UNIVERSITY OF SOUTHAMPTON

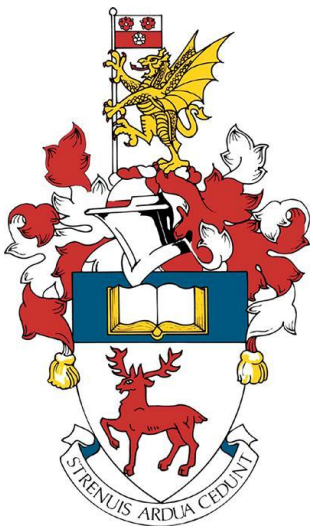
FACULTY OF NATURAL & ENVIRONMENTAL SCIENCES

Centre for Biological Sciences

The role of the rhizosphere microbial community in plant chemistry and aphid herbivory in *Brassica oleracea*.

by

Flora Jane Mary O'Brien



Thesis for the degree of Doctor of Philosophy

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ABSTRACT

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THE ROLE OF THE RHIZOSPHERE MICROBIAL COMMUNITY IN PLANT CHEMISTRY AND APHID HERBIVORY IN *BRASSICA OLERACEA*.

Flora Jane Mary O'Brien

Soil microbial communities can influence plant productivity, chemistry and even diversity. Intensive farming practices have caused widespread soil degradation, raising concerns regarding soil health and need for sustainable agriculture. Although soil microbe-plant interactions have been extensively studied, the relationships between soil microbial communities and higher trophic levels, such as herbivorous insects, are poorly understood. This thesis reports the findings of a series of mesocosm experiments which used a model system of Derby Day cabbages (*Brassica oleracea* L. var. *capitata*), peach-potato aphids (*Myzus persicae*), and soil sourced from an agricultural field site. Firstly, I conducted an exploratory study of the soil microbial community response to different fertiliser regimes and cabbage growth using Next Generation Sequencing (NGS) of the 16S rRNA gene. This was complemented by concomitant measurements of the plant and aphid performance in order to identify potential soil-plant-insect relationships. The results revealed that the diversity and composition of bacterial communities were more strongly influenced by the cabbage age and fertiliser treatment than aphid herbivory. Several bacteria exhibited enhanced abundance in rhizosphere of older cabbages, including sulphur-oxidising bacteria (SOB) of the *Thiobacillus* genus. A member of this genus was then selected as an inoculant in the subsequent experiment to test its plant growth promoting potential for *B. oleracea*. Brassica plants produce a class of secondary metabolites called glucosinolates, which have multiple beneficial properties including anti-herbivory and anti-carcinogenic attributes. As this compound is rich in sulphur (S), it was hypothesised that soil inoculation with the SOB *Thiobacillus thioparus* may enhance glucosinolate production in *B. oleracea*, thereby improving its defence against aphid feeding. Analysis revealed a promotional effect of enhanced SOB populations on glucosinolate content of leaves. Although an accompanying significant effect on aphid populations was not detected, this experiment shows the potential for the modulation of plant chemical defences by the soil microbial community.

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DECLARATION OF AUTHORSHIP

I, Flora Jane Mary O'Brien, declare that this thesis and the work presented in it are my own and has been generated by me as the result of my own original research.

The role of the rhizosphere microbial community in plant chemistry and aphid herbivory in *Brassica oleracea*.

I confirm that:

1. This work was done wholly or mainly while in candidature for a research degree at this University;
2. Where any part of this thesis has previously been submitted for a degree or any other qualification at this University or any other institution, this has been clearly stated;
3. Where I have consulted the published work of others, this is always clearly attributed;
4. Where I have quoted from the work of others, the source is always given. With the exception of such quotations, this thesis is entirely my own work;
5. I have acknowledged all main sources of help;
6. Where the thesis is based on work done by myself jointly with others, I have made clear exactly what was done by others and what I have contributed myself;
7. [Delete as appropriate] None of this work has been published before submission [or] Parts of this work have been published as: [please list references below]:

Signed: .....

Date: .....

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Definitions and Abbreviations

µ	micro
α	alpha
β	beta
AMF	arbuscular mycorrhizal fungi
ANOVA	analysis of variance
ARISA	automated ribosomal intergenic spacer analysis
bp	base pairs
CAP	canonical analysis of principal coordinates
CC	Climate Change
CFU	colony forming unit
CM	chicken manure
Con	control
dbRDA	distance-based redundancy analysis
DCA	detrended correspondence analysis
DGGE	denaturing gradient gel electrophoresis
DRB	deleterious rhizobacteria
EIL	economic injury level
ET	economic threshold *or* ethylene
EDTA	ethylendiaminetetraacetic acid
FDR	false discovery rate
GBC	glucobrassicin glucosinolate
GLS	glucosinolate(s)
GNA	gluconapin glucosinolate

ha	hectare
HN	high N fertiliser treatment
IAA	indole-3-acetic acid
IBE	glucoiberin glucosinolate
ITS	internal transcribed spacer
JA	jasmonic acid
KEGG	Kyoto encyclopedia of genes and genomes
LN	low N fertiliser treatment
4OH	4 hydroxyglucobrassicin glucosinolate
4MeOH	4-methoxy-3-indoylmethyl glucosinolate
N	nitrogen
NEO	neo-glucobrassicin glucosinolate
NGS	next-generation sequencing
NMDS	non-metric multidimensional scaling
NPK	nitrogen-phosphorus-potassium (fertiliser)
n.s.	not (statistically) significant
OTU	operational taxonomic unit
P	phosphorus
PBS	phosphate buffered saline
PCoA	principle coordinates analysis
PCR	polymerase chain reaction
PERMANOVA	permutational ANOVA
PGP	plant growth promoting
PGPR	plant growth promoting rhizobacteria
PICRUSt	Phylogenetic Investigation of Communities by Reconstruction of Unobserved States

PRO	progroitrin glucosinolate
QIIME	quantitative insights into microbial ecology
qPCR	quantitative PCR
RAPH	glucoraphanin glucosinolate
RNA	ribonucleic acid
rRNA	ribosomal ribonucleic acid
S	sulphur
SA	salicylic acid
SD	standard deviation
SDS	sodium dodecyl sulphate
SE	standard error
SIN	sinigrin glucosinolate
SOB	sulphur-oxidising bacteria
STAMP	Statistical Analysis of Metagenomic Profiles
T	Time
T.t.	*Thiobacillus thioparus*
UPGMA	unweighted pair-group method with arithmetic mean
VBNC	viable but non-culturable state
WHC	water holding capacity

Chapter 1: Introduction

1.1 Food Security

Food Security, defined by the World Food Programme (WFP) as the "availability and adequate access at all times to sufficient, safe, nutritious food to maintain a healthy and active life", represents one of the greatest challenges facing the human race. As the global population continues on an upward trajectory, it is predicted that there will be 9.7 billion people on Earth by the year 2050, approximately 2.4 billion more than the 2015 population (UN, 2015). In order to meet the concomitant rising demand for food, there is mounting pressure on the agricultural industry to increase the efficiency of food production. Since the green revolution, agricultural productivity has grown rapidly as a result of heavy use of chemical fertilisers, pesticides, herbicides, irrigation and tilling (Tilman, 1998). In order to produce sufficient food to feed the 9 billion people expected to populate the world in 2050, it is estimated that fertiliser inputs will need to increase by 70 to 100% relative to the amount used in 2000 (Liu *et al*, 2016). There are major concerns regarding the impacts these conventional farming practices have on the environment, which include eutrophication of waterways, soil erosion, increased greenhouse gas emissions and pollution by run-off of agrochemicals, not to mention the associated habitat destruction resulting from the expansion of agricultural land (Tilman *et al*, 2001a). The majority of this population growth is expected to occur in developing countries, predominantly in Africa, which highlights the need for affordable, accessible and sustainable farming strategies to increase yields (UN, 2015). Another aspect which may contribute towards achieving Food Security is the enhancement of the nutritional value and health benefits of crops, often referred to as 'biofortification' (see Bouis and Welch (2010) for a review). There are a variety of ways through which this may be achieved, such as genetic modification (e.g. the enhancement of selenium uptake by transgenic *Arabidopsis thaliana* (Ellis *et al*, 2004)), and the use of bacteria which promote nutrient-uptake by plants (e.g. microbial-enhanced selenium uptake by wheat (*Triticum aestivum* L.) (Yasin *et al*, 2015)).

1.2 Soil services

Soils provide a number of functions and services, some of which can broadly be divided into the following categories:

- nutrient cycling;
- flood prevention;
- pathogen control and antibiotic production;
- degradation of toxic compounds (bio-remediation);
- carbon (C) storage.

These services are not only paramount in supporting wider ecosystem functioning, but also are vital for agricultural production. They are performed by a complex community of soil organisms, which can be grouped according to size. Soil macrofauna (500 μm- 50mm), which include earthworms and termites, are the largest members of this community, with the smallest being the microscopic organisms (1- 100 μm), such as bacteria and fungi, collectively referred to as microflora (Barrios, 2007, Wall *et al*, 2001, Swift *et al*, 1979). Soil organisms can also be placed into key functional groups, as many microbes perform the same function (functional redundancy). However, it is not always possible to assign species to a single group, since these organisms often perform multiple functions - a topic which has fuelled debate as to the importance of soil biodiversity and species richness in providing sufficient soil services for ecosystem functioning (Barrios, 2007). These functional groups include microsymbionts (e.g. nitrogen-fixing bacteria); soil ecosystem engineers (e.g. termites and earthworms); nutrient transformers (e.g. denitrifiers); decomposers (e.g. lignin degrading microbes); soil-borne pests and pathogens (e.g. root rot diseases); and micro-regulators (e.g. bacterial grazers) (Barrios, 2007). Depletions in soil biodiversity can result in loss of ecosystem functions, including those related to nutrient cycling such as plant litter decomposition and the prevention of phosphorus losses from leaching, which could have severe consequences for plant growth (Wagg *et al*, 2014). Thus the maintenance and protection of soil biodiversity is crucial to successful crop production and the functioning of the wider ecosystem.

1.2.1 Soil health in relation to agriculture

The intensification of agriculture has high costs both financially and environmentally. Conventional agricultural techniques are often detrimental to soil health and the wider environment. The use of heavy machinery for intensive farming methods, such as tilling, not only releases polluting emissions through fuel combustion, but also results in soil compaction and erosion. This diminishes the water storing capacity of the soil, thereby increasing the risk of flooding. Tilling also causes disruption to the soil profile, often causing declines in fungal abundance (Young and Ritz, 2000, Bailey *et al*, 2002). This may have negative consequences for crop yields, as fungal mycelial networks are known to enhance plant nutrient acquisition and so their destruction could result in diminished plant growth (Young and Ritz, 2000, Lambers *et al*, 2008).

Soil compaction and erosion also heightens the risk of run-off (leaching) of agrochemicals, which leads to the eutrophication (excessive nutrient enrichment) of the surrounding environment and waterways, and subsequent biodiversity losses (Powlson *et al*, 2011). Other modern agricultural practices associated with the deterioration of soil health are the production of monocultures, short fallow periods and irrigation. The degradation of soil health can lead to diminished soil services, culminating in reduced crop success which the farmer may choose to compensate for by further increasing the chemical inputs in an effort to increase production. Thus, a negative feedback loop can evolve, which poses a significant threat to the environment and Food Security.

1.2.2 Soils and Climate Change

Soils are inextricably linked with climate change (CC) largely due to the vast carbon (C) pool stored within soils. Terrestrial soils contain approximately 2500 gigatons (Gt) of carbon, constituting the second largest carbon sink on Earth after the ocean (Lal, 2004a, Ontl and Schulte, 2012). Approximately 62% of soil C is stored in organic forms, with the rest being inorganic (Ontl and Schulte, 2012). Agricultural soils alone are estimated to contain more than 20% of the total global C pool, and account for approximately 10.8% of the total soil organic C (Bommarco *et al*, 2013). Organic C exists in various forms, including soil microbes, decaying plant matter, animal faeces, and decomposition by-products (Lal, 2004b, Ontl and Schulte, 2012). Soil C-storage capacity is determined by several factors, including soil pH, temperature, nutrient status, water content/infiltration, and soil structure, all of which are affected by agricultural activities (Lal, 2004b). Given the vast quantity of carbon stored in soils, anthropogenic-induced release of carbon from soils could contribute substantially towards CC.

Soil erosion is a naturally occurring process whereby soil aggregates are displaced from their original location by natural forces such as wind and rain; however, it can also be caused by anthropogenic activity (Lal, 2003). Certain farming practices, such as tilling, increase the risk of soil erosion in addition to accelerating mineralisation rates, which are thought to generate soil C emissions amounting to as much as 1 Gt C $year^{-1}$ (Lal, 2003, Lal, 2005). It is important that sustainable farming practices are adopted which prevent soil erosion and therefore minimise the risk of C loss from soils.

Soil microbial communities are involved in the fluxes of several atmospheric greenhouse gases, such as carbon dioxide (CO_2), methane (CH_4) and nitrous oxide (N_2O) (Bardgett *et al*, 2008). Methane-producing microbes are a group of archaea collectively known as methanogens, whereas methanotrophs are methane-consuming (oxidising) bacteria (Singh *et al*, 2010). N_2O is produced as a result of both denitrification and nitrification, with N_2O from the latter process being produced mainly by autotrophic ammonia-oxidising bacteria from the class *Betaproteobacteria* (Teske *et al*, 1994). Consequently, any shifts in the abundance of these microbial groups as a result of anthropogenic activities could have significant consequences with regard to CC. However, the future feedback effects of CC on soil C dynamics remains a strongly debated issue. One school of thought is that increased temperatures may cause terrestrial soils to shift from being carbon sinks to carbon source as a result of increased soil microbial respiration and decomposition of organic matter, whilst others believe that a CC-related increase in C-sequestration by vegetation will outweigh any acceleration in soil C-losses (Melillo *et al*, 2002, Bardgett *et al*, 2008). Nevertheless, there is a wide consensus that the effects of CC will vary across different habitat types and regions, and the impact it will have on the global C budget is highly complex (Singh *et al*, 2010).

Land-use change can lead to dramatic changes in the soil organic C pool, with conversion from natural to agricultural land depleting the organic C store by as much as 60% in temperate regions and over 75% in the tropics (Lal, 2004a). To put this into perspective, the total amount of C released to the atmosphere as a result of agricultural land conversion during the post-industrial era (1850 to 1998) is estimated to be equivalent to half the amount produced by fossil-fuel combustion over the same period (Lal, 2004a). On a positive note, it is possible to recover lost soil C stores to some extent through the restoration of degraded soils and by the re-vegetation of marginal agricultural land (Lal, 2004b). The carbon-sequestering ability of soils can be promoted by adopting sustainable farming practices which involve high inputs of organic matter and biomass, minimal disturbance to soil structure, and enhancement of the activity of beneficial soil organisms (Lal, 2004a). There is a need, therefore, to promote the adoption of sustainable farming practices which increase yield on a per hectare basis in order to avoid the increased C emissions resulting from conversion to cropland.

1.2.3 Sustainable farming practices

The Food and Agriculture Organisation (FAO) defines sustainable agriculture as following five key principles:

(i) Improved efficiency in resource use (e.g. water and fertilisers);
(ii) Actions to conserve, protect and enhance natural resources (e.g. freshwater environments and soils);
(iii) Promotion of rural livelihoods, equity and social well-being (e.g. fair employment conditions);
(iv) Enhanced resilience (i.e. to extreme weather events and market volatility) of people, communities and ecosystems;
(v) Responsible and effective governance (FAO, 2014).

There are several types of sustainable farming systems, ranging from the most stringent, organic farming, which prohibits the use of any agrochemicals (e.g. fertilisers and pesticides), to more integrative systems, such as low-input systems, which employ a mixed approach involving reduced chemical inputs in combination with organic practices. Although organic farming typically produces crop yields which are 20% lower than in conventional systems, the price premium placed on organic produce means that the profits are comparable and its popularity has risen over recent years in European countries such as Spain and Austria (Mäder *et al*, 2002, Forster *et al*, 2013, de Ponti *et al*, 2012). In the UK, organic produce was valued at £97 million in the period between 2000 - 2001 (Watson *et al*, 2002), however there has been a decline in organic farming following the financial crash of 2008/9. In 2015, 521 thousand hectares (3%) of the UK's total agricultural land was dedicated to, or in the process of being converted into, organic farming, which represents

a 30% reduction from the 2008 statistic (Defra, 2016). The decline in UK organic agriculture has been widely attributed to the high production costs, in addition to the increasingly strict regulations.

Organic farming typically utilizes longer-term solutions to improving soil fertility and health, in contrast to conventional agricultural management which uses more fast-acting strategies (Watson *et al*, 2002). The main principles of organic farming relate to the use of natural (biological) resources as fertilisers and for the control of pests and weeds, as opposed to the alternative synthetic, chemical varieties. Organic fertilisers include animal manures, composts and mulches, whilst biocontrol methods for pests and weeds typically utilise natural enemies of the pests, such as ladybirds and weevils (Louda *et al*, 2003, van Diepeningen *et al*, 2006). Crop rotation, cover crops (green manures), minimal (or zero) tillage and intercropping are also common features of sustainable farming as they promote soil fertility, soil structure and can help to reduce the incidence of weeds, pests and soil-borne plant diseases (Sumner *et al*, 1981, Abawi and Widmer, 2000, Watson *et al*, 2002). Sustainable farming is generally less reliant on machinery than conventional systems, to the extent that it can reduce energy inputs (on a per dry weight of crop, or per land area basis) by more than 50% (Mäder *et al*, 2002). This corresponds to lower greenhouse gas emissions, reduced soil compaction and, usually, lower financial cost to the farmer. Organic farming may be regarded as one branch of ecological intensification, an umbrella term which encompasses other less rigorous forms of low-input farming, whereby negative environmental impacts are minimized by the reduction, but not necessarily exclusion, of anthropogenic inputs such as chemical fertilisers and irrigation (Bommarco *et al*, 2013). Thus, other forms of ecological intensification share many practices with organic farming, such as intercropping with legumes to improve soil fertility (Rusinamhodzi *et al*, 2012).

1.3 Soil microbes

1.3.1 Diversity and functioning of soil microbes

Soil functioning and the provision of its services would not be possible without soil microorganisms. Soil microorganisms mediate between 80 and 90% of all processes which take place in the soil environment, many of which are essential to plant growth (Nannipieri *et al*, 2003). The soil microbiome comprises an enormous diversity of microbes (bacteria, archaea, fungi, protozoa, actinomycetes, and algae), with some studies reporting a single gram of soil to contain an estimated 52, 000 different species and up to 1 billion cells of bacteria (Roesch *et al*, 2007). This diversity is not uniform however, with soils exhibiting strong heterogeneity in terms of microbial species composition and abundance in response to a multitude of biotic and abiotic factors.

Soil microbes play a crucial role in the formation of soil structure which occurs predominantly at the root-soil interface. The microbial production of polymers and secondary metabolites promotes the development of soil aggregates, which also result from drying-wetting events (Powlson *et al*, 2011, Lynch and de Leij, 2001). This contributes to the formation of a porous soil structure which enhances gas exchange and water retention in the soil, thus contributing towards flood prevention (Lynch and de Leij, 2001). Soil structure can also influence soil tilth, plant root penetration, erosion risk, and is a major factor determining the formation of organic matter (Miller and Jastrow, 2000).

Nutrient cycling is vital to all forms of life. Soil microsymbionts perform a variety of nutrient transformations, including nitrogen (N)-fixation by bacteria such *Rhizobium*, phosphorus (P) solubilisation by arbuscular mycorrhizal fungi (AMF), and sulphur oxidation by bacteria such as *Thiobacillus* (Tourna *et al*, 2014, Barrios, 2007). Many of these bacteria display plant growth promoting properties, which are discussed in detail later in this chapter.

1.3.2 Determinants of soil microbial community composition

The composition of soil microbial communities is influenced by a variety of abiotic and biotic factors, such as soil moisture, texture, temperature, nutrient (C, N and P) content, vegetation cover and organic matter content (Kowalchuk and Stephen, 2001, Bates *et al*, 2011, de Vries *et al*, 2012b). Plant functional group and soil chemistry have also been identified as major driving forces of microbial community composition in some soils, with many studies reporting that organic matter content also shapes the structure of the soil microbiome (Cui *et al*, 2016, Hartmann *et al*, 2015). However, soil pH is frequently identified as the strongest abiotic determinant of soil microbial diversity (Lauber *et al*, 2009, Fierer and Jackson, 2006, Hartmann *et al*, 2009, Rojas *et al*, 2016, de Vries *et al*, 2012b). The optimal pH for bacterial growth varies between bacterial groups, with some (e.g. *Gemmatimonadetes*) thriving in alkaline soils, whilst others (e.g. *Acidobacteria*) prefer more acidic environments (Hartmann *et al*, 2009).

1.3.3 Farming management and the soil microbiome

Organic and conventional farming methods can result in distinctly different soil microbial communities (Edwards *et al*, 2015). According to the literature, the effects of different farming systems on the soil microbiome are varying and subject to a host of environmental factors. Nevertheless, there is a substantial body of evidence indicating that in comparison to conventional farming systems, soils treated with organic amendments (e.g. animal manures and sewage sludge) exhibit increased microbial activity, diversity, and biomass (Ghorbani *et al*, 2008b, Ge *et al*, 2008, Peacock *et al*, 2001, Mäder *et al*, 2002, Sun *et al*, 2004, Reeve *et al*, 2010, O'Donnell *et al*, 2001, Lazcano *et al*, 2013, Fließbach and Mäder, 2000). This may result from the organic inputs

providing a greater and more durable pool of organic C as a substrate for microbial growth (Peacock *et al*, 2001). Additionally, it may be indirectly caused by the effect of the organic matter on the abiotic conditions of the soil environment (Dolfing *et al*, 2004).

Fertiliser inputs can alter microbial-mediated nutrient transformations. Indeed, soils under organic and low-input management have been found to have a greater abundance or activity of several functional genes encoding for various nutrient pathways and enzymes such as ureases, phosphatase, nitrification, dinitrogen fixation and xenobiotic degradation (Reeve *et al*, 2010, Xue *et al*, 2013, Mäder *et al*, 2002). This may partially explain why, in comparison to synthetically fertilised soils, organically managed soils often have higher levels of nitrogen (N), carbon (C), sulphur (S), and phosphorus (P), in addition to increased humic acid content and water holding capacity (Reeve *et al*, 2010, Pimentel *et al*, 2005, Peacock *et al*, 2001, Ghorbani *et al*, 2008a, Brown *et al*, 2000, Drinkwater *et al*, 1995). The pH of fertilisers also affects microbial populations, with synthetic ammonium-nitrate fertilisers having been reported to have acidifying effects, and organic soils having a marginally higher pH (Peacock *et al*, 2001, Mäder *et al*, 2002, Jangid *et al*, 2008).

The effect of fertiliser inputs on bacterial:fungal ratios in soils has been long established in the literature. Fungal abundance tends to respond negatively to synthetic NPK fertilisers, with elevated nitrate-N levels corresponding with declines in arbuscular mycorrhizal fungi (AMF) populations (Detheridge *et al*, 2016, Hartmann *et al*, 2015). Correspondingly, bacterial biomass has been shown to be greater in the rhizospheres of plants with high foliar N content, whereas low N plants tend to be associated with fungal dominated communities (de Vries *et al*, 2012b).

There is substantial evidence indicating that the form of N, inorganic or organic, may be the crucial determinant as to the impact of a fertiliser on soil microbial communities (Ge *et al*, 2008). In a long-term fertiliser experiment, Ge *et al* (2008) demonstrated that the structure and diversity of bacterial communities of soils treated with organic manure were significantly different to those which received various chemical fertilisers (NK, PK, NP and NPK). The organically fertilised soils exhibited significantly lower abundances of the phyla *Proteobacteria* and *Acidobacteria*, and greater abundances of *Bacteroidetes* and *Firmicutes* than soils which received synthetic fertilisers (Ge *et al*, 2008). Hartmann *et al* (2015) also identified a positive association between organic inputs and bacteria within the *Firmicutes* phylum, including the genera *Bacillus*, *Ureibacillus*, *Solibacillus*, *Thermobacillus* and *Clostridium*. The authors note that many of these contain thermophilic species which are known for their ability to degrade organic substrates such as manure (Charbonneau *et al*, 2012).

The impacts of organic fertiliser regimes appear to be highly durable. Dolfing *et al* (2004) used PCR-DGGE to demonstrate that the difference in the microbial profiles between manure-amended soils and those which did not receive any organic treatments were still detectable after 50 years of

being stored in an air-dried state. This implies that changes in soil microbial communities induced by organic amendments are long-lasting and far from transitory (Dolfing *et al*, 2004).

1.3.4 Poultry manure

Chicken manure is commonly used both as feed for cattle (in the USA) and as an organic fertiliser for crops (Himathongkham and Riemann, 1999). In comparison to chemical fertilisers, poultry manure has a low nutrient content and generally has to be applied to crops in greater quantities. However, this may be compensated for by the numerous benefits to soil health associated with poultry litter applications. Soils amended with poultry litter have been shown to be less acidic, have greater potential C and N mineralization, biomass C and higher concentrations of extractable nutrients in comparison to inorganically fertilised soils (Jangid *et al*, 2008).

As with all faecal-based substrates, poultry manures comprise diverse and rich microbiomes. In comparison to synthetically fertilised soils, poultry litter-amended soils have been shown to have significantly higher levels of bacterial diversity in terms of both species richness and evenness (Jangid *et al*, 2008, Sun *et al*, 2004). Several groups of bacteria have been positively associated with chicken manure fertilisers, including several *β*- and *Δ-Proteobacteria*, *Brevibacterium*, *Brevundimonas*, *Brachybacterium*, *Enterococcus* and *Zimmermannella* (Yang *et al*, 2016, Dumas *et al*, 2011, Jangid *et al*, 2008). Bacteria which have been found to have diminished abundances as a result of poultry manure additions include *Acidobacteria* and *Υ-Proteobacteria* (Jangid *et al*, 2008). These changes in microbial diversity could be mediated by the effect of poultry manure on soil chemistry (Jangid *et al*, 2008).

However, there are concerns regarding the harmful bacteria associated with poultry litter, which incidentally has been shown to increase the abundance of antimicrobial-resistant bacteria in soils (Yang *et al*, 2016). The application of organic manures to crop land is thought to be one of the main routes responsible for food-borne illnesses in the UK (Nicholson *et al*, 2005). Poultry manure has been found to harbour a spectrum of human and avian pathogens, including *Arcobacter* spp., *Campylobacter* spp., *Clostridia* spp., *Bordetella* spp., verocytotoxic *Escherichia coli*, *Salmonella typhimurium* and *Listeria monocytogenes* (Martin *et al*, 1998, Himathongkham and Riemann, 1999, Lovanh *et al*, 2007). Several genera of toxic fungi have also been detected in poultry litter including *Aspergillus*, *Fusarium* and *Penicillium* (Viegas *et al*, 2012).

The risk of spreading pathogenic bacteria can be reduced by implementing a storage period before spreading the manure onto the field, particularly if left as solid manure heaps which can reach high temperatures (>55°C) that are effective in reducing pathogenic bacteria populations (Nicholson *et al*, 2005). In the UK, manure can only be sold as organic fertiliser providing it satisfies the minimum requirements of being partially processed using heat (70°C for at least 60 minutes) or

pressure, before undergoing tests to validate that it has sufficiently low levels of pathogenic bacteria such as *Enterococcus faecalis*, *Escherichia coli* and *Salmonella* spp., and viruses such as *Pavovirus* (Defra, 2014). Sterilised poultry manure is commonly available in a dried, pelleted form, usually having a fairly alkaline pH ranging between 6.5- 8.0.

1.4 The Rhizosphere

The rhizosphere was first defined by Hiltner (1904) as 'the soil compartment influenced by the root', where interactions occur between microbes. These microorganisms can be beneficial or deleterious to the plant through their effects on plant nutrition and health (Hinsinger and Marschner, 2006). In beneficial relationships, microbes often deliver nutrients to the plant in exchange for carbon, whereas non-symbiotic microbes utilise the plant as a carbon source without delivering any benefit, nutrients or otherwise, to the plant (Lynch and de Leij, 2001), possibly with pathogenic effects. The rhizosphere can be subdivided into three zones (**Figure 1**):

- the **rhizoplane**: the surface of the root, including the root epidermis and mucilage;
- the **endorhizosphere**: the inner root cell layers, comprising the root cortex and endodermis where microbes can occupy intracellular spaces;
- the **ectorhizosphere**: the outermost zone of the root which includes the soil directly surrounding it (Lynch and de Leij, 2001).

