The Ecological Impacts of a Novel Nematode Control Agent

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April 2017

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A thesis submitted to Lancaster University in fulfilment of the requirements for the degree of Doctor of Philosophy.

Centre for Global Eco-Innovation

This project was supported by the Centre for Global Eco-Innovation and is part financed by the European Regional Development Fund.

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DECLARATION STATEMENT

I declare that the work presented in this thesis is my own, except where acknowledged, and has not been submitted elsewhere for the award of a degree of Doctor of Philosophy.

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April 2017

ABSTRACT

Plant-parasitic nematodes are prevalent in many soils and impose an economic burden worldwide on agriculture, through yield reductions and cost of control. There is a prominent need for the development and implementation of sustainable control mechanisms, to reduce the widespread use of hazardous nematicides. The incorporation of less hazardous nematicides has been suggested as a possible step to move towards this. This study aimed to address commercially relevant and ecological important questions on the use of a potential novel nematode control agent, BGT, as developed by Arcis Biotechnology Ltd.

In laboratory assays, bacterial species and a yeast showed different levels of susceptibility to BGT exposure. It is difficult to extrapolate the concentrations used to that at which the product would be in the soil but these studies suggest that soil application would likely cause changes in the microbial community structure. Soil samples were taken from experimental plots on a UK potato field following treatment applications to explore both immediate impacts and recovery of microbial communities. The changes in functional bacterial diversity and metabolic potential were estimated using community-level physiological profiling (CLPP). Additionally, taxon-specific quantitative PCR was used to detect changes in the bacterial and fungal community structure.

The BGT treatments resulted in changes in the diversity of substrate utilisation as recorded by CLPP. Differences in utilisation patterns indicate repeated BGT treatments of 4 L/ha are likely to change the bacterial community structure. The use of qPCR showed that BGT treatments at 8 L/ha led to changes in the relative abundance of bacterial and fungal taxon groups. Although no significant changes in total abundance of bacterial communities were detected and current theory suggests that a small loss of diversity may not have a major impact on current soil functioning, it may impair long term soil health and ultimately productivity.

In controlled toxicity assays, BGT was found to be of relatively low toxicity to earthworms, suggesting it is of low risk to terrestrial organisms. When applied directly to seed of wheat and tomato, BGT caused some reduction in seedling growth but treatments did not appear to cause any phytotoxic effects on wheat plants in glasshouse trials or on potato crops in field trials following both pre-planting and repeated application during plant growth.

ACKNOWLEDGEMENTS

This work has been supported by the Centre of Global Eco-Innovation and Arcis Biotechnology Ltd. I would also like to extend thanks to those at Barworth Agriculture Ltd for the provision of the field trial and the collection of the agronomy data.

I would like to give a sincere thank you to both of my supervisors, Dr Mike Roberts and Professor Ken Wilson for giving me this opportunity, and their continuing advice, patience and support.

During my time, studying and working at the Lancaster, I have been fortunate to receive the support and friendship of many staff and students including but not limited to; the members of lab A14, members of the Insect and Parasitology Group (IPEG), the inhabitants of PGR office B31a and all those in LEC's Enterprise and Business Partnerships team.

I owe so much to my family, Mum, Dad and Richard a.k.a. Team 238. I do not know where I would be without their continual love, support, advice, humour and cups of tea. In addition, I am so grateful to have received the encouragement and support from so many friends who have provided a listening ear and cheered me on to completion.

My final thanks go to my most dedicated supporter, Jonathan, for never letting me believe I was not able to succeed.

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1. GENERAL INTRODUCTION

Plant-parasitic nematodes (PPN) are prevalent in many soils and cause a significant loss of profits worldwide in agriculture, forestry and horticulture, through yield reductions and cost of control. Many economically important and life sustaining crop plants including wheat, rice, sugarcane, cotton and soybean are susceptible hosts (Sasser and Freckman, 1987). Their management presents significant challenges to sustainable agricultural practices and has relied heavily on synthetic chemical nematicides, many of which are known to pose significant risks to environmental and human health (Barker and Koenning, 1998; Zasada et al., 2010).

The industrial partner to this PhD project, Arcis Biotechnology, have developed a potential novel nematode control agent, named here as BGT, which contains a novel mixture of broad spectrum antimicrobials, and non-ionic and cationic surfactants It is thought that given the known properties and relatively low toxicity of the products' components, the product may offer a less hazardous form of control. This thesis therefore addresses this hypothesis, as well as potential secondary benefits of the product for plant growth.

This introductory chapter outlines the importance of sustainable agriculture and the difficulties in achieving this in the context of the control of PPN and the reliance on potentially hazardous pesticides. The release of synthetic chemicals into the terrestrial environment may result in a number of changes

and effects. The current regulatory risk assessments for terrestrial ecosystems for the registration of plant protection products are carried out in defined categories of: terrestrial vertebrates, bees, other arthropods, soil organisms, and non-target plants (European Commission, 2002). There is discussion amongst ecologists as to whether these tests are appropriate and sufficient to fully explore the environmental risks posed by existing and novel products, particularly in regards to the soil microbial community (Karpouzas et al., 2016; Jacobsen and Hjelmso, 2014). However, it is widely recognised that there are still many gaps in the knowledge regarding soil microbial communities and functioning which hinder our ability to assess the effects of agricultural practices (Bardgett and van der Putten, 2014; Baveye et al., 2016; Benbrook, 2017). Section 1.9 of this introduction discusses the importance of soil microbial communities and current opinions of how this is related to vital functioning processes.

1.1 Sustainable Agriculture and Crop Pests

As the global population continues to increase, there is an increasing need to ensure that the agricultural sector will continue to provide food for all (FAO, 2016). A critical focus in this aim is to establish agricultural practices that do not have adverse effects on the environment, but are also accessible and effective for growers (Godfray et al., 2010). Plant pests and diseases are a major challenge in this quest. It is estimated that there are close to 9000 species of insects and mites, 50,000 species of plant pathogens and 8000

species of weeds that threaten crop production across the globe (Pimentel, 1997). The potential lack of controls, but also the resulting adverse effects from control attempts, threaten the sustainability of food production. There are increasing regulatory as well as some consumer pressures to reduce the use of synthetic pesticides and initiate greater usage of integrated pest management (IPM). The Food and Agriculture Organization of the United Nations (FAO) defines IPM as “The careful consideration of all available pest control techniques and subsequent integration of appropriate measures that discourage the development of pest populations and keep pesticides and other interventions to levels that are economically justified and reduce or minimize risks to human health and the environment” (FAO, 2017).

1.2 PESTICIDE USAGE

Pesticides play a major role in modern agricultural systems. Eighty-five percent of global pesticide production is used in agriculture (Tilman and Clark, 2015). Many farming practices have co-evolved for so long with the availability of effective synthetic chemical pesticides so it is difficult to evaluate what large-scale commercial production would be like without them. One estimate suggests that without pesticide application agricultural yields could decline by 78% in fruits, 54% in vegetables and 32% in cereals (Cai, 2008). Pesticides can be defined as any bioactive, toxic substance that is released with the intention to kill or control an organism, although typically the term pesticide is used to refer to synthetic chemical substances.

In 2011, statistics reported by the FAO, showed the highest amount of pesticide usage per year was in Asian countries (approx. 2 million tonnes), followed by countries in North and South America (approx. 900,000 tonnes) and European countries (approx. 400,000 tonnes) (FAO, 2014). Within Europe, Italy, Germany, Spain, France and the UK are the major pesticide consumers (FAO, 2014).

Many traditional synthetic chemical pesticides present a major risk to both environmental and human health. It is estimated that pesticides are responsible for 20,000 acute poisoning deaths each year, 99% of which occur in developing countries due to weaker safety and environmental regulations (WHO, 1990). There are also likely just as many, or more, cases of milder intoxications and illness as a result of chronic exposure (Ecobichon, 2001). Many pesticides are environmentally-hazardous and are a cause of concern for the pollution of groundwater (Mauffret et al., 2017), pollution of possible sources of drinking water (WHO, 1990) and the decline of beneficial insect populations (Tilman and Clark, 2015).

The European Union directive 91/414/EEC greatly affected the availability of pesticides in the EU, by increasing restriction limits on the registration of new pesticides and the phase-out of conventional products characterised by a high environmental impact (European Commission, 1991). Additionally, the EU Directive 2009/128/EC strongly encourages the use of non-chemical methods in agriculture (European Parliament, 2009). Despite this, many pesticides that have proved to be hazardous are still widely used. In a recent study, Hossard et al. (2017) found that despite the adoption of an environmental plan to

reduce pesticide usage in France, introduced in 2008, there are no clear indications that practices are actively being changed. There was no change in pesticide sales on a national scale between 2001 and 2014, and there has been no detected reduction in pesticide water pollution, which is detected in 93% of French water courses (Hossard et al., 2017). This is indicative of the wider pesticide usage across Europe. Although some countries have shown a decline in usage, from 2011 to 2014, overall pesticide sales increased from 381,261 tonnes to 395,768 tonnes, across the current 28 member states of the European Union, Norway and Switzerland (Statistical Office of the European Communities, 2017).

The control of plant parasitic nematodes has relied heavily upon chemical pesticides or nematicides which provide reliable control in intensive farming systems, but there is an increasing need for growers to move towards less hazardous, sustainable control strategies. Many agricultural systems have developed with reliance on an array of broad-spectrum pesticides and it is unlikely there will be a rapid transformation to systems that do not rely on any chemical input. Less hazardous pesticides may offer less reliable control, but they may offer a half-way solution with the increasing introduction of more integrated pest management (IPM), which incorporates a wider range of alternative forms of control.

1.3 PLANT PARASITIC NEMATODES

Nematodes are the most numerous multicellular animals on Earth, with nearly 20,000 species within the recognised phylum Nematoda. They occupy a wide range of niches within soil, and fresh and salt waters. Nematodes are the most abundant metazoan in the soil, closely interacting with many other soil organisms and acting as a vital component in soil food webs (Thorne, 1961). Their activity affects primary production, energy flows and nutrient cycling (Sochova et al., 2006). Some are free-living, feeding on microorganisms, whilst others attack and parasitize living plants and animals (Thorne, 1961; Neher, 2010).

1.3.1 Ecology of Plant Parasitic Nematodes

Plant parasitic nematodes (PPN) are obligate parasites mostly within the order Tylenchida and a small number in the order Dorylaimida (Blaxter et al., 2004). Very little is known of their natural distribution, as areas studied have been disturbed by the global transportation of plant root material (Coomans, 2002). Soil nematodes are between 30-1000 µm in length and 15-35 µm wide. PPN may be distinguished by the possession of a mouth spear. This is typically a hollow structure but some have a solid modified spear. They move through the soil using water films surrounding soil particles to move through existing pore spaces (Thorne, 1961). In brief, the life cycle of a nematode consists of four juvenile stages each followed by a moult, and adult stage when reproduction occurs. There is some growth throughout the juvenile stages but juveniles are similar in appearance and biology to adults. The life cycle under optimal

conditions may be two to four weeks from egg to egg but this is longer in cooler temperatures (Neher, 2010).

Some phytoparasitic species act as ectoparasites, moving from host to host feeding externally on plant cells through spears or stylets. This can cause root morphology abnormalities and reduce root function. Endoparasitic nematodes, typically enter one host plant in their life cycle where they can cause major morphological and physiological abnormalities in the root. They enter the plant tissue and disrupt cell walls as they migrate, causing major changes to root cell development to initialise a feeding site. In addition to direct injury to the plant, nematode infestations are often associated with secondary infections of pathogenic bacteria and fungi that are able to invade a weakened plant (Neher, 2010; Barker, 2003).

PPN are most abundant in the top 15 to 30 centimetres of soil where their distribution is usually irregular and clustered around roots of susceptible hosts. This is due to the attraction to the plant exudates in the rhizosphere and the rapid reproduction permitted by the high food supply. There are also significant population fluctuations over time (Neher, 2010; Neher et al., 1995). Some nematode species are known to be influenced by substances that stimulate hatching, but most eggs have been found to hatch freely in water without any special stimulus (Perry and Wesemael, 2008). Some are also known to use quiescence as an adaptation for survival in fluctuating environmental conditions or absence of a suitable host (McSorley, 2003).

1.3.2 Damage Caused by Plant Parasitic Nematodes

Although many PPN's have specialised parasite-host interactions, most of the symptoms, particularly aboveground, are general, non-specific signs of plant stress including stunting, wilting and chlorosis. The only unique attributable visible character is the possible presence of root galls or cysts (Barker, 2003; Kommedahl, 1991). Subsequently, infestations may go undiagnosed at low levels. There are also few useful morphological and anatomical diagnostic characters to distinguish between species due to their small size and simple anatomy which can hinder specific diagnosis, although advances in molecular methods are being used to further developments in this area (Zasada et al., 2010). Perhaps ironically, it was not until the introduction of fumigant nematicides in the 1940s, when farmers and scientists began to fully realise the damage and crop losses caused by PPN (Barker and Koenning, 1998).

As with many other agricultural issues, there are a range of estimates of the yield losses and cost attributed to PPN. Most nematology publications cite the international opinion survey of 371 nematologists carried out in 1987 which estimated an imposed yield loss of approximately $78 billion US dollars worldwide (Sasser and Freckman, 1987). In 2003, an inflation-adjusted figure of $125 billion US dollars was quoted based on the 1987 estimation (Chitwood, 2003). This is thought to equate to 5-12% loss of crop yields worldwide. Precise predictions of losses due to PPN can be difficult to predict as nematode survival and reproduction is subject to many environmental factors and nematode-host interaction. Subsequently, damage may vary temporally and spatially across climatic regions and soil types (Zasada et al.,

2010; Sasser and Freckman, 1987). The expansion of global trade has increased the importance of sustainable crop and pest management systems and associated pest quarantines. Shipments of equipment and agricultural produce risk new infestations of diverse PPN species globally (Barker and Koenning, 1998; Smith et al., 1992; McNamara, 2004; Nicol et al., 2011).

Notable groups include the *Meloidogyne* genus, commonly named the root-knot nematodes. They are the most widely distributed group of plant parasitic nematodes (Nicol et al., 2011; Sasser and Freckman, 1987). *M. javanica*, *M. arenaria*, *M. incognita* and *M. hapla* contribute to more than 90% of the crop losses caused by the group of 55 root-knot nematodes (Greco and Esmenjaud, 2004). *Meloidogyne* sp. have particularly wide host ranges; the majority of flowering plants are thought to be susceptible hosts and species are able to thrive in a diverse range of habitats and soil types. Only one other nematode (*Rotylenchus reniformis*) and certain arbuscular fungal species have similarly wide host range as phytoparasites (Nicol et al., 2011).

Two genera, *Globodera* (potato cyst nematodes) and *Heterodera* (soybean and cereal cyst nematodes) are considered to be the most economically important due to their prevalence and hosts (Jones et al., 2013). They are both endoparasitic and pose a challenging and serious threat to modern agriculture due to their successful life history strategies, and the lack of effective sustainable management strategies (Eves-van den Akker et al., 2015; Nicol et al., 2011). In the next section I principally discuss the *Globodera* nematodes and their threat to UK potato production.

1.3.3 Plant Parasitic Nematodes and UK Potato Production

Two *Globodera* species, *G. rostochiensis* and *G. pallida*, are potato parasites, both prevalent across soils in temperate climates (Greco and Esmenjaud, 2004). Alongside potato blight (*Phytophthora infestans*), they are considered the biggest threat to UK potato production. The UK is the world's 11th largest producer of potatoes. In 2007, the harvest was 5.6 million tonnes, with average yields exceeding 40 tonnes per hectare which is currently lower than domestic demand (Gartner and Blok, 2016; Trudgill et al., 2014).

Globodera rostochiensis and *G. pallida* are both thought to originate, like the potato, in South America and were introduced to the United Kingdom around 1850 (Jones et al., 1970). The two species differ in their host interactions. There are also different known strains or pathotypes within each species which are distinguished by their response to their host and in their virulence (Kort et al., 1977; Trudgill et al., 2014).

At the start of the last century there were increases in potato production across the UK, leading to a peak growing area of 520,000 hectares in 1948, due to the increase in demand for domestic produce during the Second World War (Minnis et al., 2002). This also aided the widespread distribution of potato cyst nematodes (PCN). Intensification practices meant PPN numbers increased beyond a natural threshold level. At low population levels, numbers are regulated within the soil ecosystem and host crops can tolerate the small amount of parasitism as one of the many symbiotic relationships with the soil community without significant impacts on plant growth (Trudgill et al., 2014; Neher, 2010).

The area across the UK used for growing potatoes has halved since 1948 but PCN populations only show small declines in the absence of a host crop as cysts remain dormant in soils. Dormancy is commonly 7-10 years but viable cysts have been found in fields up to 20 years after the planting of a known susceptible host (Gartner and Blok, 2016). Infestation may have been further exacerbated as reductions in growing areas may have resulted in shorter crop rotations. In 1998, a survey of potato growing areas found PCN present in 64% of 484 sites sampled across England and Wales (Minnis et al., 2002). This was an increase from the last comparable study (Hancock, 1996).

Free-living nematodes (FLN) are a lesser but still significant concern for potato growers. They affect a relatively smaller area but marketable yield losses of 30% are common. *Trichodorus* sp. and *Paratrichodorus* sp. are common in sandy soils in parts of the UK that are used for potato production (Minnis et al., 2002). Whilst they cause direct feeding damage to plant roots they are a large concern as vectors of tobacco rattle virus (TRV). The virus results in reduced quality and subsequently value of the crop as they cause visible brown arcs known as ‘spraing’ on the tubers (Cooper and Harrison, 1973).

Within the European Union, PCN are considered well-established and are a high biosecurity risk in international trade. Quarantine control and movement is regulated by more than 100 countries (Smith et al., 1992). Regulatory actions imposed to minimise spread have included banning host crop production and promotion of integrated management systems (Greco and Esmenjaud, 2004). PCN will be discussed in greater detail in Section 4.1.3.

1.4 CONVENTIONAL NEMATICIDES

There are four main groups of chemicals that have been traditionally used in nematode control: halogenated hydrocarbons, isothiocyanates, organophosphates and carbamates. The first two are commonly grouped as fumigant nematicides and the latter two, non-fumigants. For many years, halogenated hydrocarbons were the main agents used and provided a highly efficient form of control but with some serious consequences. They are injected into soils before planting and act by disrupting the function of membranes and nervous systems. Due to their mode of action they have a broad range of toxicity (Zasada et al., 2010). The use of 1, 2-dibromo-3-chloropropane (DBCP) was banned almost worldwide in 1985, after it was linked in several cases to human male sterility (Hofmann et al., 2006; Olsen et al., 1995). Methyl bromide (CH_3Br) was widely relied upon in plant protection, yet it acts as a powerful ozone-depleting substance. Following the Montreal Protocol in 1992, all countries involved agreed to completely phase out the use of the substance as an insecticide by 2005 for developed countries and 2015 for developing countries (Thomas, 1996; UNEP, 1992).

Non-fumigant nematicides are usually cholinesterase inhibitors, which act by preventing the breakdown of acetylcholine in synapses. As a result, many products are considered to be of high risk to human health (Vandekar et al., 1971). The past decade has seen many products of this type withdrawn or be subjected to increasingly restricted usage.

Table 1.1 shows the nematicide products currently available for the use on potatoes. Notably, most of the products require a long harvest interval, which is the mandatory interval between treatment application and harvest to avoid residues in food crops. In 2014, a fatal industrial accident in the production of Vydate® 10G and subsequent closure of the production plant, meant farmers were forced to find alternatives for PCN control in spring 2016. Products with longer harvest intervals such as fosthiazate can be challenging for some growing early season potatoes which meant it was not a viable substitute for some growers at short notice (Briggs, 2015).

Changing products and management strategy can be costly for growers as it can require changing machinery, rotors and calibration. It is also not without risk that the product will not provide adequate control in their field leading to crop failure. In 2015, a novel biopesticide, NEMGuard®, based on garlic extract was given emergency approval for PCN and FLN control on potatoes, due to lack of control options (HSE, 2015). This was followed by full registration in 2016 (Certis, 2016). This incident highlights the prominent need for the development of safe, effective nematode control methods.

Nematicides in general, reduce the nematodes' ability to locate plant roots or cause a temporary inhibition of the hatching of cysts. They can also reduce the level of root invasion by PCN larvae. In most cases, nematicides are not relied upon to completely eradicate a PCN population, but instead maintain parasitism levels below a tolerable threshold at crucial times (Barker, 2003). Subsequently, the potato plants have several extra weeks of growth without invasion. This means they are stronger and more tolerant of nematode

damage when the nematicide dissipates or degrades. The use of nematicides allows for potato production in fields with higher PCN infestation levels than would otherwise not be viable. It is estimated that without the use of nematicides and further successful control intervention, potato growers with PCN infested fields would see a 60% decline in yield within 10 years (Clayton et al., 2008).

Table 1.1: Commercial products currently available in the UK with registered approval for control of potato cyst nematodes (PCN) and free-living nematodes (FLN) on potatoes.

Product	Active substance	Harvest interval
Vydate® 10G (DuPont-Dow Industries)	Oxamyl (10 % w/w)	80 days
Nemathorin® 10G (Syngenta)	Fosthiazate (10% w/w)	119 days
Mocap® 15G (Certis Europe)	Ethoprophos (15% w/w)	80 days
NEMguard® PCN Granules (Certis Europe)	Garlic extract	NA

1.5 NON-CHEMICAL NEMATODE CONTROLS

Many possible alternatives to synthetic chemical nematicides have been documented. Over a century ago, in 1889, there were reports on the potential use of sterilisation of soil, rotation with non-host plants, trap crops and botanical based soil amendments (Barker and Koenning, 1998). However, the success of these methods is often more variable than the broad spectrum chemical nematicides that many modern systems have become reliant on. Some of the methods are briefly discussed here, principally in relation to their success in controlling potato cyst nematodes.

1.5.1 Crop Rotation

Crop rotation is a crucial part of PCN management. Pest population growth is slowed or halted without a suitable host and populations of potato cyst nematodes can decline at an approximate rate of 20-30% each year a suitable host is not present (Clayton et al., 2008; Lechenet et al., 2014; McSorley, 2011; Atkins et al., 2003; Davis et al., 2003; McSorley, 1996). However, population numbers swell dramatically when a susceptible potato crop is planted. Evidence of efficacy of crop rotation as a management strategy is still mixed and depends heavily on initial population numbers as well as rotation length (Clayton et al., 2008; Lechenet et al., 2014; McSorley, 2011; Atkins et al., 2003; Davis et al., 2003; McSorley, 1996). Adequate rotation length is crucial and can place financial pressure on growers. The sole use of crop rotation has been found to manage PCN population levels below a damage threshold on infested fields when potatoes are only used in 1 in 12 rotations.

However, this would only allow 3 potato crops in a 25-year period (Wright et al., 2017).

1.5.2 Trap Cropping

The use of *Solanum sisymbriifolium* (sticky nightshade) as a trap crop is documented as a possible PPN control. The trap crop stimulates the hatching of eggs. The nematodes then attempt to feed on the roots which contain substance toxic to the nematodes. This method imposes a large cost to establish and may result in further income losses due to reduction in growing space, time and resources which may otherwise produce a cash crop. For this reason, it may be difficult to implement on a large commercial scale (Hauer et al., 2016; Dias et al., 2012; Scholte and Vos, 2000; Scholte, 2000).

1.5.3 Steam Sterilisation

Soil steam sterilisation is a technique that has been used for decades in both open fields and greenhouses (Zasada et al., 2010; Greco and Esmenjaud, 2004; McSorley, 1996). The hot steam causes the physical degeneration of cells. It has commonly been a suggested replacement for the soil fumigant methyl bromide (Greco and Esmenjaud, 2004; McSorley, 1996; Zasada et al., 2010). There is some variety in method which may involve injection of superheated steam (180 – 200°C), sheet steaming using insulation fleece or depth steaming with the use of drain and vacuum systems. Steam sterilisation provides a quick and secure method for removal of pests but is non-selective method. Further inputs may be required to re-introduce microorganisms that support plant growth. Whilst useful for nursery crops it is impractical for large

areas and would place an increased carbon cost on potato production (Runia and Molendijk, 2010).

1.5.4 Resistant Cultivars

Long-term planting of resistant cultivars can cause shifts in nematode species or sub-species and can lead to the occurrence of multiple nematode species in one field. Resistant cultivars may have inferior quality or lower yield compared to susceptible cultivars (Young, 1992). The first British potato cultivar with resistance to *G. rostochiensis* pathotype Ro1 was introduced in 1966 (Clarke and Shepherd, 1966). The repeated use of the resistant cultivar was successful in reducing the damage caused by *G. rostochiensis* populations but this then led to selection favouring *G. pallida*, for which there are only 'tolerant' potato varieties (Brodie, 1996; Minnis et al., 2002). It is estimated that a quarter of the UK land used for potato growing is infected by both *G. rostochiensis* and *G. pallida*, so the use of a cultivar resistant to only one is not a long term viable strategy, but could be a useful tool especially used alongside crop rotation. Increased efforts may also be needed to make resistant cultivars that are commercially competitive with more desirable, productive but susceptive cultivars (Davies and Elling, 2015).

1.5.5 Biopesticides and Bio-Based Soil Amendments

Biopesticides are recognised as an important part of integrated pest management (IPM) systems but there has been a relatively poor uptake in the UK with relatively few products successfully commercialised (Chandler et al., 2011). There have been several different organisms and natural extracts

identified with the potential to act as a PPN control (Chitwood, 2002; Chitwood and Meyer, 2014). However, very few have resulted in a registered control agent for widespread use. As listed in Table 1.1, a garlic-derived polysulfide product was given emergency approved for control of PCN on potatoes in 2015, followed by full registration in 2016. The use of nematophagous fungi is widely explored and *Paecilomyces lilacinus* 251 is registered as an active substance for the control of nematodes with a few products now approved for select usage in Europe. There are no products based on the fungi currently available for use in the UK. Whilst biopesticides offer a promising alternative control, there are many reasons cited for the lack of development of products, including inconsistent performance in the field, which slow and prevent widespread implementation. There are also attempts being made to improve the regulatory process for biopesticides as currently they are assessed under the same criteria as synthetic chemicals which many deem unsuitable (Villaverde et al., 2014; Chandler et al., 2011).

1.6 THE TEST PRODUCT: BGT

BGT contains a novel mixture of broad spectrum antimicrobials and non-ionic and cationic surfactants (Table 1.2). The product was initially developed in a group of antibacterial products by Arcis Biotechnology. In a series of experiments to explore applications, researchers at Arcis Biotechnology found the product to have nematicidal activities and began to investigate the potential of BGT as an agricultural nematicide (personal communication). At the beginning of this PhD project there had been a small number of trials

carried out but the product is not a registered nematicide. The mode of the nematicidal action is not completely understood and it is believed it could be a combination of the antimicrobial and surfactant properties of BGT.

The components of BGT have been commonly used in many other applications but not within an agricultural product. Previous uses and studies of the components, many of which are discussed in this section, help to inform the possible effects of the whole product, but it is important to test these in combination as would be supplied as it is likely that the components may affect the activity and persistence of one another. The studies in Chapter 2 seek to address this.

1.6.1 Surfactants

Some of the BGT components are known to have surfactant properties. Surfactants, surface acting or wetting agents as they are referred to across relevant literature, may be defined as substances with molecules containing both a hydrophobic and hydrophilic group, which alter the energy relationships at the interfaces between two media, reducing surface tension (gas-liquid interfaces) and inter-facial tension (liquid-liquid and solid-liquid interfaces) (Parr and Norman, 1965; 1964). There are a broad range of substances with surfactant properties that are now widely used in almost every modern industry. They are commonly found in a wide range of products across food, cosmetic and industrial cleaning industries as agents to promote solubility or preservation (Banat et al., 2000).

Table 1.2: The composition of BGT and the volume of active ingredients.

Percentage active refers to the percent at which the substance is present in the product solution (Column 2) it is commercially available in.

Chemical	**Product used**	**Percentage active**	**Amount used in 100% BGT (mg/ml)**	**Active component in BGT (mg/ml)**
Polyhexamethylene biguanide (PHMB)	Vantocil™ IB	20	20	4
Benzalkonium chloride (BAC)	Barquat® 50-65	50	20	10
Alcohol ethoxylate (AE)	Surfac UN65	100	4	4
Dimethyloctadecyl [3-(trimethoxysilyl) propyl] ammonium chloride (Si-QAC)	AEM5772 Antimicrobial	100	8	8
Alkyl polyglucoside (APG)	TRITON™ CG-50	50	16	8

In agriculture, surfactants are used to dilute and aid even dispersion of a range of agrochemicals, as they lower surface tension which promotes better coverage. Surfactant compounds are also found to extend shelf life and reduce pesticide degradation due to sunlight exposure (Yusoff et al., 2016). In herbicides particularly, they are used to promote penetration and foliar uptake of an active substance into plant tissues to improve efficacy (Forster et al., 2004). Surfactants are commonly classified as anionic, cationic, non-ionic, or ampholytic (capable of acting either as anionic of cationic), depending on the nature of the electrical charge, or absence of ionization, on the hydrophilic portion of the molecule.

1.6.2 Test Product Components

Poly (hexamethylene) biguanide hydrochloride (PHMB) (CAS Nos. 32289-58-0 or 27083-27-8, INCI Polyaminopropyl Biguanide), acts as a broad spectrum cationic antimicrobial agent. BGT contains Vantocil™ IB Antimicrobial; a 20% aqueous solution of PHMB (Lonza, 2017b). Cationic surfactants can be readily adsorbed into soil and sediment due to the opposing electrical charge. They can also be used to modify the soil surfaces and promote the sorption of hydrophobic organic compounds (Brownawell et al., 1990; Wagner et al., 1994). It has a wide range of activity, effective against Gram positive and Gram negative bacteria, fungi, and yeast (Allen et al., 2004; Mashat, 2016).

There are no reported cases of acquired bacterial resistance to PHMB. It is freely soluble and stable in solution, so is commonly applied in a wide range of water-based products susceptible to microbial growth (ECHA, 2011). It has been used in several applications including; as an antimicrobial agent in

treated dressings, eye drops, contact lens cleaning solution, and swimming pool water treatment as it is effective against *Legionella pneumophila*, the bacterium which is the cause of Legionnaire's disease (Gao and Cranston, 2010; Kusnetsov et al., 1997; Roth and Brill, 2010). It is also used a preservative in cosmetic products. It induces cell death by disrupting the ion balance in cell membranes (Kaehn, 2010).

A review by the US Environmental Protection Agency (US-EPA) noted that with the exception of occupational users, the polymer has a very low aggregate risk of adverse health effects to the public or the environment (US-EPA, 2005). However, there is some concern as to its possible adverse effects as a contaminant in stream water as it has a relatively high toxicity to some aquatic species (Lucas, 2012).

The next component, Barquat® 50-65, is a blend of benzyl ammonium chlorides (BAC) (CAS No. 68439-45-2). It is a 50% aqueous solution of Alkyl (C12 67%, C14 25%, C16 7%, C8 10%, C18 < 3%), a quaternary ammonium compound which is known to act as both a biocide and a cationic surfactant (Attwood, 1985; Lonza, 2017a). Dissolution in water is slow but it is mostly supplied in aqueous solutions, for ease of use. The aqueous solution foams when shaken which causes BGT to foam. Like PHMB, Barquat® 50-65 is found in many consumer and industrial products including cosmetics, sanitisers and hard surface disinfectants as both a biocide and preservative (Gainor et al., 1997). BAC also acts to disrupt the cell membrane structure. It has been found to be fast acting with moderately long duration of action (Dyer et al., 1998).

Benzalkonium ammonium chloride (BAC) is listed as an unapproved active substance in the European Commission's pesticide database. However, it would appear that it has only previously been used in products used to disinfect surfaces and equipment used for food preparation rather than as a plant protection product applied to the growing crop or soil. In 2012, it was found that the products were leaving detectable residues on food, which is categorised under pesticide residues. Following this, new approaches to monitoring and enforcement of maximum residue limits (MRLs) were outlined in 2015 (European Commission, 2014).

Dimethyloctadecyl[3-(trimethoxysilyl) propyl] ammonium chloride (Si-QAC) (CAS No. 27668-52-6), added to the BGT in the form of AEM 5772 antimicrobial, is also a quaternary ammonium compound, which acts as an antimicrobial and cationic surfactant. It is produced by the addition of silane to a quaternary ammonium compound which creates greater surfactant bonding when applied to the surface creating a longer lasting antimicrobial (Isquith et al., 1972). It is commonly used in textile manufacturing as a biocide and as a surfactant to support the processing. It has also been used commonly as a surface disinfectant in food and medical sectors (Siddiqui et al., 1983). Si-QAC is listed as a corrosive chemical, however, the United States Environmental Protection Agency (EPA) reported that there was no suspected risk to human health (EPA, 2007).

Alcohol ethoxylates (AEs) (CAS No. 27668-52-6), added to BGT in the form of Surfac UN65, are non-ionic surfactants widely used in laundry cleaners and to a lesser extent in household cleaners, cosmetics and process industries such

as textile and paper production (Tolls et al., 2000). In 2009, it was reported that the current usage of AEs does not pose a risk to humans or the environment with regards to surface water and soil contamination, and are not deemed to be genotoxic, mutagens or carcinogenic. Surfac UN65, contains a C9-11 alcohol with 6.5 moles/litre ethylene oxide in a 65% aqueous solution to improve handling at cooler temperatures (Surfachem, 2017).

Alcohol ethoxylates have been found to undergo rapid and easy biodegradation in laboratory and field studies (Ang and Abdul, 1992; Dyer et al., 2006). This may be considered an environmental benefit of the product if the antimicrobial components remain in the soil for the duration needed to be effective against hatching plant-parasitic nematodes, but do not persist to cause long-term adverse effects on the soil community or subsequently fresh water communities. The product works to reduce the surface tension of the formulation, improving the emulsifying, spreading and wetting properties. It is included in BGT to act as a solvent to aid the inclusion of Si-QAC.

C8-C10 alkyl polyglucoside (APG) (CAS No. 68515-73-1) is a mild non-ionic surfactant used widely in household and industrial detergents. It is added to BGT in a 50% aqueous solution, TRITON™ CG-50. APG is derived from fatty alcohol and glucose derived from recyclable starch (Foley et al., 2012). It is considered to be non-toxic and easily degradable due to the biodegradable sugar compounds it is derived from, which has made it desirable for use. Zhang et al. (2011) describe it as “environmentally friendly”. Due to the low toxicity, it is often used to solubilise biological membranes (Santonicola et al.,

2008). It has previously been used in pesticide application, notably to enhance glyphosate in herbicide applications (Castro et al., 2014).

In single species laboratory testing APG is reported with low toxicity, yet a study in freshwater pond mesocosms suggested that APG-containing chemicals increase biological oxygen demand in lentic systems due to rapid biodegradation, which may have implications for freshwater community structures (Sutton and Cohen, 2012)

1.6.3 Previously Recorded Nematode and Plant Responses to BGT

Prior to the start of this PhD project, tests undertaken by the industry partner, Arcis Biotechnology Ltd, had found BGT to be lethal on a range of juvenile plant parasitic nematodes during *in vitro* mortality assays (personal communication). Following this, in February 2011 a series of trials were undertaken at the Agri-Food and Biosciences Institute (AFBI), Belfast, Northern Ireland on behalf of Arcis, to assess the efficiency of BGT on reducing PPN numbers in turf grass root-zones and examine any phytotoxic effects on turf grass (confidential, unpublished data).

In glasshouse dose-response trials, the efficacy of the agent was compared on four different soils: USGA (United States Golf Association) sand root-zone, 70:30 sand soil root-zone, loam soil and peat based compost. BGT was applied at concentrations from 0.001% to 10% at 500 l/ha, to inoculated dampened root-zones containing juveniles of nematodes belonging to the following genera: *Helicotylenchus*, *Tylenchorhynchus*, *Heterodera*, *Meloidogyne* and *Trichodorus*.

The results showed no difference in susceptibility to BGT across the different genera but there were significant differences in the numbers of nematodes recovered from the different soil types in comparison to the control treatment. BGT was most efficient in USGA sand where a significant reduction in nematode numbers was seen at 0.01% concentration. Efficacy of BGT was lower across the other soil types, with the lowest effect seen in the compost. It was suggested that it is possible that the organic matter content of the treated soil may affect the product's efficacy, possibly due to the surfactant properties of some of the agent's components (personal communication).

