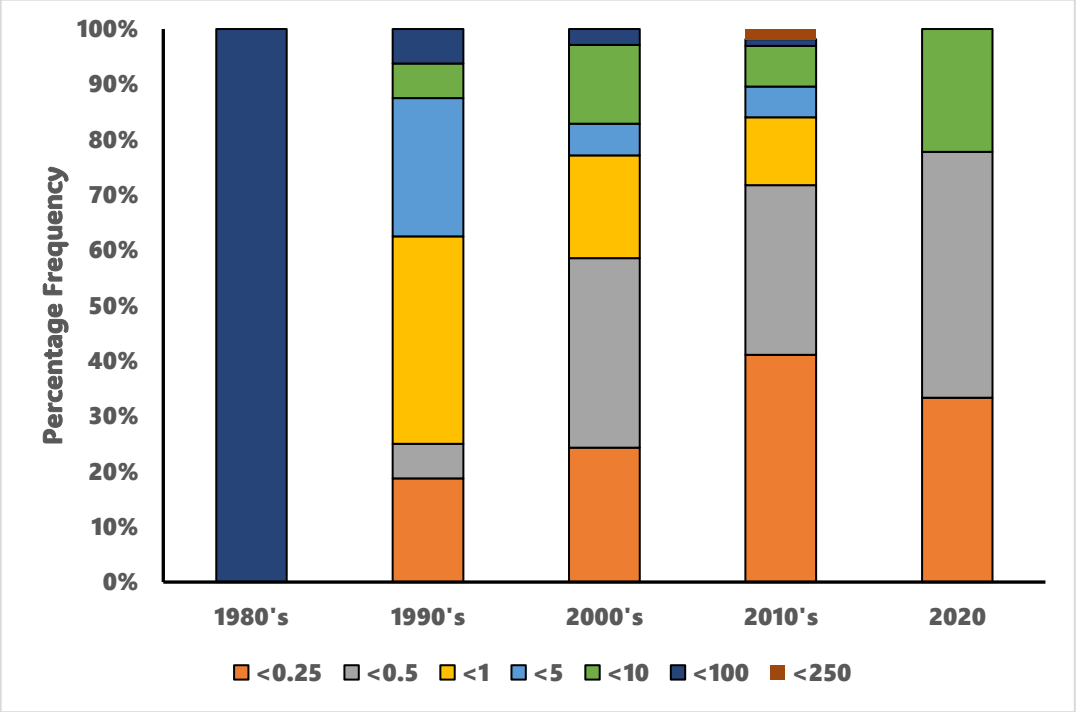

Figure 2. Frequency of soil sample size (g) used in both commercial and bespoke DNA extraction methods over time.

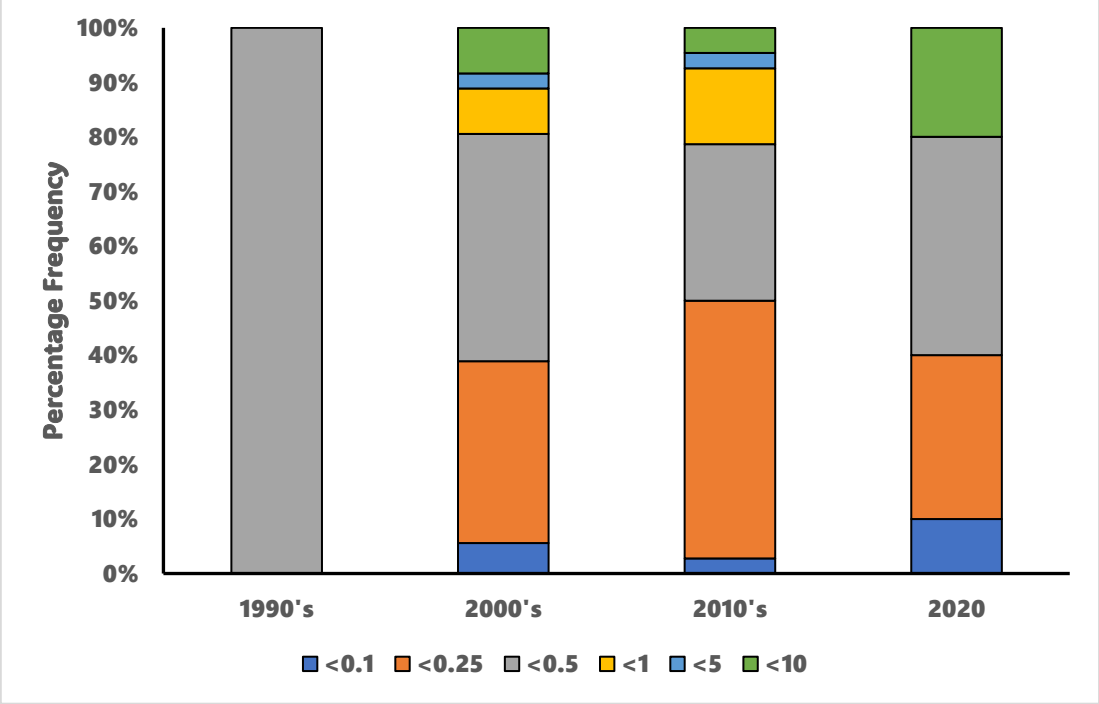

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Figure 3. Frequency of soil sample size (g) used from commercial DNA extraction kits over time

Ellingsøe and Johnsen (2002) were the first to investigate the effect of soil sample size on the assessment of bacterial communities, using DGGE fingerprinting. They tested four different sample sizes (0.01-10 g) on a single soil type (acid sandy forest soil). The largest variations in community fingerprints were seen in sample sizes below 1 g, indicating the need for sample sizes above 1 g.

In 2003, Ranjard *et al*. (2003) studied the effect sample size had on DNA yield and bacterial and fungal communities, using Ribosomal Intergenic Spacer Analysis (RISA). They tested 6 different sample sizes (0.125 g, 0.25 g, 0.5 g, 1 g, 2 g and 5 g) on three different soil types, using a bespoke method by (Martin-Laurent *et al.*, 2001). They found that DNA yield correlated with sample size in two of the soil types, but not with a clay soil. Larger soil samples achieved higher DNA yield; the study hypothesised this was due to smaller samples having a lower lysis efficiency. Bacterial communities seemed unaffected by sample size; however principal component analysis revealed sample sizes larger than 1 g were required to obtain reproducible fingerprinting analysis of fungal communities. This again highlights the importance of considering target organisms in the choice of DNA extraction protocols. This both opposes and supports the findings in the study by Ellingsøe and Johnsen (2002), which reported changes in the bacterial community in response to sample size, but supported the sentiment that samples sizes need to be larger than 1 g.

A study by Budge *et al.* (2009) investigating *Rhizoctonia solani* distribution in soils determined that due to *R. solani's* non-random distribution there were high levels of between sample variation and to correct for this larger soil samples could be used. A similar conclusion had been made by Roget and Herdina (2000) in the case of *Gaeumannomyces tritici,* where they called for a minimum of 250g. Many organisms have been found to be clustered with in fields, potentially meaning that smaller soil samples may 'miss' clusters of the target organism.

Even though these studies recommend the minimum of 1g, the results showed that 67% of studies still used under 1g (Figure 1) and the proportion of those using over 1g is getting smaller over time (Figure 2). A divide in trends between the users of commercial kits and bespoke methods was observed with 79% of those using

commercial kits using samples of less than 1g compared with 33% of those using bespoke methods.

In 2012, Budge, from the earlier 2009 paper, developed a new large scale DNA extraction method for soils, but this time working with *S. cepivora* (Woodhall, *et al.*, 2012). This method was designed to account for *S. cepivora's* hardy resting structures and low infection threshold, again indicating that target organism should be a consideration in choice of DNA extraction protocols. The method could be used to extract DNA from 250g of soil inoculated with 1,2,5,10, and 50 *S. cepivora* sclerotia and positive detection was achieved at the lowest inoculation level. The method could also be successfully used for extracting DNA, suitable for use in PCR analysis, from as much as 1kg of soil. Also in 2012, Taberlet *et al.* developed a DNA extraction method with the goal of extracting from a larger initial sample size of soil (4kg), to gain the more representative sample. They combined multiple soil cores into one sample and performed a large lysis step. They then continued the extraction using the NucleoSpin Soil kit (Macherey-Nagel, Duren, Germany), negating the lysis step.

Despite these two studies in the 2010's, the results showed that most researchers still chose smaller samples sizes; even among those using bespoke methods only 34% used over 10g (Figure 4), compared with only 6% of those using commercial kits, despite the release of larger-sized commercial kits (Figure 3). The most common sample size remains between 0.1g-0.25g, which is a concern when the minimum recommended sample size is 1g. However, when regarding only bespoke published methods, the most common sample size increases to 0.5g (Figure 4).

Dopheide *et al.* (2019) performed a study assessing the effect of DNA extraction, number of PCRs and sample size on metabarcoding of communities in soil. A larger soil sample was homogenised and then sub-samples of 1.5g, 7.5g and 15g were removed. Their results showed that all the variables tested affected biodiversity estimates. However, they consistently found that target organisms were a key factor to consider when choosing a suitable extraction method and sample size. The larger soil sample size resulted in higher biodiversity estimates for arthropods but not prokaryotes or microeukaryotes and better spatial discrimination of metazoan communities but not prokaryotes. Furthermore, the larger sample sizes showed both within and between plot discrimination for eukaryotes. Unlike the previous studies, they found that smaller samples were better for micro-organism community analyses

i.e. fungi and bacteria. However, this study used a minimum of 1.5g which is considerably larger than some of the other studies, bringing this opinion in line with that of Ellingsøe and Johnsen (2002) which recommended over 1g. Recommendations of much larger sample sizes of 250g+ (Roget and Herdina, 2000; Budge *et al.*, 2009; Woodhall, *et al*., 2012), came from studies detecting specific organisms compared with studies investigating community structure. End-use may also need to be an important factor to be considered when choosing sample size. However, the aim of this systematic review was to assess DNA extraction choices for the detection of target organisms, potentially making the findings by Roget and Herdina (2000), Budge *et al.* (2009) and Woodhall *et al.* (2012) more relevant to this study.

The suggestion by Dopheide *et al.* (2019) that larger organisms require larger starting soil sample size was supported in the study by (George *et al*., 2019) which admitted uncertainties on the ability of environmental DNA methods using small sample size to accurately characterise communities of larger organisms, during their study on national trends of biodiversity in soils. Again however, this was a community-based study.

Contradictory to the previous studies, Guerra *et al.* (2020) found that the smaller 0.05g yielded better results than the 0.2g method. Fungal DNA was not detected in the 0.2g method and this had a lower recovery of bacteria. DNA was quantified using 16S and 18S primers within qPCR. This juxtaposed the prior consensus that fungal detection requires larger soil sample size (Ellingsøe and Johnsen, 2002; Ranjard *et al.*, 2003).

These findings echo the challenges outlined in the introduction. Target organism appears to be a common variable to consider. This systematic review did not differentiate between bacterial and fungal organisms as the target organism, which may further inform sample size choice. There also appears to be a difference in findings between those performing studies looking at communities as a whole and those targeting specific organisms. Those performing targeted detection studies opted for significantly larger sample size. When you consider that this systematic review only included papers performing targeted studies, it makes the continued use of smaller sample sizes more concerning.

Despite numerous publications recommending the use of larger soil starting sample size, at least over a minimum of 1g, we see even in 2020, the continued use of <1g. It begs the question what parameter these studies are basing their decisions on, if not the published literature when it concerns the starting sample size. This decision might be swayed by the convenience and availability of commercial kits, which presently offer quantities of less than 10 g. A variable that is not considered is the size and distribution of the population that the sample is coming from. Perhaps in pot studies the smaller starting sample size are adequate, however when sampling agricultural fields or vast habitats it is much more difficult to gain the larger picture from only a “drop” in the metaphorical “ocean”.

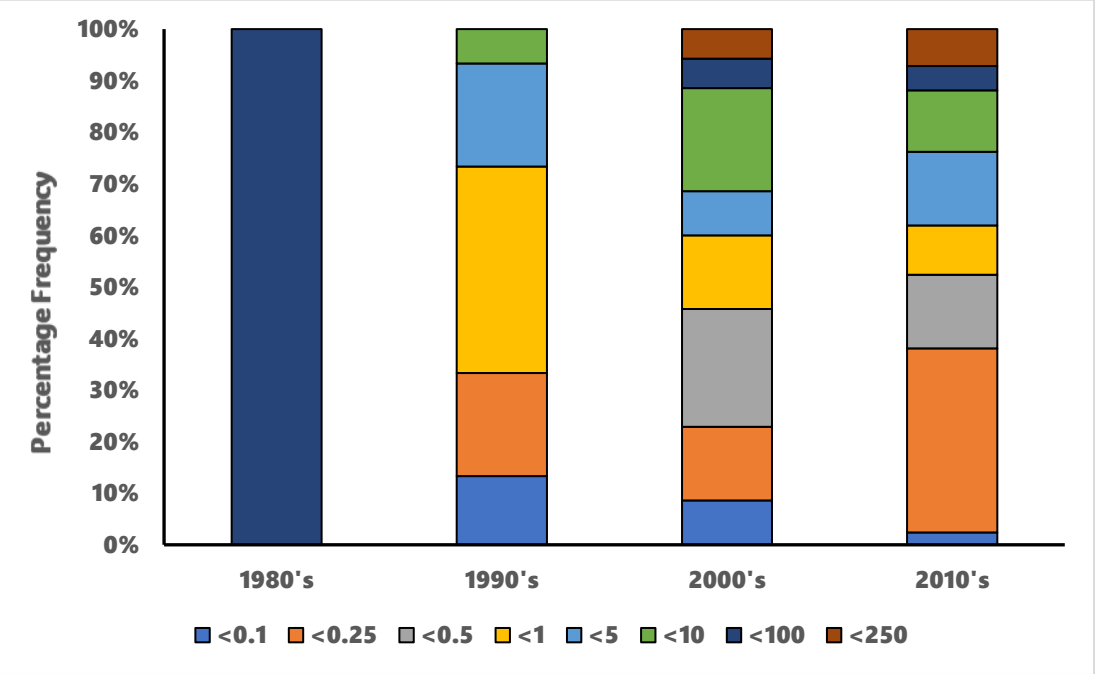

Figure 4. Frequency of soil sample size (g) used in bespoke DNA extraction methods over time

Lysis buffer

Lysis buffer composition is one of the key components of DNA extraction success (Knauth *et al.,* 2012). Lysis buffers are the solution that the freed DNA is released into and because of this it has many roles; lysing of organisms, protection of the free DNA and PCR inhibitor prevention. Lysis buffers usually consist of the combination of the following: chelating agents, detergents, salts and enzymes. Chelating agents, such as EDTA (ethylenediaminetetraacetic acid), work by chelating the metal ions minimising the activity of enzymes that break down the target DNA (DNase), leading to protection of the freed DNA. Detergents cause cell lysis by breaking down lipids in

the cell membrane and allowing the release of DNA. Examples of detergents include SDS (sodium dodecyl sulphate) and CTAB (cetyltrimethylammonium bromide). Salts neutralize the negative charge of the freed DNA making it less hydrophilic, so it does not bind to water molecules. Salts also remove any proteins bound to the DNA and keeps them dissolved in water, so they do not precipitate out with the DNA. Common salts used in DNA extraction include sodium phosphate and potassium phosphate. Finally, is the use of enzymes, such as proteinase K and lysozymes. Although chelating agents prevent the action of enzymes, some enzymes are beneficial to the extraction of DNA. They help with lysis of cells and with the removal of proteins. Proteinase K helps by degrading nucleases, protecting the freed DNA from degradation.

When assessing the use of different lysis buffers only bespoke extraction methods were considered, as buffer composition was not freely available for commercial kits. This study did not incorporate the sample size or concentration of each component, only its frequency of application. As such, only 13% of studies utilised all four of the components investigated, two or three of the components were used in 30% of studies and in 27% only one of the components was used (Figure 5). This does not mean that this was the only element of the lysis buffer in these studies, some studies included items that did not fit into the description of the four components selected for analyses. For example, milk was used in 7 studies. The wide variety of different combinations of lysis buffer, not including the variations in concentration and sample size, shows the breadth of options available and choices to be made in subsequent research.

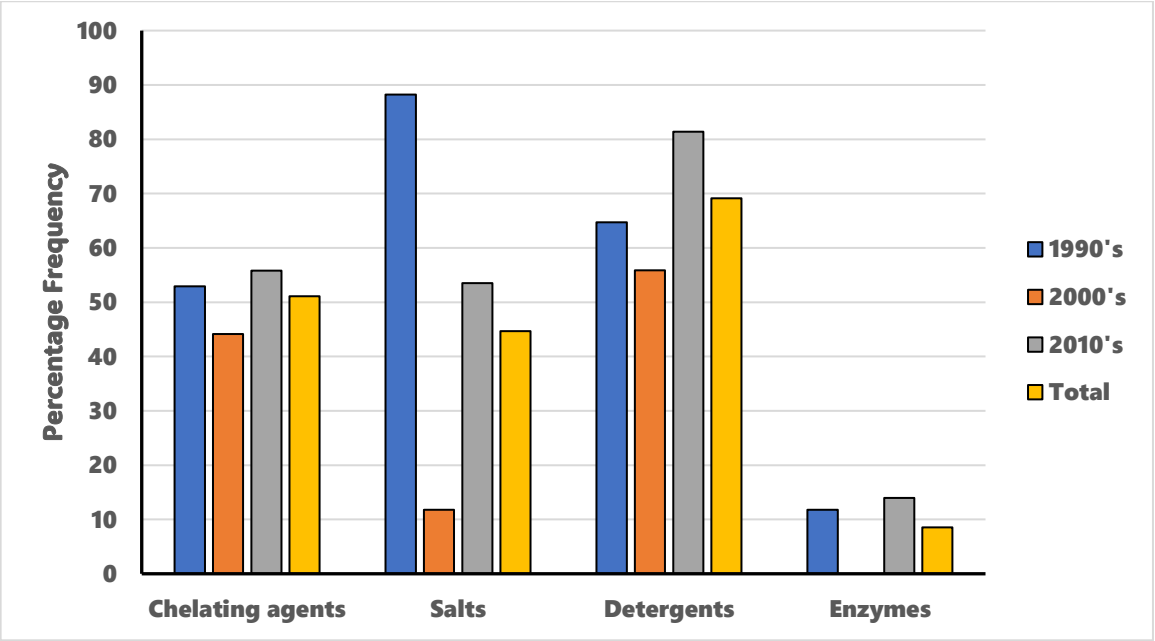

Figure 5. Frequency of studies that used different components in lysis buffers during DNA extraction from soil over time.

Just over half of studies used the action of chelating agents in their lysis buffers, and their use appears to be relatively consistent across the decades (Figure 5). The most common chelating agent was overwhelmingly EDTA, being used in 87% of studies. Krsek and Wellington (1999) tested 6 different buffers in combination with different lysis methods. All the tested buffers contained EDTA in different concentrations. They found that increased EDTA concentrations led to higher DNA yields but lower purity but had the added benefit of protecting DNA from enzymatic degradation and absorption when using harsh mechanical disruption methods. This was affirmed by their buffer with the lowest EDTA concentration not producing amplifiable DNA when used with bead beating, however amplifiable DNA was achieved with the higher EDTA concentration buffers. Lamontagne *et al*. (2002) echoed this sentiment, attributing the lower molecular size of DNA produced from their protocol to EDTA concentration, this had the lowest EDTA concentration of the methods tested (3mM). Furthermore, in 2003, Robe *et al*. confirmed Krsek and Wellington's (1999) findings that EDTA increased DNA yield but to a lower purity.

Sixty-nine % of studies used detergents in their lysis buffers, higher than those using chelating agents. In the 2010's there was an increase of detergent use in lysis buffers to 81% from 55% of studies reported in the 2000's (Figure 5). SDS (48% of reports) was the most commonly used detergent followed by CTAB (41% of reports). The combination was rarely used together, with just over 1% of studies applying this

approach. (Figure 6). Miller *et al*. (1999) recommended the use of SDS over the use of guanidinium isothiocyanate as a detergent in their study assessing the extracting capabilities of different combinations of chemical, physical and enzymatic disruption methods. This early paper's recommendations are in line with the findings that SDS was the most popular detergent used across all studies. This recommendation was corroborated in the study by Krsek and Wellington, also in 1999, which achieved higher DNA yields with SDS than in combinations without. In their study comparing different soil DNA extraction methods, Lamontagne *et al*. (2002) criticised the use of guanidinium isothiocyanate in the lysis buffer as the reason for the lower yields achieved by one of their methods tested. This condemnation of guanidinium isothiocyanate by both Miller *et al*. (1999) and Lamontagne *et al*. (2002) is echoed with in this PhD study, as it appears that none of the studies utilised guanidinium isothiocyanate as their detergent. Lamontagne *et al*. (2002) also found that buffers containing CTAB reduced humic acid contamination in their samples when compared to those not containing CTAB, encouragingly supporting the popularity of CTAB, as the second most used detergent. In 2003, Robe *et al*. claimed SDS to be the most commonly used detergent and 7 years later that still appears to be the case. Again in 2005, an extraction method containing guanidinium isothiocyanate performed worst when compared to other methods in the study by Damm and Fourie (2005) and a method containing SDS performed best. When comparing a CTAB based buffer to the SDS buffer, Damm and Fourie (2005) found that the inhibition levels were the same despite CTAB's apparent superior ability to remove humic acid contamination (LaMontagne *et al.*, 2002), however SDS came out on top with increased yields and more consistent PCR results. This was again found in 2020 when an SDS and phosphate based buffer was compared to a CTAB based buffer in the study by Guerra *et al.* (2020) which found the SDS + phosphate buffer yielded higher DNA than the CTAB based buffer.

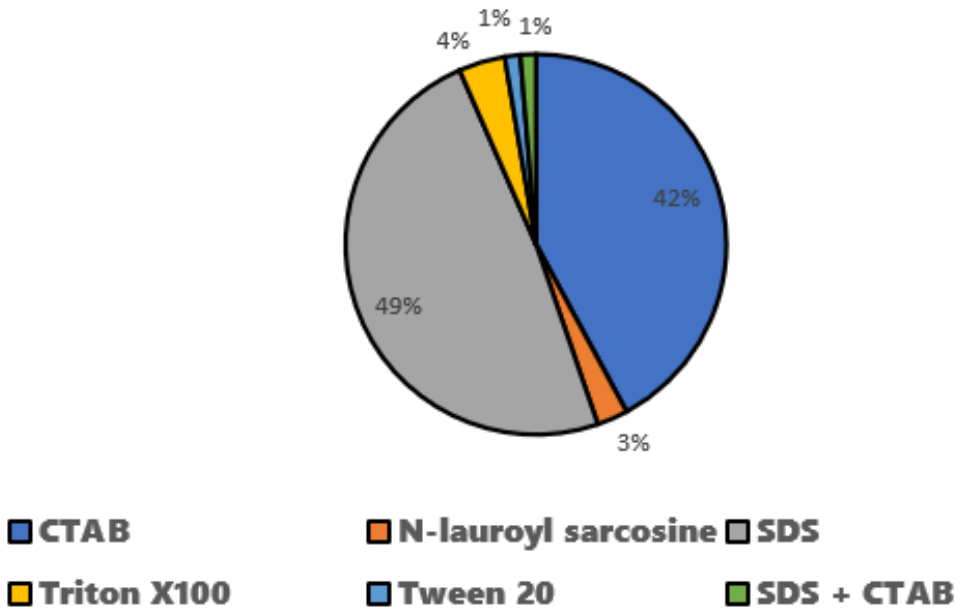

Figure 6. Relative frequency of use of different detergents in lysis buffers across all studies where they were used.

In the 1990's just under 90% of studies used salts in the lysis buffer. This dropped drastically in the 2000's to only 12%, but increased up to 54% in the 2010's (Figure 5). Vandeventer *et al*. (2012) tested different buffers, altering pH, ionic strength and salt concentration, for their ability to bind DNA. They found that for sufficient binding of DNA from soils to silica a high salt concentration is required. However, only 45% of studies used salts in their buffer solutions (Figure 5). There appears to be a high variation in the choice of salt in the studies, with 14 different combinations. NaCl is overwhelmingly most used (31% of studies). This was followed by NaCl plus sodium phosphate (21% of studies) and sodium phosphate plus sodium chloride (13% of studies). In 2002, Lamontagne *et al*., tested three concentrations of NaCl (0.1M, 0.6M and 1.1M) in a lysis buffer containing Tris and EDTA. They found that increasing salt concentration in the buffer reduced humic acid contamination in extracts from compost soil and yielded higher DNA yields than the lower salt buffers. However, Krsek and Wellington (1999) had found that increasing salt concentration in the lysis buffer increased humic acid contamination in their soil extracts, leading to Lamontagne *et al*. (2002) theorising that the optimum salt concentration may be higher for higher organic matter content soils, like composts. Salts are recognized as a key component in DNA extraction from soils, primarily due to their role in DNA precipitation (Roose-Amsaleg *et al.,*2001; Demeke and Jenkins, 2010; Vandeventer *et al*., 2012). The variety of salts used, alongside the focus on concentration rather

than type in most studies, suggests that the concentration of the salt is more critical than the specific choice of salt.

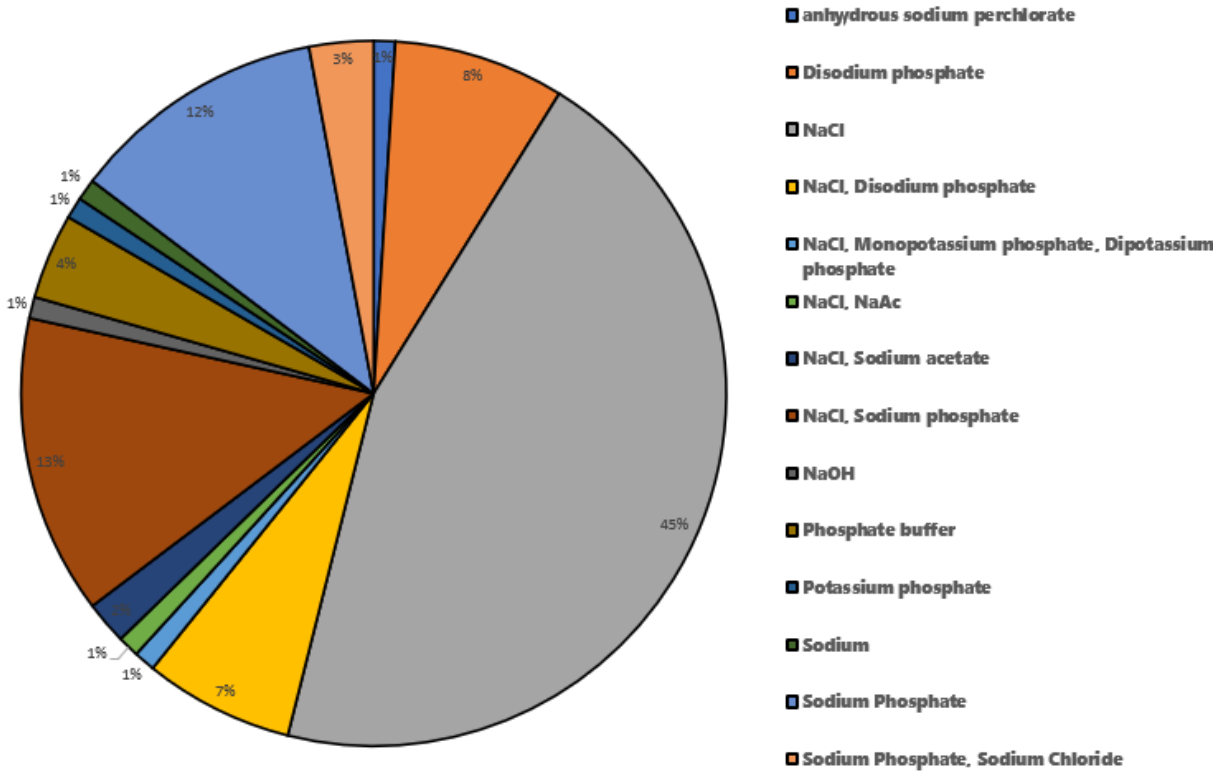

Figure 7. Relative frequency of use of different salts in lysis buffers during DNA extraction from soil across all studies where they were used.

Enzymes were rarely used (8% of studies) within lysis buffers and in the 2000's not a single study included their use. Proteinase K was the most frequently used enzyme agent, followed by RNase A and then Lysozyme (Figure 8). Enzymes were at no point used in combination. Miller *et al.*, (1999) used lysozyme as a pre-treatment, prior to the extraction of the DNA from the soil sample. This significantly reduced the DNA yield and increased humic contaminants, indicating why it was found to be the least popular choice. In contradiction, Krsek and Wellington (1999) found proteinase K to be unreliable, with non-reproducible increases in DNA yield. They recommended the use of lysozyme as it improved the purity of the extracted DNA, extracted larger fragments (40-90kb) of DNA (when used with SDS) and actively precipitated humic acids in their study. However, their recommendations may be biased since they were solely based on the extraction of DNA from soil bacteria. Lysozymes hydrolyse the glycosidic bond between c-1 of N-acerylglucosamine and the c-4 of N-acetylmuramate in the bacterial cell wall, meaning it may have been less effective when targeting other organisms such as fungi. Basim *et al*. (2020) tested 4 different DNA extraction protocols, 1 commercial kit, 2 containing proteinase K and 1

containing lysozyme. They found that the method containing lysozyme performed better having both a higher yield and lower humic contamination than those containing proteinase K. This corresponds with the findings by Krsek and Wellington (1999) where lysozymes performed better than proteinase K. However, the use of enzymes was not the only component of the extraction method altered between protocols so cannot be entirely attributed to the methods success.

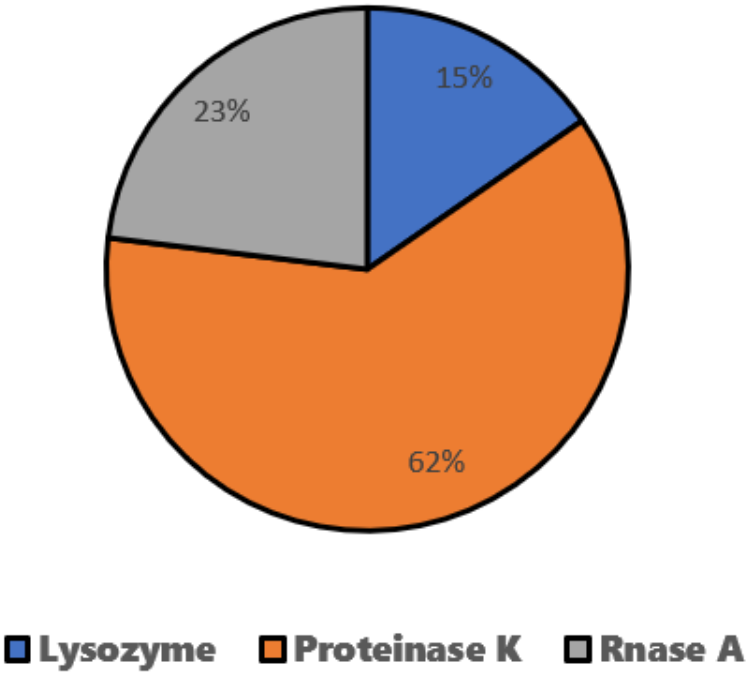

Figure 8. Relative frequency of different enzymes used during DNA extraction from soil across all studies where they were used.

Unlike with sample size, lysis buffer component choice appears to have followed the findings of early experimental recommendations. However, as mentioned previously there are detailed elements that this systematic review was unable to encompass, such as making conclusions regarding buffer concentrations, volumes and pH. For instance, Taberlet *et al.* (2012) increased buffer volume in response to the organic matter composition of the soil and Frostegård *et al.* (1999) attributed DNA yield in their study to the pH of the lysis buffer.

Mechanical disruption

Mechanical disruption is common place in soil DNA extraction protocols, aiding cell disruption, soil particle dispersion, penetration of lysis buffer and homogeneity of samples (Roose-Amsaleg *et al.,* 2001). It has been identified as one of the main factors affecting DNA extraction procedure efficiency (Dequiedt *et al*., 2012).

Mechanical disruption was used in 100% of the studies reviewed, more than one method of mechanical disruption was used in 14% of the studies and less than 2% used 3 methods of mechanical disruption in their protocol. Despite being an integral part of protocols for DNA extraction from soils, overuse of disruption can be detrimental to the end result, either through the shearing of DNA or release of contaminants (Roose-Amsaleg *et al.,* 2001; Sagar *et al.*, 2014; Xie *et al.*, 2018). Krsek and Wellington (1999), in their study testing different methods, found that their protocol not using a mechanical disruption method produced the largest fragments of DNA. Nevertheless, they still described mechanical treatments as beneficial and recommended a method that utilised mechanical disruption. This was supported in the study by Frostegård *et al*. (1999) which found that further mechanical disruption (sonication and thermal shocks) did not further aid the recovery of DNA. Furthermore, they noted with increased mechanical disruption the final extract appeared darker, indicative of increased coextraction of other unwanted compounds. They also found that their protocol featuring multiple rounds of grinding resulted in smears on agarose gels indicative of DNA degradation, showing that not only multiple types of mechanical disruption but repeated mechanical disruption can be detrimental to the DNA extract and end results of downstream DNA analysis. The effect of repeating a single mechanical disruption method was not evident within this systematic review. Basim *et al*. (2020) testing soil DNA extraction methods recommended a protocol that utilised 3 different methods of mechanical disruption: shaking, bead beating and freeze-thawing. This study investigated oily soils, a particularly difficult soil environment, perhaps echoing the ongoing sentiment that successful DNA extraction may depend on soil type. End use may also influence decisions, for example if larger fragments are needed for the unbiased end analyses of a community. Overall, using a single method of mechanical disruption is in line with most recommendations within the literature.

The most popular method of mechanical disruption used to date was bead beating, whether used alone (66%) or in combination with other methods (a further 9%) (Figure 9). This was followed by grinding (6%) and vortexing (6%) (Figure 9). The opinion that bead beating is the best method is echoed throughout the literature. Kuske *et al*. (1998), in their study comparing different lysis methods (freeze-thaw, hot detergent and bead beating) found that bead beating was the only method capable of successfully releasing DNA from *Fusarium moniliforme* conidia and *Bacillus globigii.*

Similar findings of bead-beating being superior, in terms of DNA yield and fragment size, when compared to other methods were recurrent throughout; Miller *et al*., (1999) when compared with freeze-thawing, Krsek and Wellington, (1999) when compared with sonication and shaking, Bakken and Frostegård (2006) when compared to grinding and freeze-thawing, Lakay *et al.,* (2007) when compared to microwaving and liquid nitrogen, to name a few. This systematic review did not evaluate physical characteristics like bead size and material, which would likely introduce further variability in the efficiency of mechanical disruption methods.

Grinding was found to be the second most popular method of mechanical disruption. A study by Frostegård *et al*. (1999) tested different combinations of mechanical disruption either alone or in combination with grinding, sonication or thermal shock. They found that grinding increased DNA yield compared with no lysis treatment but that further disruption with sonication was not beneficial to DNA yield. The study also found that thermal shocks were unable to release viable DNA but released humic substances from the soils tested. They recommended grinding as the mechanical disruption method of choice and claimed it to be on par with bead beating. Notably, Bollmann-giolai *et al*. (2020) when comparing different soil DNA extraction methods, chose to grind all samples irrespective of the tested soil DNA extraction methods published protocol. The reasoning behind grinding being less popular than bead beating is not clear from the literature. Grinding is similar to bead beating in terms of ability to break up soil aggregates and homogenise the sample.

There was minimal literature found that investigated vortexing as a method of mechanical disruption. Damm and Fourie (2005) compared vortexing to the FastPrep instrument, a bead-beating and grinding instrument, and found that vortexing alone resulted in 10 times less DNA extract suitable for their PCR assay. Vortexing is a common method used in laboratories and is therefore freely available, unlike equipment like the FastPrep instrument (FP 120, Bio101, Savant, Farmingdale, New York) and the Minimix auto paint shaker (Merris Engineering Ltd, Ireland) used for grinding and bead beating by Woodhall *et al.*, (2012), perhaps indicating that availability rather than a proven ability to release DNA from organisms within soil aggregates is the reason for the methods popularity.

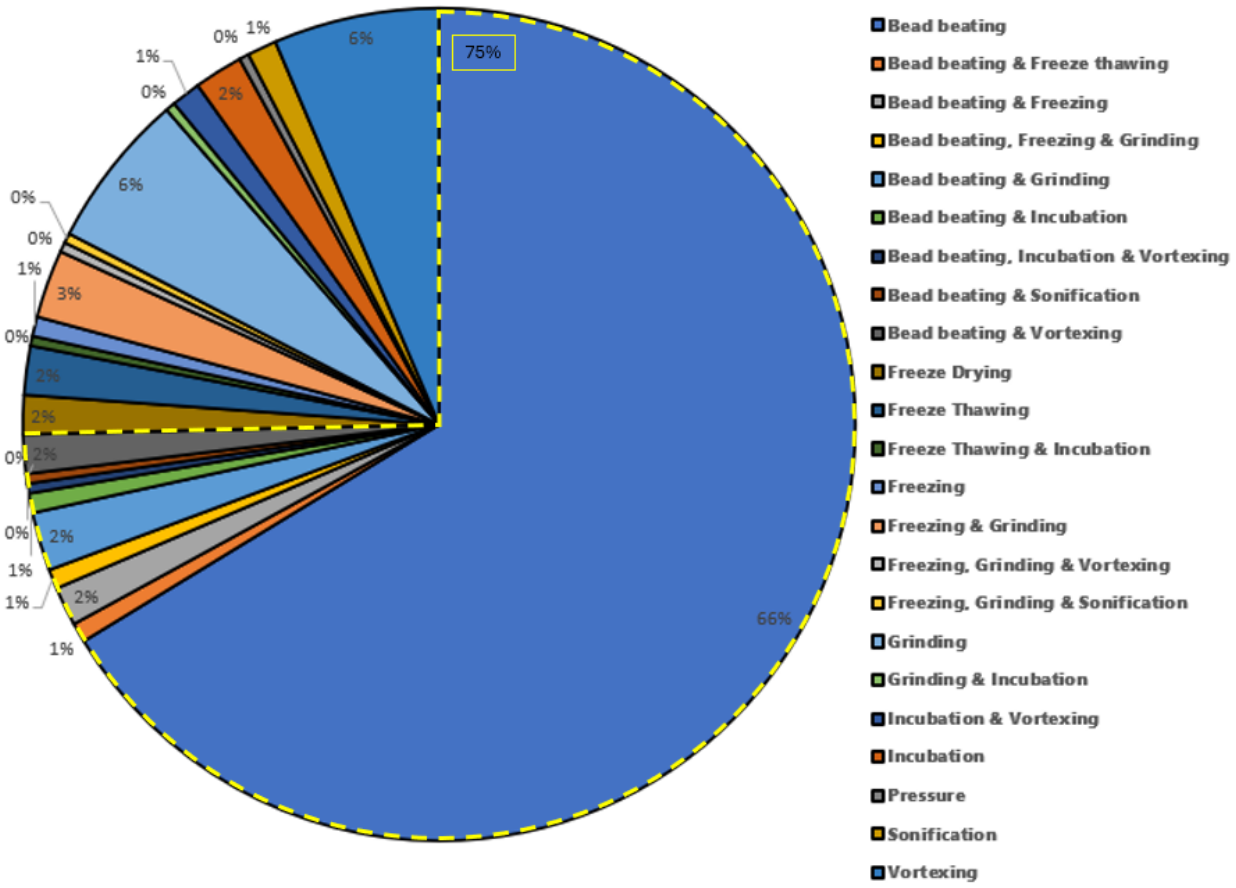

Figure 9. Relative frequency of use of mechanical disruption methods across all studies. Section containing all combinations including bead beating outlined in dashed yellow.

The popularity of these three homogenisation methods is reflected across the decades (Figure 10), with bead beating remaining popular across the decades and increasing in popularity. This increase in popularity may again be related to the increased use of commercial kits. In the 2000's commercial kits became popular, and the majority of these kits use bead beating as the method of mechanical disruption. This is reflected in the increase of studies using this method between the 1990's and the 2000's. Although there remains some methodological variability, with seven methods chosen in the 2000s, this diversity diminishes in the 2010s, where only five mechanical disruption methods were utilized, with freeze-drying only being used once. By 2020, bead beating within commercial kits emerges as the sole method of choice, reflecting a convergence towards standardized practices.

The choices of mechanical disruption method appear to be in line with those recommended in the early literature. Bead beating was consistently preferred. This review was not able to judge the effects of bead beating time or bead composition

and size. Again, end use may influence this decision as bead-beating has been associated with DNA shearing impacting DNA strand length; however it has been identified as the only method capable of releasing DNA from certain organisms, such as *Bacillus globigii* and *Fusarium moniliforme* (Kuske *et al.*, 1998).

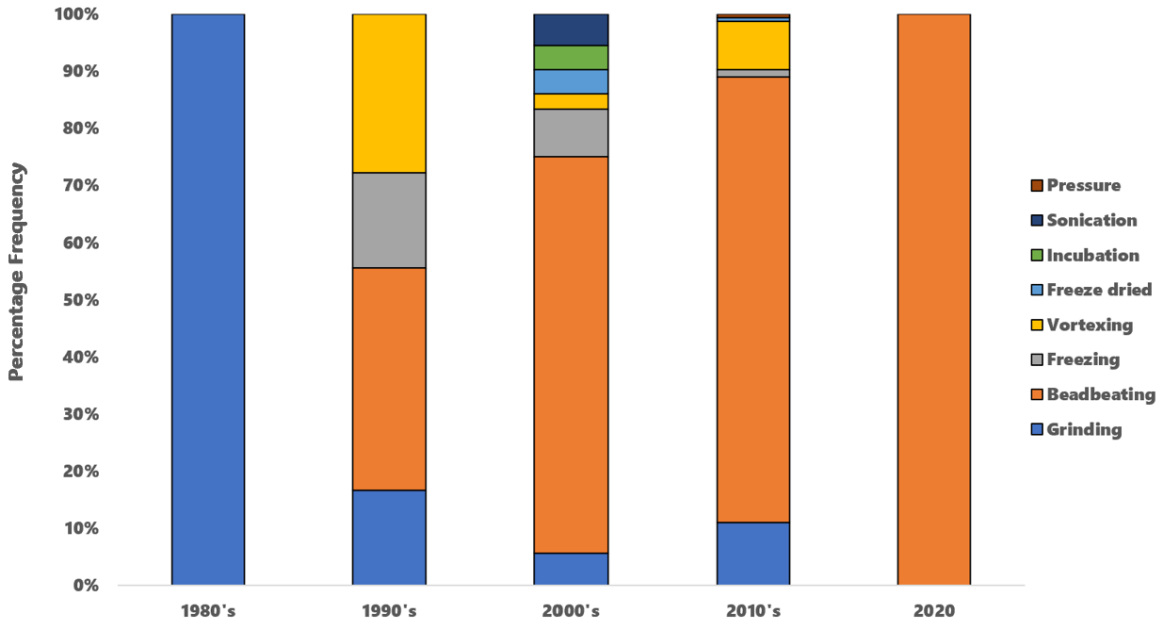

Figure 10. Frequency of first mechanical disruption methods used within each study, per decade.

Commercial kits vs bespoke methods

The choice of whether to use a bespoke DNA extraction method or a commercially available kit has been discussed throughout this review. Bespoke methods were used in 35% of studies whereas the majority (65%) chose to use commercially available kits. This popularity of commercial kits has increased over time, as shown in Figure 11. Prior to the 2000's bespoke methods dominated the studies, with commercial kits only entering the market in the late 1990's. Then in the 2000's, when commercial kits begin to dominate the market, there was a 45:65 split in the choice between commercial and bespoke methods respectively. This increase in popularity continued in 2020 (as of May) when all studies chose to use commercial extraction kits.

There have been calls for a universally used method for the extraction of DNA from soil (Martin-Laurent *et al.*, 2001; LaMontagne *et al.*, 2002; Carrigg *et al.*, 2007; Wang *et al.,* 2012; Anderson, 2016; Dopheide *et al.*, 2019). Commercial kits could offer a

solution to this as they offer ease of use and repeatability (Young *et al.*, 2014) and often utilise readily available laboratory equipment. However, they do have their disadvantages. Commercial kits are often expensive (Damm and Fourie, 2005; Guerra *et al*., 2020), difficult to automate (Budge *et al*., 2009) and their composition is often unknown and therefore is not adaptable to different scenarios (Anderson, 2016; Guerra *et al.*, 2020). Another key disadvantage of commercial kits is their removal from the market. For instance, the Ultra Clean Soil DNA Kit (MoBio) was removed from the market in 2013, impacting protocols developed by Braid *et al.* (2003), Van Den Boogert *et al.* (2005) and Gonzalez-Franco *et al.* (2009). However, they are often replaced with "improved" alternatives and continuously developed (Knauth *et al.,* 2012; Anderson, 2016). Anderson (2016) chose to compare their newly developed method to two commercially available kits; MoBio Powersoil DNA isolation kit (MoBio, US) and the SoilMaster kit (Epicenter, US), however prior to the publication of their study the MoBio Powersoil DNA isolation kit (MoBio, US) was purchased by Qiagen, possibly altering the composition and the SoilMaster kit (Epicenter, US) was discontinued completely.

Bespoke methods offer advantages and disadvantages, but countless protocols have been developed (Dequiedt *et al.*, 2012; Knauth *et al.,* 2012), each varying in their time, equipment and protocol (Kuske *et al*., 1998). Out of all the studies that used bespoke methods, only 36% referenced previously bespoke methods, implying that the remaining 64% used methods not previously used, leading to the current large variability in method choice.

The most popular commercial kit chosen was the FastDNA SPIN Kit for soil (Bio101, US) (28%) followed by MoBio Powersoil DNA isolation kit (MoBio, US) (25%) and Ultra Clean soil DNA kit (MoBio, US) (16%), all other kits were used between 1-3% of the time. Martin-Laurent *et al*., (2001) tested three different extraction methods, including two of the most popular commercial kits; Ultra Clean soil DNA kit (MoBio, US), FastDNA SPIN Kit for soil (Bio101, US) and an in-house bespoke method on 3 different soils. Both kits yielded higher 16S rRNA PCR product than the in-house method, with the FastDNA SPIN Kit for soil (Bio101, US) producing significantly higher amounts, this was the case across all three soils and their amendments (sewage sludge and farmyard manure). Similarly, in 2005, Damm and Fourie used the FastDNA SPIN Kit for soil (Bio101, US) as a 'control' when comparing 2 in-house methods. They found that the FastDNA SPIN Kit for soil (Bio101, US) outperformed

the in-house developed method in yield and was able to extract DNA from all samples, unlike the other methods, however it was not as sensitive and was unable to amplify two targeted DNA regions. They noted however that their in-house method was cheaper than the FastDNA SPIN Kit for soil (Bio101, US), but was slower. In 2007, Carrigg *et al*. performed a similar study testing 3 in-house developed methods against the Ultra Clean soil DNA kit (MoBio, US), another of the most popular kit choices. They found that the Ultra Clean soil DNA kit (MoBio, US) caused DNA shearing, unlike the other methods that produced 23kb fragments of DNA. The Ultra Clean soil DNA kit (MoBio, US) was intermediate in performance when assessed for cell lysis efficiency and extracted significantly less DNA than the other methods in some cases. However, it released less humic acids than the other method from two of the soils. The main aim of this study was to investigate the effect of DNA extraction method on bacterial community composition, they found that the kit method resulted in highly variable DGGE profiles between replicate extractions. Knauth, Schmidt and Tippkotter (2012) tested the FastDNA SPIN Kit for soil (Bio101, US) against two methods; innuSPEED soil DNA kit (IST Innuscreen GmbH, Germany) and Nucleospin soil kit (Macherey-Nagel, Germany), on 3 different paddy soils. The innuSPEED soil DNA kit (IST Innuscreen GmbH, Germany) failed to extract DNA. The key difference in this method is that it utilised silica particles to bind the DNA, as opposed to a silica membrane which is a common feature of commercial kits. The FastDNA SPIN Kit for soil (Bio101, US) caused less DNA shearing, resulting in sharper bands in electrophoresis, however the Nucleospin soil kit (Macherey-Nagel, Germany) produced purer samples. In 2019, the DNeasy PowerMax Soil Kit (Qiagen, US) was first introduced into comparative studies, this commercial kit has the largest starting sample size of 10g. Dopheide *et al*. (2019) used Illumina sequencing to assess the ability of these kits to extract DNA to evaluate communities, one of the key findings was that only 36-41% of operational OTU's were shared between extracts obtained by the different methods (MoBio Powersoil, Mobio Powermax and 2 bespoke methods), highlighting the bias introduced by each method. Then in 2020, Bollmann-giolai *et al*. compared the FastDNA SPIN Kit for soil (Bio101, US) and the DNeasy PowerSoil Pro Kit (Qiagen, US) to their developed method. The new method produced DNA fragments ranging 11.3-11.7 kb, showing low variation in fragment length, however the DNeasy PowerSoil Pro Kit (Qiagen, US) produced the largest fragments (13.9-24.4kb) but had larger variation. The FastDNA SPIN Kit for soil (Bio101, US) failed to produce fragments larger than 10kb.

Commercial kits have been frequently used to compare the extraction efficiency of new bespoke methods. Many of these comparison studies chose to use the FastDNA SPIN Kit for soil (Bio101, US) within their studies, which was the most popular commercial kit used. Martin-Laurent *et al*. (2001) and Damm and Fourie (2005) found the FastDNA SPIN Kit for soil (Bio101, US) produced the highest yields in their studies, but Damm and Fourie (2005) noted its lack of sensitivity when detecting *Phaeomoniella chlamydospora*. Similarly, Bollmann-giolai *et al.* (2020) noted that the FastDNA SPIN Kit for soil (Bio101, US) produced smaller fragment sizes than the other methods (<10kb). However, in the study by Carrigg *et al.* (2007) the FastDNA SPIN Kit for soil (Bio101, US) produced fragments of 23kb in line with the other method in the study. Perhaps these different findings are due to the different soil conditions as opposed to the performance of the kit, leading back to the issue of soil property bias. Similarly, the kits lack of sensitivity identified by Damm and Fourie (2005) might be a concern when considering end use. Ultimately, the FastDNA SPIN Kit for soil (Bio101, US) appears to be a strong choice as it performs well in a range of different comparison studies which is encouraging with it being the most popular choice in the systematic review. However, biases due to variations in soil properties, target organism, and end use remain an important factor when making methodology choices.

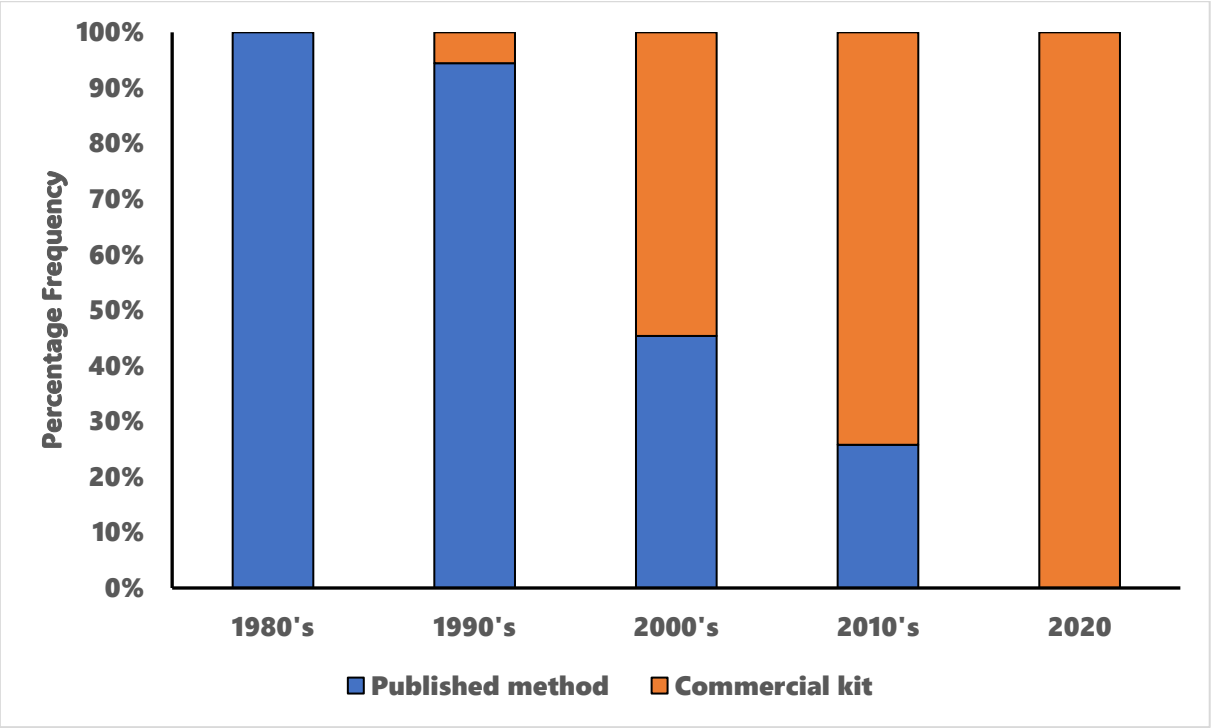

Figure 11. Frequency of bespoke published methods vs commercial kits used within each study, per decade.

Conclusion

This review aimed to give an overview of the soil DNA extraction methods used for the detection of specific organisms in soils over time and provide justification for these choices based on the experimental literature. Overall, it was found that the majority of studies opted for the method components that were most recommended by the experimental literature, when it came to lysis buffer composition and mechanical disruption methods. However, it was found that the majority of the studies used a soil starting sample size less than 1g, which was below the recommendations from the experimental literature. This review did not consider the size of the experimental area in relation to the starting sample size, although it may be an important consideration when choosing a starting sample size.

This review demonstrated the plethora of commercially available and bespoke methods available to choose from, each with many options available at each stage in the extraction process. This makes the choice of method to achieve unbiased results incredibly difficult, especially as soil type and organism bias can independently influence the success of any DNA extraction. In addition to this the range of different methods means that comparisons of results across different studies are not possible. This ability to compare across studies is of particular interest as global focus on soils

increases. The DNA extraction method has been shown to influence and be influenced by a range of different elements and therefore it is of upmost importance that we understand its role and impact in future soil biology studies.

Chapter 3- Methodology

i) Soil Sampling

Soil samples were collected from field trials across the 2018-2019 and 2019-2020 seasons. Sampling method was crop dependant on the crop sampled and according to field layout (rowing). The full sampling method for each trial is outlined in Chapter 6. Each sample was mixed thoroughly with care being taken to break up larger clusters of soil. From this the sub-sample to be extracted was removed for DNA extraction by taking small pinches of soil at random from the larger sample, making sure not to collect any stones, until the desired sample size was achieved.

ii) DNA Extraction

For field samples taken between 2018-2019, the DNeasy PowerMax soil kit (Qiagen, Germany) was used to extract DNA from 10g of sub-sampled soil. In line with the manufacturer's instructions 15ml of PowerBead solution (guanidinium thiocyanate solution) was added to the PowerMax Bead Tube, containing disrupting beads. The 10g soil subsample was then added to this tube and vortexed for 1 minute, to release DNA from the sample. C1 solution (sodium dodecyl sulphate solution) (1.2ml) was then added to this tube and vortexed for 10 minutes, aiding further with cell lysis. The sample was then centrifuged at 5000G for 5 minutes, to separate released DNA from the soil component. Supernatant was then transferred to a clean falcon tube and 5ml of solution C2 (ammonium acetate solution) was added to the supernatant and inverted multiple times to mix. This was incubated at 5^{o}C for 10 minutes to precipitate inhibitors and then centrifuged at 5000G for 3 minutes. The supernatant was again transferred into a clean falcon tube with 4ml of solution C3 (AAS-12 solution) and inverted to mix. This was incubated at 5^{o}C for 10 minutes to further remove any inhibitors and then centrifuged at 5000G for 3 minutes. The supernatant was transferred into a clean falcon tube and 30ml of solution C4 (guanidine hydrochloride & isopropanol solution) was added and inverted to mix, aiding the binding of the DNA to the silica column. The resulting suspension was then passed through DNeasy PowerMax® Soil Spin Filters (Qiagen, Germany) by centrifuging at 2,500G for 2 minutes, discarding the filtrate. This step was repeated until all the solution containing the supernatant and the C4 solution had been processed through the

column. DNeasy PowerMax soil kit C5 solution (alcool éthylique solution) (10ml) was then pipetted on to the filter column washing contaminants from the extracted DNA and centrifuged for 3 minutes at 2,500G, discarding the filtrate. The column was then centrifuged for a further 5 minutes at 2,500G to allow the silica membrane to dry. The column was then transferred to a clean falcon tube and 2ml of the DNeasy PowerMax soil kit C6 solution was pipetted onto the membrane and incubated for 5 minutes at room temperature to elute the DNA. Finally, the column was centrifuged for a final 3 minutes at 2,500g and the filtrate was collected as purified DNA in solution. Purified DNA was stored in clean 2ml tubes and stored at -18^{o}C +/- 3^{o}C.

For the 2019- 2020 season, the extraction procedure was adapted to allow DNA extraction from larger sub-samples of soil by adding additional procedures described by Woodhall *et al*. (2012). Initial homogenisation of 50g sub-samples in 100 ml CTAB buffer (120ml Sodium phosphate buffer, 20g 2% CTAB, 87.66g 1.5M NaCl, made up to 1L with distilled water, pH 8) containing 3ml antifoam B emulsion (Sigma Aldrich, US) (Woodhall *et al.*, 2012). The resulting suspensions were then disrupted by shaking in 250ml Nalgene bottles with 6 x 2cm diameter ball-bearings in a paint shaker (Merris Engineering Ltd., Ireland) for 4 minutes, to release DNA from the sample. A 50ml quantity of the disrupted sample was then centrifuged in a clean 50ml falcon tube at 5,000G for 3 minutes, to separate the released DNA from the soil. A 10ml aliquot of the supernatant was then mixed in another clean falcon tube with 9ml of 7.5M ammonium acetate, incubated on ice for 10 minutes and centrifuged at 12,000G for 3 minutes, to precipitate inhibitors from the sample. The supernatant was then vortexed with 28ml of 100% isopropanol and 2ml lysis buffer B to precipitate the DNA, leaving PCR inhibitory compounds in the supernatant (Promega Wizard™ Magnetic DNA Purification System for Food, US). The resulting suspension was then passed through DNeasy PowerMax® Soil Spin Filters (Qiagen, Germany) by centrifuging at 2,500G for 2 minutes, discarding the filtrate. This step was repeated until all the solution containing the supernatant and the isopropanol solution had been processed through the column. DNeasy PowerMax soil kit C5 solution (10ml) was then pipetted onto the filter column washing the contaminants from extracted DNA and centrifuged for 3 minutes at 2,500G, discarding the filtrate. The column was then centrifuged for a further 5 minutes at 2,500G to allow the silica membrane to dry. The column was then transferred to a clean falcon tube and 2ml of the DNeasy PowerMax soil kit C6 solution was pipetted onto the membrane and

incubated for 5 minutes at room temperature to elute the DNA. Finally, the column was centrifuged for a final 3 minutes at 2,500G and the filtrate was collected as purified DNA in solution. Purified DNA was stored in clean 2ml tubes and stored at -18^{o}C +/- 3^{o}C.

iii) qPCR

Extracted DNA was used in quantitative PCR (qPCR) employing primers and probes with specificities for different soil-borne pathogens and biocontrol agents relevant to the cropping system being investigated as well as for quantification of 16S and 18S rRNA DNA sequences for estimating DNA from whole bacterial and fungal communities.

All qPCR assays used in this project were either published in peer reviewed journals or validated by Kerr (2018) (Table 4). However, most qPCR assays are validated against DNA purified directly from cultured target organisms (Anderson *et al.,* 2003). Therefore, assays used in this project were also validated for their use in quantifying relevant targets in the range of soils studied in this thesis ((Kerr, 2018); or Chapter 4 of this thesis).

Table 4. qPCR targets, primers and probe sequences (FWD: Forward primer, REV: Reverse primer, P: Probe).

Target		Reference
Fusarium oxysporum (General)	FWD: GCTCCCCTTTCCGCGAT REV: GAATATCGCATAGAAAGAGATGTAAAG AGTTAT P:CCCCGTGCGAAACCCAAATCGAT FAM-TAMRA	Personal Communicatio n James Woodhall.
Clonostachys rosea	Confidential – Supplied from product manufacturer (Lallemand Inc., Canada)	Unpublished to date.
Stemphylium vesicarium	FWD: AGGGTCGCTACAGA CTGGGTCACT	(Graf *et al.*, 2016)

	REV: GCACTCATAAGGTTAGTAATAACTGTAGC P: CTGCTTAATGTACAGGCGAAAC FAM-BHQ	
Verticillium dahliae	FWD: CGTTTCCCGTTACTCTTCT REV: GGATTTCGGCCCAGAAACT P: CACCGCAAGCAGACTCTTGAAAGCCA FAM-BHQ	(Bilodeau *et al.*, 2012)
18S	FWD: GGRAAACTCACCAGGTCCAG REV: GSWCTATCCCCAKCACGA P: TGGTGCATGGCCGTT FAM-NFQ	(Liu *et al.*, 2012)
16S	FWD: TGGAGCATGTGGTTTAATTCGA REV: TGCGGGACTTAACCCAACA P: CACGAGCTGACGACARCCATGCA FAM-BHQ	(CDC, 2011)
Funneliformis mosseae	FWD: GGAAACGATTGAAGTCAGTCATACCAA REV: CGAAAAAGTACACCAAGAGATCCCAAT P: AGAGTTTCAAAGCCTTCGGATTCGC FAM-BHQ	(Thonar *et al.,* 2012)
Rhizophagus irregularis	FWD: TTCGGGTAATCAGCCTTTCG REV: TCAGAGATCAGACAGGTAGCC P: TTAACCAACCACACGGGCAAGTACA FAM-BHQ	(Thonar *et al.,* 2012)

qPCR reaction conditions

Irrespective of the target assay, the same qPCR reaction conditions were used throughout. All qPCR reactions were run on ViiA 7 Real-Time PCR System (Applied Biosystems), for continuity across the project. All reactions were run in 20 µl volumes in 384 well plates. Reactions were run in duplicate.

Each reaction mixture contained 10 µl TaqMan™ Environmental Master Mix 2.0 (Applied Biosystems), 4 µl molecular grade water, 0.8 µl of the forward (7.5µM) and reverse primers (7.5 µM) and 0.4 µl of the relevant dual labelled probe (5 µM).

Extracted DNA (5 µl) was added to each reaction following dilution (either 5- or 10-fold) in molecular grade water. Dilution of the extracted DNA was needed to reduce the concentration of inhibitors of Taq Polymerase that were inevitably co-extracted from the soil samples.

qPCR cycling conditions were as follows: 10 minutes initial denaturation at 95^{o}C, followed by 40 cycles of 15 seconds denaturation at 95^{o}C and 1 minute primer anneal and extension at 60^{o}C. Data is presented as cycle threshold (CT), this is the number of cycles required for the fluorescent signal to cross the threshold in a real-time PCR reaction and relates to the amount of target nucleic acid (such as DNA or RNA) present in a sample. A lower CT value indicates a higher amount of target nucleic acid in the sample, while a higher CT value indicates a lower amount.

Quantification of target DNA

For use as standards in qPCR reactions, a selection of gBlocks™ Gene Fragments (Integrated DNA technologies, US) were prepared as per manufacturer's instructions. Tubes of concentrated dried gBlock ™ Gene Fragments were initially centrifuged prior to opening to prevent the pellet from escaping. Molecular grade water was then added to achieve a final concentration of 10 ng/µl after vortexing. This was then incubated at 50^{o}C for 15-20 minutes followed by a brief vortex and centrifuge. Prepared gBlocks™ Gene Fragments were stored at -18^{o}C +/- 3^{o}C. For gBlock™ Gene Fragments design see Chapter 4.

Standard curves were created by serially diluting each gBlock™ Gene Fragment 10-fold in water. qPCR was then performed with each relevant assay to plot CT values

obtained against the known concentrations of gBlock™ Gene Fragments present in each reaction, to form a standard curve.

To quantify the amount of target DNA in a sample, the CT of the standard curve samples were plotted against the Log10 of the known pg/µl of the standard curve. From this, the equation of the trendline was used to calculate the pg/µl of the field samples ($Y = mx + c$). The quality of the standard curve was assessed by the r^2 value of the regression line, the closer the r^2 value is to 1, the better the data points fit the regression line. If the r^2 value of the line was under 0.9 then this was rejected, and the plate was repeated. The gradient of the line (*m*) equates to the efficiency of the PCR reaction, -3.3 is the optimum with 100% efficiency, a +/- 0.3 tolerance was applied (efficiency approximately 90%).

The DNA extraction method resulted in a final volume of 2000 µl of DNA, the measured amount of DNA (pg/µl) was therefore multiplied by 2000 to calculate the total amount of DNA in the whole sample, and this was then divided by the weight of soil in the extracted sample to arrive at the final value of pg/g of soil.

iv) Metabarcoding to compare general bacterial and fungal diversity of soils

Metabarcoding techniques were used to compare the relative abundance and diversity of fungal and bacterial communities in response to organic amendments applied in the field trials and the occurrence of disease. The abundance was also compared to the qPCR-quantified bacterial and fungal populations in the same samples, with the aim to identify any correlation between qPCR and metabarcoding data, for the end use of routine soil testing.

Barcode PCR

Purified DNA samples, extracted from the field trial soils as described above, were put through an initial PCR to amplify specific targeted regions. The Phusion High-Fidelity PCR kit (Thermo Scientific, UK) was used to perform the initial PCR, the combination of the mastermix was as follows:

ITS1 rRNA barcodes; 6 µl Phusion HF buffer, 0.9 µl dNTP Mix, 0.9 µl MgCl2 solution, 0.3 µl Phusion DNA polymerase, 19.1 µl molecular grade water, 0.9 µl Nex_ITS1R_Wobble (10µm) and 0.9 µl Nex_ITS1_Ky02F (10 µm) (Toju *et al.*, 2012)

(Full sequences in Appendix 2). Cycling was performed at 98ºC for 2minutes, then 30 cycles of 98ºC for 20 seconds, 54ºC for 30 seconds and 72ºC for 90 seconds, this was followed by 72ºC for 5 minutes and held at 12ºC until the next step.

For V4-16S rRNA barcodes; 6 µl Phusion HF buffer, 0.9 µl dNTP Mix, 0.3 µl Phusion DNA polymerase, 20 µl molecular grade water, 0.9 µl 806R (10um) (Apprill *et al.*, 2015) and 0.9 µl 515F (10um) (Parada *et al.,* 2016) (Full sequences in Appendix 2). PCR conditions were an initial cycle at 98ºC for 2minutes, followed by 30 cycles of 98ºC for 20 seconds, 65ºC for 30 seconds and 72ºC for 60 seconds, with a reduction of 0.5ºC per cycle down to 54ºC (22 cycles), this was followed by 72ºC for 10 minutes and held at 12ºC until the next step.

Gel electrophoresis was used to check the samples for production of the expected sized product using 1% agarose TBE gel. If the expected product was not observed (ITS1 rRNA: 150-350bp and V4-16S rRNA: 250-300bp), the initial PCR was repeated with the sample diluted in 1:10 molecular grade water. In some cases, a further dilution of 1:20 was required to achieve a product.

PCR amplified products were then purified using AMPure XP Solid-Phase Reversible Immobilization (SPRI) magnetic beads (Beckman and Coulter, UK). AMPure beads (20 µl) were added to each well of a microtitre plate and mixed thoroughly. This was then placed on a magnetic stand for 2 minutes. The supernatant was then removed from each well. The beads were washed by adding 200 µl of 80% ethanol to each well and again incubated on the magnetic stand for 30 seconds, and the supernatant removed. This ethanol wash was then repeated once more. The plate was then air dried for 5-10 minutes and removed from the magnetic stand. Dependant on the product intensity from the previous step, samples were either eluted in 4 0µl (intense) or 25 µl (weak) of molecular grade water. Samples were mixed thoroughly to resuspend the beads and incubated at room temperature for 5 minutes. The plate was then centrifuged at 280G for 20-30 seconds and placed back on the magnetic stand. Once the supernatant was clear, 35 µl or 22 µl of supernatant were transferred to a clean plate, dependant on the initial dilution volume.

Index PCR

Index PCR is performed to allow multiple samples to be pooled together and sequenced simultaneously in a single sequencing run. Unique barcode sequences, also known as indexes or primers, are added to the PCR products obtained from the

initial amplification step. These unique barcode sequences are used to tag each DNA fragment from different samples with a specific identifier.

After purifying the initial PCR product, samples underwent index PCR. Each well contained 5 µl Phusion HF buffer, 0.75 µl dNTP Mix, 0.5 µl 50mM $MgCl_2$ solution, 0.25 µl Phusion DNA polymerase, 19.1 µl molecular grade water, 2.5 µl of the sample. 5µl of the Nextera UDI indexes are added to their assigned well. PCR was then performed with the following conditions: 95^{o}C for 3 minutes, then 8 cycles of 95^{o}C for 30 seconds, 55^{o}C for 30 seconds and 72^{o}C for 30 seconds, followed by 72^{o}C by 5 minutes. It was then held at 12^{o}C until the next step.

The amplified index PCR product was then purified using the AMPure XP beads (Beckman and Coulter, UK). Beads (27.5 µl) were added to each well, mixed thoroughly, followed by incubation for 5 minutes. The plate was then placed on the magnetic stand until the supernatant was clear, which was then removed. The beads were washed by adding 200 µl of 80% ethanol to each well for 30 seconds and removed, this was then repeated. The plate was removed from the magnetic stand and air dried before being rehydrated in 17.5 µl of molecular grade water. This was then mixed thoroughly and incubated at room temperature for 5 minutes. The purified DNA sample was then transferred into a new plate.

Quantification

DNA was quantified using a Fluoroskan plate reader (Thermo Scientific, UK). Each well contained 198 µl of 99.5% 1x TE Buffer, diluted in molecular grade water using the 20x TE buffer, and 0.5% of Quant-IT Picogreen^{TM} dsDNA (Quant-iT^{TM} PicoGreen dsDNA Assay Kit, Thermo Scientific, UK), and 2µl of sample. A range of samples with high / low quantities were verified using a TapeStation 2200 (Agilent, UK) to confirm that the expected peaks were present. Two µl of sample buffer and 2 µl of prepared DNA sample was placed in each tube for the HS D1000 screentape.