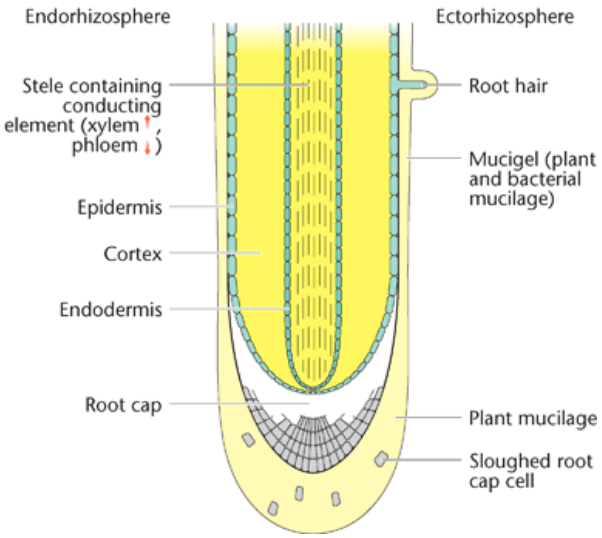


Figure 1 A plant root illustrating the sub-zones of the rhizosphere (modified from Lynch and de Leij (2001)).

The rhizosphere typically exhibits much higher microbial activity and biomass than the bulk (root-free) soil, with the number of bacterial cells in the rhizosphere being 100 to 1000 times higher than in the bulk soil (Hartmann *et al*, 2008, Bulgarelli *et al*, 2012, Lynch and de Leij, 2001, Glick, 2014). Rhizosphere soils are associated with copiotrophic bacteria, whilst bulk soil are colonised predominantly by oligotrophs (Dennis *et al*, 2010). Oligotrophs are slow-growing bacteria

commonly found in nutrient-poor environments, whereas copiotrophs are fast-growing and characteristic of fertile environments (Koch, 2001). It is often quoted that one gram of rhizosphere soil is estimated to contain up to 10^{12} cells (Lynch and de Leij, 2001). This is largely due to carbon-rich root exudations and root debris (collectively termed *rhizodeposits*) and other plant materials providing a significant energy source for microbial processes, resulting in the C concentrations of the rhizosphere being substantially higher in comparison to bulk soil (Powlson *et al*, 2011). Root exudates include water-soluble compounds such as plant hormones (e.g. auxins, gibberellins), sterols (e.g. campestrol, stigmasterol), sugars (e.g. glucose, galactose), vitamins (e.g. niacin, riboflavin), amino acids (e.g. glutamate, arginine), organic acids (e.g. acetic, lactic), phytosiderophores, enzymes (e.g. protease, amylase) and phenolic compounds (Bardgett and van der Putten, 2014; Dakora and Phillips, 2002; Jones *et al*, 2009a; Torrey, 1976; Dennis *et al*, 2010; Badri and Vivanco, 2009). Insoluble rhizodeposits include secreted mucilage, sloughed off root cap and border cells, and lysed root epidermal and cortical cells (Lynch and de Leij, 2001; Jones *et al*, 2009a). The amount and types of compounds released by roots are dependent on multiple factors, including the plant age, soil nutrient status and soil texture (reviewed by Nguyen (2003)). Microbial abundance can also be stimulated by the artificial application of these exudates to soils. The addition of maize-derived mucilage to bulk soil has been shown to promote microbial growth, increasing the number of cultivable bacteria in the soil by 450% (Benizri *et al*, 2007).

Contrastingly, plant root exudates can also inhibit microbial activity. Inhibitory rhizodeposits include antimicrobial compounds, nematicides and flavonoids which can suppress pathogens and pests (Philippot *et al*, 2013). This is the case for cowpea (*Vigna unguiculata* (L.) Walp.), which releases antifungal compounds that are effective against the fungus *Fusarium oxysporum* (Nóbrega *et al*, 2005). Glucosinolates, a class of secondary metabolites produced by many plants of the Brassicaceae family, similarly have been shown to have antimicrobial effects, such as the suppression of pathogenic *Rhizoctonia* spp. (Mazzola *et al*, 2001).

The microbial richness of the rhizosphere supports populations of protozoa and nematodes which graze on bacteria (Lambers *et al*, 2009). These bacterial grazers contribute to soil nutrient cycling by releasing ingested N in the form of ammonia (N mineralisation), which is either taken up by the plant directly or converted into nitrate by nitrifying bacteria (Bonkowski *et al*, 2001, Lynch and de Leij, 2001). The combined action of bacterial grazers and the localised effects of plant roots (on soil structure, chemistry and microbial activity) contribute towards the elevated nutrient levels of the rhizosphere, which tend to exceed those of the bulk soil (Powlson *et al*, 2011).

1.4.1 Plant growth promoting rhizobacteria

Plant rhizospheres harbour an assortment of plant-beneficial organisms such as nitrogen-fixing bacteria (e.g. rhizobia), mycorrhizal fungi and plant-growth promoting rhizobacteria (PGPR). The

term "plant growth promoting rhizobacteria" (PGPR) was first coined in the late 1970s by Kloepper and Schroth (1978) to describe bacteria that colonise plant roots and promote plant growth (Beneduzi *et al*, 2012). The use of PGPR in agriculture pre-dates the discovery of bacteria in 1683, when legumes were first observed to improve soil fertility in ancient times (Bhattacharyya and Jha, 2012). PGPR enhance plant growth either by improving plant nutrition, or by reducing the susceptibility of the plant to biotic or abiotic stress (Pineda *et al*, 2010). This may be achieved via microbial-mediated nutrient transformations, or by the production of antibiotics and phytohormones (e.g. gibberellins, auxins and cytokinins) which contribute towards plant defence against both pathogens and pests (**Figure 2**) (Brussaard, 1997, Lynch and de Leij, 2001). Other PGPR attributes include the breakdown of toxic compounds, a process termed bioremediation (Rodríguez and Fraga, 1999, van Loon, 2007).

It is generally agreed that to be classed as PGPR, bacterial strains must fulfil at least two of the following three criteria: aggressive colonisation, plant growth stimulation and biocontrol (Weller *et al*, 2002, Vessey, 2003). PGPR have been found to occur in many different genera including *Arthobacter*, *Clostridium*, *Hydrogenophaga* and *Enterobacter* (Lavakush *et al*, 2014). Many bacteria possess multiple plant growth promoting attributes, which may have both direct and indirect effects (Glick, 1995). *Rhizobium* is an example of this, as it has been demonstrated that in addition to its N-fixing properties, it can improve soil structure and help alleviate water stress during periods of drought when inoculated in sunflower plants, potentially due to the secretion of exopolysaccharide (EPS) enhancing the water holding capacity of the soil (Alami *et al*, 2000).

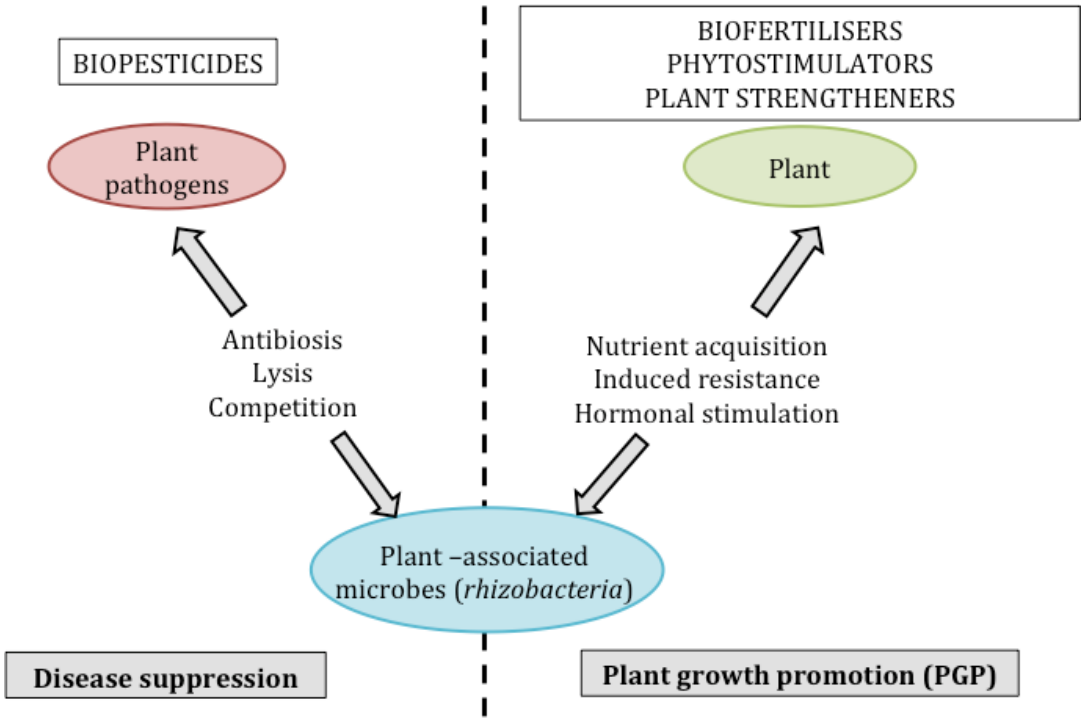


Figure 2 Mechanisms of microbial promotion of plant growth and health, and the potential agricultural applications (modified from Berg (2009)).

1.5 Direct Plant Growth Promotion

PGPR can act as biofertilisers by enhancing the nutrient status of a plant via five main routes:

i. Biological nitrogen (N_2) fixation
ii. Increasing the availability of nutrients in the rhizosphere
iii. Stimulating enlargement of the root surface area (phytohormones)
iv. Enhancing other beneficial symbioses of the host
v. A combination of the above (Vessey, 2003).

1.5.1 Nitrogen fixating microbes

Nitrogen-fixing bacteria (diazotrophs) convert atmospheric N into plant-available forms, and can be classed as either free-living or symbiotic (Rousk *et al*, 2016). Free-living diazotrophs include species of cyanobacteria, *Azospirillum*, *Herbaspirillum* and *Azotobacter* (Steenhoudt and Vanderleyden, 2000, Vessey, 2003). Free-living N-fixers have a smaller impact on plant growth, with *Azospirillum* inoculation leading to plant yield increases ranging from 5-18% (Lynch and de Leij, 2001). This limited ability of free-living diazotrophs to fix N has been attributed to the high energy demand of the process (Lynch and de Leij, 2001). Symbiotic N-fixing bacteria, which include species of *Rhizobium*, *Frankia* and *Bradyrhizobium*, are responsible for the formation of root nodules (nodulation) in leguminous plants (Lynch and de Leij, 2001, Franche *et al*, 2009). The plant-growth promoting effects of these microorganisms has been long established, with *Rhizobium* constituting the primary ingredient of the world's first patented microbial inoculum "Nitragin", which became commercially available in Germany in 1896 (Bashan, 1998, Compant *et al*, 2010).

1.5.2 Siderophore producers

Iron is one of the major factors determining bacterial growth in soils since it is required for many microbial functions, however levels in the soil are often too low to support them (Glick, 1995). Many bacteria have evolved adaptations to cope with low iron availability, one of the foremost mechanisms being the production of siderophores (Luján *et al*, 2015). Siderophores are low molecular weight ligands that forage for and bind to ferric iron-molecules (Fe^{3+}), enabling microbial cells to remotely recruit iron-molecules for assimilation which they would otherwise not be able to access (Saha *et al*, 2013, Glick, 1995). The benefits associated with siderophores are not restricted to the bacteria by which they are produced, as the sequestered iron is available for uptake by other microorganisms and plant roots in the vicinity that are capable of utilising the siderophore-iron complex (West and Buckling, 2003).

However, the increased availability of iron itself is not the most prominent PGP feature of siderophore-producing bacteria. The most valuable attribute of many siderophore-producing bacteria, which includes several fluorescent species of *Pseudomonas*, is the suppression of plant diseases such as *Fusarium oxysporum*, *Pythium ultimum* and other wilt-causing diseases (Duijff *et al*, 1993, Kloepper *et al*, 1980, Saha *et al*, 2013). There is evidence that siderophore production by *Pseudomonas aeruginosa* 7NSK2 accounts for its antipathogenic properties against *Pythium*-induced damping-off in tomatoes (*Lycopersicon esculentum*) (Buysens *et al*, 1996). This disease suppressive effect is thought to result from the bacterially-produced siderophores reducing the availability of iron for pathogens (Kloepper *et al*, 1980). However, the antipathogenic potential of siderophores is subject to numerous factors, such as soil type, crop plant species, and the phytopathogen species in question, as well as the affinity of the specific siderophore for iron (Glick, 1995). This may explain instances where inconsistent results have been observed in the anti-pathogenic effects of siderophores when transitioning from lab-based experiments to field trials (Glick, 1995).

The localized depletion of iron does not have inhibitory effects on the plant since the amount of iron required for plant growth is usually about 1000 times lower than the microbial requirements. Moreover, some plants have evolved adaptations enabling them to bind the siderophore-iron complex and remove the iron component to be taken up by the plant itself (Yehuda *et al*, 1996). This has been reported to occur in peanut, cotton (Bar-Ness *et al*, 1991), barley (Yehuda *et al*, 1996) and cucumber plants (Wang *et al*, 1993).

Another beneficial property of siderophore-producing bacteria is their contribution to the degradation of heavy metals in polluted environments, which is referred to as phytoremediation (Saha *et al*, 2013). This occurs when the siderophores bind to other heavy metals, such as copper and zinc, thereby reducing metal toxicity of contaminated soils (O'Brien *et al*, 2014). This compromises the capacity of the microbe to recruit iron molecules, and so this may be viewed as an altruistic behaviour since the bacteria are promoting plant growth at the cost of obtaining iron for their own benefit (O'Brien *et al*, 2014). Dimkpa *et al* (2008) demonstrated that *Streptomyces* increased its siderophore production in metal-contaminated soils, which the authors attributed to other metal ions competing with iron for siderophore binding, thereby inducing the bacteria to produce more siderophores in order to obtain sufficient iron. An ACC deaminase- and siderophore-producing strain of *Kluyvera ascorbata* (SUD165) that can tolerate toxic levels of nickel, lead, zinc and chromate, has been shown to enhance the resistance of canola and tomato seedlings to nickel toxicity in a pot experiment (Burd *et al*, 1998). However, in this instance siderophores were not thought to be the main cause for this growth-promoting effect since the uptake of nickel by the roots and shoots was unaffected in inoculated plants compared to controls. Instead, the authors concluded that ACC deaminase production led to a reduction in the nickel-

induced ethylene stress was a more likely agent for the improved performance of inoculated plants (Burd *et al*, 1998).

1.5.3 Phosphorus solubilisers

Phosphorus (P) is one of the primary nutrients essential for plant growth, second only to nitrogen (Sharma *et al*, 2013). Soluble (plant-available) forms of P are usually present in soils at very low levels, accounting for approximately just 0.1% of total soil P, with the rest occurring in insoluble, immobilised forms (Zou *et al*, 1992). Agricultural soils often have much higher P reserves due to the repeated application of NPK fertilisers, although a large portion of the applied P is rapidly rendered unavailable to plants as a result of the precipitation of P by metal-cation complexes (Al^{3+} and Fe^{3+}) which is subsequently immobilised (Rodríguez and Fraga, 1999, Sharma *et al*, 2013). This insoluble P is unable to be assimilated by plants, and is vulnerable to leaching. Eutrophication of surface waters by leached fertiliser-P is one of the leading causes of algal blooms, which embodies the largest single threat to freshwater life in lakes and streams (Tilman *et al*, 2001b). Furthermore, excessive P-fertiliser applications can alter soil microbial communities, although to a lesser extent than N fertilisers (Beauregard *et al*, 2010, Rooney and Clipson, 2008, Guo and Wang, 2009, Eo and Park, 2016, Cassman *et al*, 2016).

Phosphorus solubilisation has been confirmed in species of *Achromobacter*, *Agrobacterium*, *Aereobacter*, *Bacillus*, *Burkholderia*, *Erwinia*, *Flavobacterium*, *Paenibacillus*, *Pseudomonas* and *Micrococcus* (Rodríguez and Fraga, 1999). Bacterial mobilization of P is largely accomplished by the production of organic acids (e.g. lactic, isobutyric and acetic acids), which solubilise immobilised P thereby rendering them available for plant uptake (Nahas, 1996, Vazquez *et al*, 2000). Other possible bacteria-mediated mechanisms of P solubilisation include the excretion of protons in combination with ammonium ion absorption, and the secretion of phosphatase enzymes (Illmer *et al*, 1995).

Arbuscular mycorrhizal fungi (AMF) also enhance P-uptake by plants by the development of hyphal networks with plant roots (Cassman *et al*, 2016). The beneficial properties of P solubilising bacteria and mycorrhizal fungi can act synergistically, as demonstrated by the co-inoculation of Douglas fir seedlings with *Bacillus amyloliquefaciens* and the ectomycorrhizal fungi *Laccaria laccata*, and also by *B. subtilis* in combination with the arbuscular mycorrhizal fungi (AMF) *Glomus intraradices* lettuce (*Lactuca sativa* L. cv. Cherry) (Duponnois and Garbaye, 1991, Kohler *et al*, 2007). Such bacteria are often referred to as "mycorrhization helper bacteria" (MHB) based on the assumption that they stimulate plant growth indirectly via their proliferating effect on mycorrhizal root tip formation (Probanza *et al*, 2001). P-fertilisation can result in fewer AMF associations as plants are able to take up the P directly and therefore do not require AMF assistance (Bolan, 1991, Treseder, 2004).

1.5.4 Phytohormone producers

Certain microorganisms, particularly those located in the rhizosphere, are able to promote plant growth through the production of plant hormones (phytohormones) such as gibberellins, auxin, cytokinins and zeatin (Glick, 1995, Egamberdieva, 2009). Auxins are the most widely studied PGPR hormones, specifically indole-3-acetic acid (IAA). IAA can stimulate plant growth both on a short-term and long-term scale, typically by stimulating (lateral) root elongation and branching (Glick, 1995, Casson and Lindsey, 2003, Dowling and O'Gara, 1994). Elevated auxin levels have also been correlated with increased root hair formation, a beneficial trait which can enhance ion uptake and phosphorus mobilisation in soils (Wittenmayer and Merbach, 2005). IAA producing bacteria, such as *Pseudomonas aureantiaca* and *P. extremorientalis*, have been found to alleviate salt stress, increase root and shoot growth, and enhance germination rates in wheat (*Triticum aestivum* L.) cv. Residence (Egamberdieva, 2009). The effect of PGPR-produced IAA can vary between plant species to the extent that while it may benefit one plant, it may actually have negative impacts on the growth of another (Dubeikovsky *et al*, 1993). Indeed, several pathogens express this IAA-producing ability to the detriment of the host plant, by interfering with plant development through excessive production of auxin and cytokinin. Examples of this include the crown gall-inducing *Agrobacterium tumefaciens* and the olive knot-causing bacteria *Pseudomonas savastanoi* pv. *savastanoi* (Jameson, 2000).

Cytokinins are aminopurine compounds which regulate plant growth and can influence plant processes such as cell division, leaf senescence, root and shoot growth, and seed germination (Werner *et al*, 2001, Werner *et al*, 2003). Bacteria which exhibit cytokinin-synthesizing abilities include *Azotobacter vinelandii* (Azcón and Barea, 1975) and *Pantoea agglomerancs* (Omer *et al*, 2004). A cytokinin-producing strain of *Bacillus subtilis* was shown to increase growth of lettuce (*Lactuca sativa* L., cv Lolla Rossa) plants under drought conditions (Arkhipova *et al*, 2007).

Another plant hormone produced by PGPR is ethylene, which is formed from the hydrolysis of 1-aminocyclopropane (ACC) by the enzyme ACC deaminase (Glick, 1995). Ethylene is often produced as a plant response to stress and mediates various plant processes including senescence, chlorosis, leaf abscission and general plant growth inhibition (Glick, 2014). It is also responsible for the ripening of fruit and is commonly applied artificially in agricultural production to accelerate the process postharvest (Abeles *et al*, 2012). Several studies have shown that the production of ethylene by PGPR can end seed dormancy and trigger germination, thereby promoting improved seed survival and germination success (Corbineau *et al*, 2014). However, the production of high levels of ethylene can have negative effects on plant processes such as inhibited root elongation, growth deformations, leaf chlorosis, and senescence (Konings and Jackson, 1979, Abeles *et al*, 2012, Jensen and Veierskov, 1998). Ethylene is also produced by plants in response to environmental stress, such as extended photoperiods and fungal pathogens, although paradoxically

this hormone can actually exacerbate the severity of infections (van Loon, 1984, Jensen and Veierskov, 1998).

Gibberellins (GA_3) influence several aspects of plant development, including seed germination, stem and leaf growth, flowering and fruit growth (King and Evans, 2003, Pharis and King, 1985). Gibberellins can be synthesised by bacteria such as *Rhizobium meliloti*, *Proteus mirabilis,* and *Klebsiella pneumoniae* (Karadeniz *et al*, 2006, Bottini *et al*, 2004). Bacterial gibberellin production has been associated with elevated shoot and root growth in rice inoculated with the N-fixing *Rhizobium leguminosarum* bv. *trifolii* (Yanni *et al*, 2001) and also with sheath elongation growth in dwarf rice mutants treated with a strain of *Azospirillum brasilense* (Cassan *et al*, 2001).

Other plant growth regulators produced by bacteria include abscisic acid (ABA) and jasmonic acid (JA). ABA can aid water conservation in plants experiencing drought stress by inducing stomatal closure, which also serves to protect plants against invasion of pathogens such as necrotrophic pathogen *Alternaria brassicicola* and *Pythium irregulare* (Adie *et al*, 2007). However, ABA is commonly associated with an increased susceptibility of plants to pathogens, as demonstrated with the necrotrophic fungal pathogen *Fusarium oxysporum* in *Arabidopsis*, and the rice blast fungus *Magnaporthe grisea* in rice plants (Bari and Jones, 2009, Anderson *et al*, 2004, Koga *et al*, 2004). In contrast, JA production has been identified as contributing to PGPR-induced systemic resistance (ISR) against plant pathogens such as the tomato late blight (*Phytophthora infestans*) (Yan *et al*, 2002).

In addition to synthesising them, certain PGPR destroy plant hormones (Kudoyarova *et al*, 2015). This may be beneficial under certain circumstances, as exemplified by the bacterial production of 1-aminocyclopropane-1-carboxylate (ACC) deaminase, which induces the decay of ACC. ACC is the precursor of ethylene, and so it can reduce the incidence of ethylene-mediated processes such as senescence, chlorosis and leaf abscission (Glick, 2014). Consequently, ACC deaminase-containing bacteria have been hailed as a potential tool for enhancing crop yields, with evidence that it can help plants cope with environmental stresses such as flooding, heavy metal pollution and drought (Glick, 2014, Shaharoona *et al*, 2006). Bacteria capable of producing ACC deaminase include *Achromobacter piechaudii* ARV8, which enhanced growth of tomato plants under drought conditions (Mayak *et al*, 2004).

Phytohormone-producing and nutrient cycling bacteria are prevalent in the rhizosphere of many plants. A study by Fürnkranz *et al* (2009) indicated that as much as two thirds of cultivable bacteria obtained from the rhizospheres of four crop species (horseradish, sorghum, sunflower and safflower) possessed plant growth-promoting properties. They found that 66% of the 59 bacterial strains isolated from these rhizospheres exhibited at least one PGP property. Specifically, 19% were nitrogen (N_2) fixers, 41% were phosphorus (P) solubilisers, 17% were auxin (IAA) producers and 10% were ACC degraders (Fürnkranz *et al*, 2009).

1.6 Indirect Plant Growth Promotion

1.6.1 Anti-pathogenic PGPR

Indirect enhancement of plant growth is achieved by PGPR production of various metabolites (e.g. enzymes, antibiotics and volatiles) which deter plant pathogens or pests, both above- and below-ground (Pineda *et al*, 2010). This phenomenon is called 'induced systemic resistance' (ISR) and usually involves JA or jasmonic ethylene (JE) pathways (Van Oosten *et al*, 2008, Doornbos *et al*, 2011). Examples of biocontrol bacteria include members of the genera *Agrobacterium*, *Bacillus*, *Burkholderia*, *Collimonas*, *Pantoea*, *Pseudomonas*, *Serratia*, *Stenotrophomonas*, and *Streptomyces* (Raaijmakers and Mazzola, 2012). Although many of these exhibit strong antipathogenic properties, they are often not viable as commercial products owing to their poor shelf life (Haas and Défago, 2005). The majority of commercially successful PGPR biocontrol products consist of bacilli species, such as *Bacillus subtilis* strains GB03 (Kodiak; Gustafson), *B. pumilus* strain GB34 (YieldShield; Gustafson), *B. thuringiensis* subspp. *Tenebrionis* (Novodor FC, Valent BioScience Corpoation, Libertyville, IL, USA) and *B. licheniformis* strain SB3086 (EcoGuard; Novozymes) (Raaijmakers and Mazzola, 2012, Haas and Défago, 2005, Hartmann *et al*, 2015). These biocontrol traits are highly species specific, and it is not uncommon for bacterial genera, such a *Pseudomonas*, to contain both pathogenic and anti-pathogenic species (Raaijmakers and Mazzola, 2012).

The microbial control of root diseases has been attributed to several classes of antibiotic compounds, including phenazines, phloroglucinols, pyoluteorin, pyrrolnitrin and cyclic lipopeptides (Haas and Défago, 2005). Hydrogen cyanide production by pseudomonads has also been shown to suppress black root rot (Voisard *et al*, 1989). The production of antibiotics can be verified by using one of the following genetic manipulative approaches to diminish or enhance this trait (Glick, 1995): (i) the removal of the antibiotic-producing ability of the PGPR having deleterious effects on its anti-pathogenic properties; or (ii) the genetically engineered enhancement of the antibiotic-production rates of the PGPR strengthening its ability to defend the plant against the pathogen (e.g. Maurhofer *et al* (1992)).

PGPR can also inhibit the detrimental effects of plant pathogens by hydrolysing the plant-damaging compound fusaric acid which is produced by phytopathogenic *Fusarium* species (Toyoda and Utsumi, 1991). Some PGPR species take this strategy a step further and produce enzymes that lyse and digest the pathogenic fungi themselves. For instance, *Pseudomonas stutzeri* produces chitinase and laminarinase which degrade *Fusarium solani* mycelia (Lim *et al*, 1991). Other pathogens that can be suppressed by PGPR include the fungi *Rhizoctonia solani*, *Sclerotium rolfsii*, and *Pythium ultimum* (Glick, 1995). The disease-suppressing properties of PGPR may also relate to their ability to compete for nutrients and niches (as discussed earlier in relation to

siderophores), and the PGPR-mediated inducement of the host plant's systemic resistance to pathogenic fungi as demonstrated by JA-producing bacteria.

1.6.2 Biopesticide PGPR

The current use of microbes for the biocontrol of insect pests is relatively limited, and holds potential to provide more economical and sustainable alternatives to chemical pesticides. Several PGPR have been shown to have negative effects on the growth and development of foliar-feeding (phytophagous) insects (Pineda *et al*, 2010). *Pseudomonas maltophila* has been shown to have inhibitory effects on the development and growth of the corn earworm, *Helicoverpa zea* (Bong and Sikorowski, 1991). The mechanisms behind these interactions generally involve changes in the production of constitutive or induced herbivore-deterring compounds (Mithöfer and Boland, 2012). Studies have identified herbivore-deterring properties in *Bacillus pumilis*, *Pseudomonas putida* and *Flavomonas oryzihabitans* against *Acalymma vittatum*, *Diabrotica undecimpunctata* and *Bemisia argetifolii* feeding on cucumber and tomato (Zehnder *et al*, 1997). A study by Fahimi *et al* (2014) found that inoculation of cucumber seeds with strains of *Pseudomonas fluorescens* increased fruit weight and had inhibitory effects on the population growth of the cotton aphid, *Aphis gossypii* (Fahimi *et al*, 2014). Similarly, the inoculation of tomato plants with *Bacillus subtilis* has been shown to hinder the development of the whitefly *Bemisia tabaci* (Valenzuela-Soto *et al*, 2010).