It was reported that BGT concentrations of 0.1% and above significantly reduced nematode numbers recovered. Dose-response tests were also carried out in a USGA sand-based rugby pitch. It stated that the product at concentrations of 1-10% applied at 500 l/ha reduced nematode levels equivalent to a registered furfural nematicide at 0.1%. Whilst BGT may appear less effective, it should be noted that furfural caused visible phytotoxic effects in the turf grass at concentrations above 0.1%. A small-scale replicated plot experiment tested the efficacy of BGT on nematode populations (both parasitic and beneficial) in a USGA sand-based rugby pitch. The 1% BGT treatment reduced nematode population levels. The reduction was equivalent to that caused by a commercially available furfural-based nematicide at 0.1% concentration.

Alongside this, observations were taken for possible effects on turf growth. When phytotoxicity in the field plots was examined, none of those treated with

the agent showed any adverse effects from the BGT treatment applied at 1-10% in a volume of 0.5 L/ha.

Pot tests were also carried out to explore whether the agent might be viable as a soil drench. Nematode-inoculated pots were soaked to excess with 10% solutions. They were allowed to drain and irrigated daily for five days with water, and grass seed added. Germination rates were compared after 14 days. No reduction in germination levels occurred in any of the turf grass species in treated soils. When pots containing 21 to 80-day-old turf-grass plants were treated with a 1% solution, there was no subsequent sign of phytotoxicity. However, when the same treatment was applied to 7 day old seedlings there was visible growth reduction and discoloration.

Nematode levels were reduced in all treated pots. From these initial results, there was some evidence to support the potential use of the novel control agent as a nematicide; however, it should be noted that whilst it was effective in USGA sand root zone, the capacity to kill nematodes was decreased in other soil types.

Given the results of this study it was decided there was a need to look the effects of BGT when applied in soils with a greater organic matter as it appears to affect the efficacy. It was suggested that the surfactant properties of some of the components could cause the product to aggregate more around organic matter preventing it move dispersing throughout the soil (personal communication). I also wished to explore the effect of BGT on other plant species.

The industry partner, Arcis Biotechnology Ltd, was planning to carry out various nematode assays alongside the studies discussed here. Preliminary results gathered by Arcis Biotechnology had shown that a 24 h exposure to BGT at 3.33% completely inhibited the hatching of *Globodera pallida* juvenile nematodes over 6 weeks; 100% mortality of newly hatched *G. pallida* juveniles was seen after 24 hours in a 2.08 x 10^{-1} % BGT solution. As the industry partner was carrying out a related research project on the effect on target and non-target nematodes, it was not the principal focus of this study. Within this PhD study I carried out a small experiment to explore the effect of BGT on the number of nematodes recovered from field soil (Section 2.2.5).

1.7 Understanding Of Soil Microbial Biodiversity And Function

This project will aim to explore the effect of BGT on non-target populations within the soil to begin to understand how the product may affect the whole agricultural system. Soil systems are diverse and complex, containing bacteria, protozoa, fungal hyphae and numerous other organisms, all interacting in multiple overlaying systems in a heterogeneous and discontinuous structure. Their functions control and influence a number abiotic and biotic factors that contribute to agricultural productivity. Indirectly, plants rely on the decomposer microbial community for the provision of carbon and other nutrients from organic material, and the soil structure and environment which is maintained by soil biota. More directly, many organisms including

plant growth promoting (PGPR) bacteria have been shown to influence plant productivity (Bardgett, 2005).

It is widely believed that the majority of soil microbial species and their functions are yet to be studied (Fierer et al., 2007; Huang et al., 2016). The complexity and remaining unknowns of the systems create a challenge for those trying to quantify and understand changes in the soil system for which there is an increasing demand, as their importance in the supply of ecosystem services and potential decline are recognised (Bardgett and van der Putten, 2014).

Agenda 21, prepared at the United Nations Conference on Environment and Development in Rio de Janeiro, 1992, stressed the importance of biodiversity in the functionality of a system (United Nations Division for Sustainable Development, 1992). Biodiversity provides many direct ecosystem services to humans including food, fibre, fuel and income, along with other, perhaps more vital, indirect services including recycling of nutrients, regulation of microclimate, control and mitigation of hydrological processes, regulation of pests, and detoxication and remediation of noxious chemicals (Altieri, 1999). Biodiversity in soil is considered a crucial factor in the proper functioning of soils (Bardgett and van der Putten, 2014; Baveye et al., 2016; Nannipieri et al., 2003). It has been estimated that the space occupied by microorganisms in the soil is generally less than 5%, yet it is thought that 80-90% of soil processes are mediated by the microbial community (Badalucco et al., 1994; Ingham et al., 1985). Ninety percent of the energy flow in soil passes through microbial decomposers (Nannipieri et al., 2003; Nannipieri et al., 1994). Soil

health and subsequently plant health, driven by numerous factors both above and belowground, are intrinsically linked with net income in agricultural sectors (Benbrook, 2017).

It is recognised that the abundance in aboveground diversity is linked with ecosystem functioning. However, the picture belowground is not as clear (Bardgett et al., 2009; Wardle et al., 2004; Bardgett and Shine, 1999). The testing of hypotheses in soil is constrained by two intertwined limitations; our limited ability to accurately measure community species composition and a lack of understanding about species involvement in functions (Nannipieri et al., 2003).

It has been argued that a relative decrease in species richness has little effect on soil functioning because of the large amounts of transient functional redundancy of many soil organisms (Jacobsen and Hjelmso, 2014). The “everything is everywhere” hypothesis suggests that changes inflicted on microbial communities are of minor influence as there is fast adaptation (Johnsen et al., 2001). However, it has also been proposed that there is likely a minimum number of species that should be present in a system to ensure functioning under “steady” conditions and larger numbers will better ensure the stability of the system in changing environments. This idea has been referred to as the “insurance” hypothesis (Loreau et al., 2001; Nannipieri et al., 2003).

Studies have reported varying relationships between soil biodiversity and functioning, but a lack of a common methodology and setup impair full

comparisons. Laboratory studies either take constructive or destructive methodology approaches (*i.e*. by introducing or removing specific groups) which can both show limited representation of natural communities (Jacobsen and Hjelmso, 2014). The study of constructed communities through inoculation methods are limited as it can only include organisms that are culturable in the laboratory, giving an unrealistically small microbial diversity (Griffiths et al., 2000). Whereas, deconstructive methods rely on the assumption of specificity of biocides to targeted groups. The biocides can be adsorbed by the soil or used as a nutrient source for other species creating unintended population shifts (Badalucco et al., 1994).

Positive relationships have been reported between bacterial diversity levels and increased degradation of specific compounds (Fredslund et al., 2008), nitrification, denitrification and ethane oxidation (Griffiths et al., 2001b; Griffiths et al., 2001a; Griffiths et al., 2000), substrate induced respiration (Griffiths et al., 2001a), and resistance to invasion of new microorganisms (Liu et al., 2012; van Elsas et al., 2012). However, in other studies, there was no effect of reduced species diversity on carbon mineralisation, nitrification or denitrification (Nielsen et al., 2011; Wertz et al., 2006), thymidine and leucine incorporation, heat stress, nitrate accumulation (Griffiths et al., 2001a) or the rate of decomposition of organic matter (Brookes, 1995; Giller et al., 1998). It must also be considered that functional traits of microorganisms may not correlate with phylogeny. However, there are a few documented well-established links which are now being utilised in methods, notably archaea and bacteria's capability of ammonia-oxidation which has been linked with functional genes amoA and amoB (Feld et al., 2015; Jacobsen and Hjelmso,

2014; Ruyters et al., 2013). Whilst our understanding and knowledge of soil diversity has progressed rapidly in recent years, there are still gaps in our knowledge which limit our ability to investigate and understand the effects of agricultural management strategies on the long-term health and functioning of soil communities (Bardgett and van der Putten, 2014).

1.8 THESIS STRUCTURE AND AIMS

This project was designed to aid and inform the development, commercialisation and potential registration of the novel nematode control agent, described here as BGT, by generating new knowledge on the effects of its application on microbes, animals and plants. The first stage of the project, discussed in chapter two aimed to assess the relative toxicity of the substance in order to be able to compare with other relevant substances. These results were also able to inform predictions made on the impacts of BGT on a soil community structure.

The studies in Chapter 3 aimed to examine any direct impact of BGT on plant growth through controlled growth experiments. These experiments were designed to help inform potential application methods of BGT. Given the surfactant properties of BGT, experiments were also conducted to examine the effect on soil moisture loss and the impact this may have on plant growth under reduced water availability.

Based on the knowledge gathered in Chapters 2 & 3, the studies in chapter 4 aimed to assess some of the impacts of BGT application when applied in a small-scale field trial. A community level physiological profiling (CLPP) technique using Biolog Ecoplates^{TM}, and the quantitative polymerase chain reaction (qPCR) where used to assess soil extracts for changes in the bacterial community structure with regards to relative abundances and diversity of functional (CLPP) or taxonomic (qPCR) groups. Additional data, which was collected by those running the trial is analysed and presented in this thesis to show the effects of BGT application on PCN populations and potato plant growth.

The final chapter aims to collate all the knowledge gathered on the potential impacts of BGT treatments in the wider context of the development of sustainable plant pest management and the risk assessments made on substances such as pesticides released into ecosystems.

1.9 OBJECTIVES

1. Establish a minimum inhibition concentration of BGT and its components on bacterial and fungal species within an *in vitro* toxicity assay in order to understand the possible susceptibility of bacterial and fungal organisms to BGT exposure.
2. Measure the toxicity of a BGT soil treatment on soil samples naturally containing nematodes, to further understand the response of nematodes to test product.
3. Establish the toxicity (LC50) of BGT on the model invertebrate test organism, *Eisenia fetida* in a contact filter paper assay and artificial soil assay to be able to compare the toxicity of BGT in comparison to other plant protection products.
4. Establish the effect of a range of BGT concentrations on seed germination and early seedling growth to assess for potential phytotoxicity which may occur when BGT is used as a soil drench pre-planting or at time of planting.
5. Measure the effects of the surfactant properties of BGT on the rate of loss of moisture from soil.
6. Test for phytotoxic effects of a range of BGT concentrations on wheat plants grown to seed in both well-watered and reduced watered conditions.
7. Use community level physiological profiling (CLPP) technique using Biolog Ecoplates™ to monitor potential changes in the functional diversity and metabolic potential of bacterial populations in BGT treated soil samples.

8. Use qPCR to monitor potential changes in the taxonomic diversity and relative abundances of bacterial and fungal populations in BGT treated soil samples.
9. Analyse data collected from the small scale potato field trial to assess the effectiveness of the BT treatment on potato cyst nematode populations and the subsequent impact on potato plant growth and production.

2. THE DOSE-RESPONSE OF TARGET AND NON-TARGET ORGANISMS TO THE TEST PRODUCT BGT IN CONTROLLED ASSAYS

2.1 INTRODUCTION

Any product released into the environment with a biocidal intention is expected to have a range of impacts on both the target and non-target organisms. The risk assessment procedures of pesticides are commonly based on species that have been intensively studied and are amenable to laboratory toxicological assessment (Jansch et al., 2006). The studies in this chapter aimed to begin to quantify the toxicity and dose-response of the product BGT on a range of species, including, bacteria, nematodes and earthworms (Objectives 1-3, Section 1.9). This will provide information on the biocidal activity of BGT and inform predictions made on the likely ecosystem impacts on the soil microbial community. Assays were carried out in controlled environments to compare the relative toxicities of test substances.

2.1.1 Bacterial and Fungal Response

Due to the combinations of broad spectrum antimicrobials and surfactants in BGT, it is expected that the product will exhibit a range synergistic component effects which will also be likely impacted by a range of environmental factors. Previous uses and studies of the components, many of which are discussed

previously, help to inform the possible effects of the whole product, but it is important to test these as would be supplied in combination as it is likely that the components may affect the activity and persistence of one another.

The first set of studies in this chapter aimed to assess the minimum inhibition concentration of BGT and its components on bacterial and fungal species within an *in vitro* assay, in order to understand the possible susceptibility of bacterial and fungal organisms to BGT exposure and toxicity. The assay explores the dose-response to BGT as both a biostat and a biocide. A *biostat*, often known specifically as a bacteriostatic agent in the case of bacteria, is defined by its ability to prevent growth or reproduction of an organism. This contrasts with a *biocide* that has the ability to kill the organism. The two effects may be separated as one would expect the continuation of growth of reproduction of an organism once a biostatic agent is removed whereas no further growth or reproduction would occur after contact with an agent at a biocidal dose.

2.1.2 Earthworms as an Ecotoxicological Model Organism

The model test organism *Eisenia fetida*, was used to begin to establish the possible environmental effect of the test product. Any biocide released into the environment is likely to have an adverse effect on non-target organisms. Earthworms are key organisms within soil ecosystems and decomposer communities (Datta et al., 2016; Edwards and Bohlen, 1992). They ingest large amounts of soil, creating close relationships with many other soil biomasses. They have a significant role in nitrogen cycling and soil formation processes (Wang et al., 2012). It has been suggested that the abundance of

earthworms may be representative of the health of a soil ecosystem (Doran and Zeiss, 2000; Bertrand et al., 2015).

The Environmental Risk Assessment Scheme for Plant Protection Products outlined by the European Plant Protection Organization (EPPO) includes earthworms as a standard test organism due to their importance in terrestrial systems (EPPO, 2003). The main exposure route to earthworms and other soil invertebrates is through contaminated soil pore water, so it is relevant to examine the effects of BGT which is applied in solution. The use of *Eisenia fetida* as an Eco-toxicological model organism is well supported and approved by the European Union (EU) and the Organisation for Economic Co-operation and Development (OECD) (Edwards and Bohlen, 1992; Gupta et al., 2011; .Wang et al., 2012).

2.2 METHODS

2.2.1 Preparation of the Test Solution

The test product used in all the experiments was made in the laboratory of the industrial partner. This was primarily done by staff there but I was able to assist in the production of some of the batches. All the components included are supplied from manufacturers in mixed aqueous solutions (Table 1.2). The components are all water soluble and can be readily mixed at room temperature, except for Si-QAC which is solid at room temperature. It is therefore heated to 40°C and mixed with APG before mixing with other components. Sterile deionised water is added to make up BGT solution to 1 litre. For the assays in my study I began with a batch of the 100% BGT (Table 1.2) which was then diluted further as needed, in tap water or sterile deionised water (when sterile conditions were required).

As there was a small amount of continuing product development occurring alongside some of the initial work, there was some changed to the concentration in which BGT concentrate was supplied. This meant that what had previously been referred to as 100% was then considered to be x15 concentrate. For clarity all the concentrations used in these studies have been standardised. This meant that some concentrations are now given as a non-integer, irregular percentage number. Where necessary, for clarity, these have been shown to two decimal places standard form.

For testing the components individually and in various combinations (Table 2.1), the individual solutions were made to the concentration at which they

occur in the 100% BGT. Si-QAC could not be used alone as it needs to be first combined with APG. Sterile deionised water was then added to make up to the required volume.

2.2.2 Culture Conditions

A selection of cultures of laboratory strains of bacterial and fungal species were grown from stocks previously stored at -80°C, donated from stocks kept by the Plant Stress Biology group at Lancaster University. The Bacterial species *Escherichia coli* XL1 Blue (Bullock et al., 1987), *Pseudomonas syringae* T1 Avr Pto (Salmeron and Staskawicz, 1993), *Rhizobium radiobacter* GV3101 (Holsters et al., 1980), *Pseudomononas fluorescens* 89B61 (Yan et al., 2002), *Bacillus pumilus* SE34 (Yan et al., 2002), *Bacillus pumilus* INR-7 (Zehnder et al., 1997) and *Variovorax paradoxus* 5C-2 (Belimov et al., 2005) were grown in Luria Bertan (LB) broth. LB broth was prepared using 10 g Bacto-tryptone, 5 g Bacto-yeast extract and 10 g sodium chloride (NaCl). The pH was adjusted to 7.5 with sodium hydroxide (NaOH) with sterile deionised water to give a final volume of 1 litre. The medium was sterilised by autoclaving at 121°C for 20 minutes. *Saccharomyces cerevisiae* BY4742 (Brachmann et al., 1998) was grown in Yeast Peptone Dextrose (YPD) broth, which was prepared using 10 g Bacto-yeast extract, 20 g Bacto-peptone, 20 g glucose with sterile deionised water to give 1 litre of medium. The medium was sterilised by autoclaving as before. Cultures were grown in a shaking incubator at 29°C or 37°C (*E.coli*) at 200 rpm. When growing *E.coli* XL1 Blue, 50 µg/ml of the antibiotic tetracyclin was added to ensure the sole growth of the desired E.coli strain instead of any possible contamination. The worms *(E.*

fetida) were cultured in a commercially available polyethylene wormery (430 mm x 360 mm, 27 litre capacity) purchased from Original Organics, UK. The compost bedding provided was mixed with calcium oxide to prevent acidic conditions. Temperature in the surrounding room was maintained at 17 - 20°C.

2.2.3 Minimum Inhibitory Concentration Assay (MIC)

Test cultures of each bacterial and fungal species were created from freshly-grown stock liquid cultures. A spectrophotometer was used to measure the optical density at 600 nm of the liquid cultures to allow a final concentration of approximately 1 x 10^6 CFU/ml. A two-fold serial dilution of BGT was prepared in sterile LB or YPD broth. The required optical density was approximated using the assumption that an OD_{600} for 1 x 10^6 CFU/ml equals 0.0022. To these mixtures, 1 ml of the test culture at 1 x 10^6 CFU/ml was then added to give a final volume of 2 ml at a two-fold serial dilution range from 10% - 0.002% BGT. Two additional samples, one without inoculation at 0.002% and one without test product, were also included alongside each assay to provide positive and negative controls for microbial growth.

Cultures were incubated for 20 h in an incubated shaker at 29°C after which time the optical density of each solution was read. The MIC was recorded as the lowest concentration of BGT at which there was no change in optical density between the control and treated sample. All assays were repeated in triplicate.

Following the assay, cells were recovered from the liquid cultures from the serial dilution for plating. This was done to examine if the cultures which had not grown in the presence of BGT, were recoverable to reveal if BGT was bactericidal or bacteriostatic, as previously defined. To do this, 1 ml of the solution was removed and placed in an Eppendorf tube. The solution was centrifuged for 90 s at 13,200 rpm. The excess liquid was remove and 1 ml of LB broth added. This was then vortexed and centrifuged again for 90 s. The excess liquid was removed and 50 µl of LB broth was added. This was then vortexed and then plated on LB agar. The LB agar was prepared in the same way as the LB broth with the addition of 20 g/L agar. Plates were incubated at 29°C or 37°C (*E.coli*) for 24 h.

The same assays were also carried out with oxamyl, an active carbamate substance in a commercially available nematicide, Vydate 10G®, at a concentration of 10% w/w (mass/mass) or 0.1 mg/ml oxamyl. The assays were carried out with two-fold series dilution beginning at 0.01 mg/ml; one tenth of the oxamyl concentration in Vydate 10G®.

2.2.4 Field Soil Collection

Soil samples were collected on 30th January 2013 from an uncultivated field at Lee Farm, Myerscough College, Lancashire, UK. The site was chosen as the site staff anecdotally knew the field to be infested with PPN and it had been historically used for potato growth. Soil was collected in woven polypropylene bags. Soil was sieved using a perforated plastic tray to removes stones and other debris, and stored at 4°C.

2.2.5 Baermann Funnel Experiment for Nematode Extraction

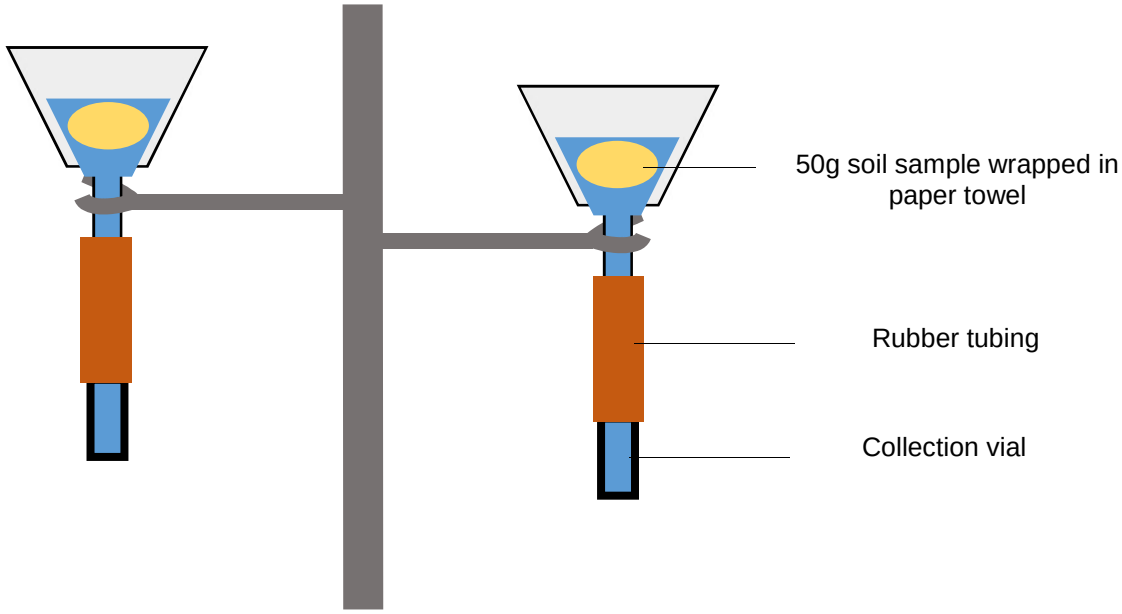


Figure 2.1: Baermann funnel system. *6 funnels were set up for the extraction of nematodes from soil samples.*

A system was set up to act as a Baermann funnel (van Bezooijen, 2006) for the extraction of nematodes from soil samples (Figure 2.1). Rubber tubing (12.5 mm bore x 150 mm length) was attached to high-density polyethylene (HDPE) funnels (140 mm top diameter) and secured upright in metal clamp stands. Plastic vials (5 ml) were attached to the bottom of the rubber tubing and secured with a rubber band where necessary. Clean tap water was then added to completely fill the vial, tubing and neck of the funnel to ensure there were no air pockets or leaks in the tubing. For extraction, 50 g soil samples were wrapped in a sheet of paper towel and placed inside the funnel. The water level was then topped up to completely cover the soil sample. Active, mobile nematodes could then pass out of the soil into the water and eventually sink into the vial below.

After some initial time-interval testing, it was found that no further nematodes were collected beyond 72 h. The equipment was regularly checked for leaks and the water level was topped up when necessary. After 72 h, the tubing was clamped above the vial and the vial removed. The contents were then counted in suspension under a dissecting microscope in a gridded Petri dish. Both active and inactive nematodes were counted as it was assumed that only live nematodes were able to pass out of the soil.

2.2.6 Field Soil Treatment

Two plastic bags were filled with 1 kg of the field soil. Three 50 g samples were taken from each bag, wrapped in paper towel and placed in the funnels for extraction. One bag was then treated with 10% BGT at 15.38 ml/kg and the other with 10 ml tap water. The solution was mixed throughout the soil. Bags were stored at 4°C. After 72 h, the vials were collected from the funnel and 3 new samples taken from each bag. This process was repeated such that samples were taken at 0, 3, 6 and 9 days after treatment. This assay was also repeated but instead with samples taken at 0, 7, 21 and 35 days after treatment. For statistical analysis, the nematode counts were log-transformed to remove over–dispersion which would otherwise violate the assumption of the model. A generalised linear model (GLM) with a quasi – Poisson error distribution and a log link function was applied. A quadratic term was applied to account for non-linear variation across the sample days.

2.2.7 Filter Paper Contact Toxicity Test

A filter paper contact toxicity test adapted from the OECD Guideline for testing of Chemicals was used to give an initial indicator of the toxicity of the substances to earthworms and inform further tests (OECD, 1984). Median lethal concentration (LC50) is defined as the concentration of the test substance which kills 50% of the test animals within the test time-period.

Worms were collected from the wormery, gently rinsed with sterile deionised water and laid in plastic trays lined with clean dampened filter paper. Trays were covered with perforated film and incubated in darkness at 20 (± 1 °C), for 4 h to allow worms to void their gut contents (OECD, 1984). All worms were then rinsed, dried and weighed. Individuals were determined as mature for assay use with a visible clitellum and a mass of 300 - 600 mg. Petri dishes (90 mm diameter) were lined with 180 µm filter paper treated with 1 ml of each test solution. BGT was tested in a two-fold serial dilution (Table 2.1). The equivalent exposure concentration is based on the volume of BGT (assuming 1 µl = 1 µg) per cm^2 of the 9 mm diameter filter paper. This allows the results to be compared with other studies of pesticide toxicity.

Ten control treatments were carried out with deionised water. The filter paper was air dried and remoistened with 1 ml of sterile deionised water. Ten replicates of one worm per Petri dish were carried out per treatment level. Dishes were incubated in darkness at 20 (± 1 °C). Mortalities were counted at 24 and 48 h. Mortality was assumed if there no physical movement response to a gentle mechanical stimulus. The median lethal dose (LD50) was calculated using the ‘dose.p’ function provided in the ‘MASS’ package

(Venables and Ripley, 2002) within the R software (Version 3.2.2)(R Core Team, 2016).

Table 2:1: Range of BGT concentration used in filter paper contact toxicity test. *The percentage BGT solutions were diluted from a 100% stock solution composed as in Table 1.2. The equivalent exposure concentration is based on the amount of product added in a 1 ml solution per cm^2 of the (9 mm diameter) filter paper.*

Percentage BGT solution (%)	Equivalent concentration exposure ($\mu g/cm^2$)
12.500	1006
6.250	503
3.125	252
1.563	126
0.000	0

2.2.8 Artificial Soil Assay

An artificial soil mixture was created as described by the OECD guidelines, containing 10% Sphagnum peat, 20% kaolin clay and 70% fine sand (OECD, 1984). All moisture was previously removed from the peat by drying at 105 °C until a constant mass was reached. For each assay, 300 g (dry mass) of the mixture was placed in individual one pint (568 ml) glass containers. The soil was moistened with the test solutions to give an overall moisture content of approximately 35% of the dry mass. Solution concentrations were determined

to give final test BGT concentrations of 0, 0.67, 6.67 and 66.67 mg/kg in the soil. Worms were collected from the wormery, gently rinsed and laid in trays lined with clean dampened filter paper. Trays were covered with perforated film and incubated in darkness at 20° ± 1 °C for 4 h to allow worms to void their gut contents. All worms were then rinsed, dried and weighed. Individuals were classed as mature for assay use with a visible clitellum and a mass of 300 - 600 mg. All individuals were then conditioned for 24 h in a large tray of the artificial soil mixture covered with perforated film in darkness for 20 h at 20 (± 1 °C). After 24 h, five individuals were placed on the surface of the test soil in each container. The glasses were covered with perforated film and incubated at 20 (± 1 °C). The assays were performed under continuous light to ensure that the worms remained in the soil for the duration of the assay. After 7 d, the worms were rinsed, patted dry with paper towel and weighed. Mortalities were assessed, again determined if there was no physical movement response to a gentle mechanical stimulus. The live worms were then again left on a tray of dampened filter paper as before to void their gut contents before weighing.

2.2.9 Statistical Analysis

Unless stated otherwise all statistical analysis of the data in this chapter was conducted using the R software (Version 3.2.2) (R Core Team, 2016). All analyses in this chapter were conducted with the base package functions. Graphical plots were created using ‘ggplot2’ package (Wickham, 2009). Regression and generalised linear models were carried out using the base package following checks for normality and homogeneity of residues. Where

applicable (as described in relevant sections), the response data was transformed to fit the assumptions of the models.

2.3 RESULTS

2.3.1 Minimum Inhibitory Concentrations on Bacterial and Fungal Species

The minimum inhibitory concentration of BGT was determined using a range of bacterial species and a yeast, *S. cerevisiae* (Table 2.2). All assays were repeated in triplicate. There was no variation in MIC of the technical replicates of each species. The MIC for the species tested ranged from 0.125% to 0.0078%. For the majority of the species, the MIC was found to be 0.0078%. The most tolerant species were the bacterium *P.syringae* T1 Avr Pto and the yeast *S. cerevisiae* BY4742.

After the initial assay, the samples were spun, rinsed and plated to examine if there were still viable cells where BGT concentration appeared to inhibit growth. In all cases, there was some colony growth from the solutions taken from the MIC but this was visibly reduced from that of the control. The lowest concentration at which no growth was recovered can be seen in the final column of Table 2.2, which could be deemed as the minimum lethal concentration. The minimum lethal concentration for the species tested ranged from 10% to 0.25%. There is no correlation between observed inhibitory concentrations and lethal concentrations across the species.

It is not possible to infer whether BGT acted as a biocide or as a biostatic but, whichever it is, it is likely that treatment with BGT will cause shifts in the soil microbial community structure. In the range of oxamyl concentrations included in the assay no growth inhibition was observed.

To examine BGT further, a set of serial dilution assays were carried out on *B. pumilus* SE34, one of the bacterial species with the greatest sensitivity to the whole product, with the different combinations of the 5 individual components. PHMB appeared to be the strongest active component, as it was bacteriostatic at the lowest concentration, followed by BAC and AE (Table 2.3). The mixture of Si-QAC and APG alone did not have an effect on growth at the highest concentration tested. The addition of Si-QAC and APG to PHMB resulted in a lower MIC than PHMB alone suggesting an interactive effect of the components on the bacteria. The addition of Si-QAC and APG to BAC or AE did not change the MIC compared to when APG and BAC were tested individually. The solution with all the components included, excluding AE, had the same MIC as the complete product on *B. pumilus* SE34.

Table 2.2: Minimum inhibitory concentration of BGT on selected microbial species and their general characteristics.

Species	Minimum inhibitory concentration (%)	Minimum lethal concentration (%)	Characteristics	Phylum
Escherichia coli XL1 Blue (Bullock et al., 1987)	0.0078	0.50	Gram negative, rod shaped coliform bacterium.	Proteobacteria
Pseudomonas syringae T1 Avr Pto (Salmeron and Staskawicz, 1993)	0.1250	0.50	Gram negative, rod shaped bacterium. Plant pathogen.	γ–Proteobacteria
Pseudomonas fluorescens 89B61 (Yan et al., 2002)	0.0078	10.00	Gram negative, rod-shaped bacterium. Plant growth-promoting rhizobacteria (PGPR).	α-Proteobacteria
Rhizobium radiobacter GV3101 (Holsters et al., 1980)	0.0078	0.25	Gram negative, rod shaped bacterium. Plant pathogen; cause of crown gall disease.	α-Proteobacteria
Bacillus pumilus SE34/ INR-7 (Yan et al., 2002)	0.0078	10.00	Gram positive, rod shaped, spore forming bacillus. PGPR.	Firmicutes
Variovorax paradoxus 5C-2 (Belimov et al., 2005)	0.0078	10.00	Gram negative, rod shaped bacterium. PGPR.	β-Proteobacteria
Saccharomyces cerevisiae BY4742 (Brachmann et al., 1998)	0.0313	10.00	Yeast	Ascomycota

Table 2.3: Minimum inhibitory concentration of BGT components on B. pumillus SE34. *The mg/ml concentration is based on the concentration of the ingredients in each solution (Table 1.2) rather than the equivalent BGT concentration.*

Solution	**Equivalent BGT MIC (%)**	**MIC on *B. pumillus* SE34 (mg/ml)**
Si-QAC+APG	> 1.0000	> 2.40×10^{-2}
PHMB	0.0313	6.25×10^{-4}
PHMB + Si-QAC + APG	0.0078	3.30×10^{-4}
BAC	0.0625	1.25×10^{-3}
BAC + Si-QAC+ APG	0.0625	2.75×10^{-3}
PHMB + BAC	0.0078	3.10×10^{-4}
PHMB + BAC + Si-QAC + APG	0.0039	2.50×10^{-4}
AE	0.5000	2.00×10^{-3}
AE +Si-QAC + APG	0.5000	1.40×10^{-2}

2.3.2 The Effect of BGT on the Number of Nematodes Extracted from Infested Field Soil

Some small trials were carried out to explore the potential effect of BGT on field soil containing nematodes in which there was no measurable effect on the number of nematodes recovered from the soil (Section 2.25). There was no significant change due to the treatment ($p = 0.586$) or sample day ($p = 0.061$) across the 9 day experiment (Table 2.4) (Figure 2.2a). Across 35 days, there appears to be a significant reduction in the number of nematodes recovered from the BGT samples compared to the control samples ($p=0.022$) (Table 2.4) (Figure 2.2b). However, there is a large amount of variation across a relatively small number of sample replicates which makes it difficult to make any definitive interpretations of these data.

Table 2.4: Analysis of deviance table from a quadratic generalised linear model (GLM) with a quasi-Poisson distribution and a log link function to assess the possible changes in number of nematodes recovered from field soil samples treated with BGT over 9 days and 35 days.

	Variables	D.F.	Residual D.F.	Deviance	p value
9 Days	Day	1	22	0.058	0.061
	Treatment	1	21	0.005	0.586
	Day^2	1	20	0.034	0.148
	Day:Treatment	20	19	0.036	0.139
35 Days	Day	1	22	0.081	0.121
	Treatment	1	21	0.010	0.581
	Day^2	1	20	0.003	0.765
	Day:Treatment	20	19	0.175	**0.022**

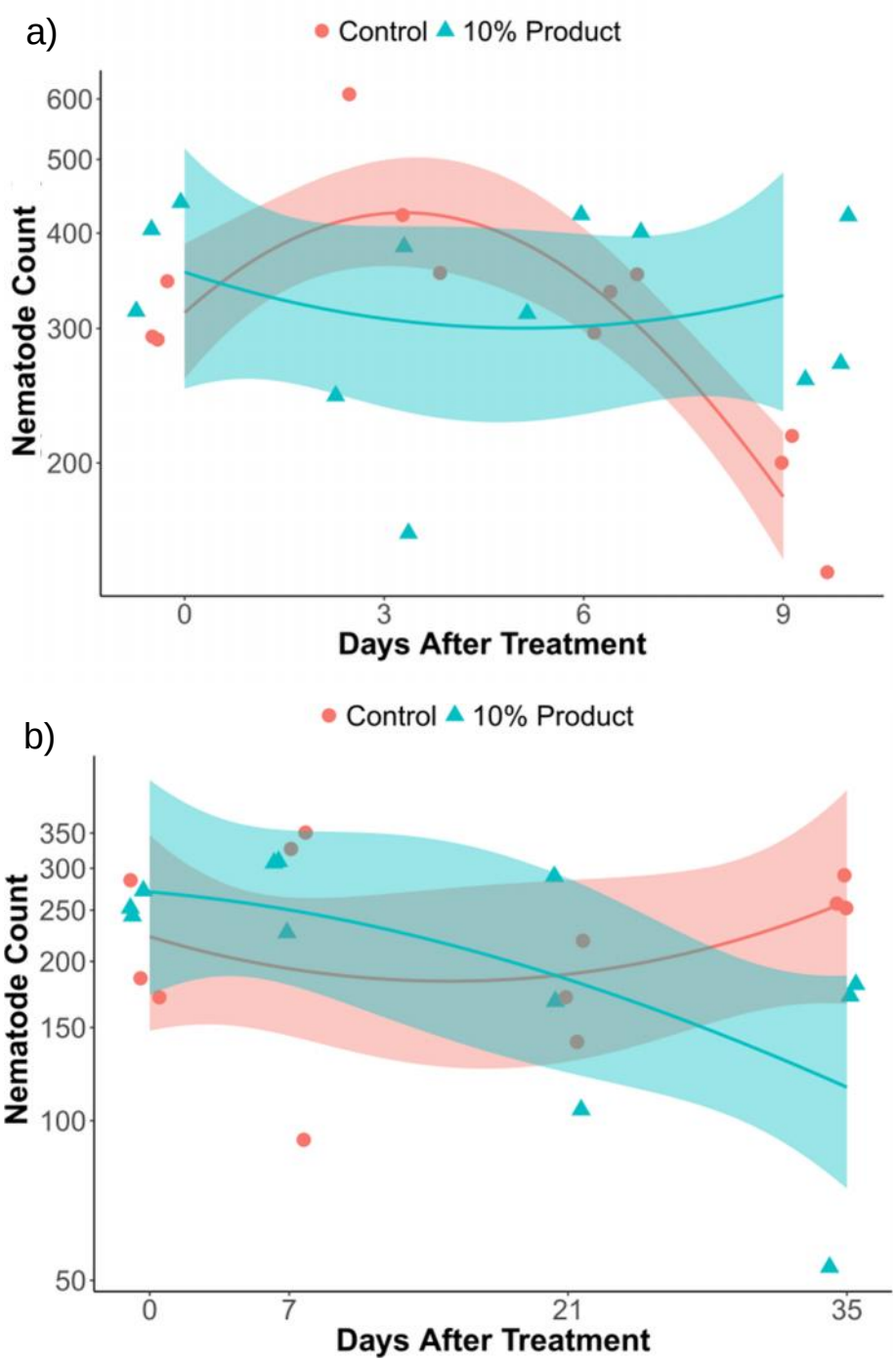


Figure 2.2: Number of nematodes recovered from field soil a) 9 days and b) 35 days after following BGT treatment. *The Y axis is shown in log scale. The lines indicate fitted values of a quadratic GLM with quasi - Poisson error distribution and a log link function. The shaded areas indicate 95%*

2.3.3 The Median Lethal Dose of BGT on *Eisenia fetida*

Eisenia fetida were placed on a range of treated filter papers to establish a median lethal concentration. One hundred percent mortality was achieved at 12.5% BGT (Figure 2.3). Most of the individuals exposed to this concentration exhibited morphological changes after 24 h including body swelling, constrictions and some mucus secretion (Figure 2.4). Some bulging was seen to a lesser extent in the individuals exposed to 6.25% BGT solution. In lower concentrations, there was less than 50% mortality and there were no visible morphological changes in the dead worms. With the exception of the control, all concentrations tested caused some mortality. A binomial distribution was fitted to the survival proportion using a generalised linear model (GLM with a log link, tested against the Chi square (χ^2) distribution (deviance = 39.19, D.F. = 3, $p < 0.001$). From this a median lethal concentration (LC50) was calculated to be 4.379% ± 0.605. This is the equivalent of 359 µg/cm^2 based on the application to a 9 mm diameter filter paper.