Sample Pooling and Sequencing

Samples were then pooled to create a 20nM pool. The amount of each sample added was calculated to create even concentrations of each sample in the pool, this was based upon the predicted base pair length and the quantification result. Concentration of the pool was measured using the Qubit and the high sensitivity double stranded kit (Thermo Scientific, UK)

The Agilent TapeStation 2200 was again used to check that the pooled samples had a peak between 200-800 bp and at a size close to the expected amplicon size. Also, that there was no peak at 100 bp indicating primer dimers. The pool was then diluted to 4 nM. The 4 nM pool (5 µl) was then added to 5 µl of 0.2N NaOH, mixed and then incubated at room temperature for 5 minutes. Next 990 µl of the HT1 buffer from the Illumina MiSeq Reagent Kit v3 was then added and placed on ice. Separately, 5 µl of 4nM PhiX pool was combined with 5 µl 0.2N NaOH (20% PhiX) and incubated at room temperature. The DNA pool (270 µl) was added to 30µl of the PhiX and 300 µl of HT1 buffer, to create the 10pM library pool. The Illumina Miseq and a V3 600 cycle cartridge were prepared as per manufacturers protocol. 600 µl of the prepared library pool was then pipetted into the cartridge reservoir and placed in the Illimuna Miseq to perform the sequencing analysis.

Chapter 4- Method refinement

A series of preliminary experiments were performed to assess and optimise the performance of various molecular methods for use throughout the investigations reported later in this thesis. From the results of these experiments, a standard methodology was adopted. This chapter details these experiments and their outcomes.

Statistical Analysis

All analyses were conducted using IBM SPSS Statistics version 26. Data normality was evaluated using the Shapiro-Wilk test ($p > .05$), and homogeneity of variance was assessed with Levene's test. If the data met the assumptions of normality and homogeneity, parametric tests were applied, including analysis of variance (ANOVA), linear regression, and Pearson's correlation.

If the data violated these assumptions, a Log^{10} transformation was applied, and the normality and homogeneity tests were repeated. If the transformed data met the assumptions, parametric analyses (e.g., ANOVA) were conducted on the transformed dataset.

In cases where the data continued to violate the assumptions after transformation, non-parametric alternatives were employed on the original untransformed data. These included the Kruskal-Wallis test for group comparisons and Spearman's rank correlation for correlation analysis.

For the ANOVA analyses (one-way, two-way, etc.), post hoc comparisons were performed using Tukey's test to identify significant differences between group means. In addition to the post hoc tests, descriptive statistics and effect size estimates were calculated to further interpret the results. In some cases, to account for temporal variability, time was included as a covariate in the analyses, allowing for the control of its potential influence on the outcomes.

Statistical significance was determined at a threshold of $p < 0.05$.

i) Comparison of DNA Extraction Methods

Introduction

As discussed in Chapter 3 there is a plethora of both published and commercial methods for extracting DNA from soil, therefore choosing the correct method is critical. Decisions regarding DNA extraction method need to consider a range of variables, including soil type (Hu *et al.*, 2010; Dequiedt *et al.*, 2012; Young *et al.*, 2014; Schulze *et al.*, 2016), organism bias (More *et al.*, 1994; Berthelet *et al.,* 1996; Kuske *et al.*, 1998; Robe *et al.*, 2003; Bakken and Frostegård, 2006), method bias (Kuske *et al.*, 1998; Frostegård *et al.*, 1999; Sagar *et al.*, 2014) and end use (Van Elsas *et al.,* 1997; Kuske *et al.*, 1998; Roose-Amsaleg *et al.*, 2001; Vandeventer *et al.*, 2012; Sagar *et al.*, 2014; Basim *et al.*, 2020; Guerra *et al.*, 2020). All of the factors impact the efficiency of the DNA extraction and downstream analyses.

After initial analysis of samples from the 2018-2019 experimental season, concerns were raised regarding the capability of the DNeasy PowerMax Soil Kit to release DNA from the targeted organisms *S. cepivora* and *V. dahliae* due to their hardy structures, as positive and high values were expected, but not observed from the field trials. This was reinforced during preliminary testing for *S. cepivora* using the DNeasy PowerMax Soil Kit. Soils that were previously inoculated with *S. cepivora* which were subsequently allowed to enter dormancy (no active hyphae growth, only resting structures) tested negative by qPCR, despite the high initial inoculation rate. These soils had previously tested positive when hyphae were present. This indicated that the mechanical lysis method used by the DNeasy PowerMax Soil Kit was not adequate for lysing these resting structures and releasing their DNA.

Combination of a large scale soil DNA extraction method developed by Woodhall *et al.* (2012), for the detection of *S. cepivora,* with the DNeasy PowerMax Soil Kit procedure aimed to combine a larger starting volume and a more robust disruption method with the high throughput and ease of the DNeasy PowerMax Soil Kit. In comparison, the DNeasy PowerMax Soil Kit uses 0.7mm garnet beads and vortexing to disrupt the sample, whereas the Woodhall *et al.* (2012) method utilises 25mm steel ball bearings and a paint shaker.

Method

Soils were collected from two fields in the UK: from the daffodil and onion field trials respectively and prior to any experimental treatments (Table 5). Each sample was mixed thoroughly prior to DNA extraction to encourage homogeneity of the sample.

Table 5. Location and soil properties from the Daffodil and Onion field trial sites.

TRIAL	**ONION**	**DAFFODIL**
LOCATION	Bedford	Norfolk
SOIL	Clay Loam	Sandy Silt Loam
PROPERTIES	pH: 6.6	pH: 8.3
	OM%: 14.4	OM%: 3.4
	Mg: 114.0	Mg: 88.5
	K: 261.0	K: 84.2
	P: 33.8	P: 13.2

Three soil DNA extraction methods were compared. Firstly, method 1 (Figure 12), the DNeasy PowerMax Soil Kit, performed as per the manufacturer's instructions DNeasy PowerMax Soil Kit Handbook (Qiagen 11/2018 HB-2259-002) and described in Chapter 3; ii).

Method 2 (Figure 12) involved beating 50g of soil in 250ml Nalgene bottles with 6 steel balls (1-inch diameter, Grade 316), in a paint shaker (Merris Ltd.) for 4 minutes, in 100 ml of Powerbead solution with 6 ml of buffer C1, both from the DNeasy PowerMax Soil Kit, acting as a lysis buffer. The sample was then centrifuged in a falcon tube for 30 seconds at 5000 G. Next 20 ml of this sample was then put through the DNeasy PowerMax Soil Kit from step 7 of the DNeasy PowerMax Soil Kit Handbook

Method 3 (Figure 12) combined the large-scale extraction method (Woodhall, *et al.*, 2012) and the DNeasy PowerMax Soil Kit, which increased initial starting volume but utilised the cleaning and purification from the DNeasy PowerMax Soil Kit. See Chapter 3; ii) for full method description. Each sample was extracted using each method with 4 replicates.

Total bacterial and fungal populations in each sample were quantified using total 16S and 18S rRNA qPCR assays (for method see Chapter 3; iii), however samples were not diluted. All reactions were run on a single plate so that CT values could be compared without quantification with a standard curve.

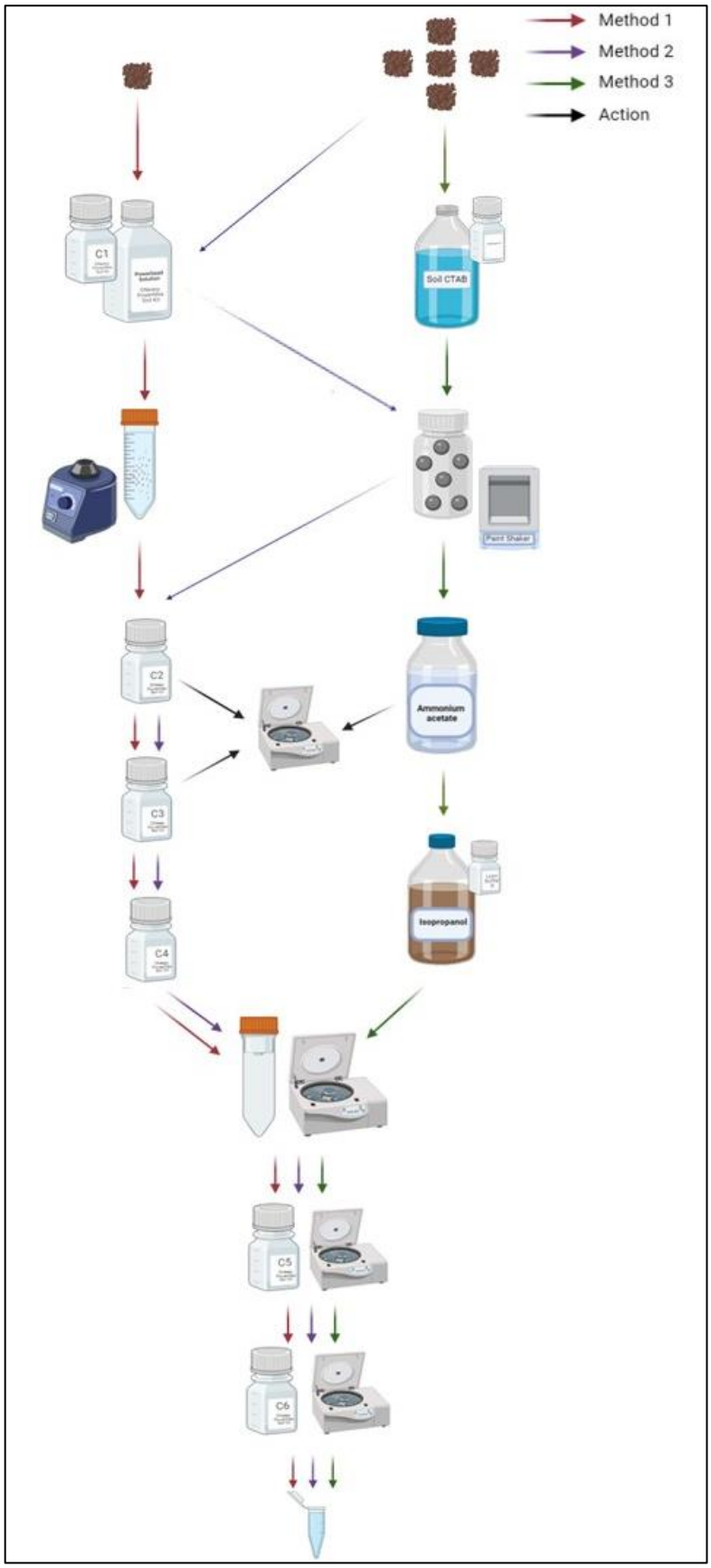

Figure 12. Diagram of the method flow of the three methods tested. Red arrow: Method 1, Purple Arrow: Method 2, Green Arrow: Method 3. Created using Biorender.com

Results and discussion

Method choice appeared to have no significant effect on the CT values derived from each sample, apart from soil from the Daffodil site when analysed with the 16S rRNA qPCR assay (Figure 13) (p=0.158-0.856). This effect was analysed using a one-way ANOVA. In this case method 3 resulted in the lowest mean CT of 16.05, compared to 17.2 and 16.6 from methods 1 and 2 respectively. Conversely, this method resulted in higher CT values than the other methods when the same sample was tested using the 18S rRNA assay (Figure 14). The insignificance of method found may be due to the small sample selection tested and that there were only 4 repeats. Due to this insignificance decisions based on what method to use for the remainder of the project were primarily based on logistical capabilities.

There was higher variation between 18s rRNA assay results when using extraction method 1, when compared to the other methods. Furthermore, this method used the lowest sample volume. Although sample volume appeared to have minimal effect when quantifying total bacterial and fungal populations, this may impact the likelihood of detecting target organisms that may be less abundant and less homogenous in soil samples. This is also in line with the findings in Chapter 2 (Review of methods for extraction of DNA from soils), where larger samples were favoured to smaller sample sizes.

Both methods 2 and 3 used 50g of soil as their starting volumes, making them favourable due to their larger starting volume. From a logistical perspective, method 2 utilised a larger volume of the solutions than what was provided by the manufacturer of the kit and therefore relied on the excess reagents from previous uses. Currently (2021), it is not possible to purchase these solutions separately and their formula is protected, further evidencing the dilemma of using manufactured kits in a standardised procedure, as discussed in Chapter 2. Due to the reasons discussed above, method 3 was chosen to be used for the remainder of the experiment. In summary this is due to its larger starting volume, more robust disruption method and accessibility to reagents. Method 3 also has the additional incentive of it being the best method in the only significant combination.

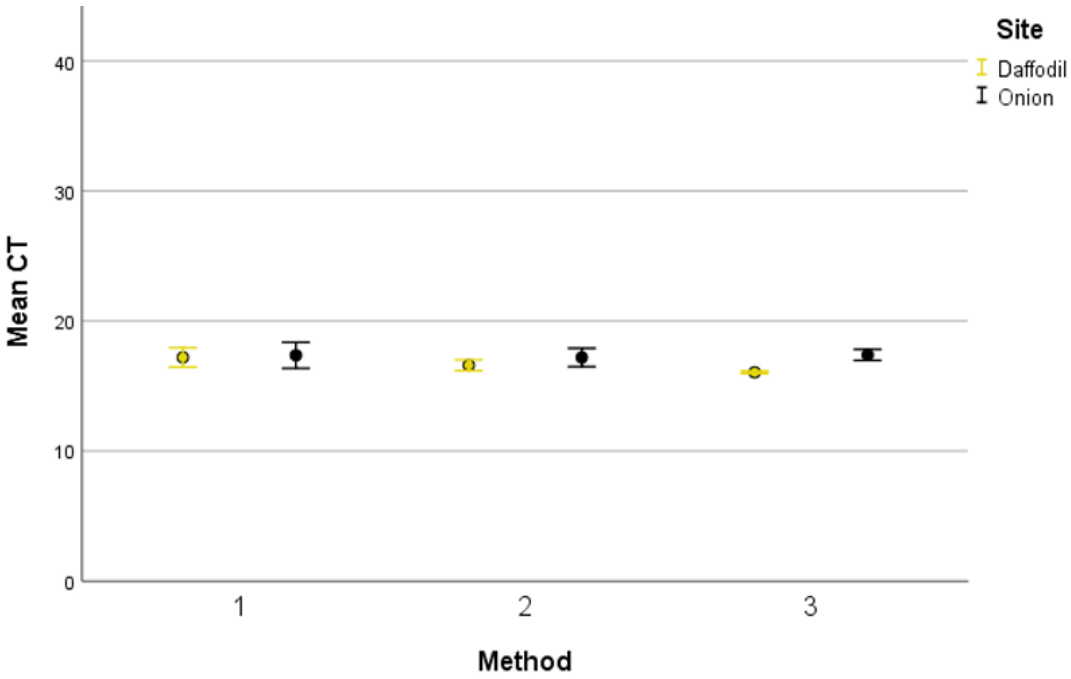

Figure 13. Mean CT values obtained with 16S rRNA positive control assay, for samples collected from the onion and daffodil field trials. A 3-fold increase in CT denotes an approximately 10-fold decrease in DNA target concentration. Error bars= +/- 2 SD (n=14).

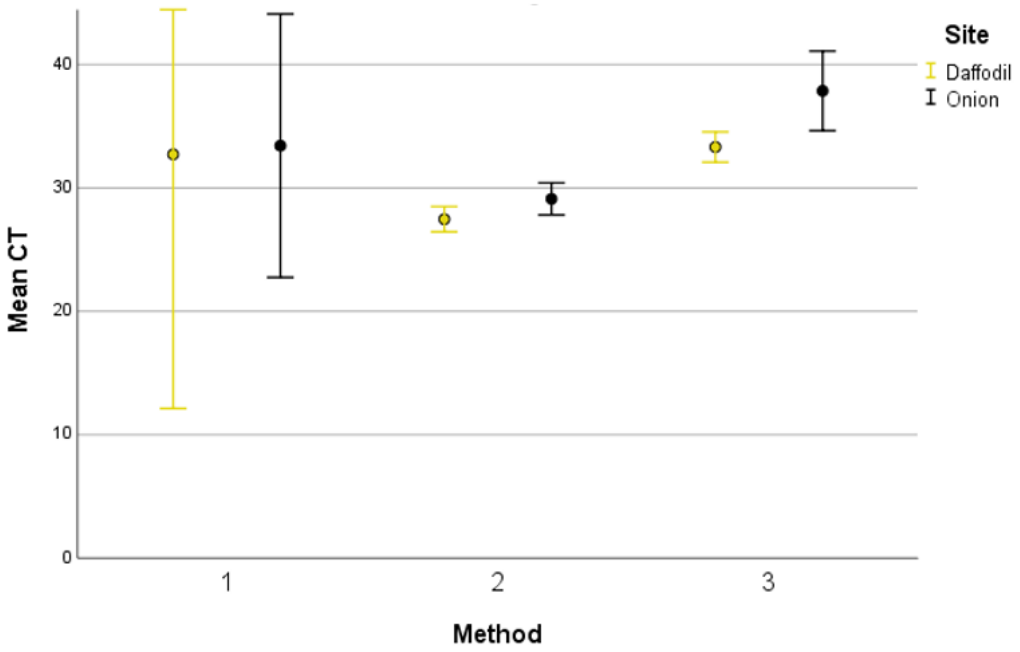

Figure 14. Mean CT values obtained with 18S rRNA positive control assay for samples collected from the onion and daffodil field trials. A 3-fold increase in CT denotes an approximately 10-fold decrease in DNA target concentration. Error bars= +/- 2 SD (n=14).

ii) Purification of DNA samples using Powdered Activated Charcoal

Introduction

DNA extracted from soils often contains substances that can inhibit downstream analyses. Activated charcoal is known for its ability to remove humic substances (Starek *et al.,* 1994; Barbaric *et al.,* 2015) and is often used in air and water filtration, leading to its potential for use in DNA extraction. It is a highly porous material and its large surface area increases its ability to adsorb large amounts of humic substances (Verma and Satyanarayana, 2011). The meso- and macropores in activated charcoal adsorb humic substances (Starek *et al.,* 1994). Activated charcoal has previously been used at different stages in the DNA extraction process: Barbaric *et al.* (2015) used powdered activated charcoal (PAC) to remove PCR inhibitors from the final DNA extract and Verma and Satyanarayana (2011) and Sharma *et al.* (2013) also utilised PAC in the lysis stage of DNA extraction. In this experiment, we chose to assess the ability of PAC to remove PCR inhibitors from extracted DNA.

Method

To continue on from the work completed in the Chapter 4, i) Comparison of DNA Extraction Methods, repeats of the onion site samples from each DNA extraction method underwent further purification using activated charcoal.

For each sample, 200 µl of extracted DNA were added to different amounts (2%, 5% or 10%) of powdered activated charcoal (PAC), to try to remove humic substances from the final DNA sample and improve PCR efficiency. After vortexing and incubation at 30 ºC for 1 hour (modified from (Atkins and Clark, 2004), each sample was centrifuged at 16000G for 5 mins and the supernatant then transferred to a clean tube. Each sample was tested by qPCR using a 16S rRNA TaqMan assay to estimate total bacterial populations. Results were compared with extracts not treated with activated charcoal, and the same extracts diluted 1:5 in water.

Results and discussion

The use of activated charcoal for removal of inhibitors from extracted DNA did not improve qPCR analyses of DNA extracted from samples tested (Figure 15). Improvement was assessed by comparison of CT value compared to the undiluted sample, with a lower CT indicating better performance. Statistical analysis (ANOVA) showed there was no significant difference of the effect of activated charcoal

between all samples on CT (p= 0.089-1.000), apart from in one interaction Undiluted and 5% PAC (p=0.016). It appeared that 5% PAC performed the worst for all three methods and undiluted performed consistently well, with low CT values. In conclusion, the additional clean with PAC offered no benefit when compared the undiluted sample.

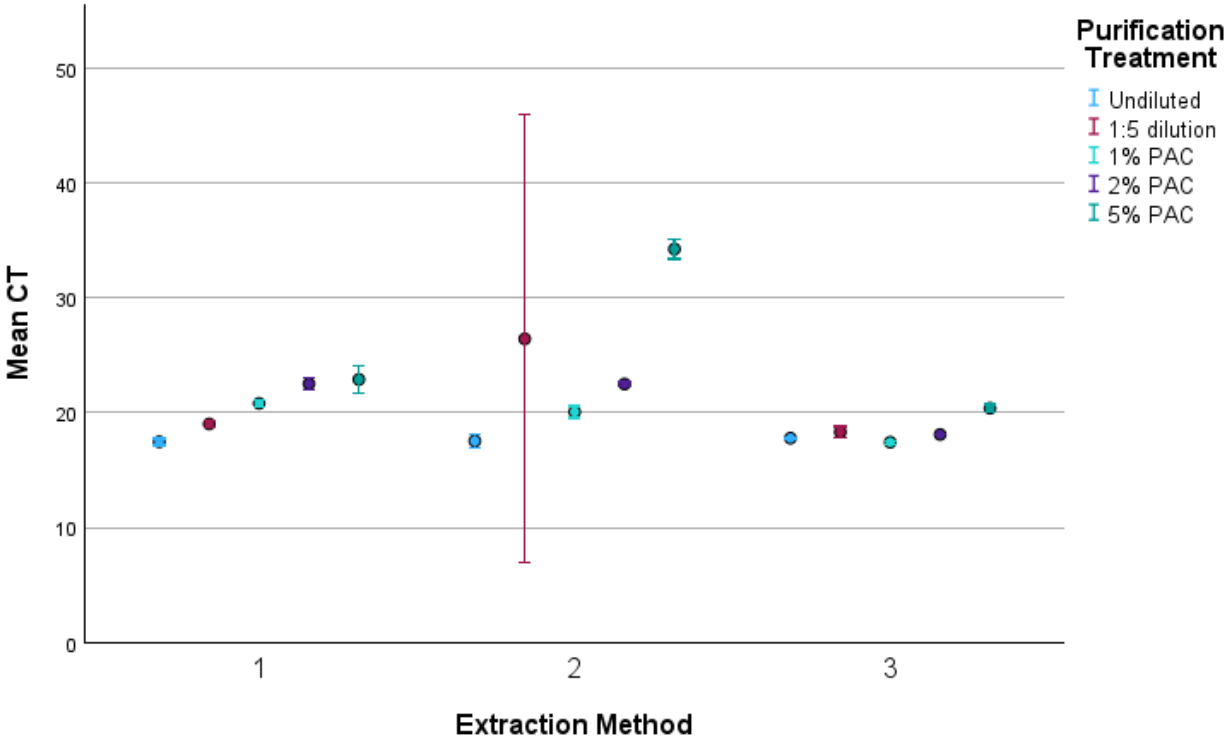

Figure 15. Mean CT values obtained by qPCR assays targeting 16S rRNA gene DNA extracted by three methods, either after purification with PAC or after aqueous dilution (Error Bars +/- 2 standard deviation) (n=15).

iii) Addition of Activated Charcoal and $MgCl_2$ to the DNA Extraction Method for improved inhibitor removal

Introduction

As discussed in the section above activated charcoal can be used to remove PCR inhibitory substances during the DNA extraction process. Verma and Satyanarayana (2011) found the addition of PAC during the lysis of microbial cells significantly reduced co-precipitation of humic substances along with DNA for the end use of restriction digestion.

In addition to the use of activated charcoal within DNA extraction, $MgCl_2$ has also been tested in this study. $MgCl_2$ binds with DNA to protect it from DNase activity and blocks negative charges of lipoproteins, and therefore it is commonly used in the lysis stage of DNA extraction. $MgCl_2$ has been used previously by Hu *et al.* (2010) and Braid *et al.* (2003) to remove humic substances from the final DNA sample and by Sharma *et al.* (2013) in the lysis stage. Magnesium has been shown to increase coagulation of humic substances when used to remove humic substances from water (Vik, 1988). Sharma *et al.* (2013) found that the combination of $MgCl_2$ and activated charcoal yielded highly pure DNA free from humic acids and other contaminants, with minimal DNA loss in comparison to other purification methods. In line with these studies the addition of activated charcoal and $MgCl_2$ to the lysis buffer was tested to minimise inhibitors in extracted DNA.

Methods

Three soil samples were selected at random from different field trial sites to test if $MgCl_2$ and PAC could be used to improve the purity of DNA extracted. The soil from the Raspberry trial was a sandy silt loam with a pH of 8.2 and organic matter of 2.2%, the soil from the Daffodil trial was sandy silt loam with a pH of 8.3 and organic matter of 3.4% and the soil from the onion trial (pre-treatment) was a clay loam with a pH of 6.6 and organic matter of 14.4%.

Each sample was extracted twice, once using the standard method 3 protocol and again with the addition of 1% of activated charcoal and 10 mmol/L $MgCl_2$ to the soil cetyltrimethylammonium bromide (CTAB) buffer.

Samples were then analysed, using the 16S and 18S rRNA qPCR assays, method as described in Chapter 3; iiI). Samples were also diluted in a 10-fold series to

evaluate any potential effect of humic acid inhibition on the qPCR results. All qPCR reactions were run on a single plate so that CT values could be compared without quantification with a standard curve.

Results

The addition of $MgCl_2$ and activated charcoal to the CTAB buffer mix had no significant effect on the CT values achieved for the 16S rRNA assay (p=0.845) or the 18S rRNA assay (p=0.888) and, in most cases, produced higher CT values for all dilution levels (Figure 16). There was no significant interaction between dilution and the addition of $MgCl_2$ and activated charcoal for either 16S rRNA (p=0.874) or 18S rRNA (p=0.968). This means that for these samples, at the levels tested, the combination of $MgCl_2$ and activated charcoal within the soil CTAB buffer did not improve inhibition control within the qPCR reaction (inhibition was confirmed by an observed decrease in CT after diluting the extracted DNA when analysing using the 16S rRNA qPCR assay (Figure 17)).

When assessing the effect of dilution on CT for the 18S rRNA assay the 10-1 dilution performed the best as it achieved a lower CT than the undiluted samples (Figure 17) (p=<0.001), this may be due to the 18S rRNA (or fungi) assay being more sensitive to soil characteristics (like pH, structure and organic matter content) and inhibition. Further dilution did not improve the CT value meaning that the optimal dilution in this series to limit inhibition was 10^{-1}; further dilutions diluted the sample below limit of detection. For the 16S rRNA assay the undiluted sample had the lowest CT values and this was significant according to a two-way ANOVA (p=<0.001). Further dilution did not improve the CT values, as seen in the 18S rRNA assay results.

These results indicated that the 18S rRNA assay (fungi) was more sensitive to inhibitors than the 16S rRNA assay (bacteria), due to it requiring dilution to overcome inhibition. Due to the results of this experiment all subsequent DNA extracts were diluted 10^{-1} as standard to minimise any qPCR inhibition.

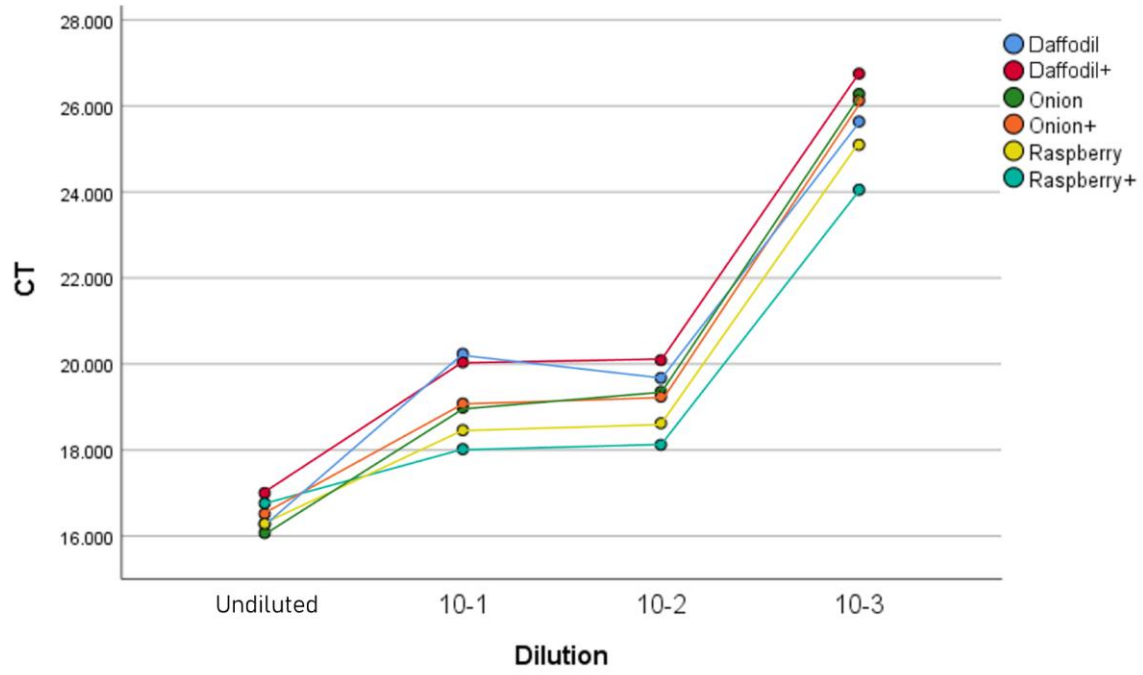

Figure 16. Mean CT values obtained by 16S rRNA qPCR after DNA extraction from 3 soils with or without addition of $MgCl_2$ and activated charcoal before and after dilution of the extracted DNA ('+' denotes the addition of $MgCl_2$ and activated charcoal) (n=12).

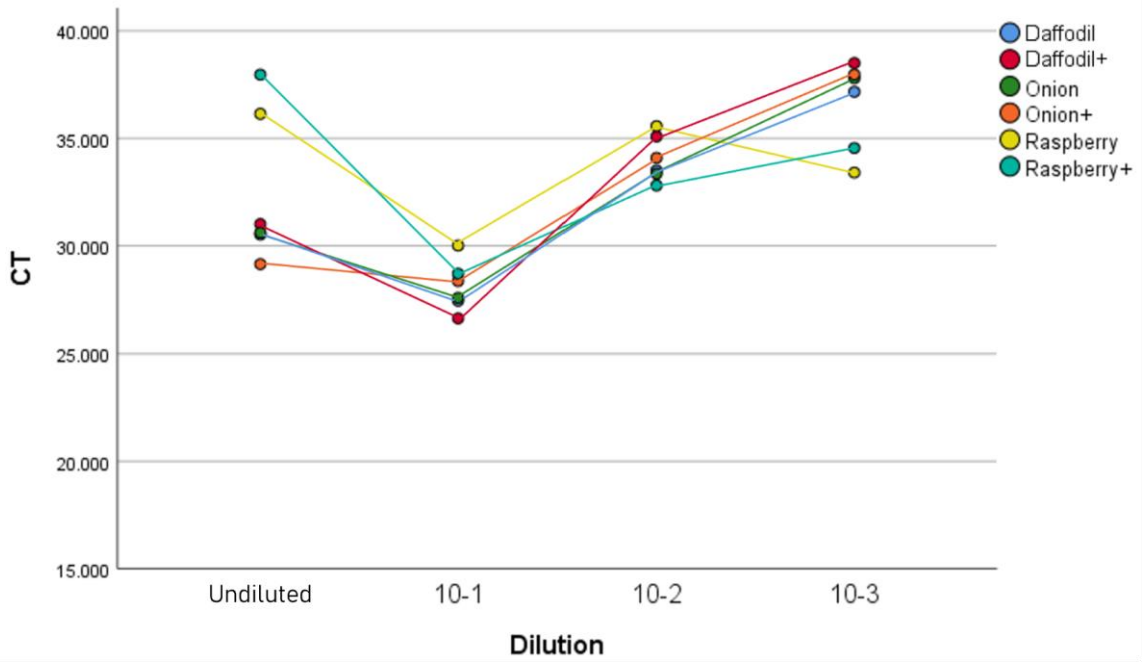

Figure 17. Mean CT values obtained by 18S rRNA qPCR after DNA extraction from 3 soils with or without addition of $MgCl_2$ and activated charcoal before and after dilution of the extracted DNA ('+' denotes the addition of $MgCl_2$ and activated charcoal) (n=12).

iv) Addition of Bovine Serum Albumin to qPCR reaction to limit PCR inhibition

Introduction

To limit the effects of PCR inhibition many papers describe extensive DNA extraction methods in an attempt to purify the extracted DNA. Inhibiting substances include: proteins, polysaccharides, non-target nucleic acids, polyphenols, heavy metals, soil and cell debris, and fulvic and humic acids (Saleh-Lakha *et al.*, 2005; Bakken and Frostegård, 2006; Gibson *et al.*, 2012; Wang *et al.,* 2012; Hargreaves *et al.,* 2013; Sharma *et al.,* 2013; Sagar *et al.*, 2014; Hebda and Foran, 2015), and can impede downstream uses, such as qPCR and cloning, through a variety of different mechanisms such as outcompeting nucleic acids (Vik, 1988; Roose-Amsaleg *et al.*, 2001; Lakay *et al.,* 2007; Hu *et al.*, 2010; Wang *et al.,* 2012; Hebda and Foran, 2015).

These additional purification steps risk loss of DNA, risk quantification errors, as well as increasing expense and time of the procedure (Kreader, 1996; Wang *et al.,* 2007; Plante *et al.*, 2011; Cao *et al.*, 2012). Therefore recommendations have been made to relieve interference by inhibitory substances at the end use stage, rather than attempting their removal (Kreader, 1996; Plante *et al.*, 2011). This can be achieved by either the addition of certain compounds to the PCR reaction or by dilution of the crude extract.

Dilution of the crude extract in water is regularly used to limit the effects of inhibitors. Cao *et al.* (2012) used a 1:5 dilution to reduce inhibition when testing for *Enterococcus* in DNA extracted from water samples, this was successful in 78% of samples in the study. Dilution works by reducing concentrations of the inhibitors in the sample, however this in turn dilutes the target DNA in the sample, risking it falling below the limit of detection (Wang *et al.,* 2007; Schriewer *et al.,* 2011; Cao *et al.*, 2012). This can be particularly detrimental in cases where the target is in low concentrations.

An alternative to dilution is the addition of compounds to the PCR reaction. These include, Dithiothreitol (DTT), T4 gene 32 protein (GP-32), glycerol and Bovine Serum Albumin (BSA). DTT is a reagent used to stabilize enzymes, it works by protecting sulfhydryl groups of cystine residues, this is of particular importance in PCR as Taq DNA polymerase contains four cysteine residues (Nagai *et al.,* 1998); GP-32 is a

single stranded DNA binding protein, it may assist PCR by binding with denatured strands of DNA and prevent reannealing (Kreader, 1996); Glycerol has been linked to boosting hydrophobic interactions between proteins, lowering strand separation temperatures, raising protein transition temperature and denaturing secondary structures (Nagai *et al.,* 1998); and BSA is a protein used as a stabilizing agent in enzymatic reactions (Nagai *et al.,* 1998; Plante *et al.*, 2011; Cao *et al.*, 2012) and furthermore enables coordination of the polymerase, target DNA and magnesium ions, that are all fundamental in PCR (Schriewer *et al.,* 2011).

Nagai *et al.* (1998) tested the effects of BSA, DTT and glycerol on detecting *E. coli* using PCR. They recommended concentrations of 1mg/ml BSA, 10mM DTT and 5% glycerol. They found that not only the individual use of these additives improved PCR performance, but they had a complementary affect when used together. They hypothesised that this indicated that the three additives utilise different mechanisms.

Kreader (1996) tested the efficacy of BSA and GP-32 on limiting inhibition effects of PCR in faeces, freshwater and marine water. Samples were inoculated with *Bacteroides distasonis* and *Bacteroides vulgatus* and inhibited at different levels with various inhibitors. In most cases BSA and GP-32 reduced inhibition when compared to undiluted but was unsuccessful in all cases when inhibited with 1mM of EDTA. Unlike with BSA, DTT and glycerol, the combination of the two added no further benefit. Kreader, (1996) recommended between 200-400 ng/µl for BSA and 100-150 ng/µl GP-32 for humic acid inhibition.

BSA has a high success rate in a range of matrices for instance as used by Garland *et al.* (2010) when detecting *Batrachochytrium dendrobatidis* from the skin swabs of amphibians, where 72.5% of swabs that were previously completely inhibited produced signal when BSA was used. Similarly, Wang, Olson and Chang (2007) found increased amplicon brightness with the use of 100 ng/µl BSA when detecting the gene associated with the enzyme hydrogenase A (a gene found within the Clostridia genome) in environmental sludges and also by Plante *et al.* (2011) when testing for multiple human virus pathogens in water from rinsed vegetables, they also showed the addition of BSA restored positive signal in all samples.

BSA was investigated here for its use in qPCR analysis of DNA extracted from soil samples to minimise effects of inhibitors, as a relatively low cost PCR additive at approximately £3 per mg For instance, Kageyama *et al.* (2003) assessed the

efficacy of three different forms of BSA; BSA Wako, BSA fraction V and BSA Fraction F (fatty acid free), at a rate of 0, 200, 400 and 800 ng/µl, on the detection of soil-borne pathogens in soil samples. There was no difference observed between the types of BSA used and the effect of concentration was dependant on the soil type and target. For example, BSA enhanced the amplification of *Pythium ultimum* in one soil type and was essential for detecting *V. dahliae* in another soil type but had no effect on detecting *Plasmodiophora brassicae* in the third soil type.

However, the use of BSA comes with its own risks. Schriewer *et al.* (2011) evaluated BSA's effect on qPCR in water samples detecting *Acinetobacter baylyi* ADP1. Samples were spiked with humic acid standards and the bacterium *Acinetobacter baylyi* ADP1. They found that BSA improved CT (lower values) but worsened PCR efficiency. Although detection was improved, copy numbers detected were down which may impact the application of this approach for quantification. Furthermore, they noted melting temperatures were altered, which was supported in the study by Wang *et al.* (2007) which also found melting temperatures were altered by the use of BSA which in turn altered gene copy number. Schriewer *et al.* (2011) theorised that BSA may be most effective in the initial PCR stages, when polymerase and magnesium ions are abundant and the target DNA is the limiting factor, leading to the exponential phase being reached sooner. However, at the later stages when the concentration of facilitators and target are reversed, the effect of BSA appears to lessen. They theorised that this may be due to the degradation of the BSA molecules in response to the temperature cycles or the simple steric inhibition by the bulkier humic molecules. This finding that BSA is most effective at the early stages of PCR was substantiated by Farell and Alexandre (2012).

Method

Across the project field trials 5 soil DNA extracts were chosen to include an example of both extraction methods from each site (Table 6). A further 2 samples were taken from the preliminary soil testing from glasshouse experiments, these were 'Rothamsted Prescription Mix' compost (75% L&P medium peat, 12% ST loam, 10% Grit, 3% vermiculite) that had been inoculated with *V. dahliae* at two different levels and extracted using method 3 (method outlined Chapter 4; i).

Table 6. List of samples tested, the year collected, and extraction method used, for assessment of the application of BSA to PCR reactions to limit inhibition.

Site	Year	Extraction Method
Raspberry	2019	DNeasy PowerMax soil kit (Qiagen, Germany)
Raspberry	2020	Method 3 (method outlined Chapter 4; i)
Daffodil	2019	DNeasy PowerMax soil kit (Qiagen, Germany)
Daffodil	2020	Method 3 (method outlined Chapter 4; i)
Onion	2019	DNeasy PowerMax soil kit (Qiagen, Germany)

Each sample was tested using 16S rRNA, 18S rRNA, *F. oxysporum* and *V. dahliae* specific qPCR assays (Table 4. qPCR targets, primers and probe sequences (FWD: Forward primer, REV: Reverse primer, P: Probe). Each well contained 10µl Applied Biosystems TaqMan™ Environmental Master Mix 2.0, 0.8µl of the 7.5µM forward and 7.5µM reverse primers and 0.4µl the 5uM dual labelled probe, plus one of the 5 treatments (Table 7).

Table 7. List of treatments and PCR reaction protocol for testing of BSA in PCR reaction to limit inhibition.

Treatment	PCR reaction protocol
Undiluted	5µl Sample + 4µl Molecular grade water
10:01 Dilution	5µl 1:10 diluted Sample + 4µl Molecular grade water
0.2 BSA	5µl Sample + 4µl 0.2 µg/µl BSA
2 BSA	5µl Sample + 4µl 2 µg/µl BSA
20 BSA	5µl Sample + 4µl 20 µg/µl BSA

The qPCR reactions were run on ViiA 7 Real-Time PCR System by Applied Biosystems, for continuity across the project. All samples were run on 20µl 384 well microtitre plates in duplicate. The cycling conditions were as follows; 10 minutes at 95^{o}C, followed by 40 cycles of 95^{o}C for 15 seconds and 1 minute at 60^{o}C.

Results

Among the 84 combinations of BSA, target, and site, only three instances resulted in a notable improvement (lowering of the resulting CT in comparison with the reactions without BSA). Treatments with improved PCR efficiency were: (0.2 BSA: Daffodil 2019 (18S), Onion (*F. oxysporum*), Raspberry 2019 (*V. dahliae*) and 2 BSA: Onion (*F. oxysporum*), when compared to the undiluted sample. Conversely, 54 combinations showed no detection, and 27 exhibited a higher CT than the undiluted sample (Table 8).

qPCR on DNA extracts diluted 1:10 demonstrated enhanced detection in 16 out of 28 cases, reinstating signal in all instances where no detection had occurred in the undiluted extracts. This finding aligns with the recommendations of previous studies in the studies by Cao *et al.* (2012) and Plante *et al.* (2011), which advocated 1:5 and 1:10 dilutions respectively. However, it is crucial to note that dilution, while aiding in signal restoration, may result in reduced sensitivity, potentially explaining the observed worsened sensitivity of detection in certain cases with 1:10 dilutions compared to the undiluted extracts (16S: Daffodil 2018 and 2019, Onion, Raspberry 2019; *F. oxysporum*: Daffodil 2018, Onion; *V. dahliae*: Daffodil 2018) (Table 8).

Effects of dilution and BSA additions varied according to type of soil sampled and also the qPCR target. Guy *et al.* (2003) reported in their study that the addition of BSA removed inhibitory effects from some samples but not others, indicating that the effect of BSA may be dependent on the sample, this may be due to its composition and/or the inhibitors it contains (Schriewer *et al.* 2011). Furthermore, the effect of BSA seemed to depend on PCR assay composition, Plante *et al.* (2011) also experienced that the effects of BSA were dependant on primer-probe combinations. The 18S rRNA assay appeared to improve greatly with extract dilution at all sites, which is in line with the previous work earlier in this chapter, (Chapter 4: iv), namely that the 18S rRNA assay appeared to be more sensitive to inhibition and therefore benefited from the dilution (Figure 17). This may be attributed to the fact that BSA has been shown to alter melting temperatures of assays (Nagai *et al.,* 1998;

Schriewer *et al.,* 2011). Guy *et al.* (2003) found that the addition of BSA reduced PCR amplification of the COWP gene to 70%, but did not affect the β-giardin assay PCR efficiency. Similarly, (Clark and Hirsch, 2008) found that in their study the PCR amplification using Clostridum Hyd A and *C. butyricum* Hyd A primers were enhanced by the addition of BSA due to the alteration of melting temperatures, increasing observed peak height. However, enhancement of the melting temperatures is not expected in all cases and BSA supplementation may also be detrimental, dependant on primer-probe composition.

Due to the above findings, it was decided that standardised procedures to be used in this project would use extract dilution over the use of BSA for all subsequent qPCR analyses. Furthermore, BSA affects the PCR efficiency and resulting target copy numbers which therefore has a direct impact on the ability to quantify the initial targets (Garland *et al.*, 2010; Plante *et al.*, 2011), a key aim of this research. This impact on quantification was discussed in the study by Garland *et al.* (2010), which described it being problematic to correct the variation in effect by BSA when comparing different skin swabs, each with different levels of inhibition. This difficulty to correct for the varying effects of BSA may be linked to the uncertainty surrounding its mode of action. In the study by Cao *et al.* (2012), which investigated the use of various Taq mixes/methods (TaqRegular, TaqFast, TaqFastfast, TaqEnviron and ScorpionN), recommended dilution in combination with reagents designed specifically for use in environmental samples. As such, the final method selected for use in this thesis used TaqMan™ Environmental Master Mix 2.0 (Applied Biosystems, US), which is designed for use with environmental samples with high levels if inhibitors.

Table 8. Achieved CT value for each sample tested under different PCR reactions treated with different concentrations of BSA, 10:01 dilutions or undiluted. (+ = CT lower, - = CT higher, ND =CT>40).

Target	Site	Undiluted	10:01	0.2 BSA	2 BSA	20 BSA
16S	Daffodil 2019	16.17	19.06(-)	16.21(-)	16.32(-)	27.51(-)
	Daffodil 2018	13.5	16.67(-)	13.54(-)	13.62(-)	19.49(-)
	Inoculated Compost (medium)	34.47	22.18(+)	36.27(-)	37.12(-)	ND
	Inoculated Compost (high)	21.29	20.79(+)	34.96(-)	38.89(-)	ND
	Onion	15.39	18.59(-)	15.45(-)	15.61(-)	25.84(-)
	Raspberry 2019	15.98	16.94(-)	16.08(-)	16.28(-)	25.32(-)
	Raspberry 2018	ND	16.46	ND	ND	ND
18S	Daffodil 2019	32.77	28.01(+)	32.68(+)	35.62(-)	ND
	Daffodil 2018	25.37	24.96(+)	27.41(-)	29.45(-)	ND
	Inoculated Compost (medium)	ND	32.73(+)	ND	ND	ND
	Inoculated Compost (high)	ND	29.78(+)	ND	ND	ND
	Onion	27.92	27.59(+)	33.03(-)	35.89(-)	ND
	Raspberry 2019	34.03	26.49(+)	ND	ND	ND
	Raspberry 2018	ND	27.032(+)	ND	ND	ND
FO	Daffodil 2019	ND	37.1(+)	ND	ND	ND
	Daffodil 2018	34.15	36.76(-)	34.36(-)	34.95(-)	36.64(-)
	Inoculated Compost (medium)	ND	36.9(+)	ND	ND	ND
	Inoculated Compost (high)	ND	36.66(+)	ND	ND	ND
	Onion	32.61	34.85(-)	31.91(+)	32.12(+)	36.83(-)
	Raspberry 2018	ND	37.01(+)	ND	37.74	ND
	Raspberry 2019	ND	36.7(+)	ND	ND	ND
VD	Daffodil 2019	ND	38.8(+)	ND	ND	ND
	Daffodil 2018	37.09	37.95(-)	38.04(-)	39.36(-)	ND
	Inoculated Compost (medium)	ND	35.32(+)	ND	ND	ND
	Inoculated Compost (high)	ND	32.06(+)	ND	ND	ND
	Onion	39.66	38.34(+)	ND	ND	ND
	Raspberry 2019	39.57	34.12(+)	38.04(+)	ND	ND
	Raspberry 2018	ND	37.53(+)	ND	ND	ND

v) Design and validation of gBlock™ gene fragments for improved accuracy of target quantification when using qPCR.

Introduction

qPCR is widely used to quantify target DNA from environmental samples. This is often done by comparing signal intensity of the template to a reference and extrapolation from a standard curve (Atkins and Clark, 2004; Marmiroli and Maestri, 2007), as described in Chapter 2; ii). These standard curves are often derived from the results of qPCR performed on serially diluted target DNA derived from positive control material that has been quantified via other means, for example NanoDrop spectrophotometry or Qubit Fluorometric Quantification, or according to direct measurement of the initial sample i.e. spore counting or weight of mycelium (Deora *et al.*, 2015). Papers which have used diluted positive control material to create a standard curves include: Madigan *et al.* (2019) in human related organisms, and by Filion *et al.* (2003), Atkins and Clark (2004), Budge *et al.* (2009) and Woodhall *et al.* (2012) for plant pathogens in soils.

Ideally the DNA used for preparation of the standard curve should have a similar amplification efficiency to that of the target organism requiring quantification in the sample (Marmiroli and Maestri, 2007), this has been reported to have led to a 4-fold error when the PCR efficiencies only differ by 0.4 (Schriewer *et al.* 2011). Extraction efficiencies are rarely considered and pose a challenge when quantifying with standard curves, however some have proposed mathematical solutions. Standard curves, generated with positive control material extracted from target organisms, hinge on the effective extraction of DNA from the material. The efficiency of this extraction process is critical, as it can inadvertently introduce inhibitory substances that may influence the subsequent amplification efficiency. Consequently, any inaccuracies in the initial quantification of the positive control material may be further compounded by the presence of these inhibitory substances.

Furthermore, Marmiroli and Maestri (2007) stated that ideally the source of positive control DNA used to create a standard curve needs to be as close as possible to both size and sequence of the qPCR target. When quantifying the positive control material for use as a standard curve, quantifying the whole genome of the target rather than just the region of interest can lead to over estimation of the population size in the sample, particularly with assays based upon regions with high gene copy numbers.

gBlocks™ are double stranded gene fragments that are synthetically produced at known copy numbers, allowing for more accurate quantification by qPCR. gBlocks™ are becoming more prominent in their use as standard control materials when quantifying DNA from environmental samples. For instance; Balážová *et al.* (2020) used gBlocks™ to quantify zoonotic pathogens, as did Yang *et al.* (2021) and Billones-Baaijens *et al.*, (2018) for detection of plant pathogens, and by Sauvageau *et al.* (2019) for detection of plant pathogens in soils. A further benefit of using a gBlock™ is that they are specifically designed for each qPCR assay allowing optimum binding and removing the risk of mutations which limit binding when genomic DNA is used.

Furthermore, gBlocks™ produce lower values for quantification than positive control standard curves, this is due to positive control standard curves quantifying the whole genome from the target organism, whereas gBlocks™ only quantify the primer targeted region of the genome (~100bp). This can be further extrapolated in cases where the gene copy number is known in the target, allowing for even more accurate population estimates.

Design

For use as standard positive controls in qPCR assays, gBlocks™ were designed using the same target DNA sequences to which each specific TaqMan assay was originally based.

If the DNA sequence of the target region of the assay it was designed against was known this was used. If the region was unknown the assay sequence was searched using the Basic Local Alignment Search Tool from The National Center for Biotechnology Information, and the highest relevant match was used (Camacho *et al.*, 2009). The TaqMan assay was then aligned to the target region using Mega X (Kumar *et al.*, 2018).

Once the assay was aligned with the corresponding sequence, three bases either side of the forward and reverse primers were included in the gBlock™ design as well as the spaces between the primers and the probes, as demonstrated in Figure 18. gBlocks™ were then produced by Integrated DNA Technologies, Inc. (Coralville, Iowa). (Designed gBlocks™ sequences are available in Annex 1.)

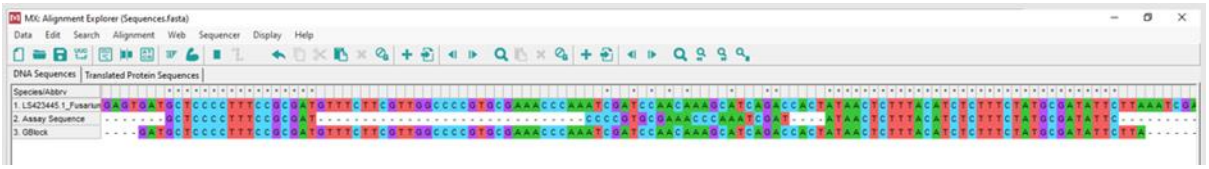

Figure 18. Alignment of the *Fusarium oxysporum* CaM gene for calmodulin, strain ITEM17647, isolate CN087 sequence (LS423445) (top) against the target Taqman assay (Personal communication, James Woodhall). Created using Mega X (Kumar *et al*., 2018)

Validation

All gBlock™ and their dilution series (Table 9) were validated for their use as a standard curve using qPCR. qPCR (method outlined in Chapter 3; iii)) was performed on each gBlock™ dilution series using their corresponding TaqMan assay. The log of the known concentration was plotted against the CT achieved for each of the dilutions and the line of best fit applied (Figure 19). R^2 was calculated and all gBlock™ standard curves achieved an R^2 of over 0.9.

Table 9. Dilution series and known concentration of gBlock™ used to create standard curves for quantification.

Dilution series	*Known concentration (pg/ul)*
10^{-1}	25
10^{-2}	2.5
10^{-3}	0.25
10^{-4}	0.025
10^{-5}	0.0025

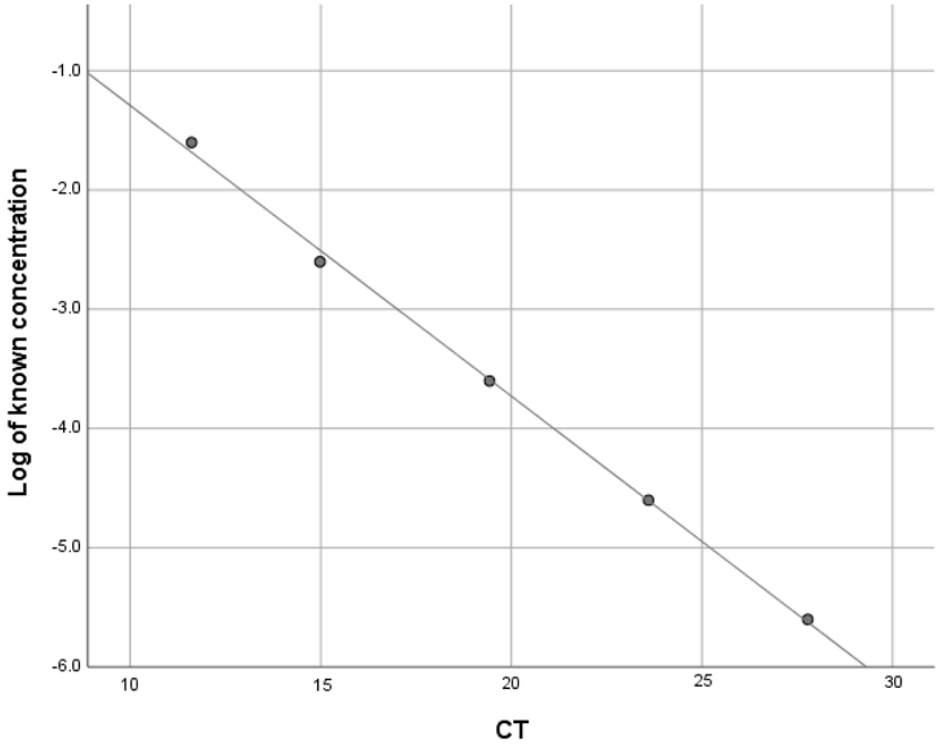

Figure 19. Log of known DNA concentration of the18S rRNA assay gBlock™ plotted against CT. R^2= 0.998

vi) Validation of Molecular Methods in Targeted Soils and their ability to detect specific organisms

As discussed in chapter 3, soil properties can interfere with extraction of total soil DNA (Hu *et al.*, 2010; Dequiedt *et al.*, 2012; Young *et al.*, 2014; Schulze *et al.*, 2016) and downstream qPCR analysis (Hu *et al.*, 2010; Dequiedt *et al.*, 2012; Young *et al.*, 2014; Schulze *et al.*, 2016). The soils tested in this thesis were chosen to represent a variety of soil properties, with pH ranges from 6.6 – 8.3 and organic matter contents from 2.2-14.4%.

pH is regularly cited as a factor affecting DNA extraction from soil (Robe *et al.*, 2003; Lakay *et al.,* 2007; Hu *et al.*, 2010; Dequiedt *et al.*, 2012) as it affects the adsorption of free DNA into the soil matrix (Young *et al.*, 2014; Guerra *et al.*, 2020). For instance, it has been found that soils below a pH of 5.5 have the greatest capacity for adsorption, with decreasing adsorption as pH increases (Guerra *et al*., 2020).

Similarly, organic matter content has been identified as a factor impacting DNA extraction efficiency. Van Elsas *et al*. (1997) found that the higher organic matter soils (30% w/w) in their study required multiple purification steps to achieve amplifiable DNA. Kuske *et al*. (1998) tested soils with a range of organic matter contents which were inoculated with the bacterium *Pseudomonas putida and* found that the DNA yield achieved from each of the soils was dependent on the organic matter content. Therefore, for the current studies, it was necessary to verify the efficiency of the chosen DNA extraction method across the selected soils in order to ensure a consistent yield of amplifiable DNA from all of the soils.

Successful DNA extraction can also vary according to the target organisms. For instance gram negative bacteria are more easily lysed than gram positive bacteria, due to the thickness of their peptidoglycan cell wall layer (Robe *et al.*, 2003; Bakken and Frostegård, 2006). Similarly, the shape and size of the bacterium effects its ability to be lysed, for instance rods are more readily ruptured than cocci and larger cells more so than small ones (Berthelet *et al.,* 1996; Bakken and Frostegård, 2006). This extraction bias is also seen across fungal organisms (Paplomatas *et al.*, 1992; Damm and Fourie, 2005; Thrall *et al.*, 2015; Habib *et al.*, 2017). For example, Tien *et al.* (1999) were unable to extract *Pythium aphanidermatum* and *Fusarium solani*

DNA, from inoculated soil, using their methods but successfully extracted DNA of other fungal organisms from the same soils.

A key objective of this thesis was to be able to analyse soils of various types for a range of soilborne plant pathogens relevant for different cropping systems using a range of qPCR assays, each able to specifically detect and accurately quantify a given pathogen. Some of these assays have been previously validated across different soils when first published (Table 4. qPCR targets, primers and probe sequences (FWD: Forward primer, REV: Reverse primer, P: Probe).), including in a recent MSc thesis by Kerr (2018). However, many of these assays were initially validated on DNA purified from pure cultures of the target organisms, rather than from DNA extracted from mixed communities in soil (Anderson *et al.,* 2003). The following validation experiments were therefore conducted to ensure the suitability of the selected DNA extraction method and specific qPCR assays for use across the soils sampled from the various field trials studied in this thesis.

Methods

Samples of soils were taken from each of the raspberry, daffodil and onion field trial sites (Table 10) and a sample of the compost in the glasshouse trials. Each soil sample was spiked with known amounts of relevant target organisms prior to DNA extraction (Table 11).

Table 10. Locations and soil properties of soils tested.

TRIAL	**RASPBERRY**	**ONION**	**DAFFODIL**
LOCATION	Norfolk	Bedford	Norfolk
SOIL	Sandy Silt Loam	Clay Loam	Sandy Silt Loam
PROPERTIES	pH: 8.2	pH: 6.6	pH: 8.3
	OM%: 2.2	OM%: 14.4	OM%: 3.4
	Mg: 50.9	Mg: 114.0	Mg: 88.5
	K: 85.0	K: 261.0	K: 84.2
	P: 51.8	P: 33.8	P: 13.2

Sub-samples (50g) of each of the soils were spiked with each target organism, as shown in Table 11. Reference cultures of *V. dahliae* and *F. oxysporum* were grown via the method outlined in Chapter 5. Fungi were cultured on PDA agar plates and

transferred into bags containing autoclaved 1:5 polenta and silver sand. These were then incubated for 2-3 weeks at room temperature in the dark. *C. rosea* was applied to the soil sample as the bio-fungicide product Prestop (Lallemand, Canada) at the concentration of 5 g/L and the Arbuscular mycorrhizal fungal (AMF) species (*Funneilformis mossae* & *Rhizophagus irregularis*) were applied to the soil sample as a commercial AMF product (PlantWorks Ltd., UK) in granular form.

Each inoculum, weighing 5g, was diluted into 45g of the corresponding soil and mixed thoroughly. A further two dilutions were then made by taking 5g from the first dilution and transferring into a further 45g of the corresponding soil. Three replicates were created.

DNA was extracted from each of the dilutions in each soil using the method outlined in Chapter 3, ii) and analysed using qPCR using the method outlined in Chapter 3 iii).

Table 11. Showing the starting amount of each organism inoculum in each of the soils.

Soil	Amount of initial inoculum
Raspberry	5g *V. dahliae inoculum*
	8ml *C. rosea (Prestop)*
Compost 1	5g *F. oxysporum inoculum*
	0.2g R. *irregularis (AMF product)*
	0.2g *F. mossae (AMF product)*
Compost 2	5g *V. dahliae inoculum*
	8ml *C. rosea (Prestop)*
Onion	5g *F. oxysporum inoculum*
Daffodil	5g *F. oxysporum inoculum*
	0.2g *R. irregularis (AMF product)*
	0.2g *F. mossae (AMF product)*

Results

Both target pathogens (*F. oxysporum* and *V. dahliae*) were detected in each of the relevant soils and CT increased in increments as expected as it became more diluted (10^{-1} dilution series), as shown in Figure 20 and Figure 21. The potentially beneficial organism *C. rosea* was also successfully detected in the relevant soils, again

increasing in increments expected with a 10^{-1} dilution, this is shown in Figure 22. However, the two AMF species (*R. irregularis* and *F. mossae*) were not detected in any of the soils. This may be due to the AMF being applied using the AMF product (PlantWorks Ltd., UK) used in the field trials instead of active mycelium. This may have meant that the organism was not given the opportunity to become active in this experimental situation, either because of time or the lack of an activating plants to grow to detectable levels, as they have been detected in the daffodil field trial experimental samples.

This trial validated the ability of the methods chosen to detect actively growing *F. oxysporum, V. dahliae* and *C. rosea* in soils at a range of different levels, including those used in the glasshouse trials. However, the two AMF species could not be detected using the methods employed.

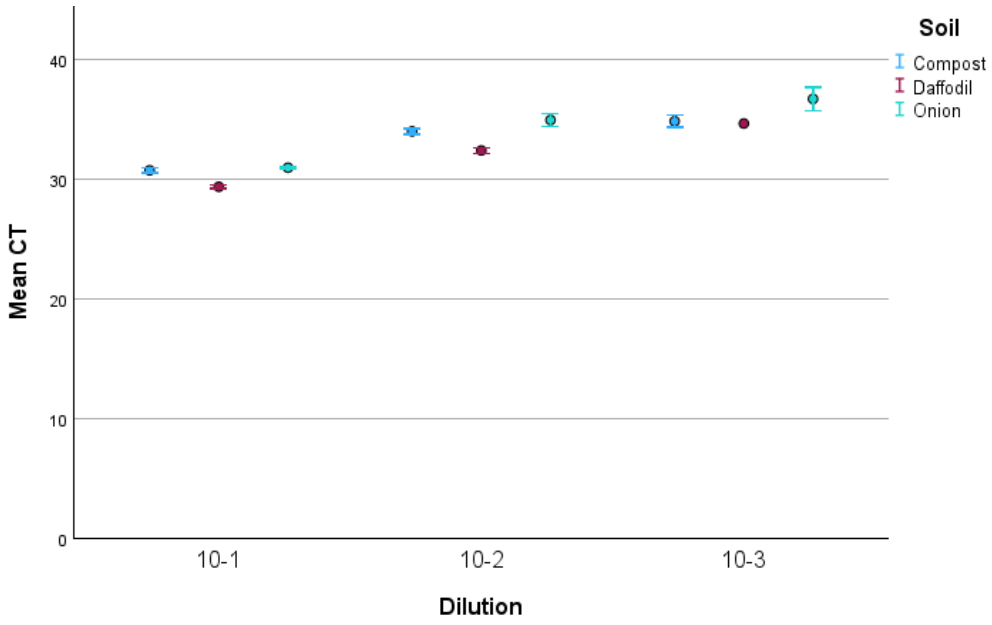

Figure 20. Average CT obtained after qPCR testing of DNA extracted from 3 soil types inoculated with a dilution series of *Fusarium oxysporum.* (Data is presented as mean ± standard deviation)

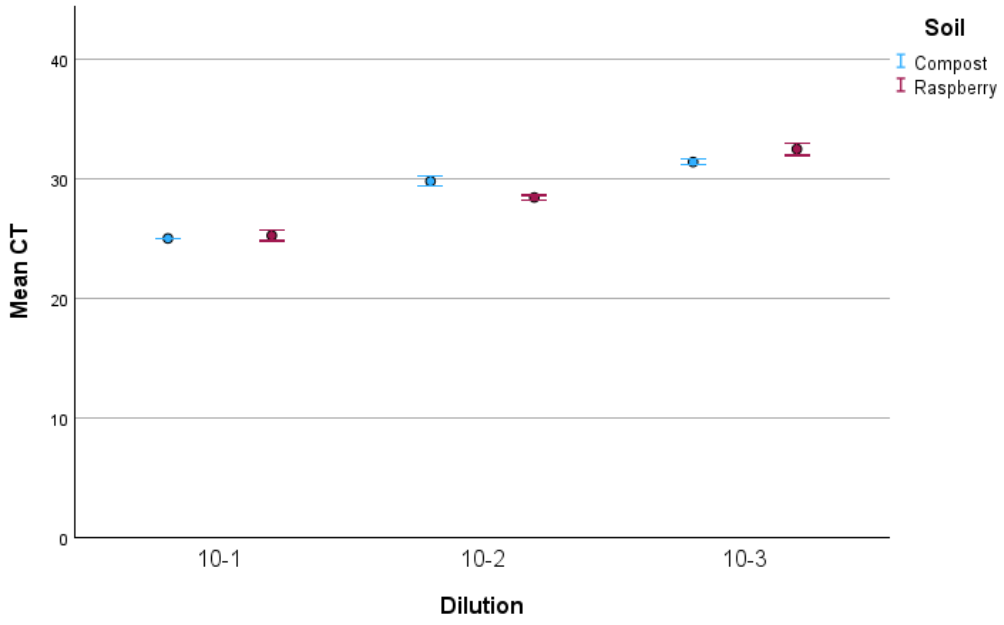

Figure 21. Average CT obtained after qPCR testing of DNA extracted from 3 soil types inoculated with a dilution series of *V. dahliae*. (Data is presented as mean ± standard deviation)

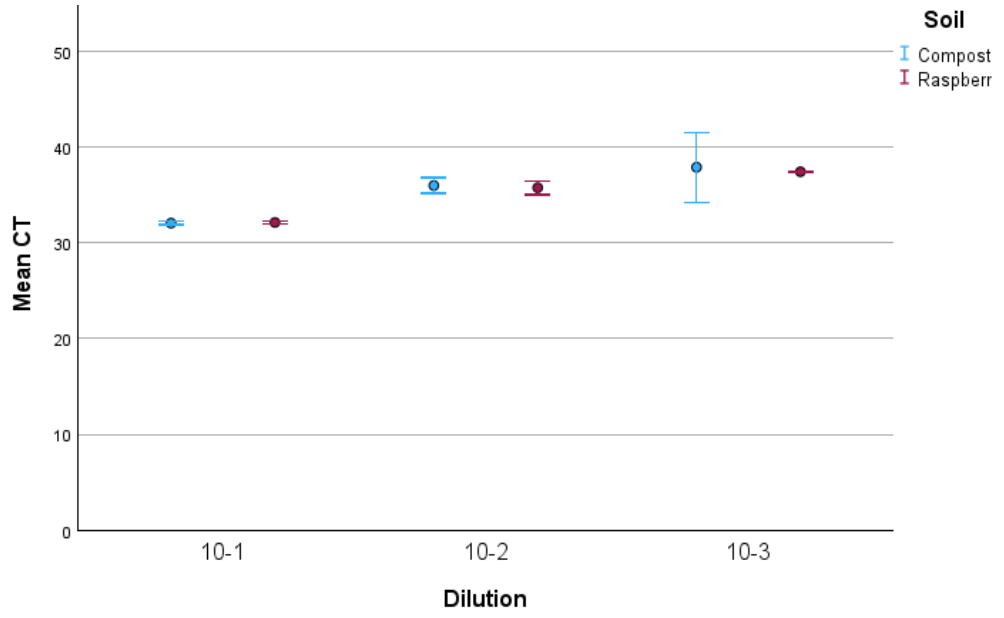

Figure 22. Average CT obtained after qPCR testing of DNA extracted from 3 soil types inoculated with a dilution series of *Clonostachys rosea.* (Data is presented as mean ± standard deviation)

vii) Summary

The experiments outlined in this chapter served as groundwork for refining the methodologies employed in this thesis for monitoring microbial communities in soils using PCR. This encompassed enhancements in DNA extraction techniques, mitigation of reaction inhibition, and method validation.

Key findings:

- Comparison of DNA extraction methods identified ‘method 3’ as the preferred way forward, due to its larger starting volume and more robust disruption method and accessibility to reagents.
- Additional DNA extract purification steps using activated charcoal did not significantly reduce inhibition in samples, therefore no further purification steps were performed beyond the DNA extraction method chosen.
- The addition of the PCR reaction additives; activated charcoal, MgCl2 and Bovine Serum Albumin, did not significantly improve PCR reactions by reducing inhibition and often lead to higher CT values than the undiluted samples. 1:10 dilution was found to be the most effective method of overcoming inhibition in samples for qPCR analyses.
- The use of gBlocks™ allowed successfully quantification of target organisms when used to create a standard curve, minimising potential bias from the use of standard control material.

The selected methods were subsequently validated across various soil types included in this thesis. This demonstrated that methods selected performed as expected under the experimental conditions of this thesis.

Chapter 5- Monitoring soilborne populations of the plant pathogen *Verticillium dahliae* and the biocontrol agent *Clonostachys rosea* in relation to strawberry plant growth and disease development under controlled greenhouse conditions.

i) Introduction

Improved methods for soilborne pathogen surveillance and the ability to predict disease likelihood before planting, can lead to knowledge based management and more sustainable farming (Yuen and Hughes, 2002; Saleh-Lakha *et al.*, 2005; Strange and Scott, 2005; Savary *et al.*, 2012; Bebber and Gurr, 2015; Yuen and Mila, 2015; Newbery *et al.,* 2016; Kettles and Luna, 2019). Before we can begin to predict disease in the field, the relationship between inoculum and disease incidence/severity needs to be established (Xiao and Subbarao, 2007). Inoculum density – disease incidence (ID-DI) relationships have been studied since the 1970's, continuing as technology advances. Measurement of disease development in crops grown in different levels of inoculum, either in controlled environments or in the field, can be used to predict the probability of disease under given environmental conditions.

ID-DI relationships have been investigated for many soilborne fungal pathogens, for instance; *R. solani* was shown to have ID-DI correlations in cabbage, radish and sugar beet (Keinath, 2007; Kinsbursky and Weinhold, 2007; Schulze *et al.*, 2016); *Pythium* and *Phytophthora* have also had ID-DI relationships established in cabbage, peanut, soybean and cotton (Mitchell, 1978). This study focusses on *V. dahliae*, a soilborne fungus where ID-DI relationships have previously been established across a range of crops, including; cotton, cauliflower, artichoke, olive and tomatoes (Paplomatas *et al.*, 1992; Berbegal *et al.,* 2007; Xiao and Subbarao, 2007; Roca *et al.*, 2016). For instance, Nicot and Rouse (2008) found disease incidence (the proportion of potato stems infected) increased with inoculum level, however disease progressed in infected plants at the same rate independent of inoculum level. Nevertheless, in contrast to the findings of Nicot and Rouse (2008), Xiao and Subbarao (2007) found that higher inoculum density lead to earlier disease onset in cauliflower. They also observed a positive exponential model between *V. dahliae*

inoculum and disease incidence. This was also observed by Ashworth, Jr. *et al*., (2008) where the rate of infection increased in tomato as the inoculum density of *Verticillium* increased. However, by the end of the season all plants were infected at all inoculum densities studied (0.1-27 microsclerotia/g soil).