The efficacy of the inoculants depends to some degree on how specialized the insect is to the host plant and its feeding style (e.g. chewing or phloem-feeding) (Nalam *et al*, 2013). For instance, the inoculation of *Arabidopsis* plants with *P. fluorescens*, which triggers the JA/ET defence pathways, deters feeding by the generalist herbivore *Spodoptera exigua*, but has no impact on the larvae of the specialist herbivore *Pieris rapae* (Van Oosten *et al*, 2008). PGPR which convey anti-herbivory properties through altering the production of plant secondary metabolites are more likely to affect insects which chew on plant foliage rather than phloem-feeders which use their stylet to pierce the phloem sieve tubes of the plants and so are less likely to encounter these compounds (Nalam *et al*, 2013).

Rhizobacterial inoculations can produce undesirable effects on populations of phytophagous pests and their natural enemies. The inoculation of *Arabidopsis* plants with *P. fluorescens* bacteria-treatment, for instance, produced conflicting results. The bacteria triggered changes in the plant's volatile production via the JA-signalling pathway, which elicited a positive response from the phloem-feeding aphids *Myzus persicae*, but led to reductions in the parasitoid (*Diaeretiella rapae*), which is a chewing insect (Pineda *et al*, 2013). The genotype of the host plant can determine whether a plant-growth promoting rhizobacteria has a positive or negative effect on insect performance. This was demonstrated in the relationship between the PGPR *Pseudomonas aeruginosa*, barley (*Hordeum vulgare*) and the grain aphid *Sitobion avenae*, with the inoculation

having opposing effects on the aphid population when different genotypes of the plant were compared (Tétard-Jones *et al*, 2012).

1.6.3 Commercial PGPR products

In light of the growing need for sustainable agricultural practices, there has been a recent surge in research into potential PGPR-based products to enhance crop yields. Indeed, bacterial agricultural products have been hailed by some as a revolutionary opportunity for agriculture to move away from its reliance on chemical products (Glick, 2014). This may be achieved through the development of microbial-based products, such as biofertilisers or biopesticides, which harness the plant growth promoting properties of the microbes, thereby reducing, or even eliminating, the need for chemical inputs (fungicides, herbicides, pesticides and fertilisers). The global market for microbial inoculants is estimated to be growing at an annual rate of 10% (Berg, 2009). For an inoculant to be successful, the PGPR must be able to establish and maintain a population in the soil environment. The critical colonization level for achieving successful rhizosphere colonization has been estimated at 10^5–10^6 colony-forming units (CFU) g^{-1} of root in the case of *Pseudomonas* spp. (Haas and Défago, 2005). This represents one of the major challenges in developing commercial microbial inoculants, as prototypes which performed well in laboratory or greenhouse based studies often fail to replicate these yield-promoting effects in the field. This inconsistency in the performance of PGPR products when transferring their application from the laboratory to the field may be related to one or more of the following factors:

- ***Host plant species:*** Microbial inoculants may not be effective on all crops since plant-associated microbes display a degree of host specificity (Berg and Smalla, 2009). In order to colonise the rhizosphere successfully, bacteria must have the ability to both utilise the host plant's rhizodeposits for growth and to compete with other microbes for resources (Dennis *et al*, 2010). These traits are linked to the growth rate and motility of bacteria, with the latter playing a vital role in chemotaxis - the movement of bacterial cells towards carbon compounds (Dennis *et al*, 2010).
- ***Multiple species inoculants:*** dual or multiple inoculants are attractive in their potential to provide multiple PGP benefits simultaneously or to enhance the benefits provided by a single species inoculant. In some cases relating to biopesticide PGPR, however, it has been demonstrated that inoculating with a single species has a stronger impact on insect performance than using multiple species (Trabelsi and Mhamdi, 2013).
- ***Introduction of non-resident species:*** Inoculating soils with microbial species which are not already present in the community, or are not naturally associated with the host plant, is liable to being either unsuccessful in the long-term, or producing inconsistent results. Gadhave *et al* (2016) postulate that this may be due to the introduced microbial species surviving in the soil for a limited time which is insufficient to incur positive functional

(PGP) effects on the plant. Furthermore, the new species may have detrimental impacts on the abundance and diversity of native microbial species due to increased competition for niches and resources (nutrients).

Genetic engineering is often used to create novel PGPR-based inoculants for agriculture with enhanced growth promoting traits. Genes that confer the ability to degrade certain xenobiotic compounds (e.g. herbicides or pesticides) can be inserted into the genome of the PGPR to enhance their ability to persist and grow in the soil environment, giving them a competitive advantage over other (possibly pathogenic) microorganisms which do not possess this function (Glick, 1995). The ability to either synthesize or tolerate antibiotics can also be introduced to PGPR through genetic manipulation, although the transfer of antibiotic resistance genes to other (possibly undesirable) soil organisms is a significant risk in the latter approach (Glick, 1995). Similarly, genes denoting herbivory-resistance can be transferred from one PGPR bacterium to another. This has been demonstrated by the insertion of a toxin gene originating from *Bacillus thuringiensis* into a PGPR strain of *Pseudomonas putida*, thus enabling *P. putida* to induce resistance against the sugarcane borer (*Eldana saccharina*) (Herrera *et al*, 1994). Another genetic manipulation approach involves expanding the range of siderophores a PGPR strain can assimilate, which may have the added benefit of improving its competitive advantage over pathogenic microorganisms. However, there are significant drawbacks to some genetic manipulations, such as the introduction of N-fixing genes to a non-diazotrophic PGPR species, since these processes are highly energy-demanding (ATP), so could reduce the overall competitiveness of the PGPR (Glick, 1995).

PGPR can be applied to agricultural crops in a variety of ways. Seed coating is one of the most commonly used methods to inoculate plants with PGPR and has been shown to be effective in many crops. The coating of carrot seeds with the PGPR *Serratia entomophila* (Family: *Enterobacteriaceae*) has been shown to serve as an effective deterrent against the New Zealand grass grub (*Costelytra zealandica* (White)) (Scarabeidae: Melolonthinae) (Wright *et al*, 2005). N-fixing *Rhizobia* is another popular seed inoculant for legume plants (Deaker *et al*, 2004). Seed treatment with a strain of *Pseudomonas putida* (GR12-2) has been shown to promote root elongation in canola (*Brassica campestris*), with the addition of phosphorus to the soil augmenting the PGPR's beneficial effects (Lifshitz *et al*, 1987). Alternatively, PGPR may be applied to the soil either in a solid (granules or powders) or liquid (cell suspensions) form (Haas and Défago, 2005). Seed coating is often regarded as the preferable method, however, since it requires smaller quantities of bacteria to be effective and also can be applied at the time of sowing using pre-existing machinery (Taylor and Harman, 1990).

Agriculture is not the only sector which may benefit from PGPR. The majority of antibiotics in medicinal use have their origins in soil, with many ground-breaking discoveries being made since Selman Waksman designed a systematic approach to testing soil bacteria for antimicrobial properties in the 1940s (Raaijmakers and Mazzola, 2012, de Vrieze, 2015, Pawlowski *et al*, 2016,

Lewis, 2012). Antibiotic-producing PGPR may represent an important source of novel pharmaceuticals, which is highly pertinent given the growing threat of antimicrobial-resistance in human-associated pathogens in conjunction with the decline in the development of new antibiotics (Compant *et al*, 2010, Raaijmakers *et al*, 2002).

1.6.4 Deleterious rhizobacteria

Many harmful organisms also reside in the rhizosphere, such as soilborne pathogens and pests, which have negative effects on plant growth (Raaijmakers *et al*, 2009). Deleterious rhizobacteria (DRB) (Suslow and Schroth, 1982) are rhizosphere-dwelling microorganisms which impede or diminish plant growth without causing visible disease symptoms (Lynch and de Leij, 2001). Phytotoxins, such as cyanide, produced by deleterious rhizobacteria are believed to be the major cause for this stunted growth (Lynch and de Leij, 2001). Other mechanisms through which DRB can lead to yield losses are the production of phytohormones, competition for nutrients and the suppression of mycorrhizal function (Nehl *et al*, 1997).

1.7 Plant effects on soil microbiomes

Plant community composition can exert a strong effect on rhizosphere microbiomes to the extent that plant genetic variation (phenotypes) can influence soil microbial communities (Marschner *et al*, 2001, Marschner *et al*, 2004, Wieland *et al*, 2001, Kuske *et al*, 2002, Van Nuland *et al*, 2016, Peiffer *et al*, 2013). The influence of plant taxonomy and phenotype on soil microbial community composition has been reported to occur at a variety of scales, from greenhouse experiments (Marschner *et al*, 2001) to tropical forests (Aleklett *et al*, 2015). This may result from the influence of plant genotype on various factors relating to the physiology, morphology and chemistry of a plant (such as the nutrient acquisition rates) (Van Nuland *et al*, 2016). However, it is also proposed that plants actively recruit beneficial microbes, for instance bacteria which aid pathogen defence or promote nutrient availability (Revillini *et al*, 2016). The mechanisms behind this formation of host plant (genotype)-specific rhizosphere communities are not fully understood, one hypothesis being that bacteria are attracted to the root via chemotaxis stimulated by the release of rhizodeposits (e.g. photoassimilates such as benzoxazinoid and glucosinolates) from the plant root (Bulgarelli *et al*, 2013, Bressan *et al*, 2009). These species-distinguishing microbiomes are even detectable in the epiphytic microbial communities of seeds, as demonstrated in wheat (*Triticum* spp.), canola (*Brassica* spp.) and corn (*Zea* spp.) (Links *et al*, 2014, Johnston-Monje and Raizada, 2011, Weiss *et al*, 2007).

1.7.1 Plant growth effects on the soil microbiome

There is widespread evidence that the pool of plant-associated microbes changes over the course of the plant's growth, with some suggesting that plants actively select for different bacteria according to their specific requirements at different stages of development (Chaparro *et al*, 2014, Baudoin *et al*, 2002). These changes are hypothesized to be controlled by variation in the type and amount of exudates produced during the growth of plants (Baudoin *et al*, 2002). In comparison to later developmental stages of plant growth, root exudates released in early growth stages have been shown to be richer in carbohydrates which are easily degraded, suggesting that this may correspond to the period of greatest microbial activity (Hamlen *et al*, 1972, Lynch and de Leij, 2001). The carbohydrate exudates released by older plants tend to be more recalcitrant and therefore less accessible for microbial growth (Lynch and de Leij, 2001).

1.8 Tritrophic interactions between the soil microbiome, plants and phytophagous insects in relation to fertilisers

Interactions between rhizosphere microbial communities and phytophagous insects can occur via their shared host: the plant. These interactions can be bi-directional, with belowground microbes and foliar-feeding insects exerting plant-mediated feedback effects on each other. The potential for soil microbes to influence plant defence against insects has already been discussed (see **Biopesticide PGPR**), so this section will mainly focus on the bottom-up effects of phytophagous insects on soil microbial communities.

Aboveground herbivory has been shown to influence the soil microbial community in several studies (Hamilton *et al*, 2008, van Dam, 2009). This may caused indirectly by herbivore-induced changes in plant root biomass (Ayres *et al*, 2007) and rhizodeposition rates (reviewed by Bardgett *et al* (1998)), which consequently affects the resource availability for soil microbes. In some cases, herbivory can induce recruitment of beneficial bacteria in the rhizosphere which may enhance plant defences (Yang *et al*, 2011, Lee *et al*, 2012). Insect herbivory can have long-term impacts on the soil microbial community via alteration of the host plant root exudation. This was demonstrated in a study where the infestation of ragwort (*Jacobaea vulgaris*) with cabbage moth larvae (*Mamestra brassicae*) significantly altered the soil fungal community composition (Kostenko *et al*, 2012). This resulted in a legacy effect, whereby the chemistry of plants grown subsequently in this soil was altered in such a way that the performance of *M. brassicae* and its parasitoid were affected (Kostenko *et al*, 2012). Another study by Bezemer *et al* (2013) also reported an effect of aboveground herbivory on soil fungal communities, whilst a long-term field experiment by Macdonald *et al* (2015) found that the exclusion of invertebrate grazers (insects and mollusc) were associated with lower bacterial biomass as well as reduced AM fungi in comparison to treatments which included invertebrate grazers.

Recently, it has been discovered that plants can actually communicate warnings of oncoming insect attacks. This was shown by Babikova *et al* (2013), who showed that broad bean plants (*Vicia faba*) subjected to herbivory by pea aphids (*Acyrthosiphon pisum* Harris) can send signals to neighbouring plants via their connected mycelial network. The arbuscular mycorrhizal fungal mycelia appeared to be acting as a conduit for the infested plant to transfer signal molecules to neighbouring plants, warning them of possible impending aphid attack and thus enabling these to prime themselves for attack (i.e. instigate chemical defence systems prior to attack). Furthermore, these volatile organic compounds (VOCs) act as attractants to parasitoid wasps (*Aphidius ervi*), thereby enhancing the defence potential of the plant against aphids. It has been proposed that this communication is instigated by the triggering of the jasmonate (JA) pathway (Song *et al*, 2014).

The diversity of the soil microbiome can influence herbivore performance. Hol *et al* (2010) showed that reducing the population of rare soil microbes (by re-inoculating sterilised soil filtrates from field soil at various concentrations) had a positive impact on both the biomass and nutritional quality of two crop plants (*Beta culgaris* and *Brassica oleracea*). This in turn resulted in a positive correlation between reduced abundance of rare soil microbes and aphid body size. The authors attribute the improved plant performance in the (near) absence of rare soil microbes to a possible reduction in microbially-produced phytotoxins, although it could also have been caused by a reduction in soil-borne plant pathogens. The plants with larger populations of rare microbes exhibited higher concentrations of defensive compound which is likely to account for the reduction in aphid performance on these plants. Thus this study promotes the idea that a reduction in soil microbial diversity may result in greater plant growth and nutritional quality, but may also lead to larger pest infestations.

Fertiliser regimes can play an important role in determining the outcome of insect-plant-microbe interactions. N-fertilisers are typically associated with enhanced performance of phloem-feeding insects. Indeed, it is well established that the performance and population growth rate of generalist aphids is greater on synthetically fertilised plants whose phloem has higher amino acid content (Kos *et al*, 2015, Awmack and Leather, 2002, Stafford *et al*, 2012, Hosseini *et al*, 2010, Sauge *et al*, 2010, Patriquin *et al*, 1988). Phytophagous insects may affect soil microbes by inducing changes in plant chemistry and resource allocation, which varies to some extent according to the nutrient status of the plant. Vestergård *et al* (2004) demonstrated that the effect of aphid herbivory on rhizosphere-dwelling bacteria differed according to fertiliser use and the reproductive stage of the plant. In the early stages of barley growth, aphid-infested plants had depleted soil bacterial populations in comparison to un-infested plants, with corresponding declines in root growth. The authors hypothesised that this may have been caused by a diminished allocation of photoassimilates to root exudate production as a result of the plant having to compensate for the photoassimilates (phloem sap) removed by the aphids. This reduction in rhizodeposit production represents a loss of C and, therefore, a depletion of resource availability for soil-dwelling microorganisms which may

account for the lower bacterial abundance. Another theory was that the aphids induced the production of plant defence compounds which may have inadvertently inhibited bacterial growth. However, at later growth stages, fertilised plants infested with aphids exhibited increased bacterial rhizosphere populations, whilst the aphid infested unfertilised plants showed no change in numbers of bacteria (Vestergård *et al*, 2004). This suggests that dynamic and complex multitrophic interactions exist between soil bacteria, plants and aphids which are influenced to some extent by the nutrient status of the soil.

In order to examine these multitrophic relationships further, I have conducted a series of mesocosm (glasshouse) experiments using a model system of Derby Day cabbages (*Brassica oleracea* L. var. *capitata*), peach-potato aphids (*Myzus persicae*), and soil sourced from an agricultural field site. This system was first used to explore soil microbial community responses to different fertiliser regimes, cabbage growth and aphid herbivory using Next Generation Sequencing (NGS) of the 16S rRNA gene. This was complemented by concomitant measurements of the aboveground plant and aphid performance in order to identify the effect of the different fertilisers on plant quality and aphid herbivory, in addition to potential soil-plant-insect relationships. Following on from this study, a species of sulphur-oxidising bacteria (*Thiobacillus thioparus*) found to be closely associated with *B. oleracea* in the 16S rRNA NGS experiment, was used as an soil inoculant to test its potential to enhance the chemistry and defence abilities of cabbages. Finally, I drew on aspects from both of these experiments to perform initial investigations into the effects of varying N and S availability on cabbage growth and aphid performance.

1.9 Model organisms

The model biological system used in experiments throughout this project consisted of the Derby Day cabbage (*Brassica oleracea* L. var. *capitata*) and one of its herbivores, the green peach aphid (*Myzus persicae*). These organisms and their characteristics relevant to this thesis are discussed in turn below.

1.9.1 Brassicas

The Brassicaceae (Cruciferae) family, belonging to the order Capperales, contains around 375 genera and 3200 species, many of which are major crop vegetables, often referred to as crucifers (Rancé, 2003). The genus *Brassica* is the most economically important member of the Brassicaceae family, comprising around 159 species (Zhang *et al*, 2003, Branca and Cartea, 2011). Commonly cultivated *Brassica* crops include those derived from *B. rapa* (turnips, swede and Chinese cabbage), *B. nigra* (mustards) and *B. napus* (oilseed rape) (Lowe *et al*, 2004, Ishida *et al*, 2014). Another species, *B. oleracea* L. (**Figure 3**), has been cultivated over many decades to produce a broad spectrum of marketable crops including varieties of cabbage, broccoli, cauliflower,

kale and Brussels sprouts (Kushad *et al*, 1999). *Brassica* crops are rich in vitamins (e.g. folic acid), minerals (e.g. zinc and magnesium), carbohydrates (e.g. sucrose), amino acids (e.g. L-glutamine), and an array of phytochemicals (e.g. phenolics and phytoalexins) (Jahangir *et al*, 2009).

Figure 3 *Brassica oleracea* L. var *capitata* Derby Day variety.

Crucifers are valued not only for their nutritional value, but also for their medicinal health benefits, potential as a biofuel, and biocontrol properties (Ahuja *et al*, 2010). There are three main oilseed species of *Brassica* (*B. napus*, *B. rapa* and *B. juncea*) which collectively are ranked as the third most imported source of vegetable oil globally (Zhang and Zhou, 2006). Oilseed *Brassica* spp. have an additional use as a renewable energy source, with fatty acid methyl esters extracted from oilseed rape (*B. napus*) and *B. carinata* being used for the development of biodiesel (Del Gatto *et al*, 2015, Cardone *et al*, 2003).

Insect pests are a significant problem in *Brassica* crop production. These include specialist and generalist species of Lepidoptera (e.g. the diamondback moth, *Plutella xylostella* L.), Hymenoptera (e.g. the turnip sawfly, *Athalia rosea* L.), Diptera (e.g. the cabbage root fly, *Delia radicum* syn. *brassicae* L.), Coleoptera (e.g. the crucifer flea beetle, *Phyllotreta cruciferae* Goeze), weevils (e.g. the cabbage stem weevil, *Ceutorhynchus pallidactylus* Marsham) and Homoptera (e.g. the cabbage aphid, *Brevicoryne brassicae* L.) (Ahuja *et al*, 2010). Brassicaceae have evolved various defence mechanisms against these pests, the most noteworthy being the glucosinolate-myrosinase complex.

1.9.1.1 Glucosinolates and insect herbivory

Glucosinolates play an important role in plant defence, acting as "natural pesticides" against many herbivorous insects (Hanschen *et al*, 2015). Glucosinolates are non-volatile, non-toxic compounds, which are normally stored in a chemically stable, inactive state within the plant cell vacuole. The toxic potential of GLS is only realised upon wounding or herbivory-related damage to plant tissue, so that the otherwise compartmentalised glucosinolates are released and brought into contact with the myrosinase enzyme (Wittstock and Halkier, 2002). Myrosinase is a thioglucoside glucohydrolase, which hydrolyses the glucose moiety of the glucosinolate (Halkier and Gershenzon, 2006). This reaction yields a variety of breakdown products, including isothiocyanates (mustard

oils), nitriles and thiocyanates (Fahey *et al*, 2001). These hydrolysis products account for the toxicity of these plants to certain generalist insect herbivores, as well as to fungi and bacteria (Winde and Wittstock, 2011). This property has led to the use of *Brassica* plants in agriculture as biopesticides and biofumigants for the control of pathogens (e.g. *Gaeumannomyces graminis* var. *tritici* (take-all wheat) and *Rhizoctonia* spp.), nematodes and weeds (Halkier and Gershenzon, 2006, Angus *et al*, 1994, Mazzola *et al*, 2001).

The effect of herbivory on GLS production is partially determined by the feeding guild of the insect and its specificity for the host plant (Mewis *et al*, 2006). Herbivory by leaf-chewing insect pests causes substantial damage to plant tissue, which is likely to induce a rapid defence response (e.g. altered GLS production) in the plant. Caterpillars of the beet armyworm (*Spodoptera exigua*), for instance, have been found to induce a two-fold increase in total GLS of Arabidopsis (Columbia) plants after just one day of feeding (Mewis *et al*, 2005). In contrast, phloem-feeding insects, such as aphids, tend to elicit a different response as their phloem-piercing stylet causes minimal damage to leaves. These insects may even avoid activating the glucosinolate-myrosinase defence complex, as supported by the finding that *M. persicae* secrete considerable quantities of intact, inert aliphatic GLS in their honeydew (Malka *et al*, 2016, Barth and Jander, 2006, Khan *et al*, 2010). This may explain the variability in the effect of *M. persicae* on total GLS concentration. Kim and Jander (2007) noted an overall reduction in total GLS production following *M. persicae* feeding on Arabidopsis, whilst Mewis *et al* (2005) reported that both *M. persicae*- and *B. brevicoryne*-infested Arabidopsis (Columbia) leaves accumulated significantly higher levels of total GLS than undamaged controls.

The variation in plant responses to differential insect feeding strategies may be explained in part by the defence signalling pathways they activate. The main plant defence pathways are regulated by the stress compounds salicylic acid (SA), jasmonic acid (JA) and ethylene (ET), and they are thought to mediate GLS profiles and concentrations (Soler *et al*, 2012, Kuśnierczyk *et al*, 2007). Phloem-feeders affect the salicylic acid (SA) pathway, whilst leaf-chewers are associated with induction of the jasmonic acid (JA) pathway (De Vos *et al*, 2005, Ludwig-Müller *et al*, 1997, Vogel *et al*, 2007, Walling, 2000). These pathways are highly complex and overlapping, having both synergistic and antagonistic effects on each other (Koornneef and Pieterse, 2008). Indole GLS levels increase sharply in response to exogenous JA application, a common experimental technique used to imitate insect herbivory (Fritz *et al*, 2010), which supports the many reports of leaf-chewing insects eliciting increased indole GLS levels (Mewis *et al*, 2005). The leaf-chewing insect *Pieris brassicae*, for example, has been found to cause significantly lower concentrations of total GLS, sinigrin, glucoiberin and glucobrassicin, but increased indole GLS (Velasco *et al*, 2007, Sotelo *et al*, 2014). Another specialist letpidopteran species *P. rapae* also induces higher indole GLS levels in white cabbage (*Brassica oleracea* var. *alba* L.) (Mewis *et al*, 2006, Poelman *et al*, 2008). Root-feeders have been found to elicit a similar response, with the turnip root fly (*Delia*

floralis Fall) being reported to cause up to a 17-fold increase in the root content of the indole GLS 1-methoxy-3-indolylmethyl (neoglucobrassicin) glucosinolate (Birch *et al*, 1992).

Activation of the SA pathway by phloem-feeding aphids can lead to suppression of the JA pathway to the extent that aphid-infested plants can have JA levels 10-fold lower than undamaged plants (Soler *et al*, 2012). Furthermore, molecular studies indicate that the saliva of phloem-feeders may in fact contain compounds which actively suppress JA (De Vos *et al*, 2005, De Vos *et al*, 2007, Zhang *et al*, 2009). This can render the plant more susceptible to attack by other insects, as leaf chewers have been shown to perform better on plants previously infested by aphids (Soler *et al*, 2012).

Insects display a diverse range of adaptations to their host plants. The majority of insects are specialised to feed on a small number of plants, so are referred to as 'specialists' (Vogel *et al*, 2007). A few insect species, termed 'generalists', are able to feed on and (to some extent) tolerate the defences of a wide variety of plants (Vogel *et al*, 2007). Elevated GLS levels tend to elicit negative responses in generalist feeders, whereas the performance of specialist herbivores tends to be less affected or even improves in some cases (Giamoustaris and Mithen, 1995, Van Der Meijden, 1996, Cole, 1997). Specialist herbivores, such as the phloem-feeding cabbage aphid *Brevicoryne brassicae* and the leaf-chewing *Pieris rapae*, have evolved a variety of mechanisms which enable them to tolerate glucosinolates or even use them to their advantage. These include the detoxification of GLS; inhibition of (iso)thiocyanate formation by nitrile-specifier proteins (Ratzka *et al*, 2002, Wittstock *et al*, 2004); and the sequestration of GLS (Müller, 2009). In some cases the glucosinolate compounds can stimulate feeding or oviposition by these specialists (Ratzka *et al*, 2002). This is true for the small white cabbage butterfly (*Pieris rapae*) for which GLS act as a stimulant for both larval feeding and adult oviposition (Miles *et al*, 2005). *P. rapae* also possesses nitrile-specifier proteins that divert the breakdown of GLS from formation of toxic isothiocyanates to less toxic nitriles instead, which it is able to digest and later excrete (Wittstock *et al*, 2004). Similarly, the diamondback moth (*Plutella xylostella*) is able to digest the toxic substances using a sulphatase gut enzyme which deactivates the glucosinolate hydrolysis system, whilst also using the glucosinolates as a host recognition cue for oviposition (Sun *et al*, 2010, Kliebenstein *et al*, 2005). Some specialists, such as larvae of the turnip sawfly *Athalia rosea*, have developed the ability to bypass the myrosinase hydrolysis step and instead accumulate intact GLS in their haemolymph to use for their own defence against predators (Kliebenstein *et al*, 2005, Muller *et al*, 2001). The specialist cabbage aphids *Brevicoryne brassicae* (L.) and *Lipaphis erysimi* (Kaltenbach) not only possess the ability to safely sequester GLS, but they use it to their own advantage by producing their own myrosinase so that the two compounds act together as a chemical defence against natural enemies (Kazana *et al*, 2007).

Consequently, the accumulation of certain GLS can have contrasting effects on the performance of generalist and specialist feeders, with the latter group tending to show a positive response. This has

been exemplified by reaction of the specialist cabbage aphid (*Brevicoryne brassicae*) and the generalist green peach aphid (*Myzus persicae*), to indole GLS. The authors found that 3-methoxyindolyl caused reduced fecundity in *M. persicae*, whereas another indole GLS, 3-indolylglucosinolate, was positively associated with *B. brassicae* reproduction rates (Cole, 1997). Likewise, Kim and Jander (2007) reported that 3 days of *M. persicae* herbivory on *Arabidopsis thalania* stimulated higher production of another indole GLS, 4-methoxyindol-3-ylmethyl (4MeOH), which also had a negative impact on aphid fecundity (Kim and Jander, 2007). Specialist insects do not always respond positively to GLS, however (Mewis *et al*, 2005). Increased concentrations of aliphatic GLS in *Arabidopsis thalania* plants have been found to negatively correlate with feeding by both the generalist herbivore *Spodoptera exigua* and the specialist *Pieris rapae* (Kos *et al*, 2012).

The effect of insect herbivory on GLS is not restricted to the site of damage, meaning that feeding by one insect may alter the susceptibility of the rest of the plant to attack by other insects. Below-ground herbivory, for instance, can have significant implications for foliage-feeding insects (Van Dam *et al*, 2004). Root damage by *Delia floralis* has been shown to result in the foliage having decreased indole GLS and significantly higher aliphatic GLS contents (Birch *et al*, 1992). Another root herbivore, *Delia radicum*, also induced higher foliar concentrations of the aliphatic GLS sinigrin in black mustard (*Brassica nigra*) (Soler *et al*, 2005). This may account for the reduced performance of above-ground foliar herbivores *Pieris brassicae*, its parasitoid (*Cotesia glomerata*), and hyperparasitoid (*Lysibia nana*) which was observed after the root damage was inflicted (Soler *et al*, 2005). Similarly, aboveground herbivory can affect root herbivores through altered GLS accumulation. For example the specialist aphid *Brevicoryne brassicae* has been show to increase total GLS in the bulb of *Brassica rapa* (Sotelo *et al*, 2014).