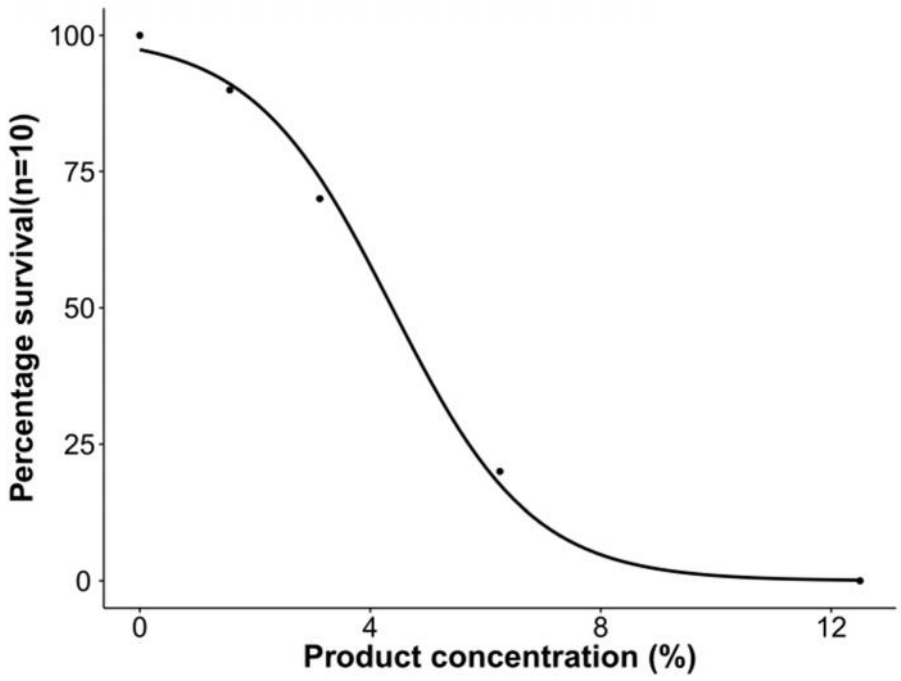


Figure 2.3: Percentage survival of E. fetida (n = 10 worms per treatment) exposed to BGT (1.56 - 12.5%) at 48 hours. *LC50 = 4.379% ± 0.605.*

Figure 2.4: Body constrictions and swelling could be seen in some of the individuals exposed to 12.5% BGT after 48 hours during filter paper contact assay.

2.3.4 The Sub-Lethal Effects of BGT Treatment on *Eisenia Fetida* in an Artificial Soil Assay

No mortality of *E. fetida* earthworms occurred in the test concentration range 0 - 10,000 mg/kg in the artificial soil assay (Section 2.2.8). The total mass of the five worms included in each container was recorded on day 0 and day 7. There was a greater mass loss in the worms exposed to the higher BGT concentrations compared to the control worms. This effect was found to be significant when fitted to a polynomial regression model ($F_{2, 9}$ = 9.328, p = 0.006; Figure 2.5). This suggests that there may be some sub-lethal physiological effects on the worms. None of the worms exhibited any signs of morphological changes as were observed in the contact filter paper assay.

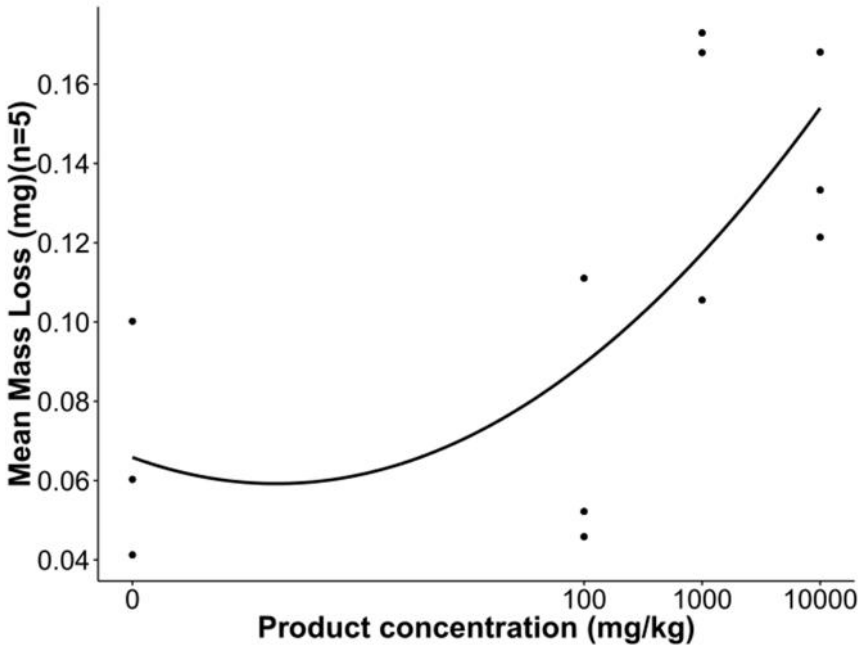


Figure 2.5: Mean individual mass loss of E. fetida individuals per container (n = 5 worms) after 7 days of exposure to BGT in artificial soil. *Three containers per treatment. The line represents the fitted polynomial regression model.*

2.4 DISCUSSION

2.4.1 The Effect of BGT Treatments on Individual Bacterial and Fungal Species

A range of bacterial species and a yeast species readily cultured in the laboratory were exposed to a serial-dilution of BGT to begin to explore the antimicrobial effect. The yeast, *S. cerevisiae* BY4742, was the most tolerant to exposure to BGT along with *P. syringae* T1 Avr Pto. There was no correlation between the sensitivity of the species to BGT and their phylum (Table 2.2). Nor was there a notable difference in the response of Gram-positive or Gram-negative bacteria. There was also no correlation between the minimum inhibitory concentration and minimum lethal concentration of the species; in all cases, the minimum lethal concentrations were much higher than the inhibitory concentration. I attempted to carry out a comparable assay with a further fungal species, *Botrytis cinerea*, but a successful method was not established. From these results (Table 2.2), it is not possible to predict how BGT might affect particular species, taxonomic or functional groups, but it is likely that due to differences between species in their susceptibilities there will be shifts in the soil microbial community in BGT treated soils. This is explored further in Chapter 4.

When examining the effect of the individual components of BGT on the growth of *B. pumillus* SE34, it was evident that some of the components had particularly high antimicrobial activity (Table 2.3). Polyhexamethylene biguanide (PHMB) is a well-established broad spectrum antimicrobial agent

(Allen et al., 2004) and, despite being present in the lowest active volume in BGT (Table 1.2), it was bacteriostatic at the lowest concentration. This was followed in strength by the other principle biocide component, BAC. The combinations of PHMB and BAC, and PHMB, Si-QAC and APG were sufficient to give the same minimum inhibitory concentration as was seen when *B. pumillus* was exposed to the complete product. Si-QAC and APG did not act as a bacteriostat at the concentration tested. However, they had an additive effect when included with PHMB, but not with the other components. This may be expected with APG, as it is a non-ionic surfactant used as a detergent and mixing agent in products, rather than as a biocide. Si-QAC would have been expected to have a biocidal effect, as it has previous application as an antimicrobial (Siddiqui et al., 1983).

The assays indicated the importance of using the product formulation in toxicity testing rather than just relying on toxicological information of individual components. Whilst BGT is expressed as a blend of anti-microbials, many plant protection products rely on one principal active substance which is labelled as the ‘active ingredient’. It is assumed that the ‘active’ substance will be the main source of the biocidal action but it is often wrongly assumed that the other components are inert when introduced into the environment. In the case of the glyphosate based herbicide Roundup®, it was found that the commercial formulations, exhibited significantly higher mammalian toxicity than glyphosate alone (Castro et al., 2014).

2.4.2 The Effect of BGT Treatments on Nematodes

Some small trials were carried out to explore the potential effect of BGT on field soil containing nematodes (Figure 2.2). Unfortunately, the levels of nematodes in the field soil is highly variable due to the behaviour of the nematodes, which often mass together in 'hotspots' (Neher, 2010). The extraction was left for a prolonged period of 72 hours to account for possible varying sedimentation and recovery times of different nematodes (Viglierchio and Schmitt, 1983). With the setup created, it was only possible to process six relatively small 50 g soil samples at any time which made it difficult to create a highly robust experimental setup, particularly with the natural variation. Whilst it may be assumed that the identical funnels have similar extraction efficiencies, this was not calculated as another extraction and counting method was not available. The Baermann funnel relies on a behavioural response from the nematodes. It would have to be assumed that BGT only caused nematode mortality or left them unable to move out of the soil into the surrounding water and subsequently in the vial. If there were any non-lethal behavioural effects of BGT on the nematodes this may impact on the reliability of the method when comparing with other treated or controlled samples. In other nematode studies, it is more common to perform this type of assay with inoculated soil so the extraction efficiency can be estimated. This was not pursued in this collection of studies as the primary focus was to examine the wider impacts on the community rather than the specific target pests. The industry partner and related research projects were undertaking other research to explore the impacts on nematodes more specifically. The results of the impact of BGT on nematode populations in a field trial will be discussed in Chapter 4.

2.4.3 The Effects of BGT Treatments on Earthworms in Laboratory Assays

Two different assays were carried out to explore the impact of BGT on *Eisenia fetida* as a model species to begin to explore possible effects on the soil ecosystem. Through the filter paper contact test, the median lethal concentration (LC50) was found to be 4.379% (Figure 2.3). This is the equivalent of 359 µg/cm^2 based on the application to a 9 mm diameter filter paper. This would appear to be a relatively high concentration given that in preliminary field trials, field treatment applications between 1-10% were sufficient to reduce the number of nematodes recovered. It could be assumed that the expressed percentage of a field treatment concentration is much higher than the likely final percentage concentration as some is adsorbed by soil particles. Wang et al. (2012) carried out the filter paper contact toxicity test on *E. fetida* to assess 45 common pesticides, including 9 insecticides, 2 acaricides, 23 herbicides and 11 fungicides. Although none of the products are specific nematicides, the study assessed the toxicity of some carbamates and organophosphates, which are groups of chemicals that have been traditionally used as nematode control (Zasada et al., 2010). Across the substances tested, the calculated LC50 values were between 0.28 – 566.1 µg/cm^2. Fourteen of the substances assessed had LC50 values of > 1000 µg/cm^2. Based on the LC50 values, the pesticides were classified on a scale set out by Roberts and Dorough (1984) as follows: super toxic (< 1.0 µg/cm^2), extremely toxic (1–10 µg/cm^2), very toxic (10–100 µg/cm^2), moderately toxic (100–1000 µg/cm^2), or relatively nontoxic (> 1000 µg/cm^2). Based on this

scale, BGT would be classified as moderately toxic, ranking as 23rd most toxic alongside the 45 substances tested in the study by Wang et al. (2012).

In the artificial soil assay, no mortality was seen in the tested BGT concentration range from 100 mg/kg to 10,000 mg/kg. However, the worms exposed to the highest concentrations showed increased mass loss after 7 days of exposure (Figure 2.5). This means that BGT has very low relative toxicity in soil when compared to the substances tested in the same manner by Wang et al. (2012). In their study, the LC50 values calculated were between 9.22 – 1035 mg/kg after 7 days of exposure. Three substances had an estimated LC50 of > 1000 mg/kg.

The contact filter paper test is commonly used as a simple, cheap and quick method of assessing and comparing chemical toxicity. It exposes the earthworm to the substance both by contact and in the aquatic phase, however, in the natural earthworm environment, the chemicals are mainly absorbed by the gut. In a study comparing the contact filter paper test, artificial soil test and an artisol test (whereby worms are placed in an artificial substrate consisting of silica, water and glass balls), Heimbach (1984) found a poor correlation between the results of the contact filter paper test and those of the other two tests. This is thought to be due to the different mode of uptake, as in both the artisol and artificial soil test the main expected mode of uptake may be through the gut.

In a further study, Heimbach (1992) found a good correlation between the assessment of pesticide toxicity on *E. fetida* using the artificial soil test and the

assessment of toxicity in field tests conducted on perennial grassland plots. Pesticides which showed low toxicity to earthworms in the laboratory tests were found to have no, or negligible, effects on earthworm populations in the field. The results of my work suggest that BGT is of relatively low risk to earthworms. However, there were some sub-lethal effects measured in the form of increased mass loss at the higher concentrations. Over time this could begin to have significant consequences to the population if it began to affect the success and movement of the worms and must be considered in the ecological risk assessment of pesticides, particularly when there may be chronic exposure (Jensen et al., 2007; Liu et al., 2011). The artificial soil assay is limited as it can also not account for other behavioural responses of the worm such as avoidance behaviour, which may have ecological consequences (Pereira et al., 2010). Additionally, these laboratory tests call for adult earthworms to be used for standardisation but juveniles may be more sensitive to some contaminants (OECD, 1984; Zhou et al., 2011).

2.4.4 Conclusions

These studies show that there is a range of dose responses of microbial organisms to the test product BGT, which indicate that there should be caution and consideration given to its release into the terrestrial ecosystem. However, in comparison to many other plant protection products it would appear to be of moderately low toxicity. The assay in this chapter showed little effect of BGT on nematodes extracted from soil but this may be primarily due to the large amount of variation given the small number of samples examined.

These types of laboratory assays explore the effect of acute doses of a substance but cannot reflect the potential effects of chronic low exposure to a contaminant which may be more relevant in understanding how some soil organisms will be effected. Whilst toxicity may appear low in single-pesticide experiments they cannot reflect the exposure to multiple substances in the field (Wang et al., 2012). For wider assessment of the effects of pesticides we must take into the account the use of mixed pesticides and other soil additives (Zhou et al., 2011). Single-species, single-product laboratory experiments can be very useful in beginning to explore the effect of pesticides and provide a controlled environment to allow a wider range of comparison to other products, however, using them alone may lead to an underestimation of the effects on the soil community (Vanstraalen and Denneman, 1989; Wang et al., 2012).

3. THE EFFECTS OF THE TEST PRODUCT BGT ON PLANT GROWTH AND SOIL MOISTURE DYNAMICS

3.1 INTRODUCTION

BGT comprises of components that had not been commonly used in plant protection products before, so there was not a large amount of information on the possible direct effects it would have on plants when applied in the soil. It is not uncommon for plant protection products to be found to be phytotoxic within a particular concentration or stage in a crop's growth (David, 1965; Zasada and Walters, 2016). Careful consideration of doses and application timings are used to help mitigate this to try to ensure a crop remains protected from potential pests without inflicting direct crop damage. BGT was previously trialled on turf grass to test for potential phytotoxicity (unpublished results) (Section 1.6.3). The aim of the studies in this chapter was to further examine the impact of BGT on crop seedlings to explore possible toxicity both when in solution and applied to the soil (Objectives 4 & 5, Section 1.9). Additionally, due to the surfactant properties of the product's components it was hypothesised that BGT would alter the soil moisture dynamics and be advantageous to crops when there is low water availability (Objective 6, Section 1.9).

Wheat and tomato plants were selected for these experiments as these were known to successful grow in the available conditions within the laboratory

research group and provided growth endpoints which were quick and easy to measure.

In these assays, plants were grown in sterilised soil to help understand the direct effects of product treatment on the soil properties or plant growth rather than as a possible result of reduced parasitism.

3.1.1 Surfactants in Agricultural Systems

Surfactants (as previously described in Section 2.1.2) work to reduce soil moisture loss by decreasing capillary flow. This is attributed to the alteration of the wetting angle between the solid soil particles and liquid water (Cavalli et al., 2000). This also better enables infiltration into water repellent growing media. Surfactants are widely used in agricultural products to act as agents to improve the solubility and dispersion of other agrochemicals as well as on their own to promote dispersion and retention of water, increasing irrigation efficiency. There are a wide range of wetting agents products that have been developed, particularly for horticultural use on recreational turfs as it is common for uneven patches of water-repellent soil to occur (DeBano, 2000). They can help control the movement and leaching of other products applied and reduce soil erosion (Miyamoto and Bird, 1978). Whilst it is evident they have many potential benefits to plant growth, if used incorrectly they may pose a risk to plant growth. At a cellular level, surfactants have been recorded to cause solubilisation of membranes, affect cell permeability and inactivate plant enzymes (Horowitz and Givelberg, 1979).

3.2 METHODS

3.2.1 Germination and Seedling Growth of Treated Seeds

All test solutions were prepared as described in Section 2.2.1. In this experiment, plastic salad trays lined with filter paper soaked in a range of BGT concentrations were used to grow wheat (*Triticum aestivum* L. cv. Ashby) and tomato (*Solanum lycopersicum* L., cv. Moneymaker) seeds to test the effects of BGT on germination and early seedling growth. BGT concentrate was diluted in distilled water to give final test solutions of 3.33×10^{-1}%, 6.67×10^{-2}%, 3.33×10^{-2}%, 1.33×10^{-2}%, 6.67×10^{-3}%, 3.33×10^{-3}%, 6.67×10^{-4}%, 3.33×10^{-4}%, 6.67×10^{-5}% immediately prior to the seed treatments. Distilled water was used as an experimental control. Twenty seeds of wheat or tomato were added to individual 30 ml universal tubes containing 20 ml of the test solution, to ensure all seeds were equally covered and exposed to the solutions. The solution and seeds were poured into plastic salad trays lined with filter paper. The tray lids were loosely fitted and trays were incubated in a plant growth chamber (Percival Scientific, Perry, Iowa, USA) set at 20 ± 1 °C/ 22 ± 1 °C with a 16h light cycle provided by Osram fluora lamps delivering 100 ± 20 µmol m^{-2} s^{-1}. Each day, the number of completely germinated seeds was counted. Germination was recorded as complete when both the radicle and coleoptile had visibly emerged with leaf growth beginning at the coleoptile tip (Zadoks et al., 1985). At six days, longest root length and shoot length of the wheat seedlings were measured, as this was shortly before the seedlings grew too large for the container. At 14 days, longest root length and shoot length of the tomato seedlings were measured

3.2.2 Early Wheat Growth in BGT Treated Soil

In this experiment, wheat seeds were planted in soil treated with a range of BGT solution to explore the effect of the test product on seedling growth in soil. BGT concentrate was diluted in tap water to give final test solutions of 6.67 x 10^{-1}%, 6.67 x 10^{-2}%, 6.67 x 10^{-3}%, 6.67 x 10^{-4}% and 6.67 x 10^{-5}%. Tap water was used as an experimental control. Pots (13 x 13 cm top diameter) were filled with equal amounts of Levington M3 bedding compost. Compost was previously sterilised in an autoclave to remove any possibility of indirect biocidal effects of BGT in soil on the plant growth. Through prior testing, it was found that 300 ml was the approximate holding capacity of the soil; a sufficient volume to drench the soil without excessive loss of water through the filtration holes in the base of the pots. Therefore, two pots were treated with 300 ml of each solution. Five wheat seeds were sown in each pot and cultivated in a controlled environment glasshouse (min 17 ± 2°C, max 20 ± 3°C) with supplementary lighting (Osram Greenpower 600 W high-pressure sodium lamps) to a minimum 250 ± 25 µmol m^{-2} s^{-1} photosynthetically active radiation at the canopy. A minimum 16 h photoperiod was maintained. Pots were watered from the trays beneath to minimise leaching of the treatment from the soil. At 20 days, the wheat shoots were harvested. Plants were patted dry with paper towel and placed in individual paper bags in a drying oven at 80 °C.

3.2.3 Soil Moisture Retention

To explore the effect of BGT on soil moisture retention, six pots containing 200 g of compost were drenched with 6.67 x 10^{-1} % BGT, 6.67 x 10^{-2} % BGT and tap water as a control (18 pots in total). This concentration range was

chosen as the industrial partner were undergoing glasshouse trials on PCN in this product range, following 100% mortality (after 24 hours) on *Globodera pallida* juveniles (potato cyst nematode) *in vitro*. This studies were carried out by a researcher at Arcis Biotechnology and are not included in this thesis.

The pots were left uncovered on a lab bench away from direct lamplight in a randomized block design. Soil moisture was measured at various intervals across 140 hours using a Delta-T ML3 Theta Probe to give volumetric soil moisture content (mV). These values were converted to be expressed as percentage soil moisture in generalised organic soil using a polynomial conversion as according to the user manual (Delta-T Devices Ltd, 2013):

$$\Theta = -0.039 + 0.802V + 0.819V^2 - 9.556V^3 + 23.823V^4 - 23.997V^5 + 8.833V^6$$

where V is the ML3 output in Volts.

3.2.4 Wheat Growth and Partial Drought in BGT Treated Soil

This experiment was set up to examine the effect of BGT both on the growth of wheat till harvest and the possible effects it may have when water is limited. BGT concentrate was diluted in tap water to give final test solutions of 3.33 %, 1.67 %, 8.33 x 10^{-1} %, 4.17 x 10^{-1} %, 2.08 x 10^{-1} % and 0 %. Sixty 13 x 13 cm pots were filled with equal amounts of Levington M3 bedding compost. Ten pots were treated with 300 ml of each solution and arranged in a blocked design to minimise any possible effects caused by bench position due to position of overhead lighting or distance from the glasshouse fan. All plants were sub-

sequently watered through the bench matting to avoid excessive leaching of the treatment from the soil.

At five weeks, all plants had visibly reached the tillering stage. At this stage, all pots were retreated with 300 ml of the test solutions. Following this, no additional water was added to half of the pots from each treatment group for three weeks. The remaining pots were still watered through the bench matting. After the three weeks, for the remainder of the trial, all plants were then watered both overhead and through the bench matting as the top layer of soil began to dry out. All plants were harvested at 20 weeks when spike heads and straw had dried out on the plant. Final numbers of tillers and spikes were counted for each individual plant before spikes and straws were harvested and dried at 80 °C. Dry straw mass, dry spike mass (including seeds) and seed mass was measured (Table 3.1).

Table 3.1: Growth parameters measured and recorded from wheat grown as described in Section 3.2.4.

Measured parameter	Description
Plant mass	Sum of seed mass, spike mass and straw mass of individual plant
Seed mass	Total fresh mass of seeds from individual plant
Spike mass	Total dry mass of spike heads on individual plant not including seeds
Straw mass	Total dry mass of shoots from individual plant not including seeds or spike heads
Tiller number	Number of shoots on individual plants
Proportion of flowering tillers	Number of spikes divided by number of tillers on individual plants

3.2.5 Statistical Analysis

Unless stated otherwise all statistical analysis of the data in this chapter was conducted using the 'R.V.3.2.2' statistical software (R Core Team, 2016). All analyses in this chapter were conducted with the base package functions. Graphical plots were created using 'ggplot2' package (Wickham,2009). Regression models and generalised linear models were carried out using the base package following checks for normality and homogeneity of residuals.

Where applicable (as described in relevant sections), the response data were transformed to fit the assumptions of the models.

3.3 RESULTS

3.3.1 Tomato and Wheat Seedling Growth in BGT Solution

Wheat and tomato seeds were germinated and grown in plastic salad boxes lined with filter paper soaked in a range of BGT concentrations (Section 3.2.1). There was no difference across the treatments in the timing of germination onset; however, seeds exposed to 3.33×10^{-1} % did not germinate, as there was no visible emergence of the radicle or coleoptile. Counts were made of germinated seeds. Concentrations above 3.33×10^{-2} % increased the probability of seed failure, and seeds coated with a 3.33×10^{-1} % solution were completely inhibited (Figure 3.1) (GLM with a log link, tested against the Chi-square distribution (χ^2) (deviance = 117.22, D.F. = 8, $p < 0.001$). A polynomial regression model with a log transformation was performed to examine the effect of BGT concentration on wheat shoot (primary leaf) and radicle root length on 7-day old plants (Figures 3.2 & 3.3). Increased concentration of BGT solution caused a significant reduction in wheat shoot length ($F_{2,169} = 230.6$, $p < 0.001$; Figure 3.2) and wheat root length ($F_{3, 168} = 317.2$, $p < 0.001$; Figure 3.3).

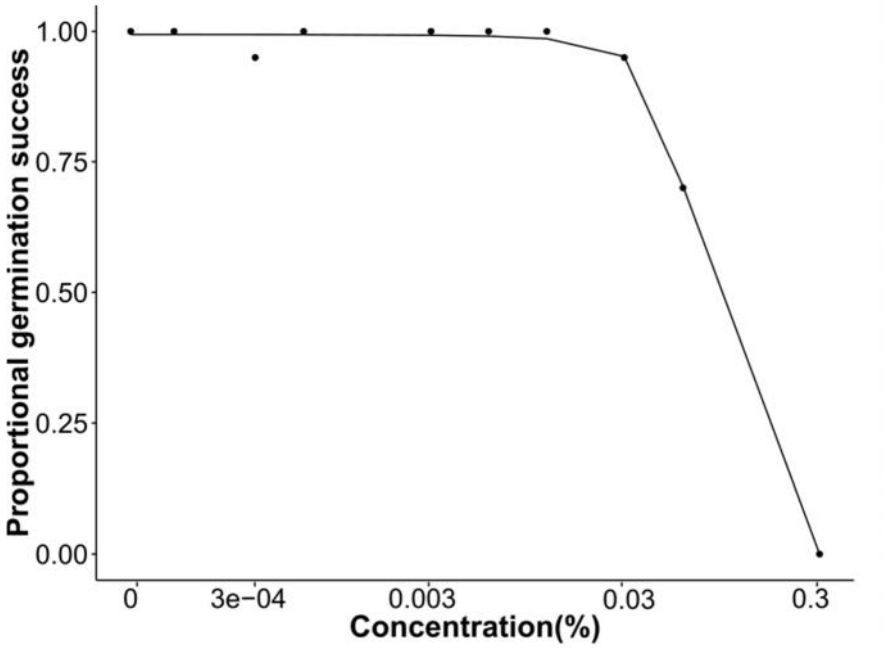


Figure 3.1: Effect of BGT concentration on wheat seedling germination after 7 days (n = 20 seeds per treatment). Line shown represents the fitted binomial generalised linear model with a log link.

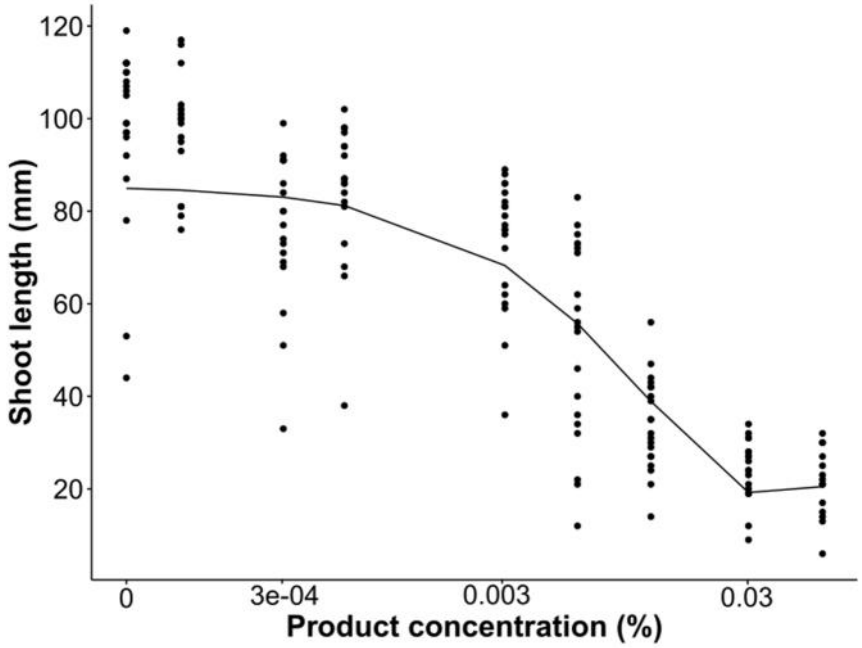


Figure 3.2: Effect of BGT concentration on wheat shoot length (n = 20 seeds per treatment). *The line represents back-transformed fitted values from a polynomial regression model.*

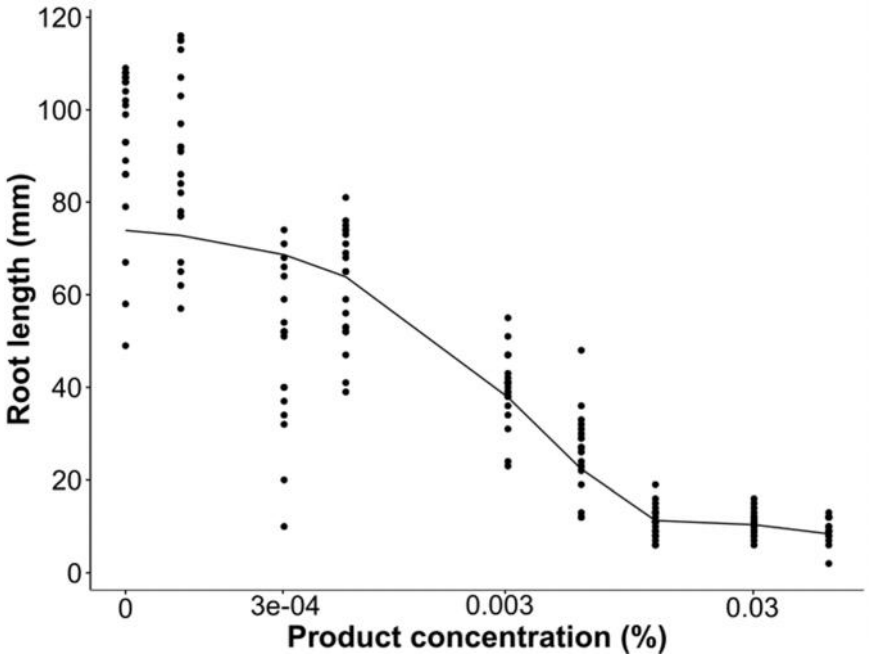


Figure 3.3: Effect of BGT concentration on wheat root length (n = 20 seeds per treatment). *The line represents back-transformed fitted values from a polynomial regression model*

The same treatment range that had been applied to the wheat was then applied to tomato seeds also in salad trays lined with filter paper. More than half the seeds were inhibited in the 1.33 x 10^{-2} % solution (GLM with binomial errors and log link: deviance = 136.43, D.F. = 8, $p < 0.001$; Figure 3.4). At 14 days, shoot and root length were measured. The impact of treatment concentration on seedling growth at 6.67 x 10^{-4}% and below is minimal, but increasing concentration does ultimately cause a reduction in shoot (LM: $F_{2, 102} = 11.9$, $p < 0.001$; Figure 3.5) and root ($F_{2, 102} = 88.39$, $p < 0.001$; Figure 3.6) growth.

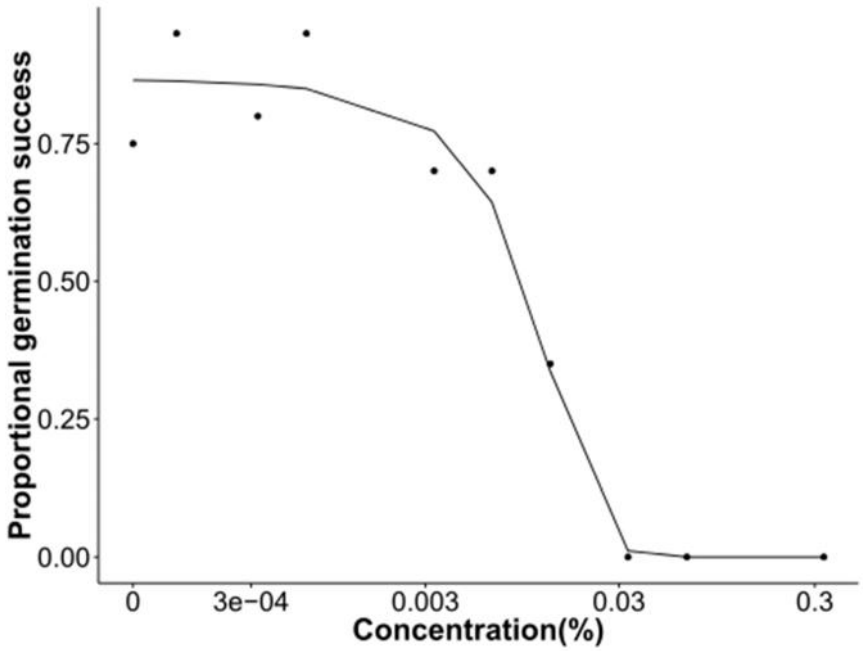


Figure 3.4: Effect of BGT concentration on tomato seedling germination after 7 days (n = 20 seeds per treatment). *Line shown represents the fitted binomial generalised linear model with a log link.*

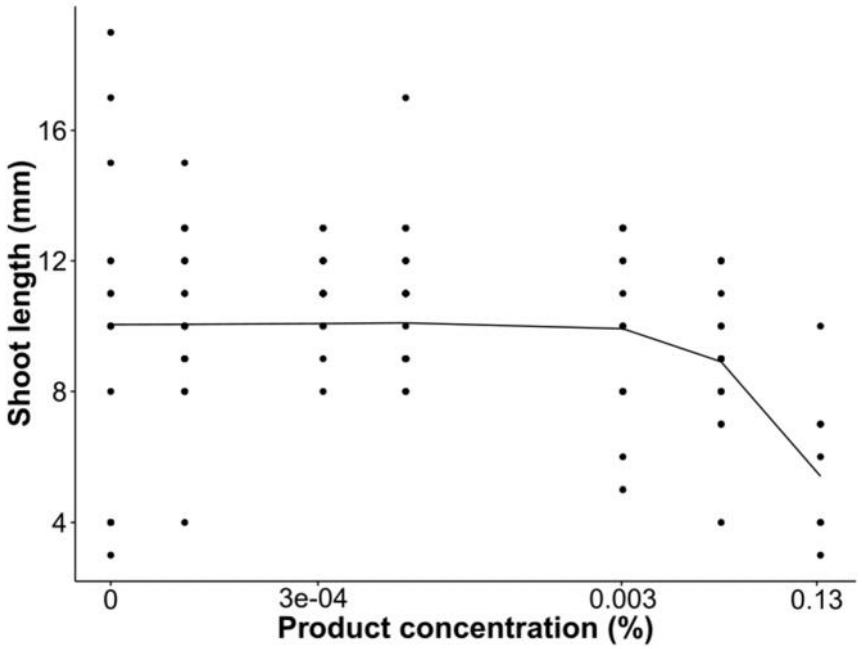


Figure 3.5: Effect of BGT concentration on tomato shoot length (n = 10 seeds per treatment). *The line represents back-transformed fitted values from a polynomial regression model.*

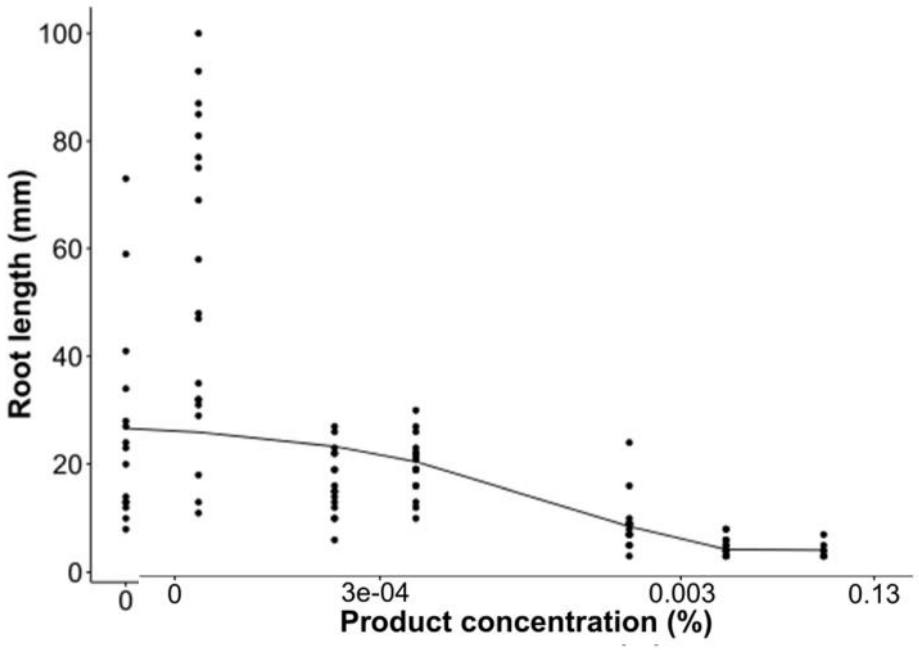


Figure 3.6: Effect of BGT concentration on tomato root length (n = 10 seeds per treatment). *The line represents back-transformed fitted values from a polynomial regression model.*

3.3.2 Wheat growth in BGT Treated Soil

Across the 18 pots, 85 of the 90 seeds were viable. There was no relationship between the proportion of viable seeds that germinated and treatment concentration. There was a large amount of variation of wheat shoot mass within treatments (Figure 3.7). However, most of the BGT treatments did not result in significant change in wheat shoot growth with the exception of the highest concentration 6.67 x 10^{-1} % which led to a small reduction (ANOVA: $F_{5,79} = 6.7002$, $p < 0.01$) (Table 3.2).