Many environmental factors play a role in disease occurrence, leading to variation in ID-DI relationships. In the study performed by Nicot and Rouse (2008), it was found that measurable *V. dahliae* density in soil did not always lead to infection, despite identifying an ID-DI relationship. They believed this was down to interacting environmental parameters, like soil type and air temperature. Soil type has also been shown to alter ID-DI relationships of *R. solani* in radish seedlings (Kinsbursky and Weinhold, 2007). Bacterial and fungal communities have been associated with soils with particular pH, nitrogen and phosphorus contents where soil pH is believed to have the strongest influence on bacterial communities (Chaparro *et al.*, 2012). Schulze *et al.* (2016) showed that resistance of a variety to a disease affects ID-DI relationships, as the more susceptible variety of sugar beet showed increased infection rates to *R. solani* AG2-2IIIB compared to its more resistant competitor. There are so many variables that can alter ID-DI relationships, it is difficult to quantify and model their effects. Crowe *et al.* (1979) reported that 1.0 sclerotia per gram of soil of *S. cepivora* led to 100% disease in onion and garlic, whereas Adams and Papavizas (1971) reported 5.0 sclerotia per gram of soil of *S. cepivora* caused only a 50% disease in onions. These discrepancies can be down to a range of factors, for example, the environment, soil type and variety susceptibility. Performing these studies in a controlled environment allows us to begin to understand these complex relationships.

Verticillium

Verticillium fungi are notorious phytopathogenic species, namely *V. dahliae* and *V. albo-atrum,* responsible for causing vascular wilts in a wide range of host plants, including artichoke, cotton, pepper, strawberry, and raspberry (Bhat and Subbarao, 1999). Interestingly, these pathogens can infect multiple hosts but are most pathogenic on the host of origin (Bhat and Subbarao, 1999). The ability of *Verticillium* fungi to survive in soil for extended periods, with microsclerotia persisting for more than 20 years, poses considerable challenges in eradication efforts (Schnathorst, 1981). *Verticillium* wilt is considered a single cycle disease, and consequently

inoculum density at planting becomes a critical factor (Paplomatas *et al.*, 1992; Berbegal *et al.,* 2007).

Various strategies have attempted to control *Verticillium* wilts, including avoiding fields with previous susceptible crops, increasing crop diversity in rotations, and using soil fumigants (Mirmajlessi, 2017). Inoculum density as low as 2 microsclerotia/g of soil can result in 100% wilt in strawberries (Mirmajlessi, 2017). Managing *Verticillium* wilt effectively requires knowing the amount of *V. dahliae* in the soil, as the relationship between inoculum density and disease incidence is dependent on the host plant (Mirmajlessi, 2017). *Verticillium dahliae* is particularly challenging to control due to its long-term persistence, broad host range, and scarcity of resistance in host germplasm (Klosterman *et al.*, 2009). Host resistance is considered the most practical and economical control strategy, but resistance is not available in many crops (Klosterman *et al.*, 2009). *Verticillium* fungi pose significant challenges to crop health and global agricultural production. Their ability to persist in soil for extended periods, wide host range, and adaptability demand innovative and sustainable control measures. Developing host resistance and understanding the dynamics of *Verticillium* inoculum in soil are essential components of effective management strategies to mitigate the impact of *Verticillium* wilt on crop yields and food security.

Biocontrols – *Clonostachys rosea*

Biocontrols are often living microorganisms or metabolites that express antimicrobial activity towards target pathogens (Köhl *et al.,* 2019) and may offer a more environmentally friendly alternative to chemical pesticides (Shahzad *et al*., 2017). Successful biocontrol agents should share ecological niches with the target pathogen, enabling them to persist in the same environments where they can express their particular competitive modes of action, e.g. via antibiosis or mycoparasitism, against the target pathogen (Deketelaere *et al.*, 2017; Köhl *et al.,* 2019). Potential biocontrol agents can often be isolated from the rhizosphere or root surface of the host plant (Nakayama, 2017).

Many biocontrol agents have been identified with activities against different soilborne plant pathogens, with some examples shared in Table 12. However the effectiveness of these biocontrol agents appears to be variable under field conditions and often is not comparable to the efficacy of chemical pesticides (Elshahawy *et al*., 2018).

Another challenge for biocontrol agents is their stability in natural soil environments (Rahman *et al*., 2021) where temperature fluctuation (Tut *et al*., 2021), nutrient availability and competition with native soil microbes leads to instability of biocontrol populations and their resulting levels of pathogen suppressiveness (Cao *et al*., 2011).

Table 12. Examples of biocontrol organisms that have shown efficacy against certain plant pathogens and corresponding references.

Biocontrol Organism	Pathogen	Reference
Pseudomonas corrugata IDV1	*Ralstonia solanacearum*	(Overbeek *et al*., 2002)
Pseudomonas fluorescens UA5-40	*Ralstonia solanacearum*	(Overbeek *et al*., 2002)
Aspergillus versicolor Im6-50	*Spongospora subterranea f.sp. subterranea*	(Nakayama, 2017)
Pseudomonas fluorescens	*Fusarium species*	(Abd-El-Khair *et al.,* 2019)
Trichoderma species (T. harzianum, T. viride, and T. virens	*Fusarium species*	(Abd-El-Khair *et al.,* 2019)
Bacillus amyloliquefaciens	*Fusarium species*	(Shahzad *et al*., 2017)
Bacillus subtilis	*S. cepivora, Fusarium species*	(Elshahawy *et al*., 2018), (Cao *et al*., 2011)
Bacillus pumilus	*S. cepivora*	(Elshahawy *et al*., 2018)

There have been a few potential biocontrol agents tested against the soilborne pathogen *Verticillium*. For instance, Stadler and von Tiedemann, (2014) showed *Microsphaeropsis ochracea* had strong biocontrol potential against *V. longisporum* (51-100% mortality) in vitro and in sterile sand. However, this biocontrol activity completely disappeared when tested in the field, demonstrating the instability of biocontrol agents in the natural environment. In another study, the application of *B. subtilis* successfully reduced the incidence of *Verticillium* wilt in cotton in the field (Lang *et al*., 2012), and the authors attributed its success to the presence of unique beneficial fungi not found in the other treatments (*Humicola sp., Metarhizium anisopliae,* and *Chaetomium sp*.), indicating that complex community relationships can be key to success or failure of biocontrol applications.

This study investigated the potential of *C. rosea* for the control of *V. dahliae*. The potential of this biocontrol agent against *Verticillium* has been reported by Rahman *et al*. (2021) and Mirmajlessi (2017), as has its effectiveness against *Septoria* (Egel *et al*., 2019) and botrytis (Tut *et al*., 2021). *Clonostachys rosea* is a saprophytic filamentous fungi that survives on organic matter in the soil and as an endophyte in the roots and stems of host plant (Tut *et al*., 2021). Mode of actions that have been reported for *C. rosea* include antibiosis, reduced pathogen germination, competition and mycoparasitism (Deketelaere *et al.*, 2017).

In the study by Mirmajlessi (2017) *V. dahliae* was sprayed on an agar plate and a disc of *C. rosea* was placed in the centre. They found that in vitro *C. rosea* inhibited growth up to 94.3% and they attributed this to the production of hydrolytic enzymes, chitinases, β1,3-glucanases and antimicrobial compounds by *C. rosea. Clonostachys rosea* has also been reported to produce a perilipin protein encoded by the Per3 gene, which is involved in enhanced mycoparasitic activity (Tut *et al.*, 2021); However, this study was limited to the laboratory. Rahman *et al.* (2021) tested the application of *C. rosea*, via the commercially available product Prestop (Lallemand, Canada) for potential biocontrol of *Verticillium* on tomato. They found that the application of Prestop yielded inconsistent results in the field.

In this study we utilised qPCR to quantify the populations of both *V. dahliae* and *C. rosea* and their interaction over time in artificially inoculated soils planted with strawberries, in relation to the amount of disease which developed under carefully controlled conditions. This allowed a deeper understanding of the interactions and potential of the biocontrol agent, *C. rosea* for the control of the soil-borne pathogen *V. dahliae*. Strawberry was chosen due to its use as a model crop (Amil-Ruiz *et al.*, 2011), short growth period and susceptibility to *V. dahliae*, as well as its value to the UK market, with 134,795 metric tonnes being grown in the UK in 2019 and a year on year 5.2% production increase since 2014 (Food and Agriculture Organization of the United Nations., *FAOSTAT Statistical Database* [Accessed 21/01/2024]).

ii) Methods

Selection of a *Verticillium dahliae* isolate

Cultures of *V. dahliae*, isolated from potato (CC 1802), chrysanthemum and hops were obtained from the Fera culture collection. Isolates were grown on agar plates (28 g/L Dehydrated Culture Media CM0003 (Oxoid Ltd., Canada)) for two weeks.

To test pathogenicity of the three isolates on strawberry, Alpine strawberry plantlets propagated from healthy strawberry plant runners at Fera Science Ltd. (York, UK) were used. Plantlets were maintained in a glasshouse between 20ºC – 24ºC in pots filled with 'Rothamsted Prescription Mix' compost (75% L&P medium peat, 12% ST loam, 10% Grit, 3% vermiculite). Cuts were made in the base of 3-week-old Alpine strawberry plants and swabs of each *V. dahliae* culture were pressed into the wound. This was repeated twice for each culture. After 10 days each plant was assessed for disease by calculating the percentage of diseased leaf area. This was achieved by placing a grid over each leaf and the number of squares that the leaf made contact with were counted and used as the leaf area; the number of squares with disease symptoms were then used to calculate the % diseased leaf area.

The potato isolate was chosen for use in subsequent experiments as it consistently caused the highest percentage diseased leaf area (25%). Further plates of *V. dahliae* were then grown for 2 weeks at 20ºC in the dark as recommended by (Sagova-Mareckova *et al*., 2008) to encourage microsclerotia growth.

To grow *V. dahliae* inoculum for the experiments, 1kg of silver sand and 5g of polenta were combined in autoclave bags, sealed and autoclaved. In a fume cabinet, each bag was inoculated by thoroughly mixing a quarter of an agar culture plate of the *V. dahliae* isolate, and the contents dampened and sealed. Bags were then incubated in a controlled environment room for 2 weeks to produce a master inoculum for further soil inoculations (Figure 23).

Figure 23. Image showing fungal growth of *Verticillium dahliae* on polenta and silver sand mix after 2 weeks. Black microsclerotia are visible within the white mycelium.

For *V. dahliae*, three 10-fold dilutions of inoculated compost were required for use in subsequent trials. The amount of compost needed for each inoculation level across the trials was calculated and placed in a barrel for mixing. Then 10%, 1% and 0.1% of the total volume of master inoculum was added to each barrel. The barrels were then rotated and shaken to distribute the inoculum throughout the compost. This soil was then distributed into pots, each holding 300g of inoculated compost. Uninoculated compost was used for controls.

Disease Assessment

Disease severity was measured using image analysis of all leaves per plant. Images were captured under consistent illumination, camera mounting properties and camera settings for each leaf. Exposure was set at 2.0 and ISO 800. Trimble eCognition software was used for analysis of the images. The images were loaded into Trimble eCognition Workspace, and all analysed using the same Rule Set.

Each image was analysed in turn. Initially the image was segmented into individual pixels so each pixel could be analysed separately. The first task was to identify only leaf area, removing all background information (labels, shadow, residue). This was done by calculating a general image brightness layer (Eq. 1), resulting in pixels

ranging from 0 – 255 (the digital number range for unsigned 8-bit image data). Using this brightness layer all pixels with a value ≥ 220 were removed (removing bright paper background) from any further analysis and classified as NoData. The remaining pixels were classified as Leaf, both NoData and Leaf pixels were then merged into sperate multi pixel objects, ideally one object for a single leaf and all the NoData class pixels merged into a single object. Some Leaf class objects were still classified incorrectly, these consisted of very small objects where the labels were written and large objects beyond the paper background, these were both removed by simply removing objects much smaller or much larger than the leaves present in the image. This analysis produced a separate, single object for the individual leaves classified as Leaf and all the background information (then removed from all further analysis classified as NoData).

$$\text{Eq.1} \quad \frac{(Red+Green+Blue)}{3}$$

The second stage was to re-segment the leaf objects back to individual pixel level and determine a sensible threshold between healthy and unhealthy leaf area. After multiple scenarios were tested it was noted that spectral reflectance between Red and Green were showing promise between healthy and unhealthy leaf area. The Green-Red Vegetation Index (GRVI) has advantages as a phenological indicator for the detection of early phase of leaf green-up and the middle phase of autumn colouring (Motohka *et al.*, 2010). The use of the Normalised Difference Vegetation Index (NDVI), which is often a good indicator of leaf health, was not applicable here as no Near-Infrared (NIR) band was present, however, Motohka *et al.* (2010) noted that while NDVI remained nearly constant as leaves turned gradually dark green from initial green up, GRVI gradually decreased. Chen *et al.* (2019) also note the GRVI yielded better results than NDVI in recognising phenological crop changes, especially senescence, therefore picking up the subtle changes in leaf colour change. As GRVI can detect these subtle changes in phenological change and the disease presence symptoms in the leaves imaged are similar to senescence then GRVI was calculated (Eq.2). A threshold of 0.07 was used with values below or equal to the threshold representing diseased leaves and values above the threshold representing healthy leaves (Figure 24).

$$\text{Eq.2} \quad \frac{(Green-Red)}{(Green+Red)}$$

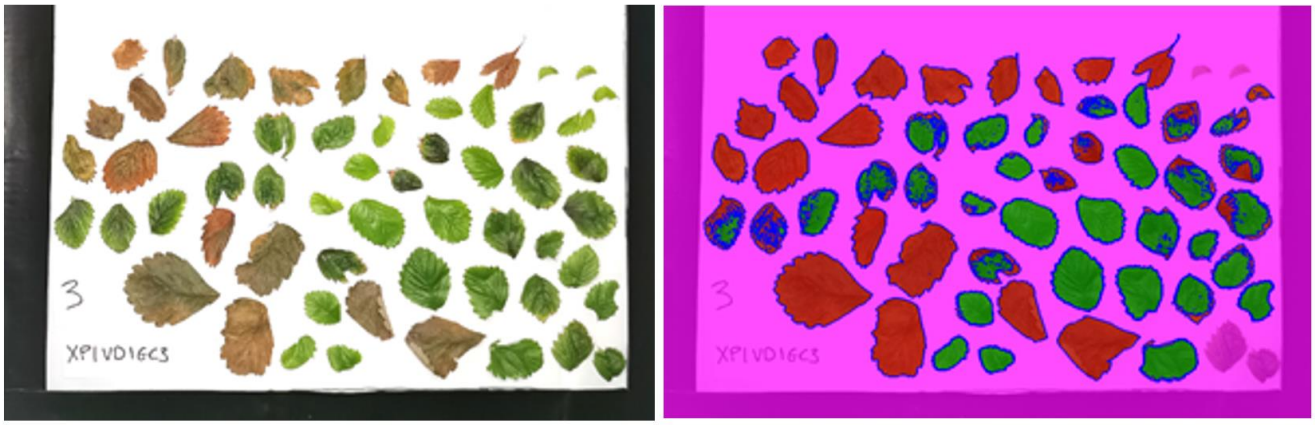

Figure 24. Example of image showing healthy and unhealthy leaves (left) and the results of the analysis Rule Set (right), pink indicates area of NoData, green indicates healthy and red indicates unhealthy.

Experiment 1: Establishing relationship between *Verticillium dahliae* inoculum level and *Clonostachys rosea* dosage at different levels in the soil and the impact on disease and plant growth

Twenty pots with each soil inoculum level (10%, 1%, 0.1%) were planted with 3-week-old Alpine strawberry plantlets. These were propagated from healthy strawberry plant runner stocks maintained at Fera Science Ltd. (York, UK). Within each inoculum level, five plants were then treated with three doses of *C. rosea*, in the form of the biocontrol product Prestop (Lallemand, Canada). The doses were 0.5 g/L, 5 g/L and 50 g/L, representing 10% below the recommended dose, the recommended dose and 10% above the recommended dose, according to the manufacturer's instructions. Each plant was treated with 50ml of the solution as a soil drench. The treatment combinations are shown in Table 13. The strawberries were kept at the following glasshouse conditions: temperature 20-24ºC and lighting between 04:00-20:00 hours when ambient light levels were low.

After 8 weeks, all of the plants were assessed for disease. Disease was assessed by all leaves being removed from each plant and placed within a 35x35cm white square and photographed. These images were then analysed using eCognition (Trimble Germany GmbH. 2021. *eCognition* (10.2) [Software]).

Samples of 10g of compost from the rhizosphere of three plants per treatment combination were collected for DNA extraction (Chapter 3, i) and qPCR analyses (Chapter 3, ii)) to detect and quantify *V. dahliae* and *C. rosea* populations in the

rhizosphere soil. Plant and root, both wet and dry weight, were also measured. Plant and root matter were separated, washed, and then dried in an oven at 60^{o}C overnight and weighed.

Table 13.Treatment combinations for Experiment 1

V. dahliae **Inoculum level**	***C. rosea*** **dose**
0	0 g/L
0	0.5 g/L
0	5 g/L
0	50 g/L
0.1%	0 g/L
0.1%	0.5 g/L
0.1%	5 g/L
0.1%	50 g/L
1%	0 g/L
1%	0.5 g/L
1%	5 g/L
1%	50 g/L
10%	0 g/L
10%	0.5 g/L
10%	5 g/L
10%	50 g/L

Experiment 2: Comparing population dynamics of *Verticillium dahliae* and *Clonostachys* in the rhizosphere soil over time after planting of the strawberry plants.

Twelve pots of each inoculum level of *V. dahliae* (10%, 1%, 0.1%) were planted with 3-week-old Alpine strawberry plantlets. These were propagated from clean strawberry plant runners maintained at Fera and grown in glasshouse conditions (as above). All 12 plants were treated with *C. rosea*, in the form of the biocontrol product Prestop (Lallemand, Canada) at the recommended rate (5 g/L). Each plant was treated with 50ml of the solution as a soil drench. The strawberries were kept at the

following glasshouse conditions: temperature 20-24ºC and lighting between 04:00-20:00 hours when ambient light levels were low.

Every two weeks, 10g of compost from the rhizosphere was sampled using a straw to collect ‘cores’ from three replicate plants, these were then removed from the sampling pool. The soil samples were frozen at -20ºC for later DNA extraction. This was continued until week 8, by which point all plants had been sampled. Plants were then destructively sampled to assess disease, as described above, and plant and root, wet and dry weight were measured. Plant and root matter were separated and washed, they were then dried in an oven at 60ºC overnight and weighed. DNA was extracted from the collected compost samples and tested using qPCR for *V. dahliae* and *C. rosea* as described in Chapter 3.

Experiment 3: Assessing the impact of organic soil amendments (anaerobic digestate) on the population dynamics of *Verticillium dahliae* and *Clonostachys rosea* in the soil environment.

As part of the wider thesis organic amendments were applied to field trials, therefore application of organic amendments, namely anaerobic digestate, were applied under controlled conditions to establish potential relationships between application and the target organisms. Sixty pots were planted with 3-week-old Alpine strawberry plantlets as described above. Combinations of three treatment options (Table 14) were applied: the presence of *V. dahliae*, the application of *C. rosea* and the addition of solid anaerobic digestate (AD). There were 10 replicates per treatment. The *V. dahliae* inoculum level of 1% was used in this experiment (prepared as in previous experiments). *Clonostachys rosea* was again applied as a 50ml drench at a rate of 5 g/L. For the addition of AD, compost was placed in a barrel (separating those inoculated with *V. dahliae*) and AD was added at a rate of 10% of the volume. This was mixed thoroughly by rotating and shaking the barrel. The strawberries were kept at the following glasshouse conditions: temperature 20-24ºC and lighting between 04:00-20:00 when ambient light levels were low.

Table 14. Treatment combinations in Experiment 3. '+' denotes the presence of the treatment and '-' denotes the absence.

V. dahliae	***C. rosea***	**Anaerobic digestate**
+	+	+
+	-	+
-	+	+
+	+	-
+	-	-
-	+	-

After 8 weeks the plants were assessed for disease as described above. Samples of 10g of compost from the rhizosphere of three plants per treatment combination was collected for DNA extraction (Chapter 3, i) and qPCR analyses (Chapter 3, ii) for detecting and quantifying *V. dahliae* and *C. rosea.* Plant and root, wet and dry weight were also measured. Plant and root matter were separated and washed, they were then dried in an oven at 60^{o}C overnight and weighed.

Statistical Analysis

All analyses were conducted using IBM SPSS Statistics version 26. Data normality was evaluated using the Shapiro-Wilk test ($p > .05$), and homogeneity of variance was assessed with Levene's test. If the data met the assumptions of normality and homogeneity, parametric tests were applied, including analysis of variance (ANOVA), linear regression, and Pearson's correlation.

If the data violated these assumptions, a Log^{10} transformation was applied, and the normality and homogeneity tests were repeated. If the transformed data met the assumptions, parametric analyses (e.g., ANOVA) were conducted on the transformed dataset.

In cases where the data continued to violate the assumptions after transformation, non-parametric alternatives were employed on the original untransformed data. These included the Kruskal-Wallis test for group comparisons and Spearman's rank correlation for correlation analysis.

For the ANOVA analyses (one-way, two-way, etc.), post hoc comparisons were performed using Tukey's test to identify significant differences between group means. In addition to the post hoc tests, descriptive statistics and effect size estimates were calculated to further interpret the results. In some cases, to account for temporal variability, time was included as a covariate in the analyses, allowing for the control of its potential influence on the outcomes.

Statistical significance was determined at a threshold of $p < 0.05$.

iii) Results

<u>*Verticillium dahliae* Isolate selection</u>

All three isolates caused disease on the strawberry plants, but the potato isolate was most consistently aggressive causing an average of 25% leaf area with symptoms. The potato isolate was therefore used in all subsequent experiments. (Table 15).

Table 15. Results from *Verticillium dahliae* bait test showing % leaf area affected after inoculation with each test isolate.

Isolate		*Total leaf area*	*Total diseased leaf area*	*Percentage diseased area*
Hops	1	320	56	18%
	2	756	66	9%
Potato	1	336	85	25%
	2	310	78	25%
Chrysanthemum	1	384	46	12%
	2	143	49	34%

<u>Impact of *Verticillium dahliae* and *Clonostachys rosea* inoculum dosages on subsequent disease development and plant growth</u>

This experiment was conducted to assess the effects of different *V. dahliae* inoculum levels as affected by *C. rosea* biocontrol treatment (applied as Prestop), on the growth of strawberry plants and disease progression over 8 weeks. Populations were only measured at the end of the 8-week trial.

There was a significant difference between the 0.1% *V. dahliae* inoculum treatment and the other treatment on the final *V. dahliae* population detected using qPCR (Figure 25), as assessed by ranked ANOVA (p= 0.004-0.008) (data was normally distributed). No other significant relationship was identified (p=0.996 -1.000).

There was no correlation between the initial *C. rosea* dosage and the final measured *C. rosea* population at the end of the trial (Figure 26), as assessed by ranked ANOVA (p=0.305-1.000) (data was normally distributed).

After 8 weeks, the population *V. dahliae* detected by qPCR decreased as inoculum level increased. Significantly higher levels than detected followed the initial inoculation rate of only of 0.1%, although large errors were observed between replicate samples. This trend was also observed with *C. rosea* population which also decreased by the end of the trial as dosage increased, however this was less pronounced in *C. rosea*.

A moderate negative relationship was observed between measured *V. dahliae* populations and applied *C. rosea*, this was found to be significant (p= 0.007) when assessed by spearman correlation (-0.399) (Figure 27).

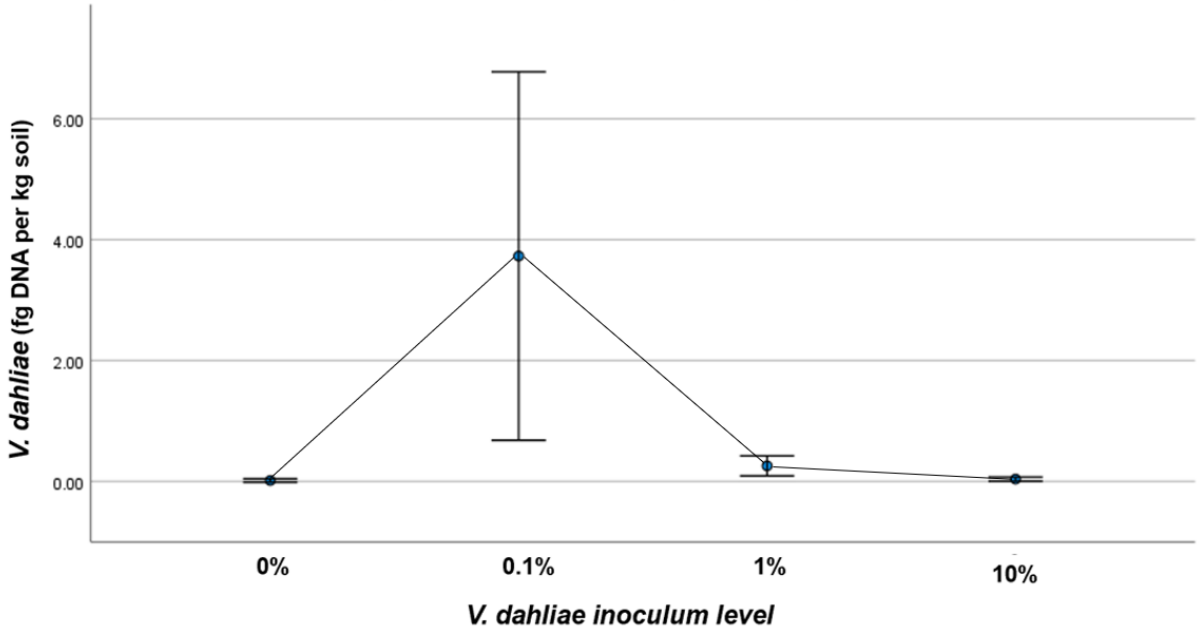

Figure 25. *Verticillium dahliae* population (as measure by qPCR), estimated 8 weeks after inoculation by qPCR at each initial *Verticillium dahliae* inoculum level. Error Bars 95% CI.

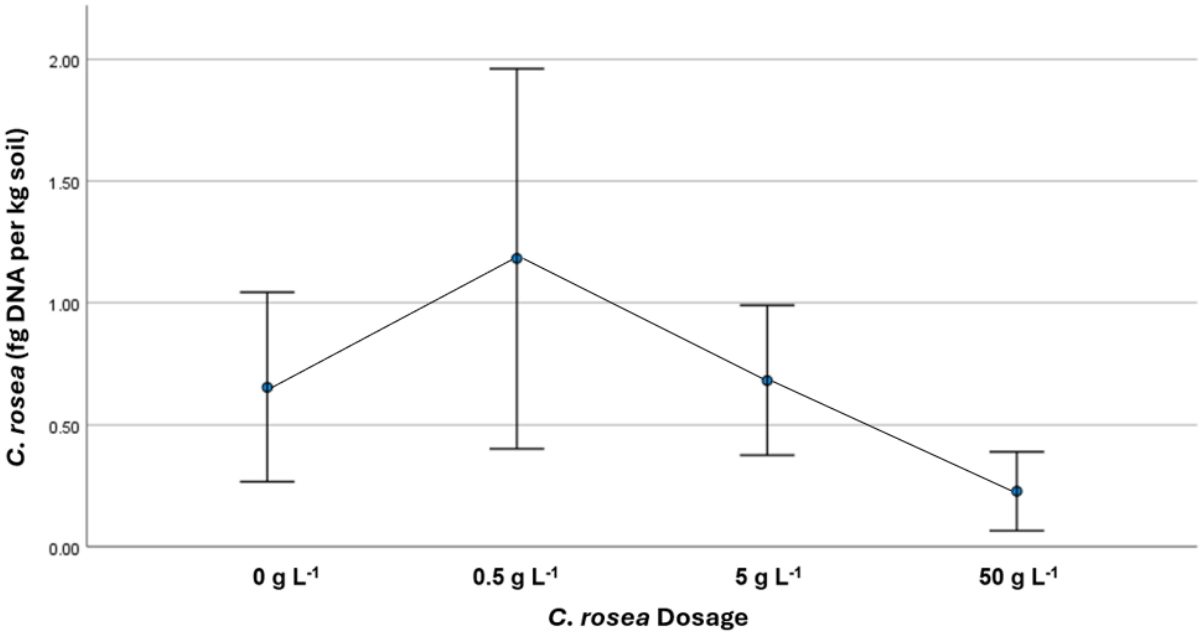

Figure 26. *Clonostachys rosea* population, estimated 8 weeks after inoculation by qPCR at each initial *Clonostachys rosea* dosage. Error Bars 95% CI.

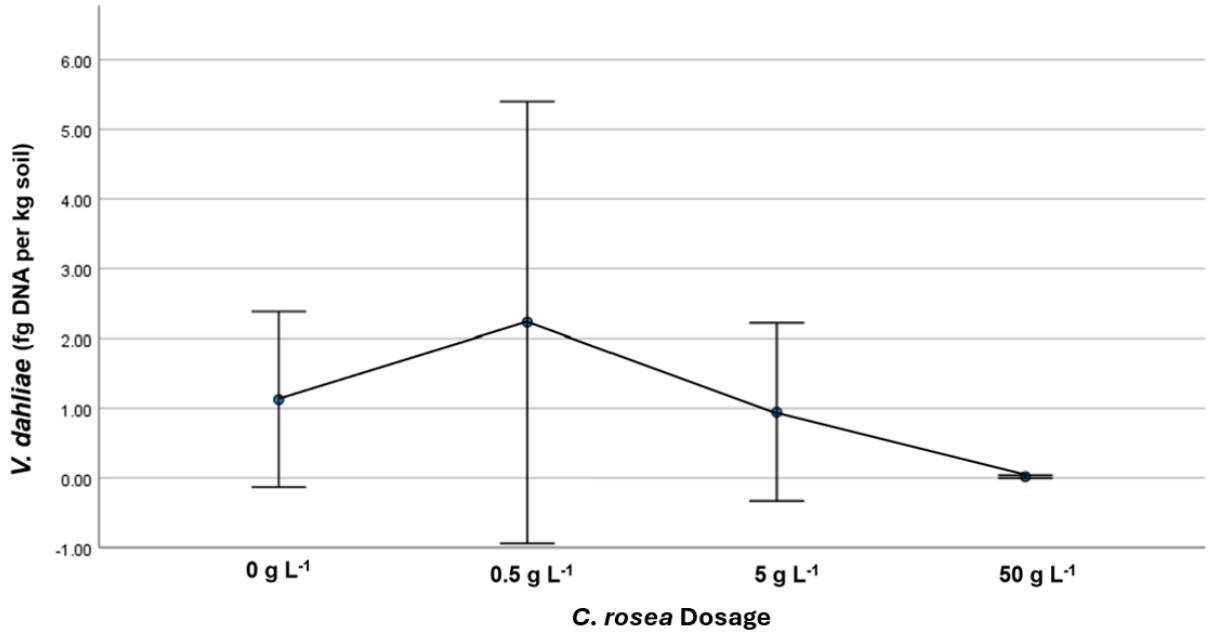

Figure 27. *Verticillium dahliae* population, estimated 8 weeks after inoculation by qPCR at each initial *Clonostachys rosea* dosage. Error Bars 95% CI.

i) Disease severity

A ranked ANOVA was conducted to determine if disease severity observed in the plant was related to treatment (*V. dahliae* inoculation * *C. rosea* dosage). Senescence observed in control plants was deemed natural, anything significantly different to control plants was deemed in response to treatment.

Data was normally distributed for each group, as assessed by Shapiro-Wilk test ($p > .05$). The control plants had a mean percentage senescence (the symptom measured of *Verticillium* wilt) of 59.2% and the lowest standard deviation of 2.79. The application of either *V. dahliae* or *C. rosea* triggered a response, though it was not consistent, as evidenced by the large deviations observed in the treatment samples.

Application of 1% *V. dahliae* and 0.5 g/L (VD1%CR0.5g/L) was significantly different from the control and multiple other treatments (VD0%CR0.5g/L, VD0%CR5g/L, VD0%CR50g/L,VD0.1%CR0g/L, VD0.1%CR0.5g/L, VD10%CR0g/L, VD10%CR5g/L, VD10%CR50g/L) (Figure 28) ($p=<0.001$-0.050). This treatment (VD1%GC0.5g/L) had a mean percentage disease of 99.8%. The only other significant interaction occurred between 0% *V. dahliae* and 50 g/L (VD0%CR50g/L) and 0.1% *V. dahliae* and 0.5 g/L (VD0.1%CR0.5g/L). Disease appeared to increase as *V. dahliae* inoculum level increased until 10% where it decreased (Figure 29), however a significant difference was only observed between 0% and 1% ($p=<0.001$), as assessed by a ranked ANOVA.

There did not appear to be a correlation between *V. dahliae* dosage and disease severity under these conditions. There was no significant interaction between *C. rosea* dosage and disease.

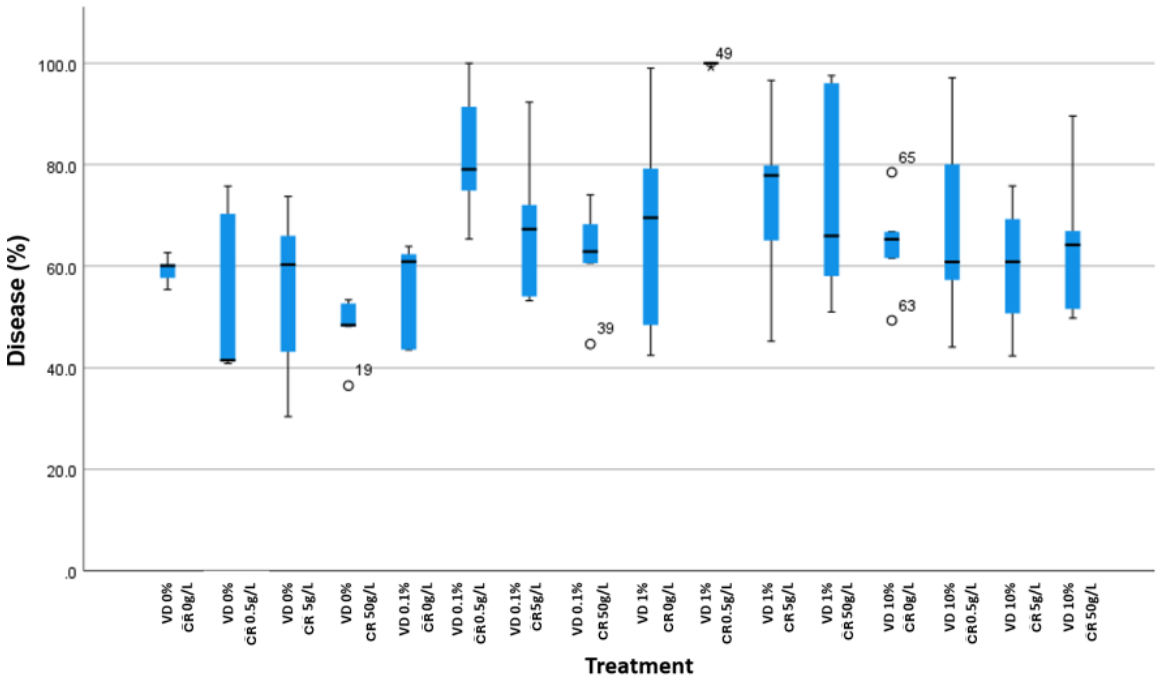

Figure 28. Percentage disease severity observed in strawberry plants against treatment (*Verticillium dahliae* inoculation * *Clonostachys rosea.* Data is presented as interquartile range and median.

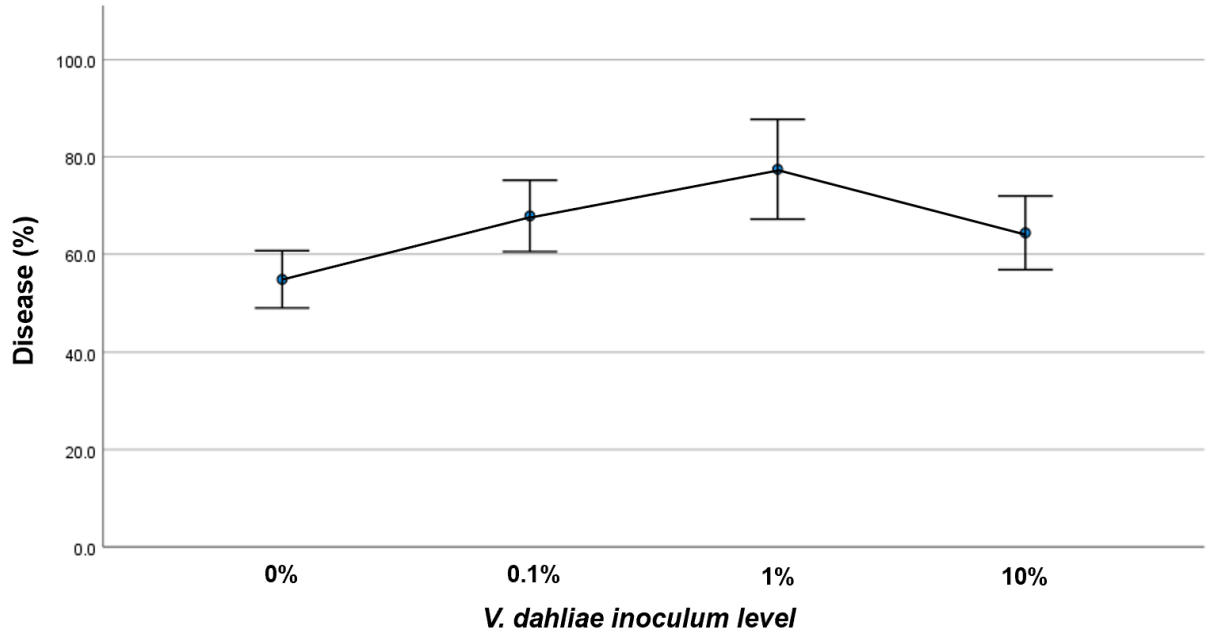

Figure 29. Percentage disease severity observed in strawberry plants per initial *Verticillium dahliae* inoculum level. Data is presented as mean ± standard deviation.

ii) Plant dry weight

A ranked ANOVA was conducted to determine if plant dry weight was related to treatment (*V. dahliae* inoculation * *C. rosea* dosage). Data was normally distributed for each group, as assessed by Shapiro-Wilk test ($p > .05$). Compared to the control (VD0%CR0g/L), treatments VD0.1%CR0g/L, VD0.1%CR0.5g/L, VD0.1%CR50G/L, all three of the 1% *V. dahliae* treatments (VD1%CR0g/L, VD1%CR0.5g/L, VD1%CR5g/L, VD1%CR50g/L) and VD10%CR0.5g/L were significantly different (Figure 30). Those with no *V. dahliae* applied were significantly different to those inoculated with 0.1% and 1%, however it was only significant at the 10% level with 0.5 g/L *C. rosea* applied. All combinations of treatments and their significance is shown in Table 16.

Independent from the *C. rosea* treatment *V. dahliae* dosage was assessed for significance using a ranked ANOVA, 0%, 0.1% and 1% were all significant from each other, however 10% failed to reach significance from 0% ($p=0.842$). *V. dahliae* levels 0%, 0.1% and 1% all reduced plant dry weight independent of *C. rosea)* dosages, however at 10% the plant dry weight did not significantly decrease from the control. This correlates from the amount of *V. dahliae* detected in this treatment using qPCR (Figure 25). There was no significant effect of *C. rosea* dosage on plant dry weight when assessed independently of *V. dahliae* inoculum.

Table 16. Table denoting the significance values of plant dry weight between treatment combinations (***** denotes those that are significant to the 0.05 level).

	VD0% CR 0.5g/L	VD0% CR5G/L	VD0% CR50G/L	VD0.1% CR0G/L	VD0.1% CR0.5G/L	VD0.1% CR5G/L	VD0.1% CR50G/L	VD1% CR0G/L	VD1% CR0.5G/L	VD1% CR5G/L	VD1% CR50G/L	VD10% CR0G/L	VD10% CR0.5G/L	VD10% CR5G/L	VD10% CR50G/L
VD0%CR0G/L	0.847	0.588	0.562	0.001*	0.001*	0.052	0.014*	0.001*	0.001*	0.001*	0.001*	0.908	0.024*	0.569	0.877
VD0%CR0.5G/L		0.463	0.440	0.001*	0.001*	0.033*	0.008*	0.001*	0.001*	0.001*	0.001*	0.757	0.015*	0.462	0.969
VD0%CR5G/L			0.969	0.004*	0.001*	0.156	0.052	0.001*	0.001*	0.001*	0.001*	0.671	0.076	0.920	0.487
VD0%CR50G/L				0.005*	0.005*	0.167	0.057	0.001*	0.001*	0.001*	0.001*	0.643	0.082	0.947	0..463
VD0.1%CR0G/L					0.191	0.135	0.335	0.092	0.057	0.375	0.316	0.001*	0.331	0.17	0.001*
VD0.1%CR0.5G/L						0.006*	0.025*	0.699	0.536	0.671	0.757	0.001*	0.030*	0.001*	0.001*
VD0.1%CR5G/L							0.588	0.002*	0.001*	0.019*	0.014*	0.067	0.655	0.257	0.037*
VD0.1%CR50G/L								0.009*	0.005*	0.067	0.052	0.019*	0.949	0.111	0.009*
VD1%CR0G/L									0.816	0.418	0.487	0.001*	0.012*	0.001*	0.001*
VD1%CR0.5G/L										0.298	0.355	0.001*	0.007*	0.001*	0.001*
VD1%CR5G/L											0.908	0.001*	0.073	0.002*	0.001*
VD1%CR50G/L												0.001*	0.058	0.001*	0.001*
VD10%CR0G/L													0.031*	0.639	0.787
VD10%CR0.5G/L														0.142	0.017*
VD10%CR5G/L															0.482

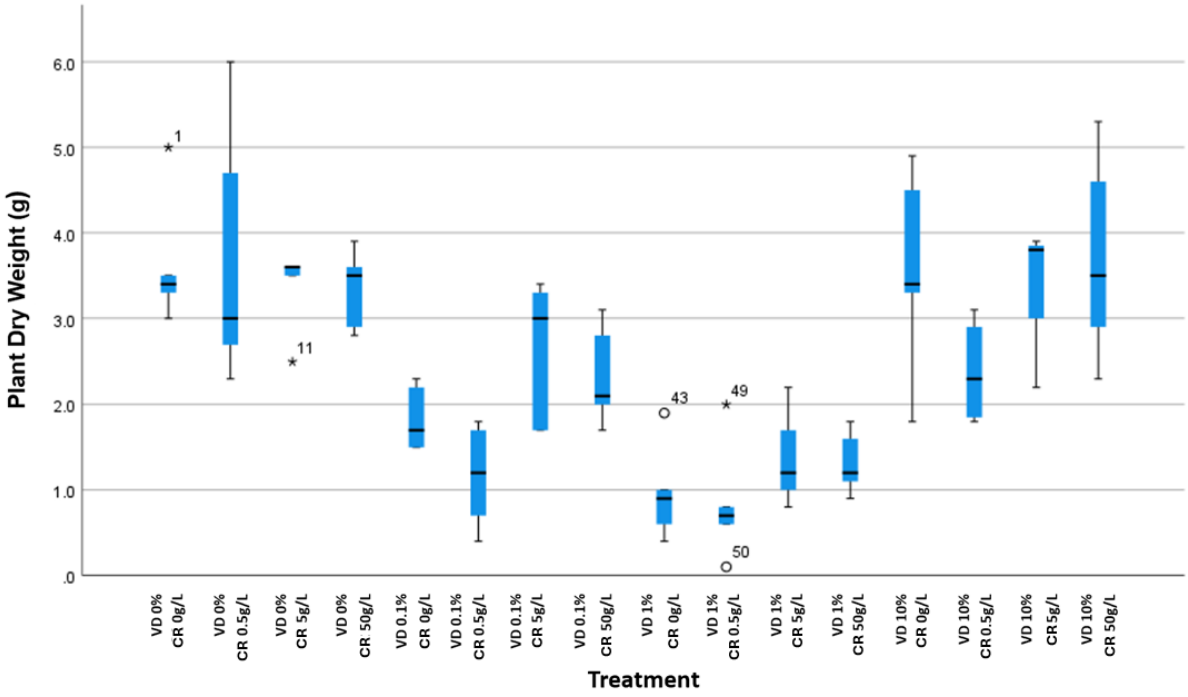

Figure 30. Plant dry weight observed in strawberry plants against treatment (*Verticillium dahliae* inoculation * *Clonostachys rosea* dosage). Data is presented as interquartile range and median.

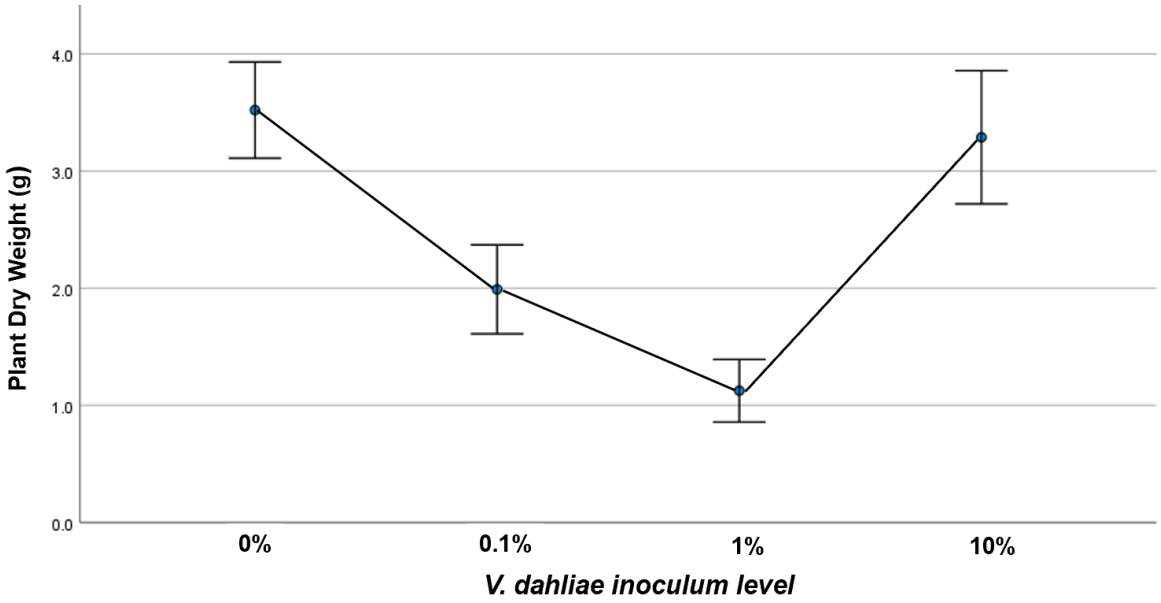

Figure 31. Plant dry weight observed in strawberry plants in response to different *Verticillium dahliae* inoculum levels. Data is presented as mean ± standard deviation.

iii) Root Dry weight

A ranked ANOVA was conducted to determine if root dry weight was related to treatment (*V. dahliae* inoculation * *C. rosea* dosage). Data was normally distributed for each group, as assessed by Shapiro-Wilk test ($p > .05$). Significant differences were observed between some treatment groups, however there was no clear pattern of relation to the treatments applied (Table 17). When compared to the control group VD0.1%CR0.5g/L, VD0.1%CR50g/L, VD1%CR0g/L, VD1%CR0.5g/L, VD1%CR5g/L and VD10%CR0.5g/L were all significantly lower (Figure 32). Root dry weight did decrease in the presence of *V. dahliae*, however 10% had higher root weight than the lower inoculum levels (Figure 33).

Table 17. Table denoting the significance values of root dry weight between treatment combinations (* denotes those that are significant to the 0.05 level).

	VD0% CR0.5g/L	VD0% CR5g/L	VD0% CR50g/L	VD0% CR0g/L	VD0.1% CR0.5g/L	VD0.1% CR5g/L	VD0.1% CR50g/L	VD0.1% CR0g/L	VD1% CR0.5g/L	VD1% CR5g/L	VD1% CR50g/L	VD1% CR0g/L	VD10% CR0.5g/L	VD10% CR5g/L	VD10% CR50g/L
VD0% CR0g/L	0.220	0.491	0.522	0.097	0.003*	0.118	0.033*	0.001*	0.001*	0.026*	0.844	0.220	0.013*	0.203	0.588
VD0% CR0.5g/L		0.588	0.554	0.005*	0.001*	0.006*	0.001*	0.001*	0.001*	0.001*	0.302	0.016*	0.001*	0.022*	0.079
VD0% CR5g/L			0.961	0.021*	0.001*	0.026*	0.006*	0.001*	0.001*	0.004*	0.622	0.058	0.002*	0.064	0.220
VD0% CR50g/L				0.023*	0.001*	0.029*	0.006*	0.001*	0.001*	0.005*	0.657	0.065	0.002*	0.070	0.239
VD0.1% CR0g/L					0.156	0.921	0.622	0.065	0.014*	0.554	0.065	0.657	0.331	0.864	0.259
VD0.1% CR0.5g/L						0.130	0.350	0.657	0.280	0.403	0.002*	0.065	0.710	0.162	0.012*
VD0.1% CR5g/L							0.554	0.052	0.011*	0.491	0.079	0.730	0.287	0.932	0.302
VD0.1% CR50g/L								0.170	0.047*	0.921	0.021*	0.350	0.609	0.550	0.107
VD1% CR0g/L									0.522	0.203	0.001*	0.023*	0.430	0.077	0.004*
VD1% CR0.5g/L										0.058	0.001*	0.001*	0.166	0.022*	0.001*
VD1% CR5g/L											0.016*	0.302	0.676	0.495	0.088
VD1% CR50g/L												0.156	0.166	0.831	0.491
VD10% CR0g/L													0.166	0.309	0.044
VD10% CR0.5g/L														0.309	0.418
VD10% CR5g/L															

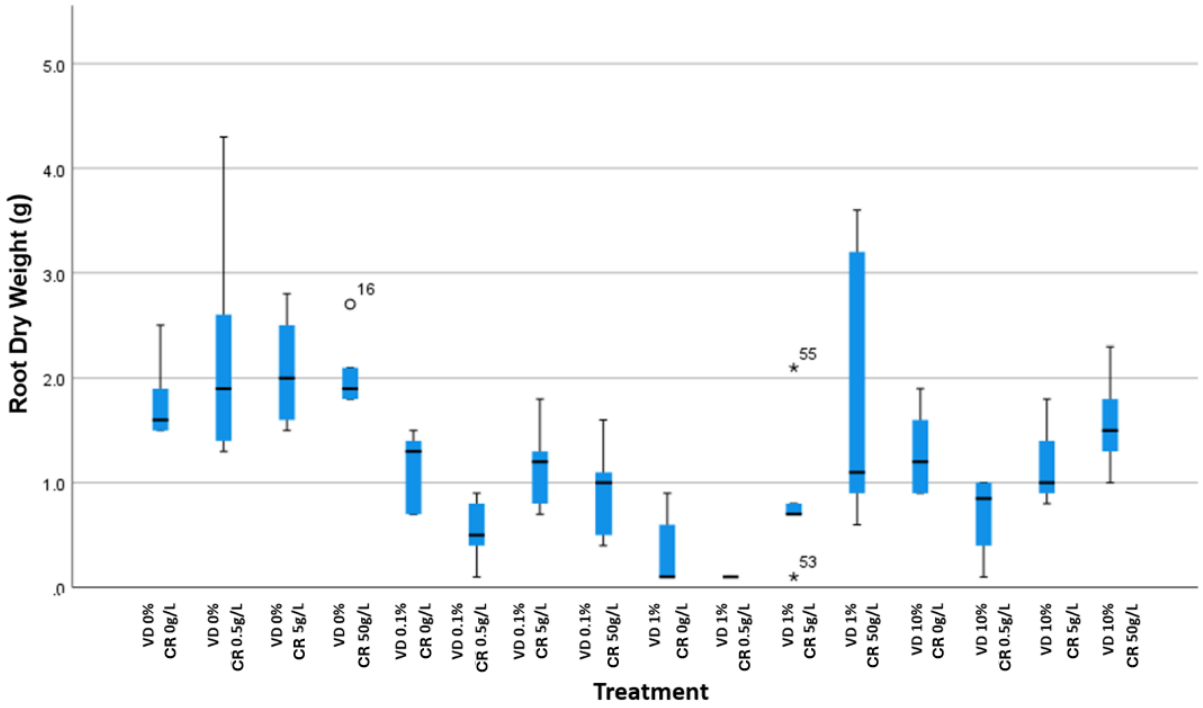

Figure 32. Root dry weight observed in strawberry plants against treatment (*Verticillium dahliae* inoculation * *Clonostachys rosea* dosage). Data is presented as interquartile range and median.

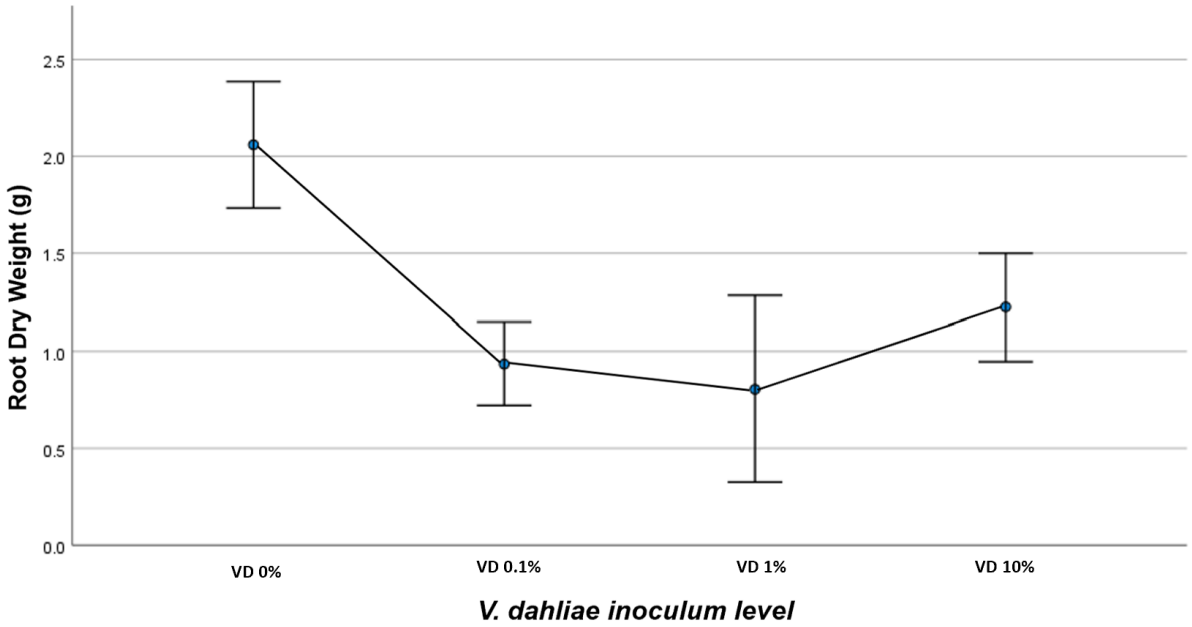

Figure 33. Root dry weight observed in strawberry plants per *Verticillium dahliae* inoculum level. Data is presented as mean ± standard deviation.

Assessing the relationship over time between *Verticillium dahliae* and *Clonostachys rosea* in the soil environment and the effect on disease and plant growth

This experiment was conducted to assess the effects of different inoculum levels and *C. rosea* treatment, in the form of Prestop, on the growth of strawberry plants and disease progression at 2-week intervals over 8 weeks. Measured populations of *C. rosea* significantly reduced over the time of the trial to undetected at 8 weeks (Figure 34) (assessed using a ranked ANOVA, p=<0.001). Populations of measured *V. dahliae* did not significantly change over the period of the trial (p=0.323) (Figure 35). Populations did appear to reduce between the weeks for 0.1%, 1% and 10%, reducing to undetectable by week 6 for 1% and 10% and all were undetectable by week 8. The 0% inoculum treatment produced a signal in week 6 but was undetectable by week 8, this may be explained by contamination in the samples, as measured levels were very low. There was no relationship observed between measured *C. rosea* and *V. dahliae* inoculation level as assessed by ranked ANOVA (p=0.84).

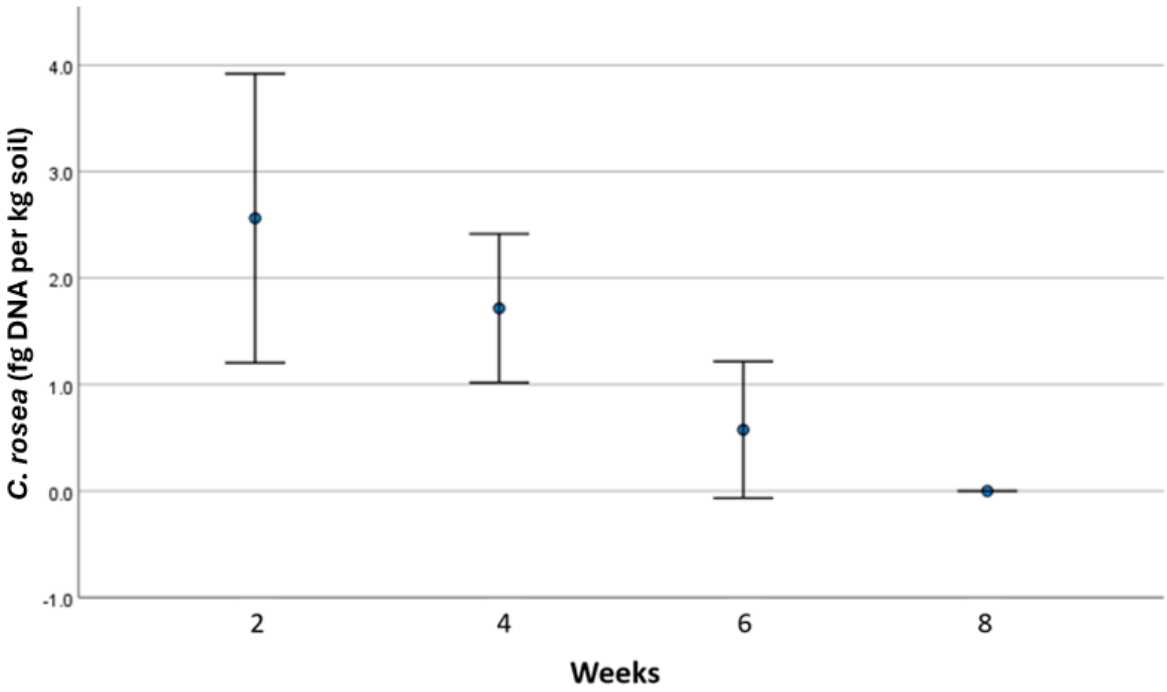

Figure 34. Plot of mean measured *Clonostachys rosea* quantities at each time point. Data is presented as mean ± standard deviation.

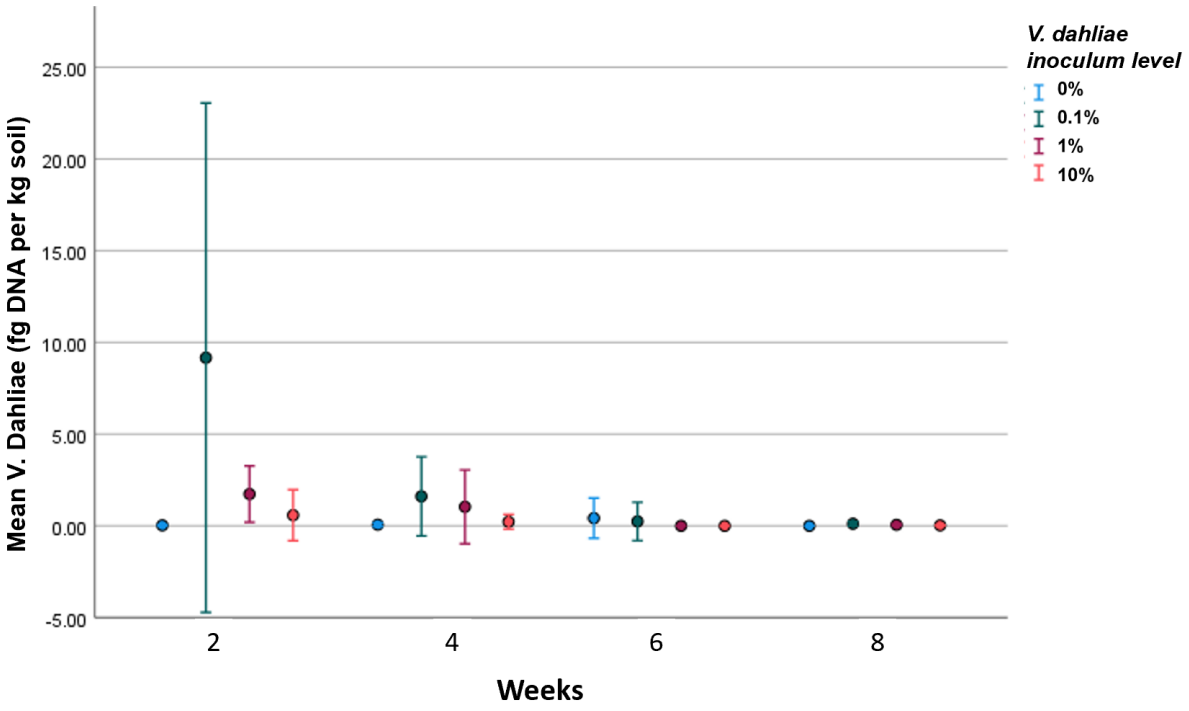

Figure 35. Plot of mean measured *V. dahliae* quantities by qPCR for each inoculum level at each time point. Data is presented as mean ± standard deviation.

i) Disease severity over time

The impact of treatment and time on disease severity was assessed using a two-way ANOVA. Data passed tests of normality as assessed by Shapiro-Wilk test ($p > .05$). The interaction between treatment and time on disease severity was found to not be significant ($p= 0.259$), nor were they significant in isolation ($p=0.791$ And 0.869 respectively). At 2 weeks, disease severity increased as initial *V. dahliae* inoculum increased however disease severity began to fluctuate as time increased. Although after 8 weeks, disease severity was highest for the 0.1% and 10% *V. dahliae* inoculum treatments and lowest in the 1% and control treatments (Figure 36).

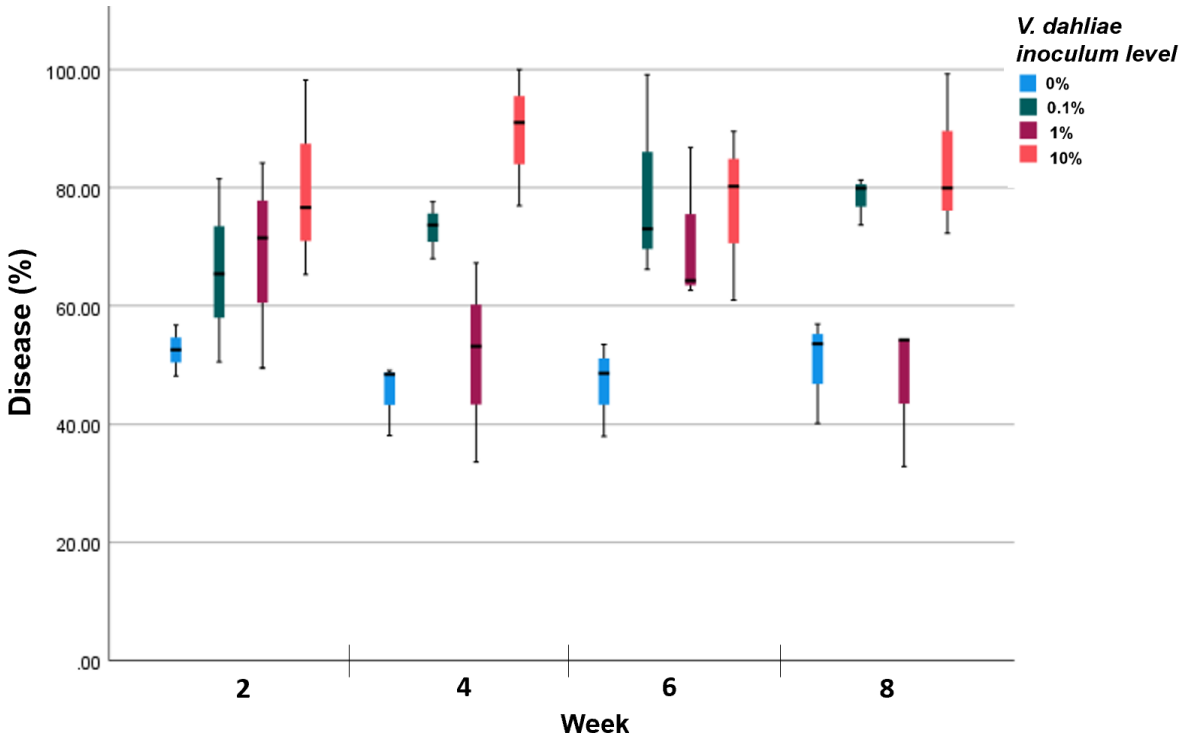

Figure 36. Percentage disease severity observed in strawberry plants against treatment over time (*Verticillium dahliae* inoculation * time). Data is presented as interquartile range and median.

ii) Plant Dry Weight

The impact of treatment and time on plant dry weight was assessed using a two-way ANOVA. Data passed tests of normality as assessed by Shapiro-Wilk test ($p > .05$). The interaction between treatment and time on plant dry weight was found to not be significant ($p= 0.323$), nor were they significant in isolation ($p=0.650$ And 0.327 respectively). There was high variation in plant dry weight across the trial, however the 0% inoculation treatment appeared to have a higher plant dry weight than other treatments (Figure 37 & 38). A correlation between measured *C. rosea* and plant dry weight was assessed using linear regression, no correlation was observed ($p=0.891$).

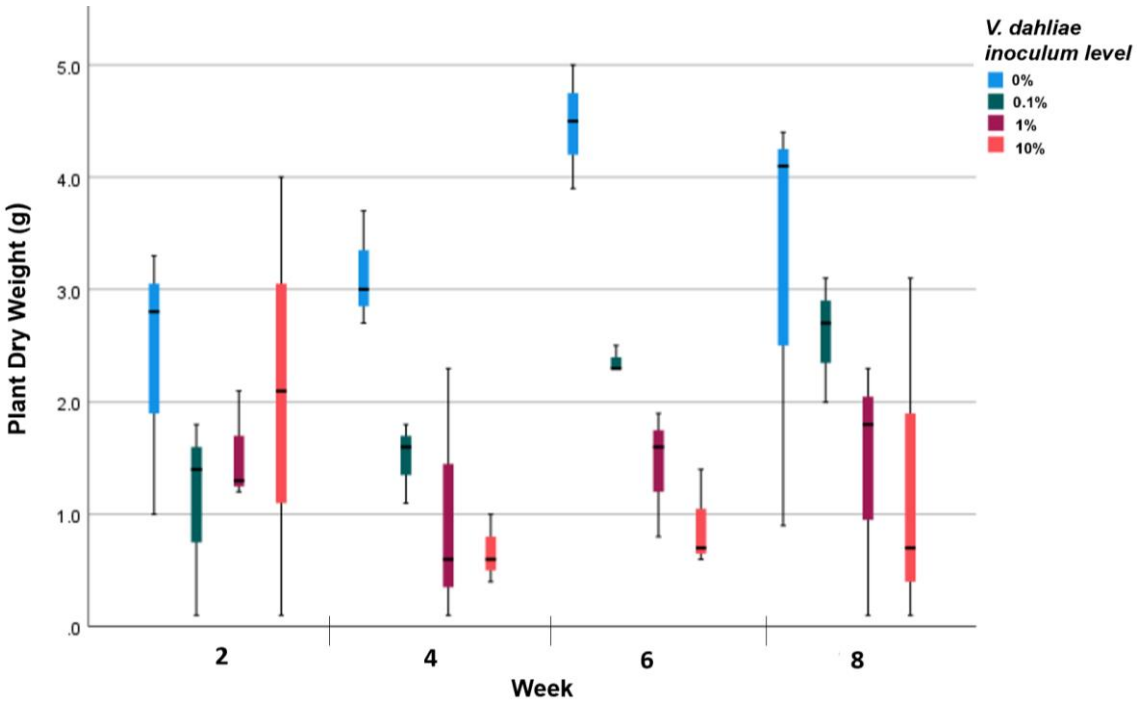

Figure 37. Plant dry weight observed in strawberry plants against treatment over time (*Verticillium dahliae* inoculation * time). Data is presented as interquartile range and median.

Figure 38. Image of strawberry plants at 8 weeks, side by side comparison of 0% *Verticillium dahliae* (Left) and 10% *V. dahliae* (Right).

iii) Root dry weight

The impact of treatment and time on root dry weight was assessed using a two-way ANOVA. Data passed tests of normality as assessed by Shapiro-Wilk test ($p > .05$). The interaction between treatment and time on root dry weight was found not to be significant ($p= 0.151$), nor were they significant in isolation ($p=0.271$ And 0.259 respectively). There was a high variation of root dry weight across the samples and no correlation with *C. rosea* treatment was observed ($p=0.294$) (assessed by linear regression) (Figure 39).

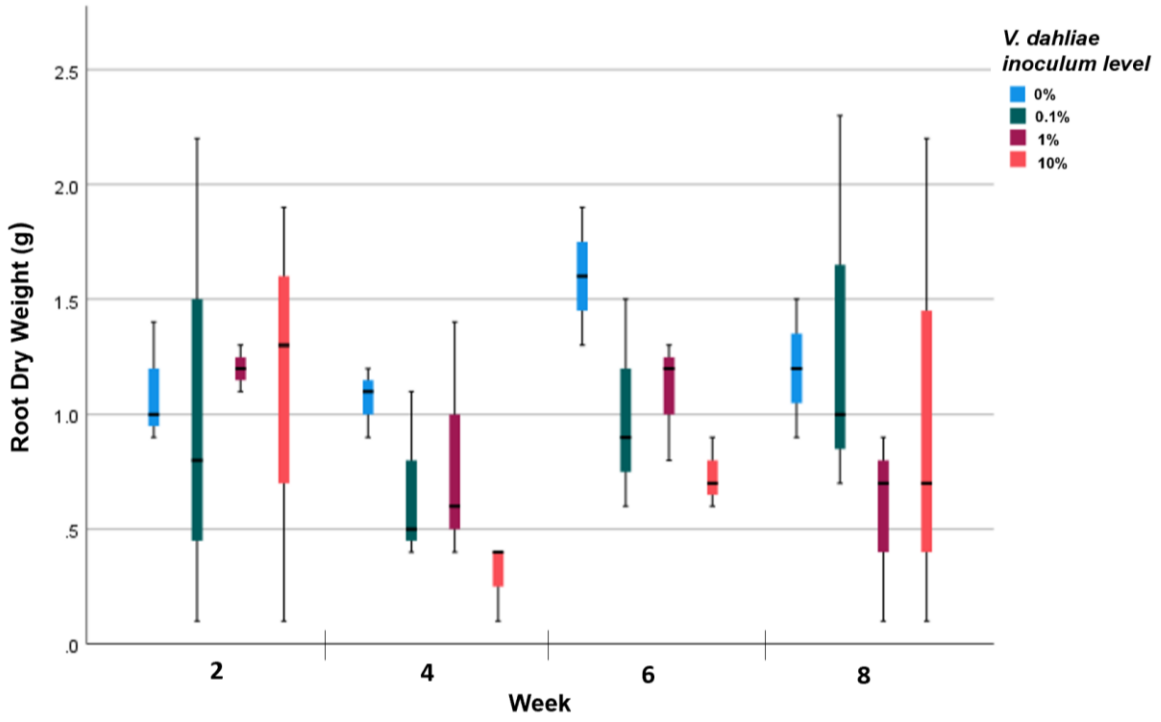

Figure 39. Root dry weight observed in strawberry plants against treatment over time (*Verticillium dahliae* inoculation * time). Data is presented as interquartile range and median.