1.9.2 Aphids

Aphids are a common agricultural and horticultural pest worldwide, comprising approximately 4000 species within the Aphidoidea superfamily of the order Hemiptera (Guerrieri and Digilio, 2008, Dixon *et al*, 1987). They are phloem-feeding insects, possessing a highly modified mouthpart called the stylet which they insert into the sieve tubes of their host plant to extract the sugar-rich, nitrogen-poor phloem sap (Behmer, 2009, Guerrieri and Digilio, 2008). The high pressure within the sieve elements allows most aphids to feed passively on the phloem, rather than actively sucking the sap, despite these pests often being referred to as "sucking" insects (Guerrieri and Digilio, 2008). Aphids produce two different types of saliva which are released at different stages of feeding (Miles, 1999). Upon initial searching for phloem sap, aphids release a dense, protein-rich 'gel' saliva which aids in the penetration of the plant epidermis and cortical layer by forming a protective sheath around the stylet, and may serve a secondary role in reducing plant defences by preventing the plant's natural response of sealing the sieve plates where they have

been punctured by the stylet (Will and Vilcinskas, 2015, Will and van Bel, 2006). Once the stylet has successfully reached the phloem, the aphid produces a second enzyme-containing 'watery' saliva which is injected into the plant (Guerrieri and Digilio, 2008). Proteins contained within this watery saliva interact with calcium in plant tissues, thereby preventing the sealing of the wound site which would be the plant's normal defence response (Guerrieri and Digilio, 2008).

Aphids can inflict damage to crops by various means. The removal of phloem sap may lead to water stress and wilting, which reduce the yield and marketability of crops (Blackman and Eastop, 2000). The saliva injected by aphids into the plant during feeding can also be phytotoxic, causing leaves to thicken and curl (Dedryver *et al*, 2010). However, the predominant cause of aphid-induced crop loss is through their transmission of approximately 275 plant diseases. Aphids are the most common vectors of plant disease, accounting for the transmission of almost 50% of all insect-borne plant viruses (Nault, 1997, Ng and Perry, 2004). Chemical control methods targeting aphids have had limited success. Increases in the frequency of aphid outbreaks have been attributed by some to the detrimental effects of broad-spectrum insecticides on natural enemy populations and the wider environment, in addition to the development of insecticide resistance among aphids (Hasken and Poehling, 1995, Blackman *et al*, 1996). Consequently, the focus is shifting to integrated and biological control strategies as an alternative to chemical approaches (Van Emden *et al*, 1969).

1.9.3 Aphid life history

Aphids exhibit a variety of life cycles and morphs which may vary within a single species. Adult aphids can be either apterous (wingless), or alate (winged). When a plant becomes crowded with aphids, alate morphs develop which can then disperse to colonise other plants. Aphids with holocyclic life cycles exhibit both asexual and sexual modes of reproduction (**Figure 4**) (Zhang *et al*, 2001). The asexual process, known as parthenogenesis, occurs during the summer whereby viviparous females give birth to live young known as fundatrigenia. These offspring are all female and identical clones of the mother. In autumn, abiotic cues (photoperiod and temperature) trigger a switch from parthenogenetic to oviparous (sexual) reproduction. Apterous females (oviparae) migrate to their primary host plant and mate with alate males, producing diapausing eggs which overwinter and hatch in spring (Moran, 1992, Cocu *et al*, 2005). Some species are capable of anholocycly, whereby they reproduce by parthenogenesis all year round and parthenogenetic females hibernate over winter on weeds or winter crops such as oilseed rape (*Brassica napus*) (Blackman, 1974, Margaritopoulos *et al*, 2002, Cocu *et al*, 2005). Anholocycly is more common in areas with warmer climates, or areas where the host plant is absent (Cocu *et al*, 2005). In temperate regions *M. persicae* has been found to use both reproductive strategies, usually determined by the severity of the previous winter and the availability of the primary host (Blackman, 1974, Margaritopoulos *et al*, 2002).

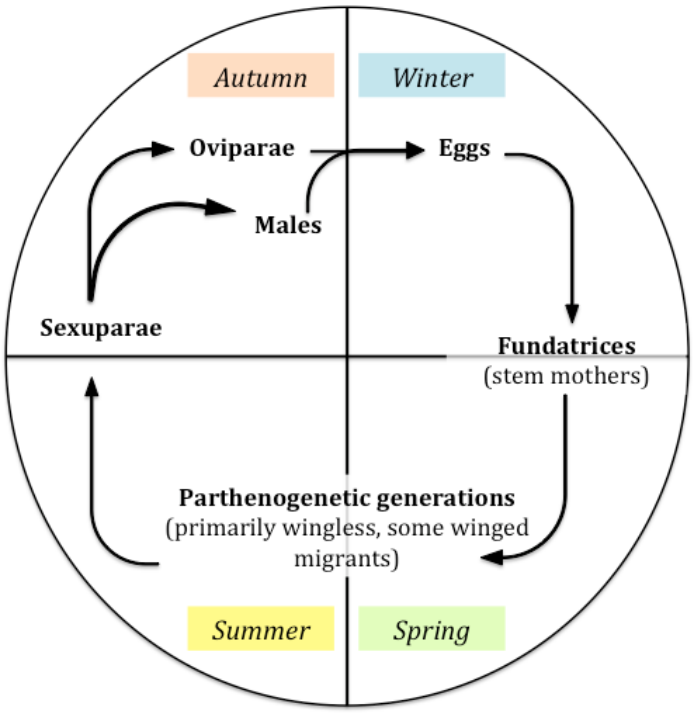


Figure 4 The simplified annual life cycle of a holocyclic aphid population, modified from Williams and Dixon (2007).

1.9.4 *Myzus persicae*

The green peach (or peach-potato) aphid, *Myzus persicae* Sulzer (1776) (Hemiptera: Aphididae) (**Figure 5**), is a common crop pest with a worldwide distribution (Blackman, 1974). The primary host plant for *M. persicae* is the peach tree, *Prunus persica* L. (Rosaceae) (Moran, 1992). *M. persicae* is a generalist (polyphagous) herbivore, reported to feed on over 400 plant species distributed across 40 different families, which include several economically important crop plants such as sugar cane, potatoes, tobacco and brassicas (Van Emden *et al*, 1969, Costello and Altieri, 1995, Quaglia *et al*, 1993). *M. persicae* is a vector of over 100 plant diseases, including virus yellows disease of sugar beet, potato leaf roll virus, and tomato aspermy virus (Qi *et al*, 2004, van den Heuvel *et al*, 1994, Chen and Francki, 1990). Foliar feeding by *M. persicae* can induce increased production of oxylipins by (Arabidopsis) plant roots (Nalam *et al*, 2012). Oxylipins are a class of signalling molecule produced by plants in response to tissue damage-induced stresses such as wounding and pathogen infection (Blée, 2002). However, as this defence mechanism can having counter-productive effects, as Nalam et al (2012) showed that these fatty acid compounds are positively correlated with infestation by *M. persicae*, with the aphids actually promoting oxylipin production and its translocation from the roots to the plant shoot.

Figure 5 An apterous (wingless) *Myzus persicae* adult.

1.10 Thesis Aims

This thesis investigates interactions between rhizosphere soil microbial communities, host plants, and *Myzus persicae* performance, with a view to enhance yield and chemical defences in *Brassica oleracea*. In order to achieve this, a mixture of deep-sequencing, molecular, chemical ecology and manipulation of the soil microbial community were used.

Chapter Two reports the aboveground component of a wider tri-trophic investigation into the responses of soil microbial communities, *Brassica oleracea* and the generalist aphid *Myzus* to different fertiliser regimes. The aboveground aspects of this system are well studied in the literature, and the purpose of this study was to confirm that what has already been reported to occur in the field was true under controlled environmental conditions in order to enable the identification of potential links to changes in the belowground (rhizosphere) community which correspond to plant-insect dynamics. This entailed a pot experiment under controlled environmental conditions. *B. oleracea* plants were grown in soil collected from a field site to which either organic (chicken manure pellets) or chemical fertilisers were added at two different nitrogen levels. The performance of the plants and aphids under each treatment was assessed using a variety of parameters (rate of intrinsic increase and mean relative growth rate for aphids, and biomass, leaf area, chlorophyll content, total foliar N and S for plants). The hypotheses were:

> **Hypothesis 1:** Cabbages treated with synthetic fertilisers would have higher plant performance metrics (i.e. biomass, leaf area and chlorophyll content) than organically fertilised plants.
>
> **Hypothesis 2:** Plants receiving higher N inputs would have higher foliar N concentrations, and would experience higher rates of aphid infestation than plants receiving lower N inputs.
>
> **Hypothesis 3:** The aphids would exhibit faster and more abundant growth on synthetically fertilised plants, with a higher performance on synthetically fertilised plants in comparison to organic plants.

Chapter Three reports on the belowground (soil) investigation, which was undertaken as part of the same experiment as described in Chapter Two. It employs high-throughput sequencing of the 16S ribosomal RNA (rRNA) gene to characterise the bacterial communities of the bulk soil and rhizosphere of *B. oleracea*. The effects of different fertiliser treatments, plant development and aphid herbivory on the soil microbiome are also investigated, and key differences between them, in terms of composition and diversity, are explored. The following hypotheses were addressed:

> **Hypothesis 1:** Organic and synthetic fertilisers will have differential effects on the soil microbial community, with the organic treatment promoting bacterial diversity; whereas higher amounts of N supplied to the soil will alter the soil microbial community and reduce diversity.
>
> **Hypothesis 2:** The rhizosphere community will differ in structure and abundance in comparison to the bulk soil, and will alter with plant age.
>
> **Hypothesis 3:** Herbivory by the aphid *Myzus persicae* will impact the rhizospheric microbial community, possibly due to changes in the chemistry of the plant and altered root exudation rates (not measured).

Chapter Four takes a more controlled approach, using the sulphur-oxidising bacteria (SOB) *Thiobacillus thioparus* as a potential PGPR candidate to enhance the glucosinolate production in *B. oleracea*. This entailed another greenhouse experiment, with a variety of inoculation approaches. Several hypotheses were tested:

> **Hypothesis 1:** *T. thioparus* inoculation will be more successful in sterilised soil than in untreated soils owing to the reduction in competition from resident microbes.
>
> **Hypothesis 2:** Enhancing the sulphur oxidising bacterial population will enhance the production of glucosinolates in *B. oleracea* given the characteristic sulphur component of these metabolites.
>
> **Hypothesis 3:** *Myzus persicae* populations will be reduced on plants with SOB enriched soils, owing to the enhanced glucosinolates in these plants, as predicted in the previous hypothesis.

This experiment was followed up with a short investigation of the effects varying sulphur and nitrogen inputs on cabbage-aphid dynamics. The *T. thioparus* inoculation was also used in combination with N fertilisers. It was hypothesised that adding N would impact S uptake by the plant, which consequently may affect aphid herbivory owing to the changes in GLS production.

Chapter 2: Aboveground biotic effects of fertilisers

2.1 Introduction

2.1.1 Brassica responses to fertilisers

Brassicas (family Brassicaceae) are a group of plants comprising many economically important vegetable crops, such as kale, oilseed rape, broccoli and cabbage. They contain compounds called glucosinolates (GLS) which are normally compartmentalised in a non-toxic form. However, upon tissue damage, caused by insect herbivory for instance, they undergo hydrolysis which produces various toxic products, including isothiocyanates (Sarwar *et al*, 1998). These compounds possess anti-carcinogenic and antimicrobial properties, but they are primarily known for their role in herbivory defence (Sarwar *et al*, 1998, Verhoeven *et al*, 1997, Textor and Gershenzon, 2009).

There are contrasting reports regarding the effect of organic and synthetic fertilisers on glucosinolates in plants. Whilst foliar N concentrations (Aqueel *et al*, 2015) and plant biomass (Aber *et al*, 1993, Lemus *et al*, 2008) tend to be enhanced by the application of N, glucosinolate concentrations have been found to decline with increasing N input (Chen *et al*, 2004, Chun *et al*, 2015 (In Press)), although this is not always the case (Staley *et al*, 2010). Brassicas grown in organic fertilisers have been shown to have up to three times higher levels of the glucosinolates in comparison to those treated with synthetic fertilisers (Hsu *et al*, 2009, Staley *et al*, 2010). Additionally, organic fertilisers are associated with lower foliar N concentrations than synthetic fertilisers (Phelan *et al*, 1995, Staley *et al*, 2010). This combination of elevated glucosinolate concentration with reduced foliar N content have been shown to culminate in lower abundances of the generalist aphid *M. persicae* on cabbage (*Brassica oleracea*) relative to synthetically-fertilised plants, although the trend was reversed for the specialist aphid *Brevicoryne brassicae* which has evolved a tolerance to the cabbage-derived defence compounds (Staley *et al*, 2010).

2.1.2 Insect responses to fertilisers

Nitrogen (N) is a growth-limiting macronutrient for most phytophagous insects (Douglas, 2006), and consequently many insects respond positively to increasing N availability (Meyer and Root, 1996, Throop and Lerdau, 2004). Elevated plant N levels are generally associated with improved insect herbivore performance as indicated by insect fecundity, growth rate, development times and survival (Mattson, 1980, White, 1984). Indeed, aphids have been shown to exhibit increased adult body weight, longevity and population growth in response to N additions (Zehnder and Hunter, 2008, Hosseini *et al*, 2015, Aqueel and Leather, 2011, Hosseini *et al*, 2010, Cisneros and Godfrey, 2001, Nevo and Coll, 2001). However, the response to increased N is not uniform across insects.

Müller *et al* (2005), for example, reported no effect of NPK fertiliser additions on the colony size of the aphid *Aphis jacobaeae*, although this may be largely owing to the fact that the fertiliser treatment also had little impact on the plant quality (e.g. plant height, biomass, number of flowers). Additionally, increases in N levels may alter the balance of other nutrients and the production of plant defence compounds, which may in turn influence insect abundance (Tao and Hunter, 2012). Overall, it seems that for most herbivorous insects there is an optimal foliar N concentration, above which further N additions may result in reduced insect performance (Mattson, 1980, White, 1984).

Organic fertilisers, such as animal manures, have been shown to reduce the incidence of crop pests in some instances (Staley *et al*, 2010, Culliney and Pimentel, 1986). This may be attributed to the slower release of N from organic fertilisers in comparison to synthetic fertilisers, thereby rendering the plants less appealing to insects due to their lower N availability. However, field trials have produced inconsistent results, with insect pest populations showing negative, neutral and positive responses to organic fertilisers (Bengtsson *et al*, 2005, Garratt *et al*, 2011).

Aims

This chapter reports the findings from the aboveground component of a mesocosm study which investigated linkages between aboveground (plant and insect) and belowground (soil microbial communities within the rhizosphere) biotic interactions under different fertiliser regimes. This study aimed to compare the effects of fertiliser quality and quantity on the performance of the host plant *Brassica oleracea* and the generalist herbivore *Myzus persicae*. This was achieved through pot experiments performed under controlled environmental conditions. The synthetic and organic fertilisers (NPK and chicken manure) were administered to the plants at equivalent concentrations of total N to enable the comparison of different fertiliser types. The synthetic fertiliser was also applied at a higher N dosage to investigate the effect of increasing N availability on these performance parameters. A series of hypotheses were tested:

- *(i) The addition of fertilisers would alter plant growth and chemistry;*
- *(ii) The two types of fertiliser applied - organic or synthetic - would have differential effects on the cabbage;*
- *(iii) Increasing the amount of N applied to the plant would enhance plant performance metrics (biomass, leaf area, chlorophyll content, and foliar N levels);*
- *(iv) M. persicae would perform better on synthetically fertilised plants than organically fertilised plants;*
- *(v) M. persicae population growth rates would be positively correlated with foliar N content and therefore would be greater on plants receiving a higher N dose.*

The methods and results of the belowground component to this study are reported in **Chapter Three**.

2.2 Materials and Methods

2.2.1 Soil collection

Soil samples were collected from an organic agricultural field in Ipsden, South Oxfordshire (51°32'59.559" N, 1°05'8.43" W) which is located at an altitude of 105m above sea level (**Figure 6**). The field was on a rotation of 2-year wheat followed by 1-year oil seed rape. This location was chosen to enhance the agricultural relevance of the study, and because the soil was well characterised in a previous study as part of the EU Seventh Framework funded SOILSERVICE project (de Vries *et al*, 2012a) (**Table 1**). The soil in this field has previously been characterised as a Brown Calcareous Earth (Cambisol) soil type, with a calcareous loam texture. The composition of the soil was reported as 15% sand, 56% silt and 29% clay. Soil pH was measured using a subsample of air-dried (10g, <2mm) soil and 50ml deionised water. This solution was shaken for 1 minute and left to settle for 30 minutes before repeating this procedure once more. The pH was determined using a calibrated pH meter (Jenway Model 3505) which was held in the solution until a stable reading was obtained. This process was repeated twice more, and an average of the three readings was taken (**Table 1**).

Figure 6 The location of the sampling sites for the SOILSERVICE study, indicated on the map by green dots, and the red dot being the field site for soil sampling in this study.

In March 2013, soil samples were collected from the top 15cm of the soil horizon in a zig-zag pattern. Upon arrival at the University of Southampton, all soil samples were stored in plastic bags in the dark at 4°C before usage. The soil was homogenised, air-dried and sieved (2mm) to remove stones and plant debris. Dry matter content was determined by calculating the loss of weight after oven-drying weighed fresh soil samples (2mm) at 105°C for 24 hours, and re-weighing to then complete the formula:

$$Dry\ matter\ (DM\%) = \frac{soil\ dry\ weight\ (g)}{soil\ fresh\ weight\ (g)} \times 100\%.$$

The water holding capacity (WHC) of the soil was determined volumetrically using the formula:

$$WHC\ (ml\ 100g^{-1} oven\ dried\ soil) = \frac{2A+MC\%}{soil\ DM\%} \times 100\%,$$

where *A* is the average volume of water retained by 50g of fresh soil and *MC* is the moisture content (determined from the dry matter content). The soil was later re-wetted to achieve 60% WHC. Nutrient analysis of the soil samples was performed by NRM Ltd. Laboratories, Berkshire (**Table 1**). Total carbon (C) and nitrogen (N) content of the soil was measured by Dumas combustion which involves total combustion of the samples in an oxygen-enriched atmosphere, passing the resultant gases through an oxidation catalyst and separating the carbon and nitrogen products through a chromatographic column. Finally, the sieved, homogenized soil was potted up into 0.4 litre pots (4" diameter).

Table 1 Soil properties of the intensive wheat field and adjacent grassland (†data obtained from Simon Mortimer, private correspondence).

Soil measurement	Intensive wheat field†	Adjacent extensive grassland field†	Soil for this study
pH	8.1	7.4	8.12
Loss on Ignition (%)	6.5	9.6	n/a
Total C (% w/w)	9.65	11.5	10.8
Total N (% w/w)	0.27	0.455	0.34
P (mg/l)	28	14	n/a
K (mg/l)	170	260	n/a
Mg (mg/l)	170	190	n/a
S (mg/l)	30	31	n/a

2.2.2 Fertiliser treatments

A literature review was conducted to gauge the range of N-application rates typically used in similar studies. The total N field application rates used in studies ranged from 50 to 500 kg ha^{-1}, whilst the maximum rate for cabbage recommended by the Department for Food and Rural Affairs (Defra) ranged from 100 to 325 kg ha^{-1} (Chakwizira *et al*, 2015, Defra, 2007 , Defra, 2010). The two rates of N application used in this experiment were 0.16g and 0.32g N $litre^{-1}$ soil, which equate to approximately 68 and 136 kg ha^{-1} respectively. They are hereafter referred to as Low N (**LN**) and High N (**HN**) for the synthetic fertiliser treatments. These were comparable to the rates used in another study using *B. oleracea* L. var. *capitata* by Staley *et al* (2011). The chemical composition of the fertilisers was analysed by NRM Laboratories Ltd. The synthetic fertiliser (Chempak ® Formula No. 3 – Fully Balanced Feed (NPK 20-20-20)) contained 20.5% total N (w/w: 12.2% ureic N, 3.75% ammoniacal N, 4.56% nitric N), 20.6% water soluble P (as P_2O_5) and 21.2% K (as K_2O)). To achieve the specified Low N and High N dosages, Chempak was administered at 0.31g and 0.62g pot^{-1} respectively. The organic fertiliser used was pelleted chicken manure (New Horizon Organic Poultry Manure Pellets), which contained 3.91% total N; 2.93% total P (as P_2O_5);

and 2.47% total K (as K_2O). Due to the low N content of the chicken manure (referred to hereafter as **CM**), and the unfeasibility of adding much larger quantities, it was applied at the Low N rate only, which equated to 1.64g pot^{-1}. The fertiliser treatments were applied in aqueous solution (50ml pot^{-1}), with the chicken manure pellets firstly being ground to a powder using a pestle and mortar. An equal volume of tap water was added to control pots. The pots were kept in trays (6 pots per tray) to prevent any fertiliser-containing leachate reaching non-target pots. This experiment was conducted under controlled environmental conditions (16:8 hour light:dark, 20°C, and 70% relative humidity) at the University of Southampton (**Figure 7**). The trays were rotated weekly to account for variations in airflow which may influence soil moisture content.

Figure 7 *B. oleracea* in the controlled environment plant growth room at the University of Southampton.

2.2.3 Plant cultivation

Brassica oleracea L. var. *capitata* cultivar Derby Day seeds (Moles Seeds, UK, Ltd.) were sown after fertiliser application, with 5 seeds pot^{-1}. These pots (18 per fertiliser treatment, total of 72 pots) were used only to germinate the plants and were thereafter discarded (i.e. no environmental DNA was extracted from these pots). The most vigorous of the successfully germinated individuals were subsequently transplanted into the experimental pots (one plant per pot) containing the corresponding fertiliser treatments (30 plants per treatment, total n=120 plants). This was done to allow for variances in germination success, and to ensure that any plant-specific effects on the soil microbiome were not confounded by differing numbers of germinated seeds in each pot. Plants were watered with tap water as necessary. After 9 weeks of growth, 10 cabbages per treatment were destructively harvested to obtain rhizosphere soil samples and plant biomass measurements (stage (iii) **Figure 8**). This left 20 plants per fertiliser treatment for the final stage (iv) of the experiment, half of which were infested with *M. persicae* for the final 14 days prior to harvesting at 12 weeks (with (+) and without (-) aphids plants, **Figure 8**). The same fertiliser treatments were applied to an additional set of 120 plants, referred to as batch 2, using the same methods. These batch 2 plants were harvested after 12 weeks, and were used to obtain more insect and plant performance data only (all soil samples taken exclusively from batch 1).

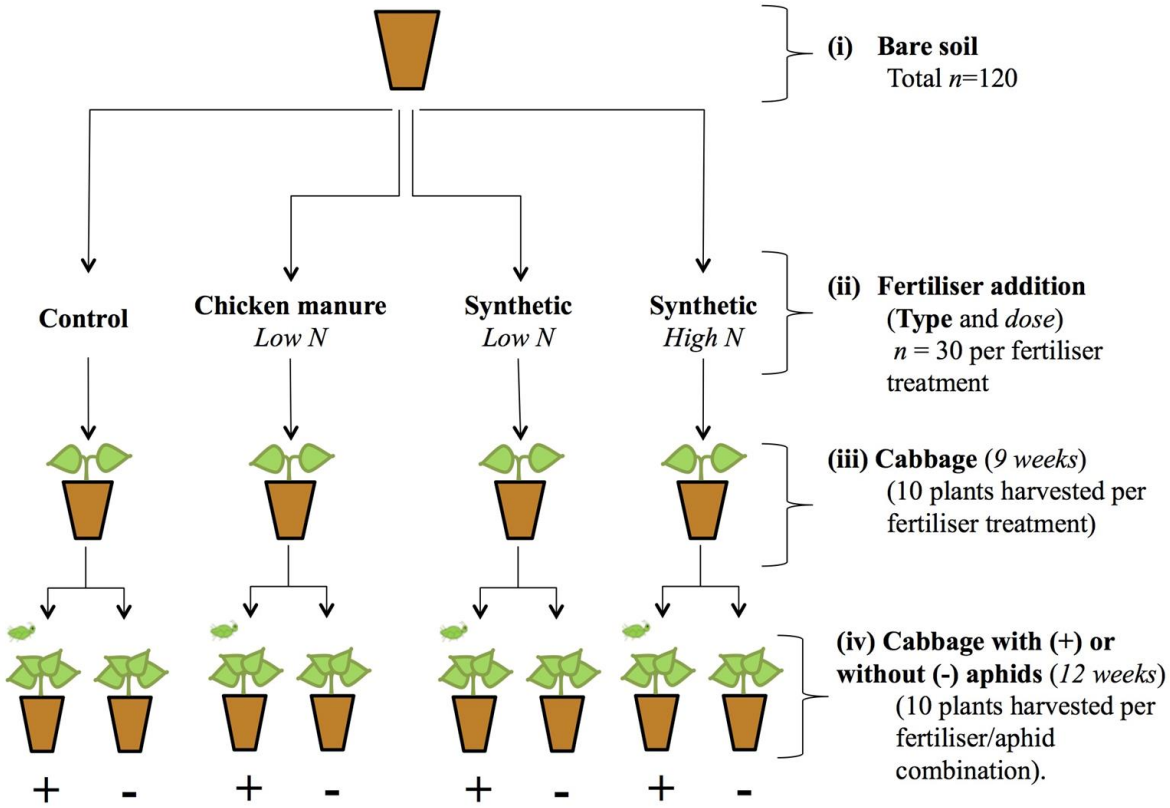


Figure 8 Schematic of the experimental set-up for the investigation of aboveground and belowground dynamics in response to different fertiliser treatments (batch 1), indicating the stages at which data was collected for soil microbial communities (i, ii, iii and iv), plants (iii and iv) and aphids (iv). The sample numbers refer to the total number of pots/plants per treatment at each sampling stage (as opposed to the number of DNA samples taken). At stages (iii) and (iv) plants were destructively sampled, and therefore these pots were removed from the experiment. During the experiment there were 3 plant deaths in the High N treatment group (batch 1) and 2 plant deaths in the Chicken Manure group (batch 2).

N.B. The fertiliser treatments are hereafter denoted as **CM** for chicken manure; **LN** for low N synthetic; **HN** for high N synthetic and **Con** for control.

2.2.4 Plant performance

A variety of plant growth parameters were measured throughout the experiment. Chlorophyll content was measured using a hand-held Opti-Sciences CCM-200 Chlorophyll Content meter. The device estimates the chlorophyll content of the tissue by measuring absorbance and gives readings in Chlorophyll Concentration Index (CCI) units which are proportional to the amount of chlorophyll in the sample. Chlorophyll measurements were taken in triplicate from each plant after, from which the mean was taken. The results report the average chlorophyll content of 9-week old

plants (*n*=30 per treatment, except HN *n*=27 owing to 3 plant deaths). Chlorophyll measurements were not taken thereafter, due to the time-consuming nature of the aphid measurements.

Total leaf area was calculated for harvested plants using ImageJ software to analyse photographs of the leaves flattened against graph paper for scale. The average leaf area was then calculated for each plant. This was done for all plants at the 9-week harvest time-point (*n*=30, except HN *n*=27), and a subset of aphid-infested plants at the 12-week harvest (*n*=10). After harvesting, the aboveground plant biomass was oven-dried at 70°C for 72 hours to attain a constant mass and weighed to obtain the dry weight aboveground biomass (g) for a subset of the aphid-infested 12 week-old plants only (*n*=5). The fresh biomass is reported for all plants harvested at 9 (*n*=10, except HN *n*=7) and a subset of those harvested at 12 weeks (without aphids *n*=3; with aphids *n*=5).

Three non-infested, 12-week old plants from each treatment were analysed for total foliar N and C content by NRM Laboratories Ltd. This was accomplished via the Dumas method, which involved total combustion of the dried and ground (<0.5mm) plant samples in an oxygen enriched atmosphere, the products of which were then passed through a thermal conductivity detector. The electronic signal produced by the detector signifies the amount of N and C present. These plants were also used for the fresh biomass measurements for 12-week old, uninfested plants.

2.2.5 Aphid culture and inoculation

The *M. persicae* colony was reared on Chinese cabbage *Brassica rapa* L. spp. *Pekinensis* (Lour) Cv. Wong Bok (Kings Seeds, Surrey, UK) in Perspex cages (70 x 69 x 45 cm) under controlled environment conditions (20±3°C, 16:8hour light:dark). Five apterous adult aphids were added to each of the cabbages in the aphid-infested treatment group using a paintbrush. After 24 hours, neonate nymphs (<24 hours old) were collected and placed within a clip-cage (**Figure 9**) on each plant to be used for mean relative growth rate (MRGR) and fecundity measurements. For each fertiliser treatment group, all plants were placed inside two large ventilated Perspex cages, keeping infested and non-infested plants separate to prevent the cross-contamination the aphids to control (aphid-free) plants (i.e. 10 plants per cage). Clip cages were also attached to non-infested plants to account for any cage-related effects on the plants, as they have been previously shown to reduce leaf growth (Moore *et al*, 2003). The clip cages were constructed from two pieces of acrylic sheet (6 x 3.5cm) with a central hole (2.5cm diameter) covered with fine mesh/muslin to allow for ventilation. The two pieces were placed either side of a leaf, and secured using an aluminium bulldog clip as shown in **Figure 9**. The cages were checked at a minimum of 3-day intervals over the two-week infestation period.