Table 3.2: Analysis of variance test assessing the significance of the effect of the BGT concentration on the dry plant mass of 20-day wheat plants compared to untreated controls. *Concentrations which caused a significant change in plant growth are highlighted in bold.*

Concentration (%)	Parameter estimate compared to control	Std.Error	t-value	p value
6.67×10^{-5}	**-15.600**	**5.728**	**-2.723**	**0.008**
6.67×10^{-4}	-4.600	5. 829	-0.789	0.432
6.67×10^{-3}	-3.000	5.728	-0.524	0.602
6.67×10^{-2}	3.900	5.829	0.669	0.505
6.67×10^{-1}	**-26.183**	**6.075**	**-4.310**	**< 0.001**

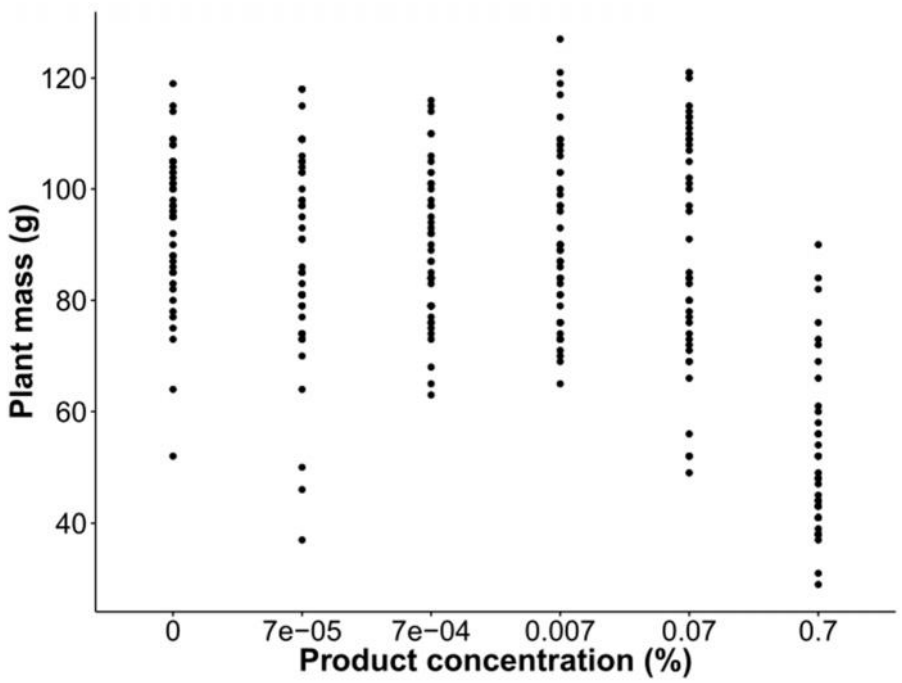


Figure 3.7: Effect of BGT concentration on aboveground mass of 20-day-old wheat plants (n = 15 plants per treatment).

3.3.3 The Effects of BGT Soil Treatments on Soil Moisture Retention

Based on observations made during the soil treatments, and due to the surfactant properties of some of the product components, it was predicted that BGT might be able to prevent soil moisture loss. To test this, soil pots without plants, were drenched with BGT treatments and left exposed on a lab bench to test the impacts of treatments on soil moisture loss. A multiple regression model was fitted with an additional quadratic hour effect term. At 0.07 %, the BGT treatment significantly reduced the rate of soil moisture loss compared to the control pots ($F_{5, 102} = 11.14$, $p = 0.001$; Figure 3.8).

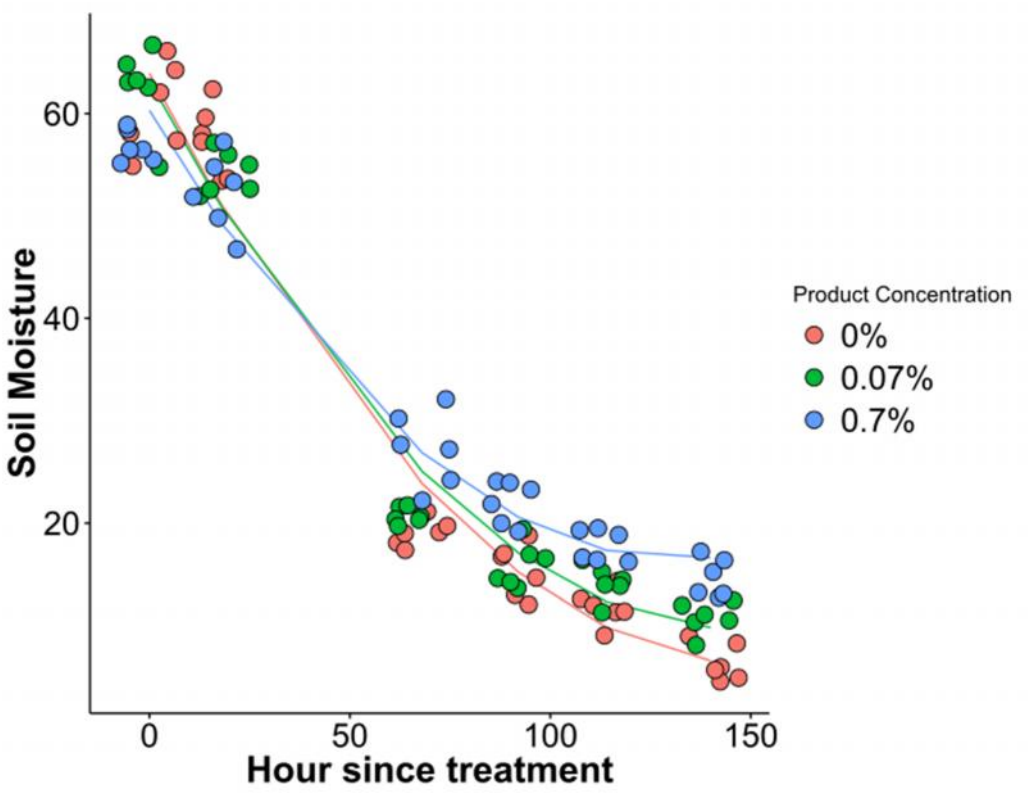


Figure 3.8: Percentage soil moisture reduction of unplanted pots treated with: Water (0%), 0.7% BGT and 0.07% BGT (n = 6 pots per treatment). *The lines represent the fitted values from a polynomial multiple regression model.*

3.3.4 Wheat Growth with Reduced Water Availability in BGT Treated Soil

The pot trial (Section 3.3.3) indicated that the BGT treatment reduced the rate at which soil moisture was lost. This may be of benefit to crop growth when water is limited in the environment. An experiment was designed to examine whether product treatment had any impact on growth of wheat plants grown to maturity, but also if the BGT treatment would be of any benefit to a plant under

a reduced watering regime (Section 3.2.4). All plants were harvested at twenty weeks and several parameters measured (Table 3.1).

As would be expected, growth of all individual plants under the reduced watering regime was significantly reduced compared to those consistently watered (LM: $F_{1, 56} = 684.142$, $p < 0.001$) (Fig.3.9). There was a significant interaction between BGT concentration and watering regime on total plant mass. (LM: $F_{1, 56} = 4.724$, $p = 0.034$) (Table 3.3). When plotting the model graphically it would appear that increased concentration caused a slight reduction in final plant growth of the continually-watered plants (Figure 3.9). However, under reduced-watering there is no reduction in plant growth and there is reduced variation in final plant mass of those treated at higher concentration (Fig.3.9). This demonstrates that under reduced water availability BGT treatment may be advantageous to plant growth despite a small amount of potential phytotoxicity. Whereas, when water is not limited an increased BGT concentration may be overall detrimental to the plant. It would also appear that increased BGT concentration reduces variation amongst the individuals under the same watering regime.

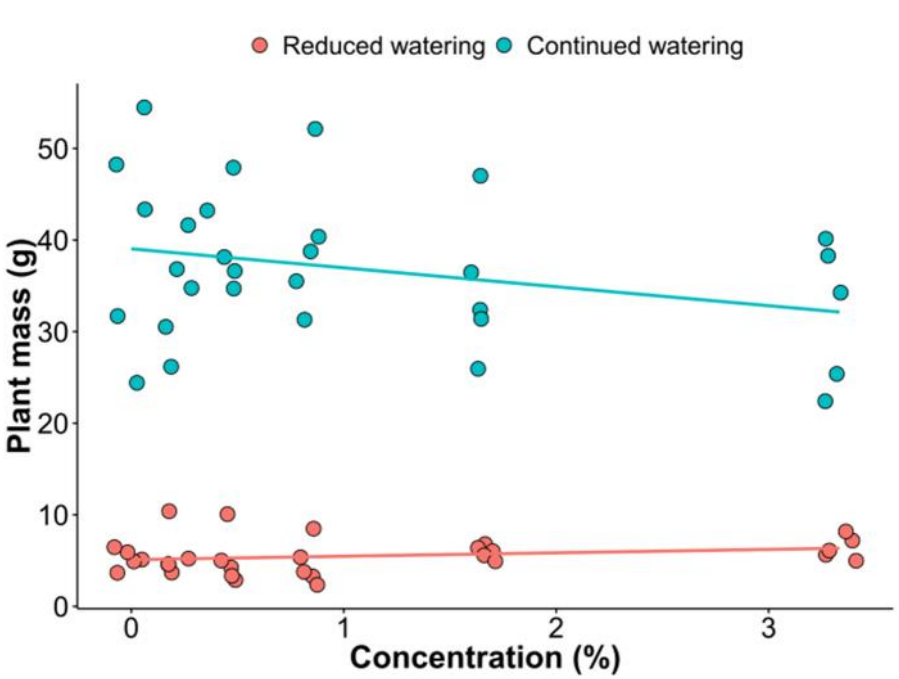


Figure 3.9: Effect of BGT concentration and watering regime on wheat plant mass (n = 5 plants per treatment). *Lines represent fitted values from regression model as specified in Table 3.3.*

Increased BGT concentration led to a significant increase in seed mass ($F_{2, 27}$ = 12.05, p = 0.001; Figure 3.10) and in complete spike mass ($F_{1, 28}$ = 11.27, p = 0.002; Figure 3.11) of those under reduced watering, but did not have a significant effect on the seed mass ($F_{1, 28}$ = 0.326, p = 0.573; Figure 3.10) or spike mass ($F_{1, 28}$ = 0.384, p = 0.540; Figure 3.11) when water was not limited. There was a significant interaction between watering regime and BGT concentration on seed ($F_{1, 55}$ = 14.123, p = 0.038) and spike mass ($F_{1, 56}$ = 10.058, p = 0.002) (Table 3.3).

Table 3.3: Effect of BGT concentration and watering regime on wheat growth parameters.

Parameter and Model Structure	Variables	D.F	F- value	p value
Plant mass Log(Plant mass) ~ Concentration * Regime	Concentration	1	0.134	0.716
	Regime	1	684.142	**< 0.001**
	Concentration:Regime	1	4.724	**0.034**
	Residuals	56		
Seed mass Log(Seed mass + 0.01) ~ Concentration * Regime + I(Concentration^2)	Concentration	1	9.687	**0.003**
	Regime	1	759.15	**< 0.001**
	Concentration:Regime	1	14.123	**0.031**
	Concentration^2	1	4.605	**0.036**
	Residuals	55		
Spike mass Log(Spike mass+0.01) ~ Concentration * Regime	Concentration	1	6.3049	**0.015**
	Regime	1	667.879	**< 0.001**
	Concentration:Regime	1	10.058	**0.002**
	Residuals	56		
Straw mass Log(Straw mass+0.01) ~ Concentration * Regime + I(Concentration^2)	Concentration	1	19.647	**< 0.001**
	Regime	1	316.373	**< 0.001**
	Concentration:Regime	1	7.502	0.341
	Concentration^2	1	0.924	**0.008**
	Residuals	55		
Number of tillers Tiller number~Concentration * Regime + I(Concentration^2)	Concentration	1	27.473	**< 0.001**
	Regime	1	0.512	0.477
	Concentration:Regime	1	12.011	**0.016**
	Concentration^2	1	6.189	**0.001**
	Residuals	55		
Proportion of flowering tillers Proportion of flowering tillers~Concentration * Regime + I(Concentration^2)	Concentration	1	11.533	**0.001**
	Regime	1	120.311	**< 0.001**
	Concentration:Regime	1	0.166	0.685
	Concentration^2	1	9.721	**0.002**
	Residuals	55		

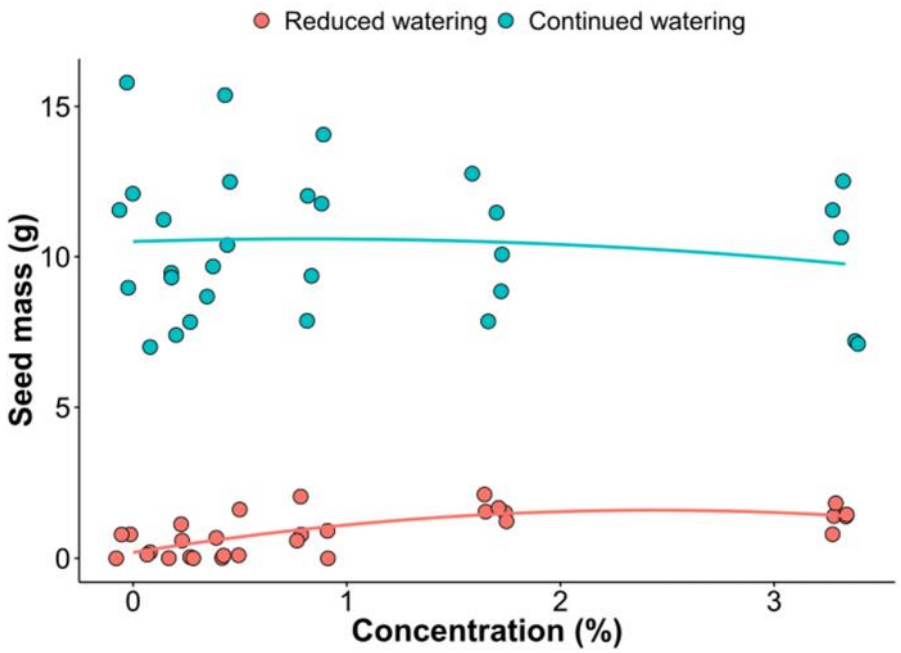


Figure 3.10: Effect of BGT concentration and watering regime on wheat seed mass (n = 5 plants per treatment). *Lines represent fitted values from regression model as specified in Table 3.3.*

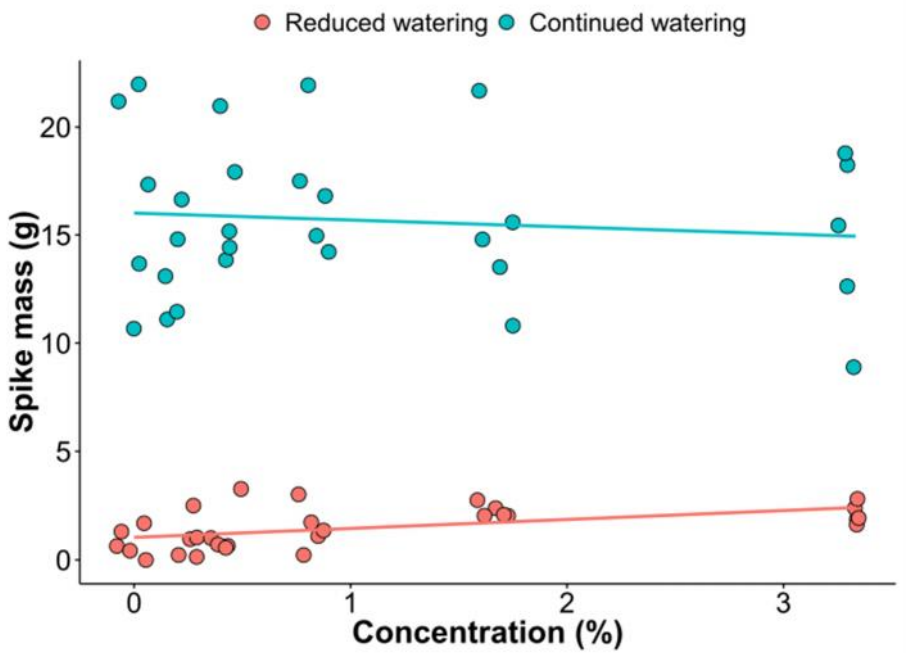


Figure 3.11: Effect of BGT concentration and watering regime on wheat spike mass (n = 5 plants per treatment). *Lines represent fitted values from regression model as specified in Table 3.3.*

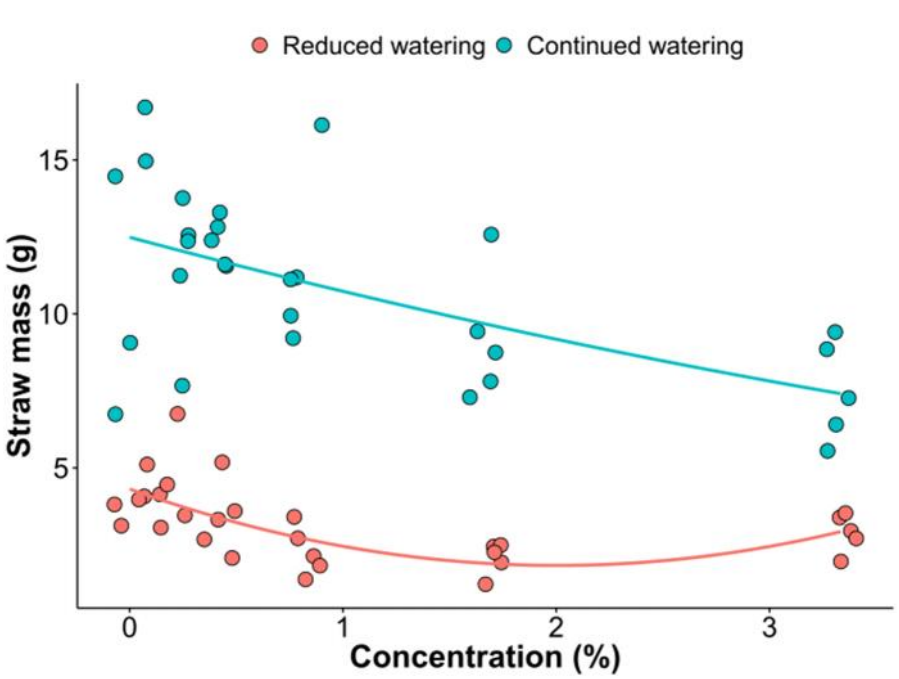


Figure 3.12: Effect of BGT concentration and watering regime on wheat straw mass (n = 5 plants per treatment). *Lines represent fitted values from regression model as specified in Table 3.3.*

Straw mass was significantly reduced with increasing BGT concentration under both watering regimes; however, the growth reduction is much greater in the plants under continued watering ($F_{1, 28}$ = 17.51, $p < 0.001$; Figure 3.12). The response of straw mass to concentration under reduced watering is not linear and introducing a quadratic term to the regression model provided a better fitting model ($F_{2, 27}$ = 12.49, $p < 0.001$).

Watering regime alone did not have a significant effect on the number of tillers counted ($F_{1, 56}$ = 0.517, p = 0.477; Figure 3.13) but there was a significant interaction between concentration and regime ($F_{1, 56}$ = 12.011, p = 0.016). The number of tillers was counted on the untreated plants under reduced watering conditions, however, these plants had some of the lowest total straw masses

which means they were producing shorter or thinner, weaker tillers. It is arguably more informative to consider the effect on the proportion of tillers which produced spike and seed (Figure 3.14). Here, there is a clear effect of both regime ($F_{1, 55}$ = 120.311, $p < 0.001$) and concentration ($F_{1, 55}$ = 11.533, $p = 0.001$), but no interaction between the two. Thus, under both watering regimes, the initial increasing BGT concentration up to 1.67% reduced the mean total number of tillers produced but increased the proportion of flowering tillers. The highest concentration of BGT used, 3.33%, caused an increase in the mean total number of tillers and reduced the proportion of successful tillers, particularly in individuals under the continued watering regime (Figure 3.14).

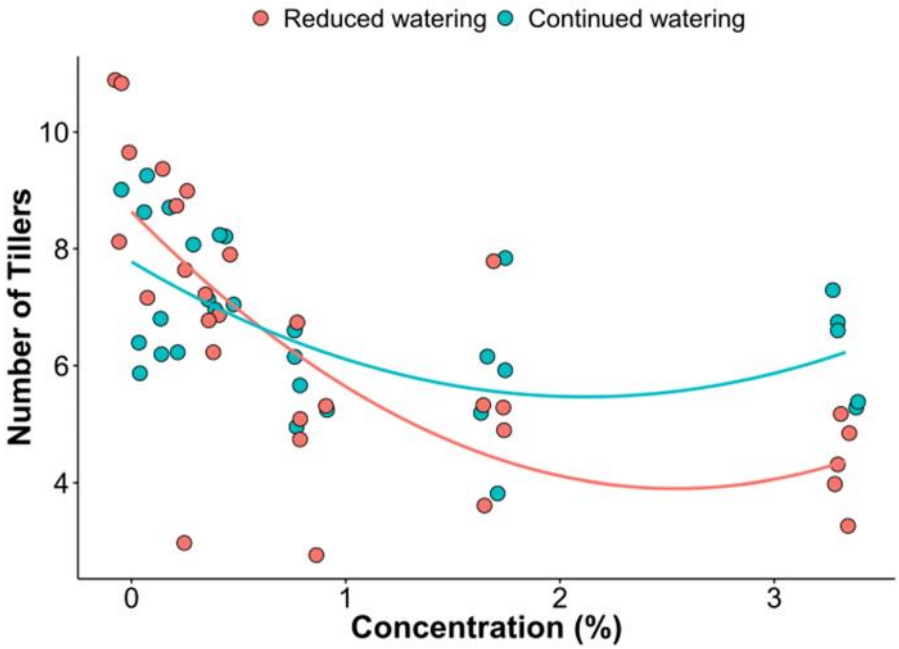


Figure 3.13: Effect of BGT concentration and watering regime on the number of tillers (n = 5 plants per treatment). *Lines represent fitted values from regression model as specified in Table 3.3*

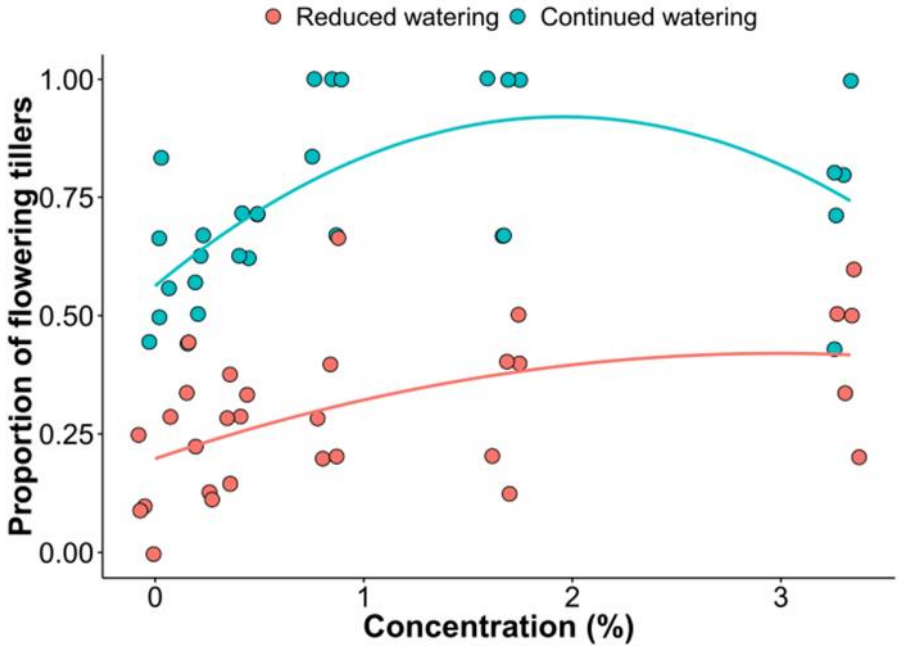


Figure 3.14: Effect of BGT concentration and watering regime on the proportion of flowering tillers (n = 5 plants per treatment). *Lines represent fitted values from regression model as specified in Table 3.3.*

3.4 DISCUSSION

3.4.1 Phytotoxicity of the Test Product BGT

The work outlined in this chapter aimed to test the impact of BGT on plant growth, as well as the interaction it has with soil moisture. In a study on turf grass, carried out prior to this PhD project, on turf grass there were no reported adverse effects on the germination levels of seeds in BGT-drenched soils, nor when a 1% treatment was applied to 21-80 day old plants (unpublished data). However, 7-day seedlings had visible growth reduction and discoloration when treated. The studies in this chapter showed BGT to have adverse effects on seedling germination of wheat and tomato seedlings (Section 3.3.1). The seeds were continually submerged in the BGT solution to act in a way BGT may if applied as a direct seed treatment or soak. This treatment exposure in these assays was acute, however the concentration applied was lower than would be expected to be used if applied as a soil treatment. Increasing treatment concentrations above 3.33×10^{-2} % and 1.33×10^{-2} % increased the probability of wheat and tomato seed failure respectively on filter paper (Figures 3.1 & 3.4), but did not affect the germination success of wheat seeds in any of the soil experiments. Small increases in BGT concentration reduced growth in 7-day wheat and 14-day tomato plants (Figures 3.2, 3.3, 3.5, 3.6). To follow this, wheat seeds were sown in drenched soil pots (Section 3.3.2). The 6.66×10^{-1} % soil treatment caused a small reduction in plant mass after 20-day growth but other treatments had no significant affect (Figure 3.7).

There are minimal studies examining the direct effects of synthetic surfactants on plants although there are some examples that report similar results as were observed in these experiments (Castro et al., 2014; Miyamoto and Bird, 1978). In one study by Horowitz and Givelberg (1979), a range of non-ionic surfactants (mostly Alkyl-polyethers) that are found as secondary ingredients in some herbicides were applied to 7-day- old sorghum seedlings (*Sorghum bicolor* L.). They tested possible root injury caused by exposure to the surfactants by measuring the release of electrolytes and amino acids, which would indicate disruption of the cell membrane. It was reported that most treatments resulted in membrane disruption within 2 to 3 hours of application, and further generic phytotoxic effects were observed in the following days.

Endo et al. (1969) tested the effects of two commercial wetting agents based on linear sulfonate and alkyl polyethylene glycol ether on variety of monocots. It was commonly found that the surfactants were more toxic to plants in solution compared to when in soil. This is believed to be primarily due to the soil sorption of the wetting agent (Valoras et al., 1969). Luxmoore et al. (1974) found that the same non-ionic surfactants penetrated the roots of barley (*Hordeum vuigare* L.) plant and caused flooding of intercellular spaces. At high treatment concentrations, the root tip meristem processes were inhibited.

3.4.2 BGT and Soil Moisture Retention

Unplanted pots drenched with a 6.66 x 10^{-1} % BGT treatment had a significantly slower rate of moisture loss than pots drenched with water alone, suggesting that BGT is able to act as wetting agent to reduce capillary flow to the surface, preventing water loss to the environment (Section 3.3.3).

Unfortunately, the concentration at which the BGT had a beneficial impact on soil moisture was found to be phytotoxic to young wheat plants in soil when weighed 20 days after planting (Section 3.3.2) and when the wheat plants were grown to seed (Section 3.3.4).

In wheat development, tillers develop sequentially giving prioritisation to the development of the older tillers to produce larger grain-bearing heads. The number of tillers is determined by genetic potential and environmental conditions. It is likely more tillers will be produced when temperature, moisture and light are favourable. Under stressed conditions such as drought it is typical that fewer tillers will be produced and initiated tillers may be aborted (Jamieson et al., 1998). In this experiment the highest numbers of tillers were counted on the untreated plants under reduced watering conditions which are assumed to be those that experienced the greatest drought stress. These plants had the lowest proportion of successful fertile tillers. Most of the plants under the reduced watering regime abandoned over half the tillers produced. If the number of abandoned tillers can be taken as a sign of stress, it would appear that the BGT treatments were able alleviate some of the environmental stress, likely due to retaining more moisture in the root zone.

Head size is determined in the mid to late tillering stage, whilst tiller, head and kernel number are all determined in the jointing stage of the plant's growth (Jamieson et al., 1998). Increased BGT concentration caused a reduction in straw mass in the plants that were continually watered but this meant an increase in proportion of fertile tillers and was not reflected in the final mass of seeds, spikes or overall plant mass. It may be possible that the reduced BGT

concentration was able to reduce some environmental stress to the plant that was not recorded here, meaning resources were given to producing fewer more successful tillers. It should also be noted that there was reduced variability in the masses of the plants under reduced watering and this was further reduced with increased BGT concentration.

For initial protection from PPN, the applied substances must be persistent in an effective concentration in the surrounding soil for the initial six to eight weeks in each growing season of susceptible crops. Unfortunately, in these studies it was not possible to determine the concentration of BGT in the soil during the plant's growth. If the product is developed it will be important to look to trace the BGT concentration levels throughout the root zone to establish the levels at which it will remain effective towards pests, perhaps providing beneficial wetting properties to the soil but below phytotoxic levels.

3.4.3 Phytotoxicity of Conventional Nematicides

Most nematicide formulations and application guidelines are created to avoid any potential risk of pesticide inflicted plant damage. However, as many nematicidal products are losing registration or applications allowances are increasingly restricted, many are looking to explore the potential alternative use of other biocides that may have been previously used at a different time in the growing season or to control different pests. This has led to more studies of the phytotoxicity of some nematicides.

1, 3-dichloropropene (1, 3-D) and other fumigant nematicides (where still used) are recommended for pre-planting, as there is a risk of phytotoxic

effects when applied to established plants. It is generally advised in the use of 1,3-D and metam-sodium that there is a waiting period between fumigation and planting, estimated at around 1 week for each 100 L/ha applied with a minimal 14-day interval recommended to avoid any damage to the crops. However, the time for breakdown and volatilization of a fumigant can vary with many factors, including temperature, soil type, application rate and formulation (Desaeger et al., 2008). A treatment applied too early, or crop planted too late may results in insufficient control of nematode population throughout the crop growth period. However, if it applied too late, growers risk plant injury, failed germination or even unsafe pesticide residues in the harvested crop.

As chemical nematode control options are reduced, some have explored the possibility of traditionally fumigant nematicidal products being used at a lower dose during crop growth. The incorporation of emulsifiers enable an application to be mixed in water and applied at a low rate with drip irrigation systems. In a trial on Chilean grapevines (*Vitis vinifera* L.), it was found that a low drip irrigated dose of soil fumigant Cordon® (containing 1,3-D) could be used without significant amounts of phytotoxicity at 200ppm, although greater concentrations significantly reduced plant growth. The post-planting 1,3-D application significantly reduced PPN numbers unlike the two non-fumigant nematicides Nemacur® 240 CS (24% Fenamiphos) and Rugby® 200 CS (20% Cadusaphos) that are more conventionally used (Aballay and Merino, 2015)

Desaeger et al. (2011) explored the potential of methomyl, a systemic broad-spectrum carbamate insecticide that is applied as both a foliar spray and to the soil, primarily used for the control of aphids, leaf miners, thrips and mites.

Methomyl was known to have nematicidal activity when it was first introduced by DuPont in 1968, however, at that time there were many superior nematicides available, particularly the fumigant nematicides, methyl bromide, 1,3-dichloropropene and metam sodium. As these products begin to be removed from the market due to environmental concerns, some are now considering the possible action of less effective controls. Across *in vitro* and pot trials it was found that at the higher applications rates methomyl gave similar amounts of nematode control compared to oxamyl at the recommended application dose, however, there was a trade-off to considered in some crops at these concentrations as phytotoxicity was observed in tomato and cotton plants.

The pre-sowing treatment of seeds with pesticides to improve germination and protect vulnerable seedlings from pest threats has long been a common practice but many compounds have been found to have phytotoxic effects (Gange et al., 1992). There is also evidence to suggest that combinations of active substances may have additive or synergistic phytotoxic effects. In some cases, it has been found that combinations of pesticides were phytotoxic to crops seeds even when the individual compounds were not (Gange et al., 1992). It has been suggested that the reactions of seeds to pesticide treatments may depend on the protein-carbohydrate composition of their food reserve as pesticides can interrupt the enzyme activity (Dalvi and Salunkhe, 1975). Tayal and Agarwal (1982) assessed the toxicity of the systemic nematicide, oxamyl and fumigant, ethylene dibromide (EDB) on a range of legumes. At the recommended dose, there was a small amount of phytotoxicity which worsened with increasing dose including a delay in

germination of seeds and inhibited radicle and plumula growth. However, the study found that the addition of Gibberellic acid could neutralise the toxic effects of the nematicides, particularly their effect on amylase activity.

Rodriguezkabana et al. (1977) explored the potential of oxamyl, carbofuran, and fenamiphos in acetone solutions, as seed treatments. They immersed wheat and rye seeds in acetone solutions of oxamyl, carbofuran, or fenamiphos which were then planted. It was found that fenamiphos was the most phytotoxic. All three nematicides were highly phytotoxic to wheat at 2.5% and 5% (w/v). Wheat seeds were able to tolerate up to 1.25% of fenamiphos or carbofuran. Rye was more tolerant than wheat to the nematicides but exhibited phytotoxic symptoms in all nematicides at 5 %.

3.4.4 Conclusions

The range of studies included in this chapter have shown that whilst the test product BGT is not benign to the plants tested, there is minimal phytotoxicity seen when a BGT solution is applied to soil. Additionally, it is generally accepted that the phytotoxic effects in the field will be less severe than those observed in laboratory or glasshouse trials (Dalvi and Salunkhe, 1975). In comparison to other commonly used nematicides, it would appear that BGT presents no abnormal risks to plants and provides more application options than those that can solely be used pre-planting. There were no trials performed to test the effects of BGT on foliar tissue as most likely it will be applied through drip irrigation or directly to soil to best target soil-borne nematodes. As predicted, the BGT treatments had an additional benefit as it was able to act as a wetting agent to retain soil moisture. This could be

particularly beneficial in application in amenity turfs where the even distribution and retention of water, as well as plant parasitic nematodes cause challenges to ground managers.

4. A FIELD STUDY TO ASSESS THE EFFECTS OF THE TEST PRODUCT BGT ON POTATO CYST NEMATODES, POTATO GROWTH AND SOIL MICROBIAL COMMUNITIES

4.1 INTRODUCTION

In spring 2014, potato field trials were set up by Barworth Agriculture Ltd on behalf on the partner company to this project, Arcis Biotechnology Ltd. The principle aim of the trials was to examine the effects of the novel test product BGT on potato cyst nematode (PCN) numbers and crop yield (Objective 9, Section 1.9). Alongside this, I took soil samples to explore potential impacts of BGT treatments on the soil microbial community, using both community level physiological profiling (CLPP) with Biolog Ecoplates™ (Biolog Inc., Hayward, CA, USA) and taxon-specific quantitative polymerase chain reaction (qPCR) (Objectives 7 & 8, Section 1.9). Given the variation in bacterial dose responses discussed in Chapter 2, I hypothesised that the BGT treatments will likely cause changes in the composition of the soil bacterial communities in the field, reducing the biodiversity with the loss or reduction of susceptible species. The CLPP will allow comparison of the functional diversity and metabolic activity of the bacterial populations in the soil samples, whilst the qPCR will allow comparison of the genetic diversity and relative abundance of the bacterial and fungal populations.