<u>Assessing the impact of organic amendments (anaerobic digestate) on the rhizosphere population dynamics of *Verticillium dahliae* and *Clonostachys rosea*</u>

This experiment was conducted to investigate any effect of soil amendment with anaerobic digestate on the population dynamics of *V. dahliae* and *C. rosea*, in relation to the growth of strawberry plants and disease progression at 8 weeks.

qPCR was unable to detect *V. dahliae* or *C. rosea* in DNA extracts from soil samples when anaerobic digestate had been applied.

i) Disease severity

A three-way ANOVA was conducted to determine if disease observed in the crop was related to treatment (*V. dahliae* inoculation * *C. rosea* application * Anaerobic Digestate) (Figure 40). Data was not normally distributed for each group, as assessed by Shapiro-Wilk test (p > .05), therefore data was transformed (Log10). There were no significant interactions identified (p=0.075-0.781), nor were they significant in isolation (p=0.062,0.113 and 0.177 respectively).

Although disease levels were higher in *V. dahliae* inoculated plants than in those without, there appeared to be no interaction of the effects of biocontrol with *C. rosea* or of soil amendment with anaerobic digestate on disease severity.

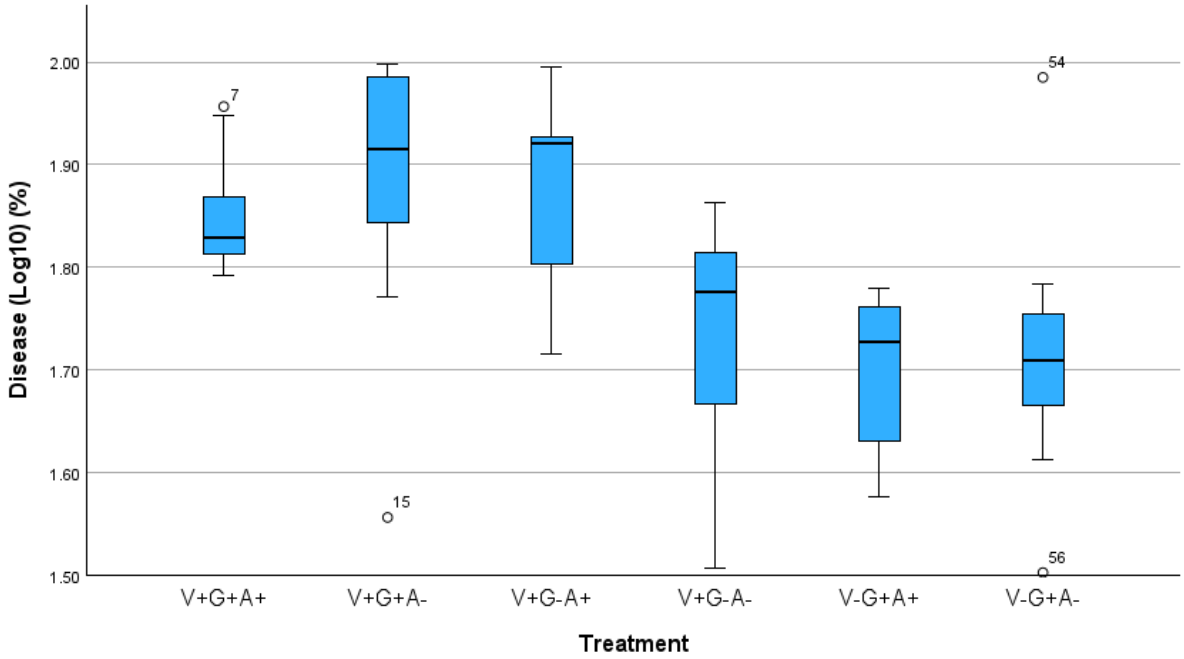

Figure 40. Percentage disease observed in strawberry plants against treatment (*Verticillium dahliae* inoculation * *Clonostachys rosea* dosage * Anaerobic Digestate). Data is presented as interquartile range and median. Note: The y-axis does not start at zero to provide a clearer view of the variation between data points.

ii) Plant dry weight

A three-way ANOVA was conducted to determine if plant dry weight was related to treatment (*V. dahliae* inoculation * *C. rosea* application * Anaerobic Digestate) (Figure 41). Data was not normally distributed for each group, as assessed by Shapiro-Wilk test (p > .05), therefore data was transformed (Log10). There were no

significant interactions identified (p=0.115-0.551). Plant dry weight in the V+C-A- treatment was significantly lower than V-C+A+ (p=0.010) and V-C+A- (p=0.012).

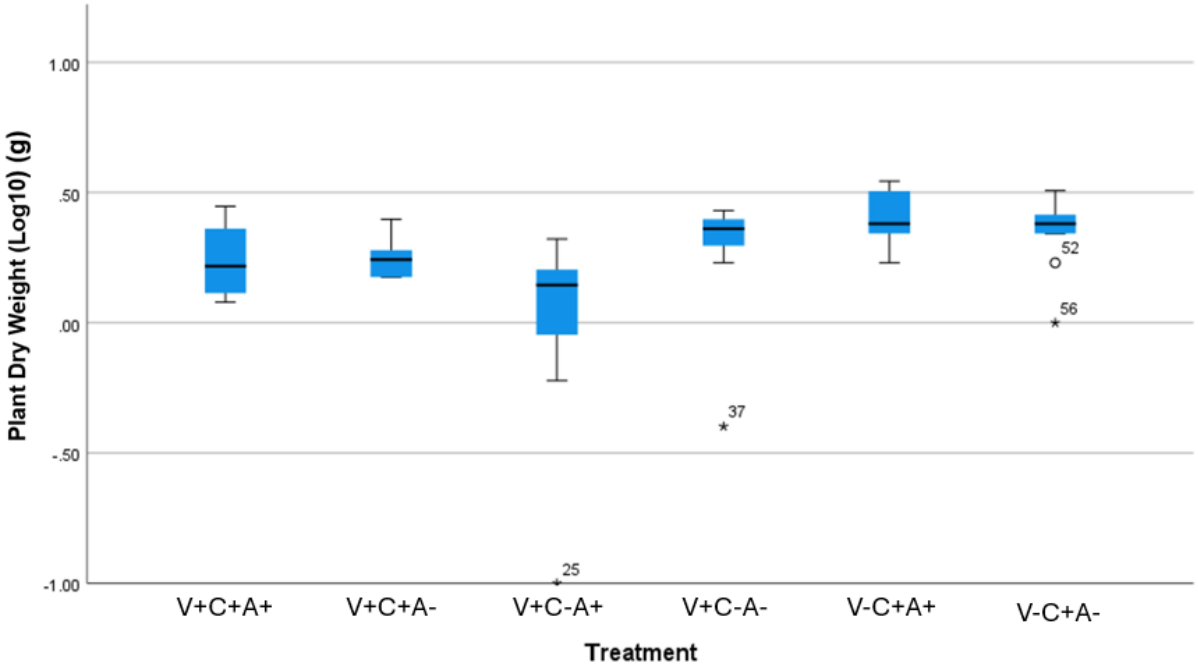

Figure 41. Plant dry weight observed in strawberry plants against treatment (*Verticillium dahliae* inoculation * *Clonostachys rosea* dosage * Anaerobic Digestate). Data is presented as interquartile range and median.

iii) Root Dry Weight

A factorial ANOVA was conducted to determine if root dry weight was related to treatment (*V. dahliae* inoculation * *C. rosea* application * Anaerobic Digestate) (Figure 42). Data was not normally distributed for each group, as assessed by Shapiro-Wilk test ($p > .05$), therefore data was transformed (Log10). There were no significant interactions identified (p=0.131-0.786). Looking at treatment in isolation, significant effect (p=0.043) was observed between V+C+A- and V-C+A+, where it increased.

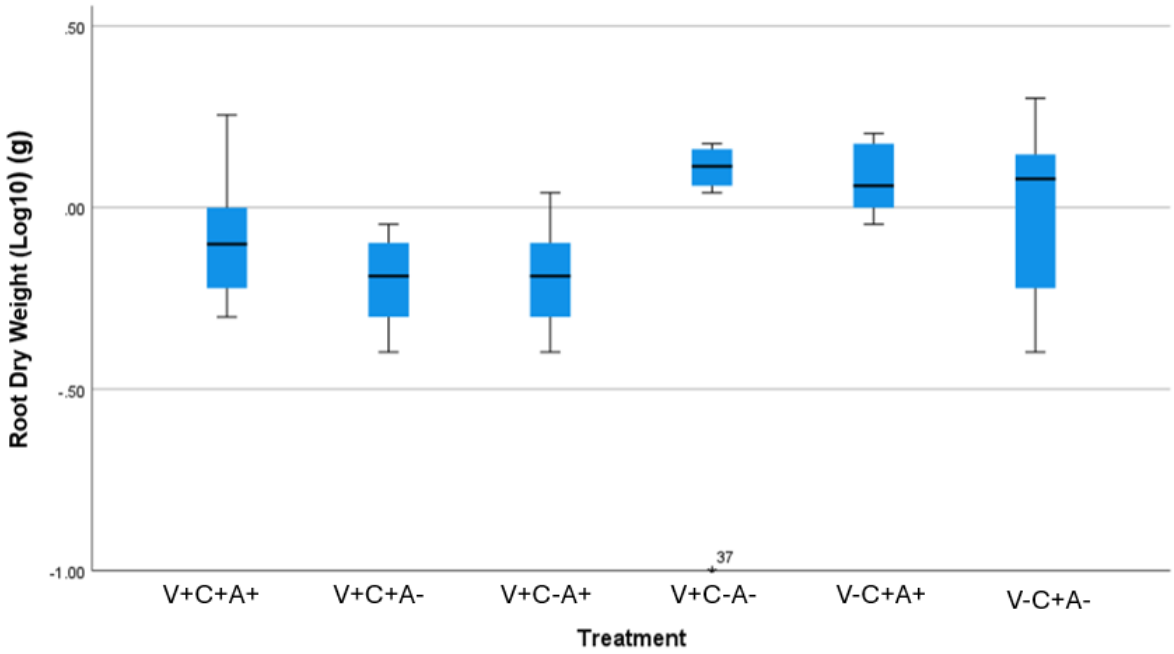

Figure 42. Root dry weight observed in strawberry plants grown in soil treated with combinations of *Verticillium dahliae* inoculation, *Clonostachys rosea* biocontrol application and amendment with anaerobic digestate. Data is presented as interquartile range and median.

iv) Discussion

Establishing relationship between *Verticillium dahliae* inoculum and *Clonostachys rosea* dosage at different levels in the soil and the impact on disease and plant growth

In the first experiment, which looked to establishing a relationship between *V. dahliae* inoculum and *C. rosea* dosage at different levels in the soil and the impact on disease and plant growth over an 8-week period, there was no observed correlation between the applied inoculum level of *V. dahliae* and the detected *V. dahliae* at the end of the trial. However, as the inoculum level of *V. dahliae* increased, its population appeared to decrease. Notably, the treatment with 0.1% inoculum level showed the highest measured population, although all treatments had a high level of deviation. By the end of the trial the 1% treatment had reduced to almost zero and at 10% it was completely undetectable. It is unclear why the highest inoculation level *V. dahliae* was unable to colonise and infect the strawberry plants and was

undetectable. It was shown in Chapter 4 (vi) that the methodology was capable of detecting these amounts of inoculum in this compost, therefore it is believed that this lack of detection is due to its absence rather than molecular shortcomings. Additionally, disease symptoms and plant senescence decreased to levels similar to the control, supporting this hypothesis. The exact reasons for the lack of colonization remain unclear, though several factors could have contributed. For example, V. dahliae requires specific soil conditions—such as pH, moisture, nutrient availability, and organic matter content—to survive and thrive, none of which were assessed during these trials. In a study by (Langendorf, 2016) they reported that various artificial attempts to inoculate soil with *V. dahliae* (drenching soil with wilt conidial suspension without root injury in soil or sandy compost, direct injection of conidial suspension in strawberry crown, root dipping in conidial suspension with artificial root injury before transplantation in autoclaved sandy compost and inoculation of wilt hyphae with colonised potato dextrose agar (PDA) media plates buried at the bottom of the pots filled up with attapulgite clay) failed to illicit wilt symptoms. This highlights the potential challenges associated with artificial inoculation with *V. dahliae* and that we do not fully understand its infection and life cycle. In this study, the need for optimisation of another wilt inoculation method to enable further research under controlled conditions was identified (Langendorf, 2016). Despite no clear linear relationship between the inoculum level and disease, the method used in this PhD study has made strides towards successful artificial inoculation of *V. dahliae*.

Likewise, to measured *V. dahliae*, no correlation was found between the dosage of *C. rosea* and its measured presence. The population of *C. rosea* decreased as the dosage increased, although this decrease was less pronounced compared to *V. dahliae*. One of the major challenges with biocontrol organisms is their ability to colonise (Pliego *et al.*, 2011), and this PhD study supported this concern as populations failed to increase or even stabilise. The success of a biocontrol agent is dependent on the interaction with the host plants and their ecological fitness (Mirmajlessi, 2017). This contributes to the lack of confidence from growers in their uptake of biocontrol methods (Moser *et al.*, 2008). However, it is worth noting that the lack of presence at 8 weeks falls in line with the recommended reapplication rate for Prestop (Lallemand, Canada). Tut *et al.* (2021) reported that formulated biocontrol agents performed better and had higher efficacies, particularly at lower doses, than unformulated organisms which was attributed to proprietary additives, including

stickers and adjuvants. This may have been due to larger doses not surviving in the soil as effectively as lower doses which was seen in this trial.

Despite the reduction in populations by the end of the trial a moderate negative relationship was identified between the measured populations of *V. dahliae* and the applied dosage of *C. rosea.* This indicates that *C. rosea* may have had a reducing effect on *V. dahliae* populations although only minor. Metcalf *et al.* (2007) also observed no additive effect when increasing dosage of *Trichoderma koningii* on suppression of white rot caused by *S. cepivorum*. In-vitro studies have shown *C. rosea* inhibiting the growth of *V. dahliae* by 94.3% (Mirmajlessi, 2017), evidencing its potential against *V. dahliae* however its efficacy depleted once used in this trial. Additionally, *C. rosea* has also been shown to be effective *in planta* (Deketelaere *et al.*, 2017).

Regarding disease progression, the control group exhibited a mean percentage disease severity (in the control 'disease severity' is being treated as natural senescence, a symptom of *Verticillium* Wilt) of 59.2% and the lowest standard deviation of 2.79. This suggests that the application of either *V. dahliae* or *C. rosea* triggered a response, although the response was unpredictable, as evidenced by the large deviations observed in the treatment samples (standard deviation range 4.24-19.28).

Disease appeared to increase with higher inoculum levels of *V. dahliae* until reaching 10%, after which it decreased. However, a significant difference was only detected between the 0% and 1% inoculum levels. This increase in disease with inoculum level is expected, although it would be expected that the 10% treatment would have the highest amount of disease if not 100% disease. Symptoms varied drastically between the plants independent of treatment. This was unexpected as direct relationships between inoculum and wilt development on a variety of crops have been reported such as; potato (Davis *et al.*, 1994; Nagtzaam *et al.,* 1997), eggplant (Cohen *et al.,* 2005), cauliflower (Xiao and Subbarao, 2007), horseradish (Khan *et al.,* 2000), cotton (Paplomatas *et al.*, 1992), olive (López-Escudero *et al.,* 2007), tomato (Grogan *et al.,* 1979), artichoke (Berbegal *et al.*, 2007) and pepper (Bhat *et al.*, 2003) and as low as 0.3g has been reported as causing 5% mortality in strawberries (Harris and Yang, 1996). However, when you consider the inoculum that was detected rather than applied, these sporadic disease symptoms observed become more understandable.

In terms of plant dry weight, significant differences were found when comparing different treatments to the control group (V0CR0). Treatments V1CR0, V1CR1, V1CR3, all three of the 1% *V. dahliae* treatments (V2CR0, V2CR1, V2CR2, V2CR3), and V3CR1 exhibited significant reduction from the control. This indicated that the application of *V. dahliae* did reduce plant growth as expected, as *V. dahliae* invades the plant vascular system diminishing plant metabolism (Bressan *et al.*, 2016). Visually, there appeared to be an increase in plant dry weight as *C. rosea* application increased with in each *V. dahliae* inoculum level, however this was not found to be significant. *C. rosea* species have been reported as promoting plant growth (Adedayo and Babalola, 2023), therefore it was expected that those treated with *C. rosea* would have a higher plant dry weight.

Regarding root weight, significant differences were observed between certain treatment groups. For example, when comparing with the control group, treatments V1CR1, V1CR3, V2CR0, V2CR1, V2CR2, and V3CR1 all exhibited significantly lower root weights. However, no clear pattern relating to the applied treatments emerged. This again may have been related to the sporadic levels of *V. dahliae* and *C. rosea* actually detected. Similarly to plant dry weight it was expected that root dry weight would be larger in those treated with *C. rosea* due to its association with aiding plant growth (Adedayo and Babalola, 2023).

This initial experiment revealed that after 8 weeks from application detectable levels of *V. dahliae* and *C. rosea* varied heavily and that colonisation by these organisms was unreliable. To understand if this undetectability and unreliability was present at all stages of the trial it was then investigated how the populations interacted over time.

Assessing the relationship over time between *Verticillium dahliae* and *Clonostachys rosea* in the soil environment and the effect on disease and plant growth

This experiment was conducted to assess the effects of different inoculum levels and *C. rosea* treatment (commercial Prestop (Lallemand, Canada)) on the growth of strawberry plants and disease progression at 2-week intervals over 8 weeks. Over the course of the trial, the measured populations of *C. rosea* significantly reduced each week eventually becoming undetectable by the 8th week. This reduction by the 8th week corroborates with the recommended dosages given by the supplier, to

reapply at 8 weeks (2 months), which was also observed in the first trial. The gradual reduction of *C. rosea* suggests its inability to colonize and survive in this environment, despite the presence of a host plant and its initial capacity to colonize the root (Rahman *et al*., 2021). Ecological fitness (Mirmajlessi, 2017) is crucial for the success of a biocontrol agent, indicating that C. rosea may be unsuitable as a long-term solution in this environment.

In contrast, the populations of measured *V. dahliae* did not significantly change over the 8-week period. However, between weeks, populations appeared (not significantly) to decrease for inoculum levels of 0.1%, 1%, and 10%, eventually becoming undetectable by week 8. Again, in this trial, at the inoculum level used, *V. dahliae* failed to colonise and survive. It is unclear as to why as *V. dahliae* was unable to flourish despite the presence of a host. Potential explanations include unfavourable environment and potentially high competition within the species for nutrients.

Despite the reduction in *V. dahliae* over the period of the trials, disease symptoms were still observed which is unsurprising due to *V. dahliae*'s reported virulence at low levels (Klosterman *et al.*, 2009; Habib *et al.*, 2017; Mirmajlessi, 2017). The interaction between treatment and time on disease was found to be non-significant. Disease levels increased as the inoculum level of *V. dahliae* increased at 2 weeks, but this linear response disappeared as time increased and disease response fluctuated week by week. By the 8th week, the 10% *V. dahliae* inoculum treatment consistently exhibited the highest disease levels, while the 0% inoculum treatment showed the lowest disease levels, consistent with what was expected. As discussed in the prior trial, linear relationships between inoculum density and disease incidence have been described for V. dahliae in a variety of crops, including strawberry. The lack of such a relationship here is surprising, though it may be linked to the inconsistency in V. dahliae detection in the soil.

In terms of plant growth, the interaction between treatment and time on plant dry weight was again found to be non-significant. Plant dry weight did not significantly change over time or vary with treatment. There was high variation in plant dry weight across the trial, perhaps leading to this insignificant result. However, the 0% inoculum treatment showed relatively higher plant dry weight compared to other treatments. This indicates that the presence of *V. dahliae* may have negatively influenced plant growth, but other factors may have also contributed to the observed

variations. Although not significant, this observation of a difference between the control and applied treatment indicates experimental success, but perhaps signifies an oversimplification of these relationships in the experimental design.

Similarly, the interaction between treatment and time on root dry weight was non-significant. High variation in root dry weight was observed across the samples, and no correlation was found between measured *C. rosea* and root dry weight. Again, this implies that *C. rosea* treatment may not have had a direct impact on root dry weight, or other factors may have influenced root development.

It was hoped that the additional measurements over the period of the trial would reveal more about the complex relationship between the soilborne pathogen *V. dahliae* and the biocontrol agent *C. rosea*. Within the first 2 weeks the two organisms of interest behaved as expected, however as time moved on detected levels fluctuated and no pattern could be discerned. Although the reasons behind this are unclear, it demonstrated the complex nature of these organisms within the wider environment, therefore further research will need to be performed. Despite this, these trials demonstrated the potential of qPCR for monitoring target organisms in soils.

Assessing the impact of organic amendments (anaerobic digestate) on the relationship between *Verticillium dahliae* and *Clonostachys rosea*

This experiment was conducted to assess the effect of the addition of anaerobic digestate in combination with different inoculum levels of *V. dahliae* and *C. rosea* treatment, in the form of Prestop, on the growth of strawberry plants and disease progression at 8 weeks. As part of the soil health movement emphasis is put on increasing organic matter in the soil (DEFRA, 2018), which can be achieved by the application of organic fertilisers like anaerobic digestate. Additionally, application of organic matter has been reported in supressing soilborne disease (Davey *et al.*, 2019). Its interaction with *C. rosea* is also of interest as it survives on organic matter in the soil (Tut *et al.*, 2021), and it was hypothesised that this may encourage colonisation of the *C. rosea*. Despite this, qPCR was unable to detect *V. dahliae* or *C. rosea* in soil samples with anaerobic digestate applied. There are two potential reasons for this, either there was no DNA of either *V. dahliae* or *C. rosea* in the soil, potentially due to the anaerobic digestate destroying these organisms, or that the anaerobic digestate inhibited the qPCR testing. It is known that soils with higher

organic matter contain more inhibiting substances such as humic acids (Dequiedt *et al.*, 2012; Liu *et al.*, 2012) and composts have been reported as containing as high as 70% organic matter content without additional organic matter applications (LaMontagne *et al.*, 2002).

In terms of disease progression, inoculation with *V. dahliae* (V+C+A+) significantly increased disease levels compared to the corresponding treatment without *V. dahliae* inoculation (V-C+A+). However, no clear pattern emerged from the disease data. The addition of anaerobic digestate did not have any discernible effects on disease progression nor did it promote effectiveness of *C. rosea.*

The addition of anaerobic digestate had minimal impact on plant dry weight. However, plants treated with *V. dahliae* and *C. rosea* but without anaerobic digestate (V+C-A-) showed significantly lower dry weight compared to both the treatment with anaerobic digestate and no *V. dahliae* (V-C+A+), and the treatment with neither *V. dahliae* nor anaerobic digestate (V-C+A-). Regarding root dry weight, a significant effect was observed between the treatment with *V. dahliae* inoculation and *C. rosea* treatment but without anaerobic digestate (V+C-A-) and the treatment without *V. dahliae* inoculation and anaerobic digestate (V-C+A+), where root dry weight increased. However, no other significant effects were observed. Again, the addition of anaerobic digestate did not appear to affect the influence of *C. rosea* or *V. dahliae* on plant development, namely root dry weight. The application of anaerobic digestate and the increase of organic matter is associated with increased plant growth due to availability of nutrients (O'Connor *et al.*, 2022; Weimers *et al.*, 2022), therefore it is surprising that there was not a significant difference between those with anaerobic digestate and those without. However, this trial assumed the need for additional nutrients from the AD.

The findings suggest that adding anaerobic digestate did not have a noticeable impact on disease progression, plant dry weight or root dry weight. Further research is needed to address the challenges posed by organic matter on DNA extraction if qPCR is to be developed as a tool for farmers in the future.

Conclusions

This series of experiments aimed to understand the dynamics between *V. dahliae*, *C. rosea*, and their interaction with soil amendments by utilising molecular techniques. The initial experiment revealed a marked variability in detectable levels of *V. dahliae* and *C. rosea* after the 8-week application period, emphasizing the unreliability of colonization by these organisms. To further probe this variability, subsequent experiments were conducted.

The second experiment focused on assessing the impact of different inoculum levels and *C. rosea* treatment (commercial Prestop (Lallemand, Canada)) on strawberry plant growth and disease progression at 2-week intervals over an 8-week span, in an attempt to understand the fluctuation seen in the first experiment. In the initial two weeks the target organisms exhibited anticipated behaviour. However, as time progressed, measurements displayed fluctuating levels with no discernible pattern. This indicates that population dynamics are more complex than the trial design allowed for, and that the simple presence of a host does not necessarily mean disease and pathogen success. Therefore, it would have been beneficial to have collected samples at timepoint 0, to establish initial inoculum behaviour within those initial 2 weeks.

The third experiment investigated the effect of adding anaerobic digestate alongside different inoculum levels of *V. dahliae* and *C. rosea* treatment (Prestop) on strawberry plant growth and disease progression at 8 weeks. The findings indicated that the addition of anaerobic digestate did not significantly impact disease progression, plant dry weight, or root dry weight. However, it highlighted the need to address challenges posed by organic matter on DNA extraction, particularly if qPCR is to be developed as a practical tool for farmers in the future.

Throughout the experiments, several key observations emerged. Firstly, that recommendations regarding application of *C. rosea* as a commercial product fell in line with observations, such as the need for reapplication after 2 months as the populations dropped below detection levels, and that application above the recommended dose did not offer further benefits. Despite this correlation with industry recommendations, an impact of *C. rosea* on disease progression and *V. dahliae* populations was not observed, although studies previously discussed indicate *C. rosea* had suppressive action against *V. dahliae*. While the presence of *V.*

dahliae was found to elevate disease levels, the specific role of *C. rosea* in disease control and its impact on plant and *V. dahliae* populations remain areas of ongoing inquiry. However, these experiments did demonstrate the potential of qPCR as a tool to monitor target populations, though further improvements will need to be made to overcome the challenge of high organic matter.

Chapter 6- Using qPCR to quantify pathogens, their relationship to disease progression in the field and their control using organic amendments in different cropping systems

i) Introduction

The significance of soilborne pathogen inoculum density to disease incidence in controlled conditions has been discussed in Chapter 5, however further value comes from understanding these relationships in the field. An important step in disease control is to be able to measure pathogen populations and distributions in field soil and predict their effect on crop diseases (Bebber and Gurr, 2015). There are existing models that begin to predict the risk of disease, for example EPIPRE which is used for controlling diseases and pests on wheat. It guides decision making processes on choice of varieties and disease control strategies throughout the growing season (Yuen and Hughes, 2002). Prediction models are based upon the disease triangle (Host-Pathogen-Environment), they work on the presence of the host, assume the presence of the pathogen and base their prediction on the environment, namely weather (Yuen and Mila, 2015). However, by quantifying the amount of pathogen, rather than simply assuming its presence, it is possible to improve prediction models based upon Inoculum Density – Disease Incidence relationships (ID-DI) (Gurr *et al.,* 2011). The ability to measure and predict not just the presence but the harmfulness of disease leads to better decision making for disease management (Savary *et al*., 2006), allowing for appropriate and potentially more sustainable interventions to be made at optimal timings (Newbery *et al.,* 2016).

Disease controls are often prescribed on the results of an early in-crop disease assessment. This requires waiting until symptoms appear. Diagnosis based upon visual symptoms can lead to misdiagnosis and then incorrect control methods are implemented, for example it has been found that Eyespot assessment early in the season is an unreliable indicator of subsequent disease development (Yuen and Hughes, 2002). Furthermore, damage to the crop and loss of yield may have already occurred, making control measures reactive rather than preventative. Therefore, to prevent losses as early as possible, decisions on managing diseases need to be made prior to planting. Being able to quantify pathogens in the soil prior to cropping may therefore prove to be a valuable tool (McKay *et al.*, 2009). Soil assays at

planting time could assist a grower in selecting cultivars (Paplomatas *et al.*, 1992), reduce the use of pesticides and change management practices.

Prediction models are constantly being improved. However, there is a need for molecular and microbial sciences to be integrated into models. Understanding the microbiome and how it interacts with the environment can help inform the environmental aspect of prediction models (Saleh-Lakha *et al.*, 2005). Molecular techniques can provide quick and reliable identification and quantification of pathogens informing decision making and modelling. Kettles and Luna (2019) predict that by 2044 sequenced based diagnostics of field samples will be possible close to real-time, which will allow monitoring of local fungal populations, their evolution and distribution. There is a call for scaling up of these sciences, so we can begin to understand what is happening at the gene, individual, community and landscape level (Standing *et al.*, 2007). This will help monitor changes over wide areas but also monitor changes in the microbiome in response to climate change. Such increased knowledge would lead to reduced management costs, reduced pesticide use and reduced crop loss, contributing to food security (Savary *et al.*, 2006).

This integration of molecular science with prediction modelling has already been proven to be successful in Australia (McKay *et al.*, 2009), where pathogen levels (e,g, *Rhizoctonia solani, Fusarium culmorum, Heterodera avenae*) are quantified using qPCR from soil samples and used to predict a range of diseases in cereals, allowing farmers to change cultivars or alter management in response. However, the threshold for the majority of critical soilborne pathogens of cereals have been established in Australia, allowing the development of risk categories, whereas this approach has not yet been applied in the majority of countries. Soil is a complex environment, adding challenges with detection, diagnosis and prediction (Mirmajlessi, 2017) even where these thresholds and risk categories have been established. Often these thresholds are established via controlled environment experiments, much like those performed in chapter 5, allowing the formation of quantitative relationships between the pathogen and host (Yuen and Mila, 2015). However these often fail to account for other impacting factors, such as weather, past field history and field/soil characteristics (Yuen and Mila, 2015) and dynamics of pathogen spread (Mastin *et al*., 2019).

For this thesis, one of the pathogens investigated within field trials is *F. oxysporum. Fusarium oxysporum* is one of the many filamentous fungi of the genus *Fusarium*

(Ma *et al*., 2013). *Fusarium* spp. are economically important within food production and agriculture, and some are known for producing mycotoxins such as fumonisins (Ma *et al*., 2013). *F. oxysporum* is known to cause disease in over 100 plant species, including onions, daffodils, asparagus, lettuce and bananas (Sasaki *et al*., 2015). Despite the large range of hosts, individual taxa within *F. oxysporum* have a narrow host range (Sasaki *et al.*, 2015; Ampt *et al.*, 2018). There are more than 120 *formae speciales* of *F. oxysporum*, for example *F. oxysporum f. sp. lactucae* that only infects lettuce (Michielse and Rep, 2009). However, many genotypes of *F. oxysporum* are non-pathogenic, so research focus has been mainly on the disease-causing isolates (Michielse and Rep, 2009; Ampt *et al.*, 2018). *Fusarium oxysporum* diseases are known by many names; e.g. *Fusarium* root rot, *Fusarium* wilt, basal rot (Sasaki *et al*., 2015) and bulb rot. Symptoms often include; stunting, yellowing of lower leaves, progressive wilting and finally the death of the plant (Michielse and Rep, 2009). In the case of bulb crops, *Fusarium* rots develop from the basal plate destroying the crop. *Fusarium* diseases impact during both production and storage of bulb crops, for instance onions (Sasaki *et al*., 2015). *Fusarium oxysporum* poses particular management challenges due to its longevity in the soil, it produces hardy chlamydospores that can survive in the soil for many years (Taylor *et al.*, 2019). Current management relies on resistant cultivars and crop rotation (Sasaki *et al.*, 2015; Taylor *et al.*, 2019), as well as fungicide and soil sterilisation which can have undesirable environmental impacts and are increasingly undergoing more restrictive regulation (Taylor *et al.*, 2019).

Fusarium oxysporum was chosen for this study due to its impact on the UK onion and daffodil market; in 2013 losses were estimated to be approximately £11million in onion (Clarkson and Taylor, 2014) and it is estimated that annual losses for daffodil are around £4.5million (Clarkson *et al*., 2019). Losses are also predicted to increase under current climate change scenarios, as disease development is favoured by warmer temperatures (Taylor *et al.*, 2019).

A second pathogen explored in field trials described here is *V. dahliae*, this pathogen has already been investigated in chapter 5 within controlled conditions. Here its behaviour in the field in response to environmental conditions is investigated. *Verticillium* wilts are single cycle diseases, so inoculum density at planting is critical to control decisions (Paplomatas *et al.*, 1992; Berbegal *et al.,* 2007). Microsclerotia survive in the soil and can persist for more than 20 years (Paplomatas *et al*., 1992;

Mirmajlessi, 2017), but other phases of the fungus life cycle are also capable of surviving environmental pressures, making it difficult to eradicate (Schnathorst, 1981). Further challenges originate from its low inoculum threshold for infection. Mirmajlessi (2017) reported inoculum densities as low as 2 microsclerotia per g of soil can cause 100% wilt in strawberries, indicating the importance of establishing populations prior to planting. A further challenge of monitoring *V. dahliae* populations is that it generally occurs in clusters or aggregated patterns, with clusters ranging in size from 2 to 4m (Xiao and Subbarao, 2007). Current efforts to control *V. dahliae* rely on fungicides (Klosterman *et al*., 2009), soil fumigation (Nicot and Rouse, 2008; Klosterman *et al*., 2009; Mirmajlessi, 2017), crop rotations (Mirmajlessi, 2017) and resistant cultivars (Habib *et al*., 2017). However, the genetic basis of resistance in the host is currently unestablished. Furthermore, there is a lack of land which does not have an existing population of *V. dahliae* and crop rotation is ineffective due to its large host range (Klosterman *et al*., 2009). *V. dahliae* is responsible for billions of dollars of annual crop losses globally (Klosterman *et al*., 2009) and has been described as having the greatest economic impact out of all *Verticillium* species (Habib *et al*., 2017).

The final soil-borne fungal pathogen explored under field conditions in this chapter is *S. vesicarium,* the causal pathogen of purple spot disease and leaf spot in Asparagus. It is also known to cause other diseases such as brown spot in pears (Graf *et al.*, 2016) and Welsh onion leaf blight (Misawa and Yasuoka, 2012). It causes premature defoliation of the asparagus fern, causing up to 52% yield loss in some cases (Graf *et al.*, 2016). *Stemphylium vesicarium* is a filamentous ascomycete, with over 30 different species recognised within the genus (Graf *et al.*, 2016). *Stemphylium* is known for a high conidial variability in different temperatures and substrates, meaning identification based on morphological assessments are often flawed, increasing the value of alternative molecular identification methods (Graf *et al.*, 2016).

Organic amendments and disease

As part of the field trials undertaken here, various organic amendments were applied in experimental plots to monitor their impact on pathogen populations and subsequent disease. The amendments studied were incorporation of cover crops or green compost at the onion field trial, farmyard manure, green compost or

mycorrhizae supplements at the daffodil field trial and, anaerobic digestate or the biocontrol product Prestop (Lallemand Inc., Canada) at the raspberry field trial.

Existing methods of controlling soil-borne pathogens included soil fumigation with methyl bromide, which has been phased out and soil heating, both of which have their drawbacks, both environmentally and regarding their cost-efficiency (Katan, 2000). Organic amendments have been associated with disease control of soilborne pathogens since the 1940's, when amendments such as nitrogen rich organic wastes and crop residues were linked to control of *R. solani*, *F. oxysporum* and *V. albo-atrum* (Bonanomi *et al*., 2018). Since then, the range of organic amendments found to have disease suppressive effects has increased, and now includes animal and green manures (Lazarovits, 2001; Klosterman *et al.*, 2009; Larkin, 2013; Bonanomi *et al.*, 2018; Mann *et al.*, 2019), bonemeal (Lazarovits, 2001), composts (Bonanomi *et al*., 2018), biochar (Bonanomi *et al*., 2018), cover crops (Abawi and Widmer, 2000; Klein *et al.,* 2011; Romdhane *et al.*, 2019) and the application of beneficial organisms (Mirmajlessi, 2017; Wilkinson *et al.*, 2019). Despite this, their integration into common farm practices for disease control has been limited, which may be due to their lack of predictability, consistency (Bonanomi *et al*., 2018) and slow rate of effectiveness (Bailey and Lazarovits, 2003). For instance, Lazarovits *et al.,* (2001) found that soil amendment with swine manure killed *V. dahliae* within a day of application only in acidic soils but was ineffective in neutral or alkaline soils. Furthermore, animal manure has also been associated with increased incidence of scab disease in potato (Bailey and Lazarovits, 2003), having the opposite of the desired effect and adding to the uncertainty surrounding the use of organic amendments for disease control. Manures have been found to supress *Verticillium*, by releasing antifungal volatile fatty acids (acetic, propionic, butyric, isobutryic, valeric, isovaleric and caproic acids), however this effect only occurs in acidic soils (Klosterman *et al*., 2009). Manures have also been attributed to increase organic matter, active carbon, water holding capacity and water stable aggregates (Mann *et al*., 2019). Similarly, composts have been found to increase organic matter and soil microbial populations (Abawi and Widmer, 2000).

Disease control through the use of organic amendments is reliant on the soil characteristics and the biological factors being conducive (Lazarovits, 2001). Many attempts at disease control using organic amendments rely on increasing organic matter and microbial biomass carbon (Davey *et al.*, 2019). The work by Lazarovits

(Lazarovits, 2001; Lazarovits *et al.,* 2001; Bailey and Lazarovits, 2003) attributes the success of high nitrogen amendments (for instance, poultry manure) for controlling soil-borne pathogens to the generation of ammonia and / or nitrous acid, following the degradation of the amendment by microorganisms. Therefore, increased biological activity is essential for the control of soil-borne pathogens (Lazarovits, 2001), for both the degradation of the nitrogen and the out-competing/antagonism of the pathogens by other microbes. This increased microbial activity has been shown to 'displace' pathogens from their natural niche within the soil microbiome, putting it under stress, removing its ability to infect and ultimately causing its death (Lazarovits, 2001; Bailey and Lazarovits, 2003; Davey *et al*., 2019). Lazarovits (2001) found that after incorporation of organic substrates in the lab, soil bacteria and fungi populations increased and 90% of *V. dahliae* microsclerotia died after 1 week. However, the soil characteristics and biological factors impact the effect of the microbial activity, for instance affecting the rate at which organic amendments, such as manure, are broken down (Klosterman *et al*., 2009).

Cover crops have been used to minimise disease impacts in crops, for instance broccoli has been used to reduce *Verticillium* populations (Larkin, 2013). They have been found to enhance soil characteristics by increasing organic matter and available nutrients and minimising soil leaching as well as inhibiting weed growth and potentially increasing yields (Abawi and Widmer, 2000; Romdhane *et al*., 2019). Cover crops, such as brassicas, are also associated with the release of glucosinolates, which are biocidal compounds (Kirkegaard and Sarwar, 1998). The efficiency of disease reduction after incorporation of cover crops can vary with the choice of species, the position in the rotation and the type of termination; spraying off (glyphosate) and incorporation (Romdhane *et al*., 2019). Romdhane *et al*. (2019) tested the impact of 4 different cover crop mixtures (a mixture of 12 different species, with or without legumes) and 3 cover crop termination strategies (frosting, rolling and glyphosate) on nitrogen and carbon dynamics, soil microbial diversity, soil structure, abundance of bacteria and archaea, N-cycling microbial guilds. They found that soil properties were modified due to cover crop management rather than species, as nitrogen and carbon was higher in treatments that received the frosted termination treatment, rather than glyphosate and rolling, and that bacterial abundance was not affected by cover crop treatment. In contrast to the findings by Romdhane *et al*. (2019) that crop species did not impact the effect of the cover crop on soil

characteristics, Klein *et al.* (2011) found that the species did affect the level of disease suppression observed in the subsequent crop. They altered soils with different crop residues; coriander, wild rocket, peppermint, rosemary, broccoli, cauliflower and sage, and monitored their ability to increase disease suppressiveness in cucumber seedlings against *F. oxysporum f. sp. radicis-cucumerinum.* All species improved suppressiveness when compared to the non-amended soils, however coriander and wild rocket reduced mortality significantly more when compared to the other species, by 20% and 80% respectively. However, as inoculum level increased the effect of wild rocket decreased, showing that initial inoculum level can impact the effectiveness of a biological control.

Building on the effect of species on disease suppressiveness, Davey *et al*. (2019) investigated the impact of incorporating different plant materials on disease suppression by comparing wheat stubble and wheat roots in a controlled environment study. They found that incorporating root residue increased populations of *R. solani AG8* and *Gaeumannomyces graminis var. tritici*, leading to higher disease incidence in the subsequent crop compared to treatments with wheat stubble. Davey *et al*. (2019) also tested these effects on three types of Australian soils and, similar to Lazarovits *et al.* (2001), suggested that abiotic factors, such as soil alkalinity, influenced the response to crop residues and disease suppressiveness. As with Klein *et al.* (2011), inoculum levels were identified as a key factor in the effect of soil amendments on disease suppression.

Another form of organic amendment for disease control is the application of beneficial organisms, this is a form of targeted control as opposed to altering the soil environment as a whole. Beneficial organisms can act through various avenues; competition, antibiosis, parasitism and predation to inhibit the growth and incidence of the pathogen, as well as promoting plant root and shoot growth (Davey *et al.*, 2019), boosting natural plant health and immunity.

Mechanisms in which beneficial organisms' function are explored in Chapter 5, however the effect of their application in the field is explored in this Chapter. Similar to the use of organic amendments, the uptake of the use of beneficial organisms for disease control in the field has been limited. This may be attributed to their variable results, often linked to their inability to establish in different soil environments and compete for nutrients with existing microorganisms (Cao *et al*., 2011). Stadler and Tiedemann (2015) studied the effect of *Microsphaeropsis ochracea* supplements on

Verticillium longisporum. They found that *M. ochracea* caused mortality of *V. longisporum* microsclerotia by 51%-100%, both in sterile soils and dead rapeseed stems in autoclaved sand. However, when this was applied in the field in natural soils or in a controlled environment with unsterile soils there was no significant effect of *M. ochracea* on the control of *V. longisporum*, indicating that the soil microbiome impacted the ability of *M. ochracea* to control *V. longisporum*. Nakayama (2017) tested the ability of *Aspergillus versicolor* Im6-50 to control potato powdery scab caused by *Spongospora subterranea f.sp. subterranean* in the field. In a three-year field trial, they found that the application of the biocontrol offered between 54-77% protection when applied to the seed potato tubers, showing that in some instances beneficial organisms can be used to control disease in natural soil environments. Elshahawy *et al*. (2018) similarly had success in controlling *S. cepivora* using beneficial organisms in the field. They tested three antagonistic bacteria: *Pseudomonas fluorescens, Bacillus subtilis* and *Bacillus pumilus* on the control of *S. cepivora* in garlic and onion. They found the combination of the three bacteria had the highest efficacy against *S. cepivora* than when used individually. In the field, the beneficial bacteria reduced disease to as low as 3% in the low inoculum density plots (40 sclerotia/kg of soil), when compared to the untreated controls where the lowest disease incidence achieved was 23.8%. However, at the higher inoculum densities the beneficial bacteria combination was not as effective, showing initial inoculum density plays a key role in the effectiveness beneficial organisms.

Within the scope of beneficial organisms are arbuscular mycorrhizae fungi (AMF). AMF are a class of beneficial microorganisms that are often found in the natural environment and are often attributed to improved health of plants via symbiosis (Weng *et al*., 2022). The use of AMF to control diseases has been less investigated than other beneficial organisms, AMF species in the Glomeromycota division of fungi are found in lower abundancies in high disease fields than in low disease fields (Mirmajlessi, 2017), perhaps indicating their role in disease supression. Matsubara *et al.* (2001) studied the effect of three AMF species, *Gigaspora margarita, Glomus fasciculatum* and *Glomas* R10, on *Fusarium* disease in asparagus. Asparagus seedlings were treated with one of the three AMF species and inoculated with *Fusarium*. When the plants were assessed 10 weeks after treatment, those treated with AMF were taller, had more shoots and higher dry matter than those without, indicating that AMF contribute to plant health as a whole and possibly disease

suppression inadvertently. The disease suppressive action of the AMF was evidenced by 90% of non-AMF treated plants showing *Fusarium* symptoms 6-weeks post inoculation, whereas only 20-50% of AMF treated plants showed symptoms. Whether the AMF species had specific disease supressing mechanisms requires further investigation. A study in 2018 by Aguk *et al.* investigated the effect of multiple organic amendments including cattle manure, compost, beneficial organisms and AMF on bacterial wilt (caused by *Ralstonia solanacearum*) in potato. They inoculated natural soils with the pathogen within potato boxes and treated them with different combinations of beneficial organisms including AMF. Area under disease progress curves were used to assess disease control of each of the treatments. All treatments including AMF species, such as *Glomus* mossae and *Rhizophagus irregularis* (previously known as *Glomus intraradices*) had significant improvements from the control (3168-3658 AUDPC) ranging from 0-871 AUDPC. However, the AMF was always applied in conjunction with other non-AMF beneficial organisms, such as *Psuedomonas sp*. and *Bacillus sp.,* and these organisms without the AMF scored between 0-44 AUDPC. Perhaps indicating the AMF may have inhibited the disease control of these organisms. A similar study was performed by Kabdwal *et al*. (2019), however this time in the field. They investigated the impact of AMF and other beneficial organisms (*Trichoderma sp.* and *Psuedomonas sp.*) on late blight, early blight, stem rot, and wilt diseases in tomato. Again, AMF was not applied on its own, meaning its actual impact on disease control was not evaluated. Nevertheless, treatments including AMF reduced disease incidence by 40-84% when compared to the untreated control. The 84% reduction was the highest achieved, but this was a combination including AMF, beneficial organisms and a chemical control, again meaning that the impact of AMF alone on disease suppression was not assessed. Despite these studies bringing into question the ability of AMF to control disease, a review by (Weng *et al.*, 2022) advocates for their use and outlines potential mechanisms. These mechanisms include promoting the growth of beneficial organisms, outcompeting pathogens, triggering plant defence mechanisms (such as modulating phytohormone concentrations, regulating signal substrate production, gene expression, and enhancing protein synthesis), as well as bolstering plant health by facilitating increased nutrient uptake, enhancing the rhizosphere, modifying plant root structures, and catalysing the synthesis of secondary metabolites. However, amongst this multitude of potential mechanisms, they concluded that it is unclear which mechanisms actually play a major role in disease suppression.

Organic amendments appear to fluctuate in their effects in controlling disease. A range of environmental factors have been shown to impede their success in controlling plant disease, from soil characteristics like pH and organic matter to the soil microbial community (Lazarovits *et al.,* 2001; Davey *et al.*, 2019). Therefore, further understanding and evaluation of their role in disease control within crop systems needs to be undertaken before they can be recommended in routine disease control strategies.

Previous applications of qPCR to monitor soil-borne plant pathogens in natural environments

Many real-time PCR assays have been developed for quantification of plant pathogens in soil, however few are routinely used in predictive diagnostic tests (Wallenhammar *et al.*, 2012). However some previous research has looked at utilising molecular techniques to monitor soil-borne plant pathogens in fields, and in some cases relate this to disease incidence seen in the crop, much like those investigated in this thesis. Burnett *et al.* (2019) investigated populations of *Phytophthora brassicae*, the causal agent of clubroot in oilseed rape, and their correlation with disease incidence and yield losses. They mapped the populations of *P. brassicae* using qPCR across 16 commercial fields and successfully detected *P. brassicae* in natural soils, although they found qPCR results did not always correlate with levels of clubroot incidence. Likewise, in 2005 Crump attempted to quantify levels of *Colletotrichum coccodes, Spongospora subterranea f. sp. subterranean*, and *Streptomyces scabies* in soils planted with potatoes and relate these to disease seen in the crop. However, they also found that DNA levels and disease levels were poorly correlated, for instance 2-12% of the time PCR results came back positive but no disease was seen in the crop and in 15-39% of the time negative PCR results resulted in positive disease. They theorised that this inconsistency could result from variation in soil properties, cropping history, crop husbandry, seed inoculum and disease history.

A similar study was performed by Almquist *et al.* (2016) quantifying *Aphanomyces cochlioides* populations in naturally infested soils using qPCR. They were able to detect as low as 1 oospore per gram of soil dependant on clay content. However, when relating these quantities to disease severity, *A. cochlioides* was only successfully detected in soils with disease severity indices above 75, and only

detected 50% of the time in soil samples with a disease severity index between 60-74%, meaning risk could only be assessed using qPCR for already high-risk areas.

Another study by Sauvageau *et al.* (2019) also attempted to use qPCR to link the concentration of propagules of the pathogen *Pythium tracheiphilum* to disease seen in commercial lettuce fields. They found variety and air temperature were strongly related to disease incidence, for instance it took 97 propagules to cause 50% reduction in the lettuce variety Estival but only 47 to cause the same in Prestige and air temperature was negatively correlated with disease. Van der Heyden *et al.* (2019) similarly studied *P. tracheiphilum* in commercial fields of lettuce, however they found 699.5 oospores per gram of soil caused 50% disease, which is considerably more than the amount reported in the study by Sauvageau *et al.* (2019). Also, in contradiction to the previously referenced study they found disease level was positively correlated with the inoculum level in the soil rather than environmental drivers.

Wallenhammar *et al.* (2012) quantified *Plasmodiophora brassicae* in naturally infested soils using qPCR. Throughout their study they found contradictions between the levels of *P. brassicae* detected and the disease seen in the crop. For instance, they observed 2 samples that had high disease severity occurred in soils where low inoculum levels were detected. Although they did not find a clear relationship between pathogen detection and disease, they were able to establish threshold pathogen levels above which disease occurred, although these thresholds varied dependant on soil type and environmental factors. They found that levels above 5 fg plasmid DNA per gram of soil lead to losses above 10% in susceptible crops. This is attributed to the increased reproduction of inoculum in soils, facilitated by the presence of a susceptible host. The assay developed in this study is now being used commercially in Sweden for guidance prior to the growing season.

Thresholds of disease were also established by Roget and Herdina (2000) for *Gaeumannomyces graminis var tritici.* Using qPCR, they established <30pg per gram of soil caused low disease severity, 30-50 pg per gram of soil caused moderate disease severity and above 50 lead to high disease severity in cereals. It is often assumed that there is a linear relationship between inoculum level and disease incidence (Crump, 2005), however this is not often found to be the case, perhaps showing that the use of thresholds may be a better tool for risk assessment.

Various studies have been previously performed with the aim of quantifying *Verticillium* and *Fusarium* spp., the target organisms investigated in this thesis. However, as of September 2022 no studies quantifying *Stemphylium* in soils had been performed prior to this thesis and very few studies quantified *Fusarium* in relation to treatment effects, with even fewer relating *Fusarium* populations to disease level in the crop. Smiley *et al.* (2016), quantified a variety of fungal populations including *F. culmorum* and *F. pseudograminearum* over 2 years across 8 trials, and related population changes to treatments. For instance, *F. culmorum* was more prominent in spring crops than *F. pseudograminearum,* where the opposite was true in winter crops and *Fusarium* species outnumbered other pathogens in rotations with wheat. In a study assessing the effect of bio-solarisation on *Fusarium solani* populations in strawberry, De la Lastra *et al.* (2018) found that inoculum density was not correlated with disease incidence, but was correlated with fruit yield, perhaps indicating that disease incidence was not the most appropriate factor when assessing pathogen impact in this case.

Multiple qPCR assays have been reported for the detection of *Verticillium* and have been used for the quantification of the pathogen in soil (Banno *et al.*, 2011). Gharbi *et al.* (2016) were able to detect as few as 2 microsclerotia of *V. dahliae* per gram in naturally infested soils using their real-time qPCR assay. Additionally, they correlated qPCR estimates of the pathogen populations to disease severity in olive. Significant differences in pathogen DNA detection were found between every two consecutive disease severity stages. This correlation between *V. dahliae* population and disease has also been in found in commercial Artichoke fields (Berbegal *et al.*, 2007). Inoculum densities between 5 to 9 microsclerotia per gram of soil caused around 50% infection in the crop. However low coefficients of determination, ranging from 0.33-0.66, were obtained in this study meaning that the reliability of predictions could be limited. In a similar study, looking at commercial sugar beet fields, *V. dahliae* and *V. longisporum* were quantified in regions of Sweden (Tzelepis *et al.*, 2017). Detected levels of *V. dahliae* and *V. longisporum* ranged from 6 fg -137.84 pg DNA per gram of soil and 5 fg – 121.62 pg DNA per gram of soil, respectively. Much like the studies above, this study called for the use of thresholds for advisory work based on qPCR results, where they proposed levels over 10 microsclerotia per gram of soil would cause high risk of disease.

Unlike for some of the pathogens mentioned above, their appears to be a consistent relationship between *Verticillium* DNA levels and crop disease development, although this may vary between crops (Berbegal *et al.*, 2007). The use of disease thresholds appears to be a favourable tool for predicting disease incidence based on quantification of pathogen levels in soils, particularly when being used as an advisory tool. However, several factors have been shown to affect the reliability of such predictive tools, including differences in cultural practices, soil types, environmental conditions and host resistance (Berbegal *et al.*, 2007).

In this chapter the potential value of qPCR is further explored to monitor and quantify different target pathogens (*Fusarium, Verticillium* and *Stemphylium*) in soils sampled from different crop systems (Raspberry, Onion, Daffodil and Asparagus). Pathogen populations were also monitored to assess their response to a range of organic soil amendments and biological supplements to assess their potential value in disease control strategies.

ii) Methods

Field trial design

As part of the AHDB Soil Biology and Soil Health Partnership (Stockdale *et al.*, 2022), three field trials were used to investigate the effects of various soil management treatments on soilborne pathogens in daffodil, onion and raspberry crops. In addition to these trials, and in collaboration with the University of Cranfield, a fourth field trial investigating the effects of soil management practices on asparagus production was used for further investigation of soilborne pathogen populations.

Soil management practices under study included cover cropping, farmyard manure, anaerobic digestate, biopesticide applications and/or mycorrhizal fungi applications, and were compared with untreated controls. Each of the AHDB Soil Biology and Soil Health Partnership sites were divided into 24 plots. Each plot was treated with one of the different soil treatments or left as an untreated control. There were six replicate blocks of the four treatments at each site. AHDB Soil Biology and Soil Health Partnership trial site overview is available in Table 18. Overview of trials, including location, variety, key dates, targeted pathogens, treatments, plot size and soil properties. Trial design for the asparagus trial is available at Maskova *et al.* (2021),

plots from the selected treatments were selected at random from the larger trial for analysis.

Table 18. Overview of trials, including location, variety, key dates, targeted pathogens, treatments, plot size and soil properties

TRIAL	RASPBERRY	ONION	DAFFODIL	ASPARAGUS
LOCATION	Norfolk	Bedford	Norfolk	Ross-on-Wye
VARIETY	Maravilla	Rumba	Carlton	Gijnlim / Millenium
PLANTED	April 2018	February 2019	August 2018	April 2016
HARVESTED	June-Sept 2018, 2019, 2020	August 2019	August 2020	Yearly between April - June
TARGET PATHOGENS	▪*V. dahliae* ▪*V. albo-atrum*	▪*F. oxysporum* ▪ *S. cepivora*	▪*F. oxysporum*	▪S. *vesicarium* ▪ *F. oxysporum*
TREATMENTS	▪Crop-based fibre digestate PAS 110-incorporated at 5 kg/m^2 ▪Biofungicide (Prestop)-Soil drench at 5 g/L (*C. rosea*) ▪Crop-based fibre anaerobic digestate + Biofungicide	▪PAS 100 green waste compost-Spread at 3 kg/m^2 ▪Autumn Cover crop (Phacelia, vetch and clover)-planted Aug 2018 at 35 kg/ha. Herbicide treated early 2019 ▪Cover crop + PAS 100 green waste compost	▪PAS 100 green waste compost - Spread at 3 kg/m^2 ▪FYM- Spread at 2.5 kg/m^2 ▪Mycorrhizae (in-furrow)- 1 g per bulb at drilling	Cover Crop (Mustard and Rye) ▪Straw Mulch ▪PAS 100 green waste compost ▪ Ridging ▪Shallow soil disturbance
PLOT SIZE	0.8 m x 8.0 m	1.83 m x 11 m	1.63 m x 10 m	1.83 m x 10 m
SOIL PROPERTIES	Sandy Silt Loam pH: 8.2 OM%: 2.2	Clay Loam pH: 6.6 OM%: 14.4	Sandy Silt Loam pH: 8.3 OM%: 3.4	

Mg: 50.9	Mg: 114.0	Mg: 88.5
K: 85.0	K: 261.0	K: 84.2
P: 51.8	P: 33.8	P: 13.2

Raspberry Trial

In 2018, a four-year project was initiated at Howes Field in Norfolk with the aim of cultivating raspberries between 2018 and 2020. Prior to this, the field had been utilized for various crops including sugar beet (2011), spring barley (2012), winter barley (2013), sugar beet (2014), spring barley (2015), potatoes (2016), and spring barley (2017).

i) Treatment application:

A crop-based fibre anaerobic digestate was incorporated in the row pre-planting at 5 kg/m^2. Prestop (Lallemand Inc., Canada) was applied as a drench at 5 g/L prepared as per manufacturer's instructions. The biofungicide Prestop, which contains *C. rosae*, was applied at planting, again 4 weeks later and once more in November. Applications were repeated at the same time in each year. There were six replicate blocks of the four treatments (Figure 43).

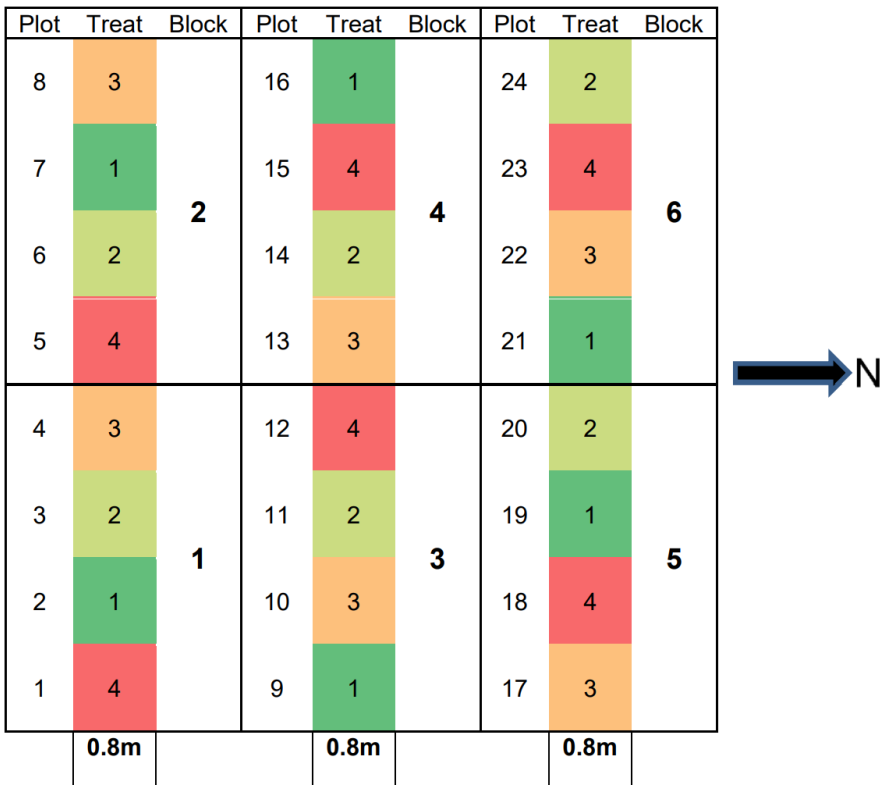

Figure 43. Treatment randomisation with two replicate blocks per bed in trial tunnel in Howes Field. Central ten raspberry stools assessed per plot (two stools discard

within ends of each 8 m plot). 1: Untreated, 2: PAS 110 anaerobic digestate, 3: Prestop Drench & 4: PAS 110 anaerobic digestate + Prestop Drench. (Wedgwood *et al.,* 2022)

ii) Soil sampling and Disease assessment:

Soil from the raspberry trial was sampled in June 2019 (within crop) and October 2020 (just prior to harvest). Soil samples were collected by taking a single 25mm core from the root ball of each of the 10 plants in each plot. Cores were combined to create a single sample per plot. DNA was extracted from each soil sample and analysed using qPCR and Illumina metabarcoding according to the methods outlined in Chapter 3. DNA was initially extracted using the DNeasy PowerMax Soil Kit and then using the improved method for all consequent testing (Chapter 4).
Plant vigour, phytotoxicity, and any signs of yellowing or wilting were evaluated concurrently with each application of Prestop drenches in May, June, and October, focusing on the central 7 meters of each plot. In 2020, due to the dense canopy (with approximately 11 canes per stool), it became challenging to distinguish individual canes, therefore assessments were conducted at approximately 0.5-meter intervals along each plot. The conclusive assessment took place in October 2020, coinciding with soil sample collection.

Daffodil (*Narcissi*) Trial

In 2018, a two-year project was initiated in Orange Field, spanning 16 hectares near Terrington St Clement, Norfolk. The field was slated for planting a commercial crop of Narcissus directly following the cultivation of wheat sown in Autumn 2017.

i) Treatment application:

PAS100 green waste compost was incorporated during cultivation prior to planting of bulbs, spread at a rate of 3 kg/m^2 so that each plot received 90 kg. Farmyard manure was applied at a rate of 3.5 kg/m^2 and was also incorporated prior to planting. Microbial product (provided by Plantworks, Sittingbourne UK) was applied at 1 g per bulb, during drilling of the bulbs. The trial area contained 24 plots, each 3.0 m wide and 10 m long (Figure 44). Each plot contained 4 rows of bulb planting. Treatments were randomised in blocks, apart from the Microbial product that was in a single row due to application protocol.

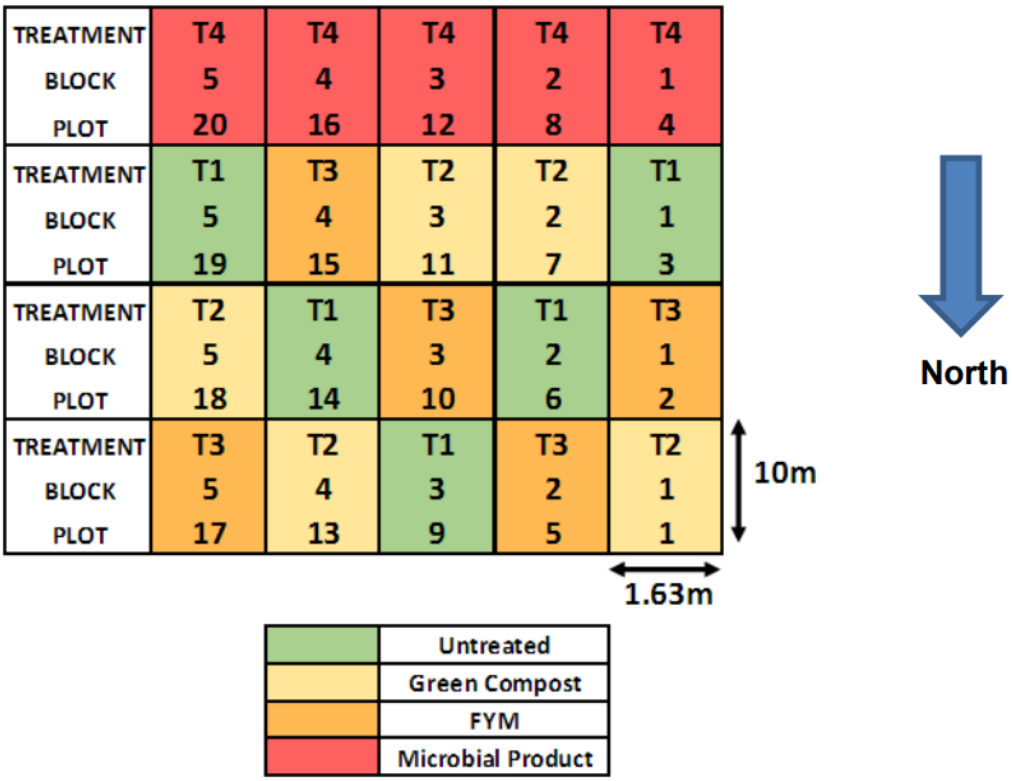

Figure 44. Trial layout for 20 plots of Narcissus cv. Carlton (with assessed area of two central rows 1.63 m x 10 m). Five replicate Blocks with Treatments T1, T2 & T3 randomized, Treatment T4 (Mycorrhiza, microbial product) plots along the South side of the trial area. (Wedgwood, Bhogal, *et al.*, 2022)

ii) Soil sampling and Disease assessment:

Soil was sampled from the daffodil trial in September 2019 (within crop) and in June 2020 (just prior to harvest). Soil samples were collected by taking 20 25mm cores to a depth of 25mm in a diagonal ‘zigzag’ pattern across the plot, which were then combined and mixed thoroughly to create a composite sample for each plot. Flowers were harvested in August 2020. Soil samples were extracted for DNA and analysed using qPCR and Illumina metabarcoding (methods outlined in Chapter 3). Samples were extracted using the DNeasy PowerMax Soil Kit in their initial testing and then using the developed method for all consequent testing (Chapter 4).

Following the emergence of leaves post-winter in both 2019 and 2020, the crop underwent examination for signs of yellowing. A secondary assessment was conducted post-flowering, prior to the onset of leaf senescence, during which the proportion of yellowed leaf area per plot was recorded.

Onion Trial

A site in Bedfordshire was chosen for its reported history of Fusarium basal rot in onion crops. The field had no onion cultivation for three years, due to previously being afflicted with Fusarium basal rot, as reported by the grower. Prior to the trial crop rotations included spring barley in 2012-2013, potatoes in 2013-2014, winter wheat in autumn 2014-2015, onions in summer 2015-2016, and winter wheat in both autumn 2016-2017 and autumn 2017-2018.

i) Treatment application:

The cover crop 'Autumn DM' (80% Rye cv. Turbogreen, 15% Vetch cv. Kwarta and 5% Phacelia cv. Stala) was sown at a rate of 35 kg/ha in August 2018, directly after wheat harvest. It was then glyphosate herbicide treated in January 2019. Green compost (30mm grade) was applied at 3 kg m^2 over wheat stubble in February 2019. The trial utilised a split strip design with alternating cover crop strips accounted for (Figure 45).

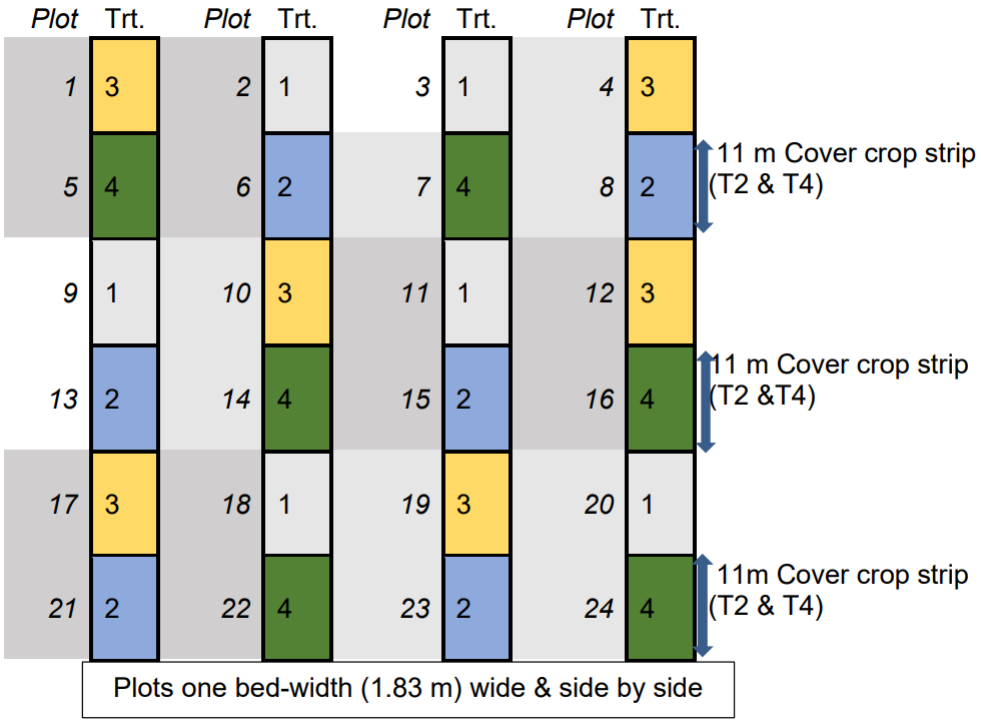

Figure 45. Layout design showcasing alternating cover crop strips amidst cereal stubble during Autumn 2018, with subsequent incorporation of green compost into half of the plots during Spring 2019 prior to onion planting. 1: Untreated, 2: Cover crop, 3: Green Compost, & 4: Cover crop + Green compost. (Wedgwood *et al.*, 2022)

ii) Soil sampling and Disease assessment:

Soil was sampled from the onion trial in December 2018 (prior to planting) and in August 2019 (prior to harvest). Soil samples were collected by taking 20 25mm cores to a depth of 25mm in a diagonal ‘zigzag’ pattern across the plot, these were then combined and mixed thoroughly to create a composite sample for each plot. Soil samples were extracted for DNA and analysed using qPCR and Illumina metabarcoding (methods outlined in Chapter 3). Samples were extracted using the DNeasy PowerMax Soil Kit in their initial testing and then using the developed method for all consequent testing (Chapter 4).

Disease assessment for onions was performed in July 2019. Initially, a random sample of 50 plants per plot from the central three rows underwent examination, with the focus on counting instances of yellowing. Observing a relatively low proportion of affected plants, a subsequent count was conducted to ascertain the total number of plants per plot exhibiting the characteristic foliar yellowing associated with early-stage Fusarium basal rot.

Asparagus Trial

PAS 100 green compost or straw mulches were applied annually in April 2018, March 2019 and March 2020 at rates of 25 t/ha for PAS 100 compost and 6 t/ha for the straw mulch. Re-ridging was performed using a tractor mounted 1.83 m double disk ridger in March 2017, April 2018, March 2019 and March 2020. Shallow soil disturbance was achieved using a winged tine. Companion crops were planted in August once the asparagus had reached full fern stage, at the rate of 150 kg/ha for Rye and 19 kg/ha for Mustard. For full trial methodology see (Maskova *et al.*, 2021).