Figure 9 Aphid-infested cabbages in a Perspex cage (left), and *Myzus persicae* adults and nymphs in a clip cage (centre and right). (Stage (iv) of the experiment, as depicted in **Figure 8**).

Unfortunately, the experiment suffered from an infestation of a leaf-chewing insect. Two *Plutella xylostella* larvae were accidentally introduced to the Low N cage in the repeat (batch 2) experiment, and two of the plants exhibited some damage. These plants were used only for plant and aphid performance assays, and were not used for the soil microbial analysis; therefore this does not affect the results reported in Chapter 3. It was later established that they had entered via the watering can, which ceased to be used to water the plants thereafter.

2.2.6 Aphid performance

The mean relative growth rate (MRGR) of *M. persicae* was assessed on ten plants from each treatment group. Three neonate nymphs (<24 hours old) were placed within a clip cage on each plant and their weight was monitored for two weeks at a minimum of 3-day intervals as a measure of their growth rate. Owing to the low weight of neonate *M. persicae*, the first weighing required all three to be weighed together, and an average taken. The MRGR was calculated as described by Leather and Dixon (1984), using the following formula:

$$\text{MRGR}\ (\text{mg}, \text{mg}^{-1}, \text{day}^{-1}) = \frac{\ln \text{W}_2(\text{mg}) - \ln \text{W}_1(\text{mg})}{\text{t}_2 - \text{t}_1}$$

where W_1 and W_2 are the birth and adult weights respectively, and (t_2-t_1) is the time taken to develop from birth to maturity (i.e. the developmental time). To determine the intrinsic rate of natural increase (r_m), a single nymph (<24 hours) was placed in a clip cage and its reproductive output monitored over the two-week infestation period. Cages were checked daily for the appearance of new nymphs, which were counted and removed from the cage. The data can be used to calculate the r_m using the following formula (Wyatt and White, 1977):

$$r_m = \frac{0.74\ (\ln F_D)}{D}$$

where F_D is the number of nymphs produced over a period of time equal to that of the pre-reproductive period (D) in days, and 0.74 is the constant defined for aphids and mites. The time to

onset of reproduction ranged from 8 days in the LN and HN plants, to more than 13 days for Control and CM plants. However, as the infestation period was limited to 2 weeks for this experiment, the r_m could not be obtained for all plants and so the overall reproductive output (i.e. final aphid population count on the whole plant, termed the 'instantaneous rate of increase' (r_i)) was used as a measure of fecundity instead.

At the end of the two-week infestation period, the final aphid population was counted on a subset of 5 plants per treatment by picking individuals off the plant using a fine paintbrush. This count was used to calculate the instantaneous rate of increase (r_i), which represents the growth of a population over a specified time, using the following formula:

$$r_i = \frac{log_e(N_t) - log_e(N_0)}{t}$$

where N_t is the final number of aphids, N_0 is the initial number of aphids used for the infestation and t is the intervening period in days (Hall, 1964).

2.2.7 Statistical analysis

Differences in aphid performance (MRGR and instantaneous rate of increase (r_i)) and plant growth (chlorophyll content, biomass (fresh and dry weight), average leaf area, foliar N and S concentrations) between treatments were compared using one-way analysis of variance (ANOVA). Shapiro-Wilks and Bartlett tests were used to test for normality and equal variance respectively. In instances where the variance was not equally distributed a Welch-corrected one-way analysis (not assuming equal variances) and Dunn's tests (with Benjamini-Hochberg correction) were used. Chlorophyll data was ln-transformed to achieve a normal distribution. A two-way ANOVA was used to test for an interactive effect of aphid herbivory and fertiliser treatment on cabbage fresh weight biomass. In cases where a significant treatment effect was detected (p <0.05), Tukey's honest significant difference (HSD) post-hoc tests were conducted to determine which treatment means differed significantly. All analyses were conducted using R v. 3.3.0.

2.3 Results

2.3.1 Plant performance

2.3.1.1 Plant biomass

Fertiliser treatments resulted in significantly different fresh biomass of plants harvested at 9 weeks (+Cabbage time-point) (one-way test (not assuming equal variances) $F_{3, 33} = 4.2261$, $p = 0.0227$) (**Figure 10**). Synthetically fertilised 9 week-old plants (LN and HN) had a significantly greater aboveground biomass than controls (Dunn's test Con-HN p=0.0450 and Con-LN p=0.0293). A two-way ANOVA also revealed a significant fertiliser effect on the fresh weight of 12-week old cabbages ($F_{3, 24}$ =31.274, p <0.001), but no effect of aphid herbivory was detected ($F_{1, 24}$ =1.986, p = 0.172). No significant interaction was detected between aphid herbivory and fertiliser treatment on plant biomass (two-way ANOVA $F_{3, 24}$ =1.702, p = 0.193). Post-hoc Tukey HSD tests showed that the control plants had a significantly lower fresh weight biomass than those treated with chicken manure (p= 0.0098), Low N (p<0.0001) and High N (p<0.0001). High N plants had a greater biomass than chicken manure (p<0.0001) and Low N (p=0.0147) plants. There was no significant difference in the fresh biomass of Low N and chicken manure plants (p=0.0761). However, when comparing the cabbage dry weight in 12 week-old aphid-infested plants there was no evidence of a treatment effect (one-way ANOVA $F_{3, 16} = 2.612$, $p = 0.0872$).

2.3.1.2 Leaf area

The average leaf areas of 9 week-old plants varied significantly between the fertiliser treatments (Welch-corrected one-way test $F_{3, 113}$ =16.437, p<0.0001). Post-hoc analysis revealed that synthetically fertilised plants (LN and HN) had significantly larger leaves than control and organically fertilised (CM) plants (Dunn's test p<0.005). The average leaf areas at the final harvest (12 weeks) reflected the observed trend for plant biomass, with HN plants having the largest average leaf area and Control plants the smallest. Average leaf area of 12 week-old, aphid-infested plants was significantly different between fertiliser treatments (one-way ANOVA $F_{3, 16} = 19.48$, p <0.0001). Synthetically fertilised plants (LN and HN) had significantly larger leaves than control and CM plants (Tukey's HSD: p<0.001).

2.3.1.3 Chlorophyll content

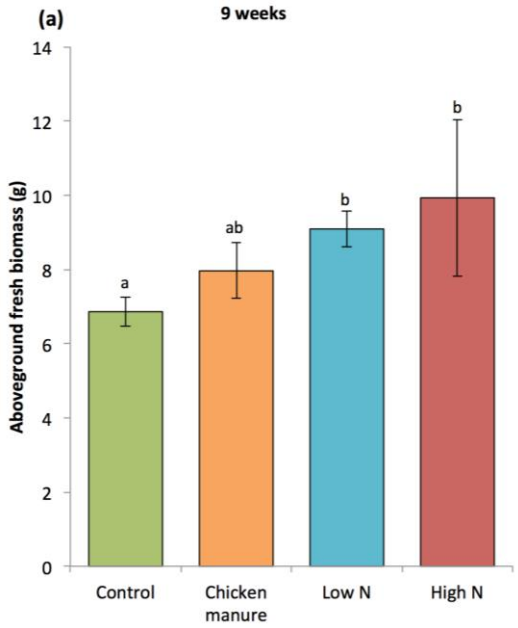
Chlorophyll content measurements were ln-transformed to obtain a normal distribution (Shapiro-Wilks p>0.05). Fertiliser additions had a significant effect on chlorophyll content of 9-week old cabbages, with the treatments following the order (highest to lowest) Low N > High N > CM > Control (Welch-corrected one-way test (not assuming equal variance): $F_{3, 113} = 6.2422$, p <0.001), with the chlorophyll content of LN plants being significantly higher than all other treatments

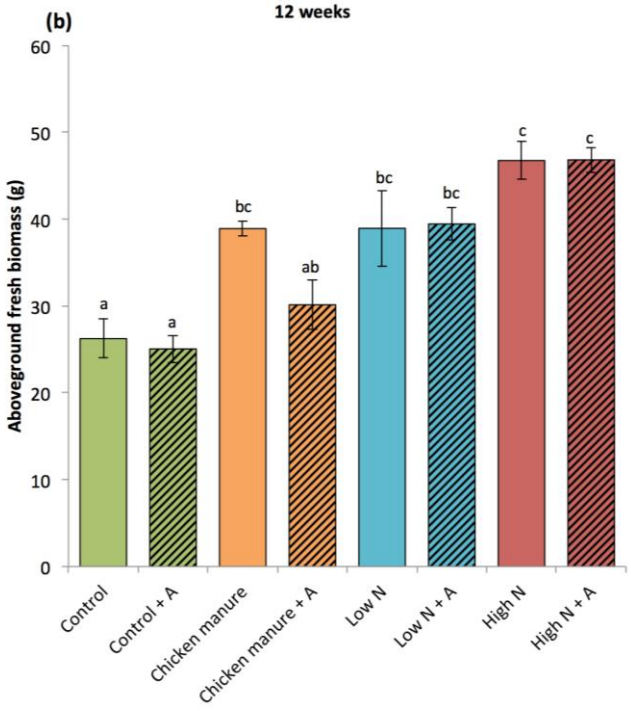
(Dunn's test: Con-LN: p = 0.0002; CM-LN: p=0.0102; HN-LN: p = 0.0490) and HN plants having a greater chlorophyll content than controls (Dunn's test Con-HN p = 0.0400). There were no significant correlations between chlorophyll content and total foliar N or S (Spearman's rank correlation p>0.05). This conflicted with reports in the literature which state that chlorophyll content is a strong predictor of total N (Heiskanen, 2005, Limantara *et al*, 2015, Liu *et al*, 2006). This may indicate that the chlorophyll meter had a poor level of accuracy, which is supported by the variation in some of the triplicated readings.

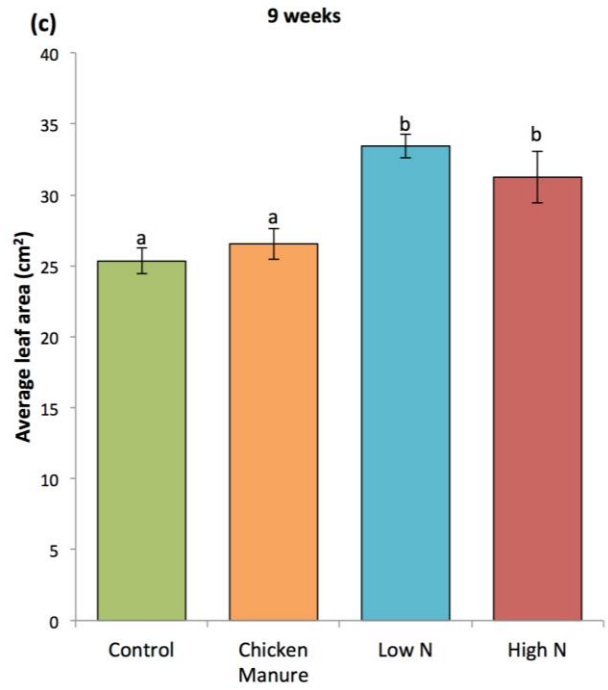
2.3.1.4 Foliar N and S concentration

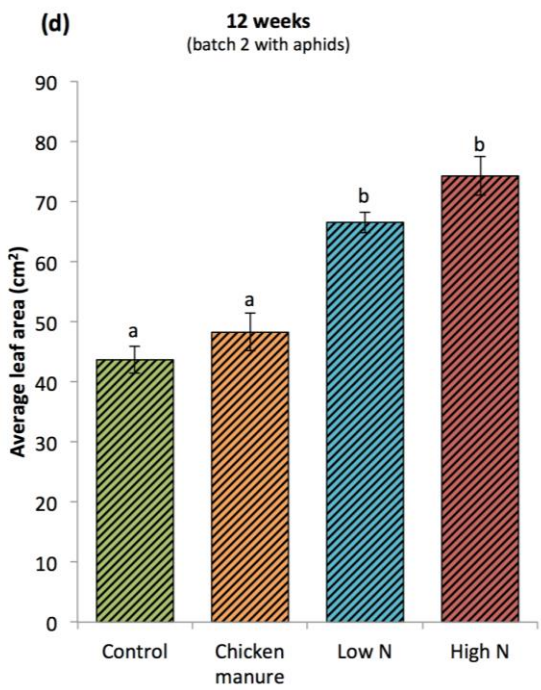
Fertiliser treatments had a significant effect on total foliar N in 12 week-old plants (**Figure 10**) (one-way ANOVA: $F_{3, 8}$ = 12.1, adjusted R^2= 0.7517, p = 0.0024). As expected, foliar N content was plants significantly higher in plants from the High N treatment than all other treatments (Tukey's HSD: p <0.05), whilst the control plants had the lowest concentration (1.4167% w/w). In comparison to controls, the average foliar N content was increased by 47.8%, 12.5% and 3.8% in HN, LN and CM plants respectively. Although slightly higher, total foliar N levels were not significantly different between the Low N synthetically fertilised plants and the organically fertilised plants (Tukey's HSD p>0.05), which suggests once again that the type, or *quality*, of fertiliser applied is less influential on plant performance than the dose, or *quantity*, since the two fertilisers were applied at the same total N rate. This lack of significance contradicts previous studies which indicated that plants treated with synthetic fertilisers have significantly higher foliar N levels than organically fertilised plants (Morales *et al*, 2001, Costello and Altieri, 1995, Staley *et al*, 2011). However, as only 3 plants per treatment group were used for the chemical analysis in this study, this absence of a significant effect may be a consequence of low sample numbers.

The sulphur (S) content of plants exhibited a reversal of this trend, with the LN and HN plants having significantly lower S content than Control plants (one-way ANOVA: $F_{3, 8}$ = 8.018, p = 0.0085; Tukey's HSD test: p <0.05 for both HN and LN comparisons with Control).


(a)
9 weeks
Aboveground fresh biomass (g)
14
12
10
8
6
4
2
0
a
ab
b
b
Control
Chicken manure
Low N
High N


(b)
12 weeks
Aboveground fresh biomass (g)
60
50
40
30
20
10
0
a
a
bc
ab
bc
bc
c
c
Control
Control + A
Chicken manure
Chicken manure + A
Low N
Low N + A
High N
High N + A


(c)
9 weeks
Average leaf area (cm²)
40
35
30
25
20
15
10
5
0
a
a
b
b
Control
Chicken Manure
Low N
High N


(d)
12 weeks
(batch 2 with aphids)
Average leaf area (cm²)
90
80
70
60
50
40
30
20
10
0
a
a
b
b
Control
Chicken manure
Low N
High N

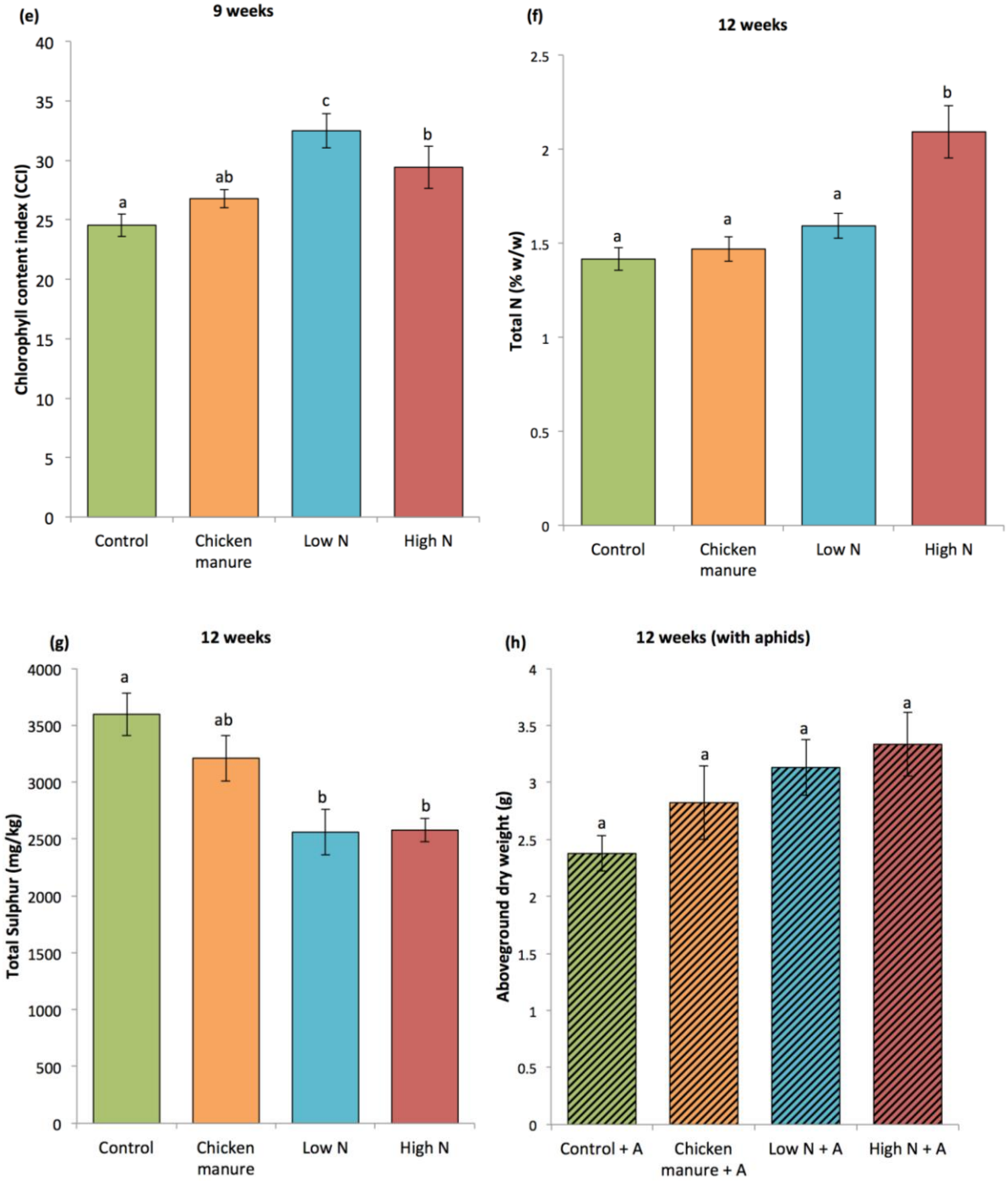


Figure 10 Comparison of the plant growth parameters under different fertiliser and aphid treatments (+A indicates plants infested with aphids) (mean ±standard error). **(a)** Aboveground fresh weight of 9 week-old plants (*n*=10, except HN *n*=7); **(b)** aboveground fresh weight of 12 week-old plants without (open bars, *n*=3) and with aphids (hatched bars, *n*=5); **(c)** average leaf area of 9 week-old plants (*n* = 30, except HN *n*=27); **(d)** average leaf area of 12 week-old plants with aphids (*n* = 10); **(e)** chlorophyll content of 9 week-old plants (*n* = 30, except HN *n*=27); **(f)** total foliar N of 12 week-old plants (*n* = 3); **(g)** total foliar S of 12 week-old plants (*n* = 3) and **(h)** aboveground dry weight of 12 week-old aphid-infested plants (*n*=5). Different letters above the bars indicate significant differences.

2.3.2 Aphid performance

As expected given the N-limited growth of these insects, the cumulative number of aphids at the end of the 14 day infestation period was highest on plants in the High N synthetic fertiliser treatment, although this difference was not significant (one-way ANOVA: adjusted $R^2 = 0.01387$, $F_{3, 16} = 1.089$, $p = 0.3821$). There was also no correlation between the final aphid population count and the aboveground plant fresh weight (Spearman's rank correlation $p = 0.6169$, S = 1172, *rho* = 0.118797) or dried weight (Spearman's rank correlation $p = 0.782$, S = 1418, *rho* = -0.06616541), or chlorophyll content (Spearman's rank correlation: $p = 0.2813$, S = 994, *rho* = 0.2526316). There was also no significant effect of fertiliser treatment on the instantaneous rate of increase (r_i) of *M. persicae* (one-way ANOVA: adjusted $R^2 = 0.0582$, $F_{3, 16} = 1.391$, $p = 0.2816$) (**Figure 11**). However, fertiliser treatment did have a significant effect on the number of offspring produced by caged aphids (Kruskal-Wallis $\chi^2 = 22.131$, df = 3, $p<0.0001$), with HN plants producing significantly more offspring than CM (Dunn's test $p = 0.0006$), Con (Dunn's test $p<0.0001$), and LN plants (Dunn's test $p = 0.0241$) (**Figure 11**).

The MRGR (which was log-transformed to achieve a normal distribution) differed significantly between treatments (one-way ANOVA: $F_{3, 36} = 6.369$, $p = 0.0014$). The MRGR of plants in both chemical fertiliser treatment groups (Low N and High N) were significantly higher than that of Control plants (Tukey HSD $p = 0.004$ for both) (**Figure 11**). An interesting observation was that CM plants generally had higher aphid infestation levels than LN plants, which had similar levels to Control plants. When comparing the number of apterous adult *M. persicae*, the only significant differences occurred between controls and CM (Tukey HSD: $p = 0.0132$) and controls and HN (Tukey HSD: $p = 0.0186$), with the unfertilised plants having significantly fewer aphids than both fertilised groups. This suggests that in terms of susceptibility to insect herbivory, the amount and, to a lesser extent, type of N applied to cabbages could be a strong determinant of the insect's performance, and there may be some advantage to using reduced inputs of synthetic fertiliser as opposed to organic inputs at equivalent N rates.

Aphids on Control and CM plants had a longer pre-reproductive period than those on synthetically fertilised (HN and LN) plants. The mean time until the onset of reproduction in caged aphids on HN and LN plants was 9.9 and 10.9 days respectively, whereas all caged aphids on control and chicken manure treated plants had a minimum pre-reproductive period of 12 days. Due to time constraints, aphid infestation periods lasted a maximum of 14 days and so the pre-reproductive period was not obtained for caged aphids which failed to produce any offspring during this period (7 chicken manure plants and 9 control plants). This concurs with previous studies which have reported a lower r_m of *M. persicae* on organically fertilised plants in comparison to those receiving synthetic fertiliser additions (Stafford *et al*, 2012). It also supports findings that development time is shortened in insects feeding on synthetically fertilised plants (Nevo and Coll, 2001).

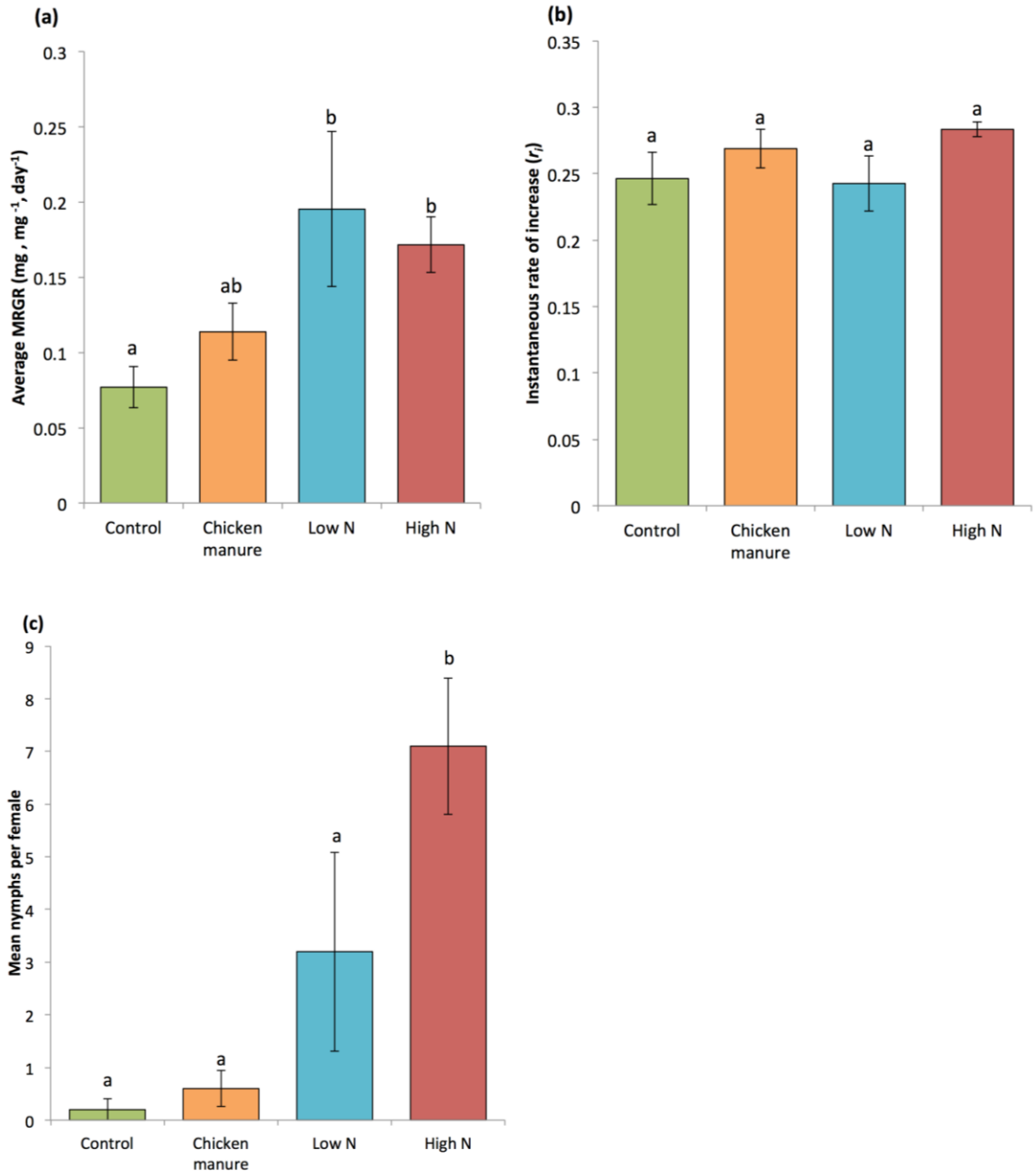


Figure 11 The effects of fertiliser treatments on *Myzus persicae* reproductive performance and growth (mean ±standard error). The graphs show **(a)** the relative growth rate (MRGR) (*n*=10); **(b)** the instantaneous rate of natural increase (*n*=5); and **(c)** fecundity (mean number of nymphs produced per plants during infestation period, *n* =10) of *Myzus persicae*.

2.4 Discussion

Hypothesis (i): The addition of fertilisers (organic or synthetic) alters plant growth and chemistry.

As predicted, supplementing *B. oleracea* with fertilisers did significantly alter plant chemistry in terms of total foliar N and S content. Plants treated with synthetic fertiliser additions had the highest foliar N concentrations, with the HN plants having an average foliar N content that was 48% higher than that of Control plants. The inverse relationship between foliar N and S content concurs with previous findings that plant S concentrations decline with increasing N additions (and vice-versa), possibly due to a growth-dilution effect (McGrath and Zhao, 1996, Janzen and Bettany, 1984, Schonhof *et al*, 2007). This apparent inhibition of S uptake in synthetically fertilised plants may have a significant bearing on the plant's defences against aphid herbivory, given that sulphur is a major component of glucosinolate compounds. This theory is supported by the findings of a field experiment by Staley *et al* (2010), in that the glucosinolate concentrations of organically fertilised plants were up to three times higher than in plants receiving mineral fertilisers. Chlorophyll measurements were not correlated with foliar N concentrations. Given the widespread support in the literature for a positive correlation between these two measures, it may be that the N input levels were insufficient to yield a marked effect on chlorophyll content.

Both synthetic and organic fertiliser additions resulted in a significant rise in cabbage fresh aboveground biomass in older, undamaged plants. This suggests that the amount of applied N exerts a stronger influence on cabbage yield than the type of fertiliser. However, in the presence of aphids, only the mineral fertiliser was associated with increased cabbage weight, whilst the chicken manure-amended plants had weights comparable to control plants. This may be interpreted to suggest that organically fertilised *B. oleracea* are more sensitive to aphid herbivory, in terms of how it affects their performance. However, there was no correlation detected between plant biomass and aphid herbivory in this study. These results are, on the whole, in agreement with the results of a *B. oleracea* field experiment by Staley *et al* (2010) and a pot experiment by Staley *et al* (2011) which used similar fertiliser treatments at comparable N rates.