4.1.1 Microbial Biodiversity in Agricultural Soils

Modern agricultural practices and anthropogenic inputs are causing significant changes in biotic communities. Impacts on aboveground diversity are well documented but there is a need for a greater assessment of belowground effects. Bacteria and other microorganisms have a high surface to volume ratio with large contact interfaces with the surrounding environment so are likely to be sensitive bio-indicators of environmental change or contaminants. However, their potential is limited by our knowledge of the regular functioning, processes and community structure (Alteiri, 1999; Bardgett and van der Putten, 2014).

In a study comparing functioning of pasture soil (20 years) to arable soil (2 years of potatoes, 3 years of wheat, 4 years of maize and 26 years of barley) it was found that ‘catabolic evenness’, measured in an assay similar to the Biolog assay, was significantly higher in pasture soil (Degens et al., 2001; Degens and Harris, 1997). The pasture soil had greater organic content, stability of aggregates and cation exchange capacity. The study also showed the conditions in the pasture soil were more resilient to the three stresses tested: decline in pH, increase in electrical conductivity, and increase in Cu concentration. This would suggest that long-used arable soils may already have lower resilience to changes compared to unmanaged soils which may make the system an easy target for new invasive pests and pathogens. Many common management strategies are reducing the ability of agricultural systems to self-maintain soil fertility and pest regulation which is at risk of

creating a negative feedback system forcing management to increasingly rely in external inputs (Swift and Anderson, 1993).

The assessment of the impact of chemicals released into the terrestrial environment is complex, not least due to the soil system alone but also due to significant interfaces with the atmosphere and aquatic systems (European Commission, 2002). It is difficult to predict the relationship between chemical structure or characteristics and the potential effect on microbial populations. The microbial responses to pesticides have shown wide variation including both depressive and stimulatory effects, as well as no measurable effects (Lo, 2010). Pesticide use may in fact cause a surge in populations that are able to degrade it (Baelum et al., 2008; Lancaster et al., 2010).

The introduction of synthetic chemicals into the terrestrial environment may result in a number of changes and effects on different aspects including:

- Capacity of a soil to act as a growth substrate for plants, including effects on seed germination,
- Organisms important for soil function and nutrient cycle conservation,
- Plant biomass production,
- Soil, aboveground and foliar invertebrates which are an important part of food webs and have crucial roles as pollinators, detritivores, saprophages and pest controls,
- Poisoning of birds and mammals,
- Accumulation of toxic compounds in food items.

The current risk assessments for terrestrial ecosystems for the registration of chemicals (specifically any chemical that is deemed to potentially have substantial contact with soil) is based on a limited set of studies. Within the EU, the ecotoxicological assessments are carried out in the defined categories of: terrestrial vertebrates, bees, other arthropods, soil organisms, and non-target plants, as outlined by the European Commission in the Guidance Document on Terrestrial Ecotoxicology Under Council Directive 91/414/EEC (European Commission, 2002). The risk assessments of a substance in soil organisms are based on results from soil carbon and soil nitrification tests (OECD, 2000b; a). The soil carbon test measures glucose-induced respiration rates as a proxy for general microbial activity and the nitrification activity is measured by the production of nitrates (Feld et al., 2015). Some argue that these tests are insufficient to ensure that the product and application doses used are not damaging terrestrial (and subsequently surrounding) ecosystems (Barnthouse, 2004; Jacobsen and Hjelmso, 2014).

Over the past few decades, the rapid development of new molecular tools has provided greater insight into soil diversity and processes and subsequently the effect of pesticides on these systems. However, the lack of standardised methodology, including experimental design, dose concentrations, and extraction and detection methods limit comparisons between independent studies (Imfeld and Vuilleumier, 2012; Jacobsen and Hjelmso, 2014).

Few studies address the effects of low but chronic pesticide exposure in soil ecosystems which may be of greater long term damage than high, acute contamination instances as ecosystems are then not given the opportunity to

repair and return to its initial state. Soil tolerance is the ability of the community to withstand potential toxic contamination and disturbance and survive in the resulting conditions. Soil resilience is the ability to return to the original state following deleterious effects (Imfeld and Vuilleumier, 2012). Some refer to the combined tolerance and resilience of soil as soil stability (Tilman, 1996). The stability of some functions may not be related to the stability of the community structure (Nannipieri et al., 2003). The tolerance of soil communities to a disturbance event is much easier to study than the resilience, particularly when there is a lack of information on the spatial and temporal patterns of soil biodiversity in regularly functioning systems (Bardgett and van der Putten, 2014). Barnthouse, (2004) suggested that risk assessment and management of plant protection products should consider the rate of recovery of populations and ecosystems following exposure.

As mentioned with other organism assays in previous chapters, product formulations of pesticide products are likely to elicit different responses than that of the pure active substance, as additives affect the movement and persistence of the substance in addition to resulting potential additive or synergistic toxicity themselves. In the field, it is of course also likely that there will not just be contamination from one solely used product, but rather a continually varying composition of agrochemicals used throughout the lifetime of an arable field.

4.1.2 Potato Cyst Nematodes

Potato cyst nematodes (PCN), *Globodera rostochiensis* (yellow cyst nematode) and *Globodera pallida* (white cyst nematode) are highly prevalent

pests in UK potato production. They are obligate endoparasites which attack potatoes along with other members of the Solanaceae family (including tomatoes), and some other root vegetables. Their prevalence and ecology (discussed in Section 1.3) makes them a challenging pest to control. In the last comprehensive study in 1998, PCN were found in 64% of potato fields surveyed in England and Wales (Minnis et al., 2002). *G. rostochiensis* was initially the most prevalent, however, the population numbers and distribution of *G. pallida* steadily increased since the 1970s with the introduction of some potato cultivars with partial resistance to *G. rostochiensis* (Minnis et al., 2002; Trudgill et al., 2014).

A developing juvenile PCN penetrates a host's root tissues with the use of a pointed stylet. The nematode will migrate through tissues, using enzyme secretions to weaken plant cell walls. It will then establish a feeding site where it will remain until maturity. Through further excretions, cells at the feeding site are modified to become a highly metabolic to act as a plant nutrient sink for the nematode to feed from. During this feeding period females become sedentary. Their bodies swell, rupturing the root tissue prior to fertilisation. The size of females at maturity has been found to correlate with the nutritional status of the plant and intraspecific competition between nematodes. Once mature, males will migrate to locate a female. After fertilisation, the female cuticle forms a tough cyst in which the eggs are laid. The hatching of both *G. rostochiensis* and *G. pallida* show a high dependence on the presence of host root exudate. However, the robust cysts mean eggs can remain viable for a long time, surviving desiccation in soil in the absence of a suitable host. Cysts may also be readily dispersed by wind, human movement and crops.

Potato varieties have varying levels of tolerance to PCN populations. King Edward potatoes, as used in this trial are known to be very susceptible to PCN but are one of the most popular crops amongst consumers due to texture and taste so are still favoured over more tolerant potato varieties. A negative correlation is typically found between the initial population of PCN (Pi), and potato crop yield and PCN multiplication. However, this may be strongly influenced by soil type and by cultivar tolerance or resistance (Trudgill et al., 2014).

4.1.3 Community Level Physiological Profiling

It has been previously shown that bacterial communities produce reproducible patterns of carbon source utilisation and the methods of measuring this to distinguish bacterial samples from a range of environments have been developed over the past 50 years. The incorporation of redox sensitive dyes now allows for rapid community profiling. In the late 1980s, Biolog Inc. introduced commercially-produced microplates to give a relatively simple and replicable study method of isolates (Garland, 1997). Since then, a variety of different plates have been produced for different applications. The assay was originally developed and described by Garland and Mills (1991) who used Biolog GN Microplates™ to measure the characteristics of a bacteria community.

The Biolog Ecoplate™ assay allows the characterisation of microbial communities based on a pattern of substrate utilization in 96-well microtitre plates containing three replicates of 31 specific carbon sources (and a control well with no substrate) (Table 4.1) (Choi and Dobbs, 1999; Insam, 1997).

Each well also contains a tetrazolium dye. As microbes utilise the carbon source, the respiration works to reduce the colourless dye to violet formazan. Following inoculation and incubation of the plates, the formation of the purple colour is measured spectrophotometrically as an indicator of sole-carbon-source utilization (Figure 4.1). Due to differences in the utilisation rate of different substrates by different bacterial groups, a high variability in colour development rate and intensity can be observed to help obtain a community-level physiological profile (CLPP). The 31 different substrates include a range of carbohydrates, polymers, amino acids, amines or amides, and carboxylic and ketonic acids, as shown in Table 4.1. It is expected that each species of bacteria present will be able to utilise more than one of the carbon sources. For example, *Bacillus thuringiensis* will grow on 13 of the 31 carbon sources (Mulcahy and Edenborn, 2007).

The Biolog assay was developed as a sensitive and powerful tool to measure temporal and spatial differences in communities based on the reproducible sole carbon source utilisation patterns. It has been used to distinguish the effect of a range of different managements and treatments on catabolic activity of bacterial communities. Recent examples of uses include the effects of herbicides (Anza et al., 2016) and fungicides (Sulowicz and Piotrowska-Seget, 2016). Unlike similar profiling tools, the Biolog assay does not require an isolation phase, making it a rapid and convenient tool for comparing diversity of whole bacterial communities (Zak et al., 1994).

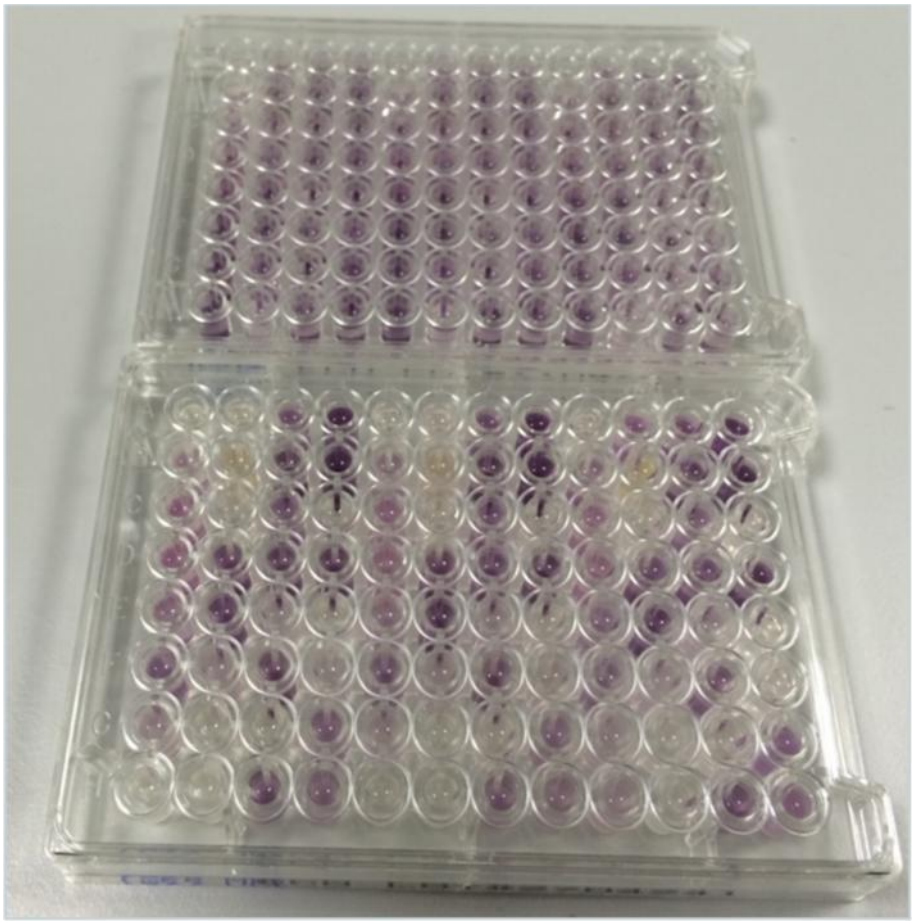


Figure 4.1: Biolog Ecoplates^{TM} following inoculation and incubation.

Table 4.1: Carbon Sources in Biolog Ecoplate™ with compound group as grouped by Weber and Legge (2009).

1 Water -	9 β-Methyl-D-Glucoside Carbohydrate	17 D-Galactonic Acid γ-Lactone Carboxylic and ketonic acid	25 L-Arginine Amino Acid
2 Pyruvic Acid Methyl Ester Carbohydrate	10 D-Xylose Carbohydrate	18 D- Galacturonic Acid Carboxylic and ketonic acid	26 L-Asparagine Amino Acid
3 Tween 40 Polymer	11 i-Erythritol Carbohydrate	19 2-Hydroxy Benzoic Acid Carboxylic and ketonic acid	27 L- Phenylalanine Amino Acid
4 Tween 80 Polymer	12 D-Mannitol Carbohydrate	20 4-Hydroxy Benzoic Acid Carboxylic and ketonic acids	28 L-Serine Amino Acid
5 α- Cyclodextrin Polymer	13 N-Acetyl-D-Glucosamine Carbohydrate	21 γ-Hydroxybutyric Acid Carboxylic and ketonic acid	29 L-Threonine Amino Acid
6 Glycogen Polymer	14 D- Glucosaminic Acid Carboxylic and ketonic acids	22 Itaconic Acid Carboxylic and ketonic acid	30 Glycyl-L- Glutamic Acid Amino Acid
7 D-Cellobiose Carbohydrate	15 Glucose-1-Phosphate Carbohydrate	23 α-Ketobutyric Acid Carboxylic and ketonic acid	31 Phenylethylamine Amine/amide
8 α-D-Lactose Carbohydrate	16 D,L-α- Glycerol Phosphate Carbohydrate	24 D-Malic Acid Carboxylic and ketonic acid	32 Putrescine Amine/amide

Using the Biolog Ecoplates™ for CLPP provides a large amount of information, which some have referred to as a metabolic fingerprint (Garland, 1997). This can be used to make a number of comparisons:

1. The overall rate of colour development indicating the abundance of bacteria in the samples.
2. The richness and evenness of the responses amongst the wells indicating the diversity in the samples.
3. The relative rate of utilisation of the substrates to demonstrate the similarities and differences in community composition across the samples.

To optimise the repeatability and discriminative power of the assay consideration needs to be given to the sample preparation and incubation conditions. There is a range of documented extraction procedures, all of which include stages for aggregate dispersion and separation of the cells from organic and inorganic particles. Calbrix et al. (2005) carried out a study to evaluate these methods to establish an effective reproducible procedure. Soil particles will have a major involvement in the optical density reading of the solution so it is important that as much as possible is removed, whilst ensuring the optimal integrity and abundance of the bacterial community extracted. Following a comparison of extraction procedures, Calbrix et al. (2005) found there was similar efficiency across all commonly used methods in the quantity of cell forming units (CFU) extracted and it was found that there was no limitation to the quickest extraction procedure based on a sodium chloride wash, which I therefore chose to use in this study. I also chose the

centrifugation speed and duration, and incubation conditions, based on the optimal recommendation from the comparative study.

As with all culture-dependent methods, the Biolog assay has the primary limitation that only the cultivable microbes that can grow in the high-nutrient assay conditions will contribute to the substrate utilisation. The tetrazolium dye used is not metabolized by fungi so they do not contribute to the profile seen on the plates (Praveen and Tarafdar, 2003).

It is not known precisely which bacterial species contribute to the utilization profile, which will be characterised by the different nutrient requests and complicated interactions between the microorganisms. It is suspected that rapid-growing bacteria, well adapted to nutrient rich environments will be more prominently represented in the assay. Whilst the 31 substrates have been specifically collated to allow the plates to be sensitive to changes in the soil bacterial community, it has been noted that the substrates may not fully represent the diversity of carbon sources available to the bacteria in their environments, which may limit the ecological relevance (Glimm et al., 1997; Hill et al., 2003). A study of the populations grown in Biolog GN plates (a similar microtiter plate with 95 carbon substrates) found that subsets of populations were selected for in the assay (Smalla et al., 1998). The possible selection of populations in the assay will depend on numerous factors, including the competitiveness of species in the cultivation conditions and their ability to oxidise the carbon source at the concentration provided. There will also be antagonistic and synergistic interactions among the populations. It is possible that the history of the community sample may also affect how the

sample is reflected in the plate growth pattern. Heuer and Smalla (1997) compared a community from an activated sludge reactor with that of a potato rhizosphere. They proposed that the community from the activated sludge would be more accurately represented in the plates as the community developed in a nutrient rich environment which selects for bacteria that can grow rapidly on readily available carbon sources, akin to the Biolog plate environment. In contrast, the potato rhizosphere community had been selected in an environment that contains diverse substrates present at low concentrations. There have been several studies of model communities that have shown that not all the community present is contributing to the utilization pattern revealed by the dye but it is not known what proportion of the species are represented (Balser et al., 2002; Smalla et al., 1998; Heuer and Smalla, 1997; Preston-Mafham et al., 2002; Stefanowicz, 2006). Heuer and Smalla (1997) also found that *Clavibacter michiganensis* can grow in some of the substrates included but only reduced the tetrazolium dye when the substrate was in high concentration giving an excess of energy. Furthermore, there are some bacteria potentially present in the soil samples that will not reduce the dye (Balser et al., 2002; Stefanowicz, 2006).

There is an underlying assumption when making inferences about the functional diversity that the colour development in each well is based solely and directly on the growth function of the proportion of the individual bacteria present in the sample able to utilize the particular substrate (Hill et al., 2003). Whilst this is not a completely unrealistic assumption, users need to be aware that colour development intensity may not be completely dependent on growth of bacteria in the environmental sample.

These limitations would be of greater concern if this method was used in an attempt to characterise soil microbial communities. However, CLPP has been recognised as a useful method for comparative research studies of soils exposed to stressing factors. Whilst in this chapter I will principally refer to diversity, metabolic activity or abundance of a soil sample for simplicity, it should be noted that the assay does not measure the actual metabolic activity of the soil community from which the sample was taken. An accurate statement on the assays measurements is given by Stefanowicz (2006) as the "metabolic potential of the part of a community which is capable of being metabolically active and growing in plate conditions".

The analysis aimed to compare the three main aspects of information provided by the Biolog assay that can be used to infer possible changes to the bacterial community: 1) Diversity of substrate-utilisation (bacterial functional diversity), 2) Average well-colour development (AWCD) (bacterial abundance), 3) Relative substrate-utilisation (the community composition and metabolic activity) to establish if any changes occur due to the treatment applied.

4.1.4 Quantitative Polymerase Chain Reaction

The development of quantitative polymerase chain reaction (qPCR), also known as real-time PCR, has provided a valuable tool for exploring soil microbial communities. It enables a quantitative assessment of the abundances of specific taxonomic groups of microorganisms in soil by amplifying specific conserved genetic sequences (Fierer et al., 2005).

The polymerase chain reaction (PCR) process amplifies a targeted gene sequence from initially low numbers (e.g. in an environmental sample), to higher, easily detectable amount. The process is made up of three main stages driven by a cycling temperature regime; 1) denaturing of the double stranded DNA in the sample, 2) annealing of oligonucleotide primers to target sequence, 3) extension of DNA sequence.

Quantitative PCR relies on the same process but with the addition of a fluorescent binding dye. In this study the qPCR assay used a SYBR® Green dye for amplicon detection. During the PCR, the *Taq* polymerase amplifies the target sequence which creates PCR products also known as amplicons. The SYBR® green dye is then able to bind to each new amplicon of double-stranded DNA which results in an increase in fluorescence proportionate to the amount of PCR product produced.

In initial cycles, as the amount of PCR product is low, there is little measurable change in the fluorescence signal. This is used to define the baseline for the amplification plot. The fluorescence intensity will increase above the baseline once the accumulated target is detected. A fixed fluorescence threshold is then set above the baseline. Each qPCR reaction is characterised by the fractional number of cycles which occur before the fluorescence passes the fixed threshold. This enables the comparison of different reactions using the parameter C_T (threshold cycle). A higher amount of the starting copy of the nucleic acid target will result in an earlier detection of the fluorescence of the attached fluorescent DNA binding dye, *i.e*. lower C_T value.

Interpretation of quantitative PCR has the underlying assumption that the efficiency of amplification is constant in each cycle. To allow comparison across reactions it is important that the experimental conditions are carefully controlled. Many factors will have an impact on the C_T. Environmental conditions such as solution pH and salt concentration can cause changes to the fluorescent emission of any molecule (D'Haene et al., 2010). The normalised reporter (Rn) is the ratio of the fluorescence emission intensity (SYBR®) to that of the passive reference dye (ROX). It is therefore also important that there are equal quantities of the same reference dye across reactions to allow comparison.

The equation below describes the exponential amplification of PCR:

$$\textit{Copy number at cycle } n = \textit{Initial copy number} \times (1 + \textit{Amplification efficiency})^n$$

At a maximum efficiency of 1:

$$\textit{Copy number at cycle } n = \textit{Initial copy number} \times 2^n,$$

meaning there will be a two-fold increase at each cycle. A decrease in efficiency would result in less PCR product produced at each stage leading to a higher C_T value (Raeymaekers, 2000). It is then possible to estimate the initial abundance of the target sequence.

The main disadvantage of the SYBR® green technology is the potential for false positive signals. The SYBR® dye binds to any double-stranded DNA present so will also bind to other non-specific sequences such as primer

dimers, which are the product resulting from the hybridisation of primer molecules. It is important that the primers are designed to ensure they do not amplify non-target sequences in the sample and that a melt curve analysis is performed (Heid et al., 1996).

In this study, I used quantitative PCR to estimate and compare the relative abundance of broad major taxonomic groups of bacteria and all fungi across the soil samples over the field trial treatment course.

4.2 METHODS

4.2.1 Field Site and Soil Collection

The trials were carried out on arable farm land near Spalding, Lincolnshire, UK (52°47'41.3" N, 0°05'23.0" E) (Figure 4.2). The soil type was silty loam (P 3.2, K 3, Mg 3.1) with an initial pH of 7.5. King Edward seed potatoes were planted on the 3rd April 2014. The trials were designed and conducted by Barworth Agriculture Ltd. The trials plots were laid out in a fully randomised block design with five replicates of six treatments as shown in Figure 4.3; 0.5L/ha BGT (the test product), 1L/ha BGT, 2L/ha BGT, 4L/ha BGT, 8L/ha BGT and 2 water controls. Each plot contained four 4 m furrows, separated from the adjacent row by 1 m. It was initially intended that one of these control plots would be used for a commercial rate application of Vydate® 10G but this was not applied for reasons beyond my control. Instead, for nematode counts and potato yield measurements, the BGT treatments were compared to four plots of the same size treated with Nemathorin®, a granular nematicide containing 10% w/w fosthiazate, within the same field, adjacent to the experimental plots.

One week after planting, the first treatments were applied in a volume solution of 500L/ha at the concentrations listed above. In the rest of the chapter this is named as day 0. Further treatments were then applied every two weeks. Soil samples were taken on the day of the first treatment prior to application and then at 2, 70 and 151 days after. Three weeks prior to harvest, the crops were desiccated with the application of a desiccant herbicide, a process sometimes

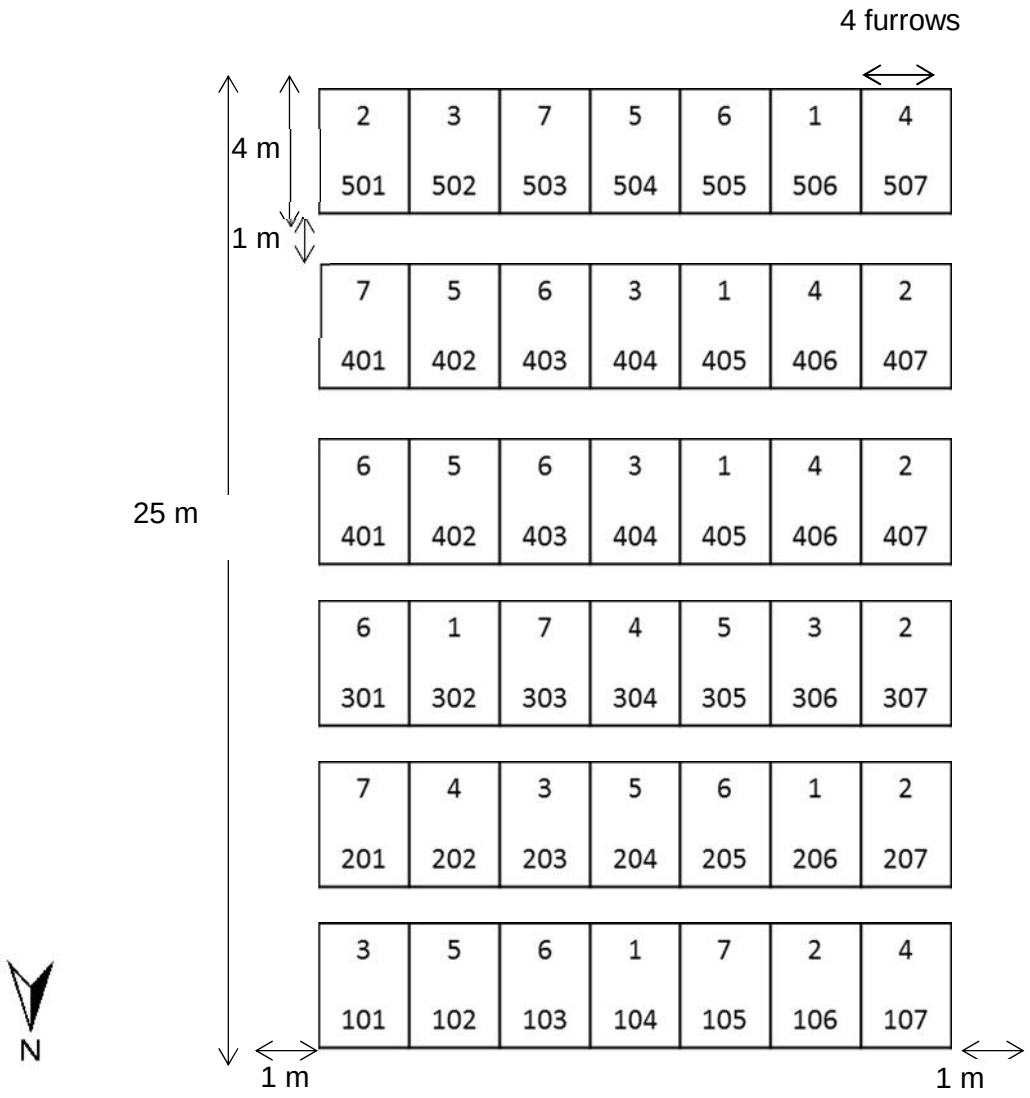
known as “burning off”. This ensures more even ripening of the tubers, allows an earlier harvest and reduces strain on harvest materials that may be caused by dense aboveground material. The full treatment and sampling schedule is shown in Table 4.2

Soil samples were taken from the middle two furrows in attempt to minimise possible edge effects caused by the adjacent plot treatment. Samples were stored at 4°C during the transportation. In a description of approaches to use of the Biolog Ecoplates^{TM}, Insam et al. (2004) advises that samples may be stored for up to 10 days at 4 °C where necessary but fresh samples are superior. In this study, due to sampling and transportation timings, the first samples were stored at 4°C for 72 h before they were processed in the laboratory. To control for this, all the remaining samples were also stored in the same way for 72 h before extraction. Soil samples to be used for qPCR were initially stored at 4°C during transportation and then were frozen in liquid nitrogen and stored at -80°C.

Table 4.2: Schedule of trial treatments and planting, April - September 2014.

Date	Trial	Days since first treatment
03 April	Seed potatoes planted	-
10 April	Initial sample and first treatment	0
12 April	Second sample	2
24 April	Second treatment	21
08 May	Third treatment	35
15 May	Ground cover count	42
22 May	Fourth treatment	49
29 May	Ground cover count	56
19 June	Third sample	70
14 August	Desiccant applied	133
21 August	Desiccant applied	140
01 September	Fourth sample	151
04 September	Harvest	154

Figure 4.2: Field trial site, near Spalding, Lincolnshire, UK at 0 (top), 70 (middle) and 151 (bottom) days after treatment.



Treatment list

1. 0.5 L/ha BGT
2. 1 L/ha BGT
3. 2 L/ha BGT
4. 4 L/ha BGT
5. 8 L/ha BGT
6. Control
7. Control

Figure 4.3: The plot layout of the BGT field trial near Spalding, Lincolnshire, UK, beginning in April 2014.

4.2.2 Preparation and Reading of Biolog Ecoplates™

Before the samples could be used to inoculate the plates they had to be processed to remove soil aggregates and debris which would interfere with the optical density in the assay. To begin, 3 g of fresh soil was mixed in 30 ml sterile sodium chloride (NaCl) solution for 3 minutes on a flatbed vortex at 1500 rpm. The solution was then centrifuged at 129 x g for 5 minutes. The soil suspension was diluted 1 in 10 parts in sterile deionised water. Using one plate for each sample, 130 µl of the soil solution was added to each of the 96 wells on the Biolog Ecoplate™. The initial optical density (OD_{590}) was read at 0 h to provide a reference value for each well using a Biotek ® Synergy™ 2 multi-mode reader. The plates were incubated in darkness at 20°C. The optical density was read at 12 h intervals for 156 h. In the final analysis 5 of the total 50 plates were not included as there was suspected contamination of one or more of the blank control wells.

4.2.3 Statistical Analysis of Community Level Physiological Profiling: Diversity of Substrate Utilisation

The changes in diversity were measured in an approach similar to that used in a study by Muniz et al. (2014) with the application of generalised additive models (GAMs) to explore the changes in diversity of the substrate utilisation during the incubation period of the plate. The mean optical density was calculated across the 3 substrate replicates on each plate at each measured time point. The mean reading was then normalised by dividing each mean reading by the initial mean OD at 0 h for the same substrate. This works to reduce the variability due to differences in inoculum densities. The diversity of

substrate utilisation (DSU) was determined using the Shannon-Weaver index (H) calculated using the formula below, where p_i is the normalised OD value calculated by dividing each OD reading by the sum of the OD readings on that plate at that time point (Muniz et al., 2014; Shannon, 1948):

$$H = -\sum p_i \ln p_i.$$

The Shannon-Weaver index (H) is a commonly used diversity measure. It allows for consideration of both the number of groups represented and the relative abundance within the groups. When applied to the data collected from the Biolog Ecoplates™, the H value quantifies the ability of community in the sample to utilise the 31 carbon sources, acting as an index of functional diversity. A higher H value will indicate communities that are able to degrade more substrates and/or degrade the substrates with similar efficiency.

The GAM models were produced using the gam function in the *mgcv* package version 1.8-16 (Wood, 2011). A GAM allows for the capture of the shape of the relationship of DSU over incubation time without prejudging, using a particular parametric from. Cubic regression splines were used as smoothers in the model, which work by fitting third order polynomials on sections of the data. The number of sections of data is determined by the number of 'knots' specified. The number of 'knots', along with the model parameter and structure was optimised to minimise the generalised cross validation (GCV) criterion and the Akaike information criterion (AIC). The final model included 13 smoothers applied over the DSU-time relationship. One smoother was applied for all samples taken on day one and then 12 individual smoothers were applied to each treatment group on each sample day so as not to assume the

relationship was the same across all samples. Treatment concentration and sample day were treated as categorical factors. The effect of sample row was also included as a model categorical model term.

4.2.4 Statistical Analysis of Community Level Physiological Profiling (CLPP): Abundance and Community Composition

The average well colour development (AWCD) of each plate was used to compare the abundance of bacteria in the samples across the treated plots. Following this, the patterns of substrate utilisation were used to explore any possible changes to the composition and metabolic activity of the community in the treated plots across the sampling days.

The AWCD was calculated from the normalised mean optical density across each plate. A two-way analysis of variance was applied to the AWCD values at 132 h after incubation to explore the interactive effects of sample day and treatment concentration. The same analysis was also carried out at 96,108,120 h, but the readings at 132 h showed the most variation between the samples and therefore was most likely to differentiate between the samples. The row from which the sample was taken was initially included as a factor in the model but it was found not to have a significant effect on the AWCD so was removed from the final model. A Tukey's HSD (honest significant difference) test was then applied to examine which sample groups were different from each other.

Following this, the recorded optical density of a substrate at 132 h was divided by the AWCD of its plate at that time. This was done to explore the relative changes in the abundance of growth on each substrate which would indicate that changes have occurred in the community composition or metabolic potential. The potential interactive effect of treatment concentration and sample day was assessed using an ANOVA followed by a Tukey HSD test.

4.2.5 Preparation of DNA for Quantitative Polymerase Chain Reaction (PCR)

The DNA was extracted from 0.25g of soil samples using the PowerSoil® DNA Isolation Kit from Mo Bio Laboratories Inc., according to the manufacturer's instructions. After extraction DNA samples were frozen at –20°C until used in qPCR.

4.2.6 Quantitative Polymerase Chain Reaction

The oligonucleotides or primers were synthesised by Eurofins genomics (Ebensburg, Germany) (Table 4.3). The oligonucleotides were initially diluted in sterile, nuclease-free, PCR grade water to a concentration of 100 µM as specified by the manufacturer. In preliminary studies, the qPCR assay was carried out on a selection of the soil samples with annealing temperatures of 55 °C, 60 °C and 65 °C with primer concentrations of 200 - 500 µM to ensure optimal conditions. The optimal primer concentration of 250 µM was chosen as the lowest concentration which resulted in the lowest Ct with minimal hybridisation of primer molecules (primer-dimers). Two additional primer pairs

to amplify Firmicutes species and Basidiomycota species were trialled but did not produce consistent results under the range of conditions tested.

Table 4.3: Group specific primers used for the qPCR assay as previously described by Fierer et al. (2005).

Primer name	Primer sequence (5'-3')	Target Group	Reference
Eub338	ACT CCT ACG GGA GGC AGC AG (20)	All Bacteria	(Lane, 1991)
Eub518	ATT ACC GCG GCT GCT GG (17)	All Bacteria	(Muyzer et al., 1993)
Alf685	TCT ACG RAT TTC ACC YCT AC (20)	α- Proteobacteria	(Lane, 1991)
Bet680	TCA CTG CTA CAC GYG (15)	β-Proteobacteria	(Overmann et al., 1999)
Actino235	CGC GGC CTA TCA GCT TGT TG (20)	Actinobacteria	(Stach et al., 2003)
Cfb319	GTA CTG AGA CAC GGA CCA (18)	Bacteroidetes	(Manz et al., 1996)
Acid31	GAT CCT GGC TCA GAA TC (17)	Acidobacteria	(Barns et al., 1999)
ITS1f	TCC GTA GGT GAA CCT GCG G (19)	All Fungi	(Gardes and Bruns, 1993)
5.8s	CGC TGC GTT CTT CAT CG (17)	All Fungi	(Vilgalys and Hester, 1990)

Table 4.4: Primer combination used in qPCR with target regions as estimated by Fierer et al. (2005).

Target group	Forward primer	Reverse primer	Approximate amplicon length (bp)
All Bacteria	Eub338	Eub518	200
α- Proteobacteria	Eub338	Alf685	365
β-Proteobacteria	Eub338	Bet680	360
Actinobacteria	Actino235	Eub518	300
Bacteroidetes	Cfb319	Eub518	220
Acidobacteria	Acid31	Eub518	500
All Fungi	5.8s	ITS1f	300

The qPCR assays were carried out in clear polypropylene 96-well plates with the Agilent Mx3000P Thermocycler. Each reaction contained: 8 µl sterile nuclease-free PCR grade water, 10 µl Brilliant III Ultra-Fast SYBR® Green QPCR Master Mix with Low ROX (Agilent Technologies), 0.5 µl upstream primer and 0.5 µl downstream primer (Table 4.4). The reactions were prepared in stock solutions for each plate in the order given above to reduce pipetting error. The solution was then transferred to the 96-well plate before the addition of 1µl sample DNA to give a final reaction solution volume of 20 µl. PCR grade water was added in place of the DNA in the no template controls. Plates were sealed with strip caps. Each plate included a reaction for each DNA sample in triplicate and a no template sterility control for each primer pair. PCR conditions were; 15 minutes at 95 °C, followed by a fast two-step process of 95°C for 15 seconds followed by 60°C for 30 seconds

repeated for 40 cycles. A dissociation step was then carried at 95°C for 1 minute, 55°C for 30 seconds and 95°C for 30 seconds. The dissociation curve (melting curve) was checked following each assay to ensure that the fluorescent reading was due to the intended specific PCR product and not a result of primer-dimers or other unwanted artefacts.