Soil samples were taken in March 2019 and July 2020 from the Cranfield University Asparagus field trial using a 25mm x 1000mm soil corer in the ridges, avoiding the wheeling areas. Each sample was a mixture of 15 cores per plot. Soil samples were extracted for DNA and analysed using qPCR and Illumina metabarcoding (methods outlined in Chapter 3). Samples from both years were extracted using the DNeasy PowerMax Soil Kit.

Statistical Analysis

All analyses were conducted using IBM SPSS Statistics version 26. Data normality was evaluated using the Shapiro-Wilk test ($p > .05$), and homogeneity of variance was assessed with Levene's test. If the data met the assumptions of normality and homogeneity, parametric tests were applied, including analysis of variance (ANOVA), linear regression, and Pearson's correlation.

If the data violated these assumptions, a Log^{10} transformation was applied, and the normality and homogeneity tests were repeated. If the transformed data met the assumptions, parametric analyses (e.g., ANOVA) were conducted on the transformed dataset.

In cases where the data continued to violate the assumptions after transformation, non-parametric alternatives were employed on the original untransformed data. These included the Kruskal-Wallis test for group comparisons and Spearman's rank correlation for correlation analysis.

For the ANOVA analyses (one-way, two-way, etc.), post hoc comparisons were performed using Tukey's test to identify significant differences between group means. In addition to the post hoc tests, descriptive statistics and effect size estimates were calculated to further interpret the results. In some cases, to account for temporal variability, time was included as a covariate in the analyses, allowing for the control of its potential influence on the outcomes.

Statistical significance was determined at a threshold of $p < 0.05$.

iii) Results

Raspberry Field trial

A one-way ANOVA was conducted to determine if the amount of *V. dahliae* DNA detected in the soil varied according to the time of sampling. Data was normally distributed for each year, as assessed by Shapiro-Wilk test ($p > .05$); and there was homogeneity of variances, as assessed by Levene's test. Change in *V. dahliae* DNA quantity over the two seasons was statistically significant ($p >0.001$). Larger variation between the soil treatments was also seen in results from 2019 compared with those

from 2020. Larger amounts of *V. dahliae* were detected in 2019 than in 2020 across all plots and treatments.

To assess the effect of soil treatments on quantities of *V. dahliae* DNA detected a factorial ANOVA was conducted to determine if *V. dahliae* DNA quantity over the season was different for each treatment (Untreated, Prestop, PAS110 digestate and Prestop + PAS110 digestate). Data was normally distributed for each group, as assessed by Shapiro-Wilk test ($p > 0.05$); and there was homogeneity of variances, as assessed by Levene's test of homogeneity of variances. There was no interaction between time point and treatment ($p=0.572$). Change in *V. dahliae* DNA quantity over the season was not statistically significant dependant on treatment ($p = 0.806$) (Figure 46).

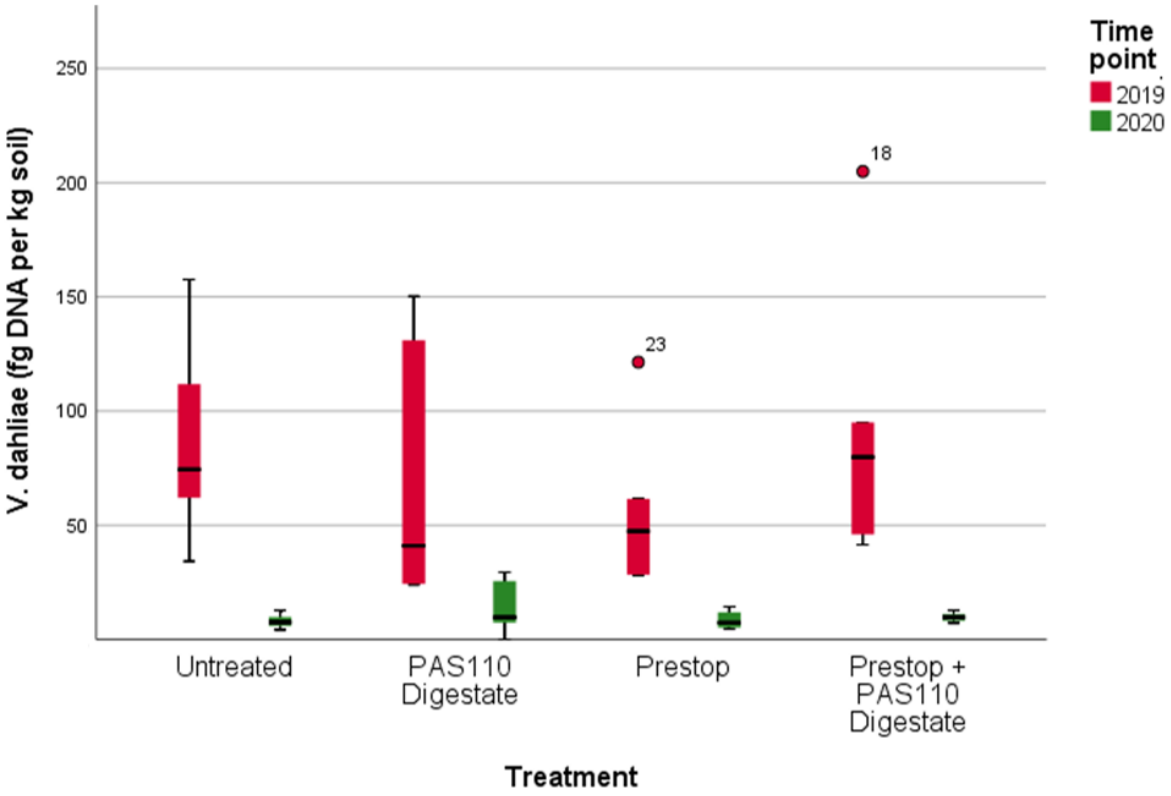

Figure 46. *Verticillium dahliae* DNA quantity from the Raspberry field site per treatment. Data is presented as interquartile range and median (n=24).

A Kruskal-Wallis H test was performed to determine any differences in detection of DNA of the biofungicide *C. rosea* (the active fungal ingredient of Prestop) between sampling times and between the various soil treatments (Untreated, Prestop, PAS110 digestate and Prestop + PAS110 digestate) (Figure 47). Soils not treated with Prestop were included in the analysis to establish if there were any background

levels of the active organism *C. rosea. Clonostachys rosea* was detected in untreated soil but not in the soils treated with PAS110 digestate alone.

Distributions of *C. rosea* DNA quantity was not similar for all treatment groups, as assessed by visual inspection of a boxplot. The mean rank of *C. rosea* DNA was not statistically significantly different between treatment groups (p=0.90). The change in *C. rosea* DNA quantity over the season was also not significant (p= 0.788). There was no interaction between time point and treatment (p=0.979).

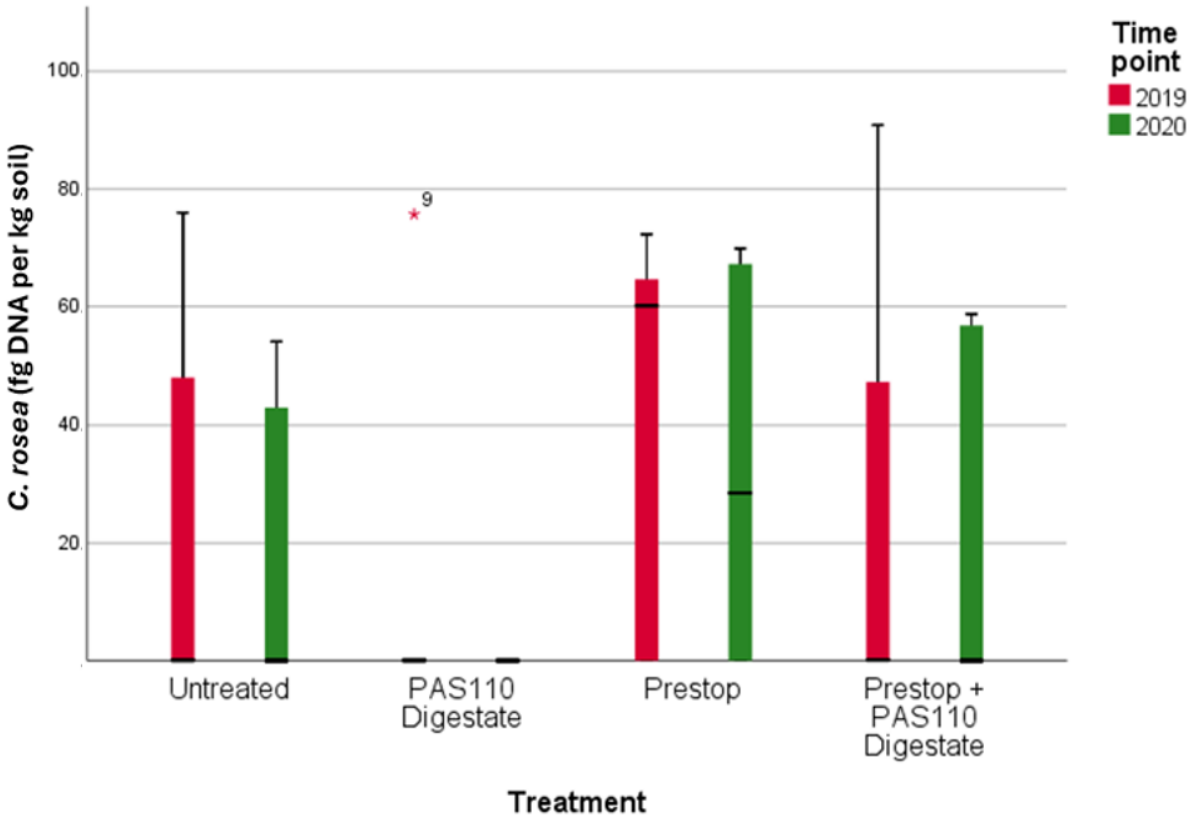

Figure 47. *Clonostachys rosea* DNA detected in soil samples from the raspberry field site after different soil management treatments. Data is presented as interquartile range and median (n=24).

No correlation was observed between the quantities of *V. dahliae* and *C. rosea* DNA (as assessed by Spearman correlation) at the time of soil sampling (p=>1.000) (Figure 48).

No disease was recorded in the crop at the end of the 2020 season.

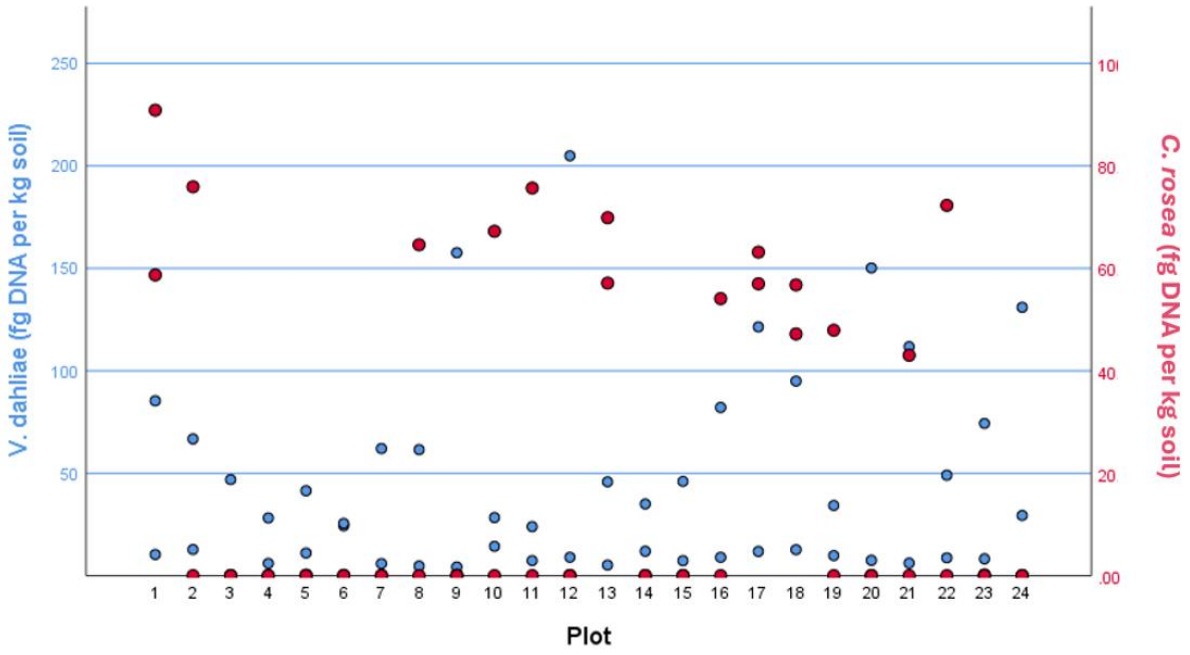

Figure 48. Quantities of *Verticillium dahliae* and *Clonostachys rosea* DNA detected in each plot in 2020.

Relationship between abundance of *Verticillium* determined through metabarcoding and qPCR quantified *Verticillium dahliae* DNA.

From the metabarcoding data performed on soil DNA extracted from the raspberry trial, *Verticillium* species were identified as *V. albo-atrum* but not as *V. dahliae.* The two species are closely related and difficult to distinguish and therefore the genus *Verticillium* was used to represent *V. dahliae* relationship to treatments and quantified *V. dahliae*.

Both the *Verticillium* metabarcoding and qPCR data failed tests of normality, as assessed by Shapiro-Wilk's test ($p > 0.05$). The data were therefore transformed using Log10. A one-way ANOVA was performed compare data between to the 2 sampling time points. No significant effect of sampling time was observed on *V. dahliae* qPCR data ($p=0.990$) or *Verticillium* abundance according to metabarcoding data ($p=0.193$).

No relationship was observed between *V. dahliae* qPCR data and abundance of *Verticillium* spp. determined by metabarcoding ($p=0.990$).

Relationship between abundance of *Clonostachys* species identified through metabarcoding and qPCR quantified *Clonostachys rosea*

Clonostachys or *Gliocladium* species could not be identified from the metabarcoding data.

Onion Field trial

Data failed tests of normality and homogeneity of variances, even after Log10 transformation, therefore a Kruskal-Wallis H test was performed to determine any differences in quantity of *F. oxysporum* DNA detected in soil amended with cover crop, PAS100 Green waste compost or the combination of both compared with untreated control soil. Distributions of *F. oxysporum* DNA quantities were similar for all treatment groups. Median *F. oxysporum* DNA quantities were not statistically significantly different between soil treatments (p = 0.991) (Figure 49).

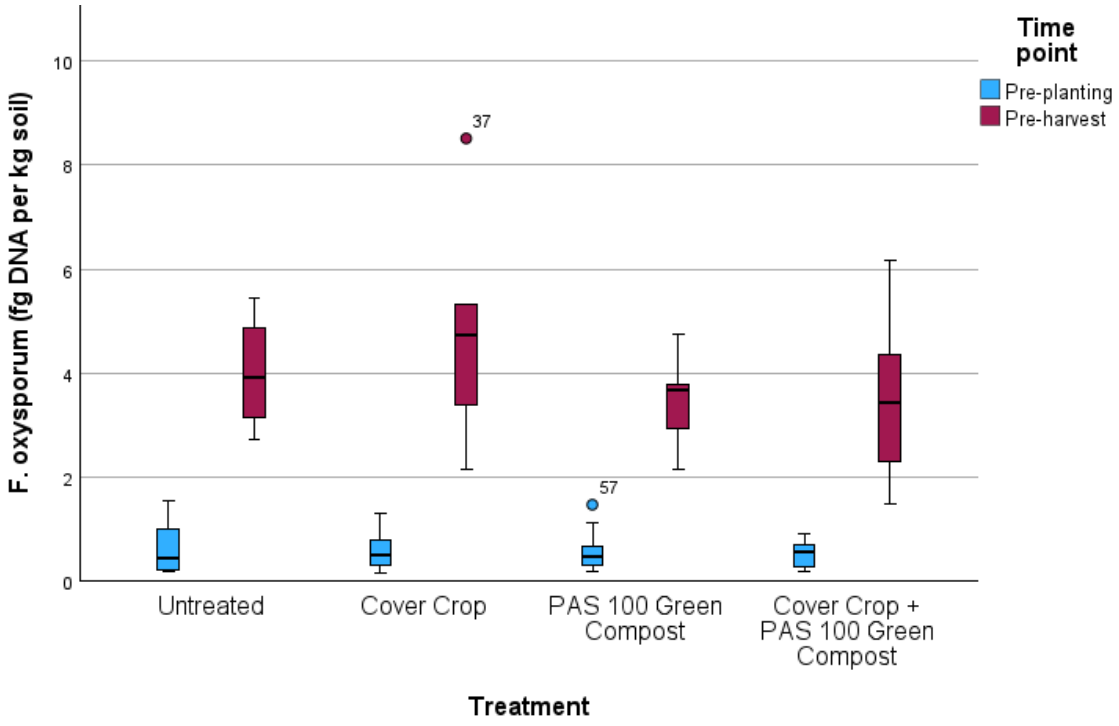

Figure 49. *Fusarium oxysporum* DNA detected in soil samples from the onion field site after different soil management treatments. Data is presented as interquartile range and median (n=24).

qPCR data was normally distributed for each group, as assessed by Shapiro-Wilk test (p > .05) and there was homogeneity of variances, as assessed by Levene's test of homogeneity of variances. A one-way ANOVA test showed a significant increase

in *F. oxysporum* DNA over the onion cropping season was observed (p = < 0.001) as shown in Figure 50. At sampling in 2020, all targets had significantly increased from estimates made in 2019 and in 23 out of 24 plots the populations had increased into the next threshold level, as shown in heat maps (Figure 51).

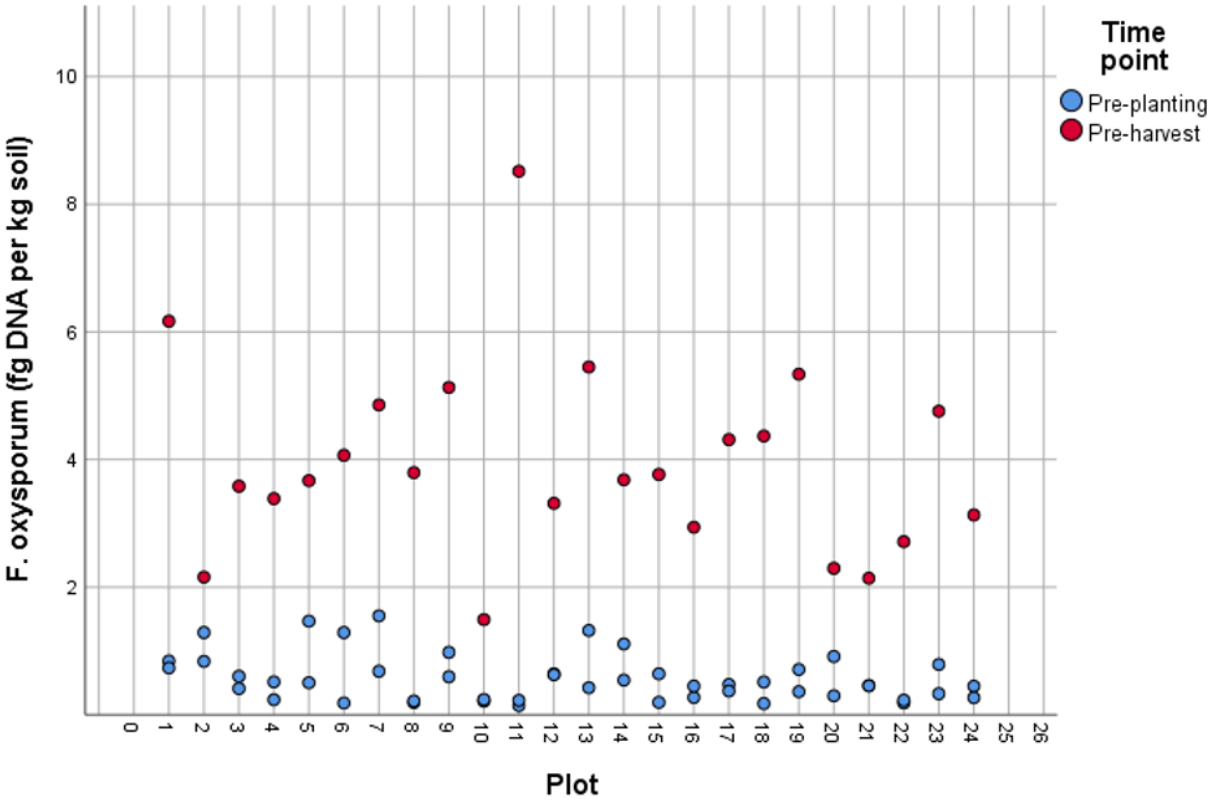

Figure 50. *Fusarium oxysporum* DNA quantity pre- and post-planting of onion across all plots.

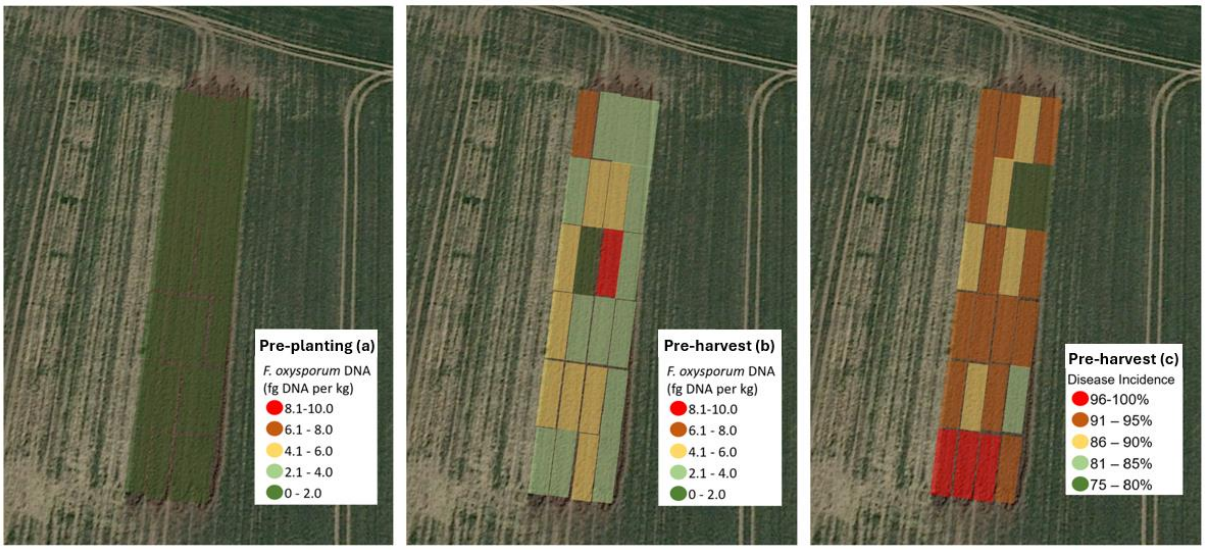

Figure 51. Heat maps from the onion field trial showing *Fusarium oxysporum* levels (fg DNA per kg) across the site before planting (a) and before harvest (b), and disease incidence across the site before harvest (c).

A further one-way ANOVA test showed that increases in *F. oxysporum* DNA over the season were not statistically dependant on any of the soil treatments applied (p = 0.432).

Disease incidence in this field trial was high, including one plot reaching 100% disease and the mean being 90.5%. Disease incidence data was normally distributed for each group, as assessed by Shapiro-Wilk test (p > .05); and there was homogeneity of variances, as assessed by Levene's test of homogeneity of variances. A one-way ANOVA test showed that the disease incidence seen in the crop at the end of the season was not statistically significant dependant on treatment (p = 0.986) (Figure 52). There was no correlation between *F. oxysporum* DNA quantity and percentage disease as assessed by Spearman correlation (p=0.179) (data not shown).

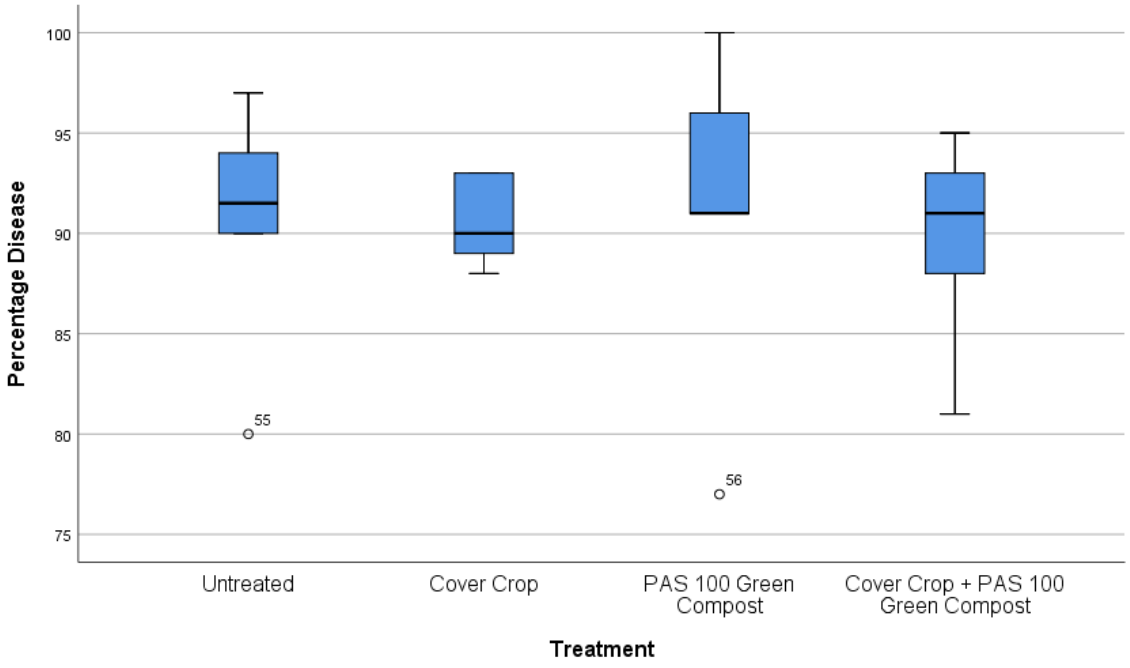

Figure 52. Percentage disease observed in the onion field site at the end of the season (2019) after different soil management treatments. Data is presented as interquartile range and median (n=24).

Relationship between abundance of *Fusarium* species identified through metabarcoding, qPCR quantified *Fusarium oxysporum* DNA and disease incidence.

Metabarcoding data on the relative abundance of *Fusarium* species passed the tests of normality whereas *F. oxysporum* qPCR data failed tests of normality, as assessed by Shapiro-Wilk's test (p> 0.05). The *F. oxysporum* qPCR data was therefore

transformed using Log10. A factorial ANOVA showed differences between to the 2 sampling time points, which was found to be significant for the relative abundance of *Fusarium* species as determined by metabarcoding (p=0.007) but not for the *F. oxysporu*m qPCR data (p=0.934). No effect of soil treatment was observed (*F. oxysporum* p=0.504, *Fusarium* p= 0.977). Nor was any interaction between time and treatment (p= 0.677).

There was also no relationship between disease incidence and relative abundance of *Fusarium* or quantity of *F. oxysporum* DNA detected.

When the data was transformed using Log10, a significant linear relationship (r = 0.471) was observed between the quantified *F. oxysporum* DNA qPCR data and the metabarcoding data on relative abundance of *Fusarium* spp.. This was assessed using Pearsons's correlation (Figure 53).

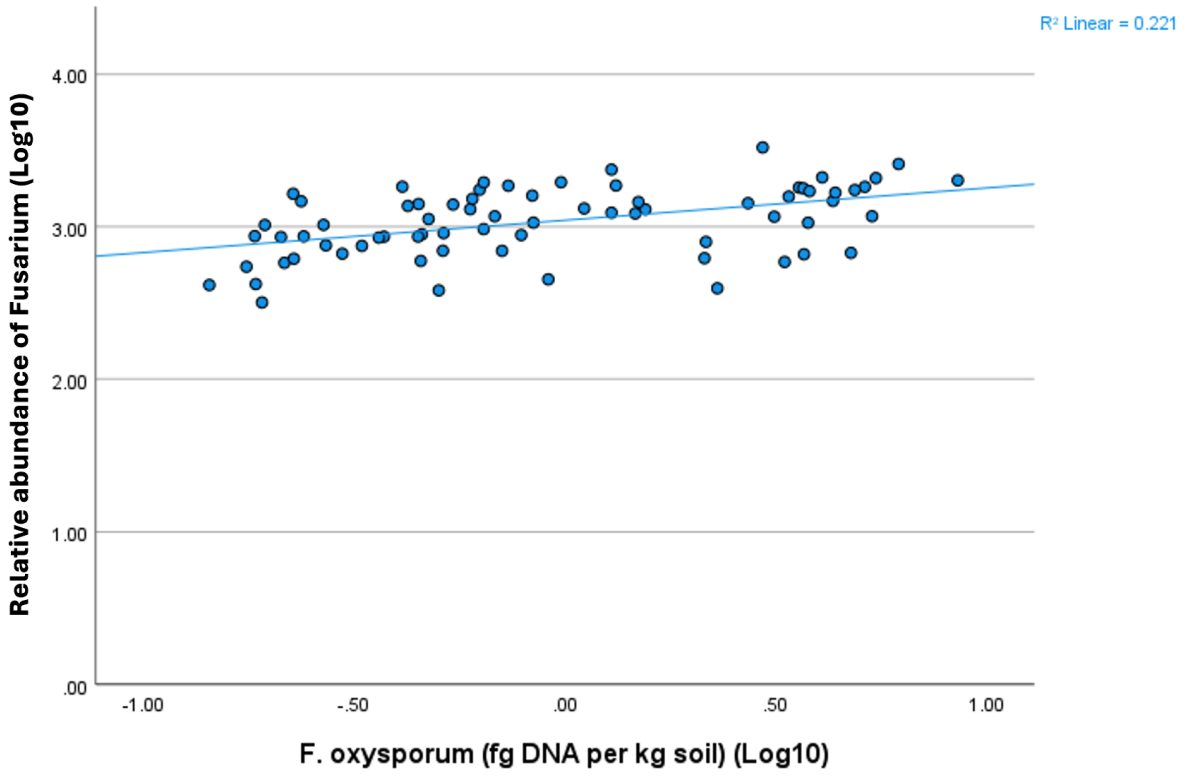

Figure 53. Positive correlation between relative abundance of *Fusarium* determined using metabarcoding and quantity of *Fusarium oxysporum* DNA quantified using qPCR. Data was transformed using Log10.

Daffodil (*Narcissi*) Field Trial

Data failed tests of normality and homogeneity of variances, even after Log10 transformation, therefore a Kruskal-Wallis H test was conducted to determine if the change in *F. oxysporum* DNA quantity was related to season. Distributions of *F. oxysporum* DNA quantities were similar for all groups, as assessed by visual inspection of a boxplot. Median *F. oxysporum* DNA quantities were not statistically different between groups (p = 0.815) (Figure 54).

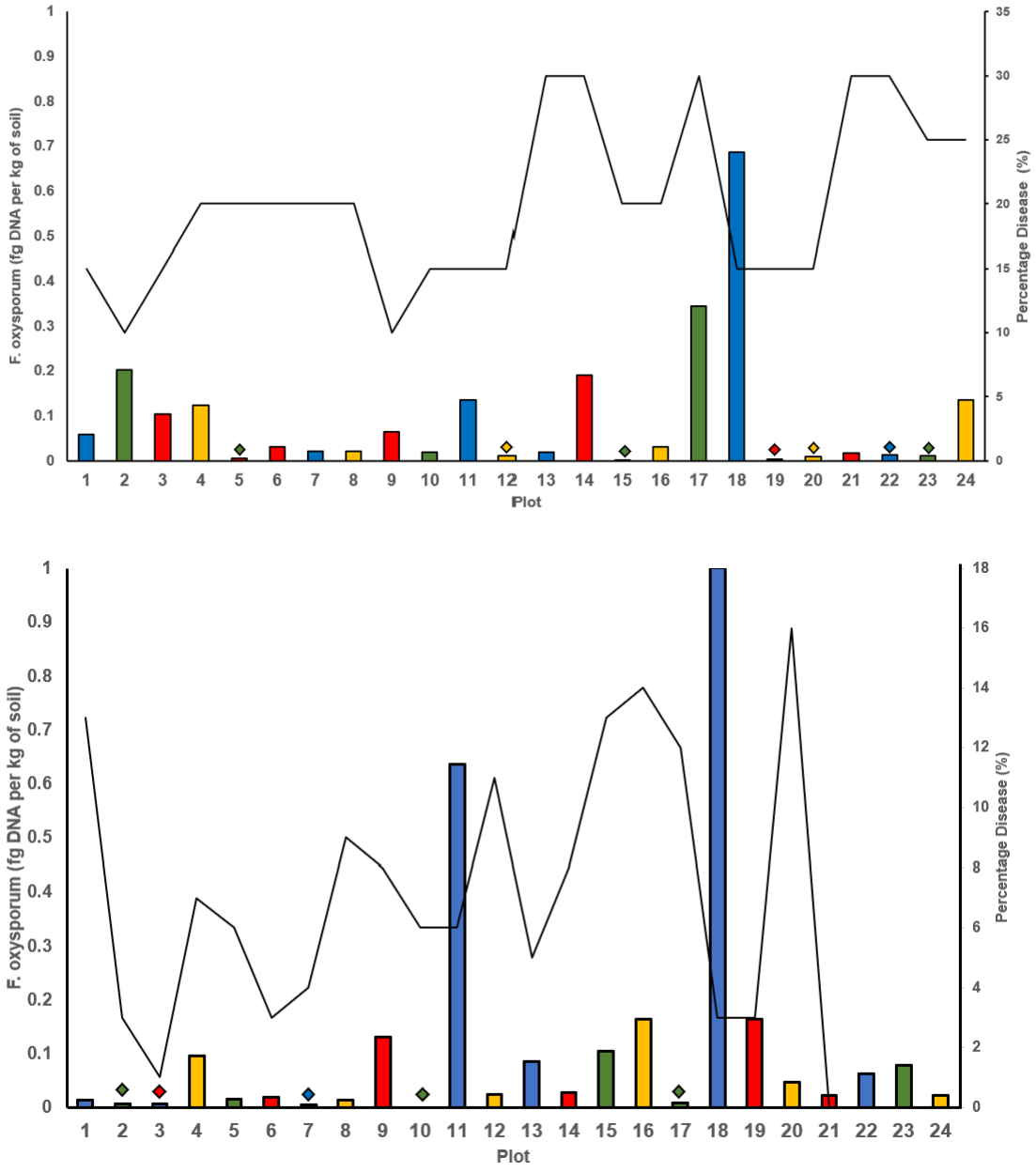

Figure 54. *Fusarium oxysporum* DNA quantity (bar) and disease incidence (line) across all Daffodil field plots (n=24). Top: 2019, Bottom: 2020. Soil treatment denoted

by colour: Blue = Compost, Green = Farmyard manure, Red= Untreated, Yellow= AMF Microbial product.

A Kruskal-Wallis H test showed that the quantity of *F. oxysporum* DNA detected between the four soil treatments (untreated, PAS100 Green waste compost, farmyard manure and AMF microbial product) was similarly distributed across all treatments. Median *F. oxysporum* DNA quantities did not differ significantly between treatments groups (p = 0.539) (Figure 55).

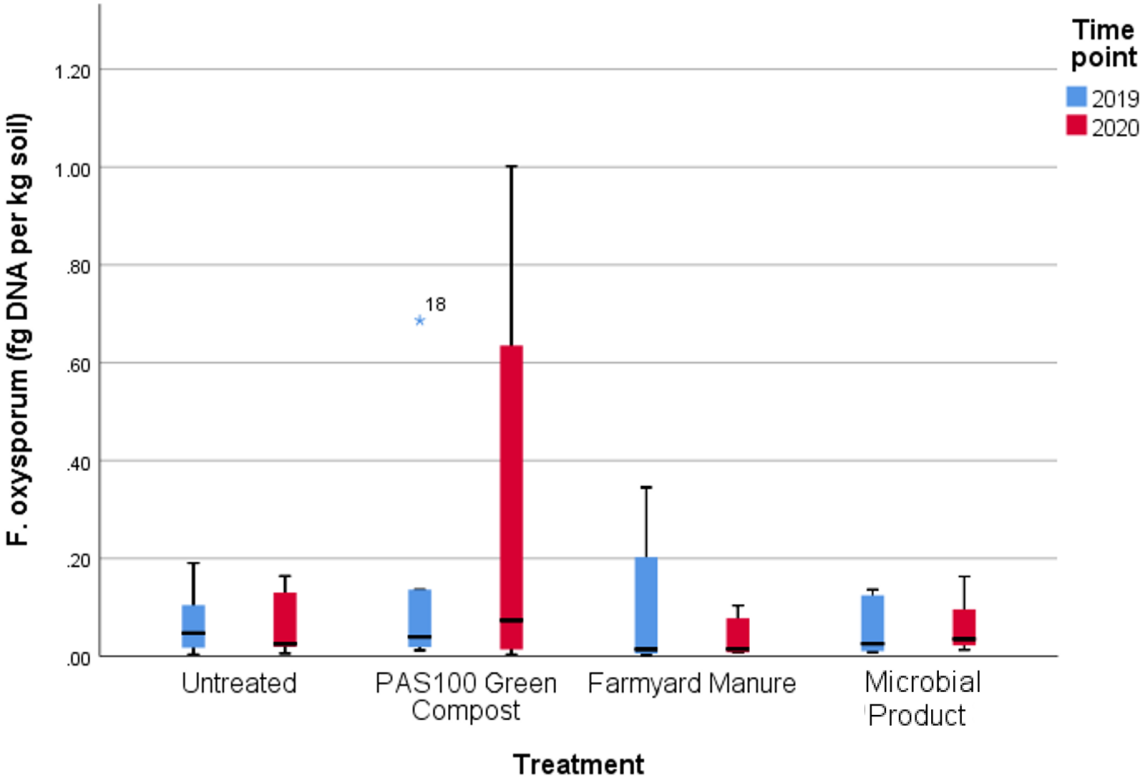

Figure 55. *F. oxysporum* DNA detected in soil sampled from the daffodil field trial. Data is presented as interquartile range and median (n=24).

A Kruskal-Wallis H test was conducted to determine if the change in *F. mossae,* a species in the microbial product, DNA quantity was related to season. Distributions of *F. mossae* DNA quantities were similar for all groups, as assessed by visual inspection of a boxplot. Median *F. mossae* DNA quantities were not statistically significantly different between years (p = 0.895).

A Kruskal-Wallis H test showed that median *F. mossae* DNA quantities detected did not differ significantly between the soil treatments (p = 0.722) (Figure 56).

Rhizophagus irregularis, a species in the microbial product, was not successfully detected in any of the soil samples.

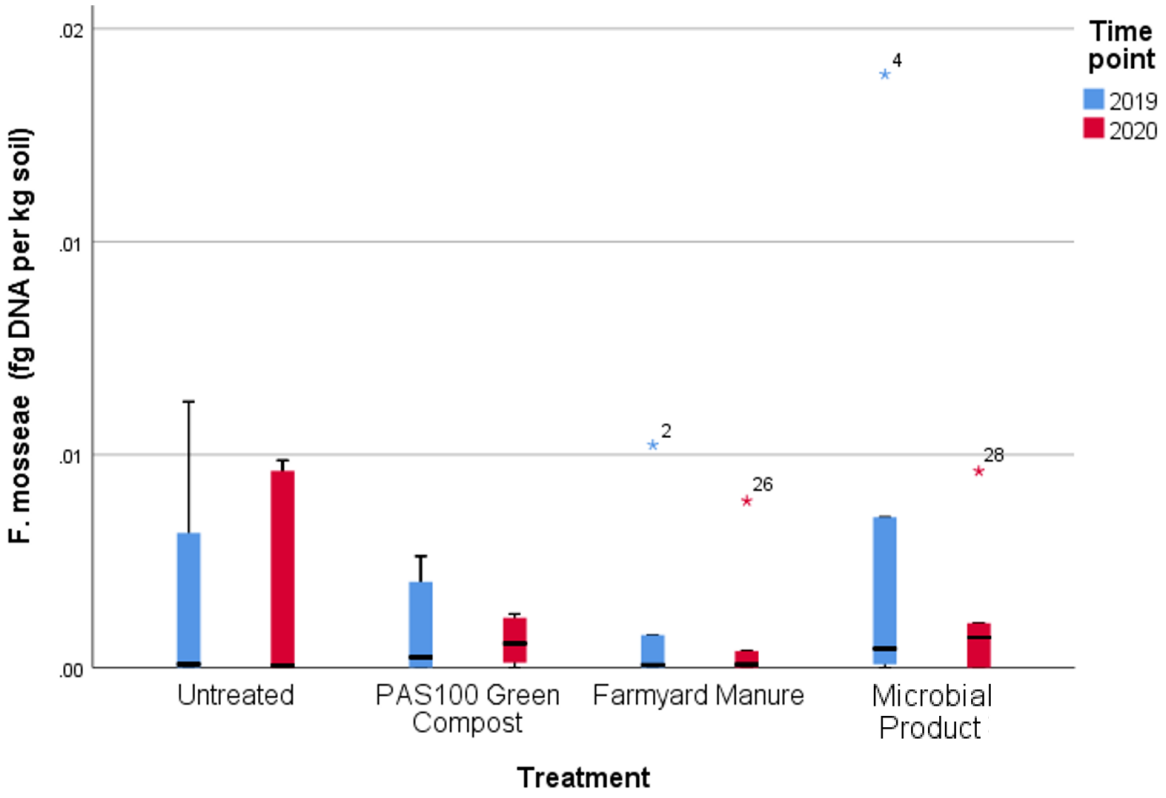

Figure 56. *F. mossae* DNA detected by qPCR in soil samples from the daffodil field trial. Data is presented as interquartile range and median (n=24).

Distributions of disease incidence were similar across all treatments. A Kruskal-Wallis H test showed that the median percentage disease incidence did not differ significantly between the soil treatments (p = 0.996) (Figure 57). There was no correlation between quantities of *F. oxysporum* DNA detected and the percentage disease incidence, as assessed by Spearman correlation (p=0.845).

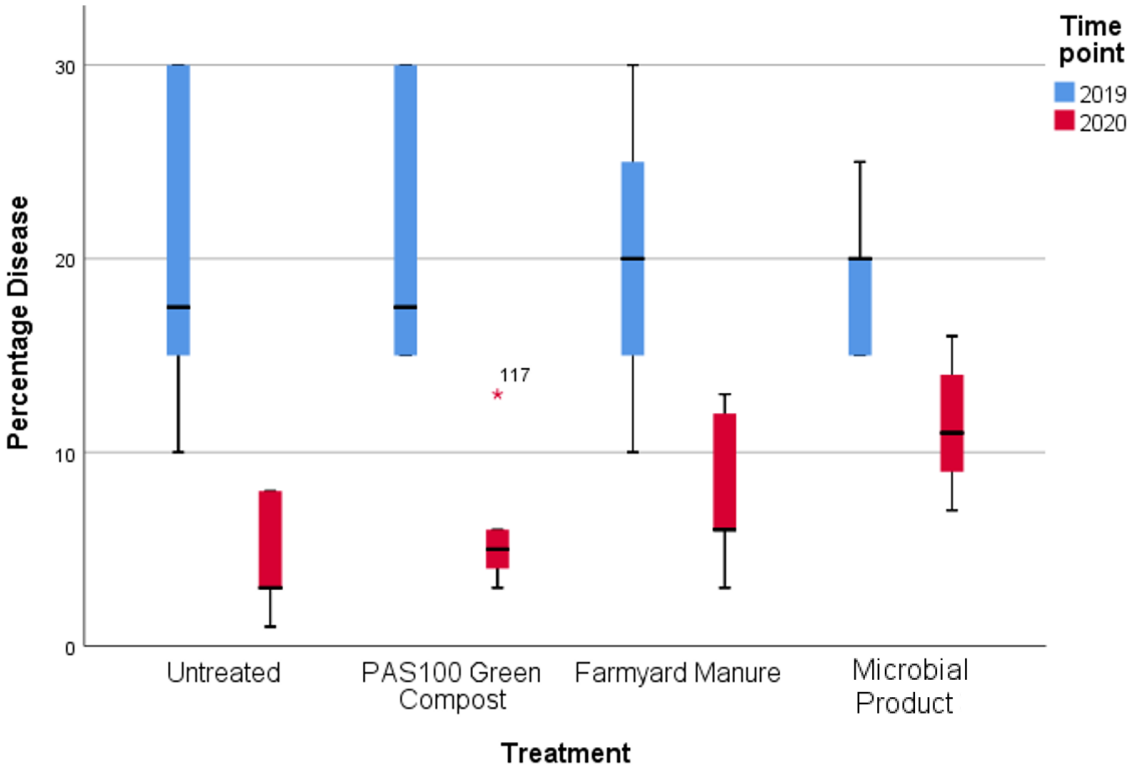

Figure 57. Effect of soil treatment on disease incidence in the daffodil trial. Data is presented as interquartile range and median (n=24).

Relationship between abundance of *Fusarium* species identified through metabarcoding and qPCR quantified *Fusarium oxysporum* DNA

Fusarium oxysporum was not identified in the metabarcoding data at the daffodil field trial, however *Fusarium acutisporum, Fusarium domesticum, Fusarium equiseti* and *Fusarium petersiae* were. Therefore, the genus *Fusarium* was used. The relative abundance of the genus *Fusarium* was estimated from the metabarcoding data, and this was compared with the qPCR data used to quantify *F. oxysporum* DNA in each soil sample. Both datasets on abundance of *Fusarium* spp. and quantified *F. oxysporum* DNA failed tests of normality, as assessed by Shapiro-Wilk's test ($p > 0.05$). All data was therefore transformed using Log10. No relationship was found between *Fusarium* species abundance and quantified *F. oxysporum* DNA.

A factorial ANOVA, performed on Log10 transformed data, showed no significant difference in the relative abundance of *Fusarium* spp. (determined by metabarcoding) between the two sampling points ($p=0.564$) or between soil treatments across both years ($p=0.258$).

Relationship between the relative abundance of AMF species identified through metabarcoding and the quantity of AMF DNA detected using qPCR

Rhizophagus irregularis was not identified from the metabarcoding data and was also not detected using qPCR.

F. mossae was detected in both qPCR and the metabarcoding data. There was no relationship observed between quantified *F. mossae* DNA and the relative abundance of *F. mossae* (assessed via Pearsons's correlation, p=0.622). Data on the relative abundance of *F. mossae* passed Shapiro-Wilk's test of normality (p> 0.05). No effect of soil treatment on the abundance of *F. mossae* was observed when assessed by one-way ANOVA (p=0.376).

Asparagus Field Trial

Both *F. oxysporum* and *S. vesicarium* were successfully detected in soil samples from the asparagus field trial using the pathogen-specific qPCR assays. No significant interactions were identified between treatment and shallow soil disturbance and ridging, not their combinations (p=0.40-0.517), when assessed using factorial ANOVA for either soil-borne pathogen. Re-ridging had no significant effect on either *Fusarium* or *Stemphylium* pathogen populations in the soil in either year, as assessed using a one-way ANOVA (Figure 58 and Figure 59). This was also the case for shallow soil disturbance (SSD) (Figure 60 and Figure 61), except in 2020 when SSD on just the bare soil plots appeared to significantly lower *F. oxysporum populations* when analysed using an ANOVA (p=0.023).

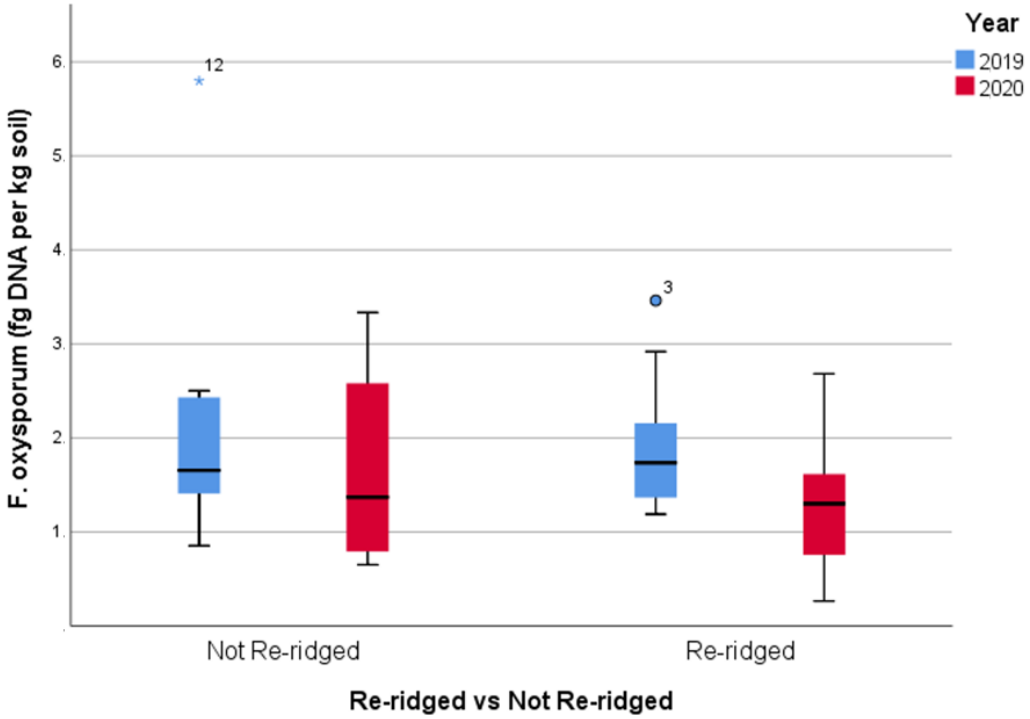

Figure 58. *Fusarium oxysporum* DNA detected in soil sampled from the Asparagus field site from plots with and without re-ridging treatment. Data is presented as interquartile range and median (n=44).

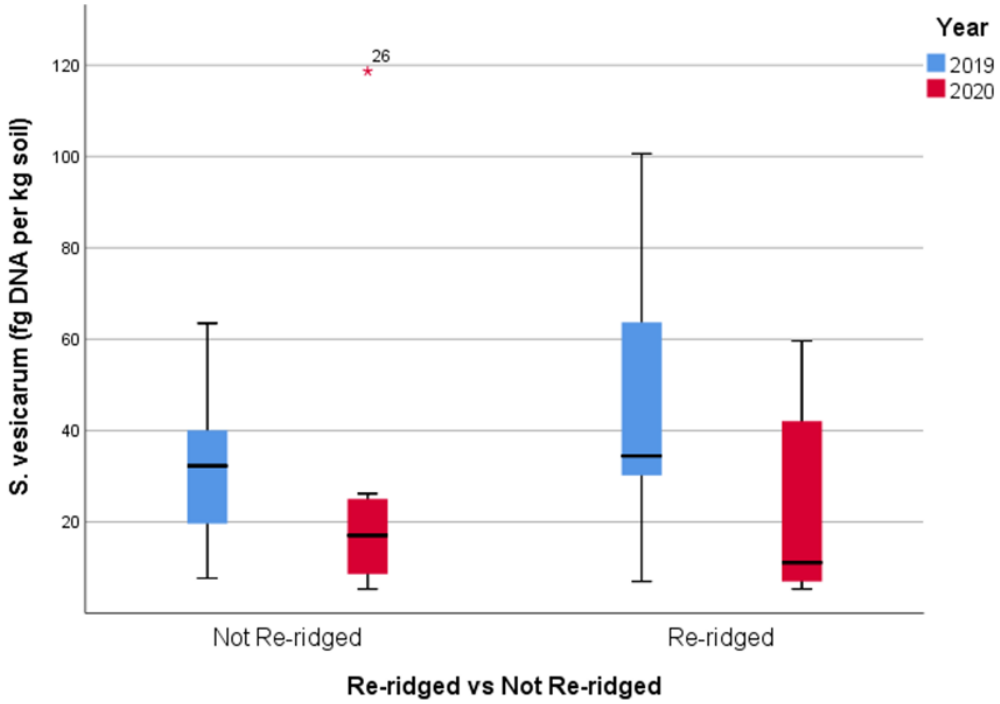

Figure 59. *Stemphylium vesicarium* DNA detected in soil sampled from the Asparagus field site from plots with and without re-ridging treatment. Data is presented as interquartile range and median (n=44).

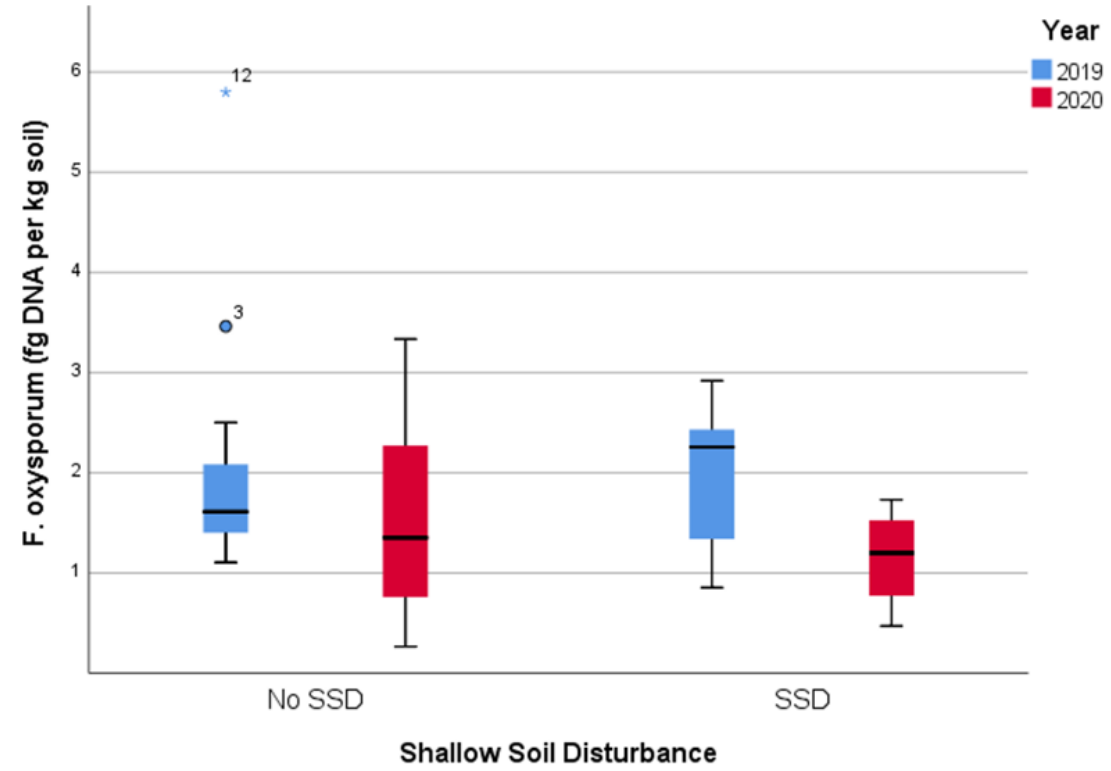

Figure 60. Effect of shallow soil disturbance treatment on *Fusarium oxysporum* DNA detected by qPCR in soil samples from the asparagus field site. Data is presented as interquartile range and median (n=44).

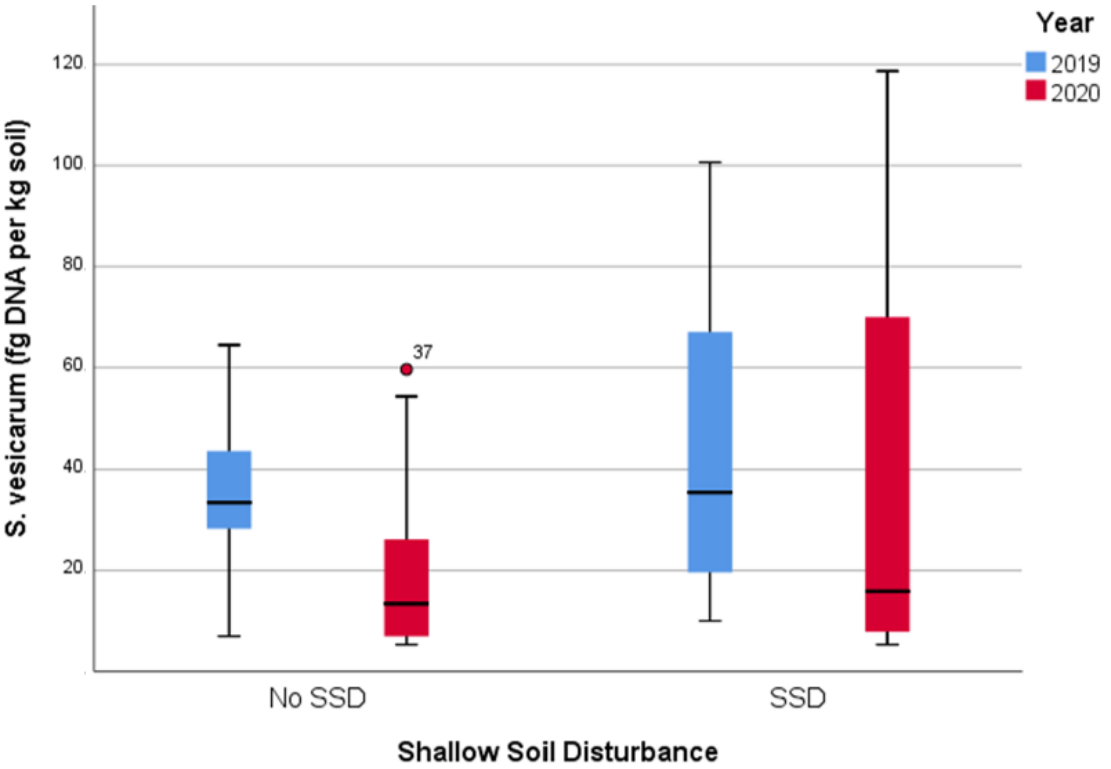

Figure 61. Effect of shallow soil disturbance treatment on *Stemphylium vesicarium* DNA detected by qPCR in soil samples from the asparagus field site. Data is presented as interquartile range and median.

Treatments were grouped according to the source of organic amendment (companion crop or compost/mulches) to investigate their impact on soil-borne pathogen populations. Changes in soilborne pathogen populations in response to treatment and time was assessed using factorial ANOVA's, there was no interaction with time (p=0.677). Between the two years, pathogen populations in bare soil did not fluctuate (Figure 62 and Figure 63). *Fusarium oxysporum* DNA was detected at a higher level in the companion crop treatment group than in the bare soil treatment groups in 2019 but not in the following year (p=0.046). Other than in this instance, the highest levels of both pathogens were detected in bare soil in both years, again not significant. Figure 63 shows significantly lower levels of *S. vesicarium* in soils with organic amendments than in the bare soil treatment (p=0.030)

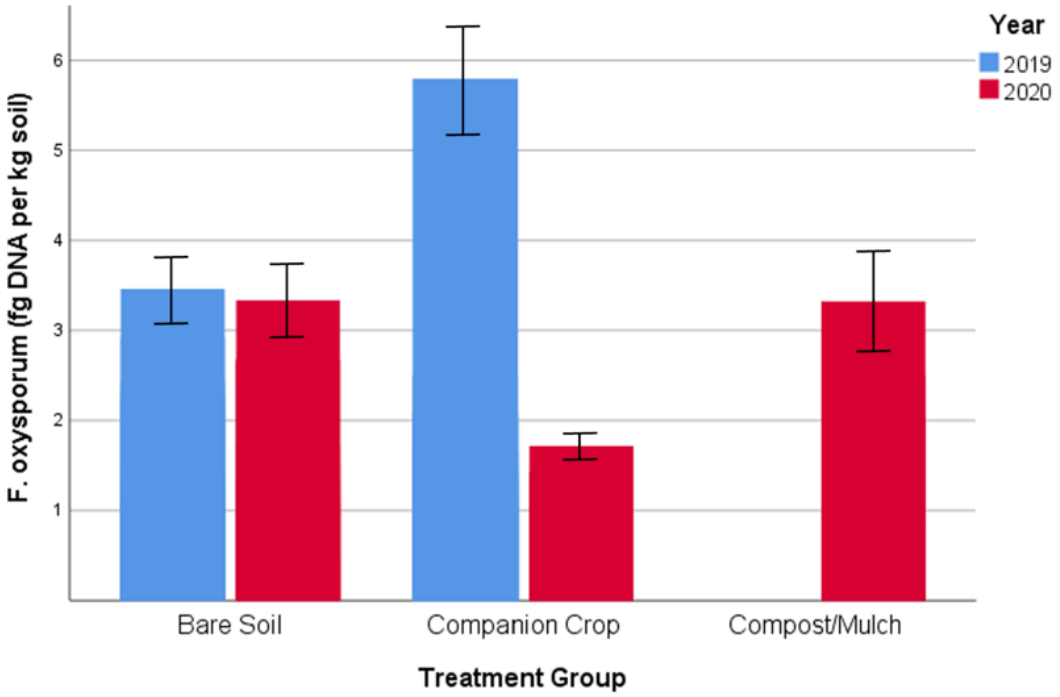

Figure 62. *Fusarium oxysporum* DNA detected in soil sampled from the asparagus field trial after different soil treatments. Data is presented as mean ± standard deviation (n=44).

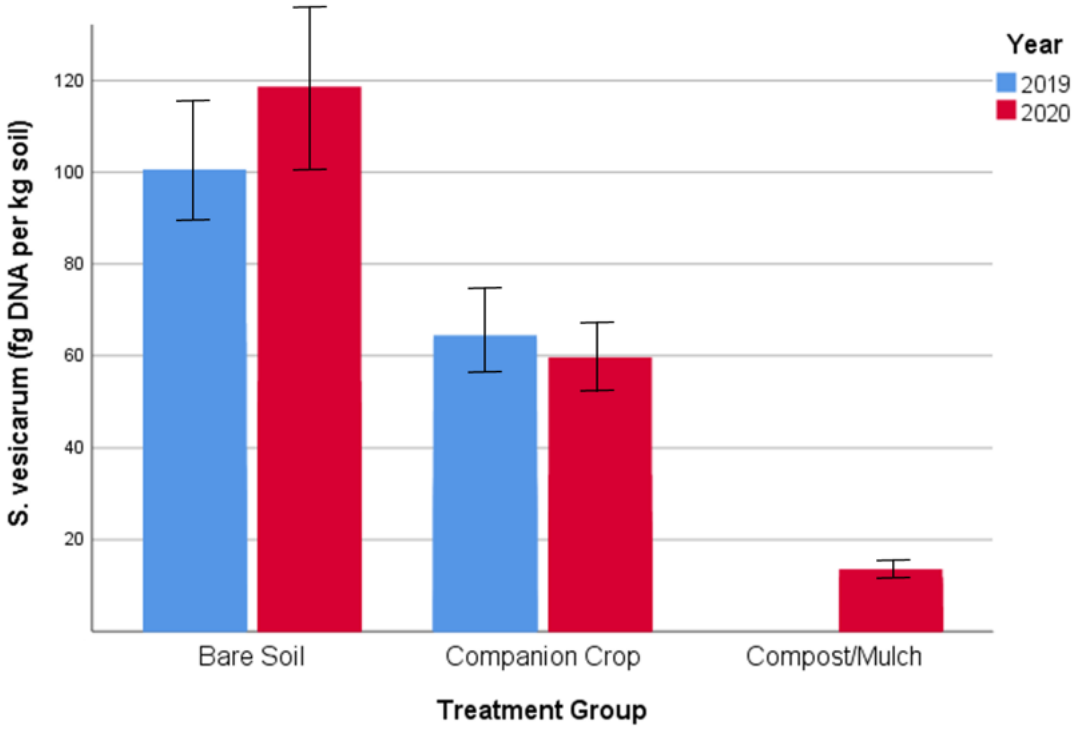

Figure 63. *Stemphylium vesicarium* DNA detected in soil sampled from the Asparagus field site after different soil treatments. Data is presented as mean ± standard deviation (n=44).

Stemphylium vesicarium populations were consistently lower in both years in soils treated with PAS100 compost or straw mulch compared with bare soil controls (Figure 63). However, neither PAS100 compost nor Straw mulch appeared to affect levels of *F. oxysporum* compared with those observed in in bare soil. The effects of incorporating Rye and Mustard companion crops were inconsistent across both years with lower levels of *F. oxysporum* DNA than in bare soil in 2020, but similar levels in 2019 (Figure 64 and Figure 65). However, none of these observations were significant when assessed using an ANOVA (p=0.119-1.000)

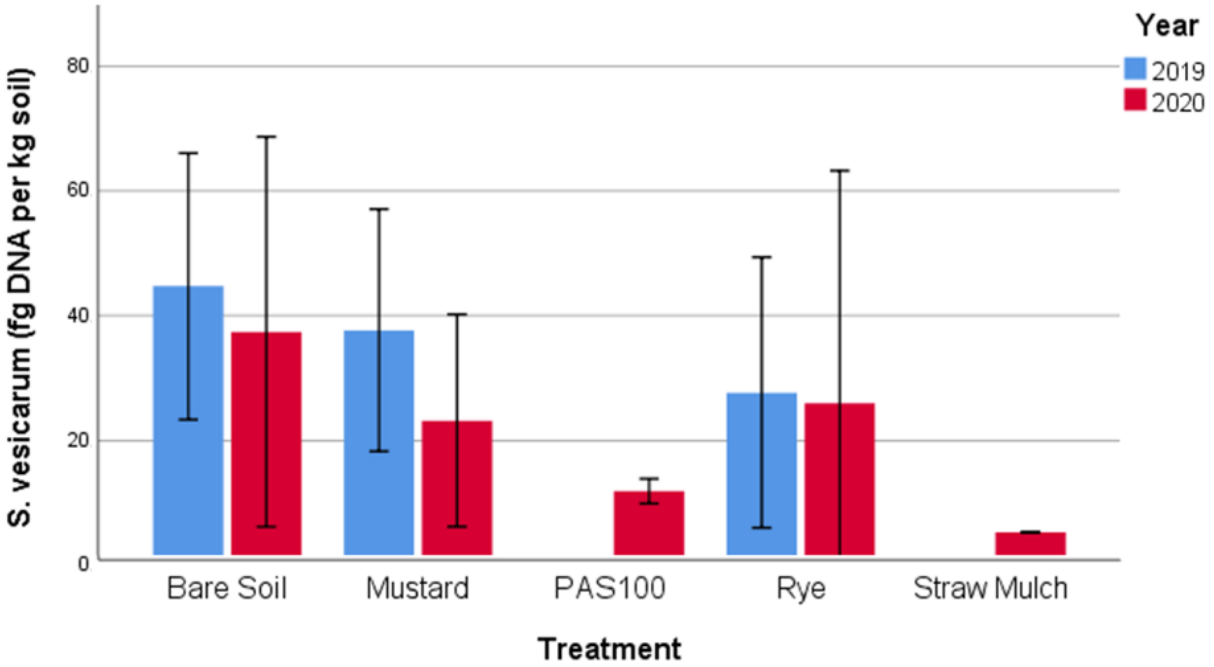

Figure 64. *Stemphylium vesicarium* DNA detected in soil sampled from the Asparagus field site after different soil treatments. Data is presented as mean ± 95% confidence limits (n=44).

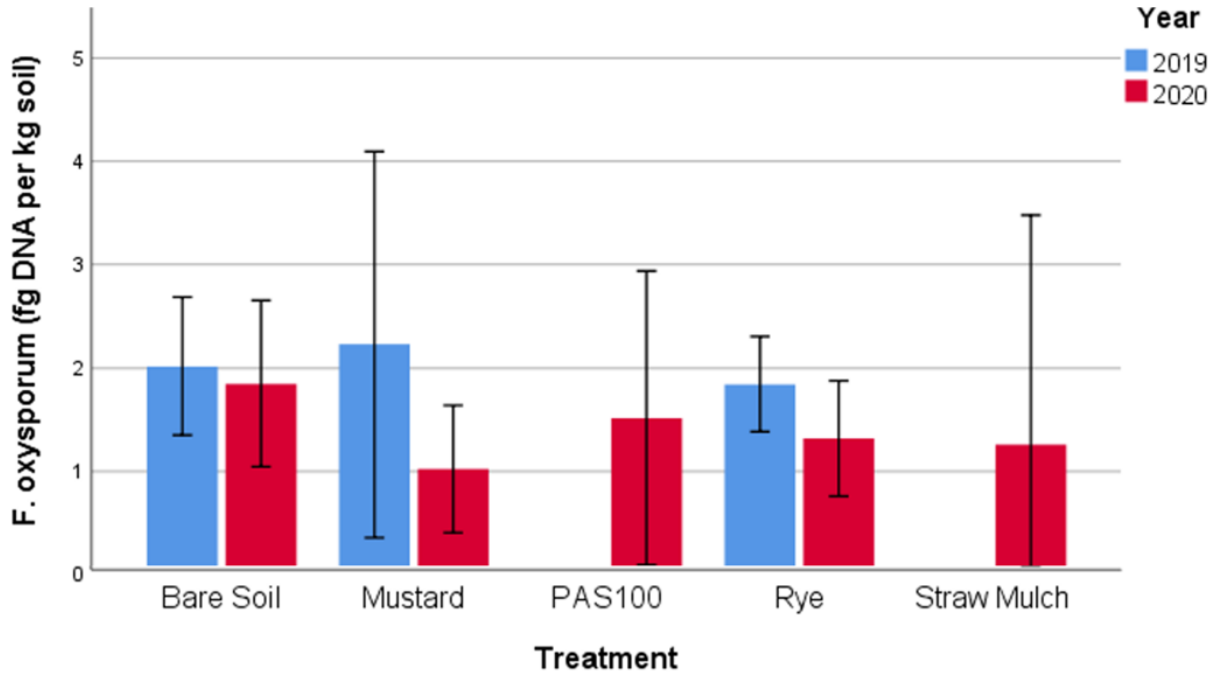

Figure 65. *Fusarium oxysporum* DNA detected in soil sampled from the Asparagus field site after different soil treatments. Data is presented as mean ± 95% confidence limits (n=44).

iv) Discussion

Detection of *Verticillium dahliae* at the Raspberry Field Trial in response to organic amendments

Verticillium dahliae was detected in soil sampled from the raspberry trial in both years. There was significant variance in the quantities of *V. dahliae* DNA detected between the years, with higher levels of detection in 2019 compared to 2020. This finding contrasts with observations made in the study by Berbegal *et al.* (2007), which reported elevated levels during a second growing season of artichoke. Changes in the DNA extraction methodology, due to the identified limitations of the initial method, between the years, may have influenced results of the current study or seasonal / environmental variables.

Although *V. dahliae* was detected in the soil during the trial, no disease symptoms were observed at the site. It was therefore not possible to relate soil inoculum to disease development from the data collected. The absence of disease symptoms was attributed to *V. dahliae* levels being too low to induce disease within the raspberry varieties grown. Various thresholds of *V. dahliae* soil inoculum have been found to induce significant crop disease, ranging from 10 microsclerotia/g soil in some crops (Tzelepis *et al.*, 2017), to as little as 1.3 microsclerotia/g in strawberry (Berbegal *et al.*, 2007) and 1 microsclerotia/g in cauliflower (Xiao and Subbarao, 2007), but not in raspberry (to date).