Hypothesis (ii): The type of fertiliser applied - organic or synthetic - would have differential effects on the cabbage.

The application of organic and mineral fertilisers at corresponding N concentrations (CM and LN) had limited effects on plant performance, with the latter resulting in significantly higher average leaf areas of 9 and 12 week-old plants. LN and CM plants produced a similar yield (fresh weight) and foliar N concentrations. This suggests that the type of fertiliser applied has negligible impact on cabbage yield.

Hypothesis (iii): Increasing the amount of N applied to the plant will enhance plant performance metrics (e.g. biomass, leaf area and foliar N levels).

Plants amended with higher N (HN) exhibited significantly greater total foliar N content than plants receiving a lower synthetic N dose. There was no significant effect of increasing synthetic N dose on leaf area, aboveground biomass or foliar S content. It seems, therefore, that the two levels of N application used in this experiment resulted in negligible differences in plant yield, but significant contrasts in the nutritional quality of the plants (in terms of N content). HN plants had significantly lower chlorophyll levels than LN plants, which would concur with the observation made by Blake-Kalff (1998) where chlorophyll degradation occurred in plants grown on high N nutrient solutions.

Hypothesis (iv): M. persicae would perform better on synthetically fertilised plants than organically fertilised plants.

Although the final population count of *M. persicae* was not significantly different between organic and synthetic fertiliser treatments, there were indications that there could be substantial long-term consequences. Firstly, synthetically fertilised plants were associated with higher rates of aphid reproduction. This is in agreement with previous reports of *M. persicae* exhibiting inferior fecundity on organically fertilised plants in comparison to those treated with ammonium nitrate (Stafford *et al*, 2012). Secondly, the growth rate (MRGR) of aphids was significantly higher on synthetically fertilised plants. Finally, the developmental time (time to onset of reproduction) was shorter for aphids reared on synthetically fertilised plants than those receiving organic fertilisers, which again corresponds with former findings (Hosseini *et al*, 2010). Overall, this supports the theory that the application of synthetic fertilisers accelerates and enhances aphid reproduction, which, over a longer period than that used in this study (14 days), may result in significantly larger aphid colonies. Indeed, when examining the final population counts, there was no difference between chicken manure-treated plants and LN plants. There are contrasting reports in the literature regarding the effect of fertiliser type (organic or mineral) on aphid performance. Some researchers, such as Costello and Altieri (1995), found a positive association between *M. persicae* abundance and organic fertilisers; some (e.g. Staley *et al* (2010)) reported mixed results; whilst others (e.g. Stafford *et al* (2012)) came to the opposite conclusion, with aphid performance (r_m) being lower in organically fertilised plants in comparison to synthetically fertilised host plants. Again, it may be that a longer infestation period than that used in this study is required to yield more conclusive results.

Hypothesis (v): M. persicae population growth rates would be higher on plants receiving increased N dose.

As predicted, overall the HN treated plants had a higher yield (as measured by biomass and average leaf area), however, this may be offset to an extent by the concomitant increase in abundance of the

generalist aphid *M. persicae* relative to the other treatments. Increasing N applications also corresponded with higher fecundity, as caged *M. persicae* reared on HN plants exhibited greater reproduction rates and faster developmental times in comparison to those on the control, LN and CM plants. This is in accordance with the Plant Vigour Hypothesis (PVH) proposed by Price (1991), and several other studies which reports increased aphid abundance in response to higher N levels (Hosseini *et al*, 2015). It may, therefore, be desirable to the farmer to reduce N inputs to a level at which yield benefits outweigh loss from aphid infestations. This is conducive to sustainable farming practices, as it involves reducing chemical inputs from both fertilisers and pesticides. According to the literature, however, the influence of nitrogen applications on aphid fecundity are not uniform, with some studies reporting no effect of nitrogen treatment on the intrinsic rate of increase of aphids (Mace and Mills, 2015). There is also evidence of a threshold N level, above which the trend is reversed and aphids are negatively affected by further increasing N applications. This was the case in a study by Sauge *et al* (2010) in which *M. persicae* numbers rose over a 30 day period on peach plants (*Prunus persicae* (L.)) supplemented with 1 - 10 mM N. However, when the N concentration was increased to 15 mM, aphid numbers were elevated over the first week, but then plateaued and declined over the subsequent fortnight. This contradicted the theory that aphid abundance correlates with plant N levels, since the15mM and 10mM treatments had highly similar total amino acid concentrations. The authors instead propose that this unanticipated result was due to the influence of elevated N availability on plant chemistry, such as the associated reduction in chlorogenic acid - a carbon-based compound which is known to enhance plant resistance against phloem-feeding pests such as the grain aphid (*Sitobion aestivum* L.) (Chrzanowski *et al*, 2012). In the fourth week, however, aphid numbers did show signs of a positive correlation with N dose as they started to increase in the highest N treatment. It would be interesting, therefore, to test whether further increases in N dosage would also lead to a decline in aphid numbers in our model system, which is briefly explored in **Chapter Four (Part II)**.

2.4.1 Study limitations

There are several aspects of this experiment which may have been enhanced had it been of longer duration. This was limited, to an extent, by the fact that the study was performed in a growth room, meaning that it is impracticable to grow the plants much beyond their size at 12 weeks. An extended experimental period would have particularly beneficial for the aphid fecundity and growth measurements. A higher sample size in the aphid-infestation part of the experiment may also have enhanced the experiment by reducing error, which was sizeable in several of the parameters measured. The plant and aphid performance measurements were taken from a subset of plants, as the time-sensitive nature of the soil DNA extractions took priority at harvest times and the availability of space in the -80° C freezers was limited. This impaired the reliability of the results, and may have introduced bias relating to the enclosure of entire treatments in a single

Perpsex box. If this study were repeated, plant and aphid performance metrics would ideally be monitored on all plants, and plants of different treatments would be integrated in each Perspex box.

The lack of correlation between chlorophyll and total foliar N suggest that the former measurements may be unreliable. The disparity in the results obtained from individual plants did seem somewhat crude at the time, and it was decided to cease using the Opti-Sciences CCM-200 Chlorophyll Content meter in subsequent experiments. Also, instead of measuring foliar content, the N and S analysis of phloem sap may produce a stronger correlation with aphid herbivory, since foliar measurements tend not to be representative of phloem nutrient content (Sandström, 2000).

Root biomass was not recorded, but on reflection may have yielded significant contrasts between infested and undamaged cabbages. Indeed, Vestergård *et al* (2004) found that aphids affected root biomass more than shoot biomass, and therefore investigating the effects on below-ground plant biomass could merit further investigation.

During the aphid-infestation stage of the experiment, infested plants of a single fertiliser treatment group were placed together in Perspex cages. This, therefore, represents pseudoreplication which may have incurred biases in the results owing to cage-specific effects (e.g. variation in temperature or light within the cage) meaning that one cage in fact represents one treatment. This is a common issue in ecological and animal behaviour experiments (as discussed by Schank and Koehnle, 2009). It was due in part to the restrictions imposed on availability of space, however, the controlled environment settings under which the experiment was performed helped to address the issue of pseudoreplication by minimising the possibility of fluctuation in abiotic factors. Yet the dangers of pseudoreplication and the lack of statistical independence were highlighted during this experiment by the inadvertent attack by the escaped *Plutella xylostella*, which is likely to have had a significant impact on the results from the affected plants. It was later discovered to have occurred through the contamination of a watering can used to water plants, which was kept in one of the controlled environment rooms in the insectary. Care was taken thereafter to exclusively use clean, transparent jugs for watering plants in order to avoid such contamination events reoccurring. The affected plants were not used for the soil microbial study (Chapter 3) and therefore, do not impact on their results. Furthermore, the experimental design allowed the aphids to move freely between plants (within a fertiliser treatment group) and so the true effect of each plant on aphid performance cannot be wholly determined. However, aphids generally avoid leaving a plant to get another plant if it involves moving across non-plant material (Poppy, 2017 – personal communication) and the plants were spaced within the cages so that their leaves were not touching, thereby reducing the likelihood of inter-plant movement of aphids. Nevertheless, these issues were addressed in subsequent experiments by placing perforated plastic bags over each plant, and interspersing the treatments by randomly assigning plants to Perspex cages, irrespective of their treatment group.

Finally, it would be interesting to test a wider range of N concentrations to see whether increasing N further eventually leads to lower aphid populations as reported by Sauge *et al* (2010). The N doses used in this study were deemed appropriate as they reflected those typically applied in the field. Further increases in N application would only be relevant up to a point, as environmental regulations prohibit excessive fertiliser applications. This is discussed further in Chapter Four (Part II).

The principal focus of this wider study, however, was to investigate the belowground (specifically soil bacterial) response to fertiliser regime, the cabbage rhizosphere and growth, and aphid herbivory. These are discussed in the following chapter.

Chapter 3: Investigating the soil bacterial communities associated with fertiliser treatments, the *Brassica oleracea* L. var. *capitata* rhizosphere, and aphid infestation using 16S rRNA NGS sequencing.

3.1 Introduction

3.1.1 Farming management and the soil microbiome

Farming management approaches can exert a strong influence on the soil microbial community. There is little consensus in the literature regarding the effect of different farming systems on soil bacterial community composition, diversity and evenness, which may be largely attributed to the many abiotic and biotic factors which also shape the soil microbiome, as well as the broad spectrum of farming systems used today. However, several trends have emerged. Conventional farming, which is generally considered to involve the use of mineral fertilisers, is often associated with bacteria-dominated soils, whereas organic farms are characterised by fungal rich soils. In a long-term field study, Hartmann *et al* (2015) found that the soil microbial communities differed in *β*-diversity according to the farming management style used. They also reported the bacterial α-diversity (i.e. species richness and abundance) of CONMIN (minerally fertilised conventional) soils was not statistically dissimilar from the unfertilised soils, but was significantly lower than soils which received some form of organic treatment (manure).

3.1.2 Rhizosphere vs. Bulk Soil microbiomes

The rhizosphere typically exhibits much higher microbial activity and biomass than the bulk (root-free) soil, with one gram of rhizosphere soil being estimated to contain up to 10^{12} cells, which is typically two orders of magnitude greater than that of the bulk soil (Lynch and de Leij, 2001). This is largely due to carbon-rich root exudations and root debris (collectively termed *rhizodeposits*) and leaves providing a significant energy source for microbial processes (Powlson *et al*, 2011). Given that rhizodeposits represent a source of C and N loss from the plant (Hunter *et al*, 2014), it may be expected that a reduction in N-stress (i.e. by fertilisation) may lead to enhanced rates of rhizodeposition.

3.1.3 Insect herbivory and soil microbiome

Soil-plant-insect interactions can occur via top-down or bottom-up forces. *Top-down* forces are regulated by their consumers (i.e. plant growth being regulated by insects); whereas *bottom-up forces* are determined by resource quantity and quality (i.e. soil affecting plant quality and thereby also affecting aphid performance) (Wardle *et al*, 2004). Microbes in the root zone can influence aboveground herbivory by affecting the quality and quantity of the host plant (Badri *et al*, 2013). For instance, root colonisation of barrelclover (*Medicago truncatula* Gaertn.) by the mycorrhizal fungal *Glomus versiforme* has been shown to affect the metabolic profile of the plant which can have important implications in herbivory defence (Harrison & Dixon, 1993). Microbes can also affect herbivory by altering soil nutrient availability and C sequestration rates (Wardle *et al*, 2004).

Likewise, aboveground herbivory of a host plant can alter plant metabolism and root exudation which may trigger changes in the soil microbiome in the rhizosphere. Cattle-grazing of bahiagrass (*Paspalum noatutm*) has been reported to alter the abundance of soil bacteriovores (Wang *et al*, 2006). In another case, artificial foliar herbivory (defoliation by clipping) of a grazing tolerant grass *Poa pratensis* L. was shown to lead to increased photosynthetic and root C exudation rates, which stimulated soil microbial activity which in turn enhanced N availability to the plant (Hamilton & Frank, 2001). This study suggested, therefore, that defoliation of *Poa pratensis* resulted in positive feedback via stimulated rhizospheric processes which ultimately resulted in higher levels of plant nutrition and photosynthesis. However, the accuracy of artificial defoliation in representing actual herbivory has been discredited in other studies (Frost & Hunter, 2004, Baldwin, 1988). Herbivory of red oak (*Quercus rubra*) by the eastern tent caterpillar (*Malacosoma americanum*) has been associated with increased soil respiration and dissolved organic C (DOC), which again was indicative of herbivory-induced enhancement of soil microbial activity (Frost & Hunter, 2004). However, herbivore damaged plants also exhibited lower total soil N. The authors hypothesised that this may be attributed to greater rhizodeposition rates stimulating microbial activity and mineralisation rates.

The influence of phloem-feeding insects on the rhizosphere community is less well understood. Aphid herbivory can influence a plant's susceptibility to soil-borne pathogens, as demonstrated by Lee *et al* (2012). Aphid-infested pepper plants (*Capsicum annuum*) were shown have significantly reduced severity of disease symptoms when exposed to the pathogenic bacteria *Xanthomonas axonopodis* pv. *vesicatoria*, which supported previous reports by Yang *et al* (2011). Furthermore, plants exposed to aphid herbivory had reduced pathogenic *R. solanacearum* SL1931 populations and recruited larger populations of beneficial rhizobacteria (*Bacillus subtilis* GB03) in comparison to controls (Lee *et al*, 2012). Nitrogen availability can exert a strong influence on the growth of plants, insects and soil microbes. It may be expected, therefore, that these three trophic levels experience competition over this nutrient.

3.1.4 Molecular methods in soil microbiology

Soil microbiological research has advanced greatly following the development of new molecular polymerase chain reaction (PCR)-based techniques and, most recently, next generation sequencing (NGS). Prior to these methods, microbiological studies relied on traditional culture-based techniques which were extremely limited, given that the portion of bacteria cultivatable under laboratory conditions (on agar plates) is estimated to be between 0.3-20% for those inhabiting bulk soil, and 1-10% of rhizosphere-dwelling bacteria (Prosser, 2002, Nannipieri *et al*, 2003, Marilley *et al*, 1998). Culture-independent methods have since enabled researchers to examine soil microbial communities in far greater detail and accuracy, thereby providing new insights into the mysterious soil "black box" and enabling the discovery of many novel bacterial taxa.

The majority of these culture-independent techniques involve the extraction of total DNA from the environment (eDNA) followed by PCR-amplification of marker genes, such as the 16S ribosomal RNA (rRNA) gene for bacteria and 18S for fungi (Kent & Triplett, 2002). The resulting amplicon libraries may then be used to determine the microbial community structure through either molecular community profiling methods, or using the more recently developed metagenomic techniques such as next-generation sequencing (NGS). The molecular community profiling approaches include denaturing gradient gel electrophoresis (DGGE), terminal-restriction fragment length polymorphism (T-RFLP) and automated ribosomal intergenic spacer analysis (ARISA). In order to obtain the gene sequence of samples, the PCR-products/fragments must be cloned into plasmid vectors (*E. coli*) and sequenced using Sanger technology. However, in comparison to NGS platforms, Sanger sequencing is time-consuming due the cloning requirements, expensive, and low-throughput (Singer *et al*, 2016, Hirsch *et al*, 2010). Microbiological research has been transformed by these modern metagenomic techniques, which encompass genomics, metabolomics and proteomics (Lynch and de Leij, 2001).

It is important to note here that this study focused on bacteria, and not fungi. This was due to the long-established knowledge that plants in the Brassicaceae family typically are not colonized by arbuscular mycorrhizal (AM) fungi, unlike the 80% of higher plants which are capable of forming these symbioses (Lundberg *et al*, 2012, Brundrett, 2009, Lambers *et al*, 2009). It is thought that this lack of mycorrhizal association is related to the production of phytoalexins, such as glucosinolates, by *Brassica* species, as these secondary metabolites have antimicrobial and antifungal attributes (Winde and Wittstock, 2011). A protein in the seed of *Brassica oleracea* isolated by Ye *et al* (2011) was also shown to inhibit mycelial growth, in addition to having antibacterial and anticancer properties. Many studies have demonstrated that the use of *Brassica* species as an amendment to fields can be effective in reducing the incidence of or suppressing fungal diseases (Farooq *et al*, 2014). However, the root exudates of brassicas have also been shown to stimulate growth of ectomycorrhizal fungi such as *Paxillus involutus*, which appears to be

caused by the action of hydrolysed indole glucosinolates (isothiocyanates) produced by the root (Zeng *et al*, 2003).

3.1.5 The 16S rRNA gene

The 16S small sub-unit ribosomal RNA (16S rRNA) gene is ubiquitous among bacteria and archaea, and plays an essential role in protein synthesis, rendering it vital for proper cell functioning. Its use as a bacterial taxonomic marker in PCR-based microbial ecology research was first performed by Woese *et al* (1985) following the sequencing of 16S genes in the mid-1980s (Vasileiadis *et al*, 2012, Lane *et al*, 1985). The 16S gene is an appropriate genetic marker as it has a relatively short sequence length of approximately 1,550 base pairs (bp) (Clarridge, 2004). Ribosomal DNA is a useful tool in studying microorganisms as ribosomes are ubiquitous to all forms of cellular life (Archaea, Bacteria and Eukarya), and the molecules contain highly evolutionarily conserved regions with intervening variable (and hypervariable) sections of sequence (Head *et al*, 1998). The 16S gene contains 9 hypervariable regions which may be used to identify the taxonomic source of the sequence down to a species or even subspecies level in some instances (Baker *et al*, 2003). Although 16S rRNA genes can undergo horizontal gene transfer, it occurs predominantly within closely-related taxa and therefore is likely to only affect taxonomic classification at the genus or species level (Tian *et al*, 2015). Another caveat regarding the use of 16S rRNA in bacterial community investigations is that the copy number varies between species, with up to 15 copies of the gene in some bacteria such as *Photobacterium profundum* (Lee *et al*, 2009). This may result in the abundance of some taxa being overestimated. Nevertheless, it remains the most commonly used marker gene in phylogenetic analyses for bacterial and archaeal taxonomic classification (Singer *et al*, 2016). Over recent decades, the 16S rRNA gene has surpassed all other taxonomic markers in terms of sequencing projects, contributing enormously to the advance of bacterial classification systems (Yarza *et al*, 2014).

3.1.6 Next-generation sequencing

Next-generation sequencing (NGS), or high-throughput sequencing, has revolutionized the field of microbial ecology research. As sequencing technologies' running costs fall and the read lengths they produce increase, 16S rRNA NGS now represents a more accessible and reliable tool for soil microbial profiling than ever before (Bulgarelli *et al*, 2013). There are many NGS technologies available, including IonTorrent (now owned by Life Technologies), PacBio (Pacific Biosciences) and SOLiD (Applied biosystems) with each technology varying in their run time, number and length of reads produced, and cost per run (Oulas *et al*, 2015, Ambardar *et al*, 2016, Glenn, 2011). Two of the most commonly used sequencing platforms are Roche 454 pyrosequencing by Life Sciences/Roche diagnostics and the Illumina systems MiSeq® and HiSeq® (Oulas *et al*, 2015). The 454 system was the original commercially available NGS machine. In brief, 454

pyrosequencing works by immobilizing DNA fragments on beads in a water-oil complex which are then amplified through PCR, and the beads are placed on a PicoTiterPlate and pyrosequenced (Oulas *et al*, 2015). 454 technology has been criticized for producing sequencing errors which lead to over-estimates diversity, in addition to biased results as a result of the noise generated by the sequencing (Oulas *et al*, 2015). Illumina sequencing platforms are now generally preferred over 454 pyrosequencing for 16S rRNA studies, largely owing to economic advantages (the Illumina MiSeq generates up to 25 million paired-end reads in a single sequencing run in comparison to 454's 1 million reads), in addition to the improved base-calling accuracy of these systems (Oulas *et al*, 2015). Illumina NGS allows users to pool multiple samples (termed multiplexing) and sequence them in parallel. A more detailed protocol is described in detail in the *Materials and Methods* section of this chapter, but, in brief, it uses barcoded primers to amplify the V4 region of the 16S rRNA gene which allows the sample-identification of each of the amplicons after they have been pooled and sequenced in parallel (Caporaso *et al*, 2012).

It is important to note that 16S rRNA NGS methods are liable to the biases inherent to PCR, such as selectivity in PCR amplification of rRNA gene, and sequencing errors (e.g. chimeras) (Head *et al*, 1998). Chimeras are produced when two or more sequences from different parents join together, resulting in the potential misidentification of novel species. However, there are bioinformatic tools available which detect and remove these chimeric sequences from sequence libraries. The extraction of environmental DNA may also contribute to biases from DNA contamination by humic substances and organic matter.

The availability of software packages and bioinformatics tools designed to analyse 16S rRNA sequencing data has expanded rapidly over recent years, each having their own pros and cons as discussed by Gonzalez and Knight (2012). Open source software packages such as QIIME (Quantitative Insights Into Microbial Ecology) and MG-RAST (the Metagenomics RAST) are designed for the analysis of microbial community sequences obtained from high-throughput amplicon and next generation sequencing, and shotgun metagenomic sequencing (Meyer *et al*, 2008). These programmes include features such as OTU picking, phylogenetic tree construction, removal of chimeras, and taxonomic assignment at the seven levels of classification: Kingdom, Phylum, Class, Order, Family, Species and Genus (Caporaso *et al*, 2010). MG-RAST incorporates multiple metagenomic tools and publicly available data sets, enabling the comparison of different soil metagenomes from diverse environments around the world (Meyer *et al*, 2008).

Aims

The results reported in this chapter are coupled with those of Chapter Two, with this chapter representing the belowground component of the experiment. This study aimed to investigate the dynamics of the soil bacterial community in response to fertiliser treatments, the growth of *Brassica oleracea* and finally, herbivory by *Myzus persicae*.

It was hypothesized that the addition of different fertilisers would alter the soil bacterial community, with the organic amendment (chicken manure) leading to more diverse communities and the mineral fertilisers resulting in a reduction in alpha diversity. The infestation of plants with aphids was hypothesised to incur changes in the belowground soil microbial community via the changes in plant chemistry and exudates which are induced by herbivory.

In order to test these hypotheses, DNA samples were extracted from the soil environment at each of the aforementioned time-points. To investigate the impact of fertiliser type (organic versus synthetic) and dosage (nitrogen content) on soil microbial communities, a synthetic NPK fertiliser was added at two N rates (high and low) and an organic fertiliser (chicken manure) was added at the lower N rate (as described in Chapter Two). DNA was extracted from the soil before and after the addition of the fertilisers (referred to as baseline and fertiliser time-points), and these constituted the bulk soil samples. After transplantation of 1week-old *Brassica oleracea* seedlings into these pots, followed by a further 8-week growth period, rhizosphere soil DNA was extracted via destructive sampling. Finally, half of the remaining cabbages within each treatment group were inoculated with *M. persicae*, which were left to colonise the plants over the final two weeks of the experiment, before they too were sampled for rhizosphere DNA, along with the non-infested plants. A schematic of the experiment is given in Chapter Two (**Figure 8**). These cabbages were grown for 12 weeks in total. The soil DNA samples were then used to characterise the soil microbiome via 16S rRNA next-generation sequencing (NGS) on the MiSeq Illumina platform. Subsequent in-depth downstream analysis of the results was performed using a range of methods and applications, as described in the following sections.

3.2 Materials and Methods

See **Chapter 2** for a full description of the methods regarding the mesocosm aspect of this study, including details of soil collection, fertiliser treatments, plant cultivation and aphid infestation. They are given in brief below.

3.2.1 Fertiliser treatments

Four fertiliser treatments were used in this study: control, organic (CM), low N synthetic (LN), high N synthetic (HN). All fertiliser treatments were applied once to each individual pot at the beginning of the experiment (after the initial set of DNA extractions of baseline samples). The synthetic fertiliser (Chempak ® Formula No. 3 – Fully Balanced Feed (NPK 20-20-20)) was applied at two N rates (0.16g and 0.32g N $litre^{-1}$ soil) which are approximately the equivalent of 68 and 136 kg ha^{-1} respectively. To achieve these specified N dosages, the synthetic fertiliser was administered at 0.31g and 0.62g pot^{-1}, representing the Low N (**LN**) and High N (**HN**) treatments respectively. The organic fertiliser used was pelleted chicken manure (New Horizon Organic Poultry Manure Pellets). Due to the low N content of the chicken manure, and the unfeasibility of adding much larger quantities, it was applied at the low N rate only, which equated to1.64g pot^{-1}. The fertiliser treatments were applied in aqueous solution (50ml pot^{-1}), with the chicken manure pellets firstly being ground to a powder using a pestle and mortar. An equal volume of tap water was added to control pots. The pots were kept in trays (6 pots per tray) to prevent any fertiliser-containing leachate reaching non-target pots. This experiment was conducted under controlled environmental conditions (16:8 hour light:dark, 20°C, and 70% relative humidity) at the University of Southampton. The trays were rotated weekly to account for variations in airflow which may influence soil moisture content.

3.2.2 Plant cultivation

Brassica oleracea L. var. *capitata* cultivar Derby Day seeds (Moles Seeds, UK, Ltd.) were sown after fertiliser application, with 5 seeds pot^{-1}. These pots (18 per treatment, total n=72) were used only to germinate the plants and were thereafter discarded (i.e. no environmental DNA was extracted from these pots). The most vigorous of the successfully germinated individuals were subsequently transplanted into the experimental pots (one plant per pot) containing the corresponding fertiliser treatments (30 pots per treatment, total n=120). Plants were watered with tap water as necessary. Plants were harvested at either 9 or 12 weeks (+Cabbage and +/- aphids time-points respectively). Half of the 12-week cohort was infested with *M. persicae* for the 14 days prior to harvesting.

3.2.3 Aphid infestation

The *M. persicae* colony was reared on Chinese cabbage *Brassica rapa* L. spp. *Pekinensis* (Lour) Cv. Wong Bok (Kings Seeds, Surrey, UK) in Perspex cages (70 x 69 x 45 cm) under controlled environment conditions (20±3°C, 16:8hour light:dark). Five apterous adult aphids were added to each of the cabbages in the aphid-infested treatment group using a paintbrush, and allowed to feed and reproduce for 14 days.

3.2.4 Extraction of bacterial DNA from soil

Total environmental DNA was extracted from 0.25g subsamples of the pooled soil samples using the MoBio PowerSoil® DNA Isolation Kit (MoBio Laboratories, Carlsbad, CA) following the methods described by the manufacturer. Briefly, cell lysis was achieved by bead-beating the samples in an SDS-containing solution. This was followed by several centrifugation and refrigeration (4°C) steps to remove non-genomic contaminants (e.g. humic acids). DNA was obtained after repeated micro-centrifugation of the samples in a high concentration salt solution with a spin filter, and lastly with an ethanol-based solution. Finally, the DNA was suspended in a sterile EDTA-free elution buffer. DNA was isolated from soil samples at each of the four stages:

(i) Baseline - *bulk soil* (*n*=8)
(ii) Post-fertiliser application - *bulk soil* (*n*=8)
(iii) Cabbage at 9 weeks - *rhizosphere soil* (*n*=8)
(iv) Cabbage at 12 weeks, with/without aphids - *rhizosphere soil* (*n*=16)

Each DNA sample consisted of pooled soil samples collected from 5 pots (within the same treatment) (**Table 2**). The samples at stages (ii) – (iv) were taken from each fertiliser treatment (Control, CM, LN and HN). Due to limitations imposed by cost and the number of samples able to be multiplexed in a single Illumina MiSeq run, duplicate (rather than the optimal triplicate) DNA samples were used per treatment at each sampling stage.

Table 2 Summary of the number of pots and DNA samples extracted at each stage of the experiment (batch 1).

Sampling stage	Total number of pots sampled	Number of DNA samples taken
Bare soil	**8**	8
Fertiliser additions	**40** (10 per fertiliser treatment)	8
Cabbage (9 weeks)	**40** (10 per fertiliser treatment*)	8
Cabbage (12 weeks) +/- Aphids	**80** (10 per aphid/fertiliser treatment)	16

*N.B. owing to plant deaths, DNA samples were taken from only 7 pots in the high N group at the +Cabbage sampling stage.