The SYBR® Green master mix allows for accelerated qPCR as it contains a mutated form of *Taq* DNA and a chemical hot start mechanism to promote faster hot start release to improve amplification specificity and faster replication. Each reaction contained 30 nM of the ROX passive reference dye which is included within the master mix. Fluorescence intensity for the reference dye does not change over the PCR reaction so enables a stable baseline to which the samples can be normalised to compensate for variations in fluorescence not due to the PCR reaction. The mix also includes a Deoxynucleotide (dNTP) mix (GATC), Magnesium (Mg^{2+}) and a buffer.

4.2.7 Statistical Analysis of Quantitative Polymerase Chain Reaction

Ct values, along with the mean and standard deviation of the technical replicates, were extracted using MxPro-Mx3005P v4.10 Build 389.Schema 85 (Stratagene, 2007). The Ct values were calculated using a common amplification-based threshold across all PCR reactions. This threshold is estimated by the software following a search for fluorescence values within the product exponential amplification to minimise Ct spread among replicates (Stratagene, 2009). For this experiment this was calculated at 21.661 Fluorescence (dR). Technical replicates of soil samples with a standard

deviation greater than 0.5 in Ct value were removed from the data set. The replicates which were removed were those with outlying, abnormally high Ct values indicating pipetting error or failed PCR reaction (D'Haene et al., 2010). For interpretation, Ct values were inversed as shown below to give a relative value attributable to the initial abundance of each target sequence.

$$Initial\ abundance = 1/\ 2^{Ct\ value}.$$

When there was no Ct value, indicating that the target sequence did not reach the detectable level in the 40 cycles, a value of 0 was given. The abundance values were used to calculate an index of diversity as described in section 4.2.3. A diversity index was calculated for each soil sample.

The abundance of the target sequence for total bacteria and total fungi were compared using a two-way ANOVA to test the possible interactive effect of sample day and treatment concentration. The initial abundance values of all other bacterial group sequences were divided by that of the total bacteria target sequence in the same sample to compare their relative abundances in the samples. These were then compared with a two-way ANOVA.

4.2.8 Crop Growth, Yield and PCN Counts

In the same trial, staff at Barworth Agriculture took several measurements over the trial period. Percentage ground cover (to a 5% accuracy) was assessed visually at 42 and 56 days (6 and 8 weeks after planting). Once harvested, tubers were graded by longest dimension and weighed. Soil samples were taken at time of planting and harvest to give initial (Pi) and final

(Pf) counts of PCN eggs per gram of soil. I was able to analyse these data alongside the other experiments I carried out. For statistical analysis, the response variables were checked for normality and homogeneity of variance. In all cases the response variables were log-transformed in the ANOVA to avoid violations of the model assumptions. I also calculated a Pearson's product moment correlation to test the relationship of potato yield with some of the other measured parameters.

4.3 RESULTS

4.3.1 Potato Cyst Nematode Population Growth Following Potential Nematicidal Treatment

Over the course of the trial, the number of PCN eggs per gram of soil increased: The mean number of eggs initially recovered (Pi) was 22.26 ± 11.24 eggs/g which increased to 265.90 ± 100.41 eggs/g in the final sample (Pf) (Figure 4.4). This is not unexpected, as the introduction of the suitable potato host plant is known to promote egg hatching and subsequently nematode development. There was no statistically significant difference in the numbers of PCN eggs recovered from the different plots before the trial (ANOVA: $F_{6, 2} = 1.728$, $p = 0.147$), however, there was a large amount of variation across samples, ranging from 3 - 50 eggs/g. In contrast, the Nemathorin® treatment appears to have had a significant effect on the number of eggs/g recovered from the plots at the end of the treatment (ANOVA: $F_{6, 32} = 6.379$, $p < 0.001$).

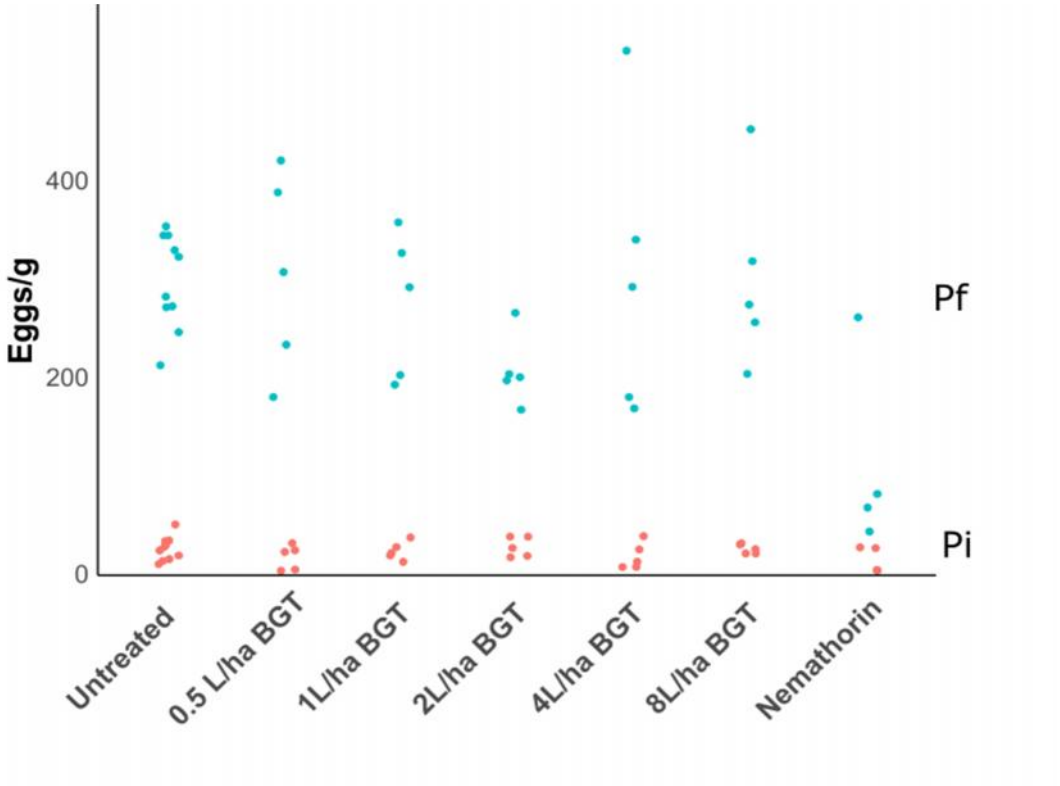


Figure 4.4: Mean number of PCN eggs per gram taken from treated plots before treatment (Pi) and at the end of the trial immediately before harvest (Pf).

However, when looking at the relationship between the number of eggs initially recovered and those recovered at the end (Pf/Pi) *i.e*. the reproduction rate, there is no significant effect of the type or concentration of the treatment (ANOVA: $F_{6,29} = 0.969$, $p = 0.463$) (Figure 4.5). This means none of the treatments caused a net reduction in the reproduction of PCN between when these samples were taken.

There were two plots treated with 0.5 L/ha which were found to have an atypically low egg density in the initial sample (Pi). However, this was not reflected in the final egg densities (Pf). This may have been due to insufficient or unrepresentative samples taken from those plots. To avoid issues caused by over-dispersion they were removed from the analysis but are shown in Figure 4.5.

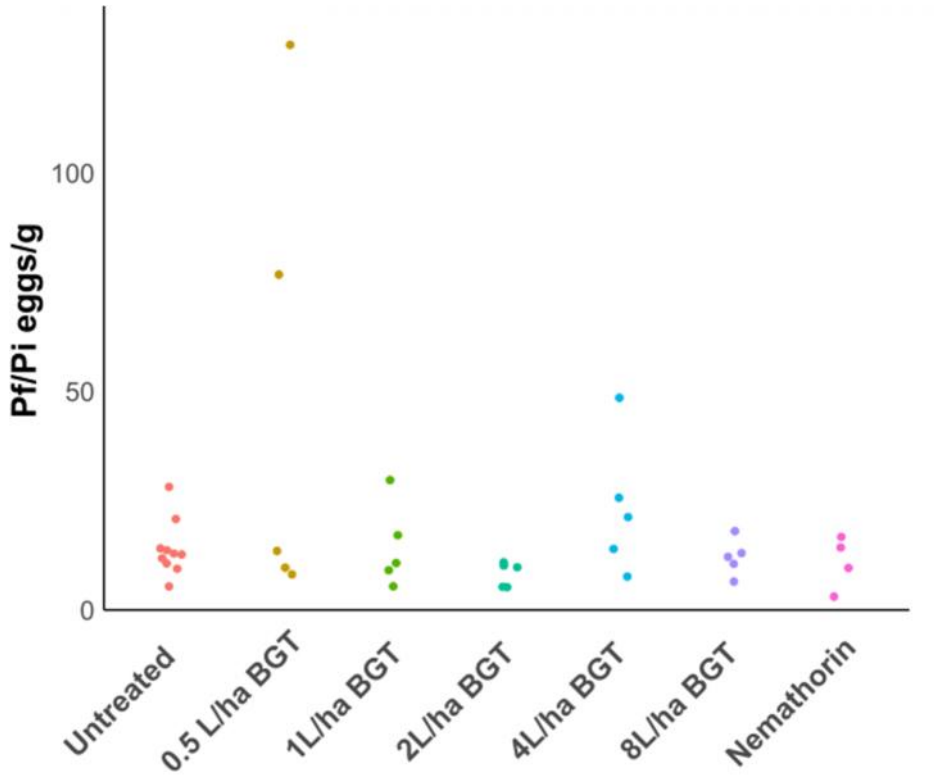


Figure 4.5: Ratio of eggs per gram from treated plots before treatment (Pi) and at the end of the trial immediately before harvest (Pf).

4.3.2 BGT Treatments did not Impact Aboveground growth of Potato Plants

Percentage ground cover of potato plant foliage was recorded for each plot at 42 and 56 days after the first treatment application (6 and 8 weeks after planting of seed potatoes) to observe the effect of the treatment on aboveground growth of the potato plants (Figure 4.6). There were no significant differences found across the plots after 42 days (ANOVA: $F_{6, 32}$ =

1.288, p = 0.291), but at 56 days, the treatment type was found to have a marginally significant effect on the percentage ground cover of plots (ANOVA: $F_{6, 32} = 2.734$, p = 0.029). The post hoc testing revealed there to be a significant difference between the Nemathorin® and BGT applied at 8 L/ha (p = 0.014). None of the other treatments were found to be significantly different from each other. It is possible this effect is due a combination of both direct

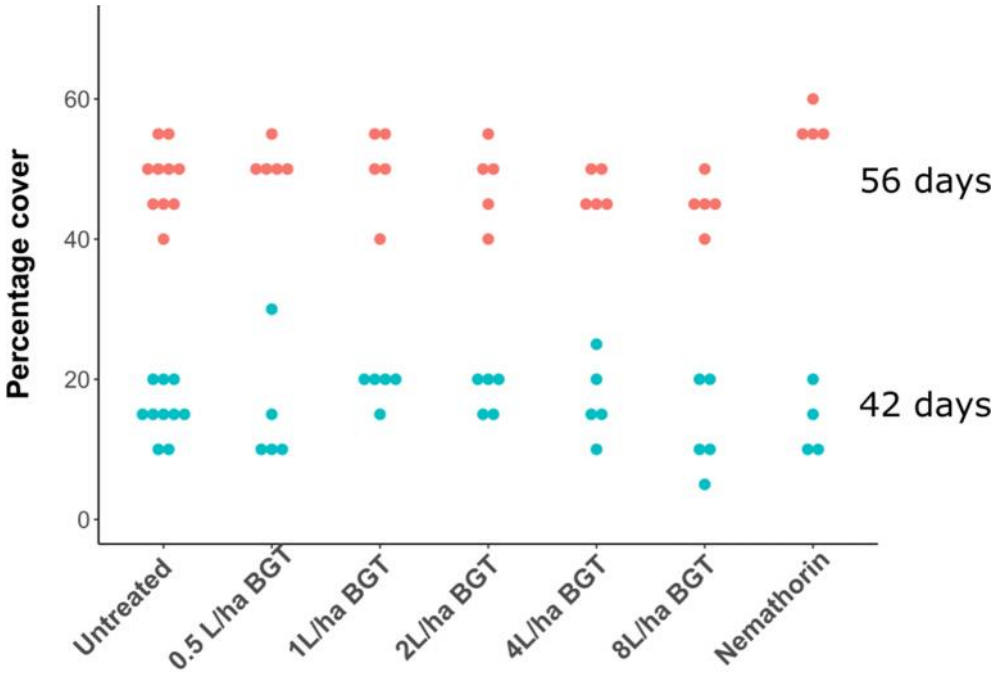


Figure 4.6: Percentage ground cover of treated plots observed at 42 days and 56 days after first treatment.

and indirect impacts of the treatment application. None of the BGT treatments caused an increase or decrease of aboveground growth compared to that of the control treatment, which suggests there was not a large phytotoxic effect of BGT. However, there is a possible small decreasing trend in ground cover in the higher BGT treatments.

4.3.3 The Effect of BGT on Potato Yields

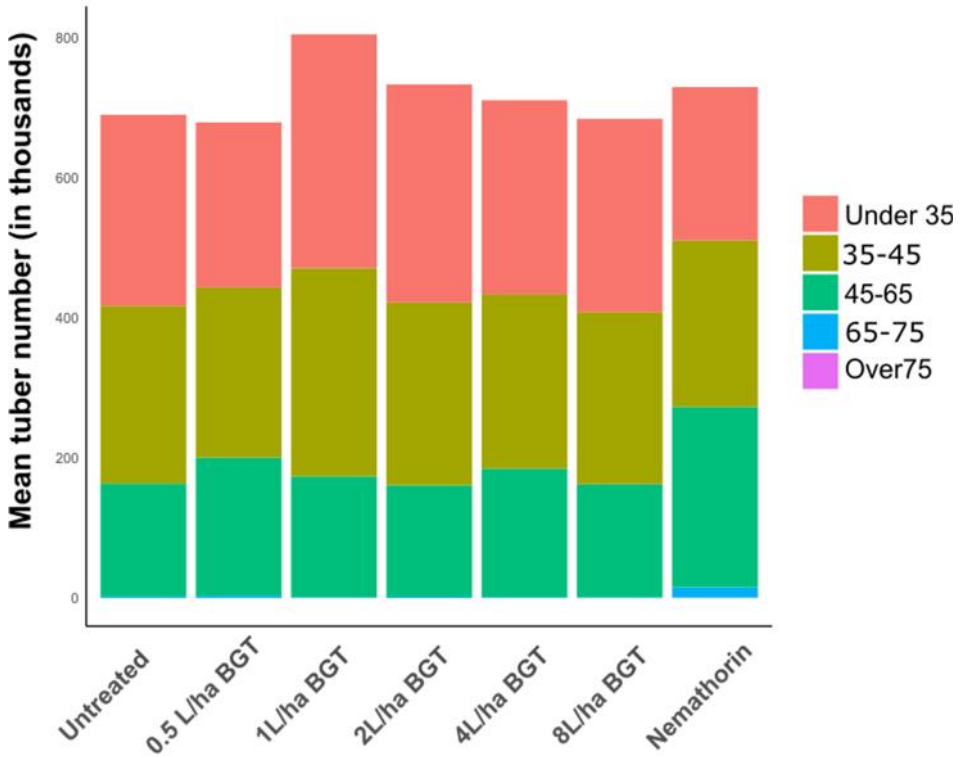


Figure 4.7: Graded mean number of potato tubers from the treated plots.

Tubers from the plots, were counted, weighed and graded based on longest length. There was no significant effect of any of the treatments on the numbers of tubers recovered (ANOVA: $F_{6, 32} = 0.673$, $p = 0.672$). However, there were differences in the grade size of the tubers collected (Figure 4.7). The Nemathorin® plots produced more potatoes of a grade above 45 mm. These were the only plots to produce tubers of a grade above 75 mm (although this is not visible on the scale of Figure 4.8). There were no significant differences in the distribution of tuber sizes amongst the other treatments. The effect of Nemathorin® treatments on tuber growth is more apparent in the comparison of weight of the tubers from the plots (ANOVA: $F_{6, 32} = 5.429$, $p < 0.001$; Figure 4.8). Post hoc testing revealed that the weight of tubers from the Nemathorin® plots was significantly greater than all other

plots. There were no significant differences between the control and BGT plots.

4.3.4 The Relationship between PCN Egg Abundance and Potato Yield

It is evident that there was a significantly greater yield (tonnes per hectare) with larger tubers from the plots treated with the granular treatment Nemathorin®. However, comparisons of the Pf/Pi ratios would imply that there was not a significant reduction in nematode population growth and reproduction. To explore these data further, a Pearson product-moment correlation coefficient was calculated to test the relationship between potato

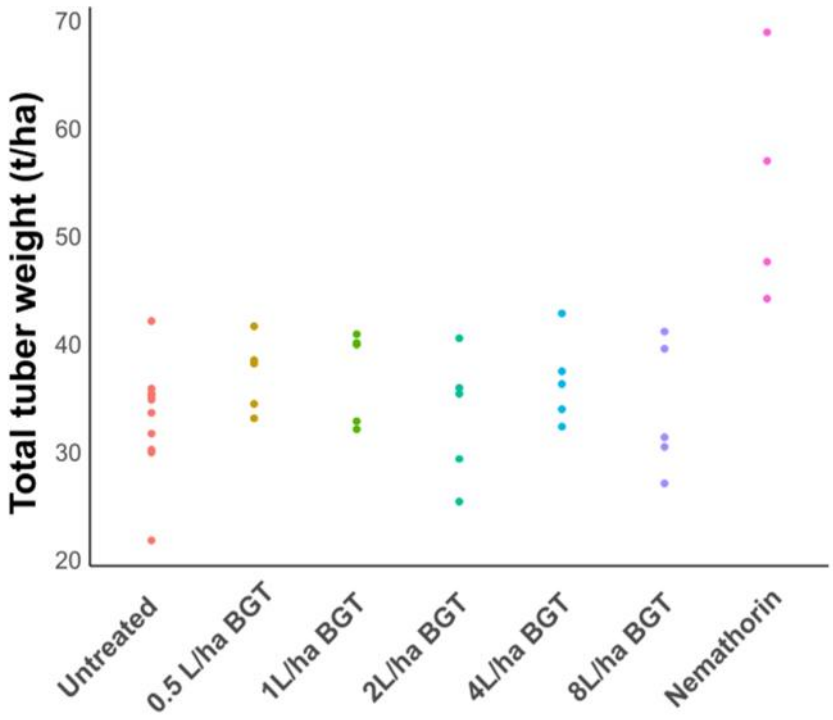


Figure 4.8: Total yield by tuber weight (tonnes per hectare) from the treated plots

yield and some of the other measured parameters.

There was a weak negative correlation between yield for both the initial PCN count ($r = -0.419$, $t_{37} = -2.808$, $p = 0.007$) and final PCN count ($r = -0.391$, $t_{37} = -2.851$, $p = 0.01$) (Figure 4.9). Notably, the two plots with highest total yield were the plots with the lowest initial number of PCN eggs. At the intermediate yield values, there is weak correlation between yield and both initial and final egg numbers. This would appear to show that yield was just as likely to be a result of the initial egg density, as the post-treatment density. Consistent with this, there was no correlation between potato yield and the Pf/Pi ratio ($r = -0.419$, $t_{37} = 0.032$, $p = 0.914$) (Figure 4.9c).

There was no correlation between final yield and ground cover 42 days after treatment (6 weeks after planting) ($r = -0.111$, $t_{37} = -0.678$, $p = 0.502$). In contrast, final yield was moderately positively correlated with ground cover 56 days after treatment (8 weeks after planting) ($r = 0.465$, $t_{37} = 3.200$, $p = 0.003$) (Figure 4.10).

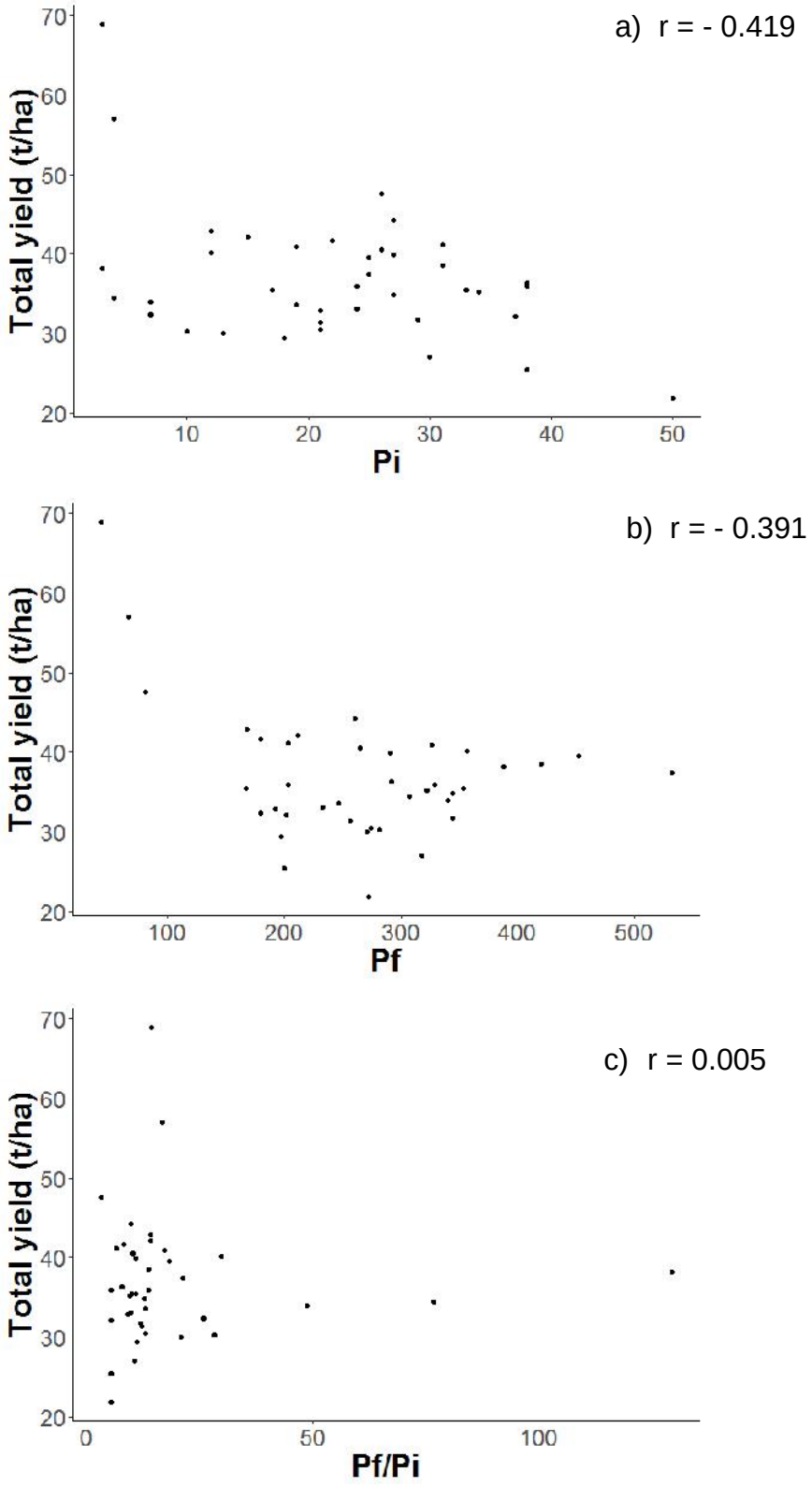


Figure 4.9: Correlation between potato yields (tonnes per hectare) of treated plots with a) Initial number of eggs per gram (Pi), b) Final number of eggs per gram (Pf) and c) Relative increase in eggs per gram during trial (PfPi).

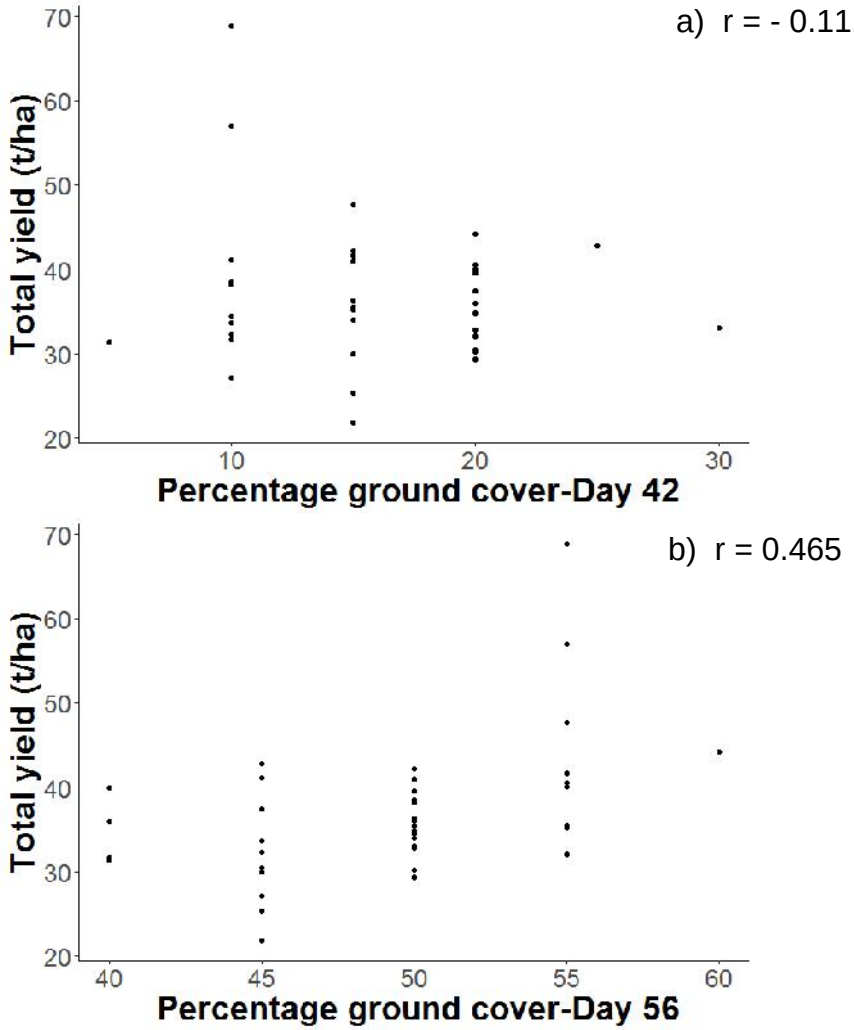


Figure 4.10: Correlation between total potato yield (tonnes per hectare) and percentage aboveground cover observed at a) 42 days and b) 56 days after treatment.

4.3.5 The Diversity of Substrate Utilisation Measured with Biolog Ecoplates^{TM} Following BGT Treatments in a Field Trial

I applied a general additive model (GAM) to compare the time-course changes in the diversity of substrate utilisation (indicated by the H value) across the sampled days and treated plots. The model showed that the time course varied with the sample day and the plot row from which the sample was taken, and a large proportion of the deviance was explained by these two factors (93.9%). The effective degrees of freedom, F value and p value of the estimated flexible effect of the non-parametric components of the GAM are shown in Table 4.5.

The DSU (Diversity of Substrate Utilisation) pattern across all the samples was qualitatively similar. As observed at day 0, pre-treatment (Figure 4.11), the diversity (H value) is highest at time zero, then gradually declines during the early stages of incubation before increasing. A large H value indicates communities that are able to degrade more substrates and/or degrade the substrates with similar efficiency. The red dotted line represents the relationship that would be seen in a hypothetical community in which all the carbon sources were utilised at the same rate, such that DSU remained constant as a function of time. The DSU at 0 h has a value of 3.47 for every sample set, as this is the H value obtained when applying the Shannon-Weaver index calculation to a normalised initial reading value of 1 in 31 samples. The highest ‘diversity’ index is achieved at 0 h, as there is consistently no growth across all the wells. All the samples show a decrease in diversity within the first 48 h of incubation. In the early hours of incubation,

fast growing bacteria begin to metabolise some of the carbon sources, giving rise to a higher diversity index. The rate of decrease is similar amongst all the sample groups. This suggests that there were no impacts on some groups of fast growing bacterial populations. The differences between sample groups occur as the diversity index begins to increase as the sources which are less easily metabolised begin to release the dye. The samples taken on day 0 and day 2 begin to show an increase in diversity after around 60 h of incubation, whereas the samples taken on day 70 do not show an increase until after 84 h at the earliest.

Table 4.6 shows which parameters had a significant effect on the DSU-time course relationship. The largest variation in communities recovered was caused by sample day. Within days, there was some variation caused by the applied treatment concentration. Although not highly significant, there is a possible effect caused by the 8% BGT treatment on day 2. The effect of the 8% treatment becomes more significant on days 70 and 151. The least diverse recovered populations were taken on day 70 from the plots treated with 4% and 8% BGT. On sample days 70 and 151, extra samples were analysed from row 3. The model estimates that the row from which the sample was taken from may have a small but significant effect on the DSU-time course relationship. This effect may be slightly exaggerated as not all groups had samples taken from both rows (Figure 4.12: f-m).

Table 4.5: Estimated non-parametric components of generalised additive model (GAM) based on the changes in diversity of substrate utilisation (DSU) changes during incubation time.

Smooth effect of variable	E.D.F.	F value	p value
s(time): day = 0	8.196	35.23	< 0.001
s(time): treatment = 0, day = 2	6.908	29.63	< 0.001
s(time): treatment = 0, day = 70	5.755	145.07	< 0.001
s(time): treatment = 0, day = 151	8.57	83.54	< 0.001
s(time): treatment = 1, day = 2	6.567	16.1	< 0.001
s(time): treatment = 1, day = 70	6.151	73.03	< 0.001
s(time): treatment = 1, day = 151	8.063	97.19	< 0.001
s(time): treatment = 4, day = 2	5.671	12.58	< 0.001
s(time): treatment = 4, day = 70	5.613	92.88	< 0.001
s(time): treatment = 4, day = 151	7.458	42.6	< 0.001
s(time): treatment = 8, day = 2	5.866	12.8	< 0.001
s(time): treatment = 8, day = 70	4.396	34.51	< 0.001
s(time): treatment = 8, day = 151	8.497	53.26	< 0.001

Table 4.6: Estimated parametric components of generalised additive model (GAM) based on the changes in diversity of substrate utilisation (DSU) during incubation time. *Significant p values (< 0.05) are shown in bold.*

Parameter	Estimate	Std.Error	t value	p value
Intercept (Day 0, Treatment 0%, Row 1)	3.140	0.004		
Treatment 1%	-0.004	0.008	-0.54	0.593
Treatment 4 %	0.011	0.005	1.956	0.051
Treatment 8%	-0.008	0.008	-0.97	0.334
Day 2	0.217	0.011	19.96	**< 0.001**
Day 70	0.195	0.006	35.14	**< 0.001**
Day 151	0.226	0.004	63.19	**< 0.001**
Row 3	-0.005	0.002	-2.26	**0.024**
Treatment 1%: Day 2	0.028	0.020	1.381	0.168
Treatment 4%: Day 2	0.022	0.012	1.81	0.071
Treatment 8%: Day 2	0.035	0.018	2.002	**0.046**
Treatment 1%: Day 70	-0.003	0.013	-0.23	0.816
Treatment 4%: Day 70	-0.021	0.008	-2.57	**0.011**
Treatment 8%: Day 70	0.027	0.012	2.288	**0.023**
Treatment 1%: Day 151	-0.005	0.007	-0.77	0.441
Treatment 4%: Day 151	0.010	0.005	1.946	0.052
Treatment 8%: Day 151	0.019	0.007	2.866	**0.004**

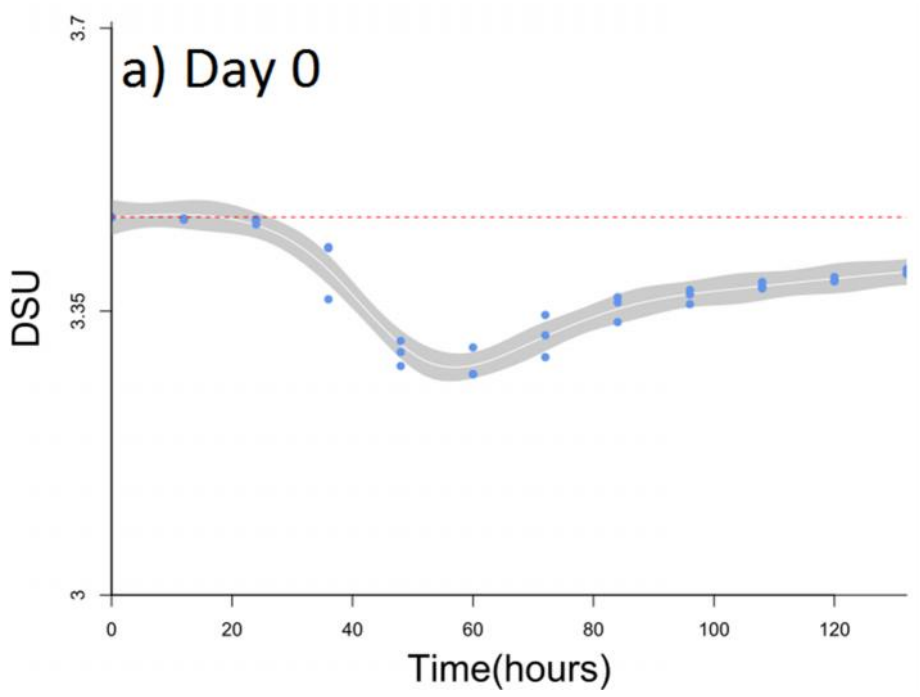
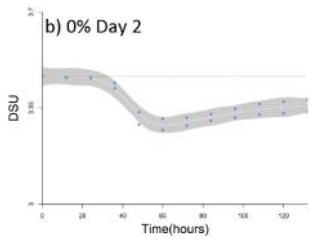
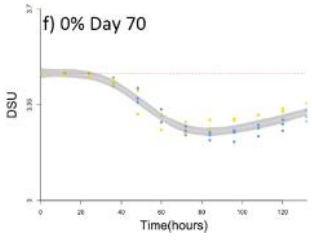
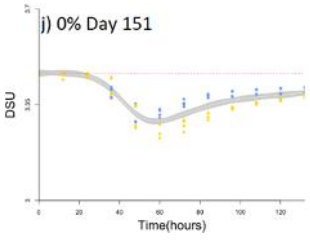
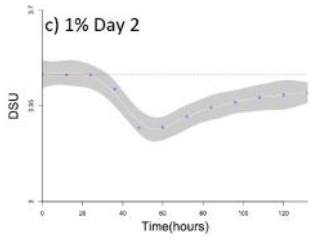


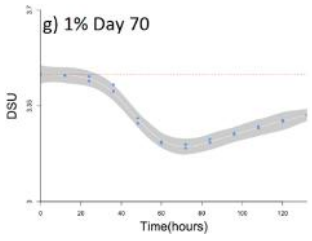
Figure 4.11 (a-m): Estimated effects of time-related changes in the diversity of substrate utilisation (DSU) of the soil sampled grouped by treatment received and day sampled. *The y-axis represents the changes in measured diversity (calculated using the Shannon-Weiner index) over incubation time (hours), obtained from a GAM. The 95%-point confidence interval is indicated by the shaded band. The data points show the mean DSU values calculated for each plate. The red dotted line at zero represents the relationship that would be seen in a hypothetical community in which all the carbon sources were utilised at the same rate such that DSU remained constant as a function of time. The smoother and the estimated degrees of freedom obtained are listed in Table 4.5. The sample row is indicated in blue (row 1) and yellow (row 3 – sample days 70 and 151 only).*

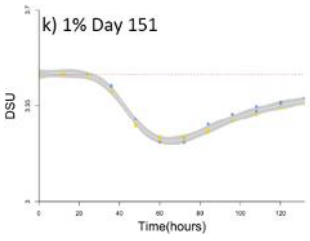

b) 0% Day 2
DSU
Time(hours)

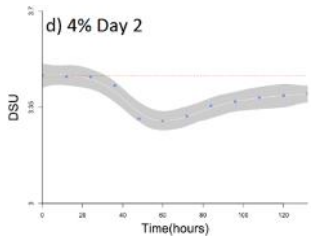

f) 0% Day 70
DSU
Time(hours)

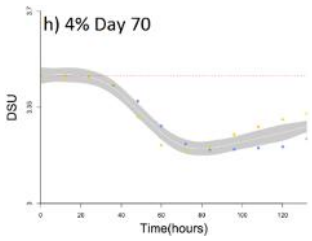

j) 0% Day 151
DSU
Time(hours)

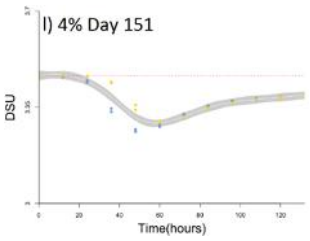

c) 1% Day 2
DSU
Time(hours)

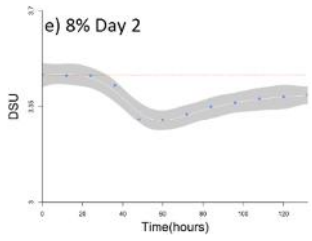

g) 1% Day 70
DSU
Time(hours)

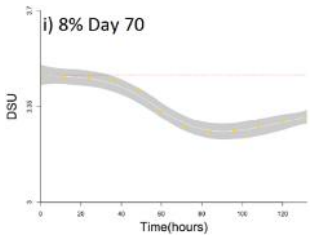

k) 1% Day 151
DSU
Time(hours)

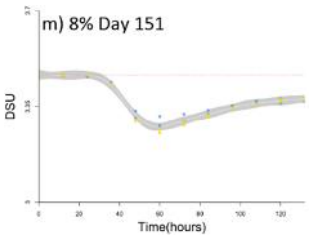

d) 4% Day 2
DSU
Time(hours)


h) 4% Day 70
DSU
Time(hours)


l) 4% Day 151
DSU
Time(hours)


e) 8% Day 2
DSU
Time(hours)


i) 8% Day 70
DSU
Time(hours)


m) 8% Day 151
DSU
Time(hours)

4.3.6 The Metabolic Potential of Soil Samples Following BGT Treatment in a Field Trial

The average well colour development (AWCD) for each plate at 132 h was compared to explore the relative abundances of bacteria in the soil samples (Figure 4.12). There was a large amount of natural variation seen in the AWCD, particularly amongst the samples taken on day 0 before any treatment was applied. The variation in the samples reduced with the beginning of the trial although there is still a lot of variation in the samples which is not accounted for by any of the controlled parameters. Despite this, there were significant changes in the AWCD across the sample days (Table 4.7). The ANOVA model indicated there was a significant difference between treatment concentration and sampling day, however, post hoc test showed that this was not between comparable plots (significant differences are indicated by different letters in Figure 4.12). The large variation in the data means definitive conclusions cannot be drawn based on AWCD alone but it would appear there may be a small increase in AWCD caused by 4% BGT on day 70.