Environmental factors, as well as crop type and variety, have been identified as significant variables influencing both *V. dahliae* inoculum density and disease incidence (Harris and Yang, 1996; Berbegal *et al.*, 2007). These factors further underscore the complexity of predicting disease dynamics and emphasize the need for comprehensive understanding of the agricultural systems when assessing disease risk.

Throughout both years of the current study, none of the soil amendment treatments significantly impacted the measurable populations of *V. dahliae* or the occurrence of disease in the crop. It was concluded *V. dahliae* inoculum levels remained below infection threshold under the experimental conditions in each year.

Detection of *Clonostachys rosea* at the Raspberry Field Trial in response to organic amendments

Prestop, a commercial product containing *C. rosea* has previously been demonstrated as a potential biocontrol agent against *Verticillium* and other pathogenic fungi. For instance, studies by both Egel *et al.* (2019) and Tut *et al.* (2021), reported suppressive effects of Prestop against blight diseases (Grey Mould, Early blight, Septoria leaf spot and bacterial spot foliar), with the latter even reporting a 77% efficacy. However, Rahman *et al.* (2021), in an assessment of Prestop's effect on *Verticillium* in tomatoes, reported inconsistent results. This PhD study found no discernible correlation between the populations of *V. dahliae* and *C. rosea* detected in the soil.

The success of biocontrol agents is often evaluated in controlled environments using artificially inoculated soils Egel *et al.* (2019) and Rahman *et al.* (2021). However, their efficacy in field conditions is frequently hindered by the complexities of natural environments (Stadler and Tiedemann, 2015). This underscores the importance of considering field conditions when assessing the practical utility of such agents in agricultural settings.

While *C. rosea* could be detected in soil sampled from Prestop-treated plots, it was also detected in untreated plots. It was not, however, detected in soil sampled from any plots amended with anaerobic digestate alone. Furthermore, it is noteworthy that in some plots across all treatments, *C. rosea* was not detected, contributing to the significant variation observed. This variability could be explained if the treatments were incorrectly applied or that their distribution was altered throughout the trial, potentially through irrigation or cultivation practices. Another hypothesis is that naturally occurring background levels of *C. rosea may* have been present across the site, and that the applied product failed to significantly influence the natural population. It is worth noting, that the Prestop treated soil consistently exhibited higher levels of *C. rosea* than in the other plots, although these differences were not statistically significant. The absence of *C. rosea* in the digestate treatment plots, along with what appears to be reduced levels in the digestate plus Prestop treatment plots, raises the possibility that the digestate impacted colonization by *C. rosea* and potentially suppressed any natural populations. If indeed this is the case, it would warrant further investigation into the combined effects of organic amendments and biocontrol agents, such as *C. rosea,* to comprehend their impact on efficacy.

However, the introduction of digestate may have inhibited the PCR reaction, as observed in Chapter 5. This effect was only noted for *C. rosea*, possibly suggesting that its lower abundance compared to the other targeted organisms contributed to the inhibition.

Detection of the biocontrol agent *C. rosea* in field soil using qPCR was demonstrated for the first time in the current study. Unfortunately, no relationship between *C. rosea* and *V. dahliae* soilborne populations or disease development in the crop could be established from the data obtained.

Detection of *Fusarium oxysporum* at the Onion field trial in response to organic amendments

qPCR testing at planting showed that *F. oxysporum* was uniformly distributed across the trial, even 3 years after the preceding infected onion crop. It was therefore concluded that the soil sampling strategy was adequate for monitoring the targeted organisms at this site. This sampling strategy matches the current recommendations (McKay *et al*., 2009) and methods used by Paplomatas *et al.* (1992), Pennock *et al.* (2006) and Mann *et al*. (2019), amongst many others. This increase is unsurprising as it is well known that the root exudates of susceptible crops trigger the germination of *F. oxysporum* microconidia (Steinkellner *et al.,* 2005). When this stimulation of *F. oxysporum* by susceptible host crops was studied by Huang *et al.* (2020), they found significantly more *F. oxysporum* in the rhizosphere than in the bulk soil indicating that germination was triggered by interaction with the root system and its exudates. It is important to note that the DNA extraction method varied between sampling dates, which may have influenced the results. However, as discussed in Chapter 4, a comparison of the two extraction methods showed no significant difference in the quantity of fungal DNA extracted, though there was a reduction in variability, minimizing its impact on the final quantified results.

Similarly to findings from the raspberry field trial, the application of soil amendments, green compost, farmyard manure or their combination, failed to affect F. *oxysporum* concentrations at the time of sampling. There was no significant change in *F. oxysporum* populations in response to any of the treatments. High initial inoculum levels of *F. oxysporum* may have overcome any impact of the treatments, in direct contrast to findings from the raspberry field trial. This is supported by the fact that

levels of disease (onion *Fusarium* basal rot) were abnormally high, with the average disease incidence per plot reaching 90.5%. This lack of response disagrees with previous reports of these amendments controlling soilborne fungal pathogens (Abawi and Widmer, 2000; Lazarovits, 2001; Lazarovits *et al.,* 2001; Bailey and Lazarovits, 2003; Klein *et al.,* 2011; Romdhane *et al.*, 2019).

Similarly, initial estimates of soilborne *F. oxysporum* populations were not related to the incidence of disease observed in the crop. For instance, the highest levels of disease, 96-100%, occurred in the bottom left corner of the field trial, however these plots corresponded with relatively low initial levels of *F. oxysporum* when compared to other plots in the field trial. Again, this inability to relate *F. oxysporum* DNA levels to disease could be related to the high and widespread initial incidence of *Fusarium* in the soil across all plots obscuring any minor differences in initial inoculum levels.

This trial successfully demonstrated detection and quantification on *F. oxysporum* in the natural field soil environment and that detected populations caused *Fusarium* basal rot disease in the onion crop. Unfortunately, it was not possible to link the high starting populations to disease levels and therefore these results could not be used to develop predictive or advisory tools for the management of Fusarium basal rot in onion. A key limitation of this trial was the uniform and high initial levels of *F. oxysporum* which masked any potential treatment effects and disease results. A further limitation of this trial was the lack of specificity of the assay which recognises all genotypes of *F. oxysporum* but not specifically *F. oxysporum f. sp. cepae,* the specific pathogen of onion. There are many other formae speciales of *F. oxysporum* that are not pathogenic to onion. Further assay specificity may increase the precision of disease forecasting for disease caused by *F. oxysporum*. Work to identify different *formae speciales* of *F. oxysporum* is in progress at Warwick University, led by Prof. John Clarkson. Their work has already identified pathogenicity related DNA probes in *F. oxysporum f. sp. cepae* (Taylor *et al.*, 2016) and *F. oxysporum f . sp. narcissi* (Taylor *et al.*, 2019) within SIX genes, however further work is needed to make these advances applicable to quantifying these pathogens in soils.

Detection of *Fusarium oxysporum* and AMF species at the Daffodil field trial in response to organic amendments

Unlike in the previous two trials, pathogen DNA levels, namely *F. oxysporum* in this case, did not change significantly between the two years. This was not expected as *F. oxysporum* is often triggered to germinate and multiply by the presence of susceptible crops (Steinkellner *et al.,* 2005; Huang *et al.*, 2020). It is unclear why this was not observed, but it could be theorised that an environmental factor, such as soil properties or weather, could have played a role. For instance, the soil at this site had the highest pH out of the trials with an average of 8.2. Growth of *F. oxysporum* has been found to be negatively correlated with pH, indicating that it may be less prolific in alkaline soils (Orr and Nelson, 2018). However, this would need to be investigated further, and other factors that could also impact the results of this study.

The application of soil amendments; green compost, farmyard manure and the microbial product, had no effect on *F. oxysporum* concentrations at the time of sampling. Much like at the onion field trial, it was expected that the treatments would affect soilborne fungal pathogen populations as previously reported (Abawi and Widmer, 2000; Lazarovits, 2001; Lazarovits *et al.,* 2001; Bailey and Lazarovits, 2003; Klein *et al.,* 2011; Romdhane *et al.*, 2019). In this case, initial *F. oxysporum* levels were lower than those reported in the onion trial and observed disease incidence varied across the site. Again, uncontrollable environmental variables probably contributed to the complex interactions occurring both under and above the soil surface.

This study was one of the first to attempt to use qPCR to monitor the role of population dynamics between AMF species and soilborne *F. oxysporum* in relation to disease development. *F. mossae*, a key active ingredient of the AMF unnamed microbial product (PlantWorks, UK), was successfully detected using a specific qPCR assay. Conversely, a second active ingredient, *R. irregularis*, was not detected using a second specific qPCR assay. It was unclear whether the assay was unable to detect the organism or whether the organisms was unable to establish in the soil and was no longer present at the time of sampling. The assay was designed and reported in the same study as the *F. mossae* assay (Thonar *et al.,* 2012), which appeared to work in this trial assigning some confidence to its specificity.

Nevertheless, neither AMF species was detected in any of the sampled soils. Assuming the assay was functional and *R. irregularis* was simply absent prompts the question: why? In the validation experiments, we attributed this lack of detection to the lack of time for the product to active and produce live mycelium, however in the field trial the product had 2 years to become active, ruling out this possibility. Another plausible explanation is that the populations remained below the limit of detection for this assay under the given conditions. Alternatively, the microbial product may have failed to colonize, and the presence of *F. mossae* detected might have been solely due to natural populations in the soil. This notion finds support in its consistent presence across all treatments, not just in plots treated with the microbial product, at similar levels.

The ability of these biocontrol agents to colonise is key to their success and their uptake by farmers, as discussed in Chapter 5. Evidence suggests that early colonisation of biocontrol agents is essential to overcome competition and prevent infection (Rahman *et al*., 2021), perhaps offering an explanation as to why the microbial product treatment failed to impact *F. oxysporum* populations and the disease seen in the crop.

Again, no relationship was established between the initial levels of soilborne *F. oxysporum* detected and the incidence of disease that developed in the daffodil crop. Furthermore, none of the soil treatments applied affected disease incidence. The lack of observed relationship between *F. oxysporum* populations detected in the soil and disease incidence may have been due to *F. oxysporum* being introduced on already infected-daffodil bulbs at planting. Clarkson *et al.* (2019) investigating *Fusarium* basal rot in a variety of crops, found that their narcissus bulbs had high background levels of *F. oxysporum* prior to planting, despite not showing symptoms. This similarly obscured their ability to discern any relationship between soilborne *F. oxysporum* and disease development.

As frequently discussed throughout this thesis, environmental variables could have played a greater role in disease development than did the initial soilborne pathogen populations. The daffodil trial site had the highest soil pH and there are many other factors that could have impacted diseased incidence, including the possibility of pre-infected planting bulbs.

It should also be noted, as with the detection of the onion pathogen, the assay used in this trial allowed generic detection of all *F. oxysporum* genotypes and was not specific for detection of *F. oxysporum f. sp narcissi*. For further understanding of these systems, a more specific qPCR assay will be required.

Detection of *Fusarium oxysporum* at the Asparagus field trial in response to organic amendments

Unlike in the previous trials, mechanical amendments as well as organic amendments were assessed for their effect on soilborne pathogens. In this trial disease symptoms in the crop were not assessed where instead only the changes in soil pathogen populations were considered. Firstly, populations of *F. oxysporum* were found to not have been affected by re-ridging. This is in contradiction to the report by Elmer (2015) where re-ridging was found to increase susceptibility of asparagus to *Fusarium oxysporum f. sp*. *asparagi.* This was also the case for *S. vesicarium* populations, where there was again no effect of re-ridging between harvests. It has been previously theorised that reducing compaction through re-ridging would reduce soil moisture through improved drainage which in turn would reduce the incidence of *Stemphylium* purple rot (Saude *et al.*, 2008).

In 2020, but not 2019, it was found that untreated plots, which did not have the soil surface disturbed, had significantly higher populations of *F. oxysporum* than untreated plots with shallow disturbance. This indicates that the shallow cultivations in the top 15cm of soil either reduced the populations of *F. oxysporum* or that populations had increased in the absence of disturbance. It could be theorised that the shallow soil disturbance disrupted the growth of *F. oxysporum* mycelium by breaking up the structures, preventing multiplication of the pathogen. Ploughing and other mechanical disruption methods have been used in disease control for decades, and have been linked to disrupting fungal hyphal networks, including pathogens (Celestina *et al.*, 2019) and AMF (Schalamuk and Cabello, 2010). However, when shallow soil disturbance was also performed in plots with either straw or green mulch applied, no significant difference between *F. oxysporum* populations was found when compared to minimum till control plots. This did not support the theory that the shallow soil disturbance disrupts growth of the pathogenic mycelium. No effect of shallow soil disturbance in the bare soil plots was observed on *S. vesicarium* populations.

Effects of soil treatments on pathogen populations were only seen in samples taken in 2020 and not in 2019. Treatments were started in 2018 with the planting of the asparagus crop and re-ridging, shallow soil disturbance and mulches were reapplied annually. Treatment effects not being observed until 2020 perhaps implies that a period of time is required for these treatments to have a significant impact on pathogen populations.

Fusarium oxysporum populations were found to be significantly reduced in plots with both rye and mustard companion crops compared to plots with bare soils. Mustard has been reported to reduce *Fusarium* levels via bio-fumigation (Cresswell and Kirkegaard, 1995; Kirkegaard and Sarwar, 1998) and rye has been reported to reduce severity of *Fusarium* rot in asparagus (Matsubara *et al.,* 2001). These treatments may therefore offer a practical means for control against *F. oxysporum* diseases. Although not statistically significant, populations of *S. vesicarium* also appeared to reduce in response to the companion crops, in particular in the rye plots. However, previous reports have identified rye as a crop that *S. vesicarium* can overwinter and survive on, despite being a common cover crop in asparagus systems (Foster, 2018).

The use of compost or straw mulches had no significant effect on *F. oxysporum* populations but did reduce levels of *S. vesicarium*. Composts and mulches are known to increase organic matter and soil microbes in general, which has been linked to reduced disease (Abawi and Widmer, 2000). It is worth noting that both mulching and compost treatments underwent shallow soil disturbance during their incorporation into the soil, but that in control plots with shallow soil disturbance alone *S. vesicarium* populations were unaffected. It was therefore unlikely that the physical effects of soil amendments with mulch or compost played a role in pathogen suppression. Further research would be of interest to improve understanding of the pathogen suppressive effect of composts and mulches to determine whether all composts and mulches would have a similar effect or whether the effect is specific to the use of amendments with straw mulch or PAS100 compost.

Using metabarcoding to monitor disease species

In addition to using qPCR to monitor soilborne pathogens, the application of metabarcoding to study their abundance metrics was assessed. *Verticillium* and *Fusarium* species are members of the Ascomycota phylum, which was the most

prevalent phylum found in the soils at the raspberry and daffodil field trials. Ascomycota dominate global soils (Egidi *et al*., 2019), limiting the value of extrapolating any changes at the phylum level in response to soil management treatments to the effects on individual genera and species. Current resolution of bioinformatic analysis of metabarcoding data is dependent on the content and reliability of sequence databases. Although these are constantly improving, at the time of analysis of the data from these studies, it was not always possible to resolve genus and species from the metabarcoding data collected. Nevertheless, it was possible to form some broad conclusions.

At the raspberry trial, despite detecting *Verticillium* at the genus level, metabarcoding could not differentiate at the species level. This contrasts with Mirmajlessi, (2017) successful use of sequencing data identifying *V. dahliae* in soil samples from areas exhibiting high disease. Similarly, in the onion trial, while *F. oxysporum* was inconsistently identified via metabarcoding, the *Fusarium* genus was consistently detected. PCR quantification of *F. oxysporum* correlated positively with *Fusarium* abundance from metabarcoding. However, without species-level resolution, the significance of this correlation remains uncertain. Again, at the daffodil trial, metabarcoding failed to identify *F. oxysporum* to species level, aligning with challenges encountered in other trials. Additionally, *R. irregularis* wasn't detected via metabarcoding, consistent with qPCR findings. Although *F. mossae* presence was confirmed by both qPCR and metabarcoding, no relationship emerged between its quantification via qPCR and its relative abundance determined from metabarcoding.

Metabarcoding offers a potential broad and valuable view of microbial communities, including pathogens, although its application to accurately distinguish between closely related species and provide quantitative data may currently be limited. Future integration of molecular techniques like qPCR alongside metabarcoding will be key for accurate and reliable detection and quantification of pathogens in soil and in understanding the population dynamics of soilborne pathogens amongst the whole soil microbiome in response to soil management.

Conclusions

It was not possible to establish a relationship between soil inoculum density and disease incidence or to relate effects of organic soil amendments to changes in soilborne pathogen populations from qPCR or metabarcoding data. Despite efforts to

mitigate the effects of environment on the molecular approaches used to monitor pathogen populations in the soil, the data collected from the field studies was not suitable for the development of predictive diagnostics.

Some significant observations were made regarding the effect of amendments on pathogens; however, these were only seen in the second year of the asparagus trial. This perhaps indicates that the impact of these organic amendments on pathogens may take longer than these trials allowed. It may therefore be valuable to continue such studies as part of a long-term trial with repeated application of organic amendments as part of the field husbandry.

A high level of variation was seen in the data obtained. It is well known that microbial communities can be spatially clustered, and in a study by Baker *et al*. (2009) looking at bacterial communities, 44% of the variation was accounted for by the spatial effect, which could also explain the degree of variation observed in the results obtained across these trials. Efforts were made via the sampling design to overcome this spatial effect of pathogen populations by pooling and mixing of sub-samples. However, this only corrected for variation across a single plot, not across the whole site, and did not account for environmental impacts across the site. Attempts were also made to minimise variation via the DNA extraction method, as outlined in Chapter 4 (i). The success of this was indicated in the raspberry trial with the 2019 method having higher levels of variation than when the improved method was used in 2020.

It will be necessary to accumulate data from a much wider range of soils and crops before a more reliable determination of the influence of soilborne pathogen populations on the risk of disease development can be undertaken. Perhaps more fundamentally, further improvements in molecular diagnostics will be required, beyond those made in this thesis, to overcome the challenges observed.

Chapter 7- Using metabarcoding and qPCR to monitor changes in bacterial and fungal communities in response to organic amendments in different cropping systems

i) Introduction

This chapter describes a molecular approach to investigate the effects of organic amendment on the overall microbiology of agricultural soils under different cropping systems. This involved the amplification of phylogenetically specific DNA ("barcoding") sequences which allows the amplification of entire bacterial and fungal communities, known as metabarcoding, and qPCR to estimate total amounts of bacterial or fungal DNA in soil samples. Effects of soil amendments on bacterial and fungal communities were assessed by comparing soils sampled from a number of field trials, in which various organic amendments had been applied (described in Chapter 6).

A key step in monitoring soil health is the monitoring of biological communities as a whole. The previous chapter focussed on the detection and quantification of specific organisms related to soil-borne plant diseases and arguably unhealthy soils. Despite this offering insight into disease dynamics, further understanding of community dynamics may offer a more comprehensive insight into soil-borne pathogen persistence and soil health in general. A new way of monitoring soil communities is through metabarcoding. Metabarcoding is the application of high throughput sequencing to identify DNA of multiple organisms from a single sample, by amplifying DNA regions such as ITS, 18S rRNA and 16S rRNA, conserved within communities of target microorganisms (fungi, bacteria etc.). PCR amplified target sequences are identified according to the specific sequences associated with that organism (Orgiazzi *et al.*, 2015). However the taxonomic resolution is often limited by the availability of curated DNA sequence information in databases, lack of genetic variation of the target sequence and quality of the sequencing reads obtained, accuracy is often limited to the phylum or genus levels (Anderson, Campbell and Prosser, 2003).

This development of high through-put sequencing overcame many of the biases associated with the previously utilised culture methods, which often only recovered a

small subset of the community (Mirmajlessi, 2017). Despite the benefits of high through-put sequencing, it also has the potential to introduce biases.

Despite this, microbiome studies using high-throughput sequencing have been increasing in popularity from 4,505 studies by December 2010 to 66,250 in February 2020, as reported by Bollmann-giolai *et al*. (2020). This could be attributed to the falling cost and accessibility of the technology. There is also an increased call to create a universal database for metabarcoding data and soil diversity, such as the EU project 'EcoFINDERS' (https://esdac.jrc.ec.europa.eu/projects/ecoFinders) and the Global Soil Biodiversity Initiative (www.globalsoilbiodiversity.org)) (Orgiazzi *et al.*, 2015). Its use in agriculture is on the rise and there is an increasing amount of genomic data on plant pathogen interactions (Sperschneider, 2019), perhaps leading to its potential use in routine farm testing. Therefore, it is key to establish protocols and understanding for its use as a future tool.

Some researchers have begun to use sequencing techniques to monitor the effect of agricultural practices on soil communities, both bacterial and fungal. As early as 2010, Yin *et al.* used pyrosequencing to study the effect of rotation and tillage on bacterial communities. From their study they generated 20,180 sequences of which 2337 were individual operational taxonomic units (OTU). Operational taxonomic units (OTU) are used to classify sequences into groups for the purpose of analysing microbial diversity, particularly in microbiome studies. They found *Proteobacteria* represented 38% of the community followed by *Acidobacteria* at 20%. Focussing within the *Acidobacteria*, they found that clusters of *Acidobacteria* Group 1 and Group 3 were more frequent in continuous wheat vs wheat- soybean rotations, whereas Group 2 were more frequent in no-till treatment and some Group 4 *Acidobacteria* were more frequent in the wheat – soybean rotation. These abundant bacteria were therefore shown to be affected by the agricultural practices they were subjected to. In another study using high-throughput sequencing to monitor soil communities Sun *et al.* (2018) looked at the effect of tillage (conventional, reduced and no tillage) on bacterial and fungal communities in the soil at different depths. They found that the tillage treatments significantly affected microbial community structure and distribution by soil depth. Within the bacterial populations identified, variation was seen as the presence or absence of different species at the different soil depths. Whereas within fungal communities, variation was seen in the relative abundance of different species, suggesting niche-based effects were more important

for bacterial than fungal communities in structuring the vertical distribution. Similarly to the previous study they found that *Proteobacteria* was the most abundant phyla (30.57%), however, *Acidobacteria* was only the fourth most prevalent genus (10.49%). As for fungi, Ascomycota was the most dominant phylum accounting for 69.69% of total reads.

When looking at the specific effects of the treatments on evenness and richness on bacterial and fungal communities, there were no significant changes in either bacterial or fungal evenness (defined using Shannon Diversity) among any of the tillage treatments, apart from one instance where fungal evenness was higher in the no tillage treatment at the 5-10cm layer. Despite this, the study showed that tillage did not appear to significantly impact evenness under these circumstances. However, tillage practices did appear to affect both bacterial and fungal richness. For instance, under conventional tillage, bacterial richness was higher in the 5-10cm layer, whereas for the reduced tillage this was in the 0-5cm layer. Which is perhaps expected due to disturbance levels at each soil depth interrupting bacterial richness. Fungal richness was highest under conventional tillage and lowest where no tillage was applied. They concluded that long-term tillage had a greater effect on the fungal community than the bacterial community due to the significant variance of fungal OTU's between tillage regimes, which was higher than those seen in bacterial communities.

Regarding studies investigating similar treatments to those investigated in this study, Celestina *et al*. (2019) used 16S rRNA and ITS Illumina MiSeq sequencing to assess the effect of fertiliser and manure treatments on bacterial and fungal communities. Principal component analysis showed microbial communities could be separated by sampling depth, and this separation was more pronounced in bacteria than fungi. This difference between sampling depth was also observed by Sun *et al.* (2018). Here, fungal community structure varied with amendment type, placement and the interaction, however this effect was smaller than that observed due to sampling depth. Over the 3 years of this study, only weak long-term effects were seen on the fungal community, and they concluded that their results did not support the hypothesis that these soil management practices would have significant lasting impacts on soil microbial communities. In another study, looking at manure amendments, Tian *et al.* (2015) compared the effects of manure composts against inorganic nitrogen on wheat-rice cropping systems over 3 years, using

pyrosequencing. They found that the manure compost increased microbial activity and gene copies of bacteria, archaea and ammonia oxidising bacteria but decreased their diversity. Whereas the inorganic nitrogen had no effect on abundance or diversity of bacteria, archaea and ammonia oxidising bacteria over the 3 years and neither amendment influenced their richness. They found that across all treatments the most frequent phyla were *Proteobacteria, Chloroflexi*, and *Acidobacteria. Proteobacteria* has previously been reported as the most frequent phyla by both Sun *et al.* (2018) and Yin *et al.* (2010). Despite *Proteobacteria* being the most frequent across the treatments, their relative abundance increased in the manure compost treatment when compared to the control, and *Chloroflexi* reduced in relative abundance. Similarly to the previous studies, Kumar *et al*. (2018) reported *Proteobacteria, Acidobacteria, Actinobacteria* and *Chloroflexi*, as well as *Firmicutes*, were the most dominant phyla, accounting for 80-85% of OTU's, in their study monitoring 16S rRNA communities in a 47-year-old long-term fertilised paddy soil. Within their study they compared the effect of nitrogen, nitrogen + potassium + phosphorus, farmyard manure, farmyard manure + nitrogen and farmyard manure + nitrogen + potassium + phosphorus on bacterial communities. Nitrogen application alone appeared to encourage *Firmicutes, Actinobacteria*, and *Nitrospira* but supressed *Proteobacteria, Acidobacteria, Cyanobacteria* as well as others including *Fibrobacteres, Spirochaetes*, *Saccharibacteria* (TM7) and GNO4 (uncultured bacteria) when compared to other treatments. The highest proportion of bacterial OTU's were recorded in the nitrogen + potassium + phosphorus treatment, suggesting that this treatment encouraged the beneficial bacterial community more so than the other treatments. The nitrogen only treatment also appeared to lower bacterial diversity as measured by the Shannon index.

In addition to using sequencing to monitor changes in overall microbial communities, studies have used the tool to specifically monitor effects on soil-borne pathogens. The abundance of antagonistic microbes and the diversity of soil microbial communities are important in their effect on suppression of soilborne pathogens. Organic amendments and tillage management have been associated with altering these antagonistic communities (Yin *et al.*, 2010). For instance, Bailey and Lazarovits (2003) found the application of composted sewage sludge after 2 years reduced populations of *Sclerotinia minor* and the incidence of lettuce drop for the next 4 years. Therefore, sequencing could be a useful tool in monitoring these changes in

relation to soilborne pathogens. Using Illumina MiSeq sequencing, Mirmajlessi (2017) observed *F. solani, V. dahliae, R. solani* and *Colletotrichum truncatum* according to metabarcoding data. *V. dahliae* was most dominant particularly in the high disease incidence soils and *Rhizophagus irregularis*, a beneficial AMF species, was more abundant in soils with healthy plants. This demonstrated that metabarcoding could be used to monitor soil-borne pathogens, including those targeted in this thesis, and relate them to changes seen in the crop. Bradley *et al.* (2020) used sequencing methods to monitor soilborne pathogenic *Fusarium*, in response to the herbicide glyphosate. They mostly identified geography, season and the farming systems as the largest drivers of microbial communities, reflecting the earlier sentiment that management practices alter the microbial communities (Yin *et al.*, 2010). However, when looking at the effect of glyphosates on the *Fusarium* they found no effect and did not detect any changes in the relative abundances of *Fusarium* sp., *Alternaria* sp., or *Macrophomina* sp., in response to glyphosates. They hypothesised that plant pathogens were in fact missed in the study and could not be differentiated from other members of the same phylum classes using the metabarcoding approach. Huang *et al.* (2020), used qPCR, amplicon sequencing and metabolomics to assess fungal community succession across bulk soil in the plant endosphere of diseased and healthy Lisianthus plants. As expected, populations of *F. oxysporum* were higher in diseased rhizosphere soils than in that from non-diseased plants and more potential antifungal microbes were present in the bulk soil and rhizosphere of healthy plants. Diversity was also found to be lower in soil from diseased plants. Fungal genera that appeared to be enriched in the soils of healthy plants included *Emericellopsis, Acremonium, Remersonia*, and an unclassified (UC) genus within *Sordariales*, whereas *Neocosmospora* and UC *Nectriaceae* were higher in the soils sampled from diseased plants. This shows how the whole microbial community can impact the disease in the crop and perhaps indicates the potential of these microbes in the control of soil-borne pathogens and improving soil health.

Metabarcoding has been shown to be a useful tool for community analyses, however it is expensive, requires complex analyses and does not offer quantitative properties (Liu *et al.*, 2012). Application of qPCR for studying the quantities of specific organisms has been discussed in chapter 6 and there is potential for it to be used to assess the overall size of soil microbial communities by quantifying 16S rRNA and 18S rRNA bacterial and fungal regions. This technique was used by Romdhane *et al*.

(2019) when assessing the effects of cover crops on the total bacterial community by quantifying 16S rRNA using real-time PCR quantification. Similarly, in 1998 Ranjard *et al*. quantified both 16S and 18S rRNA using gel electrophoresis when monitoring DNA recovery from different microenvironments in the soil.

Metabarcoding can be used to provide insights into the diversity of microbes in the soil whilst qPCR can be used for quantification of groups or individual taxa, it makes sense to use the two to complement each other and offer a fuller picture. Yin *et al.* (2010) utilised qPCR to validate the results of their pyrosequencing. When assessing the effect of rotation and tillage on bacterial communities they found that four *Acidobacteria* Group 4 OTU's dominated the wheat-soybean no till treatment, this was supported by qPCR as these samples also contained the highest population of these organisms. Similarly, Huang *et al.* (2020) used qPCR to complement their findings. When investigating the fungal community succession from bulk soil to plant endosphere in diseased and healthy Lisianthus plants, Huang *et al.* (2020) used amplicon sequencing and metabolomics in combination with qPCR to show that the relative abundance of *Fusarium* was higher in soils from diseased plants than the soil from healthy plants, further finding that the quantity of *F. oxysporum* was also higher in the diseased samples than the healthy samples.

In this study the effect of organic amendments on the soil community in 3 crop systems; raspberry, onion and daffodil were assessed. This was achieved by performing metabarcoding sequencing and qPCR molecular analyses on DNA samples extracted from soil. The relative abundance and diversity metrics were compared against the different treatment types as well as the quantified amounts of 16S rRNA and 18S rRNA to represent amounts of fungal and bacterial communities in the soil. It was assessed whether there was a direct relationship between abundance and diversity metrics and the quantities of 16S rRNA and 18S rRNA observed, that could be used to assess soil health without extensive sequencing analyses, which has not been explored previously.

ii) Metabarcoding and qPCR methodology applied to DNA extracted from field soils for microbial community analyses.

Samples from the field trials in raspberry, onion and daffodil underwent microbial community analyses, using qPCR for quantification of bacterial and fungal communities and metabarcoding to assess diversity. Full field trial descriptions, husbandry and sampling are covered in Chapter 6 (ii). DNA extracted from samples described in chapter 6 were used for the community analyses.

The raspberry trial was planted in April 2018 and harvested from June to September in 2018, 2019, and 2020. The study targeted *Verticillium dahliae* and tested several treatments, including crop-based fibre digestate (PAS 110) incorporated at 5 kg/m², a biofungicide (*Clonostachys rosea*, Prestop) applied as a soil drench at 5 g/L, and a combination of anaerobic digestate and biofungicide, on their interaction with soilborne pathogens.

The onion trial was planted in February 2019 and was harvested in August 2019. It targeted *F. oxysporum* and *S. cepivora*, this trial evaluated PAS 100 green waste compost spread at 3 kg/m², an autumn cover crop (phacelia, vetch, clover) planted at 35 kg/ha in August 2018 and herbicide-treated in early 2019, and a combined treatment of cover crop and PAS 100 green waste compost, on their interaction with soilborne pathogens.

The daffodil trial was planted in August 2018 and harvested in August 2020. The trial investigated *S. vesicarium* and *F. oxysporum* and tested PAS 100 green waste compost applied at 3 kg/m², farmyard manure (FYM) spread at 2.5 kg/m², and mycorrhizae applied in-furrow at 1 g per bulb during drilling, on their interaction with soilborne pathogens.

The asparagus trial was planted in April 2016 and harvested annually between April and June. The trial targeted *Fusarium oxysporum* and tested a range of treatments on their interaction with soilborne pathogens, including mustard and rye cover crops, straw mulch, PAS 100 green waste compost, ridging, and shallow soil disturbance, on their interaction with soilborne pathogens.

qPCR analyses

Bacterial and fungal communities were quantified using 16S rRNA (CDC, 2011) and 18S rRNA (Liu *et al.*, 2012) rRNA qPCR TaqMan assays, using the designed gBlock™ to generate a standard curve. Full description of qPCR methodology and gBlock™ design is covered in Chapter 3 (ii).

Metabarcoding

Metabarcoding was performed on each DNA extract using an Illumina MiSeq. Full molecular methodology is reported in chapter 3 (iii). Raw data was imported into the Qiime2 software (version 2018.8) (Bolyen *et al.*, 2019) before undergoing trimming with the Cutadapt software (Martin, 2011) using the 16S and ITS primers reported in chapter 3 (iii). A minimum read length of 50bp was enforced at this stage, with any reads shorter than this being excluded from further analysis. Upon completion of the trimming steps, sequences were denoised using the DADA2 software (Callahan *et al.*, 2016). This process involves removing low quality nucleotides, before truncation of each read based on the overall quality of the dataset (inferred from the Cutadapt outputs). The onion field trial samples were run separately to the samples from the other field trials, due to the maximum sample number of the protocol. For the 16S dataset, forward reads were truncated at position 253 (for both the onion and other field trial runs) and reverse reads were truncated at positions 217 for the onion field trial run and 221 for the remaining run. This was performed as the two different runs generated different quality of reads. This action did not affect the length of final merged reads (98% = 253bp, 2% 254bp). For the ITS dataset, truncation was not performed due to the variable length of the ITS region. After truncation, DADA2 performs merging of reads, detection and removal of chimeras, and produces a list of amplicon sequence variants (ASVs).

Operational taxonomic units are commonly used when reporting on sequencing data, however Amplicon Sequence Variants (ASV) were used in this study. ASVs are unique sequences inferred from the raw sequencing data without clustering. OTUs group sequences based on a similarity threshold and are less sensitive to sequencing errors but might lack resolution. ASVs represent unique biological sequences with higher resolution, allowing for more precise taxonomic identification.

Following denoising, an additional round of chimera filtering was performed with Vsearch (Rognes *et al.*, 2016). 16S sequences were filtered to remove mitochondria,

chloroplast, archaea and eukaryote sequences and any ASV's not assigned at phylum level. For ITS ASV's not assigned at either kingdom or phyla or classified as *Stramenopila* were removed. Additionally, any sample with less than 3000 sequences was removed from further analysis. Finally, sequences were classified using a naïve bayes classifier trained on either the Silva (version 138) database (Quast *et al.*, 2013) for the 16S dataset or the targeted loci ITS RefSeq database (McEntyre and Ostell, 2002) supplemented with the UNITE database (Nilsson *et al.*, 2019) (for oomycota) for the ITS dataset.

Three diversity metrics were chosen for assessment of the metabarcoding data: Shannon Diversity, Pielou Evenness and Faiths Diversity. Diversity metrics were generated using Qiime 2 (Bolyen *et al.*, 2019).

The Shannon Diversity Index is a widely used metric in metabarcoding studies to quantify the diversity of a biological community based on DNA sequence data. It takes into account both species richness (the number of different species) and species abundance (the number of individuals of each species). A higher Shannon Diversity Index indicates a more diverse community. The index considers not only the presence or absence of species but also their relative abundance, providing a more comprehensive measure of diversity.

Pielou Evenness, also known as Shannon Evenness or J Evenness, complements the Shannon Diversity Index by indicating how evenly the individuals are distributed among different species. The formula for Pielou Evenness (J) is derived from the Shannon Diversity Index. Pielou Evenness ranges from 0 to 1, where 1 indicates perfect evenness (all species have the same abundance), and 0 indicates maximum diversity but with uneven abundance. It provides insights into the equitability of species representation within a community.

While Shannon Diversity and Pielou Evenness focus on taxonomic diversity, Faith's Phylogenetic Diversity extends the analysis to incorporate the evolutionary relatedness of species in a community. This metric considers both the richness and the evolutionary distinctiveness of species. The calculation involves creating a phylogenetic tree based on genetic data and measuring the total branch length spanned by a set of species.

Statistical Analysis

All analyses were conducted using IBM SPSS Statistics version 26. Data normality was evaluated using the Shapiro-Wilk test ($p > .05$), and homogeneity of variance was assessed with Levene's test. If the data met the assumptions of normality and homogeneity, parametric tests were applied, including analysis of variance (ANOVA), linear regression, and Pearson's correlation.

If the data violated these assumptions, a Log^{10} transformation was applied, and the normality and homogeneity tests were repeated. If the transformed data met the assumptions, parametric analyses (e.g., ANOVA) were conducted on the transformed dataset.

In cases where the data continued to violate the assumptions after transformation, non-parametric alternatives were employed on the original untransformed data. These included the Kruskal-Wallis test for group comparisons and Spearman's rank correlation for correlation analysis.

For the ANOVA analyses (one-way, two-way, etc.), post hoc comparisons were performed using Tukey's test to identify significant differences between group means. In addition to the post hoc tests, descriptive statistics and effect size estimates were calculated to further interpret the results. In some cases, to account for temporal variability, time was included as a covariate in the analyses, allowing for the control of its potential influence on the outcomes.

Statistical significance was determined at a threshold of $p < 0.05$.

iii) Results

Raspberry Field Trial

For samples collected from the Raspberry field trial, a total of 244,578 reads were obtained creating 2,811 ASV's for the 16S rRNA DNA region and 1,101,144 reads and 2,875 ASV's for the ITS rRNA DNA region.

Proteobacteria, followed by *Bacteroidota, Acidobacteriota, Chloroflexi* and *Actinobacteriota* were the most frequent bacterial phyla observed (Figure 66). Data was normally distributed for each group, as assessed by Shapiro-Wilk test ($p > .05$);

and there was homogeneity of variances, as assessed by Levene's test of homogeneity of variances. Therefore, one-way ANOVA analysis was performed. Out of the 38 bacterial phyla observed at the site, 16 showed significant differences in relative frequencies between years (2019, 2020). *Proteobacteria* ($p < 0.001$), *Bacteroidota* ($p < 0.001$), *Bdellovibrionota* ($p = 0.022$), *Chloroflexi* ($p = 0.044$), *Latescibacterota* ($p < 0.001$), MBNT15 ($p = 0.002$), *Methylomirabilota* ($p < 0.001$), *Myxococcota* ($p < 0.001$), *Patescibacteria* ($p = 0.025$) and *Zixibacteria* ($p = 0.002$) significantly increased in relative frequency between years, whereas *Acidobacteria* ($p = 0.026$), *Actinobacteriota* ($p = 0.023$), *Cyanobacteria* ($p = 0.009$) and *Nitrospirota* ($p = 0.007$) significantly decreased (assessed using one-way ANOVA). There was no significant effect of treatment on the bacterial phylum observed except that *Fibrobacterota* ($p = 0.025$) was not detected in digestate or digestate + prestop treated soils, and it occurred at only a relative frequency of 0.25% in untreated plots but reached a relative frequency of 2.00% in the Prestop treatment.

Amongst the fungal phyla, identified using ITS rRNA DNA, *Ascomycota* was the most prevalent, followed by *Mucoromycota* and *Basidiomycota* (Figure 67). When comparing between years *Ascomycota* (0.003) and *Chytridiomycota* (0.004) significantly decreased between years (2019, 2020). There was no observed effect on relative frequency of any of the fungal phyla in response to the soil treatments.

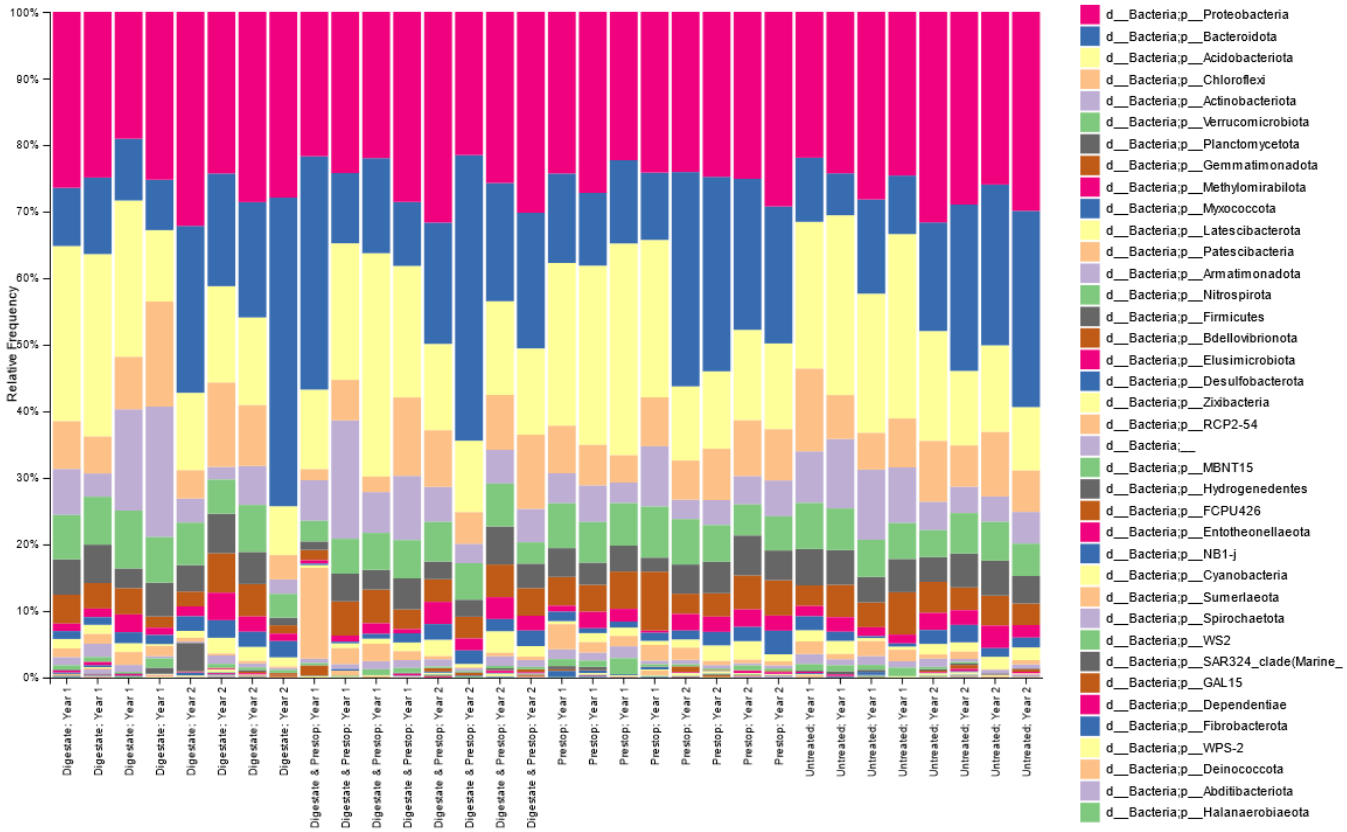

Figure 66. Relative frequency of bacterial phyla (according to partial 16S rRNA gene sequences) per treatment collected in 2 seasons from the Raspberry Field Trial. Generated using Qiime 2 viewer [accessed 26/11/2022]] (Bolyen *et al.*, 2019)

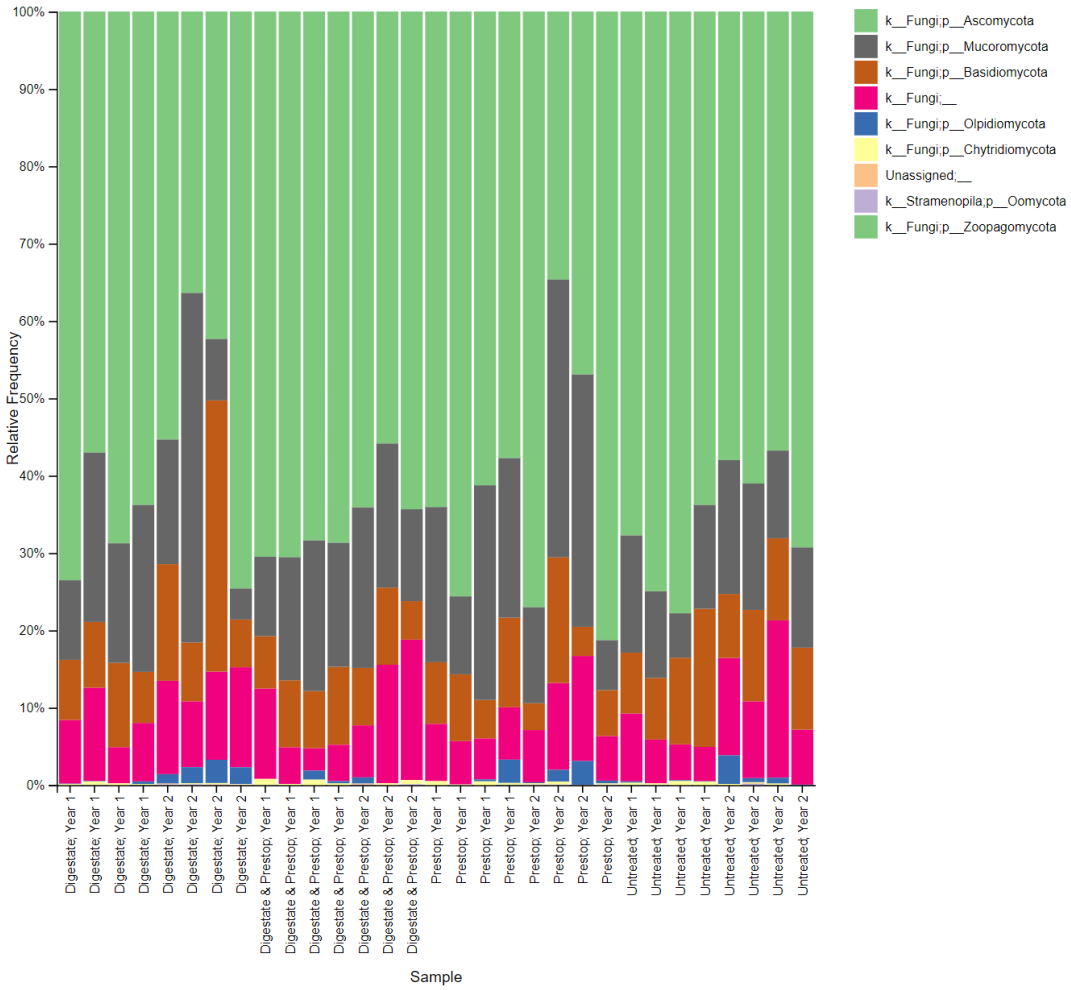

Figure 67. Relative frequency of fungal phyla (according to partial ITS rRNA sequencing) per treatment collected in 2 seasons from the Raspberry Field Trial. Generated using Qiime 2 viewer [accessed 26/11/2022] Bolyen *et al.*, 2019)

Quantification of 16S rRNA DNA, using qPCR, in response to treatment

The 16S rRNA qPCR data quantifying bacterial DNA between time points passed tests of normality, as assessed by Shapiro-Wilk's test ($p > 0.05$). The data was then assessed by factorial ANOVA. There was a significant difference between the 2 time points, independent of treatment. 16S rRNA DNA decreased between the 2 time points ($p=0.010$). There was no significant interaction between treatment and time ($p=0.349$). There was no significant effect of treatment on 16S rRNA DNA levels ($p=0.536$) (Figure 68).

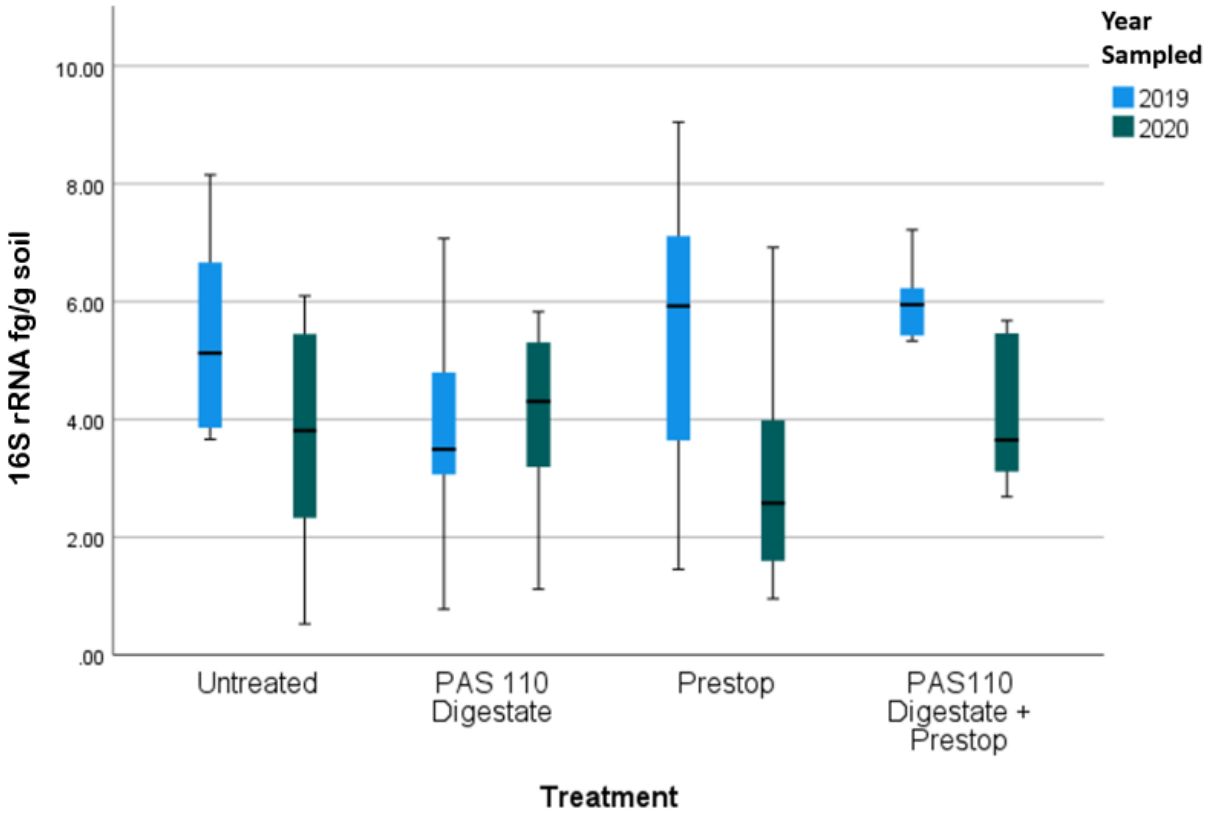

Figure 68. Quantification of total bacterial 16S rRNA DNA in soil from the Raspberry field site with or without amendment with anaerobic digestate and/or application of Prestop biofungicide. Data is presented as interquartile range and median (n=24).

Quantification of 18S rRNA DNA in response to treatment

The qPCR data quantifying 18S rRNA fungal DNA between time points failed tests of normality, as assessed by Shapiro-Wilk's test ($p > 0.05$), data was then assessed using Kruskal-Wallis H test and was not suitable for transformation. There was a significant difference between the year 1 and year 2 time points ($p=0.03$). 18S rRNA DNA decreased between the 2 time points.

Within each sampling time point, the data passed tests of normality, as assessed by Shapiro-Wilk's test ($p > 0.05$). There was no effect of soil treatment on 18S rRNA DNA at either sampling time (Figure 69).

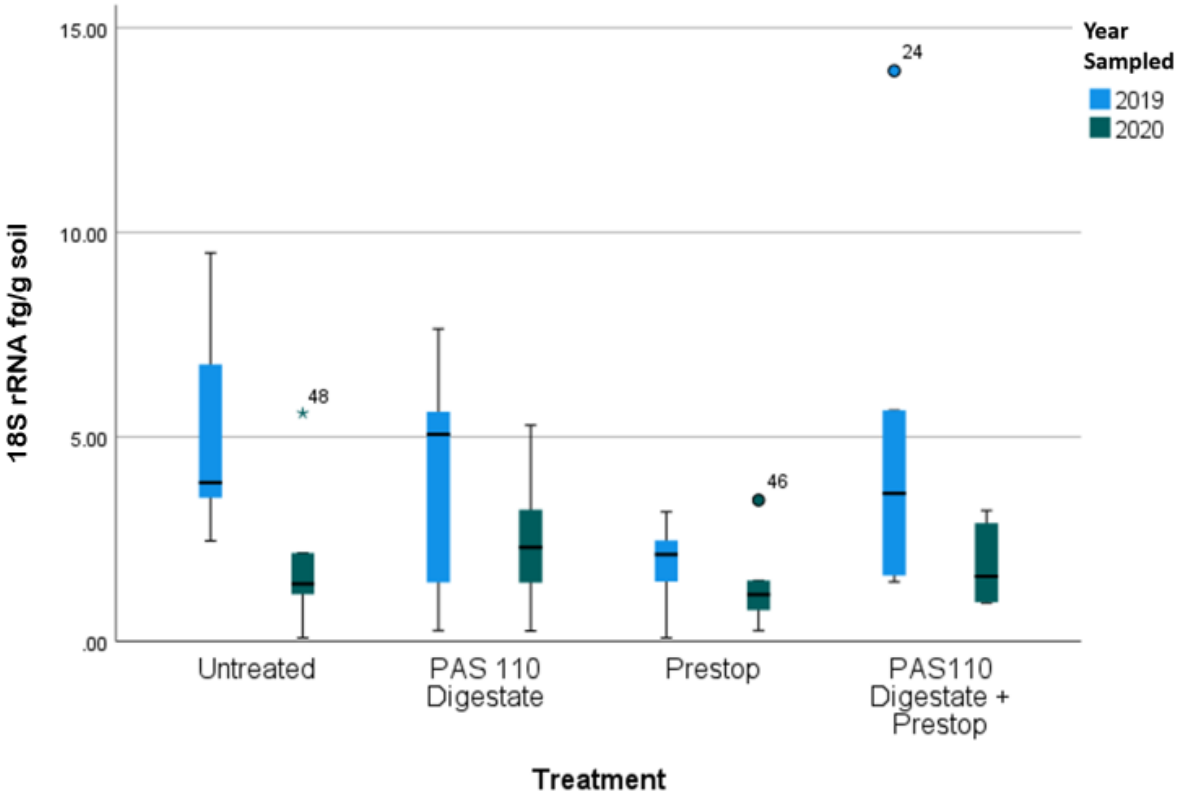

Figure 69. Quantification of total fungal 18S rRNA DNA in soil from the Raspberry field site with or without amendment with anaerobic digestate and/or application of Prestop biofungicide. Data is presented as interquartile range and median (n=24).

Assessment of Shannon diversity index in the 16S rRNA region in response to treatment

Upon examination of the Shannon diversity index within the 16S region across different time points, the dataset demonstrated normal distribution, as evidenced by the non-significant results obtained from the Shapiro-Wilk's test ($p > 0.05$). Therefore, a factorial ANOVA was conducted revealing a statistically significant difference between the two time points ($p = 0.005$). The Shannon diversity index exhibited an increase between the years. There was no significant interaction between treatment and time ($p=0.464$). There was no significant effect of treatment on Shannon diversity index ($p=0.245$) (Figure 70).

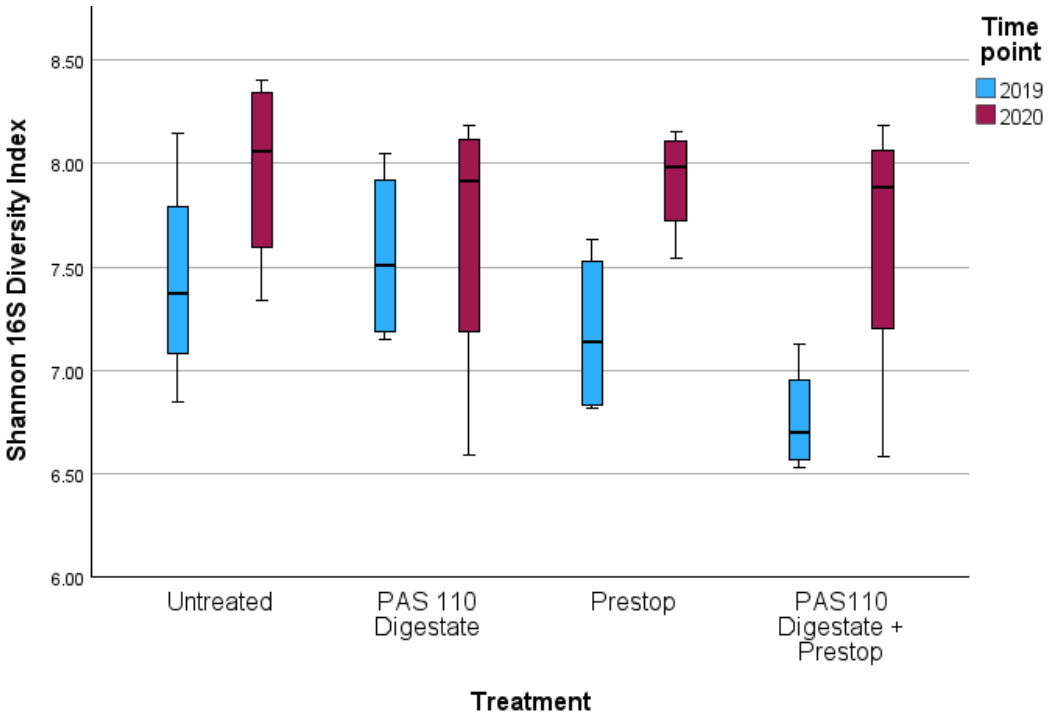

Figure 70. Shannon 16S Diversity Index in soil from the Raspberry field site with or without amendment with anaerobic digestate and/or application of Prestop biofungicide. Data is presented as interquartile range and median (n=24). Note: The y-axis does not start at zero to provide a clearer view of the variation between data points.

Assessment of Shannon diversity index in the ITS rRNA region in response to treatment

Upon examination of the Shannon diversity index within the fungal ITS region across different time points, the dataset demonstrated normal distribution, as evidenced by the non-significant results obtained from the Shapiro-Wilk's test ($p > 0.05$). Therefore, a factorial ANOVA was conducted revealing a statistically significant difference between the two time points ($p = 0.003$). The Shannon diversity index exhibited an increase between the years. There was no significant interaction between treatment and time ($p=0.091$). There was no significant effect of treatment on Shannon diversity index for fungal ITS ($p=0.784$) (Figure 71).

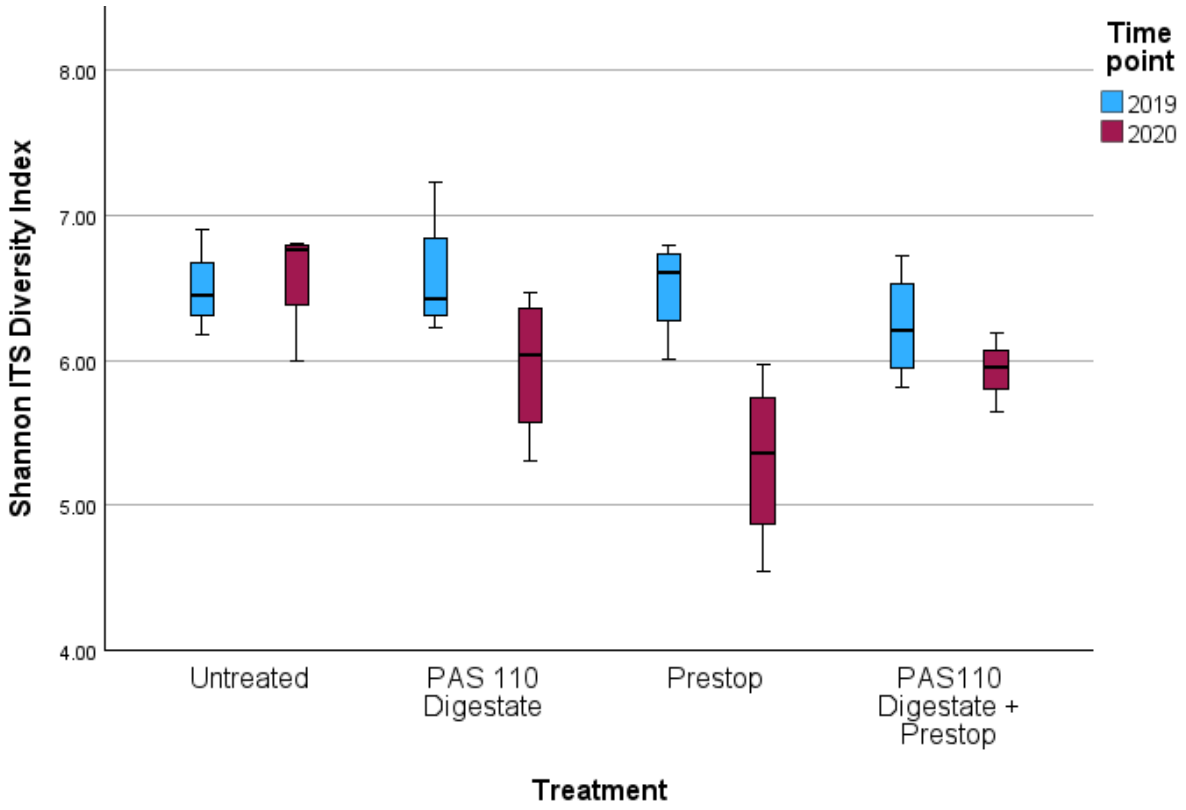

Figure 71. Shannon ITS Diversity Index in soil from the Raspberry field site with or without amendment with anaerobic digestate and/or application of Prestop biofungicide. Data is presented as interquartile range and median (n=24). Note: The y-axis does not start at zero to provide a clearer view of the variation between data points.

Assessment of Pielou evenness index in the 16S rRNA region in response to treatment

Pielou evenness index for the bacterial 16S region between the two years passed tests of normality, as assessed by Shapiro-Wilk's test ($p > 0.05$). Subsequent factorial ANOVA analysis revealed no statistically significant difference between the two years ($p = 0.659$). There was no significant interaction between treatment and time ($p=0.645$). The results indicated the absence of any significant effects between treatments ($p = 0.555$) (Figure 72).

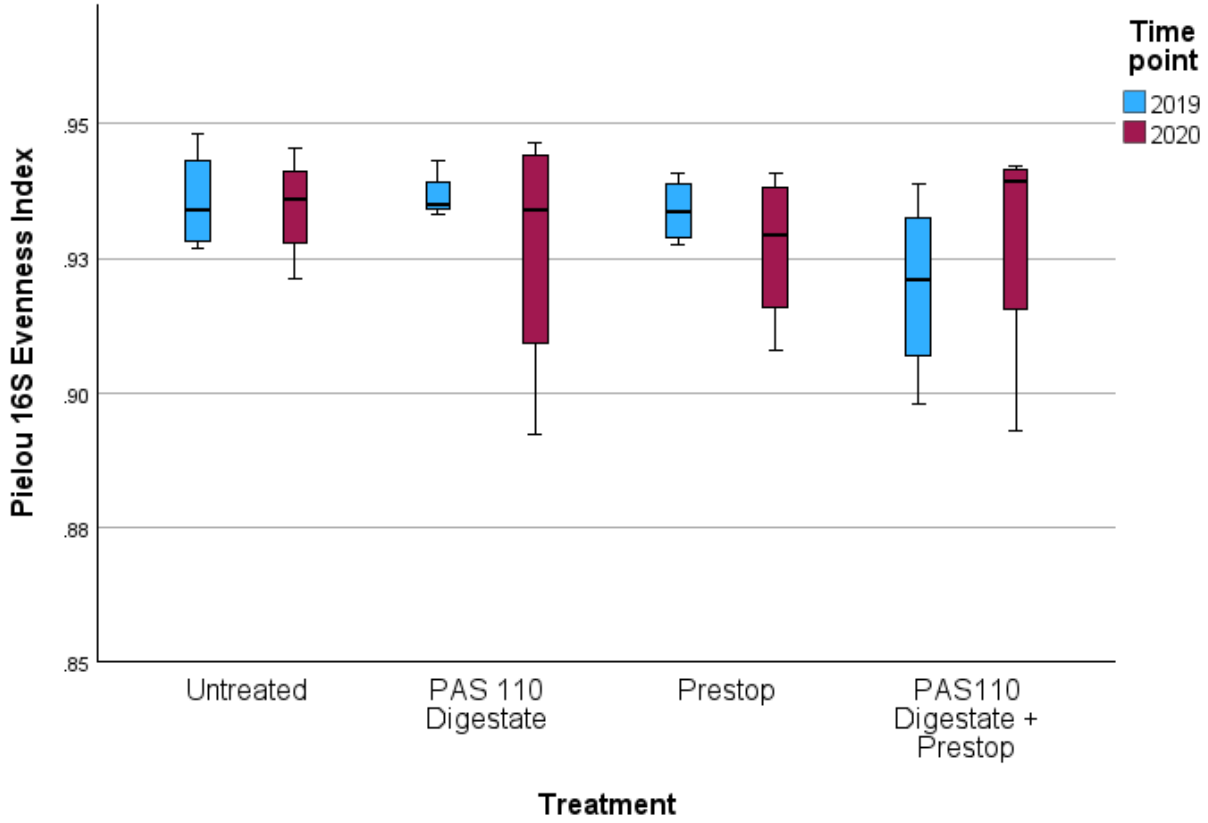

Figure 72. Pielou 16S Evenness Index in soil from the Raspberry field site with or without amendment with anaerobic digestate and/or application of Prestop biofungicide. Data is presented as interquartile range and median (n=24). Note: The y-axis does not start at zero to provide a clearer view of the variation between data points.

Assessment of Pielou evenness index in the ITS rRNA region in response to treatment

Pielou evenness index for the fungal ITS region between the two years passed tests of normality, as assessed by Shapiro-Wilk's test ($p > 0.05$). Subsequent factorial ANOVA analysis revealed no statistically significant difference between the two years ($p = 0.401$). There was no significant interaction between treatment and time ($p=0.055$). The results indicated the absence of any significant effects between treatments ($p = 0.144$) (Figure 73).

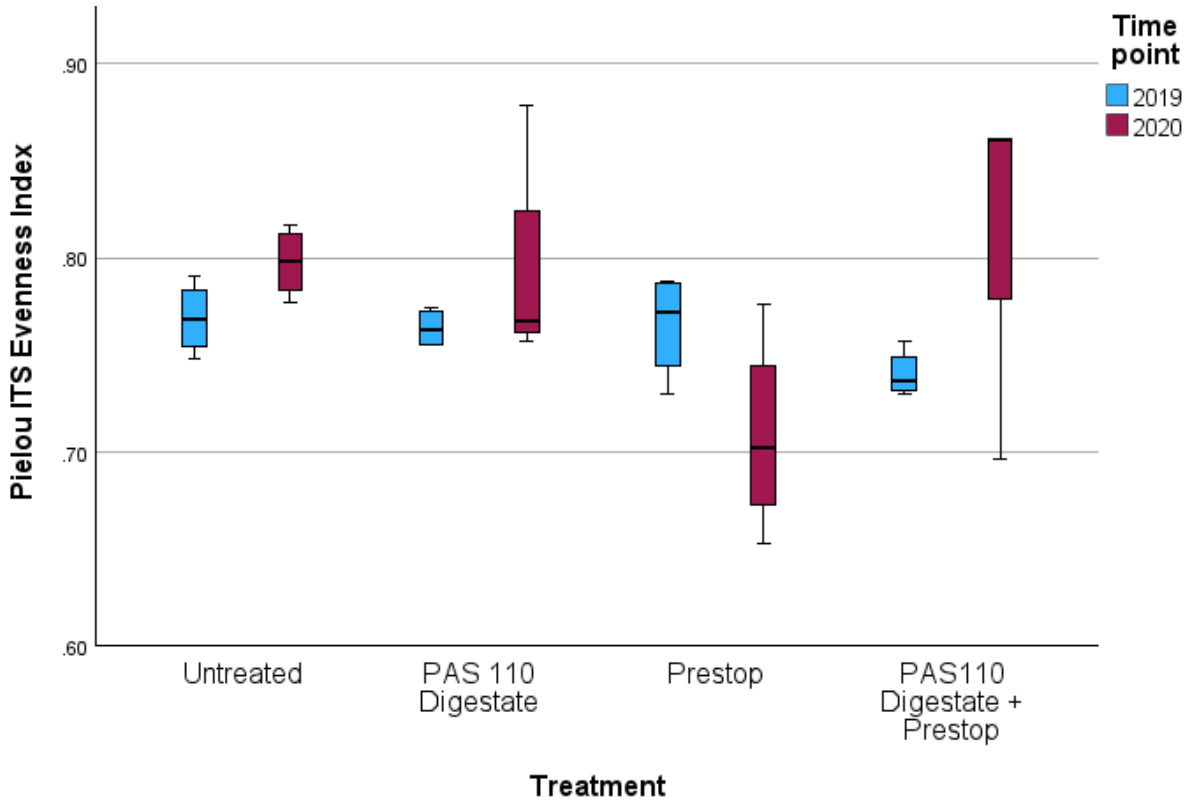

Figure 73. Pielou ITS Evenness Index in soil from the Raspberry field site with or without amendment with anaerobic digestate and/or application of Prestop biofungicide. Data is presented as interquartile range and median (n=24). Note: The y-axis does not start at zero to provide a clearer view of the variation between data points.

Assessment of Faiths phylogenetic diversity in the 16S rRNA region in response to treatment

Faith's phylogenetic diversity in the bacterial 16S region between the two years passed tests of normality, as assessed by Shapiro-Wilk's test ($p > 0.05$). Subsequent factorial ANOVA analysis unveiled a statistically significant difference between the two years ($p = 0.002$). Specifically, Faith's phylogenetic diversity in the 16S region exhibited an increase between the time intervals (Mean 2019: 24.6, 2020:31.9). There was no significant interaction between treatment and time ($p=0.406$). The results indicated the absence of any significant effects between treatments ($p = 0.321$) (Figure 74).