Owing to difficulties in obtaining sufficient DNA of adequate quality, only single DNA samples were used for the CM and HN +fertiliser sampling stage ((ii) above), and instead extra samples (*n*=3) were used for Con and HN. In the case of the CM sample, it is likely that the high organic content of the fertiliser caused the issue in the DNA isolation. For the final two sampling time-points ((iii) and (iv) above), rhizosphere soil was collected by destructively harvesting and pooling the roots of five plants per treatment, which were put in a plastic bag and shaken vigorously to remove the bulk soil from the roots. Any soil still adhering to the roots after shaking was considered as rhizosphere soil. The roots were then placed in a falcon tube with 25ml of distilled water, which was vortexed for 1 minute to separate the roots and soil solution. The roots were removed from the tube, and the solution was centrifuged at 3000*x g* for 15 minutes. This centrifugation step was repeated to further separate the soil from the water, to obtain a soil pellet, which was then used immediately for DNA extraction.

DNA quality was assessed by 0.8% agarose gel electrophoresis and the quantity and quality were determined both spectrophotometrically, based on the A260/280nm absorbance ratios using NanoDrop ND-1000 Spectrophotometer (ThermoScientific), and fluormetrically using the Qubit ® dsDNA BR Assay (Qubit, Invitrogen). DNA samples were stored at -20°C, until they were delivered (on ice) to the University of Liverpool for sequencing.

3.2.5 Sequencing library construction

The library design, PCR steps and barcoding for 16S rRNA dual-index paired-end sequencing using the Illumina MiSeq® platform were performed by the Centre for Genomic Research (CGR), University of Liverpool, UK. Briefly, the amplicon libraries for each of the 40 DNA samples were prepared through a two-step PCR amplification process to amplify the V4-region 16S ribosomal RNA (16S SSU rRNA) gene for bacteria and archaea using specific primers (**Table 3**) and Illumina flowcell adapter sequences to enable for cluster formation (Oligonucleotide sequences © 2016 Illumina, Inc. All rights reserved) (**Figure 12**). The first PCR step used oligonucleotide sequences

containing a locus-specific sequence and a universal 5' tail end (overhang adapter) to amplify the V4 region of the 16S rRNA gene. The 16S V4-specific forward and reverse primers were 501F and 806R respectively (**Table 3**), as proposed by Caporaso *et al* (2012). They amplify the region 533–786 in the *Escherichia coli* strain 83972 sequence (Greengenes accession no. prokMSA_id:470367). This PCR used 5ng of each DNA sample, 0.5µl of each primer (10µM) and 10µl of 2x Kapa Hi Fi amplification mix, to give a total PCR reaction mix volume of 20µl. The cycling conditions for this PCR were:

- 95°C for 2 minutes (hot start)
- 10 cycles of:
  - 98°C for 20 seconds
  - 65°C for 15 seconds
  - 72°C for 30 seconds
- 72°C for 5 minutes.

The resulting amplicons were then cleaned using a magnetic bead capture kit (AMPure XP) at a ratio of 1:1 and resuspended in 9µl. This was then used in the second PCR, after adding 0.5µl of each of the 8 base Nextera® indices (i7 and i5) and Illumina sequencing adapters (both at 10µM) using the Nextera DNA kit (Illumina, UK), following the manufacturer's instructions (Bartram *et al*, 2011). The adapter and barcode (index) sequences are given in **Table 4** and **Table 5** and they are complementary to the first set of primers through either the i5 or i7 sequence. This second-step PCR again used 2x Kapa Hi Fi mix (10µl) under the same PCR conditions as before, but this time increasing it to 15 cycles. During this second PCR step, the DNA is tagged with adapter sequences which are attached to both ends of the DNA, thereby allowing dual-indexed sequencing of pooled libraries on the MiSeq Illumina sequencing platform. This second-step PCR used 8 forward and 5 reverse primers, used in unique combinations for each DNA sample to enable all 40 samples to be pooled and sequenced in multiplex in a single Illumina MiSeq run (**Table 6**).

Table 3 Universal primer sequences with overhang adapters used for the first step PCR in the 16S rRNA library preparation. Field descriptions (space delimited): (i) primer pads: blue = Illumina P5 sequence (forward overhang adapter), red = Illumina P7 sequence (reverse overhang adapter); (ii) forward/reverse primer pad; (iii) forward/reverse primer linker in *italics*; (iv) forward 515F and reverse 806R primers in **bold**.

16sv4 Primer	Oligo
515F	5'-ACACTCTTTCCCTACACGACGCTC TTCCGATCT *NNNNN* **GTGCCAGCMGCCGCGGTAA**-3'
806R	5'-GTGACTGGAGTTCAGACGTGTGCTC TTCCGATCT *G* **GGACTACHVGGGTWTCTAAT**-3'

Next, the amplification products were again cleaned using 1:1 AMPure beads to remove very short library fragments, before the library normalization and pooling stages. The recovered amplicon pools were quantified and quality checked using the Qubit assay (Invitrogen) and Bioanalyzer (Agilent) DNA HS chip for peak distribution. The products were then pooled on an equimolar basis and purified using Prep (Sage Science) to select specifically for the amplicon band.

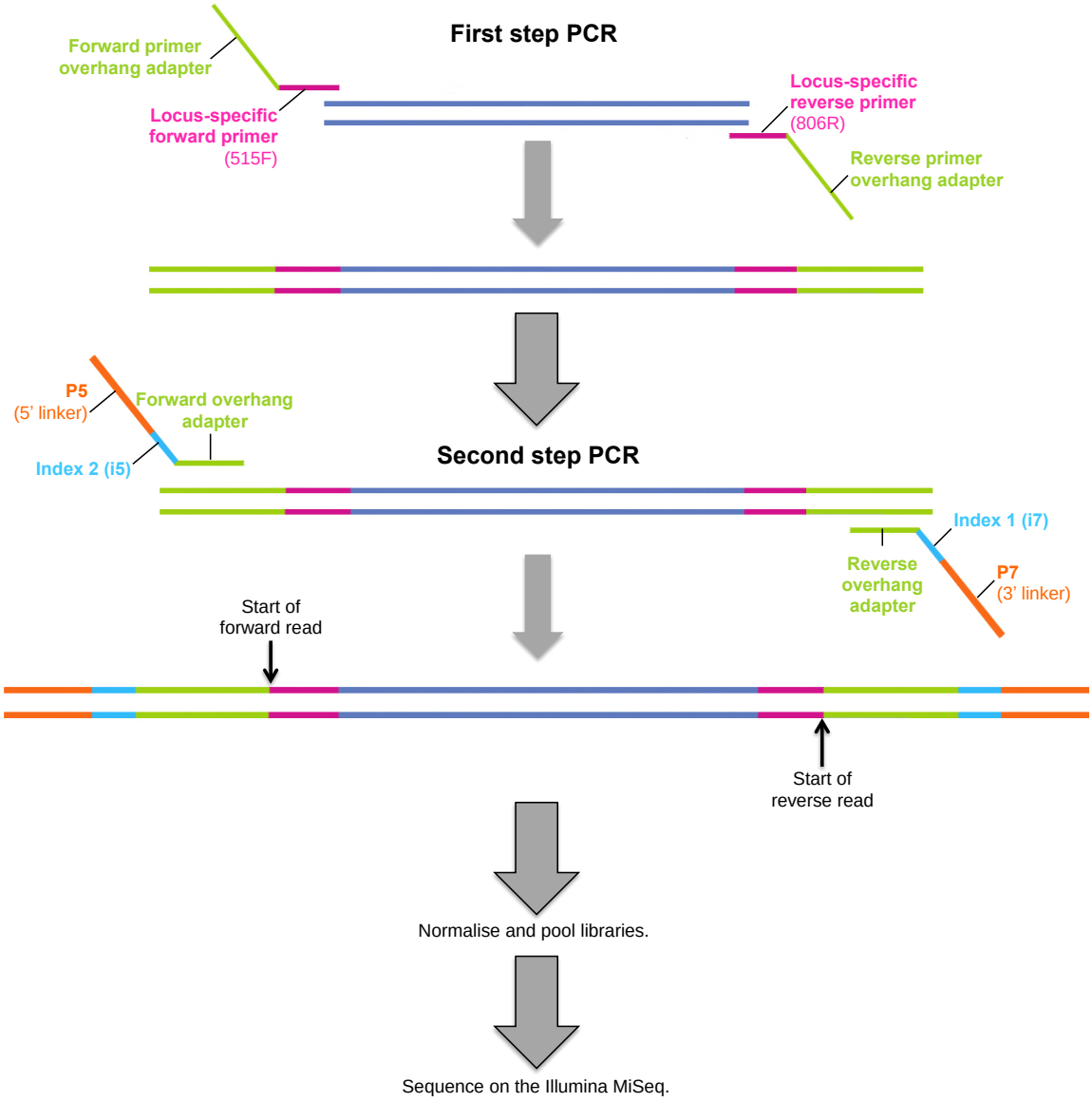


Figure 12 The two-step PCR procedure for the preparation of 16S rRNA amplicon libraries. PCR step 1 shows the attachment (ligation) of universal primers 514F and 806R with overhang adapter sequences, and step 2 shows the attachment of dual-index Nextera barcode sequences and Illumina sequencing adapters to the V4 amplicon targets, prior to pooling and sequencing on MiSeq Illumina. The sequencing produces two reads: a forward read (R1) and a reverse read (R2) for each amplicon.

Table 4 Forward primer constructs with Nextera index 2 (i5) adapters used for the second step PCR in the 16S rRNA library preparation. Field descriptions (space delimited): (i) the forward 5' Illumina adapter (P5), (ii) the 8bp forward primer linker (i5 adapter) in ***bold italic***, and (iii) the forward primer overhang adapter sequence in blue.

Index name	5' Illumina adapter (P5)	*Index 2* (i5)	Forward primer overhang adapter
N501	5'AATGATACGGCGACCACCGAGATCTACAC	***TAGATCGC***	ACACTCTTTCCCTACACGACGCTC3'
N502	5'AATGATACGGCGACCACCGAGATCTACAC	***CTCTCTAT***	ACACTCTTTCCCTACACGACGCTC3'
N503	5'AATGATACGGCGACCACCGAGATCTACAC	***TATCCTCT***	ACACTCTTTCCCTACACGACGCTC3'
N504	5'AATGATACGGCGACCACCGAGATCTACAC	***AGAGTAGA***	ACACTCTTTCCCTACACGACGCTC3'
N505	5'AATGATACGGCGACCACCGAGATCTACAC	***GTAAGGAG***	ACACTCTTTCCCTACACGACGCTC3'
N506	5'AATGATACGGCGACCACCGAGATCTACAC	***ACTGCATA***	ACACTCTTTCCCTACACGACGCTC3'
N507	5'AATGATACGGCGACCACCGAGATCTACAC	***AAGGAGTA***	ACACTCTTTCCCTACACGACGCTC3'
N508	5'AATGATACGGCGACCACCGAGATCTACAC	***CTAAGCCT***	ACACTCTTTCCCTACACGACGCTC3'

Table 5 Reverse primer constructs with Nextera index 1 (i7) adapters used for the second step PCR in the 16S rRNA library preparation. Field descriptions (space delimited): (i) the reverse complement of the 3' Illumina adapter (P7); (ii) the 8bp reverse primer barcode (i7 adapter) in ***bold italic*** (reverse 5'-3' read in brackets) and (iii) the reverse primer overhang adapter sequence in red.

Index name	3' Illumina adapter (P7)	*Index 1* (i7)	Reverse primer overhang adapter
N701	5'CAAGCAGAAGACGGCATACGAGAT	***TCGCCTTA*** (TAAGGCGA)	GTGACTGGAGTTCAGACGTGTGCTC3'
N702	5'CAAGCAGAAGACGGCATACGAGAT	***CTAGTACG*** (CGTACTAG)	GTGACTGGAGTTCAGACGTGTGCTC3'
N703	5'CAAGCAGAAGACGGCATACGAGAT	***TTCTGCCT*** (AGGCAGAA)	GTGACTGGAGTTCAGACGTGTGCTC3'
N704	5'CAAGCAGAAGACGGCATACGAGAT	***GCTCAGGA*** (TCCTGAGC)	GTGACTGGAGTTCAGACGTGTGCTC3'
N705	5'CAAGCAGAAGACGGCATACGAGAT	***AGGAGTCC*** (GGACTCCT)	GTGACTGGAGTTCAGACGTGTGCTC3'

After both PCR steps, the final sequences were as follows (i) Forward primer (5' - 3'):

AATGATACGGCGACCACCGAGATCTACAC**[i5]**ACACTCTTTCCCTACACGACGCTCTTCCGATCT*NNNNN***GTGCCAGCMGCCGCGGTAA**

and (ii) Reverse primer (5' - 3'):

CAAGCAGAAGACGGCATACGAGAT**[i7]**GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT*G***GGACTACHVGGGTWTCTAAT**

The final concentration of the library pool was determined by quantitative PCR (qPCR) with the Illumina Library Quantification Kit (Kapa Biosciences) on a Roche Light Cycler LC480II, according to the manufacturer's instructions. The template DNA was denatured according to the Illumina MiSeq protocol, before being loaded at 7pM concentration, with the addition of 15% phage PhiX to increase the complexity of the amplicon library. The PhiX spike is required to correct for the extreme base bias present in 16S amplicon samples, and it was later filtered out of the data set. The libraries were then pooled and sequenced in parallel on one flowcell of a MiSeq 2000 Illumina platform at 2x250bp paired-end sequencing with v2 chemistry.

Table 6 Summary of the sample IDs and Nextera dual-index barcode sequences (CGR, Liverpool).

Sample ID	**Barcoding Index** (i7 - i5)	**Treatment**[†]	**Sample type**
S1	TAAGGCGA - TAGATCGC	Con	Baseline
S2	TAAGGCGA - CTCTCTAT	Con	Baseline
S3	TAAGGCGA - TATCCTCT	Con	Baseline
S4	TAAGGCGA - AGAGTAGA	Con	Baseline
S5	TAAGGCGA - GTAAGGAG	Con	Baseline
S6	TAAGGCGA - ACTGCATA	Con	Baseline
S7	TAAGGCGA - AAGGAGTA	Con	Baseline
S8	TAAGGCGA - CTAAGCCT	Con	Baseline
S9	CGTACTAG - TAGATCGC	Con	Fertiliser
S10	CGTACTAG - CTCTCTAT	CM	Fertiliser
S11	CGTACTAG - TATCCTCT	LN	Fertiliser
S12	CGTACTAG - AGAGTAGA	HN	Fertiliser
S37	GGACTCCT - GTAAGGAG	Con	Fertiliser
S38	GGACTCCT - ACTGCATA	Con	Fertiliser
S39	GGACTCCT - AAGGAGTA	LN	Fertiliser
S40	GGACTCCT - CTAAGCCT	LN	Fertiliser
S13	CGTACTAG - GTAAGGAG	Con	Cabbage (9week)
S14	CGTACTAG - ACTGCATA	Con	Cabbage (9week)
S15	CGTACTAG - AAGGAGTA	CM	Cabbage (9week)
S16	CGTACTAG - CTAAGCCT	CM	Cabbage (9week)
S17	AGGCAGAA - TAGATCGC	LN	Cabbage (9week)
S18	AGGCAGAA - CTCTCTAT	LN	Cabbage (9week)
S19	AGGCAGAA - TATCCTCT	HN	Cabbage (9week)
S20	AGGCAGAA - AGAGTAGA	HN	Cabbage (9week)
S21	AGGCAGAA - GTAAGGAG	Con	Aphid (12week)
S22	AGGCAGAA - ACTGCATA	Con	No Aphid (12week)
S23	AGGCAGAA - AAGGAGTA	LN	Aphid (12week)
S24	AGGCAGAA - CTAAGCCT	LN	No Aphid (12week)
S25	TCCTGAGC - TAGATCGC	HN	Aphid (12week)
S26	TCCTGAGC - CTCTCTAT	HN	No Aphid (12week)
S27	TCCTGAGC - TATCCTCT	CM	Aphid (12week)
S28	TCCTGAGC - AGAGTAGA	CM	No Aphid (12week)
S29	TCCTGAGC - GTAAGGAG	Con	Aphid (12week)
S30	TCCTGAGC - ACTGCATA	Con	No Aphid (12week)
S31	TCCTGAGC - AAGGAGTA	LN	Aphid (12week)
S32	TCCTGAGC - CTAAGCCT	LN	No Aphid (12week)
S33	GGACTCCT - TAGATCGC	HN	Aphid (12week)
S34	GGACTCCT - CTCTCTAT	HN	No Aphid (12week)
S35	GGACTCCT - TATCCTCT	CM	Aphid (12week)
S36	GGACTCCT - AGAGTAGA	CM	No Aphid (12week)

[†]Fertiliser treatment codes: **Con** = Control, **CM** = chicken manure, **LN** = Low N (synthetic), **HN** = High N (synthetic).

3.2.6 Initial processing and quality assessment of the sequence data

The resulting 16S rRNA sequence libraries were de-multiplexed and then filtered for high-quality sequences by the University of Liverpool's CGR using an in-house pipeline (developed by Dr Richard Gregory). Briefly, indexed reads were de-multiplexed using CASAVA version 1.8.2 (Illumina) and Illumina adapter sequences were removed using Cutadapt version 1.2.1 (Martin, 2011). Adapter sequences and low quality reads and reads <10 bp were trimmed and removed using Sickle version 1.200 with a minimum quality score threshold of 20. If both pairs of a read passed this filtering process, then they were assigned to either the R1 (forward reads) or R2 (reverse reads) file. In cases where only one read passed the filtering stage, it was included in the R0 (unpaired read) file. Paired-end reads were assembled into single reads using FLASh (Fast Length Adjustment of Short reads) software version 1.2.8 (Magoč and Salzberg, 2011). Given the expected amplicon length of 253bp, sequences <200 bp and >300 bp in length were removed. A summary of the number of reads before and after trimming for each sample is provided in **Table 7**. The total number of DNA sequence reads was 11,937, 928 across all 40 samples, ranging from 207, 874 (sample 40) to 401, 311 (sample 21) sequences per sample.

3.2.7 Sequencing processing of 16S rRNA libraries

The post-processing of reads (including quality control and transfer of fastq data files), and the initial steps of the bioinformatics pipeline (quality checking of the reads and definition of operational taxonomic units (OTUs)) were done by the University of Liverpool's CGR. All downstream processing of the16S rRNA sequencing results was performed with QIIME (Quantitative Insights into Microbial Ecology) software, version 1.9.1 (Caporaso *et al*, 2010). Chimeric sequences were identified and removed, and the sequences were clustered at ≥97% similarity into groups termed 'operational taxonomic units' (OTUs) using USEARCH (Edgar, 2010). Open reference OTU-picking was performed using the open reference method (*pick_open_reference_otus.py*) with Greengenes (version 13_8) as the 16S rRNA reference database clustering at 97% sequence similarity. Taxonomy was assigned using the RDP classifier (*assign_taxonomy.py*) and this was used to construct an OTU table in the 'biom' file format. The resulting sequences were aligned and filtered (*filter_alignment.py*/ *filter_otus_from_otu_table.py*) to include only those with a minimum length of 150bp and an identity of 75%. Phylogeny was created using the *make_phylogeny.py* script. The *filter_taxa_from_otu_table.py* script was used to remove singletons (sequences which only occurred once) and reads assigned as chloroplast or mitochondria from the OTU table. The resulting data file contained a total 11,490,536 sequences, with a minimum of 198, 288 sequences per sample and a maximum of 385,712 (median = 286,604; mean = 287, 263.4 sequences/sample). In order to account for varying reads per sample, the sequencing data (OTU table) was randomly subsampled using QIIME's *single_rarefaction.py* to

198, 288 sequences per sample (the lowest number of sequences in a single sample). A script of the QIIME commands used for this study is provided in **Appendix B**.

Table 7 Summary of 16S rRNA sequence data before and after adapter and quality trimming.

Sample ID	Raw reads	Trimmed reads[1] (%)[2]	R1/R2 read pairs [1]	R0 reads[1] (%[3])
S1	679,824	679,056 (99.89)	339,147	762 (0.11)
S2	592,764	591,959 (99.86)	295,592	775 (0.13)
S3	636,418	635,623 (99.88)	317,418	787 (0.12)
S4	605,060	604,275 (99.87)	301,760	755 (0.12)
S5	666,362	665,489 (99.87)	332,318	853 (0.13)
S6	436,556	435,864 (99.84)	217,607	650 (0.15)
S7	534,280	533,572 (99.87)	266,446	680 (0.13)
S8	563,650	562,920 (99.87)	281,099	722 (0.13)
S9	558,526	557,859 (99.88)	278,598	663 (0.12)
S10	560,892	560,180 (99.87)	279,736	708 (0.13)
S11	590,062	589,411 (99.89)	294,381	649 (0.11)
S12	563,480	562,730 (99.87)	280,990	750 (0.13)
S13	767,150	766,291 (99.89)	382,717	857 (0.11)
S14	617,600	616,860 (99.88)	308,064	732 (0.12)
S15	549,566	548,922 (99.88)	274,140	642 (0.12)
S16	723,202	722,441 (99.89)	360,849	743 (0.10)
S17	562,964	562,291 (99.88)	280,810	671 (0.12)
S18	560,468	559,711 (99.86)	279,483	745 (0.13)
S19	622,918	622,089 (99.87)	310,633	823 (0.13)
S20	553,544	552,733 (99.85)	275,995	743 (0.13)
S21	816,254	815,106 (99.86)	407,044	1,018 (0.12)
S22	496,938	496,309 (99.87)	247,841	627 (0.13)
S23	567,930	567,050 (99.85)	283,136	778 (0.14)
S24	621,192	620,366 (99.87)	309,773	820 (0.13)
S25	764,352	763,450 (99.88)	381,277	896 (0.12)
S26	759,238	758,392 (99.89)	378,774	844 (0.11)
S27	600,614	600,005 (99.90)	299,698	609 (0.10)
S28	612,810	612,049 (99.88)	305,645	759 (0.12)
S29	679,910	679,126 (99.88)	339,181	764 (0.11)
S30	465,116	464,618 (99.89)	232,060	498 (0.11)
S31	722,416	721,586 (99.89)	360,379	828 (0.11)
S32	592,962	592,217 (99.87)	295,743	731 (0.12)
S33	609,650	608,956 (99.89)	304,131	694 (0.11)
S34	592,190	591,529 (99.89)	295,437	655 (0.11)
S35	649,208	648,408 (99.88)	323,810	788 (0.12)
S36	479,686	479,112 (99.88)	239,269	574 (0.12)
S37	600,832	600,120 (99.88)	299,710	700 (0.12)
S38	628,452	627,719 (99.88)	313,493	733 (0.12)
S39	592,268	591,522 (99.87)	295,398	726 (0.12)
S40	421,020	420,520 (99.88)	210,014	492 (0.12)

[1] After adapter and quality trimming; [2] Percentage of the reads after adapter and quality trimming;
[3] Percentage of the trimmed reads that are singletons.

3.2.8 Statistical Analysis

Owing to the vast depth and quantity of data produced from16S rRNA NGS experiments, a range of complex and powerful statistical tools are required in order to procure comprehensive and meaningful results. There are a number of freely available bioinformatics pipelines and software designed for the processing of 16S rRNA data which provide an array of statistical and visualisation options. These meta-analysis tools are constantly evolving and improving, and as new technologies emerge, the forms of NGS analyses become increasingly complex and computationally demanding. In this study I endeavoured to use the most relevant and sophisticated analytical tools for metagenomics data available at the time. The results presented herein utilised a variety of programmes and packages in order to strengthen the interpretation of the 16S rRNA sequencing results to reveal key temporal- and treatment-related differences in soil microbial communities. The analyses can be split into two types: firstly, broader assessments of the diversity within samples (alpha) and between groups (beta); and secondly, the identification of individual taxonomic groups that differed significantly in their relative abundance between sample groups. They are adapted to address the key questions of this study which are based on three grouping factors: (i) **plant age** (9 or 12 weeks); (ii) **aphid herbivory** (+/-aphids) and (iii) **fertiliser treatment** (control, chicken manure, Low N synthetic and High N synthetic). The questions are:

1) Does the alpha diversity (i.e. species richness and diversity within samples) of the soil microbial communities differ between groups?
2) Is the beta diversity (i.e. the presence/absence and abundance of taxa) of the soil communities distinct between groups?
3) What are the main bacterial taxa responsible for these differences, if any, in beta diversity?

The methods used to answer these key questions are discussed in turn below.

Alpha (α) diversity metrics provide an estimate of species richness or diversity within individual samples. Several α-diversity metrics (Chao1 richness, Faith's phylogenetic diversity (PD_whole_tree), observed OTUs (species)) were calculated in QIIME using the *alpha_diversity.py* command with the results of *multiple_rarefactions.py* which implemented a series of subsampling (from 10 to 195,820 sequences per sample in increments of 20,000, with 10 iterations at each increments) to an even depth of 198, 288 sequences). Chao1 gives an estimate of species richness; observed species (OTUs) metric gives a basic count of the number of unique OTUs per sample; and phylogenetic distance represents the distance between samples in the phylogenetic tree. Statistically significant differences between α -diversity metrics in different treatments or sample types were detected using the *compare_alpha_diversity.py* script which employs a nonparametric two-sample t-test with 999 Monte Carlo permutations and Bonferroni multiple test correction.

Beta (β) diversity compares the bacterial community composition and abundance between samples. This is done by quantifying distances which represent the dissimilarity of samples in a coordinate context- i.e. the more similar the samples are, the shorter the distance between them. Two distance metrics are reported in this study for comparison: UniFrac and Bray-Curtis. Essentially, UniFrac distances are based on the phylogenetic tree, whereas Bray-Curtis dissimilarity distances are determined by the taxonomic composition of the community. Thus, if the differences in community structure between groups are due to taxa that are (phylogenetically) closely related, they are more likely to be detected by Bray-Curtis rather than UniFrac distances. The two methods are discussed below.

UniFrac distances are calculated according to the distance between samples on the branches of the phylogenetic tree which may be shared or unique among samples (Paliy and Shankar, 2016). The more closely related (i.e. phylogenetically similar) the samples are, the lower their UniFrac value will be. There are two types of UniFrac distances: weighted and unweighted. Weighted UniFrac distances take into account the relative abundances of OTUs when calculating distances, whereas unweighted UniFrac distance matrices represent only the presence or absence of taxa. Generally, unweighted UniFrac distance is better at detecting changes in the abundance of rare taxa, whereas weighted UniFrac is more powerful in picking up differences in more abundant OTUs (Chen *et al*, 2012). These β diversity indices were calculated in QIIME from the rarefied OTU table using the *jackknifed_beta_diversity.py*, to a maximum depth of 190, 000. Distance matrices constructed using both weighted and unweighted UniFrac distance metrics were visualised in PCoA plots using the online NGS tool EMPEROR (Vázquez-Baeza *et al*, 2013). Additionally, UPGMA (Unweighted Pair Group Method with Arithmetic Area) trees were constructed from the weighted and unweighted UniFrac distances in Fig Tree v1.4.2. These phylogenetic trees were constructed using representative, aligned, midpoint filtered OTU sequences using the FastTree algorithm (Price *et al*, 2009).

Bray-Curtis distances are not based on phylogeny, but instead are based on community composition and the changes in abundance of the most common OTUs. Bray-Curtis metrics are a popular method for exploring large microbial ecological data sets as they account for the fact that many species are rare, and may be missing from many samples (null values in the data set). Statistical differences between the β -diversities of soil communities according to treatment and sample type were evaluated using the *vegan*, *phyloseq* and *ggplot2* packages in R.

β -diversities were examined according to the four (broad) methods recommended by Anderson and Willis (2003) when analysing multivariate ecological data: (i) an unconstrained ordination method; (ii) a constrained analysis plot; (iii) a statistical test of the main hypothesis; (iv) characterisation of the main taxa responsible for the observed effects. They are discussed in further detail over the following pages.

(i) *Unconstrained ordination*

Three different distance-based unconstrained ordination methods were employed in this study to compare their outcomes. They comprised principal coordinate analysis (PCoA), non-metric multidimensional scaling (NMDS), and detrended correspondence analysis (DCA) (Anderson and Willis, 2003). PCoA is one of the most frequently used, classical ordination analysis techniques (Paliy and Shankar, 2016). PCoA can be applied to any dissimilarity matrix whether it is based on phylogenetic distances or community composition (i.e. Bray-Curtis or UniFrac).