Table 4.7: Analysis of variance table assessing the potential interactive effects of sample day and BGT concentration on average well colour development (AWCD).

Parameter	D.F	F value	p value
Day	3	14.582	**< 0.001**
Treatment	3	1.276	0.306
Day: Treatment	8	2.765	**0.026**
Residuals	23		

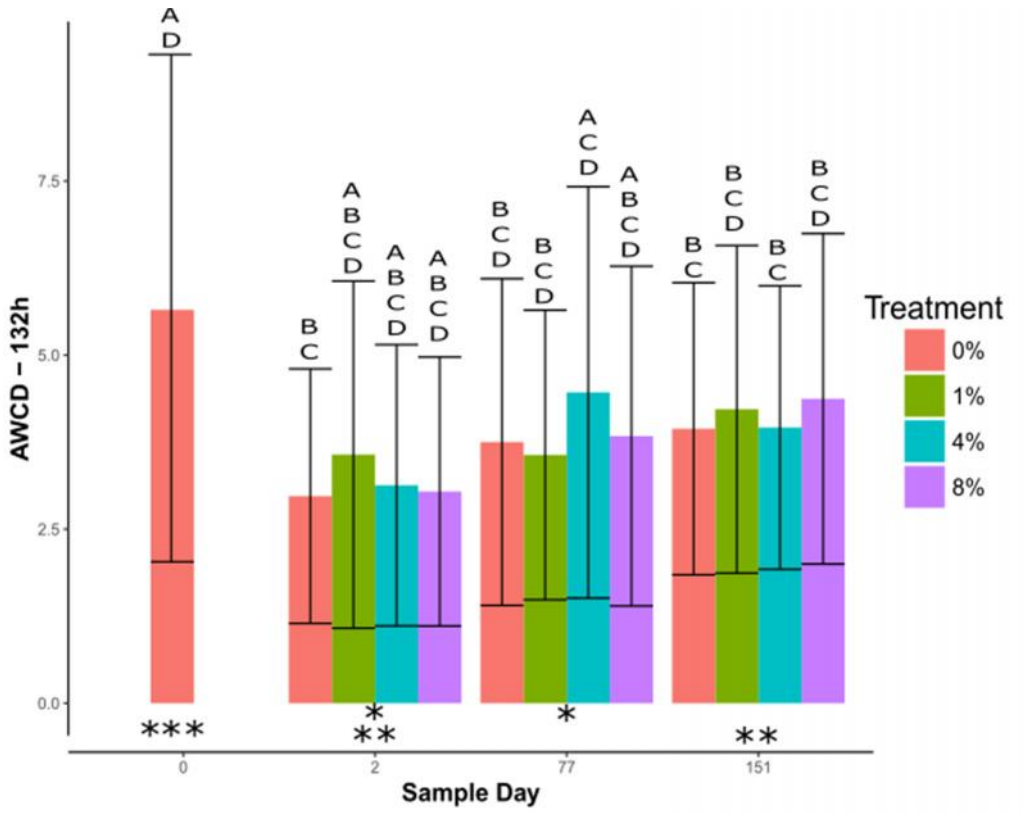


Figure 4.12: Average well colour development (AWCD) of Ecoplates™ after 132 h of incubation. *AWCD grouped by mean in sample group. Error bars show standard deviation within sample group. The individual sample groups sharing the same letter are not significantly different ($p < 0.05$). Sample days sharing the same asterisk level were not significantly different without any treatment concentration effect (i.e. No significant different between AWCD levels on day 2 and 77).*

4.3.7 The Effects of BGT Treatments on the Utilisation Pattern of Substrates on Biolog Ecoplates™

To examine changes in the bacterial community composition, 31 individual ANOVAs were applied to explore any potential differences in the relative growth on the individual substrates after 132 h of incubation (Table 4.8). The mean optical density for each substrate on each plate was divided by the AWCD of the plate. Of 31 substrates, it was found that growth varied significantly on 23 of the substrates between 2 or more sample days. The post hoc test revealed (as is also observed in Figure 4.13) that this was mostly due to a significant change in bacterial growth from the day 70 samples compared to the other days. This implies that there were significant changes in the functional composition of the bacterial community diversity across the days the field was sampled. A small number of substrates were found to have significant differences in bacterial growth due to treatment. These data support the conclusions drawn from the analysis of the diversity of the substrate utilisation, as it shows that some of the substrates (including 15, 16, 23, 29 and 30) resulted in lower than average amounts of bacterial growth, whereas other substrates (including 12, 20, 22 and 28) enabled high amounts of growth and metabolism. There were no significant changes in the growth of bacteria on substrates 6, 10, 11, 16, 19, 20, 30 or 31. There was no noticeable correlation between substrate utilisation and the type of compound (Table 4.1).

Table 4.8: Analysis of variance table assessing the potential interactive effects of sample day and BGT concentration on the relative growth of each Biolog Ecoplate™ substrate. *The relative growth values were log transformed in the model. The p values of the effects found to be significant (p < 0.05) are shown in shaded cells.*

	D.F.	F value	p value	F value	p value	F value	p value	F value	p value	F value	p value	F value	p value
Substrate		**2**		**3**		**4**		**5**		**6**		**7**	
Day	3	4.881	**0.009**	28.726	**< 0.001**	3.739	**0.025**	18.268	**< 0.001**	1.899	0.158	5.877	**0.004**
Treatment	3	0.143	0.933	0.857	0.477	1.008	0.407	0.894	0.459	0.105	0.957	0.287	0.834
Day: Treatment	8	0.381	0.920	0.956	0.493	0.699	0.689	1.599	0.180	0.511	0.836	1.620	0.174
Substrate		**8**		**9**		**10**		**11**		**12**		**13**	
Day	3	16.504	**< 0.001**	19.570	**< 0.001**	2.971	0.053	0.252	0.859	26.455	**< 0.001**	7.069	**0.002**
Treatment	3	1.313	0.294	1.784	0.178	1.241	0.318	2.951	0.054	0.480	0.700	2.067	0.133
Day: Treatment	8	1.637	0.169	3.985	**0.004**	1.361	0.265	1.613	0.176	0.611	0.760	0.935	0.508
Substrate		**14**		**15**		**16**		**17**		**18**		**19**	
Day	3	4.467	**0.013**	9.881	**<0.001**	1.487	0.245	2.844	0.060	20.714	**< 0.001**	2.718	0.068
Treatment	3	2.065	0.133	0.315	0.814	1.365	0.278	2.980	0.052	5.155	0.007	1.902	0.157
Day: Treatment	8	0.657	0.723	0.756	0.643	1.070	0.417	1.356	0.267	1,121	0.386	0.886	0.543
Substrate		**20**		**21**		**22**		**23**		**24**		**25**	
Day	3	1.143	0.353	5.354	**0.006**	4.554	**0.012**	13.984	**< 0.001**	7.929	**0.001**	7.879	**0.001**
Treatment	3	0.255	0.857	0.694	0.565	1.236	0.319	9.718	**< 0.001**	0.283	0.837	0.983	0.418
Day: Treatment	8	0.741	0.656	0.346	0.938	2.390	**0.049**	3.946	**0.005**	0.417	0.899	0.672	0.711
Substrate		**26**		**27**		**28**		**29**		**30**		**31**	
Day	3	22.111	**< 0.001**	8.473	**< 0.001**	7.404	**0.001**	17.240	**< 0.001**	3.511	**0.031**	2.647	0.073
Treatment	3	0.555	0.650	0.643	0.595	3.361	**0.036**	0.503	0.684	0.110	0.953	1.434	0.259
Day: Treatment	8	0.547	0.809	0.387	0.917	0.488	0.852	1.299	0.292	1.009	0.457	2.112	0.077
Substrate		**32**											
Day	3	5.564	**0.005**										
Treatment	3	0.990	0.415										
Day: Treatment	8	0.926	0.514										

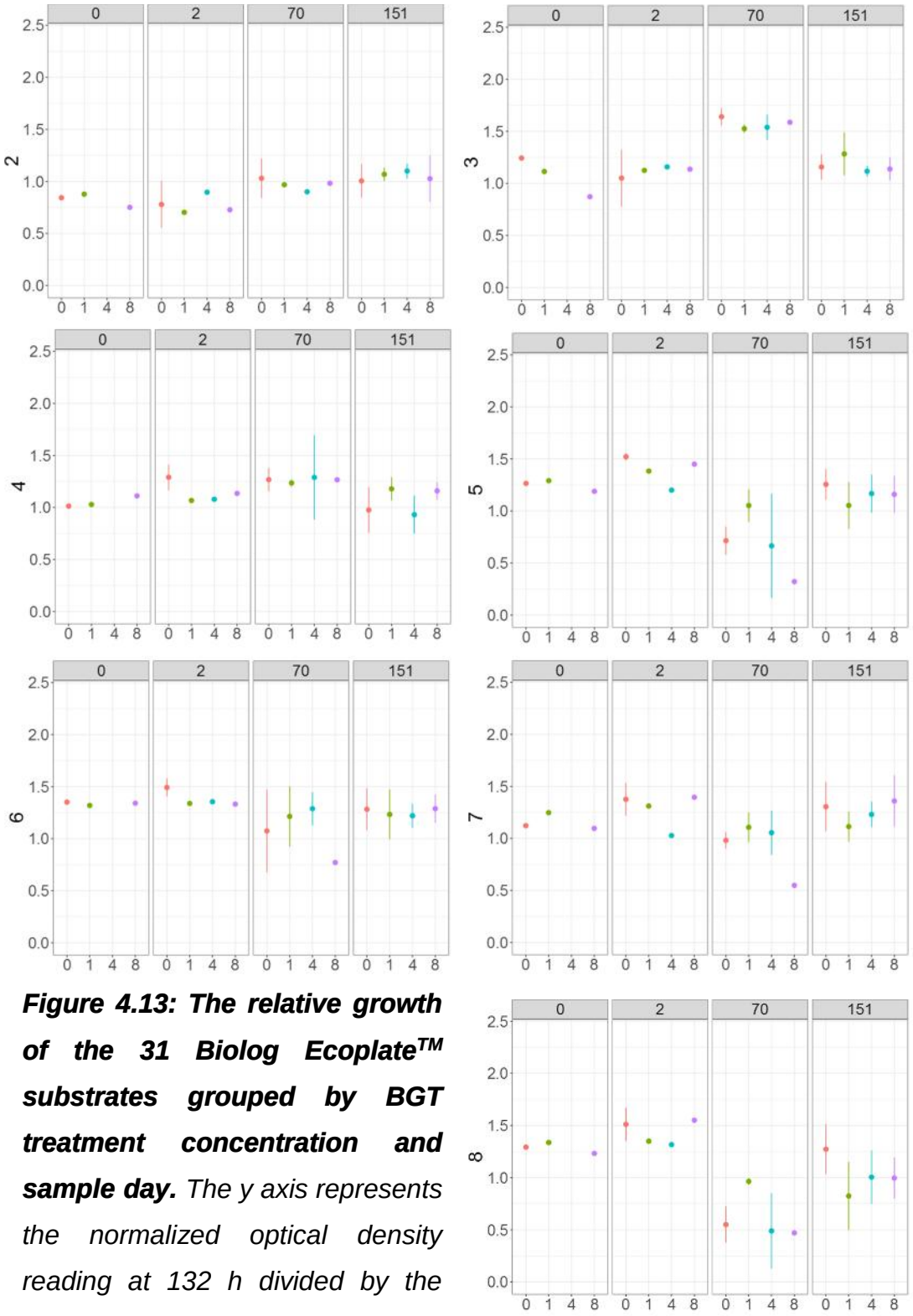
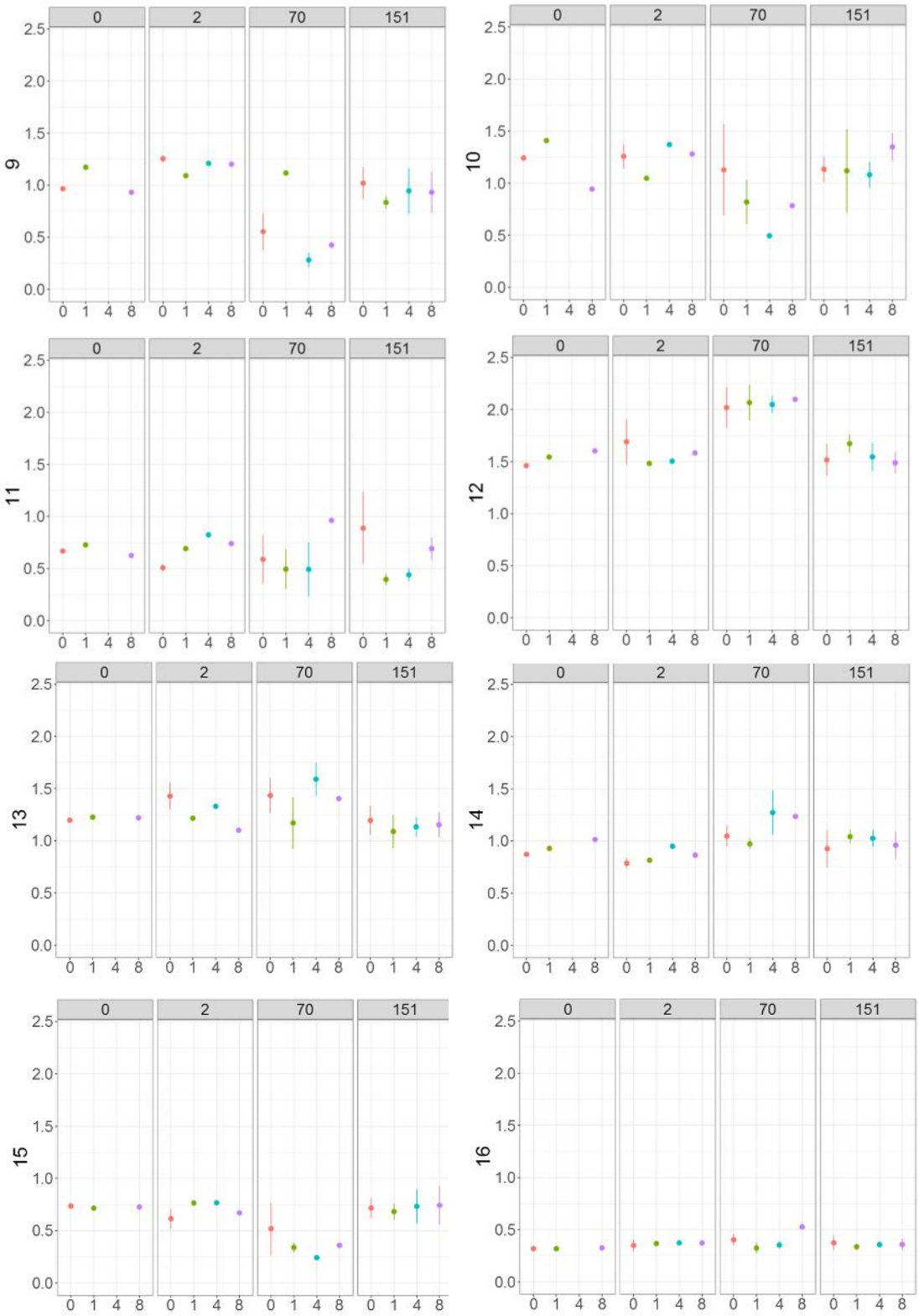


Figure 4.13: The relative growth of the 31 Biolog Ecoplate™ substrates grouped by BGT treatment concentration and sample day. *The y axis represents the normalized optical density reading at 132 h divided by the respective AWCD. The number indicates the substrate number as labelled in Table 4.1.*


0
2
70
151
9
10
11
12
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16
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0.5
1.0
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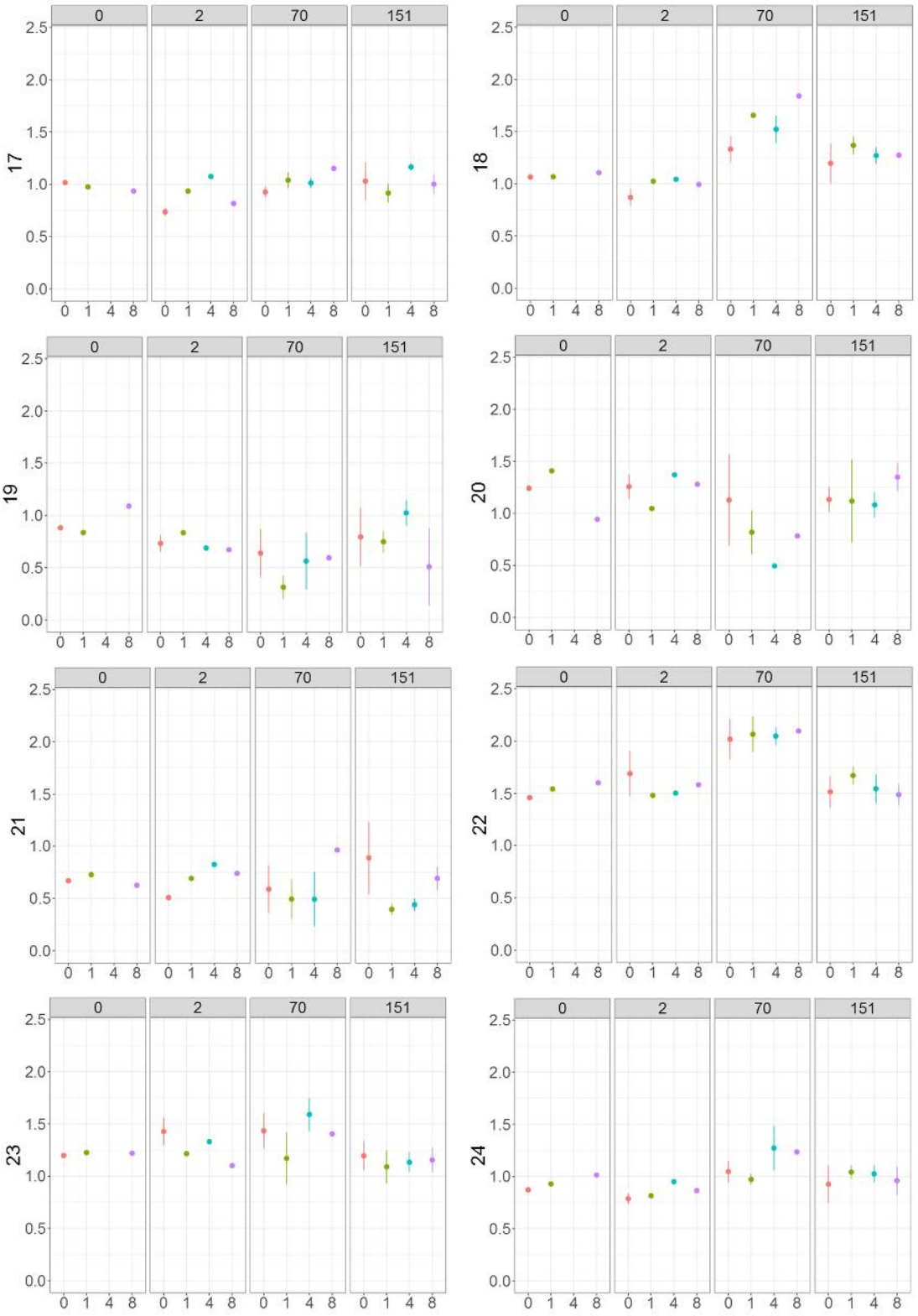
2.0
2.5
0 1 4 8


0
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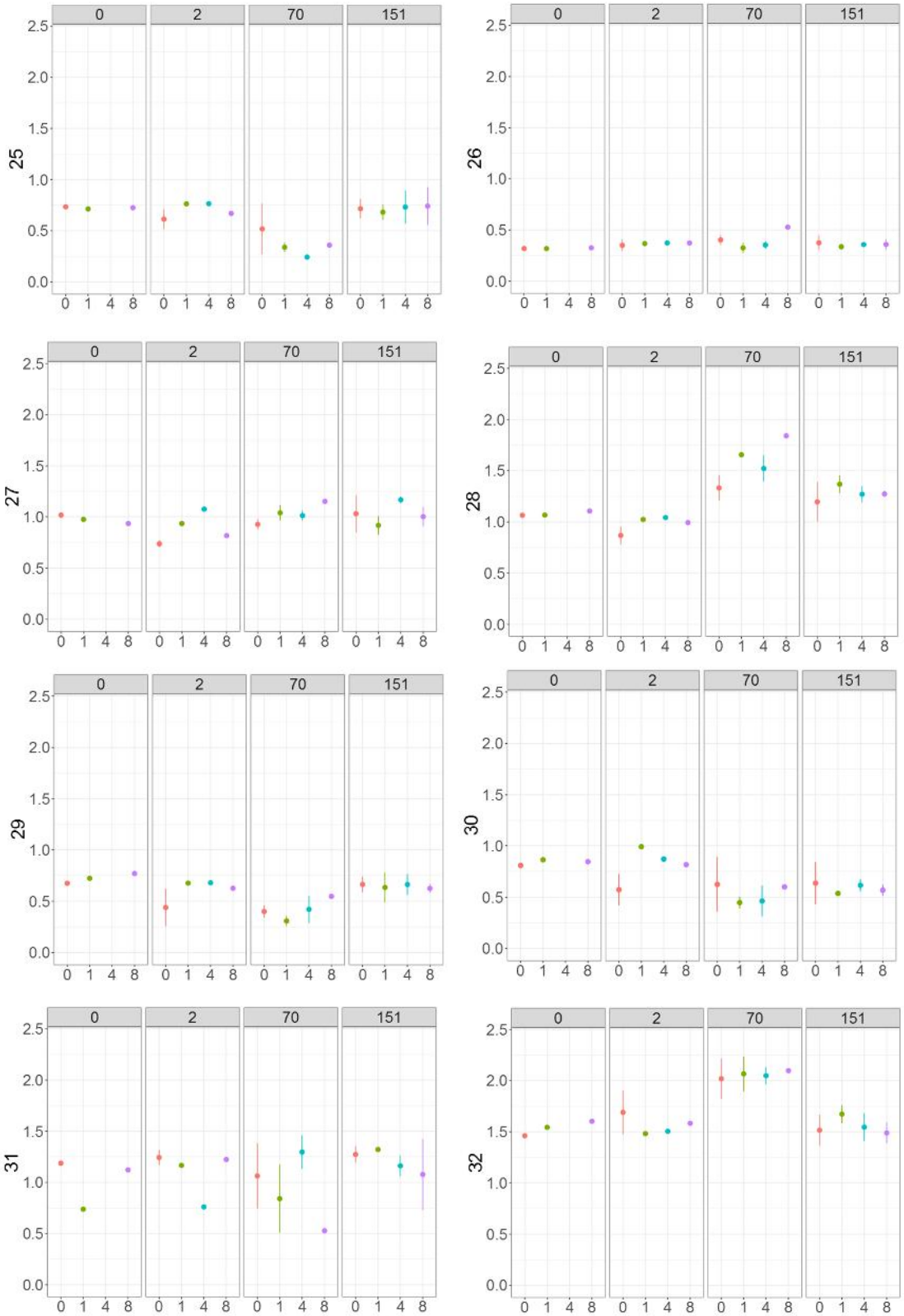
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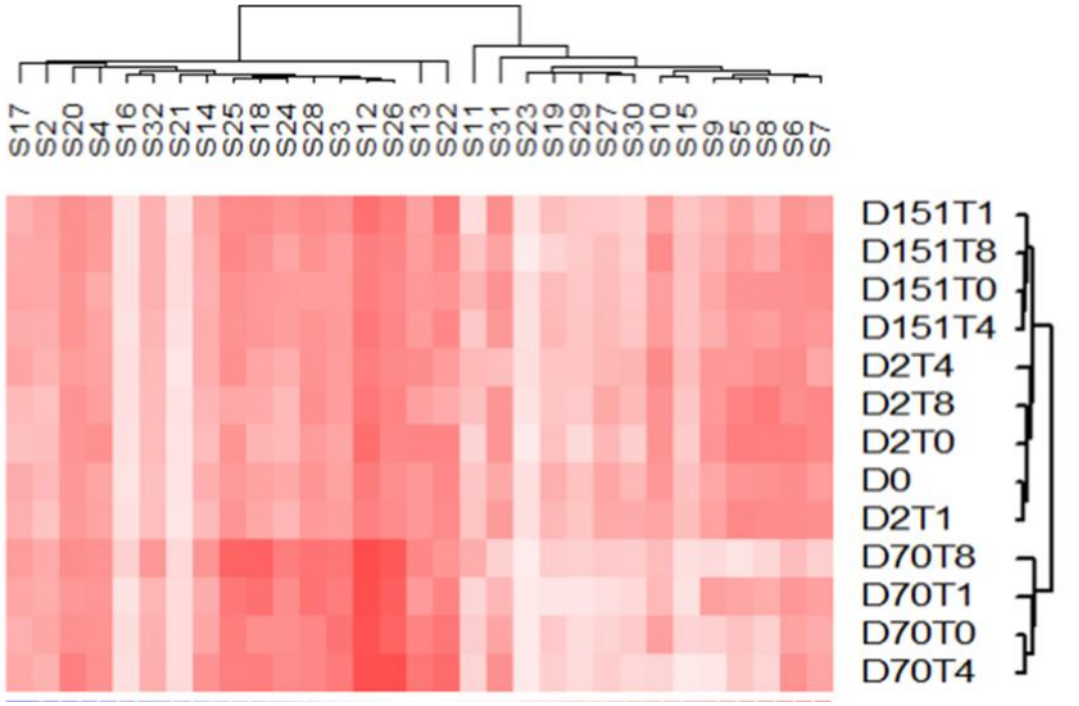


Figure 4.14: Hierarchical clustering of sampled plots based on the utilisation of the Biolog Ecoplate™ substrates after 132 h of incubation. *The colour gradient indicates the relative optical density. S= substrate number (as indicated in Table 4.1), D= sample day, T= BGT treatment concentration percentage.*

A dendrogram was produced to show hierarchical clustering of samples based on the utilisation patterns across the individual substrates (Figure 4.14). The substrates are also clustered based on those that had similar optical densities across the samples. Samples are clustered closely based on the sample day. Samples taken on day 70 are placed the furthest distance away from other samples. There is then a separation of the samples taken on day 151. Within sample day 70 the visualisation places the plots treated with 8 % BGT furthest away from the other samples on that day, which supports the findings of the other analysis methods.

4.3.8 The Diversity of Taxon Groups as Detected by Quantitative Polymerase Chain Reaction Following BGT Treatments

Soil samples taken from the experimental plots at 0, 2 and 70 days after treatment were analysed using quantitative PCR to explore changes in the genetic diversity. The calculated Ct values were inversely transformed to give a relative abundance count of the initial quantity of the target sequence of the taxonomic groupings.

In a similar method to that applied to results of the CLPP analysis, a diversity index was calculated for each soil sample (Figure 4.15). The diversity index was calculated using the values from the five individual bacterial groups and the group accounting for all fungi. In figure 4.15, the biological replicates from the different rows are shown individually. If all the individual groups had the same Ct value and subsequently the same abundance level, this would achieve the highest possible diversity index of 1.79. The samples taken on day 0 show the naturally occurring variation in the diversity. Despite this variation, changes in the diversity can been seen across the samples. The diversity indices were typically highest at the beginning of the trial. Within the samples taken 2 days after the treatment, there would appear to be a slight drop in mean diversity with increasing treatment. The samples taken 70 days after treatment were also affected by treatment: those treated with 4% and 8% BGT have a reduced diversity index relative to 1% and 0% BGT. A two-way ANOVA was applied to the complete data set, which revealed that there were statistically significant differences across sample days and treatment concentration, with a significant interaction between the two (Table 4.9).

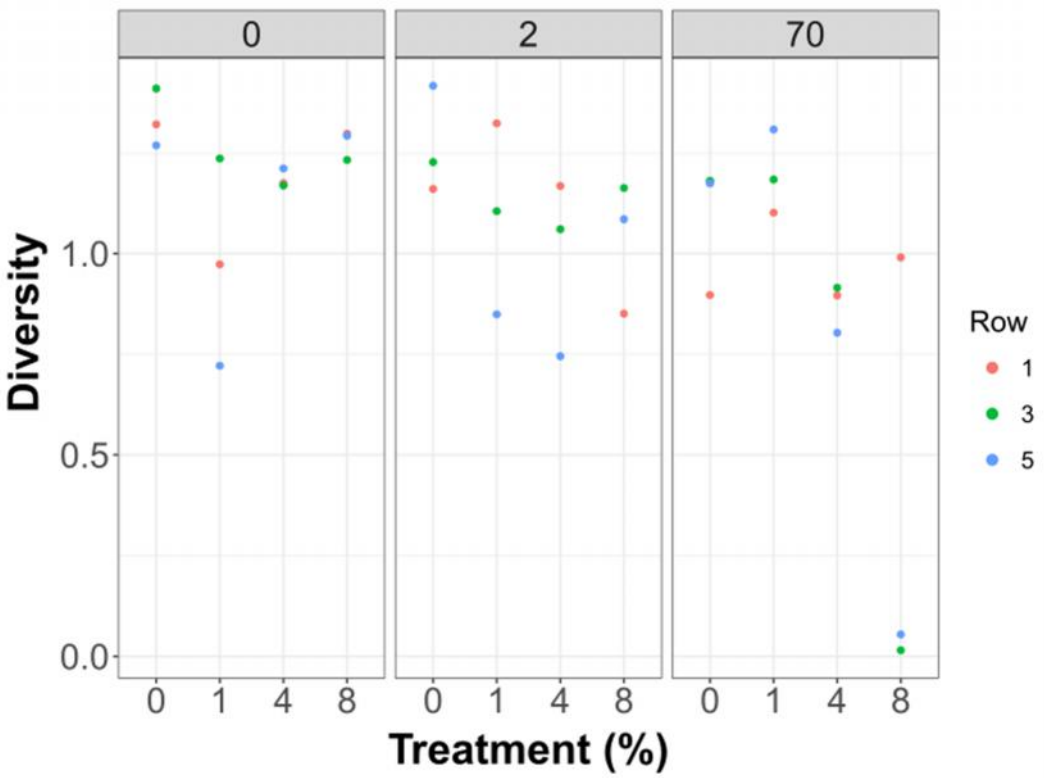


Figure 4.15: The calculated diversity of bacterial and fungal taxon groups as detected using qPCR. *Rows refer to the repeated rows in the field plot layout as shown in Figure 4.3.*

Table 4.9: Analysis of variance table assessing the potential interactive effects of sample day and BGT concentration on taxonomic diversity of soil samples as measured by qPCR.

Parameter	D.F	F value	p value
Day	2	3.875	**0.02162**
Treatment	3	6.650	**0.00504**
Day: Treatment	6	3.749	**0.00897**
Residuals	24		

4.3.9 Relative Abundance of Taxon Groups as Detected by Quantitative Polymerase Chain Reaction Following BGT Treatments

Two of the amplified target sequences in the qPCR experiment allowed for the quantification of the total abundance of bacteria (Figure 4.16a) and fungi (Figure 4.16b). The amount of bacterial DNA extracted and amplified from the samples decreased across sample days. There was a large amount of variation in the bacterial abundance before treatments were applied on day 0. There is a similar amount of variation in the control plot 2 days after treatment but the variation and mean abundance was much lower in the plots treated with 8% BGT. After 70 days, the variation and average abundance in the plots was reduced but there was no clear impact caused by the BGT treatments. The amount of fungal DNA extracted and amplified from the samples was much lower than that of the bacteria. There was a significant decrease in abundance across sample days but there no clear measured effects of treatment concentration (Table 4.10). I next compared the relative abundances of the other taxonomic bacterial groups which were targeted. The abundance values for each sample were divided by the total bacterial abundance for that sample to remove the variation already seen.

Table 4.10: Analysis of variance table assessing the potential interactive effects of sample day and BGT concentration on bacterial and fungal abundance of soil samples as measured by qPCR.