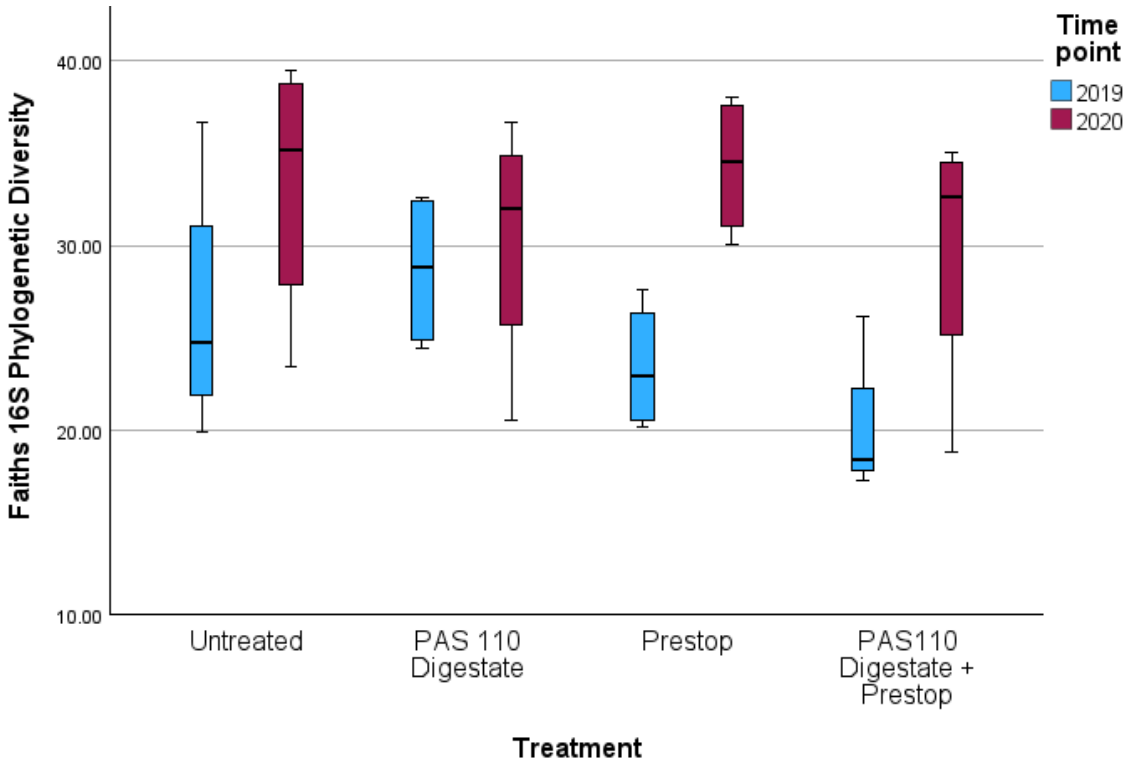

Figure 74. Faiths 16S Phylogenetic Diversity Index in soil from the Raspberry field site with or without amendment with anaerobic digestate and/or application of Prestop biofungicide. Data is presented as interquartile range and median (n=24). Note: The y-axis does not start at zero to provide a clearer view of the variation between data points.

Assessment of Faiths phylogenetic diversity in the ITS rRNA region in response to treatment

Faith's phylogenetic diversity in the fungal ITS region between the two years passed tests of normality, as assessed by Shapiro-Wilk's test ($p > 0.05$). Subsequent factorial ANOVA analysis unveiled a statistically significant difference between the two years ($p = 0.014$). Specifically, Faith's phylogenetic diversity in the ITS region exhibited a decrease between the time intervals (Mean 2019: 113.9, 2020:75.9). There was no significant interaction between treatment and time ($p=0.788$). The results indicated the absence of any significant effects between treatments ($p = 0.650$) (Figure 75).

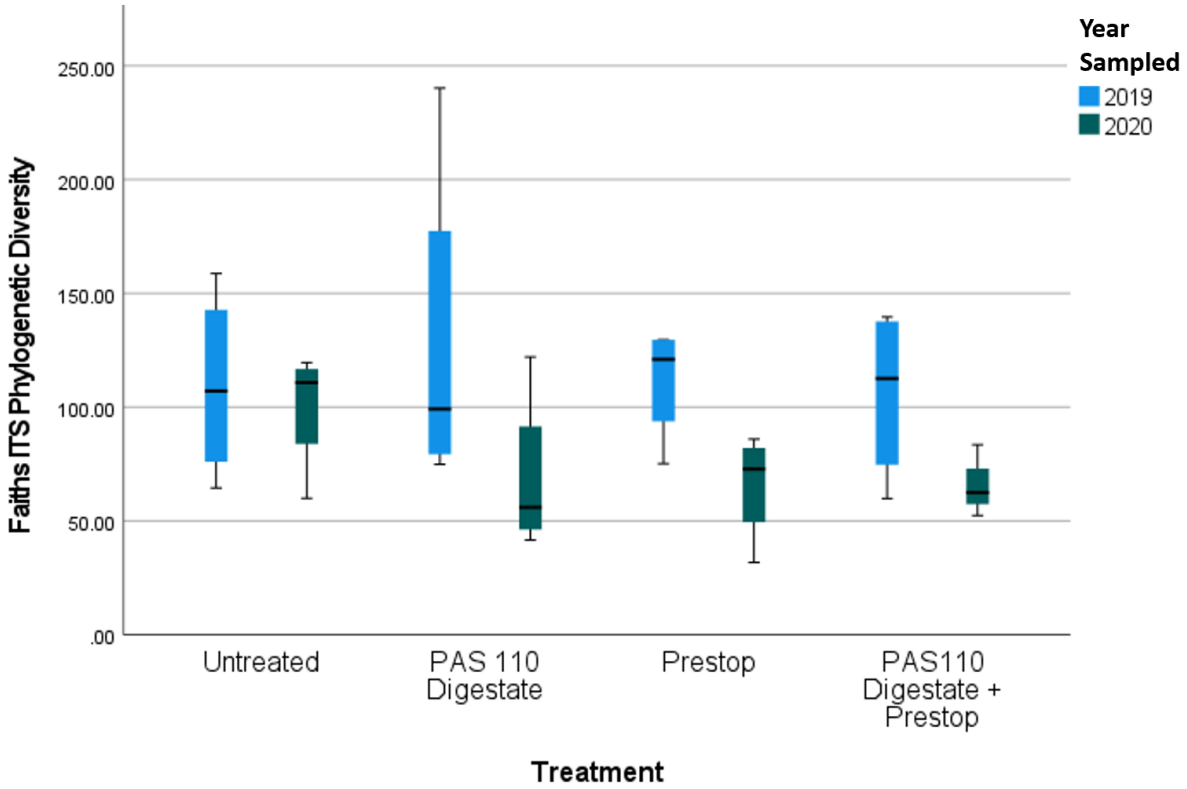

Figure 75. Faiths ITS Phylogenetic Diversity Index in soil from the Raspberry field site with or without amendment with anaerobic digestate and/or application of Prestop biofungicide. Data is presented as interquartile range and median (n=24).

Relationship between quantified 16S rRNA DNA and community indices established through metabarcoding sequencing

No correlation was observed as assessed by Pearsons's correlation (p=0.056-0.429) between the level of bacterial 16S rRNA detected by qPCR and the bacterial diversity measured according to Shannon, Faith or Pielou diversity indices.

Relationship between quantified 18S rRNA DNA and community indices established through metabarcoding sequencing

No correlation was observed as assessed by Pearsons's correlation (p=0.284-0.958) between the level of fungal ITS rRNA detected by qPCR and the fungal diversity measured according to Shannon, Faith or Pielou diversity indices.

Onion Field Trial

From the samples collected at the Onion field trial 1,019,879 reads were observed creating 3,806 ASV's for the 16S rRNA DNA region and 2,394,393 reads and 3,610 ASV's for the ITS rRNA DNA region.

Bacterial 16S rRNA metabarcoding

At the onion field trial, an analysis of bacterial phyla revealed *Proteobacteria*, succeeded by *Acidobacteria* and *Bacteroidota*, as the most prevalent groups (Figure 76). Among the 33 identified bacterial phyla, significant year-to-year changes were observed in the relative frequencies of 18 phyla (assessed via ANOVA, p=0.001-0.044), including *Acidobacteria, Actinobacteriota, Chloroflexi, Deinnococcota, Desulfobacterota, Elusimicrobiota, Fibrobacterota, Firmicutes, Gemmatimonadota, Latescibacterota, Methylomirabilota, Nitrospirota, Planctomycetota, Proteobacteria, Spirochaetota, Verrucomicrobiota, Candidatus Eremiobacterota* (previously WPS-2), and WS2.

In the first year, soils treated with green compost exhibited the lowest frequency of Nitrospirota (44.23%), while the cover crop treatment had the highest number of Patescibacteria (292.17 ASVs). In the second year, FCPU426 was only detected in soils treated with the cover crop and cover crop + green compost. Other unclassified phyla (NB1-j, WS2, and SAR324) exhibited varying frequencies across treatments and years, with distinct patterns of presence and absence. As assessed using one-way ANOVA (p=0.005-0.025).

Fungal 18S rRNA metabarcoding

When looking at fungal phyla, assessed using ITS rRNA DNA, *Mucoromycota* was the most frequent fungal phylum observed in soils from the Onion Field trial, followed by *Ascomycota* and *Basidiomycota* (Figure 77). Between years, *Ascomycota* was the only phylum that significantly increased and *Cryptomycota* was detected in the first year but not in the second (p=<0.001-0.007). There was no significant effect of any soil treatment on the fungal phyla detected, as assessed using one-way ANOVA (p=0.05-0.920).

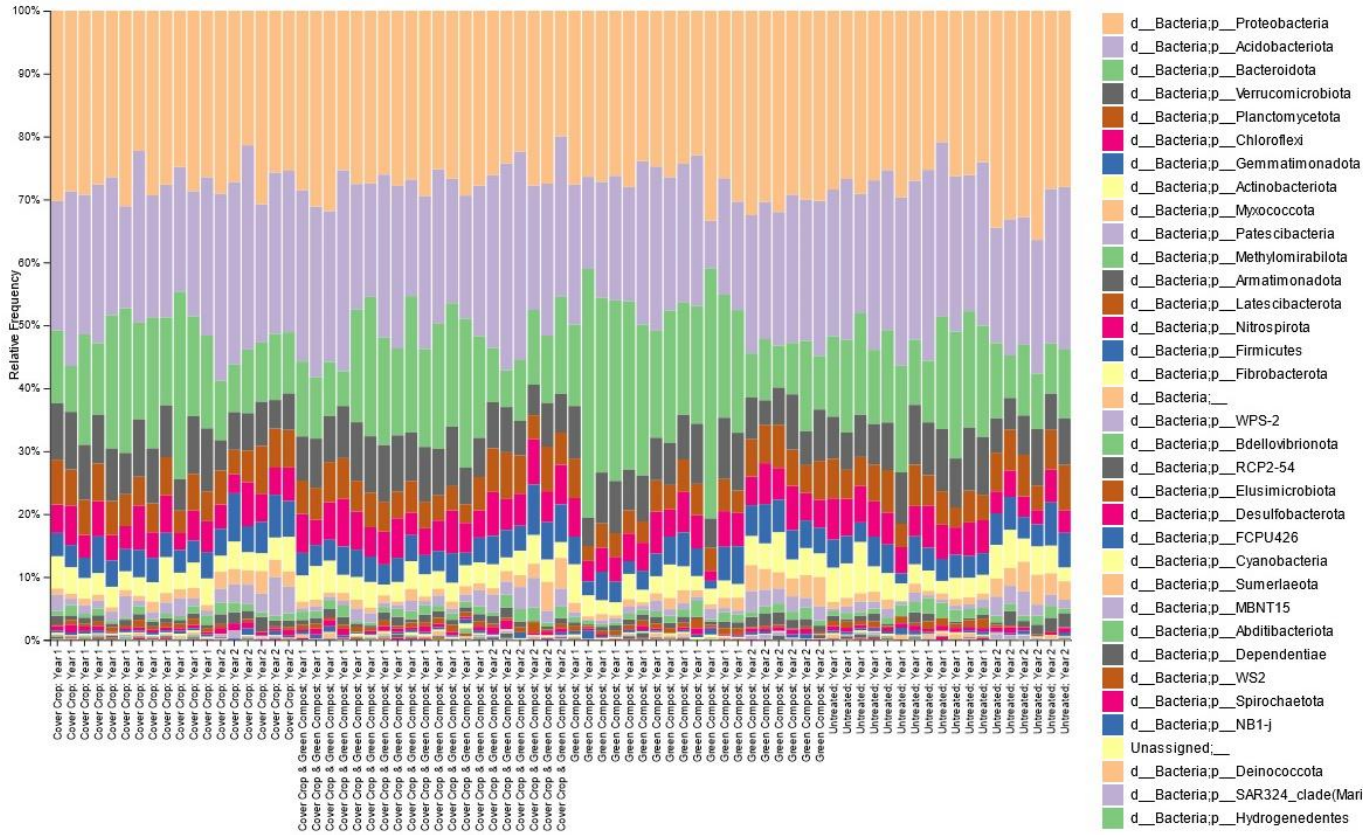

Figure 76. Relative frequency of bacteria phyla (as derived from 16S rRNA DNA) per treatment and separated by year at the Onion Field Trial. Generated using Qiime 2 viewer [accessed 26/11/2022] (Bolyen *et al*., 2019).

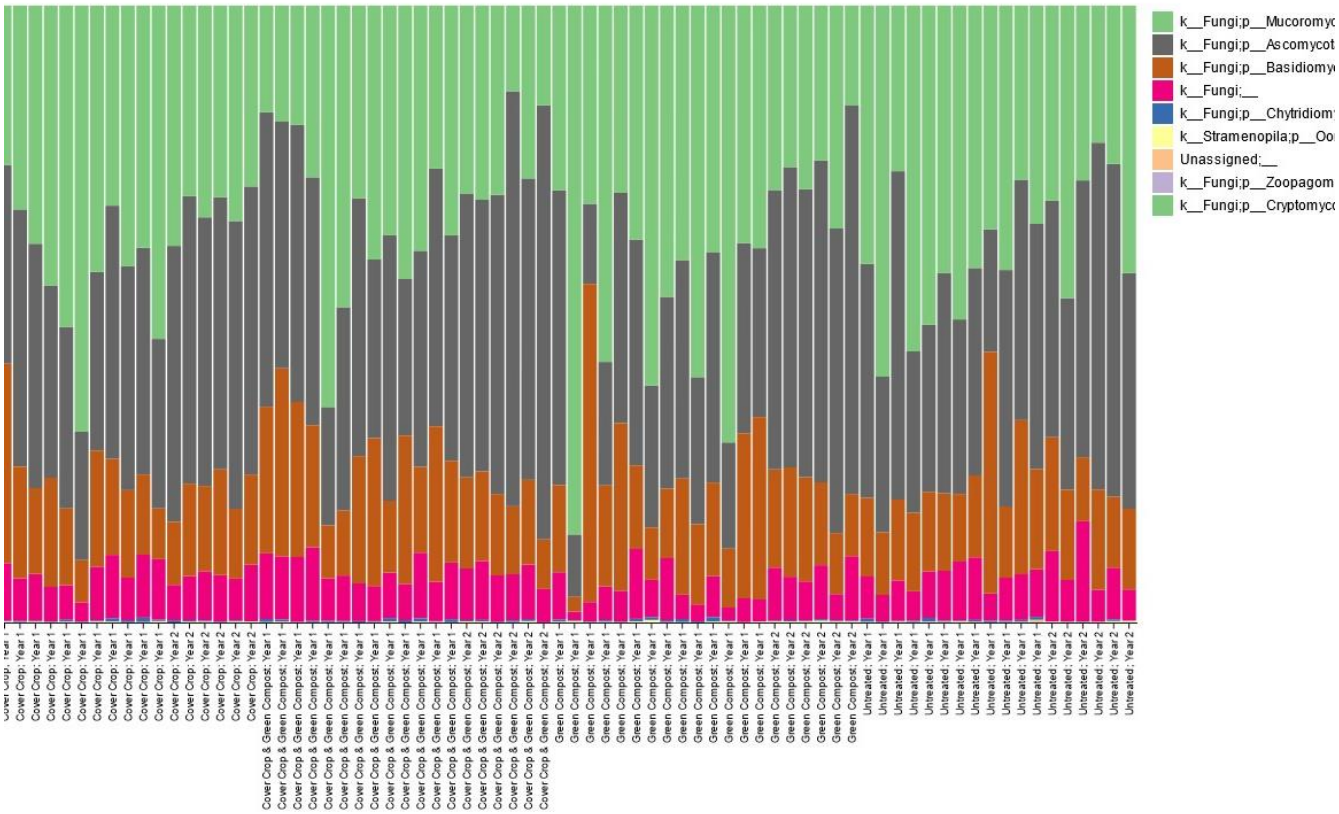

Figure 77. Relative frequency of fungal phyla (as derived from ITS rRNA DNA) per treatment and separated by year at the Onion Field Trial. Generated using Qiime 2 viewer [accessed 26/11/2022] (Bolyen *et al*., 2019).

Quantification of 16S rRNA DNA in response to treatment

Following analysis of 16S rRNA DNA using qPCR between different time points, the original data did not conform to normality, as indicated by Shapiro-Wilk's test ($p > 0.05$). To address this, a logarithmic transformation (Log10) was applied to the data. Following this transformation, a statistically significant difference was observed across the year ($p < 0.001$), signifying an increase in 16S rRNA DNA levels, as assessed by factorial ANOVA. There was no significant interaction between treatment and time ($p = 0.891$). The results indicated the absence of any significant effects between treatments ($p = 0.494$) (Figure 78).

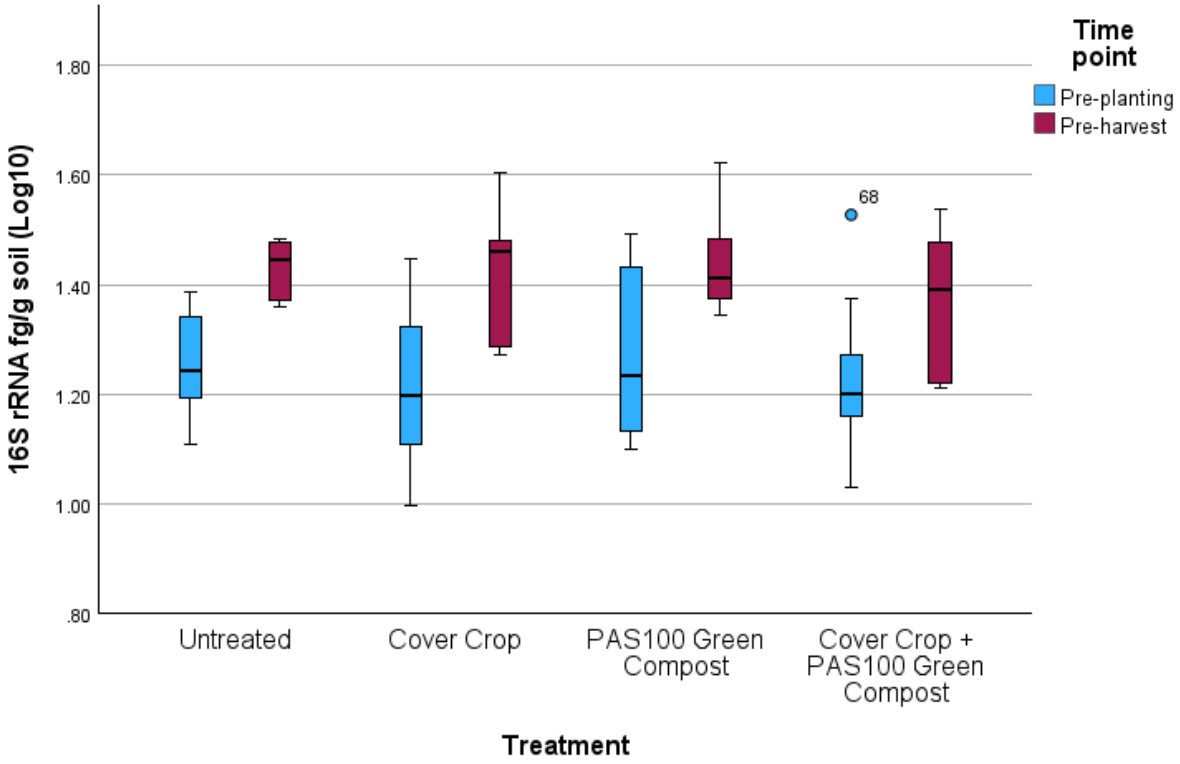

Figure 78. Quantification of total bacterial 16S rRNA DNA in soil from the Onion field site with or without amendment with Cover Crops and/or application of PAS100 Green Compost. Data is presented as interquartile range and median (n=24). Note: The y-axis does not start at zero to provide a clearer view of the variation between data points.

Quantification of 18S rRNA DNA in response to treatment

Following analysis of 18S rRNA DNA quantification using qPCR between the two time points, the data did not meet the assumptions of normality, as indicated by the Shapiro-Wilk's test ($p > 0.05$). Consequently, a Kruskal-Wallis H test was performed to assess differences between the two time points, revealing a statistically significant result ($p < 0.001$). According to medians, the 18S rRNA DNA increased by the second time point.

When analysing the effect of treatment on 18S rRNA DNA, the data did not adhere to normality and was transformed Log10. One-way analysis of variance (ANOVA) and Tukey Post-Hoc tests were performed. At pre-planting (2019), all but one combination was deemed insignificant. The green compost-treated soil exhibited significantly higher levels of 18S rRNA DNA than the cover crop treatment ($p = 0.021$).

At the pre-harvest stage (2020), fungal levels were significantly higher in green compost-treated soils compared to untreated controls ($p = 0.04$). Furthermore, the combined treatment of green compost and cover crop resulted in even higher fungal levels ($p = 0.004$) (Figure 79).

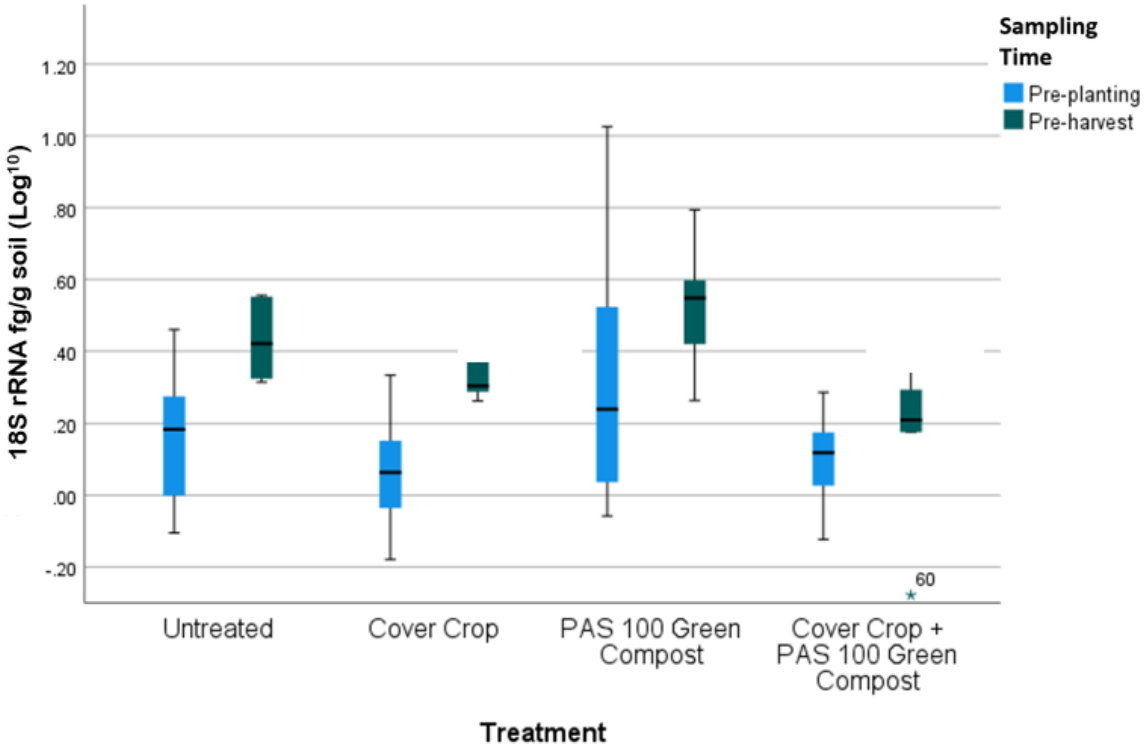

Figure 79. Quantification of total fungal 18S rRNA DNA in soil from the Onion field site with or without amendment with Cover Crops and/or application of PAS100 Green Compost. Data is presented as interquartile range and median (n=24).

<u>Assessment of Shannon diversity index in the 16S rRNA region in response to treatment</u>

Assessment of the Shannon diversity index for 16S across the year, the data did not conform to the assumption of normality, as indicated by the Shapiro-Wilk's test ($p > 0.05$). Consequently, a Kruskal-Wallis H test was performed to assess differences between the two time points, revealing a statistically significant result ($p < 0.001$). According to medians, the Shannon diversity index decreased by the second time point.

When analysing the effect of treatment on Shannon diversity index, the data did not adhere to normality assumptions. Therefore, a Kruskal-Wallis H test was performed

to assess differences between treatments across years, and no significant effect of treatment was observed (Figure 80).

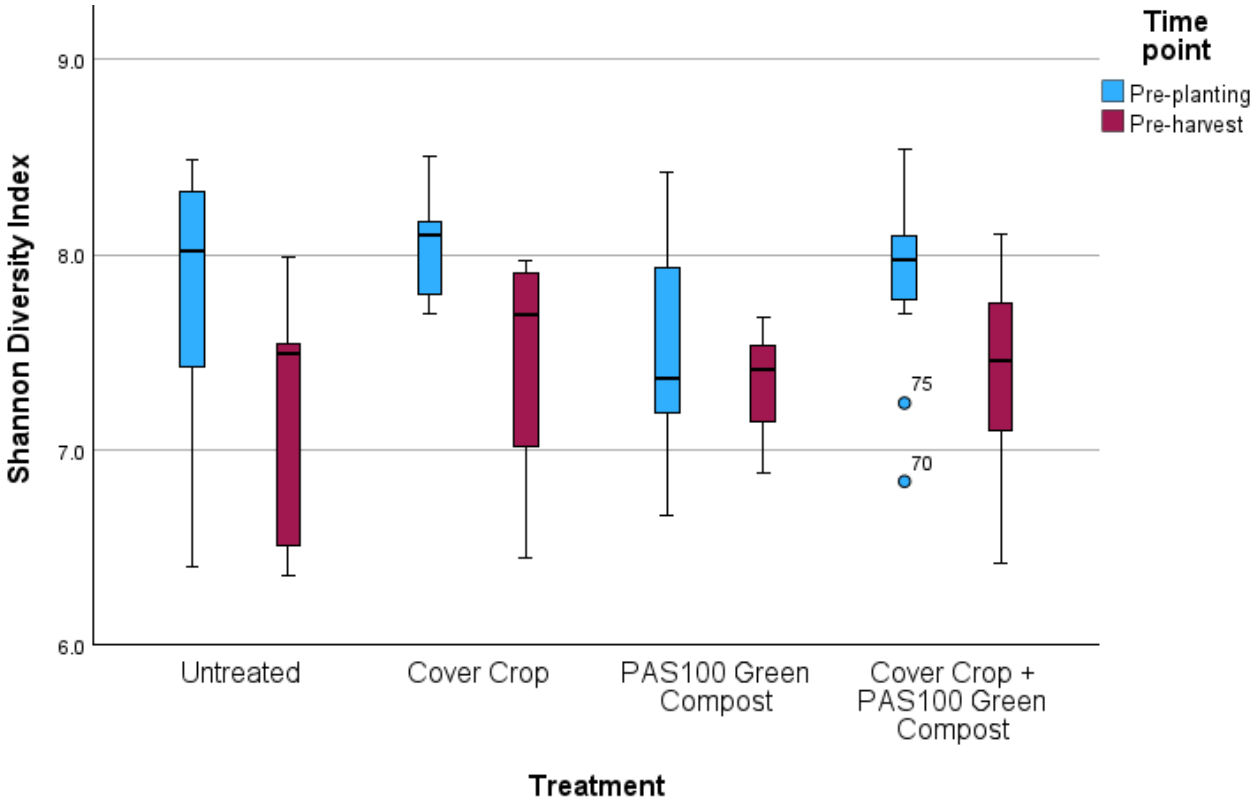

Figure 80. Shannon 16S Diversity Index from the Onion field site with or without amendment with Cover Crops and/or application of PAS100 Green Compost. Data is presented as interquartile range and median (n=24). Note: The y-axis does not start at zero to provide a clearer view of the variation between data points.

<u>Assessment of Shannon diversity index in the ITS rRNA region in response to treatment</u>

Following assessment of Shannon diversity index for ITS across the year at the Onion Field Trial, the data did not adhere to the assumption of normality, as indicated by the Shapiro-Wilk's test ($p > 0.05$). A Kruskal-Wallis H test was performed to assess differences between the two time points, and the result was found to be statistically significant ($p = 0.002$). According to medians, the Shannon diversity index increased by the second time point.

When analysing the effect of treatment on Shannon diversity index, the data did not adhere to normality assumptions. Therefore, a Kruskal-Wallis H test was performed

to assess differences between treatments, and there was no significant effect of treatment observed (Figure 81).

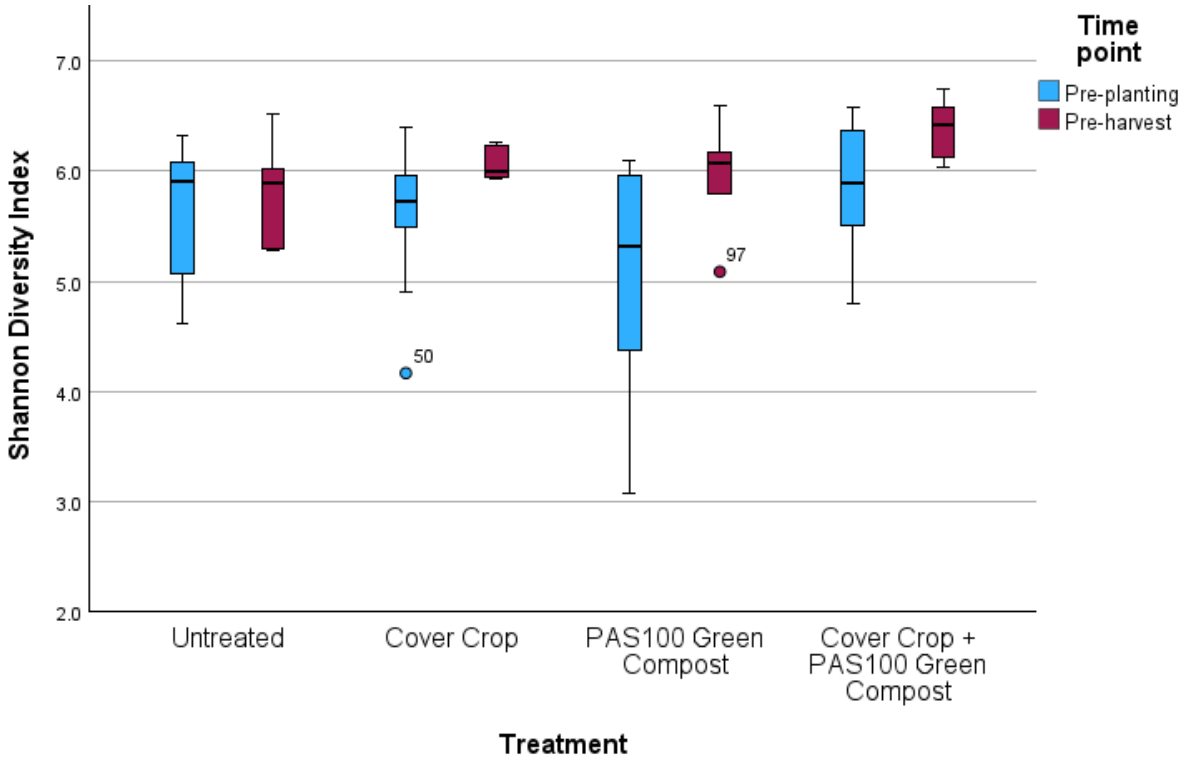

Figure 81. Shannon ITS Diversity Index from the Onion field site with or without amendment with Cover Crops and/or application of PAS100 Green Compost. Data is presented as interquartile range and median (n=24). Note: The y-axis does not start at zero to provide a clearer view of the variation between data points.

<u>Assessment of Pielou evenness index in the 16S rRNA region in response to treatment</u>

Following assessment of Pielou evenness index for 16S, the data did not meet the assumption of normality, as indicated by the Shapiro-Wilk's test ($p > 0.05$). A Kruskal-Wallis H test was performed and showed a statistically significant result ($p < 0.001$). Based on medians, the Pielou evenness index increased by 2020.

The data for individual treatments did not conform to normality as per the Shapiro-Wilk's test ($p > 0.05$), a logarithmic transformation (Log^{10}) was applied for normalization. Following this transformation, a one-way ANOVA was conducted, revealing a significant effect between treatments at the pre-planting stage (2019). Specifically, the green compost plots exhibited a lower Pielou evenness index

compared to the untreated plots (0.014) (Figure 82). The cover crop treatment plots displayed a higher index than the green compost + cover crop treatment plots (0.010) (Figure 82). No significant treatment effects were observed at the pre-harvest stage (2020).

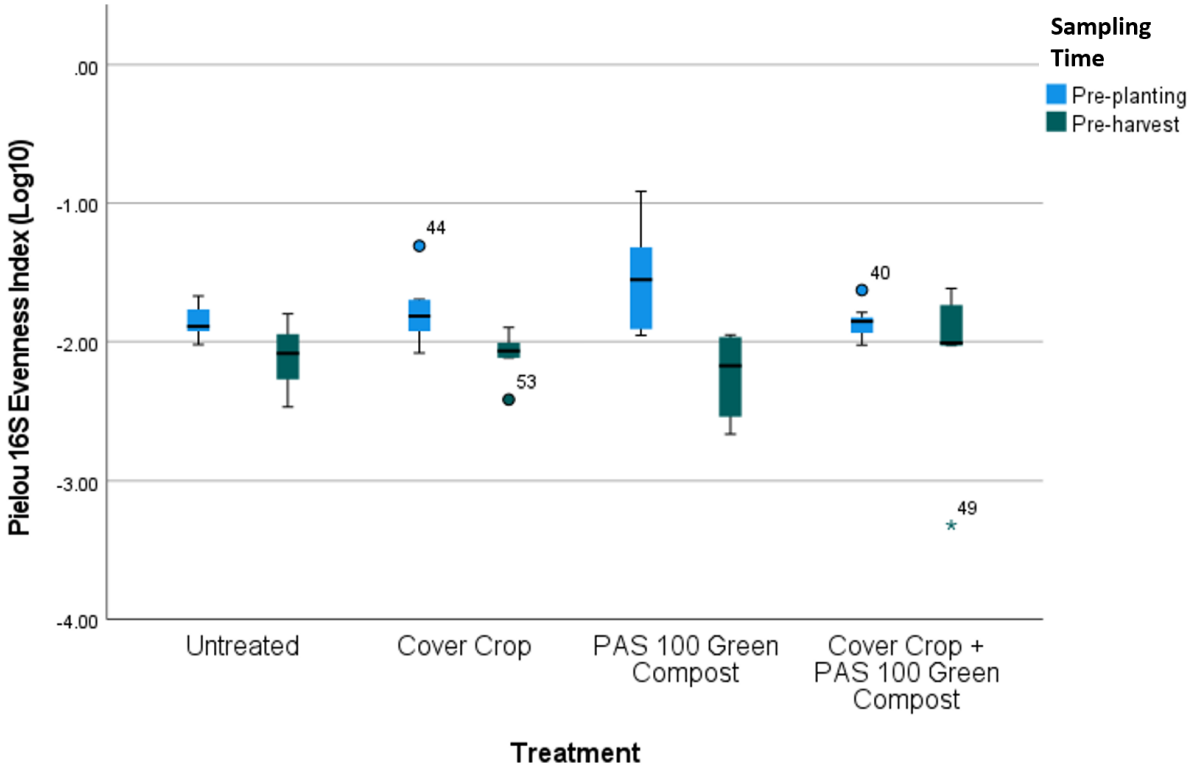

Figure 82. Pielou 16S Evenness Index from the Onion field site with or without amendment with Cover Crops and/or application of PAS100 Green Compost. Data is presented as interquartile range and median (n=24).

<u>Assessment of Pielou evenness index in the ITS rRNA region in response to treatment</u>

Following assessment of Pielou evenness index for ITS across the year, the data did not satisfy the assumption of normality, as evidenced by the Shapiro-Wilk's test ($p > 0.05$). Consequently, a Kruskal-Wallis H test was employed to assess differences between the two time points, resulting in a statistically significant outcome ($p < 0.006$). Based on the medians, the Pielou evenness index exhibited a decrease by the second time point.

When analysing the date a Shapiro-Wilk's test indicated a lack of normality ($p > 0.05$). To address this, a logarithmic transformation (Log^{10}) was applied to normalize

the data. Subsequent one-way ANOVA with Tukey Post-Hoc tests revealed no significant treatment effects, except for a significant decrease observed pre-harvest between the cover crop treatment and the cover crop + green compost treatment (p = 0.036) (Figure 83).

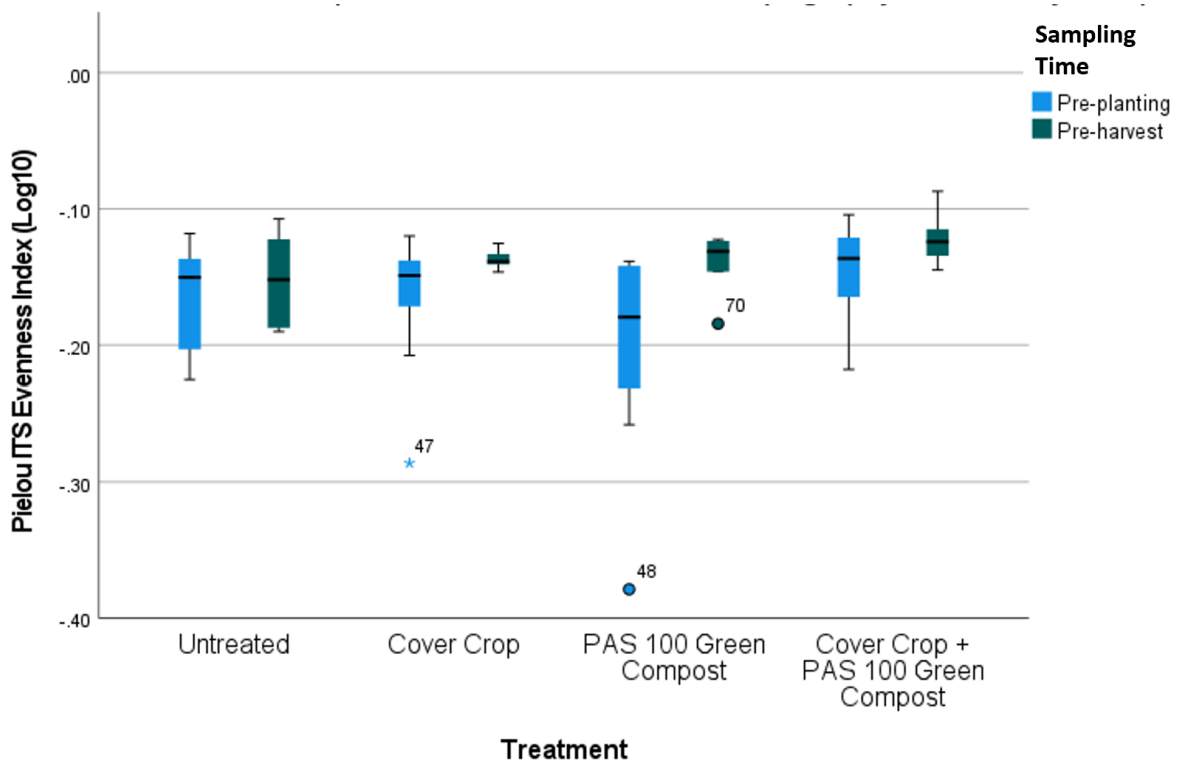

Figure 83. Pielou ITS Evenness Index from the onion field site with or without amendment with cover crops and/or application of PAS100 green compost. Data is presented as interquartile range and median (n=24).

Assessment of Faiths phylogenetic diversity in the 16S rRNA region in response to treatment

When assessing Faith's phylogenetic diversity for 16S across the year, the data exhibited normality, as confirmed by the Shapiro-Wilk's test ($p > 0.05$). Subsequent factorial ANOVA analysis unveiled a statistically significant difference between the two years ($p < 0.001$). Specifically, Faith's phylogenetic diversity in the ITS region exhibited a decrease between the time intervals (Mean 2019:26.8, 2020:20.5). There was no significant interaction between treatment and time ($p=0.889$). The results indicated the absence of any significant effects between treatments ($p = 0.356$) (Figure 84).

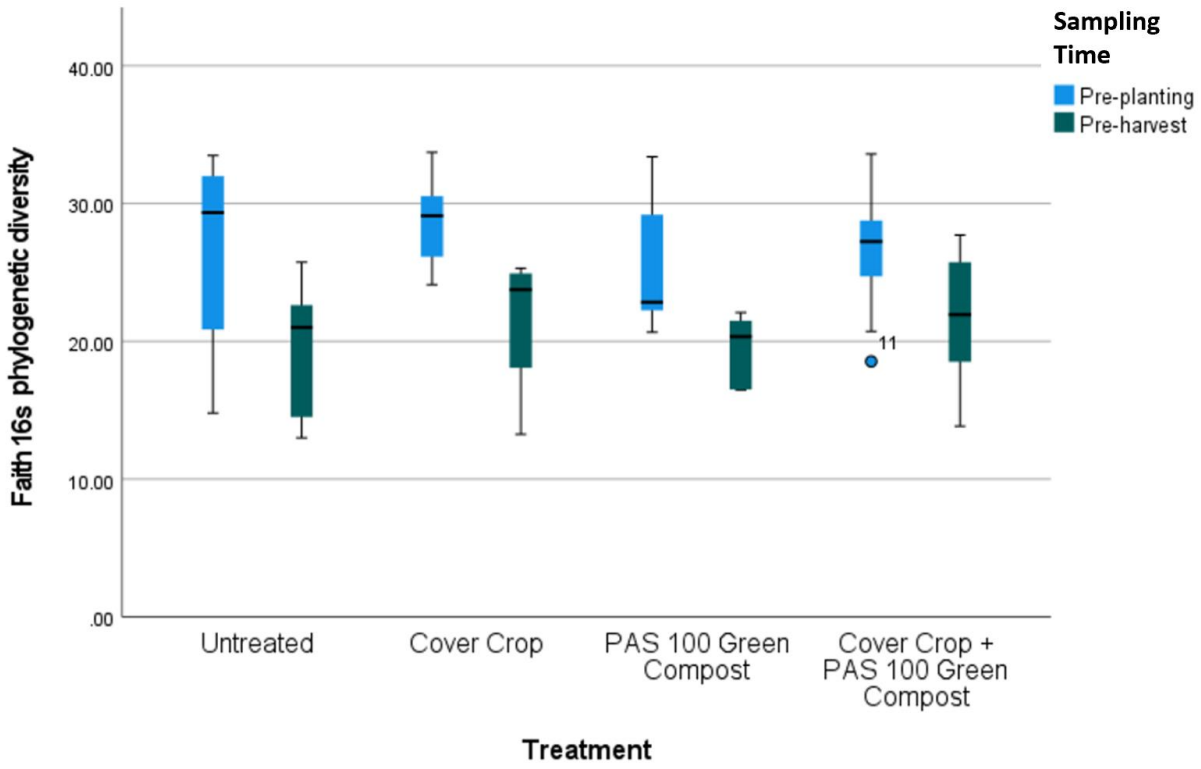

Figure 84. Faiths 16S phylogenetic diversity from the Onion field site with or without amendment with Cover Crops and/or application of PAS100 Green Compost. Data is presented as interquartile range and median (n=24).

Assessment of Faiths phylogenetic diversity in the ITS rRNA region in response to treatment

Assessment of Faith's phylogenetic diversity data across the year, the data displayed normal distribution, as confirmed by the Shapiro-Wilk's test ($p > 0.05$). Subsequent factorial ANOVA analysis unveiled a statistically significant difference between the two years ($p < 0.001$). Specifically, Faith's phylogenetic diversity in the ITS region exhibited an increase between the time intervals. There was no significant interaction between treatment and time ($p=0.547$). The results indicated the absence of any significant effects between treatments ($p = 0.716$) (Figure 85).

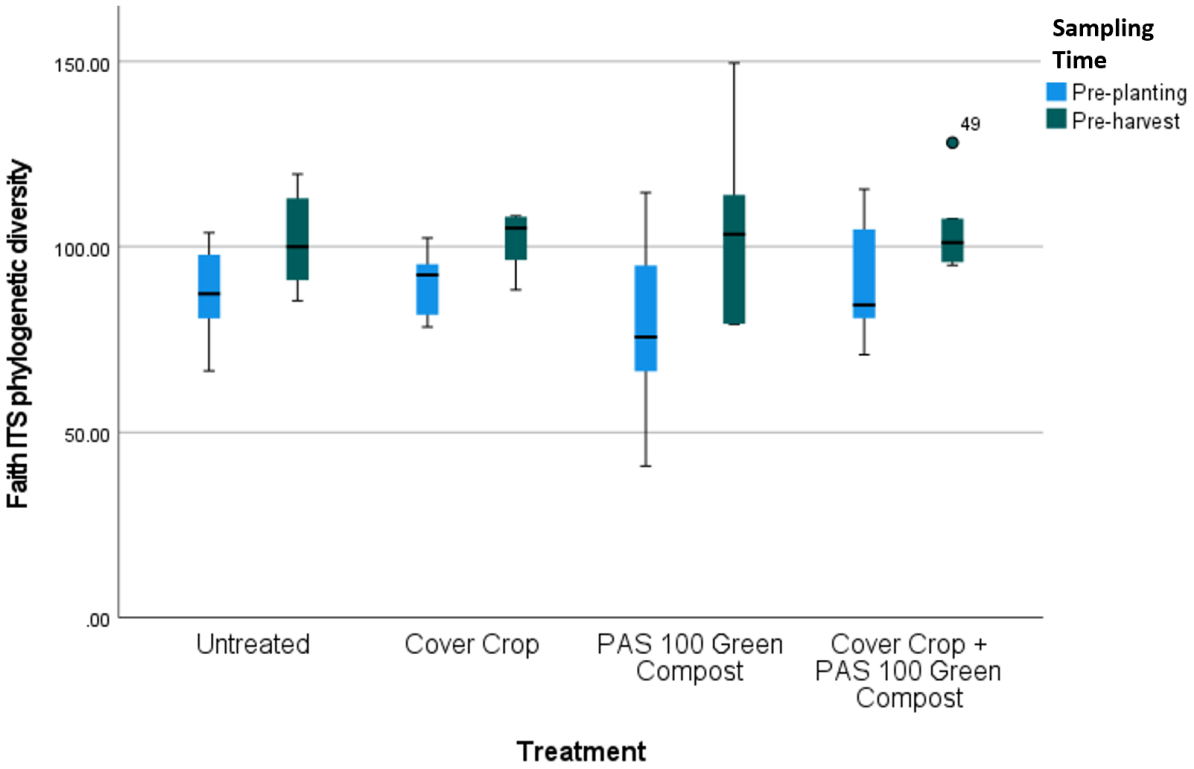

Figure 85. Faiths ITS phylogenetic diversity from the Onion field site with or without amendment with Cover Crops and/or application of PAS100 Green Compost. Data is presented as interquartile range and median (n=24).

Relationship between quantified 16S rRNA DNA and community indices established through metabarcoding sequencing

The relationship between quantified 16S rRNA DNA and the Shannon 16S diversity index was examined. Log^{10} transformation of the data was performed, and a linear relationship became evident. A negative correlation between the PCR quantified 16S rRNA DNA and the Shannon 16S diversity index (r = -0.255) (p=0.048) was confirmed using Pearson's correlation coefficient (Figure 86).

No relationship was observed between quantified 16S rRNA DNA and the Pielou 16S diversity index based upon Pearsons's correlation (p=0.521).

The relationship between quantified 16S rRNA DNA and the Faith 16S diversity index, upon Log10 transformation, the data revealed a linear relationship was apparent. A positive correlation between PCR quantified 16S rRNA DNA and the Faith 16S diversity index (r = -0.033) (p=0.006) was observed, assessed using Pearson's correlation coefficient (Figure 87).

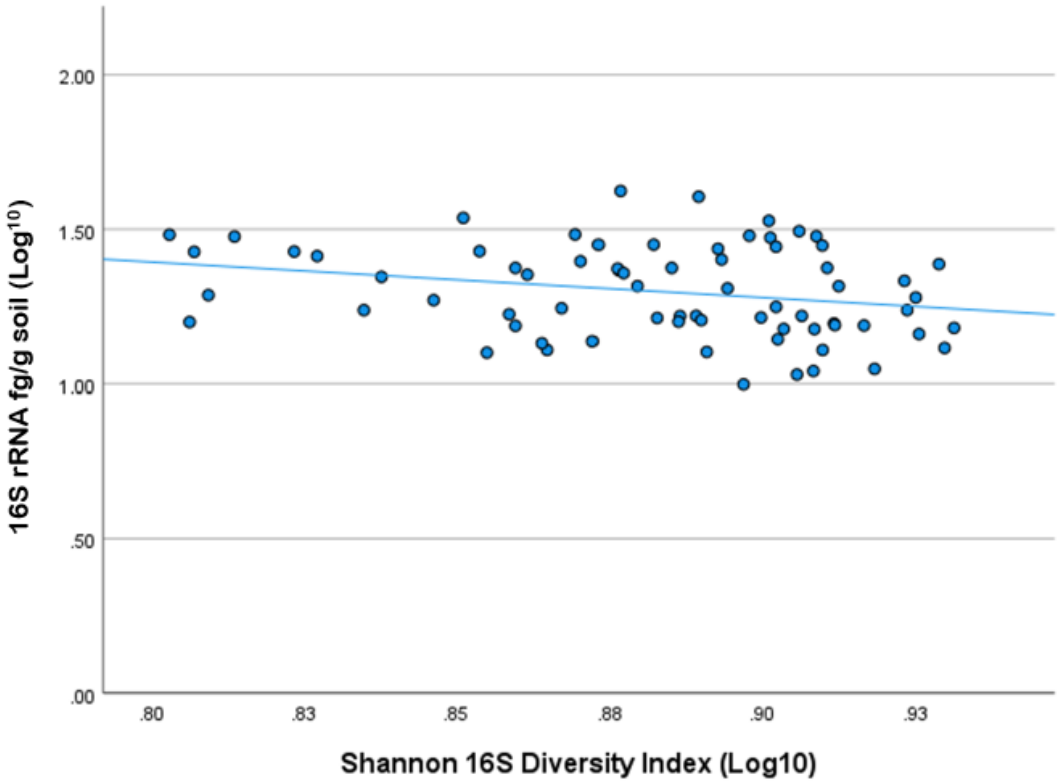

Figure 86. Scatter graph showing negative correlation between quantified 16S rRNA DNA and Shannon 16S diversity index. Data was transformed using Log10.

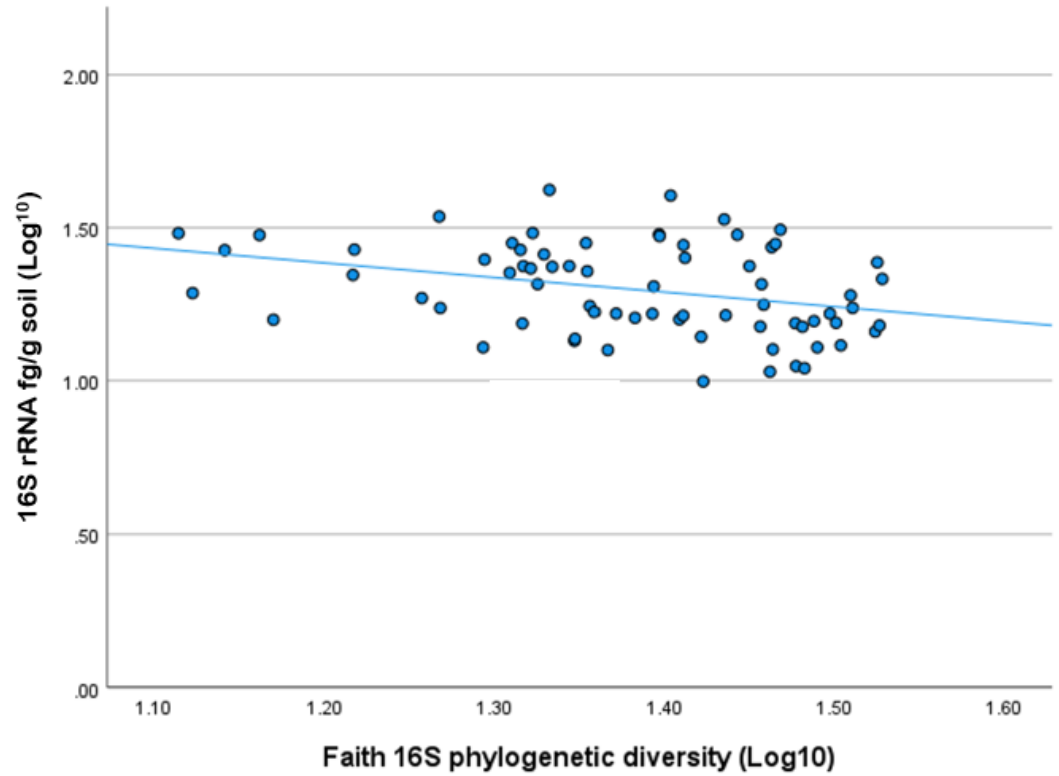

Figure 87. Scatter graph showing negative correlation between quantified 16S rRNA DNA and Faith 16S phylogenetic diversity index. Data was transformed using Log10.

Relationship between quantified 18S rRNA DNA and community indices established through metabarcoding sequencing

There was also no relationship between quantified 18S rRNA DNA and Shannon ITS diversity index, Pielou ITS diversity index or Faith ITS phylogenetic diversity index as assessed by Pearsons's correlation (p=0.062 – 0.968).

Daffodil Field Trial

From the samples collected at the Daffodil field trial 242,401 reads were observed creating 2,002 ASV's for the 16S rRNA DNA region and 889,999 reads and 1,725 ASV's for the ITS rRNA DNA region.

In the Daffodil field trial, *Acidobacteriota* emerged as the predominant bacterial phylum consistently observed across both years and all treatments (Figure 88). Proteobacteria and *Chloroflexi* followed as the subsequent dominant phyla. Between the first and second years, several phyla exhibited significant increases in their abundance. Specifically, Fibrobacterota was absent in the initial year but appeared in the subsequent year and *Firmicutes* increased 10.71%, *Latescibacterota* increased 288.95%, *Myxococcota* increased 87.62%, *Armatimonadota* increased 36.71%, *Planctomycetota* increased 331.24% and *Sumerlaeota, Zixbacteria*, and *Chloroflexi* witnessed increases of 2.6%, 15.39%, and 458.05%, respectively. Conversely, *Cyanobacteria* experienced a significant 8.68% decline. Assessed via ANOVA, p=<0.001-0.026.

Statistical analysis confirmed the normal distribution of data for each group, as determined by the Shapiro-Wilk test ($p > .05$). Additionally, Levene's test validated the homogeneity of variances across treatments. A one-way ANOVA was executed. While the majority of treatments did not show significant differences in bacterial phyla abundance, the presence of *Verrucomicrobiota* differed notably among treatments. Specifically, the microbial AMF product treatment registered the lowest frequency of *Verrucomicrobiota* at 280.38 ASV's, followed by the untreated control at 326.63 ASV's. In contrast, the green compost treatment recorded 440.38 ASV's, with farmyard manure topping the list at 443.57 ASV's. Assessed via ANOVA, p=<0.001-0.015.

When looking at fungal phyla at the daffodil field trial, assessed using ITS rRNA DNA, *Ascomycota* was the most frequent phyla, followed by *Mucoromycota* and *Basidiomycota* (Figure 89). There was no significant change observed between years or in response to any of the treatments for any of the phyla as assessed via one-way ANOVA (p=0.062-0.894).

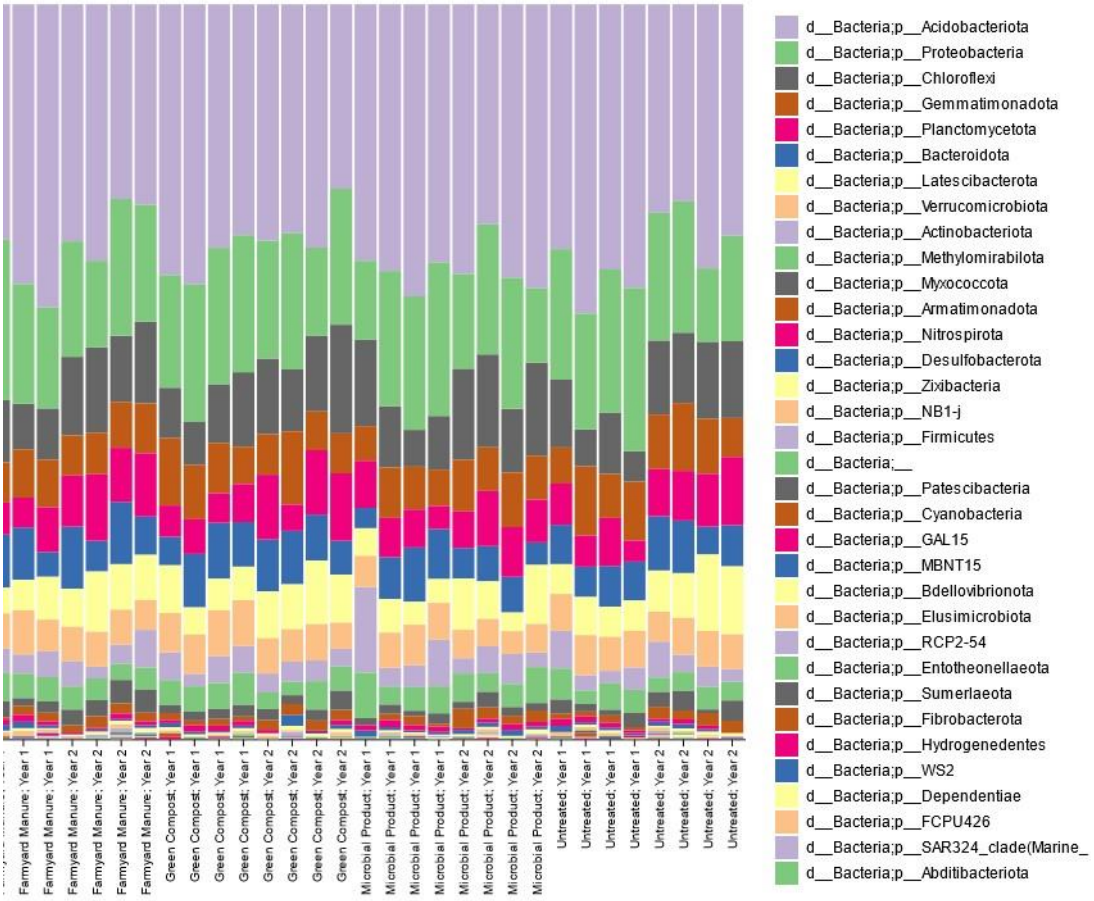

Figure 88. Relative frequency of bacteria genus (as derived from 16S rRNA DNA) per treatment and separated by year, at the daffodil field trial. Generated using Qiime 2 viewer [accessed 26/11/2022]](Bolyen *et al*., 2019).

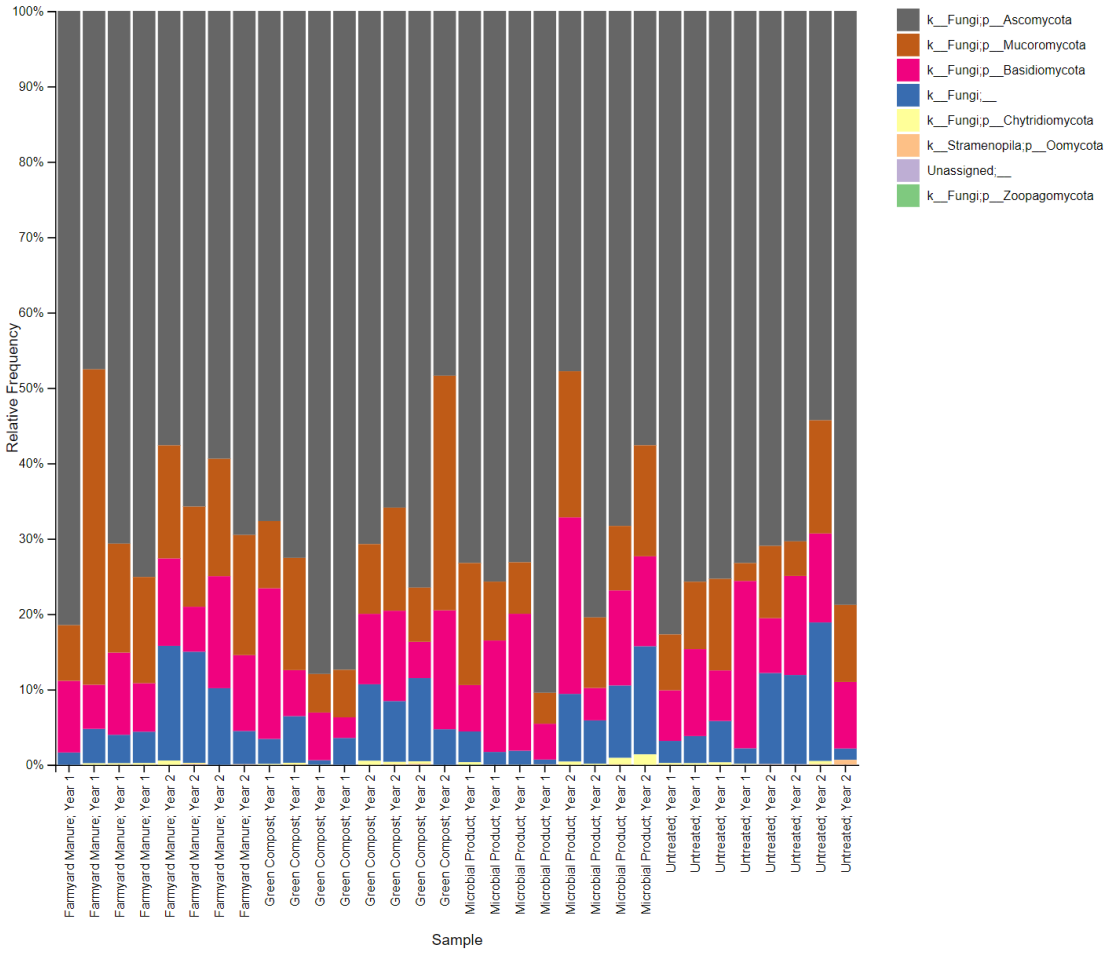

Figure 89. Relative frequency of fungal genus (as derived from ITS rRNA DNA) per treatment and separated by year, at the daffodil field trial. Generated using Qiime 2 viewer [accessed 26/11/2022] (Bolyen *et al*., 2019)

Quantification of 16S rRNA DNA in response to treatment

When evaluating 16S rRNA DNA through qPCR across different time points, the data did not exhibit normal distribution, as confirmed by the Shapiro-Wilk's test ($p > 0.05$). Consequently, the data underwent analysis using the Kruskal-Wallis H test. A significant difference was observed between the two years (Figure 90). The quantified 16S rRNA DNA displayed a decline between the first and the second year ($p = < 0.001$) (Mean 2019:5.37, 2020:1.21). Based on this significant effect, treatment was analysed by separating the time points.

Consequent analysis of the data met the criteria for normality, as evidenced by the Shapiro-Wilk's test ($p > 0.05$). The subsequent impact of treatment was evaluated

through a one-way ANOVA. No significant treatment effect was evident in either the first year (p = 0.489) or the subsequent year (p = 0.985) (Figure 90).

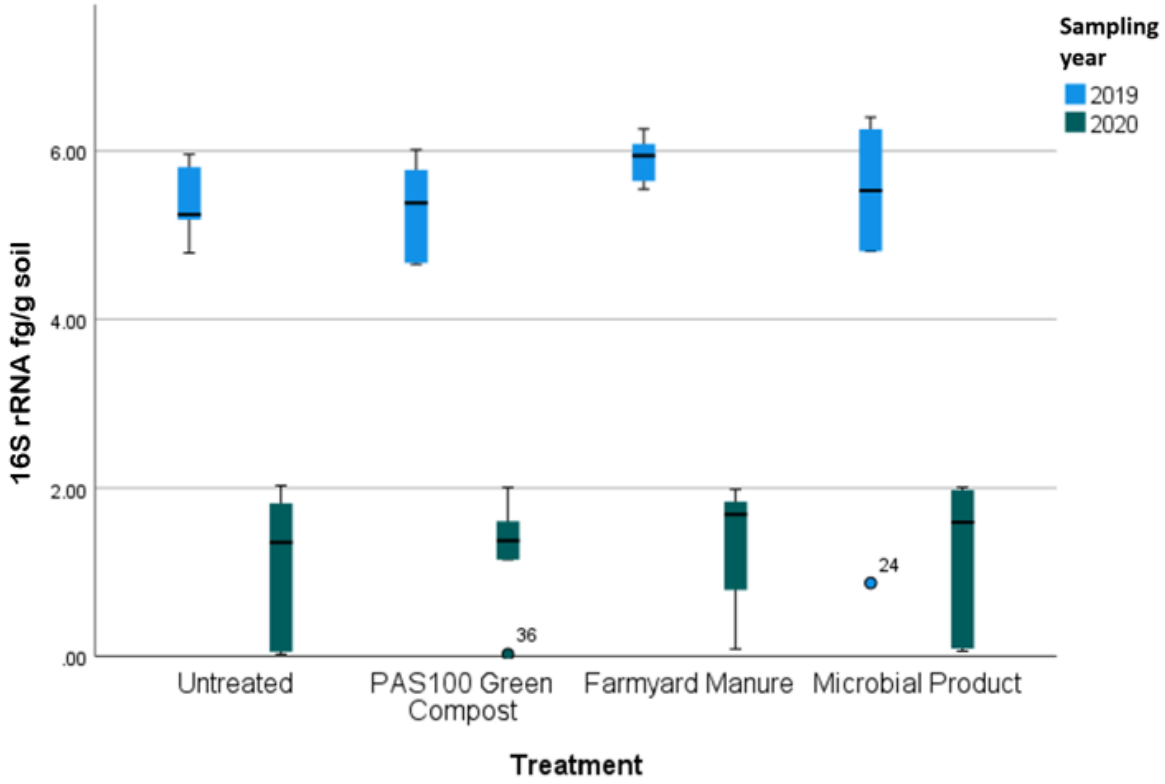

Figure 90. Quantification of total bacterial 16S rRNA DNA in soil from the Daffodil field site with or without amendment of PAS100 Green Compost or Farmyard manure or Microbial Product. Data is presented as interquartile range and median (n=24).

Quantification of 18S rRNA DNA in response to treatment

When examining 18S rRNA DNA levels via qPCR across the 2 years, data did not demonstrate a normal distribution, as indicated by the Shapiro-Wilk's test (p > 0.05). Subsequently, a Kruskal-Wallis H test was performed, revealing a significant difference between the two years. The 18S rRNA DNA levels exhibited a decrease between 2019 and 2020 (p = < 0.001) (Mean 2019:22.3, 2020:3.74). Based on this significant effect, treatment was analysed by separating the time points.

Consequent analysis of the data met the normality criteria, as confirmed by the Shapiro-Wilk's test (p > 0.05). Analysis of treatment effects was performed using a one-way ANOVA. In both the initial year (p = 0.542) and the following year (p = 0.992), none of the treatments caused a significant effect (Figure 91).

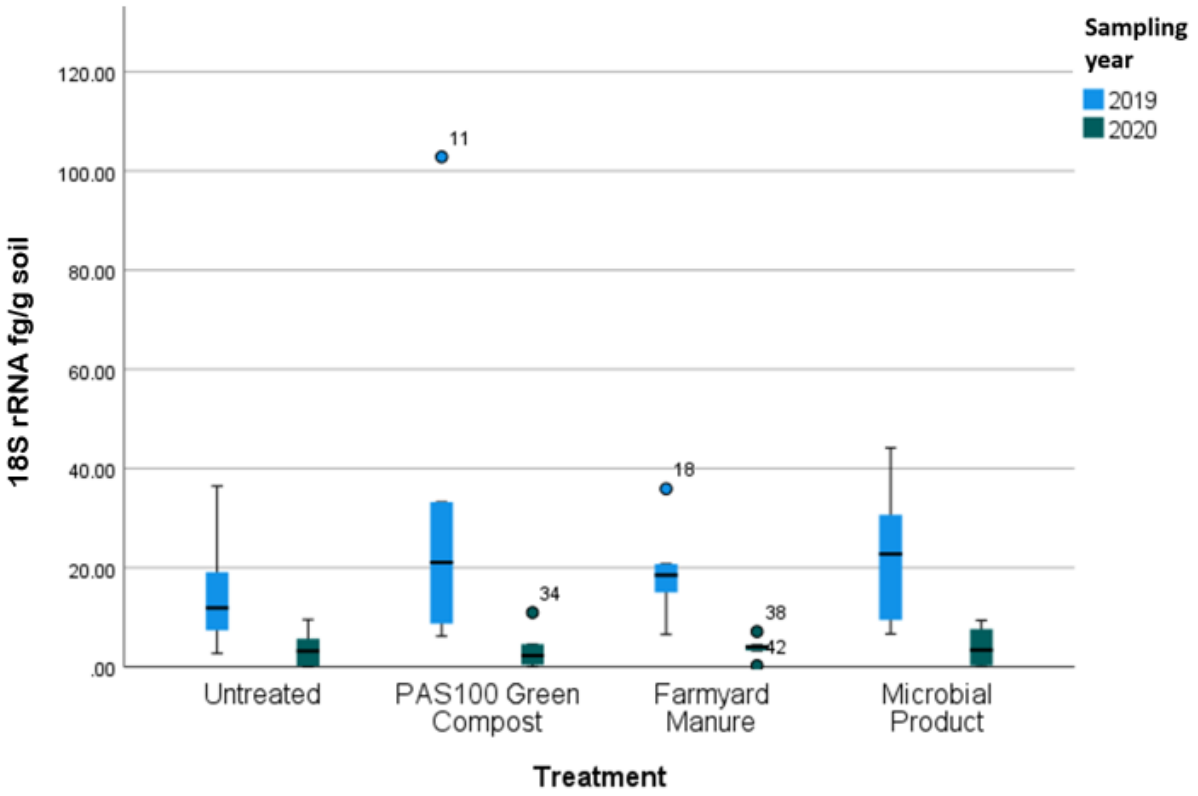

Figure 91. Quantification of total fungal 18S rRNA DNA in soil from the Daffodil field site with or without amendment of PAS100 Green Compost or Farmyard manure or Microbial Product. Data is presented as interquartile range and median (n=24).

Assessment of Shannon diversity index in the 16S rRNA region in response to treatment

When evaluating the Shannon diversity index within the 16S rRNA region across the 2 years, the data did not meet the criteria for normal distribution, as confirmed by the Shapiro-Wilk's test ($p > 0.05$). Subsequent analysis using the Kruskal-Wallis H test revealed a significant difference between the years. The Shannon diversity index increased between years ($p = 0.005$). Based on this significant effect, treatment was analysed by separating the time points.

Consequent analysis of the data met the normality criteria, as confirmed by the Shapiro-Wilk's test ($p > 0.05$). The impact of treatment was further scrutinized through a one-way ANOVA. No significant treatment effect was evident in either the initial year ($p = 0.152$) or the subsequent year ($p = 0.372$) (Figure 92).

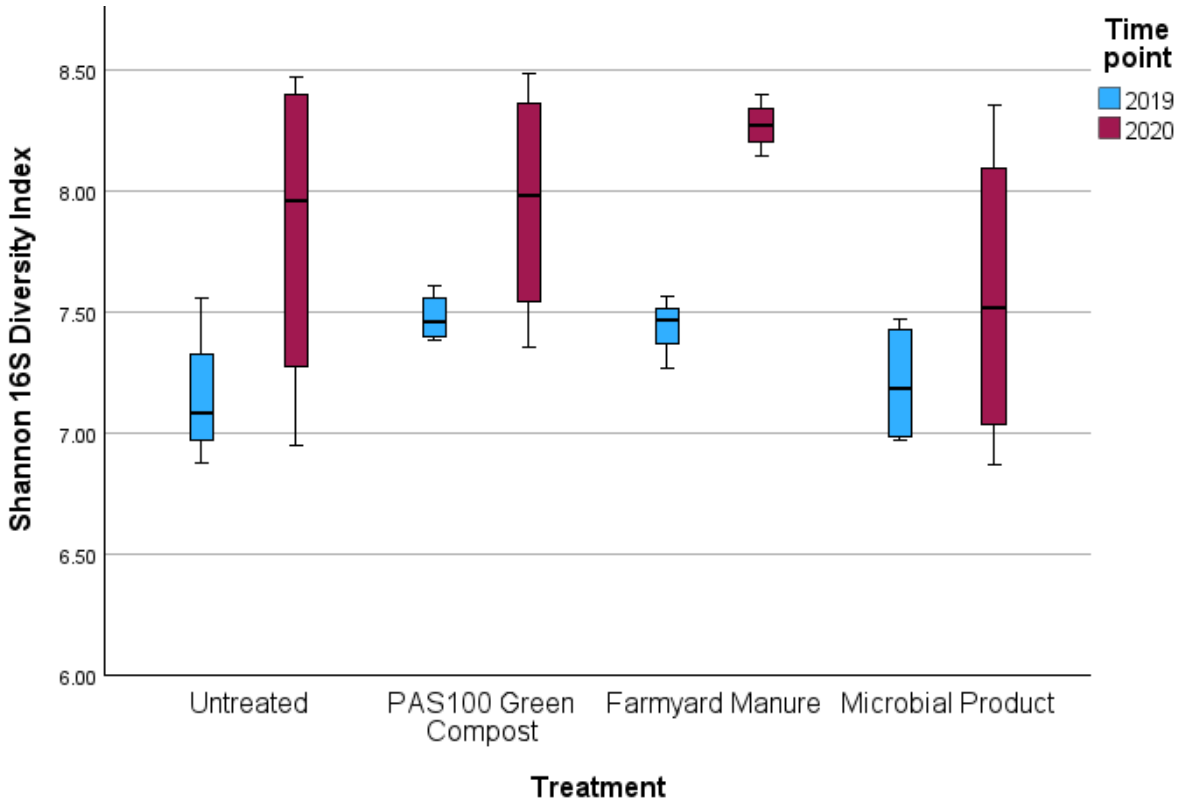

Figure 92. Shannon diversity index in the 16S region from the Daffodil field site with or without amendment of PAS100 Green Compost or Farmyard manure or Microbial Product. Data is presented as interquartile range and median (n=24). Note: The y-axis does not start at zero to provide a clearer view of the variation between data points.

Assessment of Shannon diversity index in the ITS rRNA region in response to treatment

Upon examining the Shannon diversity index within the fungal ITS region between the two years, the data did not exhibit a normal distribution, as evidenced by the Shapiro-Wilk's test ($p > 0.05$). Further analysis via the Kruskal-Wallis H test indicated no significant difference between the two time points ($p = 0.498$).

Assessment of the effect of treatment on Shannon diversity index within the fungal ITS region was assessed via one-way ANOVA. The analysis did not reveal any significant treatment effect ($p = 0.419$) (Figure 93)

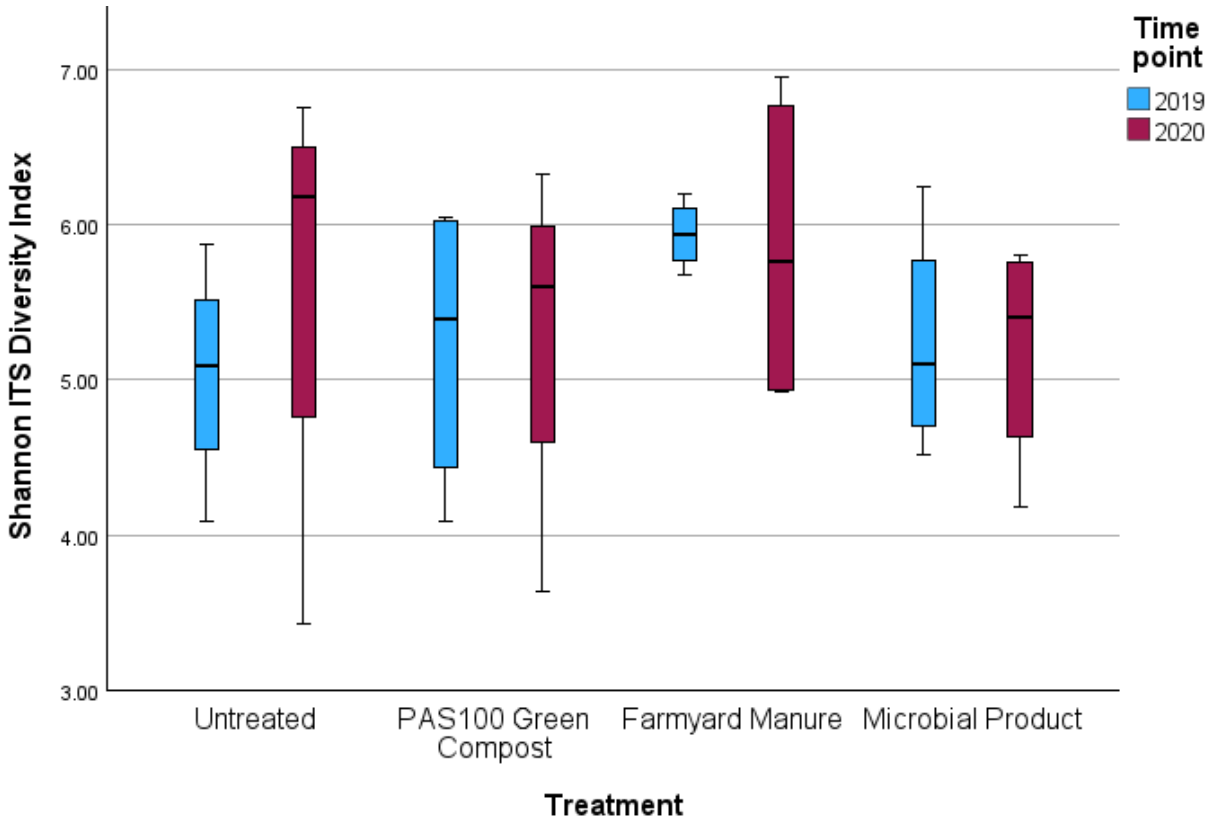

Figure 93. Shannon diversity index in the ITS region from the Daffodil field site with or without amendment of PAS100 Green Compost or Farmyard manure or Microbial Product. Data is presented as interquartile range and median (n=24). Note: The y-axis does not start at zero to provide a clearer view of the variation between data points.

<u>Assessment of Pielou evenness index in the 16S rRNA region in response to treatment</u>

When examining the Pielou evenness index within the 16S region across the 2 years, the data did not conform to a normal distribution, as confirmed by the Shapiro-Wilk's test ($p > 0.05$). Subsequent analysis using the Kruskal-Wallis H test revealed no significant ($p = 0.063$) difference between the two time points.

Assessment of the effect of treatment on Shannon diversity index within the 16S bacterial region was assessed via one-way ANOVA. The analysis did not reveal any significant treatment effect ($p = 0.939$) (Figure 94).

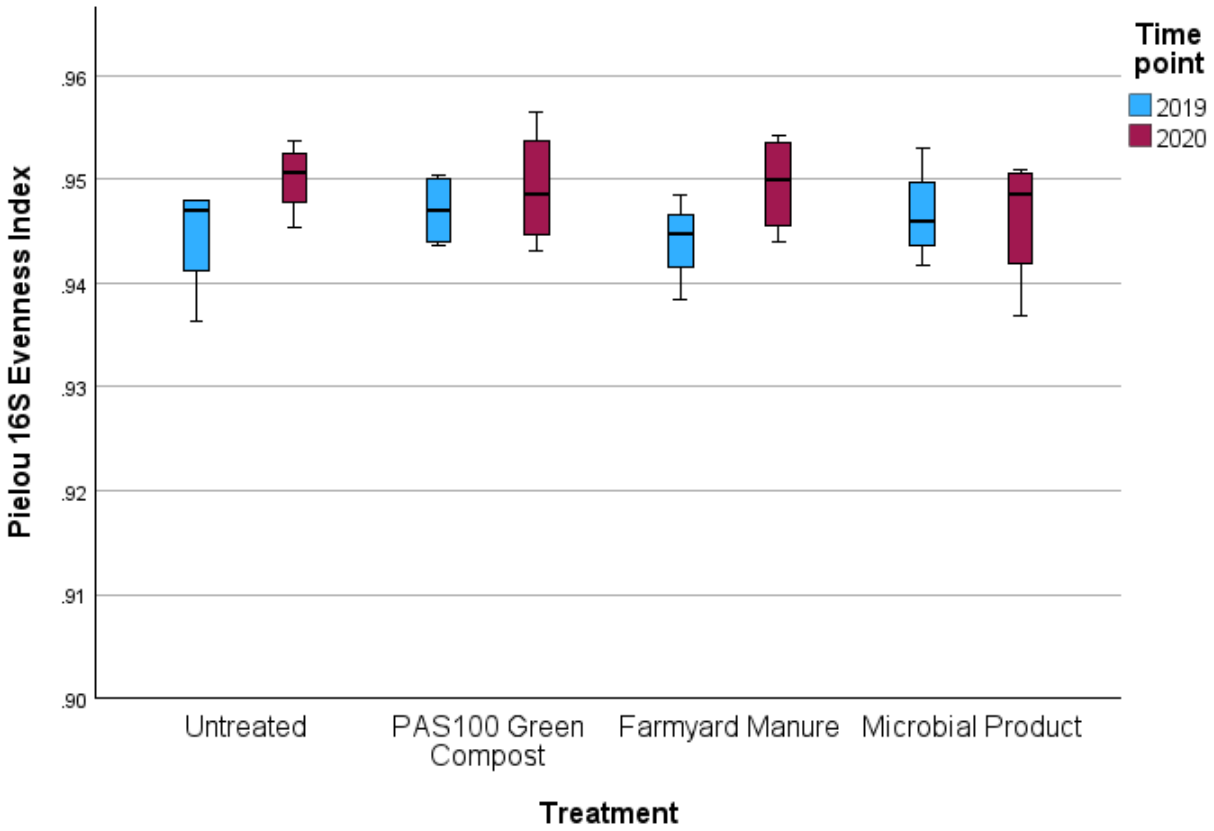

Figure 94. Pielou evenness index in the 16S region from the Daffodil field site with or without amendment of PAS100 Green Compost or Farmyard manure or Microbial Product. Data is presented as interquartile range and median (n=24). Note: The y-axis does not start at zero to provide a clearer view of the variation between data points.

Assessment of Pielou evenness index in the ITS rRNA region in response to treatment

When evaluating the Pielou evenness index within the ITS region across the 2 years, the data did not meet the criteria for normal distribution, as evidenced by the Shapiro-Wilk's test ($p > 0.05$). Therefore, a Kruskal-Wallis H test was employed for analysis. A significant difference emerged between the 2 years, with the Pielou evenness index registering an increase between 2019 and 2020 ($p = < 0.001$). Based on this significant effect, treatment was analysed by separating the time points.

Consequent analysis of the data met the normality criteria, as confirmed by the Shapiro-Wilk's test ($p > 0.05$). Assessment of treatment effects through a one-way ANOVA revealed no significant treatment impact in either 2019 ($p = 0.349$) or 2020 ($p = 0.588$) (Figure 95).

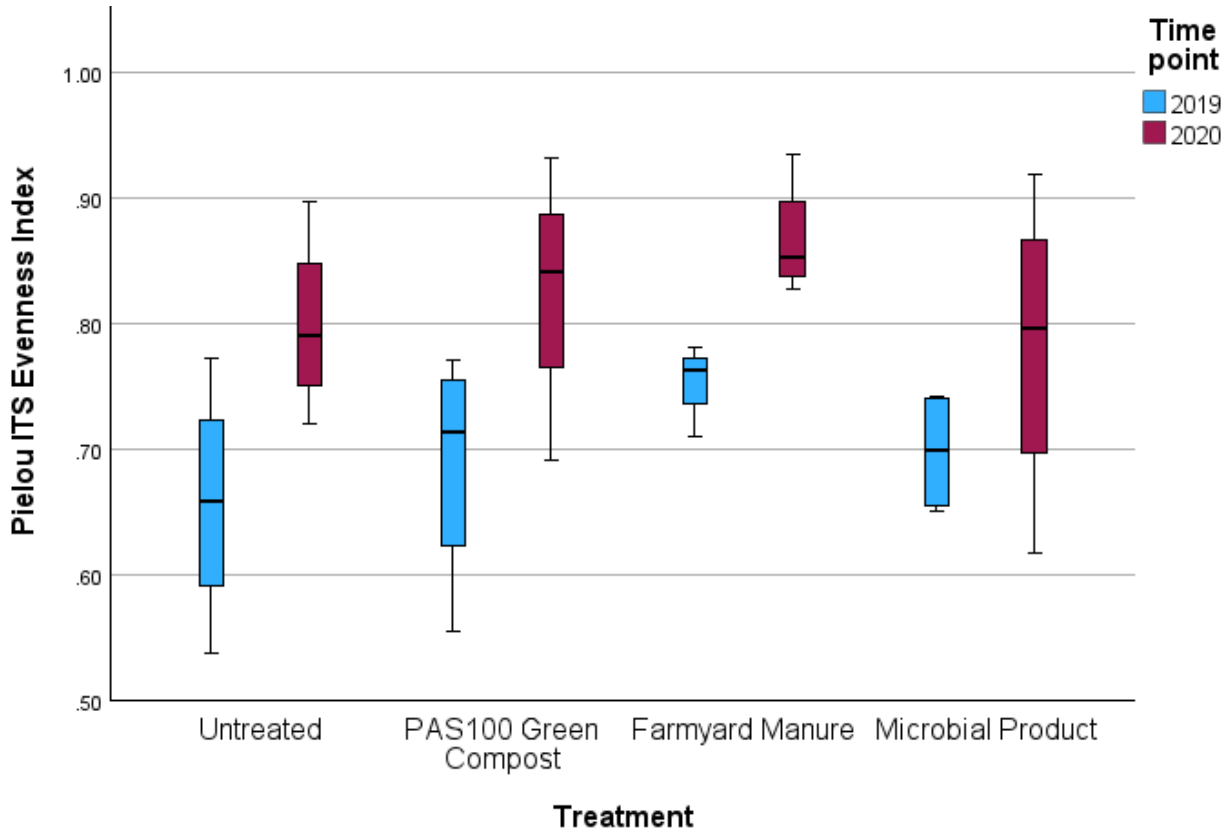

Figure 95. Pielou evenness index in the ITS region from the Daffodil field site with or without amendment of PAS100 Green Compost or Farmyard manure or Microbial Product. Data is presented as interquartile range and median (n=24). Note: The y-axis does not start at zero to provide a clearer view of the variation between data points.

Assessment of Faiths phylogenetic diversity in the 16S rRNA region in response to treatment

When examining Faith's phylogenetic diversity within the 16S region across the 2 years, the data did not demonstrate a normal distribution, as confirmed by the Shapiro-Wilk's test ($p > 0.05$). A Kruskal-Wallis H test was performed. A significant difference was observed between the 2 years, with Faith's phylogenetic diversity showing an increase from 2019 to 2020 ($p = 0.004$).

Based on the significant difference between the years, treatment was assessed with years separated. The data met the normality criteria, as evidenced by the Shapiro-Wilk's test ($p > 0.05$). The impact of treatment was evaluated through a one-way ANOVA, revealing no significant treatment effect in either the first year ($p = 0.322$) or the subsequent year ($p = 0.453$) (Figure 96).

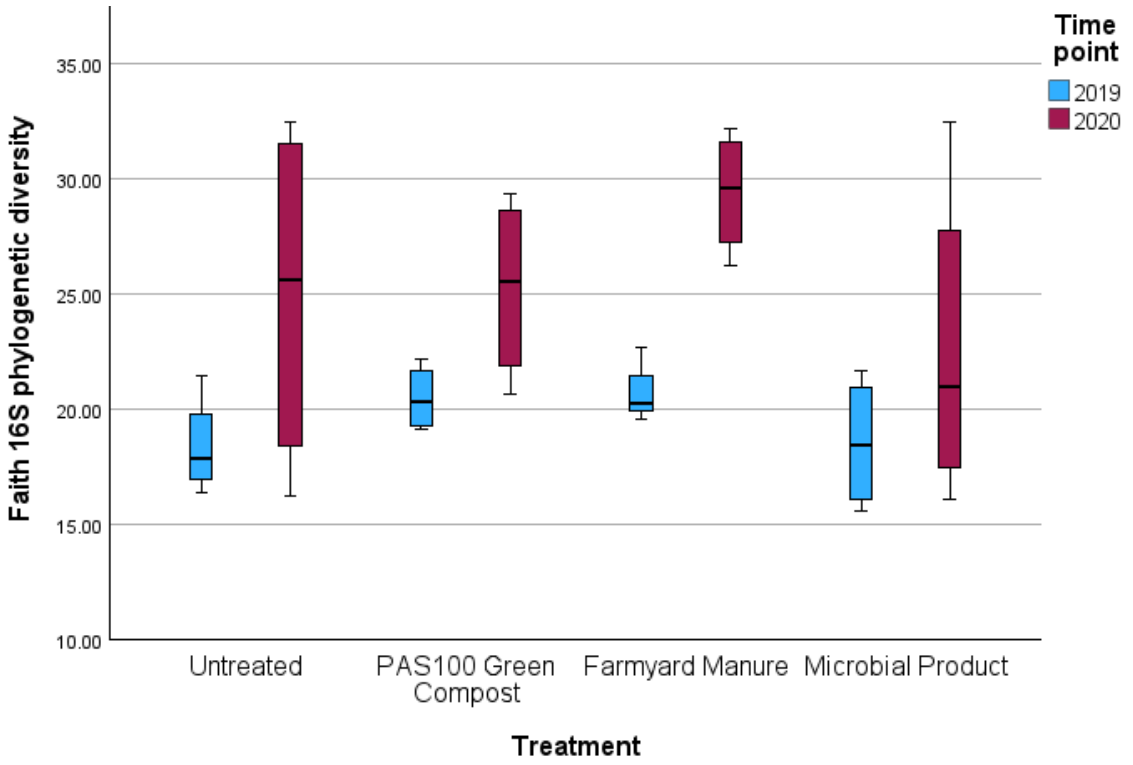

Figure 96. Faiths phylogenetic diversity in the 16S region from the Daffodil field site with or without amendment of PAS100 Green Compost or Farmyard manure or Microbial Product. Data is presented as interquartile range and median (n=24). Note: The y-axis does not start at zero to provide a clearer view of the variation between data points.

Assessment of Faiths phylogenetic diversity in the ITS rRNA region in response to treatment

When evaluating Faith's phylogenetic diversity within the ITS region across the two years, the data did not conform to a normal distribution, as confirmed by the Shapiro-Wilk's test ($p > 0.05$). Consequently, A Kruskal-Wallis H test was performed, revealing no statistically significant difference between the two time points ($p = 0.851$).

Without separating the data by year, data exhibited normality when assessed using the Shapiro-Wilk's test ($p > 0.05$). Effect of treatment was then assessed through a one-way ANOVA, which did not identify any significant treatment effect ($p = 0.429$) (Figure 97).

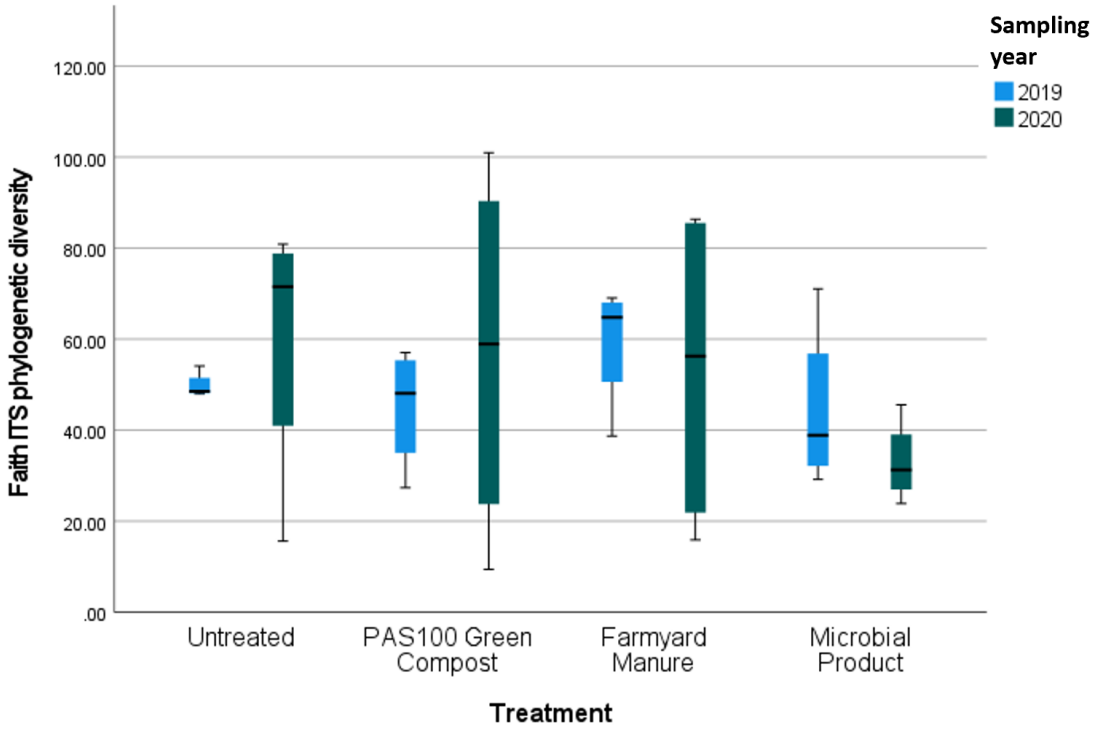

Figure 97. Faiths phylogenetic diversity in the ITS region from the Daffodil field site with or without amendment of PAS100 Green Compost or Farmyard manure or Microbial Product. Data is presented as interquartile range and median (n=24).

Relationship between quantified 16S rRNA DNA and community indices established through metabarcoding sequencing

There was also no relationship between quantified 16S rRNA DNA and Shannon 16S diversity index or Pielou 16S diversity index or Faith 16S phylogenetic diversity index as assessed by Pearsons's correlation (p=0.079 – 0.823).

Relationship between quantified 18S rRNA DNA and community indices established through metabarcoding sequencing

Data for 18S rRNA DNA and Faith ITS phylogenetic diversity index passed tests of normality and homogeneity of variance. There was no relationship between quantified 18S rRNA DNA and Faith ITS phylogenetic diversity index as assessed visually by pearsons correlation (p=0.627).

Data for 18S rRNA DNA, Pielou ITS diversity index, Shannon ITS diversity index failed tests of normality (p>0.05); therefore, the relationship was assessed using Spearman's rank correlation. 18S rRNA DNA and Pielou ITS diversity index had a negative correlation (p=<0.01), with a correlation coefficient of r_s = -0.654 (Figure 98).

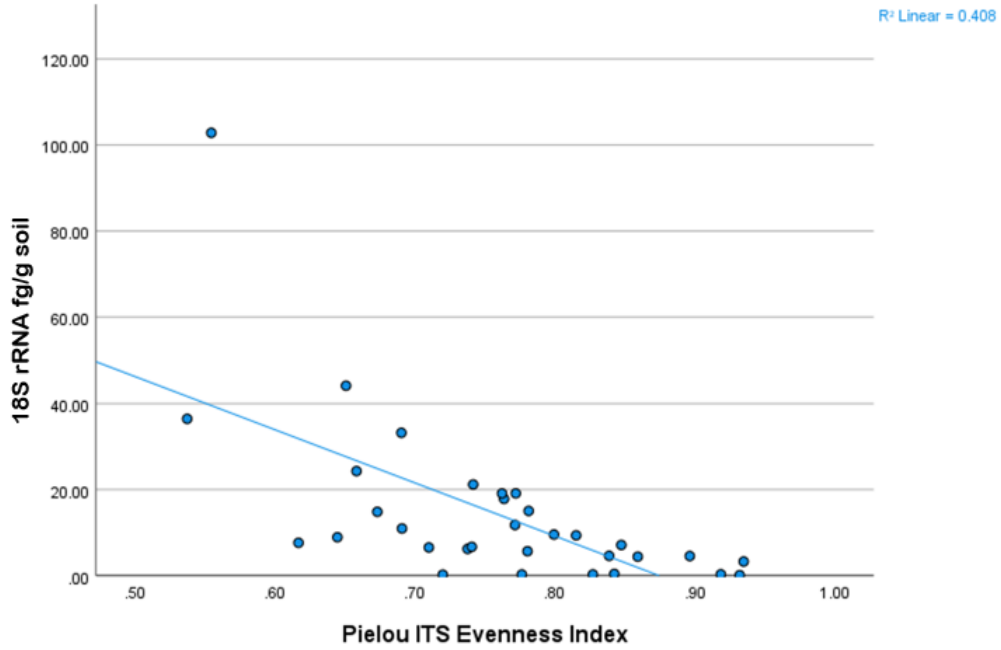

Figure 98. Scatter graph showing negative correlation between quantified 18S rRNA DNA and Pielou ITS diversity index.

Beta Diversity of bacterial and fungal communities across each site

Beta diversity for bacterial and fungal communities was assessed across all sites, and the results are visualized in the PCoA plot (Figure 100). While statistical significance was not formally tested, the PCoA reveals clear separation between the sites, indicating distinct community compositions at each location.

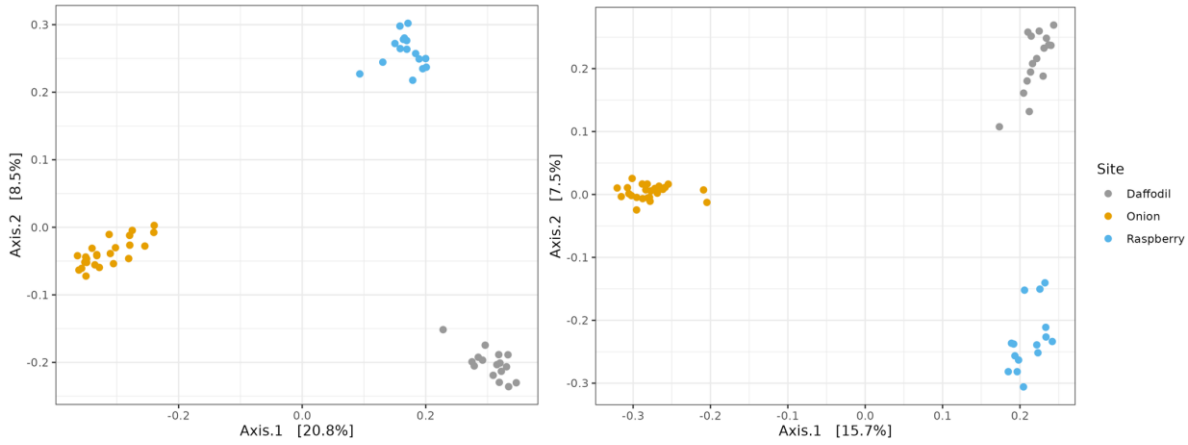

Figure 99. Analysis of beta diversity of bacterial (left) and fungal (right) communities across distinct locations (raspberry, onion and daffodil trial sites) based on 16S rRNA gene and ITS metabarcoding data from the final sampling date.

iv) Discussion

Effect of field site on communities of soil bacterial phyla

At the field sites examined, several predominant bacterial phyla were consistently identified across the three locations. Specifically, the most prevalent phyla encompassed *Proteobacteria, Bacteroidota, Acidobacteriota*, and *Chloroflexi*. Notably, *Proteobacteria* emerged as the predominant phylum in both the raspberry and onion field trials, ranking second in prevalence at the daffodil site. In the raspberry trial, the sequence of prevalence following *Proteobacteria* was *Bacteroidota, Acidobacteriota, Chloroflexi,* and *Actinobacteriota*. Conversely, in the onion trial, *Acidobacteriota* and *Bacteroidota* followed *Proteobacteria* in prevalence. Lastly, at the daffodil site, *Acidobacteriota* dominated as the primary bacterial phylum, followed by *Proteobacteria* and *Chloroflexi*. Similarly, (Yin *et al.*, 2010) found that *Proteobacteria* represented the majority of the taxa found, in their silt loam soil treated with different tillage practices and crop rotations (Manhattan, Kansas U.S) followed by *Acidobacteria* and Tian *et al.* (2015) also found the most frequent phyla to be *Proteobacteria*, followed by *Chloroflexi*, and *Acidobacteria*. The frequency of bacterial phyla did not appear to correlate with the physical and chemical soil properties measured (**Error! Reference source not found.**). Kumar *et al.* (2018), however, found bacterial community structure was significantly influenced by soil pH. Both the raspberry and the daffodil site had similar soil properties (Sandy Silt Loam, pH 8.2 & 8.3 respectively), meaning that the impact of pH could not be tested, however a different hierarchy of bacterial phyla was observed. Additionally, the raspberry and onion sites had similar bacterial phyla hierarchy but different physical and chemical soil properties. This indicates that other variables may have influenced the bacterial hierarchy.

Over the period of the field trials some significant changes in bacterial phyla were observed at each of the field trials. Notably at the raspberry site *Proteobacteria*, *Bacteroidota* and *Chloroflexi* significantly increased whereas *Acidobacteriota* and *Actinobacteriota* decreased between years, along with other significant changes in less abundant bacterial phylum. Again, at the onion field site significant changes between years were observed, including changes seen in *Acidobacteria* and *Proteobacteria.* At the daffodil site significant changes were seen in an increase in

the *Chloroflexi* phylum as well as other significant changes in less abundant bacterial phyla, including *Fibrobacterota* that was not detected in the first year.

Effect of organic amendments on communities of soil bacterial phyla

Upon examining the impacts of the organic amendment treatments across the various sites and sampling dates each year, distinct soil treatment effects were evident at each location. Specifically, at the raspberry field site a notable treatment effect was observed within the bacterial phylum *Fibrobacterota*. This phylum remained undetected in both digestate treatments, was present at minimal levels in the untreated plots, and exhibited slightly elevated but comparatively lower frequencies in the Prestop treatments, in contrast to other observed frequencies. In a study by Kumar *et al*. in 2018, *Fibrobacterota* only showed significant reduction of OTU proportion in the nitrogen only treatment and not in the farmyard manure treatments which were similar to the control. *Fibrobacterota* is an understudied bacterial phylum but is associated with breakdown of cellulose in herbivore guts, however, it is found in the environment (Ransom-Jones *et al*., 2012). It is known to prefer moist environments (Ransom-Jones *et al*., 2012; Wang *et al*., 2022) but this does not offer an explanation as to why it demonstrated significant differences between the treatments.

At the daffodil field site, only one bacterial phylum, *Verrucomicrobiota*, was significantly affected by the soil amendment treatments. Soil treated with AMF had the lowest frequency of *Verrucomicrobiota* (280.38 ASV's), followed by the untreated control (326.63 ASV's), green compost (440.38 ASV's) and farmyard manure with the highest (443.57 ASV's). *Verrucomicrobiota* are associated with triggering beneficial symbioses, limiting rhizo-competition, plant growth promotion and soil-borne disease protection and its distribution is linked to multiple factors: pH, temperature, plant root pressure, soil moisture, elevation gradient, soil depth, O_2 concentration, salinity, acidity, ion concentration, available nutrients and seasonal variability (Dash *et al*., 2020). At the daffodil field site *Verrucomicrobiota* was higher in the treatments that contain high organic matter and carbon, which is in line with previous findings (Ranjan *et al*., 2015).

At the onion field trial, more bacterial phyla showed significant differences in frequency in response to treatments. In the first year neither *Deinococcota* nor *Spirochaetota* were detected, however in the second year both were detected in all

plots apart from the untreated control, indicating that the treatments may have positively affected their frequency, however both frequencies were very low in comparison. *Nitrospirota* significantly increased in response to treatment in year 1, however the untreated control had the highest frequency by the end of the trial. Kumar *et al*. (2018) again only found a significant effect in the nitrogen treatment on *Nitrospirota* and not the organic treatment of farmyard manure. *Nitrospirota* are key in the nitrogen cycle and their encouragement could be beneficial, however it is not clear why the organic treatments may have hindered this. *Patescibacteria* also significantly increased over the trial period with the highest increase seen in the cover crop, followed by cover crop + green compost, untreated control and green compost. This indicates that green compost had a negative impact on *Patescibacteria*, and cover crop a positive impact, as it reduced the frequency seen in cover crop + green compost treated soil when compared to the sole cover crop treatment and had the lowest frequency increase overall. Tian *et al*. (2015) found that the application of compost significantly increased the relative abundance of proteobacteria and decreased the abundance of *Chloroflexi*, demonstrating that compost can have a significant impact on key bacterial phyla despite this not being seen in this study. NB1-j, WS2 and SAR324 were all not detected in the second year, perhaps indicating that either the treatments or even just presence of the crop affected their populations. The presence and absence of bacteria between treatments could offer further insight into bacterial communities. Sun *et al*. (2018) found that this was key to understanding vertical distribution of bacteria however this niche-based process was more important in bacteria than fungi.

Effect of field site on soil Fungal Phylum Community

Again, at each of the three field trial sites fungal phyla were assessed. At the raspberry and daffodil field trial *Ascomycota* was the most frequent fungal phyla, whereas at the onion field trial *Mucoromycota* had the highest frequency. *Ascomycota* are often associated with resilient and competitive traits, which may explain their high frequency (Egidi *et al*., 2019). Much like the bacteria, fungal phyla frequency did not appear to relate to soil characteristics. Fungi are thought to be less affected by soil characteristics like pH and more by environmental conditions (Egidi *et al*., 2019). Over the period of the trial *Ascomycota, Chytridiomycota* and *Stramenopila* oomycote significantly decreased at the raspberry site, whereas *Ascomycota* decreased at the onion field trial. In a study by Egidi *et al*. (2019),

looking at fungal phyla across 235 global soils, they found *Ascomycota* dominated soils globally followed by *Basiodiomycota* and 'unclassified fungi', highlighting the issues still faced by molecular techniques leading to underestimation of fungal diversity in soils. Although not the most dominant phyla, *Mucoromycota* was one of the 83 dominant fungal phylotypes found worldwide. There was no change in fungal phylum frequency between years at the daffodil site.

Effect of organic amendments on soil Fungal Phylum Community

At all of the sites no significant changes were seen in response to any of the treatments, echoing evidence from Sun *et al*. (2018) that bacteria population changes are larger compared with fungi, in response to these changes. This is also evidenced by Egidi *et al*. (2019) who found that their 83 dominant fungal phylotypes showed similar levels of relative abundance across all the sampled habitats (Boreal, forests, grassland and shrublands). However, Treonis *et al*. (2010) did find a positive effects of organic amendments (compost/biosolids vetch + straw) on fungi but only in combination with tillage, perhaps indicating that a mechanical stimulus is needed for an effect on fungi, that was not investigated in this study. Sun *et al*. (2018) also investigated the effect of tillage on microbial communities. They found that fungal communities differed strongly between tillage treatments (ploughing, moldboard ploughing, rotary tillage) and that fungal communities showed stronger responses to tillage treatments than bacterial communities.

Effect of organic amendments on quantified bacterial and fungal soil communities

In addition to monitoring bacterial and fungal communities using metabarcoding qPCR was used to quantify amounts of bacterial and fungal DNA in the soil, to see if the actual quantities fluctuated. At the raspberry and daffodil field trials both quantities of bacterial and fungal DNA decreased between the years and there was no effect of treatment on either. It was unexpected to see a decrease in fungal and bacterial DNA at the raspberry and daffodil sites, however they are the sites with the most similar soil characteristics including pH, perhaps explaining their similar response. This decrease and lack of effect was also surprising as studies have shown mostly increases in soil microorganisms in response to organic amendments (Lazarovits, 2001; Bailey and Lazarovits, 2003; Davey *et al.*, 2019). For instance with the application of manure, Tian *et al.* (2015) found increased microbial activity and gene copies of bacteria in the soil. At the onion field trial, the amount of both bacterial

and fungal DNA increased over the period of the field trial. Whilst the treatment didn't impact the quantity of bacterial DNA there were significant effects of treatment in fungal DNA quantities. The cover crop + green compost had significantly higher levels of fungal DNA than the untreated control and the green compost treatment. This lack of change in bacterial DNA quantities in response to cover crop was also seen in the study by Romdhane *et al*. (2019), which in their study similarly quantified 16S rRNA DNA in response to cover crops. The application of cover crops (rye and white mustard) actual decreased the amount of fungi (quantified via plating) in a study by Patkowska *et al.* (2016). The varying responses—increases, decreases, and lack of change—to organic amendments observed in the tested fields were unexpected. A potential explanation could be that the effects were short-lived, and the sampling regime used in this study was inadequate in capturing these short-term responses. Additionally, organic matter at the onion site was high (14%), in comparison to the other sites (raspberry 2.2% & daffodil 3.4%), so amendments to increase OM at this site may have been futile, as maximum microbial activity may have been reached, however this does not explain the lack in significant changes at the other sites, which had relatively low OM. This lack of changes could again evidence that environmental factors could be the largest driver of microbial communities in the short term.

It is believed that the incorporation of organic amendments would have a beneficial effect by increasing bacterial and fungal communities (Bailey and Lazarovits, 2003). In a study by Lazarovits (2001), investigating the application of organic amendments to control *V. dahliae*, reported 'large increases in soil populations of fungi and bacteria, supporting this belief. In a similar study by Davey *et al*. (2019) they found that organic matter inputs increased the amount of DNA of bacteria and fungi. However, both these studies were performed in artificial environments perhaps removing the uncontrollable environmental influence.

<u>Effect of organic amendments on Community Diversity and Evenness metrics in soil</u>

Various community metrics were also compared from these datasets including Shannon diversity index, Faiths phylogenetic diversity and Pielou evenness index. Diversity is often associated with improved functionality of a community and is therefore important in understanding soil microbiomes however the evenness of a community is theorised to be as valuable as a diverse community, as a species in low

abundance is unlikely to contribute highly to biomass or community interactions (Mulder *et al.*, 2004).

i) Shannon Diversity

In the raspberry field trial, fungal Shannon diversity decreased where bacterial Shannon diversity increased over the 2 years. There was no effect of soil treatment on either bacterial or fungal Shannon diversity. In a trial measuring soil restoration after application of compost and sludge (digestate) and the effect on bacterial and fungal communities (Bastida *et al.*, 2015), the application of digestate significantly decreased both bacterial and fungal diversity. This trial however took place over 10 years in comparison to the 2 in this study, perhaps indicating that time is a key parameter in the effect of organic amendments.

At the onion field trial the opposite occurred, fungal diversity increased whereas bacterial diversity decreased over the year. Again, there was no effect of soil treatments on either bacterial or fungal diversity. Similarly, Detheridge *et al.* (2016) also saw no effect of their cover crop treatments on fungal Shannon diversity in the first season, however significant effects on the fungal diversity were observed in subsequent seasons. A study investigating the short term effects of cover crop mixes (Wang *et al.*, 2020) found that their single species mix caused a significant decrease in bacterial diversity.

In the daffodil field trial, fungal diversity did not appear to change between the 2 years or in response to treatment. Kumar *et al.*, (2018) observed increases in fungal Shannon diversity in response to application of manure, however their trial was conducted over 47 years, perhaps indicating that more time was required to observe a change in fungal diversity. Bacterial Shannon diversity did however increase across the period of the Daffodil field trial, but again no effect of treatment was observed. Manure had previously been found to decrease bacterial Shannon diversity (Tian *et al.*, 2015), meaning the increase observed in the daffodil field, especially in the manured plots was unexpected. However, Celestina *et al.* (2019) reported no significant change of bacterial diversity three years after application of a manure treatment. Again, this lack of significant observation may be related to the time period after application.

Overall, Shannon diversity did not appear to be affected by the treatments applied, at any of the sites tested as part of this PhD study, for either bacteria or fungi. There

have been studies that report changes in diversity after application of similar treatments, however these often take place after a longer period than tested in these trials. The decreases, increases and lack of changes over the period of the trials work against the hypothesis that organic amendments or increases in organic amendments have an additive effect to soil microbial communities.

ii) Faith's phylogenetic diversity

Moving on to Faith's phylogenetic diversity (Faith's PD). At the raspberry site bacterial Faith's PD increased over the period of the trial, whereas fungal Faith's PD did not appear to change. Again, no effect of treatment was observed for either fungi or bacteria, nor was there a relationship between quantified DNA (either 16S or 18S rRNA) or Faith's PD. The study by Xiong *et al.* (2017) investigated the effect of fertilisers which included supplements with beneficial organisms, much like the Prestop product trialled here. They found that the Faith's PD at phylum level, for both fungi and bacterial, was higher when these beneficial organisms were supplemented than when the chemical fertiliser was used. A study looking at the effect of digestate (waste sludge) on bacterial communities found that those amended with the digestate had a significantly increased Faith's PD than controls (Rodríguez-Berbel *et al.*, 2020). Both these studies indicate that an effect of treatment on Faith's PD would have been expected at this site, however this was not observed at the phylum level. Both the published trials took place over 20 and 10 years, respectively, potentially suggesting a longer period is required for an effect to occur.

At the onion field trial both bacterial and fungal Faith's PD increased across the period of the trial however neither appeared to show a significant effect of treatment. Compost application was found by Rodríguez-Berbel *et al.* (2020) to increase Faith's PD over a 10 year study. Alahmad *et al.* (2019) in their 4-year study found an increase in fungal Faith's PD in response to cover crop application. These 2 studies demonstrated a significant effect of organic amendment on Faith's PD however this was not observed at the onion trial at phylum level. Again, these studies occurred over a much longer period of time in comparison to the 1-year onion trial. This increase in Faith's PD at the onion trial was also mirrored in the quantification of bacterial and fungal DNA, which also showed an increase over the period of the trial. This relationship was not statistically supported for fungi; however, a strong positive correlation was found between bacterial Faith's PD and quantified bacterial DNA. This relationship only occurred at the onion field trial meaning that an increase in

bacterial DNA did not always necessarily lead to an increase in bacterial phylogenetic diversity. Factors such as environmental conditions, resource availability, and microbial interactions can interfere with this relationship. One potential explanation for this relationship is that with an increase in bacterial numbers, competition for resources can intensify, leading to resource partitioning and the diversification of bacterial populations (Wilson and Lindow, 1994; Goldford *et al.*, 2018).

At the daffodil field trial only bacterial Faith's PD increased over the period of the trial whereas bacterial DNA appeared to decrease, there was no relationship between the two measurements. Again, no effect of treatment was observed for either fungal or bacterial Faith's PD. A study by Xiong *et al.* (2017), investigating the effect of different organic fertilisers, which included manure, against chemical fertilisers on microbial communities, observed that bacterial and fungal Faith's PD was higher in soils treated with organic fertilisers than with chemical fertiliser. Similarly, Ikoyi *et al.* (2020) found that Faith's PD increased in response to cattle manure after 14 weeks. Much like the other trial sites, other research indicated that these treatments had the potential to trigger a change in Faith's PD, yet this was not observed in the present study. There are many potential reasons for this including soil characteristics, environmental pressures and potentially the time period that the trials occurred over.

iii) Pielou Evenness

When assessing the Pielou evenness index at the raspberry field trial neither bacterial or fungal evenness significantly changed over the period of the trial nor in response to treatment. This was also observed in the study by Rodríguez-Berbel *et al.* (2020) which found no significant change in bacterial Pielou evenness after application of digestate (waste sludge).

At the onion field trial Pielou evenness demonstrated responses to the trial in both bacterial and fungal communities. Fungal evenness decreased by the end of the trial and there was a significant decrease between the cover crop and cover crop + green compost treatments. For bacteria, Pielou evenness index significantly increased over the period of the trial. The green compost treatment had the lowest increase in bacterial Pielou evenness index over the period of the trial, whereas cover crop + green compost had the largest increase. Rodríguez-Berbel *et al.* (2020) also

observed a significant increase in bacterial Pielou evenness when compost was applied when compared to the control.

Then at the daffodil field trial, fungal evenness increased between sampling points in each year, but no effect of soil treatment was observed. However, there was no change in bacterial evenness over the period of the trial and again no effect of treatment was observed. The study by Kumar *et al.*, (2018), investigating bacterial communities, namely Pielou evenness, found that their plots with manure applied were more even than the control plots. Interestingly the combination of manure and NPK chemical fertilisers created more even plots than manure alone. This trial occurred over 47 years in comparison to the daffodil trial that lasted 2 years, much like in the previous measurements, there may be a time element that is limiting the observation of effect. Rodríguez-Berbel *et al.* (2020) observed that the application of compost also increased the evenness of bacteria when compared to the control, again this trial occurred over a longer period than the present trial, at 10 years. At the raspberry site, fungal DNA quantities appeared to decrease whereas fungal Pielou evenness appeared to increase between the years, this was found to be a statistically significant, showing a strong negative correlation between the two. Much like the other diversity metrics, there was no consistent response at any of the sites and their treatments on Pielou evenness.

Conclusions

Based on the observations seen across the trials, and analysis of beta diversity, it would appear that site had a stronger impact at the phylum level on bacterial and fungal soil communities than the soil management treatments applied. This was demonstrated by the different dominant phyla at each of the sites. However, this did not appear to correlate with soil characteristics at each of the sites, indicating that other variables not measured could be influencing the soil microbiomes, including crop history, management history and environment. This impact of site on soil microbiome was also echoed by Bradley *et al*. (2020) where higher fungal diversity was observed at their Beltsville site than at their Stoneville site, and vice versa for prokaryotic diversity. They attributed this to differences in soil chemical characteristics and the farming system at each site. Various studies have found that tillage can significantly affect diversity of fungi (Sun *et al.*, 2018; Essel *et al.*, 2019) and bacteria (Duan *et al.*, 2022). The effects of tillage are often related to the hyphal networks created by fungal communities that may be mechanically disrupted by

tillage (Schalamuk and Cabello, 2010; Sun *et al.*, 2018; Celestina *et al.*, 2019) which also affect the vertical distribution of bacterial niches (Duan *et al.*, 2022). Tillage and crop husbandry varied in each crop system but was uniform across plots at each site.

Additionally, relative abundance and diversity metrics did not consistently relate to either treatments or quantified levels of 16S rRNA and 18S rRNA, which again appeared to be more strongly related to site and other potential factors. At this stage of global understanding and technical capability, the measurement of bacterial and fungal quantities, via qPCR analysis of 16S rRNA and 18S rRNA, is not sufficient to make judgments on microbial diversity and evenness, or in turn the health of a soil.

Finally, a factor that became apparent in the design of this study and similar studies was the effect of sampling time and duration of the trials. There was potential that sampling of the trials was not sufficiently frequent to capture the impact on the soil microbial communities, therefore it would be recommended that future studies include multiple sampling dates throughout the growing season. Or that the application of the amendments needed to be repeated for a longer duration to cause a long-term measurable effect. The raspberry and daffodil field trials occurred over a period of 2 years whereas the onion trial only occurred over 1 year. A similar trial in 1999 (Yang *et al.*, 2003) found that there was no effect of their various mulches after 1 year of application but did speculate that long term applications may have an effect. However, Wang *et al.* (2020) contradicts this finding as they observed changes in microbial diversity after one year of cover crop application. Over the period of 1 year, they recorded changes at three time points. They found that their single mix treatment decreased in diversity over time, whereas the control and multi mix fluctuated, both increasing and decreasing over the year. The only significant treatment effect observed in this study occurred in response to the cover crop at the onion field trial, suggesting that cover crops may influence soil properties within a shorter time frame. Similar short-term trials reported that a single application was unable to cause long term benefits (Celestina *et al*., 2019) and that changes are “incremental” and “slower acting” (Bailey and Lazarovits, 2003). The majority of trials reporting significant impacts of organic amendments ranged from 10 years (Bastida *et al.*, 2015; Rodríguez-Berbel *et al.*, 2020) to 47 years (Kumar *et al.*, 2018). Perhaps indicating that for a significant effect of organic amendment treatments to be observed, trials need to occur over a longer period of time.

The use of the molecular techniques, both qPCR and metabarcoding, are beginning to shed light on the complex soil microbial communities and their interactions. Although not observed significant responses from the organic amendments applied were not observed, learnings were made towards the future of these studies, including trial period, sampling frequency and the importance of monitoring soil characteristics and the environment, as well as field history, to understand long term management effects. Furthermore, the novel comparison of qPCR and metabarcoding data may help guide future testing options on farms, if a further understanding of their interaction is gleaned.

Chapter 8- General Discussion

This final chapter considers the implications of this thesis on measuring DNA of soil-borne plant pathogens and biocontrol agents and their response to organic amendments, exploring the implications of monitoring target organisms, soilborne disease management and the broader understanding of soil microbiology and future directions. The thesis had 4 main objectives:

- Validate Molecular Analysis Methods: Validate the use of qPCR approaches for comprehensive analysis of soil microbial communities, including bacterial and fungal populations.

The thesis started by systematically reviewing soil DNA extraction methodologies employed in PCR-based detection, dating back to the earliest publication in 1988. This review highlighted the vast number of different methods available and the multitude of variables influencing their efficacy. It emphasized the challenge of comparability across studies, consequently impeding a wider understanding of soil microbiology.

Molecular methods were subsequently validated for their application in this study. This validation process involved comparing soil DNA extraction methods, which included increasing the soil starting volume. However, this further contributed to the number of bespoke methods available, and the incomparability of studies. Additional DNA purification steps were tested, but they failed to significantly enhance the quality of the extract, with dilution of DNA and co-dilution of inhibitors of DNA polymerases in water proving to be the most effective method. Furthermore, the design and validation of gBlock™ gene fragments, as known standards, were carried out for improved accuracy of target quantification when using qPCR. Finally, these molecular methods were validated for use in the trial soils, demonstrating that targets of interests (excluding AMF) could be detected and quantified in their relevant soils.

- Investigate Soil Management Practices: Investigate the effects of various soil management practices, such as cover crops, manures and biocontrol agents, on soil microbial communities and soilborne pathogen populations.

At various field trials, the impact of different organic amendments on targeted organisms and the wider soilborne microbial community was assessed. The trials covered a variety of soil types and crop systems (raspberries, daffodils, onions and

asparagus), and included the application of composts, manures, digestates and biological products, relevant to the systems and diseases at each site. At each site, the target organisms were successfully detected and quantified using selected molecular methods, and metabarcoding analyses to gain further insight into the effects on microbial communities.

It was not possible to relate the effects of organic soil amendments to changes in soilborne pathogen populations from qPCR or metabarcoding data collected from the onion, raspberry or daffodil field trials. Some significant observations regarding the effect of amendments on pathogens were observed at the asparagus trial but only seen in the second year.

When assessing the impact of soil amendments on soil communities using metabarcoding, site variations had a stronger influence at the phylum level on bacterial and fungal communities compared with the effects of the soil management treatments. Diversity metrics like Shannon or Faith's phylogenetic diversity remained largely unaffected by the treatments across each study sites, for both bacteria and fungi. Notably, it was only at the onion field trial that the bacterial and fungal evenness was altered by treatments, particularly to the application of green compost. While the quantities of fungal and bacterial DNA in samples showed no significant changes in response to treatments, at the onion site, fungal DNA increased in the cover crop + green compost treatment, again highlighting the influence of green compost on the community.

- Assess Soil Health Indicators: Evaluate soil health indicators derived from molecular analyses, such as microbial diversity and pathogen abundance, as predictors of soilborne disease risk and overall soil health status.

Building on existing research investigating relationships between pathogen inoculum density and disease incidence to predict disease risk, this thesis aimed to widen understanding of the overall soil community and its influence on crop disease. Initially this was explored through the use of glasshouse trials and inoculated soils. Strawberry plants were inoculated with *V. dahliae* and treated with *C. rosea* and/or anaerobic digestate. Population levels of both *V. dahliae* and *C. rosea* were measured at intervals. Disease incidence increased with *V. dahliae* inoculum density, but neither organic amendments nor their combination affected disease severity or plant growth. Population dynamics of both fungi showed initial expected trends but

became unpredictable over the 8-week trial, suggesting complexities beyond simple pathogen presence/quantity being an indicator of disease development.

- Support Soil Health Initiatives: Support ongoing efforts to protect and improve soil health by providing scientific evidence and tools, such as molecular techniques, that can aid in monitoring and managing soil microbial communities.

Despite what constitutes a ‘healthy soil’ being up for interpretation, this thesis operated under the premise that soil exhibiting lower pathogen presence, higher microbial diversity, and a more balanced community post-treatment could be deemed 'healthier.' However, despite efforts to minimize environmental influences on the molecular techniques used to track pathogen populations, the data generated from field studies proved inadequate for developing predictive disease diagnostics and soil health indicators. Measured pathogen populations did not relate to disease seen in the crop, nor did the populations appear to respond to treatment application in the majority of cases. At the onion field trial disease incidence across the trial was uniformly high, conversely no disease was observed in the raspberry field site by the end of the trial, whereas disease incidence in the daffodil trial fluctuated throughout the trial period, showing a general decrease but with significant variability across the trial.

None of the applied organic amendments demonstrated significant effects on bacterial or fungal microbial diversity metrics (Shannon or Faith indices). The only notable impact on fungal evenness, as indicated by the Pielou index, was observed in the onion field trial following the cover crop + green compost treatments. Across all trials, fluctuations in diversity and evenness occurred between sampling periods, yet the underlying causes of these variations remained uncertain. It remains unclear whether these fluctuations were driven by crop systems, seasonal factors, or a combination thereof, given the lack of consistent responses across all trial sites.

As part of this study, the potential of PCR and quantification of total fungal and bacterial DNA was explored as a tool for monitoring soil health and inferring the presence of a 'healthy' microbiome. My findings revealed no significant correlation between sequencing-based microbial diversity and qPCR quantified DNA levels.

Refinements and Recommendations for Further Studies

Trial Duration

The duration of the trials conducted in this study, spanning between 1 to 2 years, raises important considerations regarding the time required for treatments to exert significant effects on pathogen populations and soil microbial communities. Notably, the only treatment effect on pathogen populations was observed in the second year of the asparagus trial. This delay implies that the effects of organic amendments on pathogens may necessitate longer durations than the trials allowed. Moreover, my findings regarding bacterial and fungal diversity did not demonstrate notable changes over the trial periods in response to treatments. Fluctuations in diversity observed in the onion field trial align with findings by Detheridge *et al*. (2016), where significant effects on fungal diversity were observed in subsequent seasons rather than immediately. Additionally, Kumar *et al*. (2018), documented increases in fungal diversity over nearly five decades, indicating that longer and more intensive sampling within and between seasons may be necessary to more accurately observe such changes.

Comparisons with other studies, such as the trial by Bastida *et al*. (2015) which spanned a decade, suggest that time plays a pivotal role in the efficacy of organic amendments and studies such as Rodríguez-Berbel *et al*. (2020) and Alahmad *et al*. (2019) suggested that time may be a limiting factor in observing significant effects. The majority of trials reporting significant impacts of organic amendments ranged from 10 to 47 years, emphasizing the potential necessity for longer-term studies to observe substantial effects.

Furthermore, the establishment of predictive disease models requires substantial data accumulation, frequently spanning multiple years (Paplomatas *et al.*, 1992). Studies have indicated that this process typically involves trial durations ranging from 3 to 4 years and even extending up to 7 years (Taylor *et al.,* 1981; Paplomatas *et al.*, 1992; Lawes *et al.*, 2015). Therefore, the endeavour to develop predictive models within this thesis might have yielded more successful outcomes if the trials had been conducted over longer durations.

This highlights the need for longer-term studies to understand the temporal dynamics of soil microbial communities and their responses to amendments.

More applications of amendments

To achieve long-term improvements, in addition to extending the trial duration, increasing the frequency of amendment applications may be necessary. The biocontrol agent *C. rosea,* applied as the commercial product Prestop (Lallemand Inc., Canada), was reapplied every 2 months as per recommendations at the raspberry trial, but no other amendment was repeated during the period of the trials. As per the results of the glasshouse trials (Chapter 5), *C. rosea* failed to colonise and populations were undetectable after 2 months, diminishing its ability to incite long-term benefits.

Prior studies have demonstrated the efficacy of multiple applications of organic amendments over extended periods (Lang *et al.*, 2012; Elshahawy *et al.*, 2019). For instance, Manici *et al.* (2020) repeatedly amended soils with digestate over a decade, observing increased soil capacity to support plant biomass production and reduced incidence of root infection in maize despite the presence of pathogens. Unlike in this PhD trial, these studies conducted longer-term assessments and regularly applied the organic amendment.

Similar short time trials to this thesis reported that a single application did not result in long term benefits (Celestina *et al*., 2019) and that changes are “incremental” and “slower acting” (Bailey and Lazarovits, 2003). Reapplication of amendments, like fertilisers, is common in farm management systems, therefore the effects of organic amendments should be evaluated as part of a crop rotation cycle to understand long term effects of organic amendment application and the potential for long term improvements.

Sampling Frequency

Increasing sampling frequency could have significantly enhanced the depth and breadth of insights gained from my study. In addition to longer trial durations to assess long-term effects, findings suggest that more frequent sampling intervals would have allowed for the early detection of instantaneous changes in response to organic amendments. For instance, the disappearance of *C. rosea* populations after just 8 weeks in the glasshouse trials underscores the importance of additional sampling times to observe such dynamics, and this should have been continued into field settings. Studies like Wang *et al*. (2020) have demonstrated the value of multiple sampling points, such as immediately after planting of cover crops, mowing, and just before harvest, to capture fluctuations in microbial communities. By incorporating

more sampling points seasonal and environmental influences, including factors like soil organic matter, moisture, and temperature fluctuations, on microbial communities could have better been accounted for (Dash *et al.*, 2020). Additionally, insights from Bradley *et al.* (2020) suggest that geography, season, and farming systems are crucial drivers of microbial communities, further emphasizing the need for comprehensive sampling strategies to account for these variables. Overall, increasing sampling frequency would have allowed for a more nuanced understanding of the complex interactions between organic amendments, microbial communities, and environmental factors in my study.

Future Direction of microbial studies for monitoring soil health

The series of experiments reported in this thesis aimed to understand the complex interactions between soilborne pathogen populations and soil amendments using molecular analysis. Several key findings emerged across the experiments. Firstly, there was notable variability in the detectable levels of *V. dahliae* and *C. rosea* in the glasshouse under controlled conditions, highlighting the unpredictability of colonization by these organisms. Cao *et al*. (2011) also reported a decline in *B. subtilis* over their trial period, however after 60 days it was still present. This short lifespan of biocontrol agents is also reflected in the recommended reapplication rates of commercial products; Serenade (*Bacillus subtilis*) (Bayer Crop Science, Germany) recommends every 8 weeks, T34 Biocontrol (*Trichoderma asperellum*) (Fargo Ltd., UK) recommends every 8-12 weeks and Lalstop K61 (*Streptomyces griseoviridis*) (Lallemand Inc., Canada) recommends every 2-6 weeks. This inability to colonise is a major downfall of biocontrol agents and limits their potential. Although offering a promising avenue for sustainable disease control, further research and innovation is needed, such as through improvements in formulation (Tut *et al.*, 2021). However the future is looking promising with recent works investigating application of biocontrol organism inspired RNAi (Islam and Sherif, 2020), proteins and peptides (Tóth *et al.*, 2020).

Furthermore, the impact of *C. rosea* on disease progression and *V. dahliae* populations did not correlate, suggesting the need for further inquiry into its role in disease control. Metcalf *et al.* (2007) also observed no additive effect when increasing dosage of *Trichoderma koningii* on suppression of white rot of onion

caused by *S. cepivorum.* This lack of additive effect, and remaining efficacy at lower doses (Metcalf *et al.,* 2007; Tut *et al.*, 2021), further demonstrating the complexity of the soil microbiome.

These challenges extended beyond controlled environment experiments, as establishing relationships between soil inoculum density, disease incidence, and effects of organic soil amendments on soilborne pathogen populations in the field proved difficult. Despite attempts to mitigate environmental impacts, data collected from field studies were not sufficient for developing predictive diagnostics. Disease incidence – inoculum density relationships have been successfully reported in a wide range of pathogens and hosts, including *V. dahliae* (Paplomatas *et al.*, 1992; Berbegal *et al.,* 2007; Xiao and Subbarao, 2007; Roca *et al.*, 2016). These studies utilise traditional plating methods to measure *V. dahliae* in the soil, as opposed to using molecular methods and quantifying DNA.

These traditional plating methods count the number of viable microsclerotia in the soil, ensuring the pathogen is alive and viable, however the molecular methods used here quantified all fungal structures (hyphae, sclerotia) both dead and alive, which may skew the results. There is the potential to measure RNA instead of DNA. RNA degrades faster in the environment, and extracted RNA is more likely to have come from live and viable organisms. Additionally, a study by Meyer *et al*. (2019) explored the potential of monitoring RNA communities instead of DNA to assess environmental responses. They found that RNA communities were more responsive to changes, while DNA also reflected these shifts but to a lesser extent, potentially diminishing the significance of the observed changes when using DNA alone.

The effect of organic amendments on soil health, specifically microbial diversity and evenness, appeared to be minimal or ineffective. It raises the question of what a ‘healthy’ soil is, and whether the soils tested were ‘unhealthy’ to begin with. Organic amendments have frequently been linked to responses in soil microbiomes (Lazarovits, 2001; Bailey and Lazarovits, 2003; Treonis *et al*., 2010; Tian *et al*., 2015; Davey *et al*., 2019). While organic amendments have often been associated with changes in soil microbiomes, it remains unclear whether these changes are beneficial. Defining soil health remains an ongoing challenge.

Addressing this gap, recent studies have explored the functional aspects of soil, focusing on its capacity to perform vital functions such as nutrient cycling. Maretto *et*

al. (2023) utilized qPCR to track genes associated with nitrogen, nitrite, and ammonia cycles, alongside cluster genes believed to be pivotal in bacterial adaptation to soil. Their findings revealed a higher abundance of these genes in organic systems compared to conventional ones. Similarly, Xiang *et al*. (2020) investigated functional genes linked to carbon, nitrogen, phosphorus, and sulphur cycles using quantitative microbial element cycling (QMEC) and amplicon sequencing. Their analysis highlighted distinctions between farmland and forest samples, with farmland exhibiting a lower frequency of key functional genes. In a related study, Yu *et al*. (2024) inferred functional pathways from 16S rRNA gene sequence data. Their results indicated that animal fertilization predominantly enriched nitrogen cycle pathways, whereas plant fertilizer favoured carbon cycle pathways. Shifting the focus from monitoring microbial communities to assessing soil functional capability could provide more valuable insights into optimizing soils for agricultural purposes.

Final Thoughts

Concern for the environment and the sustainability of agricultural practices has come to the forefront of public concern. Soil health has become a focus and various international campaigns have been created to prevent further degradation and begin to recover global soils (Falabiba *et al.*, 2014; DEFRA, 2018; Bodle, 2022; Gelardi *et al.,* 2023). In the UK, changes to farmer subsidies, such as the new Sustainable Farming Incentive (SFI) schemes: ‘SAM1: Assess soil, produce a soil management plan and test soil organic matter’ (DEFRA, 2023), have influenced how soils are managed and aim to encourage the use of more sustainable practices, such as organic amendments. From this there is a need to reliably measure the impact of these practices, from environmental benefits (i.e. carbon), soil health (i.e. microbial diversity) and farm productivity (i.e. disease loss/yield). However current methods are open to interpretation and comparative tests often offer widely differing results. To ensure the successful adoption of these new sustainable methods, it is crucial to thoroughly understand and quantify their impact on the environment, soil health, and farm productivity and provide implementors with the confidence to continually use these practices.

This thesis aimed to contribute to this challenge by developing predictive tools for soilborne disease and tools for monitoring soil health, building off traditional inoculum

density - disease incidence relationships and utilising new molecular techniques and analysis. Although, a singular tool or predictive model was not established in this thesis, it helped further understanding of the soil microbiome and the impact of organic amendments, whilst demonstrating the complexities of this biome and identifying the weaknesses of molecular techniques and their future potential.

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Appendices

1. gBlocks™ Sequences

Target Organism	**gBlocks™ Sequence**
Fusarium culmorum & F. graminearum	5’ ATG GCT AAA CAG CAC GAA TGC CCG ACT TGC CCC AAG GCA TTC CAA TTA AAG AAA GAC TTG AAG AGA CAT ATC TCG TGT GTT CAC GAG AAG CGC CAG CAC GCT ATG GAA GCT CGA CGT GAC CGC CAA TGT ATT TGG GGA GTG CAG CAG GAC TGC AGC TCC CAA CAC CAA GCT 3’
Gaeumannomyces tritici *& Clonostachys rosea*	5’ AAC CCA CAA GTC ACC CCA GGA AAA AAG CGT GGT TGT GTG GGC CCC TTT TCT GAA TAC GGG TCA CCT CTG GCA TGC GAG GTC CCA AAA ATG GAA AGT GGG GCG TGT GAT GGT CTG TGC AGT ATG CAC ATA ATT TTT GCA GAT GAC GCG AAA ATT CTA AGA GGA AGA GCT TGC AGG GTT
Stromatinia cepivora & Pythium violae	5’ AGT TCC GCA CAC ACA CAT TGC TGT GCA CAC ACA AGA CCA ACC TTC GTT CCT CCT CCG CCA CAG CGC AGC GTC CGA AAA CAC CAC AGT AGA AAA TTT TGA CAG AAG CAC ATC GAG AAC CTG TAA CGA GAG ATA TTA CTA CGT TCA GGA CCC AAC GGC GCC GCC ACT GAT TTT AGA GTC TGC CAT TGC TGA CAT GGA CT 3’
Stemphylium vesicarium & Bacillus amyloliquefaciens	5’ CTC GGC AGG ATC ATA CGG GAG AAA CGC GGA CTT TAC CGG TGG AGC ACA GTC ATT ATT TTG ATT TCG CCG GTC CAT AGC ACT CAT AAG GTT AGT AAT AAC TGT AGC ACC TCA TAA TGA CAT TTG CCC ATA CGG AAG AAC ATA CAA ACT TAC TTA TTA TTA ATA TAT TAA CAA TAA GCT AAA ATA ACT TTG AAT TCG TAT ATT TTA TTA GTT AAA CAC ATC TAA CTA AAA GTT TCG CCT GTA CAT TAA GCA GCA TTT CAG CCA CCC ATC AGT GAC CCA GTC TGT AGC GAC CCT TTA 3’

Verticillium dahliae	5’ GTA GGA TTT CGG CCC AGA AAC TCA ATA GGA ATA CTA GTG CAC CGC AAG CAG ACT CTT GAA AGC CAA AAC AAA TAG CGT TCA AGT ATA TAT AGT ACT TTT AAG AGT GAA ATA TAC TAA GGA CGA TAC GCT CTT TCC AGT GCA CTA AGA AGA GTA ACG GGA AAC GGC 3’
Verticillium albo-atrum & Fusarium oxysporum fsp. cepae	5’ TGC ATG ATC TAC GTG CGT CAC ATG CAG TAC GTC CAT CGC CAA TCG ACA ACA TGG CCA CCC GAA CCT CTG TCT CGC TTT TGG TCG TTC AGG TCA CCT GGA CGA TGT AGG TTT GTA TGC CTC CAA TGG AGA ATC GTT CAT AAA TGC GGT AAT TCA GTA GCG AAT TGC CAG CCC GCT CAT ACG GAG GGT TTC GGG AGA ATG TTC TAG CAT AAC CTA GAG GTT CGG CAC TAG CTC AGA TTC AGT AGA CCG CTG TTG 3’
Verticillium longisporum & Fusarium oxysporum	GCC TGG CTA TCC GGA CCT CTG TCT CTC TTT TCG TTC AGG TCA CCT GGA CGA TGT AGA TAG ATG ATG TCG CTG GCC GCA TAA GAA TAT CGC ATA GAA AGA GAT GTA AAG AGT TAT AGT GGT CTG ATG CTT TGT TGG ATC GAT TTG GGT TTC GCA CGG CGC GGC CAA CGA AGA AAC ATC GCG GAA AGG GGA GCA TC 3’
16S rRNA	5’ CGG TGG AGC ATG TGG TTT AAT TCG ATG CAA CGC GAA GAA CCT TAC CTG GTC TTG ACA TCC ACA GAA CTT TCC AGA GAT GGA TTG GTG CCT TCG GGA ACT GTG AGA CAG GTG CTG CAT GGC TGT CGT CAG CTC GTG TTG TGA AAT GTT GGG TTA AGT CCC GCA ACG 3’
18S rRNA	5’ ACG GGG AAA CTC ACC AGG TCC AGA CAA AAT AAG GAT TGA CAG ATT GAG AGC TCT TTC TTG ATC TTT TGG ATG GTG GTG CAT GGC CGT TCT TAG TTG GTG GAG TGA TTT GTC TGC TTA ATT GCG ATA ACG AAC GAG ACC TCG GCC CTT AAA TAG CCC GGT CCG CAT TTG CGG GCC GCT GGC

	TTC TTA GGG GGA CTA TCG GCT CAA GCC GAT GGA AGT GCG CGG CAA TAA CAG GTC TGT GAT GCC CTT AGA TGT TCT GGG CCG CAC GCG CGC TAC ACT GAC AGG GCC AGC GAG TAC ATC ACC TTG GCC GAG AGG TCT GGG TAA TCT TGT TAA ACC CTG TCG TGC TGG GGA TAG AGC ATT 3’

2. Metabarcoding Primer sequences

Nex_ITS1R_Wobble (10µm) (Toju *et al.*, 2012)
CWGYGTTCTTCATCGATG

Nex_ITS1_Ky02F (10 µm) (Toju *et al.*, 2012)
TAGAGGAAGTAAAAGTCGTAA

806R (Apprill *et al.*, 2015)
GGACTACNVGGGTWTCTAAT

515F (Parada, Needham and Fuhrman, 2016)
GTGYCAGCMGCCGCGGTAA