Target group	Parameter	D.F	F value	p value
Bacterial Abundance	Day	2	8.536	**0.002**
	Treatment	3	1.893	0.157
	Day: Treatment	6	1.228	0.326
	Residuals	24		
Fungal Abundance	Day	2	3.708	**0.0395**
	Treatment	3	0.979	0.4190
	Day: Treatment	6	1.250	0.3199
	Residuals	24		

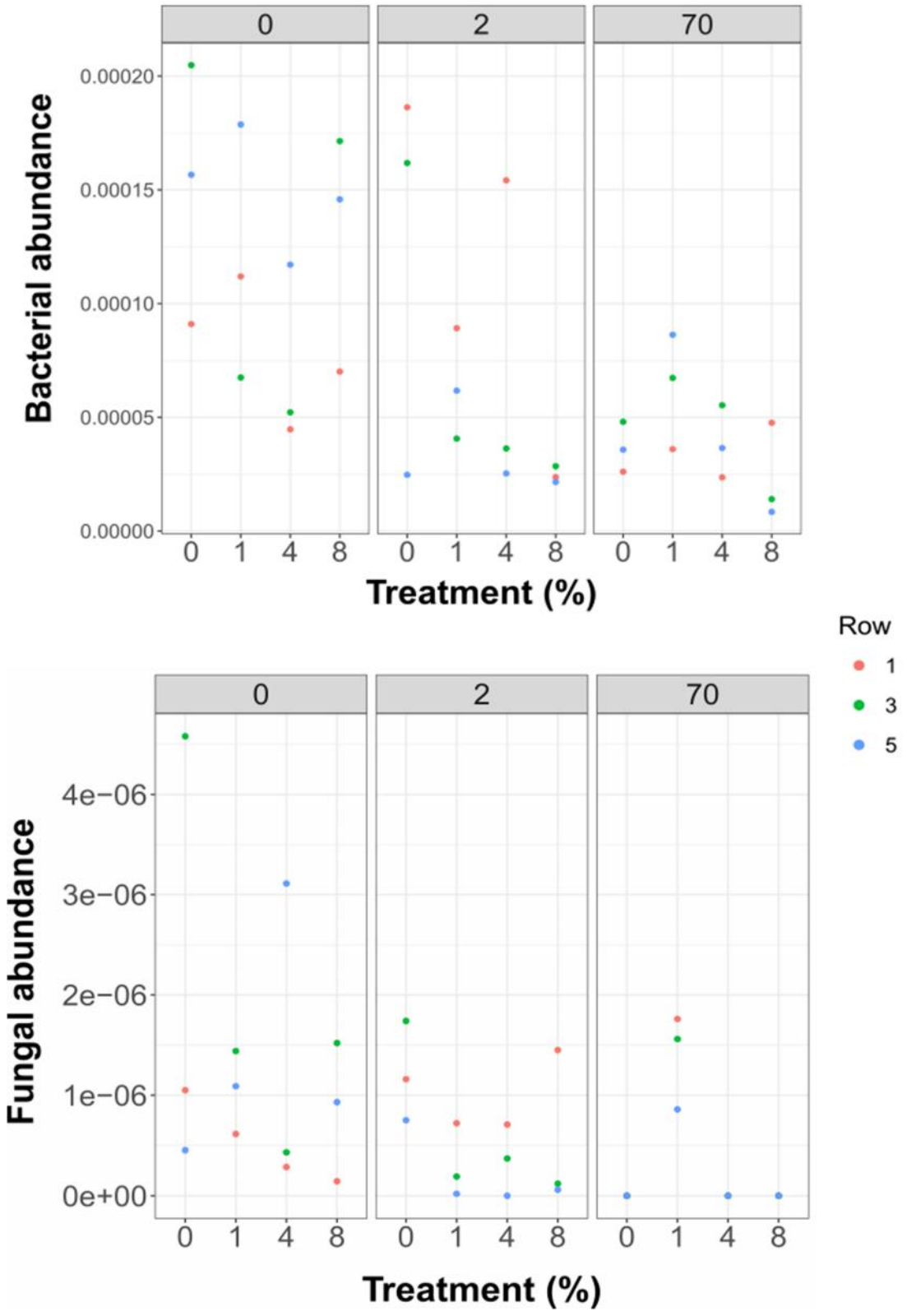
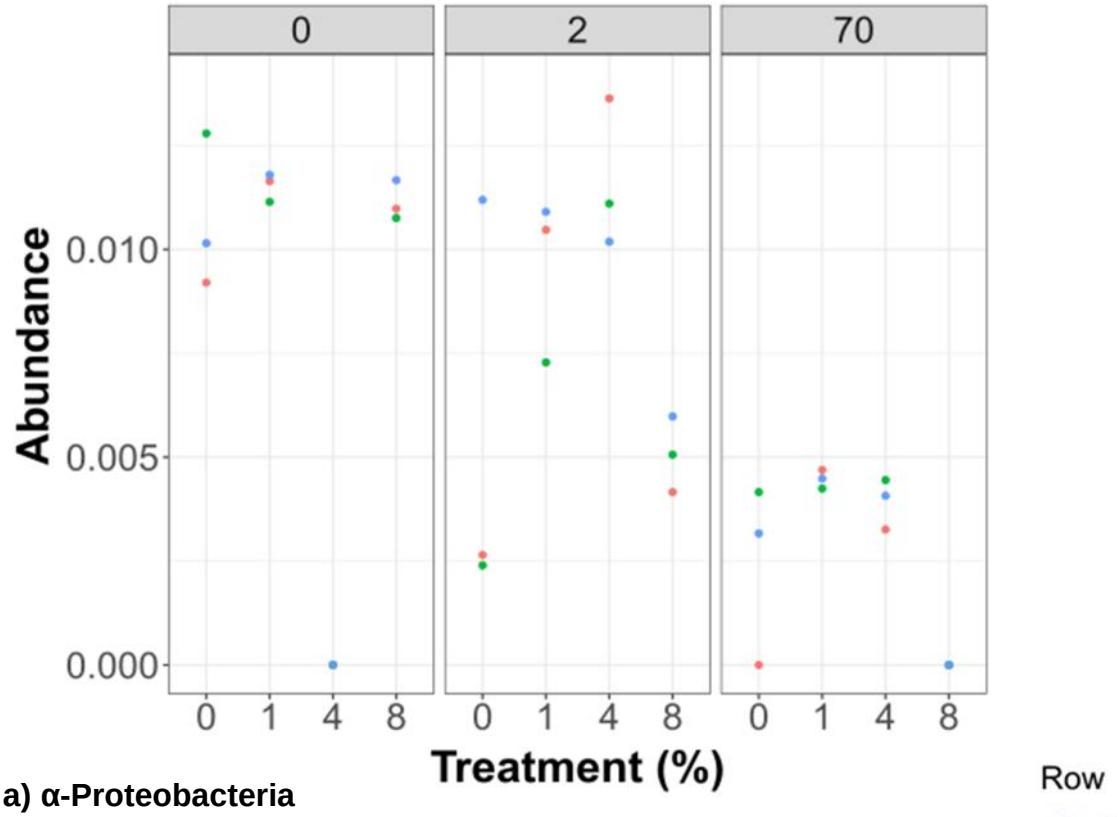
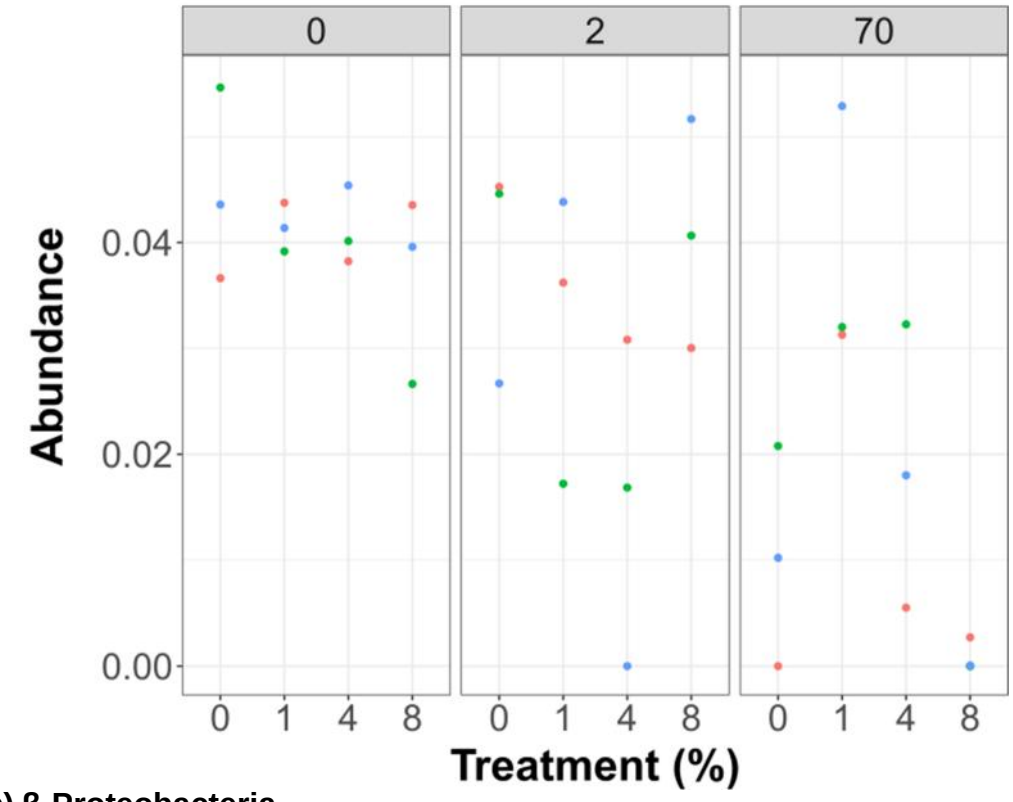


Figure 4.16: Abundance of a) Bacteria and b) Fungi as detected and quantified using qPCR, from samples taken 0, 2 and 70 days after first BGT treatment.

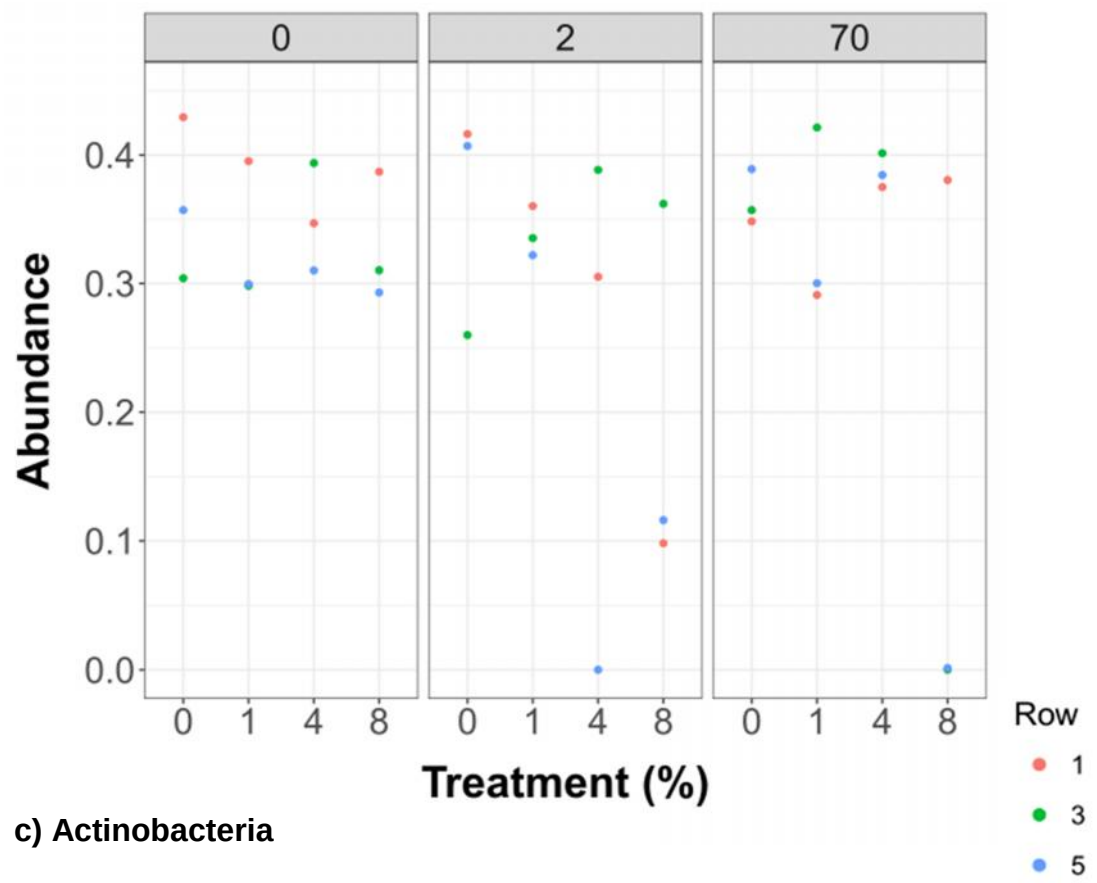


a) α-Proteobacteria

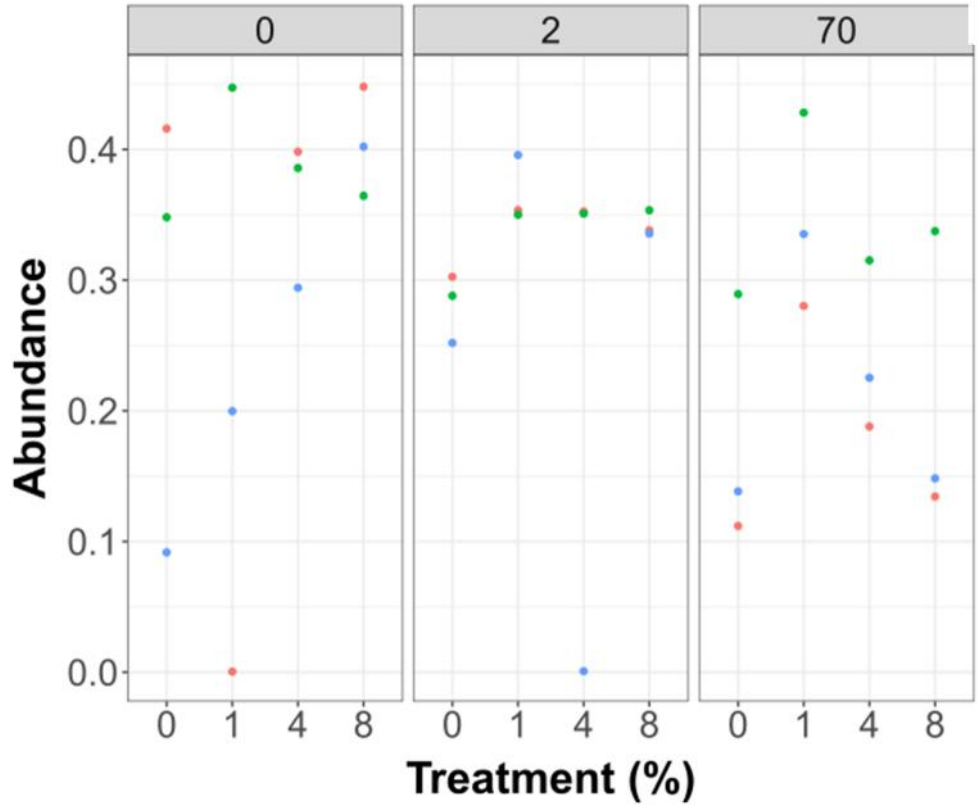


b) β-Proteobacteria

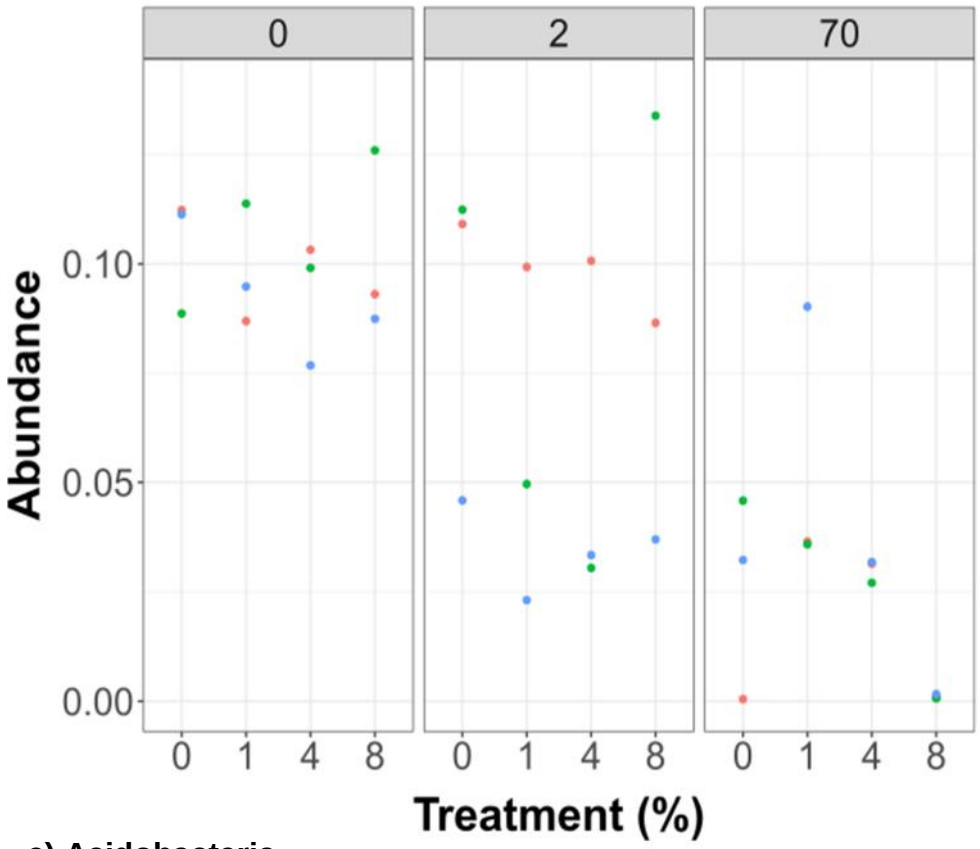
Figure 4.17 (a-e): Relative abundance of taxonomic bacteria groups as detected and quantified using qPCR, from samples taken 0, 2 and 70 days after first BGT treatment.



c) Actinobacteria



d) Bacteroidetes



e) Acidobacteria

Table 4.11: Analysis of variance table examining the changes in relative abundance of bacteria from five taxonomic groups as detected and quantified by qPCR. *Significant p values (p < 0.001) are indicated in bold.*

Target Bacterial Group	Parameter	D.F	F value	p value
α-Proteobacteria	Day	2	33.784	**< 0.001**
	Treatment	3	5.982	**0.005**
	Day: Treatment	6	16.471	**< 0.001**
	Residuals	24		
β-Proteobacteria	Day	2	16.459	**< 0.001**
	Treatment	3	2.718	0.060
	Day: Treatment	6	4.275	**0.005**
	Residuals	24		
Actinobacteria	Day	2	1.059	0.362
	Treatment	3	3.258	**0.039**
	Day: Treatment	6	1.168	0.3560
	Residuals	24		
Bacteroidetes	Day	2	1.378	0.271
	Treatment	3	0.684	0.571
	Day: Treatment	6	1.464	0.2330
	Residuals	24		
Acidobacteria	Day	2	20.036	**< 0.001**
	Treatment	3	0.461	0.712
	Day: Treatment	6	1.359	0.2710
	Residuals	24		

The relative abundances of most of the taxonomic groups varied across the sample days and at the higher concentrations of BGT treatment (Table 4.11). Three out of the five groups decreased over the sample days. Actinobacteria was the taxon with the highest mean abundance, followed in order by Bacteroidetes, Acidobacteria, β-Proteobacteria, α-Proteobacteria. The relative abundance of Actinobacteria did not change significantly over the sampled days but there was a significant effect of treatment concentration, as the 8%

BGT treatment caused a reduction in abundance in 2 of the 3 samples taken on day 2 and day 70 (Figure 4.17c). There were no statistically significant differences in the relative abundance of Bacteroidetes (Figure 4.18d) across sample days or treatments. There was a large amount of natural variation before treatment but there is notably less variation between the samples on day 2. There was a reduction in the relative abundance of Acidobacteria (Figure 4.17e) across the sample days. There is a large amount of variation across most of the samples although there is a decrease in the mean abundance with increasing BGT concentration on day 70. There was no change in the relative abundance of β- Proteobacteria (Figure 4.17b) across the sampled days but there was increased variation in the samples. Despite this variation, there is a possible deleterious effect of the 8% BGT treatment on day 70. α-Proteobacteria was the least detected taxon group in the samples and the abundance significantly decreased across the sample days (Figure 4.17a). There is possibly a reduction in the samples caused by the 8% BGT treatment, however, many of the samples had very low levels detected.

4.4 DISCUSSION

4.4.1 Main Data Conclusions

- None of the treatments, in the trial, including the commercial nematicide had a significant effect on the net growth of the nematode population, as shown by the calculated Pf/Pi ratio of eggs recovered.

- There was no phytotoxicity of the BGT treatments on the potato crops in the trial, as there were no significant reductions in aboveground growth or tuber yield compared to untreated control plots.
- The combined use of quantitative PCR and CLPP revealed potential effects of the highest BGT concentrations on the metabolic and genetic diversity and composition of the microbial populations extracted.

4.4.2 No Treatments Resulted in a Reduction in Population Multiplication of Potato Cyst Nematodes

There was evidence of significantly increased potato growth and yield in the plots that were treated with Nemathorin®, a granular nematicide containing 10% w/w fosthiazate. However, none of the treatments in the trial had a significant impact on the net multiplication rates PCN eggs as indicated by PfPi (Final count/Initial count) in the sampling interval. There are a few possible explanations for this apparent inconsistency. The four Nemathorin® plots were in the area surrounding the replicated plot layout (Figure 4.3). Two of the plots began with the lowest number of eggs/gram across all the plots in the trial. It is possible that there was experimental bias introduced at this stage as there were other environmental factors determining the distribution of PCN in the field.

It is also possible that the Nemathorin® treatment was able to supress nematode reproduction at some crucial stages in the potato's growth but this affect did not last until the final counts were taken. There is also the possibility that the Nemathorin® was able to provide some additional benefit to the

potato crop other than reducing parasitism and reproduction of PCN. Potato cyst nematodes, along with many other plant parasitic nematodes are notoriously difficult to control even with pesticides (Barker & Koenning 1998).

4.4.3 BGT Treatments Resulted in Reductions in the Diversity and Structure of the Bacterial Communities

The application of agrochemicals acts in a similar way to a natural disturbance event resulting in reduced growth or increased mortality. However, the impacts of chemical disturbance may depend less on the short-term effects of the dose-response of sensitive organisms but rather the intensity and frequency of the exposure relatives to the recovery rates of the affected populations (Barnthouse, 2004). In the field study, the effects measured by CLPP (Biolog) and qPCR after 2 days of treatment could be seen to represent the dose response of the populations to the chemical disturbance. There was a large amount of variation in the samples, so caution must be exercised when drawing conclusions, but both the CLPP and qPCR indicated a possible reduction in diversity of the organisms studied 2 days following the 8% BGT treatment when compared to the other plots sampled on the same day.

Following this, there were three further applications (of the same concentration dose) at fortnightly intervals. The next soil sample taken 70 days after treatment was 21 days after the final treatment application so could be expected to show any lasting but relatively short-term effects of the treatments. It would appear that both the 4% and 8% BGT treatments

significantly reduced the diversity of those organisms studied with CLPP and qPCR.

Samples taken on day 151 were unfortunately not analysed using qPCR but the CLPP data showed that the diversity of the 8% BGT treated plots was lower than the other plots on the same day. This may indicate that although the soil community was not completely tolerant to the repeated application of the 4% BGT treatment it has resilience abilities and was restored after no other treatments were applied.

It would have been useful to be able to combine a study on the persistence and biodegradation of BGT with the effects studied. From the data taken it is not possible to determine whether the possible changes due to BGT on day 70 was due to a build-up of the substances in the soil which finally resulted in a dose which triggered significant changes in the population, or if the changes seen were a cumulative effect of minor changes resulting from each treatment application.

The timing and persistence of the effects of the biocides on soil microbial communities varies across substance types. Some chemicals may have an immediate effect but then degrade rapidly allowing for communities to recover. Effects of many conventional pesticides have been observed to typically last between 1 to 3 months (Jacobsen and Hjelmso, 2014).

Whilst it can be assumed that all the populations extracted and cultured in the CLPP assay can be compared because they were all processed and incubated in the same way, it is very likely to only represent a small, possibly

altered subset of the true bacterial populations (Balser et al., 2002; Heuer and Smalla, 1997; Preston-Mafham et al., 2002). Equally, the DNA extraction process used for the qPCR experiment may have produced some bias (Forney et al., 2004; Frostegard et al., 1999; Martin-Laurent et al., 2001). This means that it is likely the populations represented in both assays are the same as each other or to all the populations found naturally in the field soil community. Despite this, the results suggest that there were changes in the soil community composition. If this did result in a reduction of diversity, this may not have impaired some functioning of the soil but could reduce the soil communities' tolerance and resilience to future contaminants and invasive pests (Baveye et al., 2016; Nannipieri et al., 2003; van Elsas et al., 2012).

Samples were not taken for the Nemathorin®-treated plots as it was initially intended that the BGT would be compared to the application of Vydate®, an oxamyl based nematicide. However, for reasons beyond my control, this was not applied by those running the trials. Spyrou et al. (2009) conducted laboratory and field trials to examine the effect of Nemathorin® on the soil microbial community structure by phospholipid fatty acids (PLFA) analysis. It was also compared alongside three botanical pesticides, and two fumigant products, Vapam® (metam sodium) and Enzone® (sodium tetrathiocarbonate). In the laboratory microcosms, there was no significant changes in the microbial community detected as a result of the botanical or fosthiazate treatments, however, the fumigants inhibited fungi and Gram-negative bacteria. In the field trial, fosthiazate did cause, what the author described as, "mild" changes in the microbial community compared to the control apparent 30 days after application. The effect of fosthiazate persisted

for at least 90 days for which the soil was monitored. Both fumigant treatments led to a significant reduction in the total concentration of PFLAs detected. Similar deleterious effects of fumigant nematicides on soil communities have been documented in other studies measured by Biolog® assays, denaturing gradient gel electrophoresis (DGGE) and PFLA (Ibekwe et al., 2001; Macalady et al., 1998).

To conclude, these studies have shown that despite differences, both the CLPP and qPCR analysis detected similar trends in diversity levels across sample days and treatment concentrations. It is difficult to state what would be the direct impacts of the shifts in community structure caused by the BGT treatments would have the functioning on the entire system. However, it shows that its application would have impacts on populations other than the intended targets that must be considered when evaluating the risks and benefits of the BGT treatment.

5. GENERAL DISCUSSION

There is currently a high demand for effective plant parasitic nematode (PPN) controls that will provide suitable levels of population management for growers without presenting a large risk to human or environmental health (Zasada et al., 2010; Trudgill et al., 2014; Viaene, 2014). This project aimed to assess the environmental impacts of a new agent, BGT that was identified as a potential nematode control by the industrial partner, Arcis Biotechnology Ltd. When developing a novel pest control it is important to be able to assess the potential impact the test product will have on both target and non-target organisms when released into an ecosystem. This collection of studies aimed to begin to address a number of ecologically important and commercially relevant questions regarding the use of BGT.

5.1 POTENTIAL EFFECTS OF BGT ON MICROORGANISM AND MICROBIAL COMMUNITY

In Chapter 2, controlled laboratory assays found bacterial and fungal species showed a range of tolerances to BGT in liquid growth medium (Section 2.3.1). From this, I hypothesised that the application of BGT on soil would lead to changes in the microbial community structure due to varying responses and susceptibilities of populations. Using CLPP and qPCR, I was able to explore the possible effects on both the functional (catabolic) diversity, and the genetic diversity based on a selection of broad taxonomic groups (Section 4.3). These

tests showed that after repeated application at the highest concentration of BGT tested, there were shifts in both the relative abundance of taxonomic groups and the pattern of substrate utilisation. There were declines in the diversity of the communities extracted and detected/cultured as a result of repeated BGT concentrations at 8% in both tests but there were no significant differences in the genetic abundance or catabolic potential of the community.

Much of the current understanding on soil microbial communities and diversity suggests there is a lot of functional redundancy within communities; a loss in biodiversity does not show a direct correlation with the rate and capability of many functions (Nannipieri et al., 2003; Bardgett and van der Putten, 2014; Baveye et al., 2016). There were declines in the growth on some of the Biolog Ecoplate^{TM} substrates following BGT treatment but others increased. Similar effects were observed across the taxonomic groups detected using qPCR. This suggests that some bacterial populations are able to quickly take the place of those in decline. However, this will result in a decrease in some diversity measures. Studies have shown that soils with greater biodiversity have an increased capacity to prevent invasion from pests and pathogens (Liu et al., 2012; van Elsas et al., 2012). This means that whilst some small shifts in soil microbial community structure and diversity level may not have a significant effect on soil health and functioning in the short term, these repeated small changes could have long term negative feedback effects.

Both methods used in this study to assess the soil microbial community are not without limitations and can only account for a subset of the actual microbial community present in the soil. (Smalla et al., 1998; Preston-Mafham et al., 2002; Martin-Laurent et al., 2001). Additionally, it is possible that shifts

in the community structure occur in the sample between times of sampling and analysing, dependent on the storage conditions. (Martin-Laurent et al., 2001). This somewhat limits the conclusions that can be drawn from studies on soil microbial community analysis. However, the use of these two methods in combination in this study gives increased confidence to the observed effects of the treatments as both the CLPP and qPCR indicated the same general diversity changes.

For further analysis, the effects of BGT should also be examined using more direct functionality tests including the carbon and nitrogen tests which form the risk assessment for soil microbial communities in the regulation of plant protection products (European Commission, 2002). The results from the CLPP test suggest there will not likely be significant changes in soil respiration as measure by the soil carbon transformation test (OECD, 2000b), as there were no significant differences in the average well colour development across the Biolog Ecoplates^{TM}, which indicates the overall microbial growth rates.

The registration and regulation of pesticides requires a range of standardised tests and evidence to explore the impacts the test product may have on the surrounding environment and non-target organisms (European Commission, 2002). There is increasing interest amongst practitioners and academics in the impacts agricultural inputs may be having on soil microbial communities and whether these are being effectively monitored (Dicks et al., 2013; Bardgett and van der Putten, 2014; Antwis et al., 2017). My experiments showed a large amount of variation across sample days. This was likely a result of seasonal changes as well as changes determined by the potato crop growth which results in fluctuations in the presence of root exudates, oxygen levels

and nutrient availability. If there was a greater understanding of the causes and dynamics of the communities in systems, we could perhaps better assess and understand potential changes that occur due to specific inputs such as pesticides. Rapid developments in technologies such as next-generation sequencing, metagenomics and metaproteomics will hopefully provide greater insight into soil microbial communities and their role in agricultural systems (Antwis et al., 2017).

5.2 Potential of BGT as a Nematode Control Agent

Development of BGT as a nematicide first began when a surface antibacterial product of similar composition produced by Arcis Biotechnology was found to be toxic to nematodes during laboratory testing. Following this, BGT displayed nematicidal properties on a range of endoparasitic and ectoparasitic species in trials conducted on turf root zones. It was noted that the treatment efficacy was highest in heavily sand-based soils but was impaired in soils with a higher organic matter content (Section 2.1.5). However, in my small study on sampled field soil (Section 2.2.2), and again in the potato field soil there was not any evidence of nematode control following BGT treatment (Section 4.3.1). There are further studies being carried out by Arcis Biotechnology and their other research partners on the effect of BGT on PCN but unfortunately the results could not be included in this thesis. It is not uncommon for commercial, approved nematicides to show limited control of PPN, as in the case of Nemathorin® in the field trial, particularly when there is a high initial population (Pi) and planted crops are highly susceptible (Hauer et al., 2016; Brodie, 1996; Crow and Luc, 2014). In addition, the heterogeneity and

occurrence of dense ‘hotspots’ in the distribution of nematode populations throughout the soil can mask wider population fluctuations when sampling is limited (Eves-van den Akker et al., 2015). It is possible that BGT may work as a potential nematode control in some agricultural or horticultural control systems, particularly when integrated with other control mechanisms such as resistant cultivars and careful rotation. An integrated pest management (IPM) system may be able to be used as an effective replacement of highly effective but hazardous broad spectrum fumigant nematicides. However, this requires careful and complex agricultural management, requiring a longer-term commitment which may not be appealing to risk-adverse growers (Hossard et al., 2017).

5.3 Toxicity of BGT to Plants and Non-Target Organisms

The assays carried out in Chapter 3 showed that BGT had phytotoxic effects on tomato and wheat seedlings when they were exposed to a concentrated solution (Section 3.3.1). However, there were no deleterious effects observed in wheat plants grown in BGT in soil (Section 3.3.2). The field trials discussed in Chapter 4 showed that none of the BGT treatments resulted in a significant change in aboveground crop cover or potato yield compared to that of the untreated control (Section 4.4.2). This demonstrates that phytotoxicity of BGT applied both at the time of planting and in early stages of crop growth is not likely to be of concern to growers unlike some other nematicides which may

cause crop damage with certain application timings (Desaeger et al., 2008; Tayal and Agarwal, 1982).

In addition, the results of the earthworm toxicity studies suggest that BGT is of relatively low risk to earthworms which can typically be used as an indicator of the risk is poses to other terrestrial organisms (Muniz et al., 2014; OECD, 1984) (Section 2.4.3) Further, wider testing would be needed for a full risk assessment of BGT in terrestrial systems, but these results, along with existing data on the toxicity of the BGT components, suggest that it will be less hazardous to the environment relative to conventional synthetic chemical nematicides.

5.3.1 BGT Treatments did not Result in Any Phytotoxic Effects on Potato Crops

There was no sign of phytotoxicity or reduced growth as a direct result of any of the BGT treatments in the soil compared to the untreated control plots. The treatments were applied at fortnightly intervals with the last applied on 7 week-old potato plants. This shows that BGT presents no adverse risk when applied both to soil containing seed potatoes as well as when applied to established crops. If BGT was to be used as a nematode control this would be useful as it presents growers will more options in timing application rather than the use of other traditional nematicides for which there has to be a long interval between application and planting to avoid phytotoxicity (Desaeger et al., 2008).

5.4 THE SURFACTANT PROPERTIES OF BGT

BGT contains several compounds known to act as surfactants or wetting agents. Surfactants are widely used in agriculture to aid the application, dispersal and preservation of pesticides. In some systems they are used alone to improve infiltration, water distribution, and water retention (Mobbs et al., 2012). In managed turf grass, such as golf courses, soil water repellence is a common problem impacting turf quality, which is thought to occur due to the accumulation of hydrophobic organic matter such as plant tissue (Kostka, 2000). Partially prompted by the results generated in my project which demonstrated that BGT helped maintain soil moisture (Section 3.3.2), there has been commercial exploration undertaken by Arcis Biotechnology to market BGT as a soil conditioner for amenity turf and it has been distributed in some Australasian markets.

An additional study demonstrated that BGT treatment alleviated drought stress in wheat crops in reduced watering availability (Section 3.3.4). If BGT can be used as an effective nematicide, it is likely that the growers would also be able to make use of the surfactant properties of BGT which may allow for reduced irrigation or alleviate losses due to unavoidable drought.

As the surfactant nature of the BGT has been shown to alter the soil moisture dynamics in the soil, it is highly possible that this change will have an additional effect on the soil microbial community, as soil water is a major abiotic factor. Soil water provides a habitat and a movement passage for many organisms, including beneficial and phytoparasitic nematodes. The availability of water also largely determines the nutrient and oxygen availability

which will largely effect the soil microbial community structure (Bardgett, 2005).

5.5 FUTURE OF PESTICIDES AND PEST CONTROL

There has long been disagreement over the level of risk posed by pesticide use and many have argued or assumed that without them there would be a loss of yield and profit. However, there is emerging evidence that shows that substantial reductions in pesticide (including herbicides, fungicides and insecticides) use could be made without impacting crop productivity or profitability. A recent study comparing arable farms in France, suggested that in 67% of the farms surveyed, a reduction in pesticide use would not reduce profitability. There were some conflicting situations, however, in systems producing high added-value crops, such as potato and sugar beet, both of which are heavily effected by pests and pathogens, including PCN it was found there would be a risk of loss of productivity and profit. These crops were associated both with high levels of pesticide use but also high profitability (Hossard et al., 2017). This study supports the argument presented by UN experts this year that maintains that it is misleading to claim that pesticides are vital for food security (United Nations Human Rights Council, 2017). The reports states ‘Pesticides, which have been aggressively promoted, are a global human rights concern, and their use can have very detrimental consequences on the enjoyment of the right to food.’ The report strongly supports the development of agroecology to achieve sustainable agriculture, which relies on the study of the ecological, economic and social dimensions of a food system. One listed strategy, referred to as long overdue is the

replacement of highly hazardous pesticides with less hazardous substances. Whilst this may be a viable short-term option that will not enforce huge change on the agricultural systems it cannot be seen a long-term sustainable solution.

There is increasing regulatory pressure to reduce the use of synthetic chemical pesticides. This has meant many products have now been withdrawn and there are few new products or application licenses being approved (Damalas and Eleftherohorinos, 2011). The pesticide registration process can be lengthy, costly and not without risks, which means it is not a simple process for small to medium enterprises (SME's) to endure. This may mean that the introduction of less hazardous pesticides to increase agricultural sustainability may not be a rapid or simple solution. Under the Council Directive 91/414/EEC, the Authorisation Regulation (1107/2009) states that "a plant protection product, consequent on application consistent with good plant protection practice and having regard to realistic conditions of use, shall meet the following requirements: a) it shall be sufficiently effective" and "c) it shall not have any unacceptable effects on plants or plant products" (European Parliament, Council of the European Union, 2009). Given the current evidence on the action of BGT, consideration would have to be given as to whether the level of control it can provide is sufficient to justify its use. In the case of the potato field trial discussed in Chapter 4, the poor level of control observed does not promote further use of BGT.

5.6 SUMMARY

In conclusion, these studies have shown that the proposed novel nematode control agent BGT, is of relatively low toxicity to earthworms suggesting it is of low risk to terrestrial organisms. The BGT treatments in field soil resulted in some shifts in the microbial community structure and diversity levels as measured using Biolog Ecoplates^{TM} for CLPP, and qPCR. Whilst this did not affect total abundance, and current theory suggests it may not have a major impact on many soil functions, shifts such as these caused by agricultural inputs may have long term effects on soil health and ultimately productivity. BGT treatments were not shown to cause any phytotoxic effects on wheat plants in laboratory studies and potato plants in crop trials following repeated applications both pre-planting and in early stages of plant growth. This means if used, growers would not be confined to narrow application timings to avoid crop damage, as is the case with some treatments. However, from the data that has been discussed in this thesis, BGT has not been shown to be an effective nematode control other than in pot trials of predominantly sand-based soil used for turf growth. Whilst there is a great need for less hazardous forms of nematode control, decisions to further the potential use of BGT will require careful consideration of the financial and environmental costs and benefits.

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