Non-metric multidimensional scaling (NMDS) is a strong analytical tool, which is often recommended over PCoA, as it makes no assumptions of multivariate normality and is non-parametric. It is a versatile approach as it accepts any type of similarity matrix (UniFrac, Bray-Curtis etc.) and can handle missing data (null) values. Perhaps most importantly, NMDS can reduce the data to fewer axes (2 or 3) than PCoA, thereby avoiding the loss of variation associated with ordination methods (such as PCoA) that produce many axes (Paliy and Shankar, 2016). Rather than using absolute abundances, NMDS works by assigning ranks to the distances (using the chosen metric), which are then spatially ordinated according the selected number of axes to reflect the differences in rank (Legendre and Birks, 2012). The resulting plot highlights differences in bacterial community composition between sample groups. A "stress" value between 0 and 1 is produced in association with the NMDS which represents the goodness of fit, and should lie between 0.05 and 0.3 for a good representation. As this is an iteration-based process, repeated runs of the NMDS were performed to attain the most satisfactory (lowest) stress value (Ramette, 2007). This was done using the *metaMDS* function in the vegan package in R.

The third and final method of unconstrained ordination used was detrended correspondence analysis (DCA). Again this is often a favoured community ordination approach over PCoA since it is a more robust method. DCA is an eigenvector-based technique which eliminates the so-called "arch-effect" incurred by correspondence analysis (CA) and PCoA by detrending (Paliy and Shankar, 2016). CA is based on a unimodal model - the underlying model of species distributions - and as such can be regarded as more representative of community ecology. De'ath (1999) proposed that ordination methods can be divided into two classes: "species composition restoration" (e.g. NMDS) and "gradient analysis" (e.g. DCA). The DCA plot can be used to determine the most appropriate method for constrained ordination – if the longest DCA axis is <3 then a linear method should be used.

(ii) *Constrained ordination*

Constrained ordination analysis aims to determine the axes in the data set which show the strongest associations between explanatory and response variables by *constraining* the axes of the ordination to fit the explanatory variables (Paliy and Shankar, 2016). In other words, they visualise the relationship between response variables (in this case, the soil microbial community) and predictor

variables (time-point or fertiliser treatment). Three methods of constrained ordination were compared: distance-based redundancy analysis (dbRDA), canonical correspondence analysis (CCA), and canonical analysis of principal coordinates (CAP).

Distance-based redundancy analysis (dbRDA) is a type of redundancy analysis (RDA) which incorporates dissimilarity (distance) matrices (Paliy and Shankar, 2016). RDA is linear method, meaning that it assumes a linear relationship between variables.

Canonical correspondence analysis (CCA) aims to find correlations between two variables, irrespective of whether they are explanatory or response variables (Paliy and Shankar, 2016). It uses a similar approach to RDA, with the main distinction being that instead of linear models, CCA is based on unimodal relationships between OTUs and environmental factors (Ramette, 2007). CCA is a popular choice of multivariate analysis by ecologists as it copes well with data sets containing unequal ranges, bimodal response and rare species (Ramette, 2007).

Canonical analysis of principal coordinates (CAP) uses principal coordinate analysis in conjunction with canonical discriminant analysis to compare differences between groups using constrained ordination (Anderson and Willis, 2003). An advantage of using CAP is that it can show the interactive effects of different variables on the community composition.

All of the above mentioned methods were tested in turn in an effort to detect emerging trends and to minimise the chances of missing masked effects, which can easily occur when dealing with datasets of this amplitude. This chapter reports the most informative of these methods, with the additional ordination plots being provided in **Appendix B**.

(iii) *Statistical tests*

Finally, statistically significant differences between the beta diversity distance matrices of sampling groups (time-point and fertiliser treatment) were tested for using ADONIS (a permutated ANOVA) with both types of dissimilarity matrices (Bray Curtis and both weighted and unweighted UniFrac), with 999 permutations. This was followed with a test of permutated dispersion using (PERMDISP) the *betadisper* function in the *vegan* package in R. PERMANOVA is a non-parametric test to identify significant differences between groups based on a distance matrix (Paliy and Shankar, 2016). It is a highly popular statistical method in microbial ecology (Tang *et al*, 2016). PERMANOVA was selected in preference to alternatives such as ANOSIM and Mantel, as it is considered to be a more robust test to heterogeneity, especially when supported by PERMDISP (Anderson and Walsh, 2013). In this chapter I compare the outcomes of PERMANOVA using both UniFrac and Bray-Curtis distances.

The alpha and beta diversity measures were used to test the influence of three candidate explanatory factors of the soil microbial community:

- **Cabbage age**: the influence of plant growth was explored by comparing the rhizosphere communities of plants harvested at 9 and 12 weeks (no aphids);
- **Aphid herbivory**: tested for differences in the rhizosphere soil communities of aphid-infested and uninfested 12 week-old plants;
- **Fertiliser treatment:** tested for the effect of fertiliser treatments on rhizosphere communities at 9 weeks and at 12 weeks.

(iv) *Identification of taxa responsible for differences*

The DESeq2 package (Love *et al*, 2014) was used to detect pairwise differences in taxonomic abundances based on cabbage age (9 weeks or 12 weeks no aphids), aphid presence (12 weeks, yes or no) and fertiliser treatment (pairwise between control, chicken manure, low N, high N at 9 or 12 weeks). Differences were deemed significant is they met two criteria: (i) log2 fold change threshold of 1.2 and (ii) *p*-value cut-off of 0.05 (adjusted for false discovery rate using Benjamini-Hochberg correction), thus limiting it to taxa which differed by at least 20% with a 2% chance of false positive identification.

3.3 Results

3.3.1 Soil and fertiliser nutrient analysis

The WHC for 100g of oven-dried soil was calculated to be 67.38ml at 100% WHC. The soil had a pH of 7.38 and a total N content of 0.34% w/w and total C content of 10.8% w/w (NRM laboratories).

3.3.2 16S rRNA Sequencing summary

After assembly and quality filtering, the total number of 16S rRNA sequence reads obtained from the 40 soils samples was 11, 490, 536, which comprised 82, 460 OTUs. The number of sequences per sample ranged from 198, 288 to 385,712 sequences per sample (median = 286, 604, mean = 287263.4, standard deviation = 42642.098, **Table 8**). The average number of reads per sample was 8.6% lower for bulk soil samples (mean= 271, 986.63) in comparison to rhizosphere samples (mean= 297, 447.92). Prior to further analysis, all samples were rarefied to an even depth of 198,288 sequences to account for differences in sequencing depth. There has been considerable debate among microbiologists as to whether rarefaction is appropriate in 16S rRNA data analysis (a particularly strong critique is given in McMurdie and Holmes (2014)). It was decided in this case that the disparity in sequence number between samples meant that the risk of bias merited the rarefying of the dataset. The rarefied OTU table contained 79,579 taxa across all samples, consisting of 50 phyla, 140 classes, 214 orders, 260 families, 408 genera and 117 species of bacteria and archaea (**Figure 13**). The small number of species relative to genera is caused by several factors including the inability for the OTU assignment to resolve this level of resolution in many cases owing to insufficient read lengths, and also the fact that there are many bacterial species which have yet to be identified and classified.

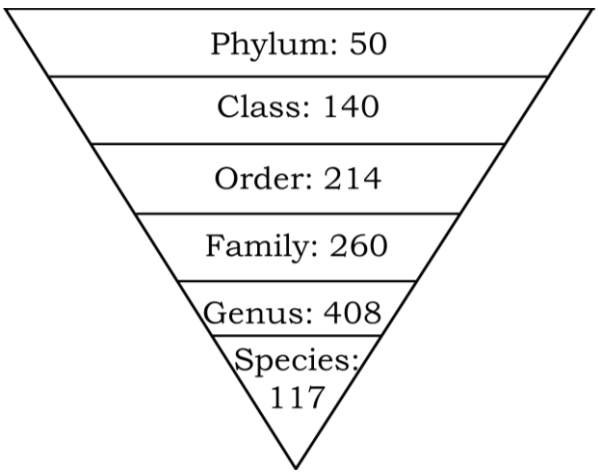


Figure 13 The number of OTUs assigned to each taxonomic rank.

Table 8 Sample descriptions and the total number of sequences in each (after filtering).

Sample ID	Treatment	Sample type	Soil type	Counts/sample	Ave. counts/sample per sample type
S1	Baseline	Baseline	Bulk	322253	
S2	Baseline	Baseline	Bulk	279937	
S3	Baseline	Baseline	Bulk	301315	
S4	Baseline	Baseline	Bulk	286962	
S5	Baseline	Baseline	Bulk	318839	
S6	Baseline	Baseline	Bulk	207419	
S7	Baseline	Baseline	Bulk	253606	
S8	Baseline	Baseline	Bulk	267539	**279, 733.75**
S10	CM	Fertiliser	Bulk	264548	
S11	LN	Fertiliser	Bulk	265572	
S12	HN	Fertiliser	Bulk	261862	
S37	Control	Fertiliser	Bulk	287524	
S38	Control	Fertiliser	Bulk	300154	
S39	LN	Fertiliser	Bulk	279857	
S40	LN	Fertiliser	Bulk	198288	
S9	Control	Fertiliser	Bulk	256111	**264, 239.5**
S13	Control	Cabbage	Rhizosphere	368191	
S14	Control	Cabbage	Rhizosphere	295311	
S15	CM	Cabbage	Rhizosphere	264732	
S16	CM	Cabbage	Rhizosphere	346837	
S17	LN	Cabbage	Rhizosphere	263452	
S18	LN	Cabbage	Rhizosphere	258574	
S19	HN	Cabbage	Rhizosphere	291736	
S20	HN	Cabbage	Rhizosphere	257557	**293, 298.75**
S22	Control	No aphids	Rhizosphere	234855	
S24	LN	No aphids	Rhizosphere	294175	
S26	HN	No aphids	Rhizosphere	364435	
S28	CM	No aphids	Rhizosphere	290459	
S30	Control	No aphids	Rhizosphere	221798	
S32	LN	No aphids	Rhizosphere	286246	
S34	HN	No aphids	Rhizosphere	284175	
S36	CM	No aphids	Rhizosphere	229438	**275, 697.625**
S21	Control	Aphid infested	Rhizosphere	385712	
S23	LN	Aphid infested	Rhizosphere	267014	
S25	HN	Aphid infested	Rhizosphere	368112	
S27	CM	Aphid infested	Rhizosphere	288203	
S29	Control	Aphid infested	Rhizosphere	324749	
S31	LN	Aphid infested	Rhizosphere	346968	
S33	HN	Aphid infested	Rhizosphere	294184	
S35	CM	Aphid infested	Rhizosphere	311837	**323, 347.375**

Fertiliser treatment codes: **Con** = Control, **CM** = chicken manure, **LN** = Low N (synthetic), **HN** = High N (synthetic).

3.3.3 Soil bacterial community composition and relative abundance

The ten most dominant bacterial phyla across all soil samples in descending order were (mean proportion, and range across all samples): *Proteobacteria* (27.22%, 23.0-37.7%), *Acidobacteria* (25.91%, 16.1-34.8%), *Planctomycetes* (9.50%, 5.8-11.1%), *Actinobacteria* (8.29%, 5.2-14.5%), *Bacteroidetes* (6.96%, 3.5-14.7%), *Chloroflexi* (5.96%, 4.7-9.0%), *Verrucomicrobia* (5.09%, 3.2-6.0%), *Gemmatimonadetes* (2.33%, 1.5-3.0%), *WS3* (2.10%, 1.2-3.3%) and *Nitrospirae* (1.37%, 0.6-1.9%) (**Table 9**). Accumulatively, these ten groups represented 94.73% of total bacterial abundance. The fertilised soils had higher proportions of *Proteobacteria* and *Bacteroidetes* in comparison to control soils (**Figure 14**). Relative to control soils, the fertilised soils had a lower abundance of *Acidobacteria*, the chicken manure soil in particular. The relative abundance of *Acidobacteria* increased over time, from a mean relative abundance of 25.97% for baseline (bulk) soil, to 26.01% in 9 week-old cabbage rhizospheres, finally rising to 28.3% in the rhizospheres of aphid-infested cabbages (**Figure 15**). *Actinobacteria* abundance was lower in the 12 week-old cabbage rhizospheres in comparison to the previous +Cabbage sampling point, whereas *Bacteroidetes* increased in relative abundance over the growth of the cabbage.

Table 9 The mean abundance and range of the ten most dominant phyla across all samples.

Phylum	Mean relative abundance (%)	Range across all samples (%)
Proteobacteria	27.22	23.0-37.7
Acidobacteria	25.91	16.1-34.8
Planctomycetes	9.50	5.8-11.1
Actinobacteria	8.29	5.2-14.5
Bacteroidetes	6.96	3.5-14.7
Chloroflexi	5.96	4.7-9.0
Verrucomicrobia	5.09	3.2-6.0
Gemmatimonadetes	2.33	1.5-3.0
WS3	2.10	1.2-3.3
Nitrospirae	1.37	0.6-1.9

The most abundant (classified) genera detected across all samples were *Candidatus Nitrososphaera* (1.0%), *Flavobacterium* (0.85%), *Rhodoplanes* (0.58%), *Kaistobacter* (0.58%), *Limnobacter* (0.57%), *Pirellula* (0.54%), *Gemmata* (0.52%), *Candidatus Xiphinematobacter* (0.48%), DA101 (0.46%) and *Lysobacter* (0.41%) (**Figure 16**). OTUs assigned to *Candidatus*, or candidate, divisions are lineages of bacteria that have not been formally ranked in the International Code of Nomenclature of Bacteria and are known only from environmental sequencing studies (Hugenholtz *et al*, 1998).

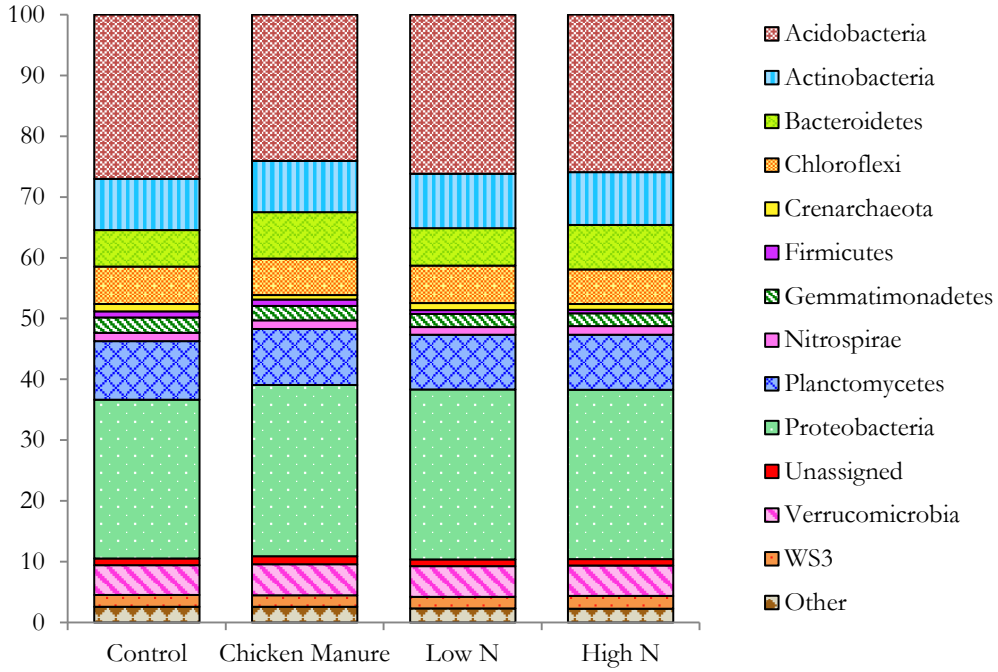


Figure 14 Mean relative abundance (%) of the major bacterial phyla in soil samples from each fertiliser treatment (+Fertiliser, +Cabbage & +/- Aphids samples). All phyla with relative abundances >1% are represented individually, with the remaining phyla grouped together as "Other".

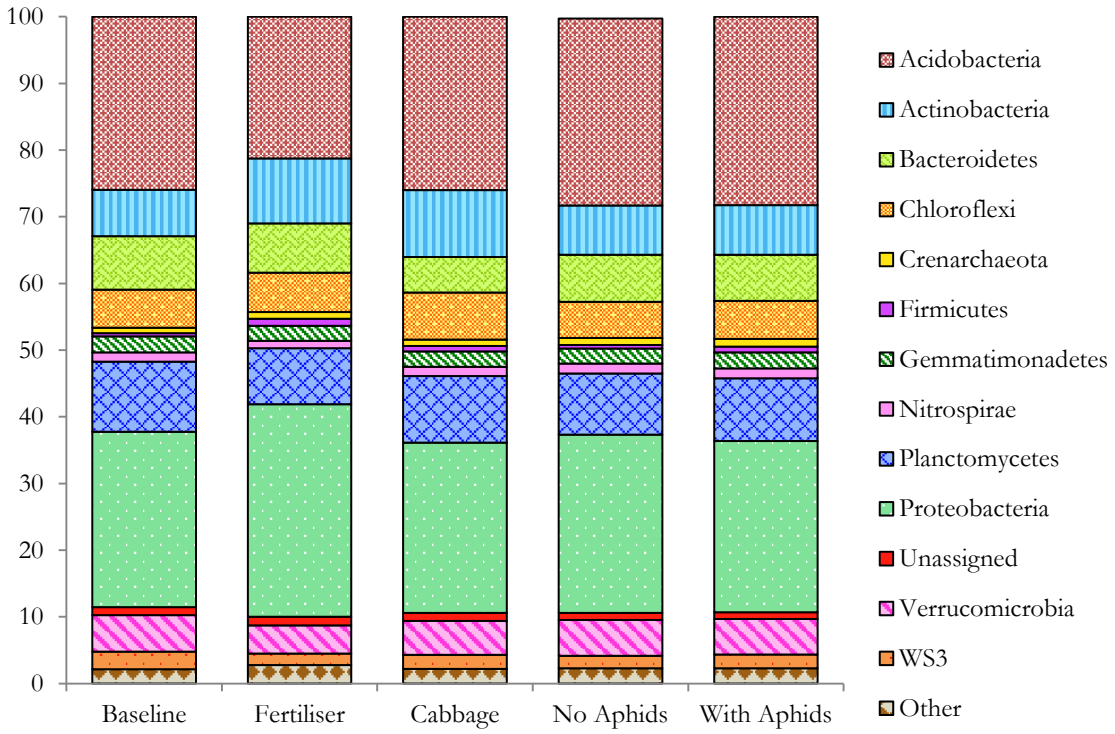


Figure 15 Mean relative abundance (%) of the most common bacterial phyla in soil samples for each sample type. All phyla with relative abundances >1% are represented individually, the rest are grouped as "Other".

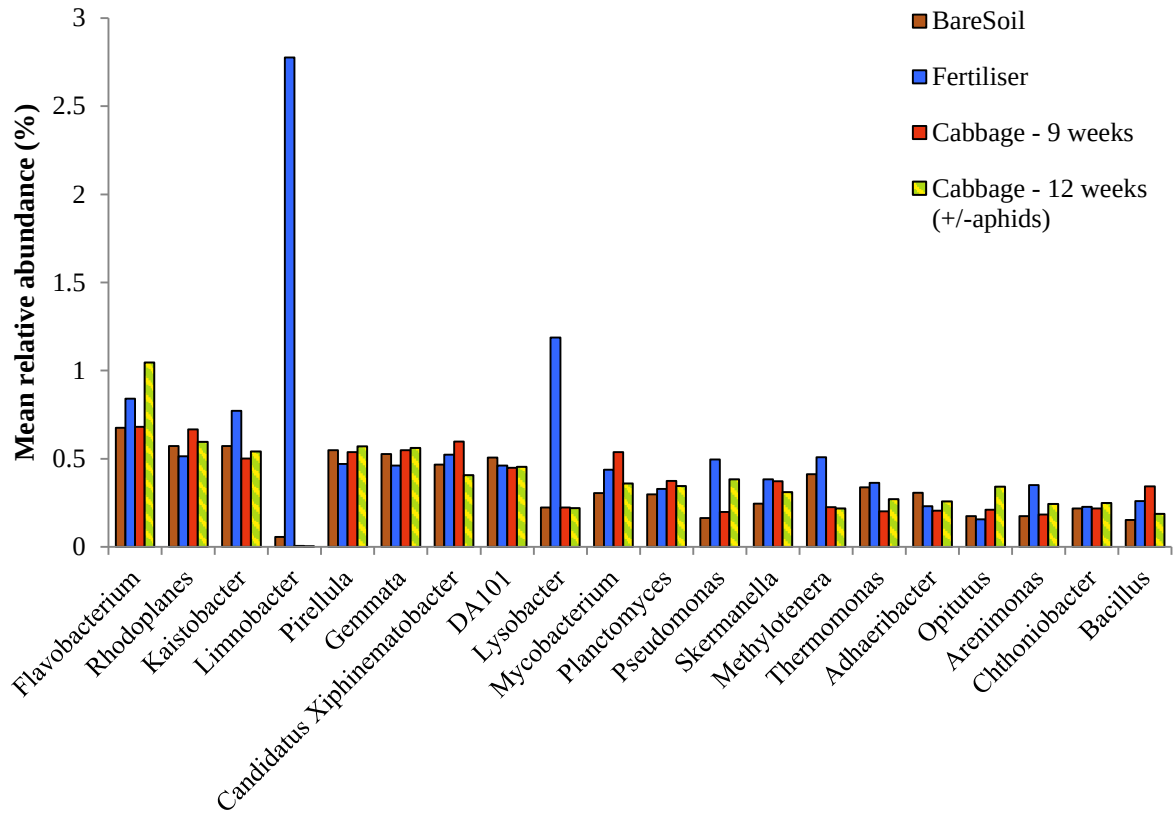


Figure 16 Mean relative abundance of the top 20 most abundant genera from each sampling stage (classified genera only).

At the genus level, soil samples taken after the fertiliser additions were particularly abundant in the genera *Limnobacter* and *Lysobacter* (**Figure 16**). The elevated abundance of *Limnobacter* in the fertilised bulk soil group was attributed to both low and high N samples (S11, S39, S40 and S12), as well as one of the controls (S9), whilst the peak in *Lysobacter* was solely attributed to the chicken manure sample (S10).

3.3.4 Diversity and richness of bacterial communities

3.3.4.1 Alpha Diversity

Alpha (α) diversity metrics, which provide a measure of species richness or diversity within samples, were calculated). There was no significant effect of fertiliser additions on α-diversity for any of the metrics employed (p>0.05), however, the Low N and High N treatment consistently had the lowest values for each α-diversity measure for 9 and 12 week-old plants respectively (**Figure 17**). There were no significant differences in the α-diversity when testing for the effects of cabbage age (**Table 10**) or aphid presence (**Table 11**). However, older (12 week) and aphid-infested cabbages were both associated with lower α-diversities in comparison to younger, aphid-free plants according to all three metrics used.

Table 10 Alpha diversity metrics at cabbage growth stages (9 and 12 week-old cabbages) using a nonparametric two-sample t-test using Monte Carlo permutations (Mean (*±SD*)).

	9 weeks		**12 weeks***		**t stat**	***p*-value**
Chao1	24194.51	*±1879.53*	22859.90	*±1950.05*	*1.3038*	0.2220
Faith's PD	811.63	*±67.71*	789.80	*± 47.85*	0.6965	0.4890
Observed OTUs	15869.56	*±1053.81*	15238.43	*±954.38*	1.1745	0.2680

* no aphids.

Table 11 Alpha diversity metrics for aphid-infested and aphid-free 12 week-old cabbages (Mean (*±SD*)) using a nonparametric two-sample t-test using Monte Carlo permutations.)

	With Aphids		**No Aphids**		**t stat**	***p*-value**
Chao1	22007.20	*±1724.79*	22821.11	*±2034.33*	*-0.8074*	0.4460
Faith's PD	770.08	*±40.33*	790.00	*±48.30*	-0.8376	0.4230
Observed OTUs	14818.43	*±833.26*	15238.49	*±957.59*	-0.8755	0.4010

There was also no significant effect of fertiliser treatment detected in soils from plants of either age cohort. However, the synthetically fertilised soils did consistently exhibit the lowest α-diversity metrics (**Figure 17**).

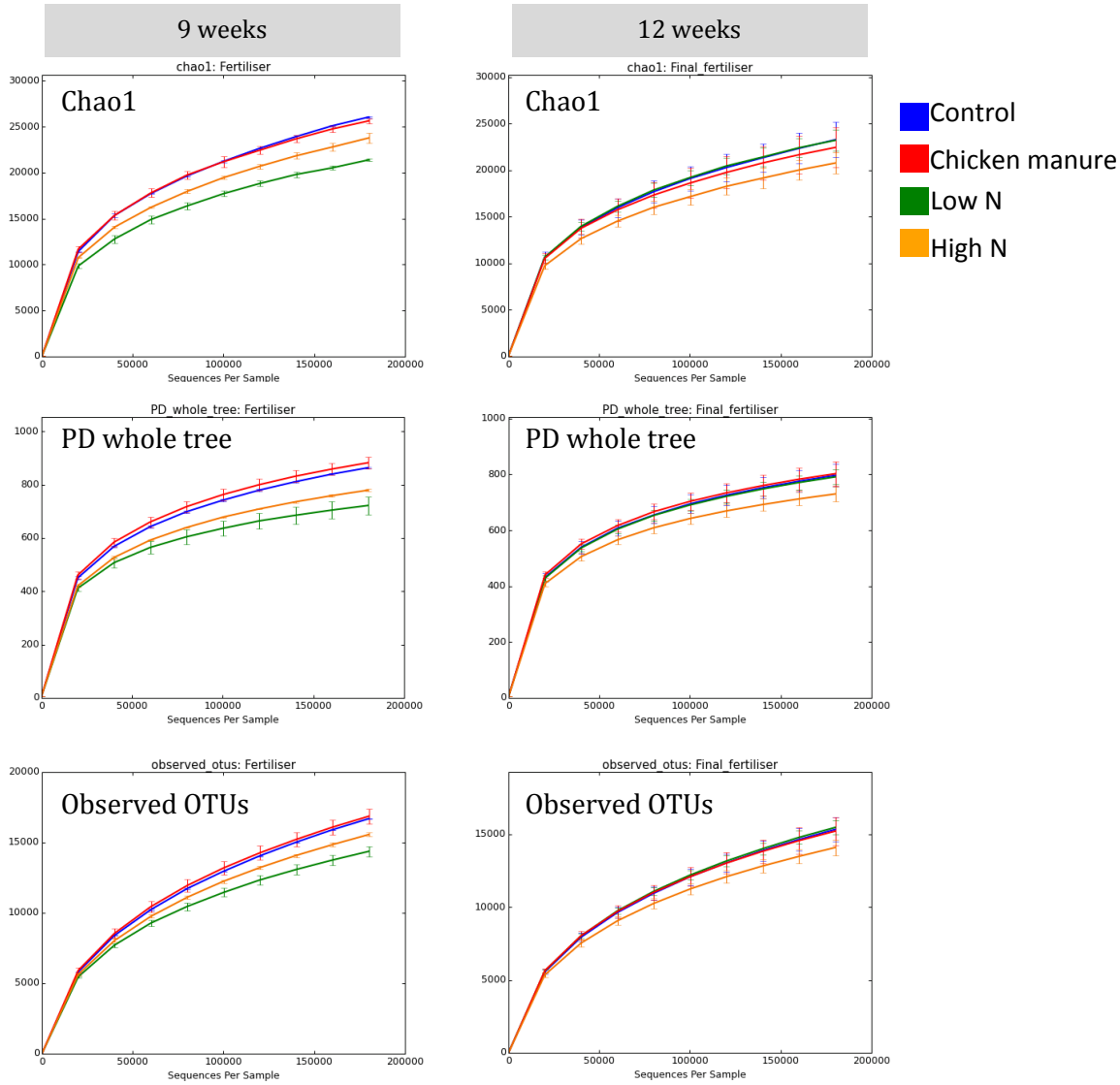


Figure 17 Rarefaction curves showing the mean values of alpha diversity metrics (Chao1 richness, observed species, phylogenetic distance (PD whole tree)) grouped by fertiliser treatment for 9 week (left) and 12 week (right) plants.

3.3.4.2 Beta Diversity

(i) Unconstrained ordination

β-diversity provides a measure of the similarities of bacterial communities from different groupings. The first *unconstrained* ordination methods used to assess beta diversity was **Principal Coordinate analysis (PCoA)** based on UniFrac distances calculated from the rarefied OTU table (198, 288 sequences/sample) (**Figure 18**). These plots were created in QIIME and visualised using EMPEROR. PCoA plots constructed using weighted UniFrac and Bray-Curtis distances are provided in **Appendix B**. Bray-Curtis distances account only for differences in species abundances

rather than their relatedness, and consequently can produce quite different results to UniFrac-based plots

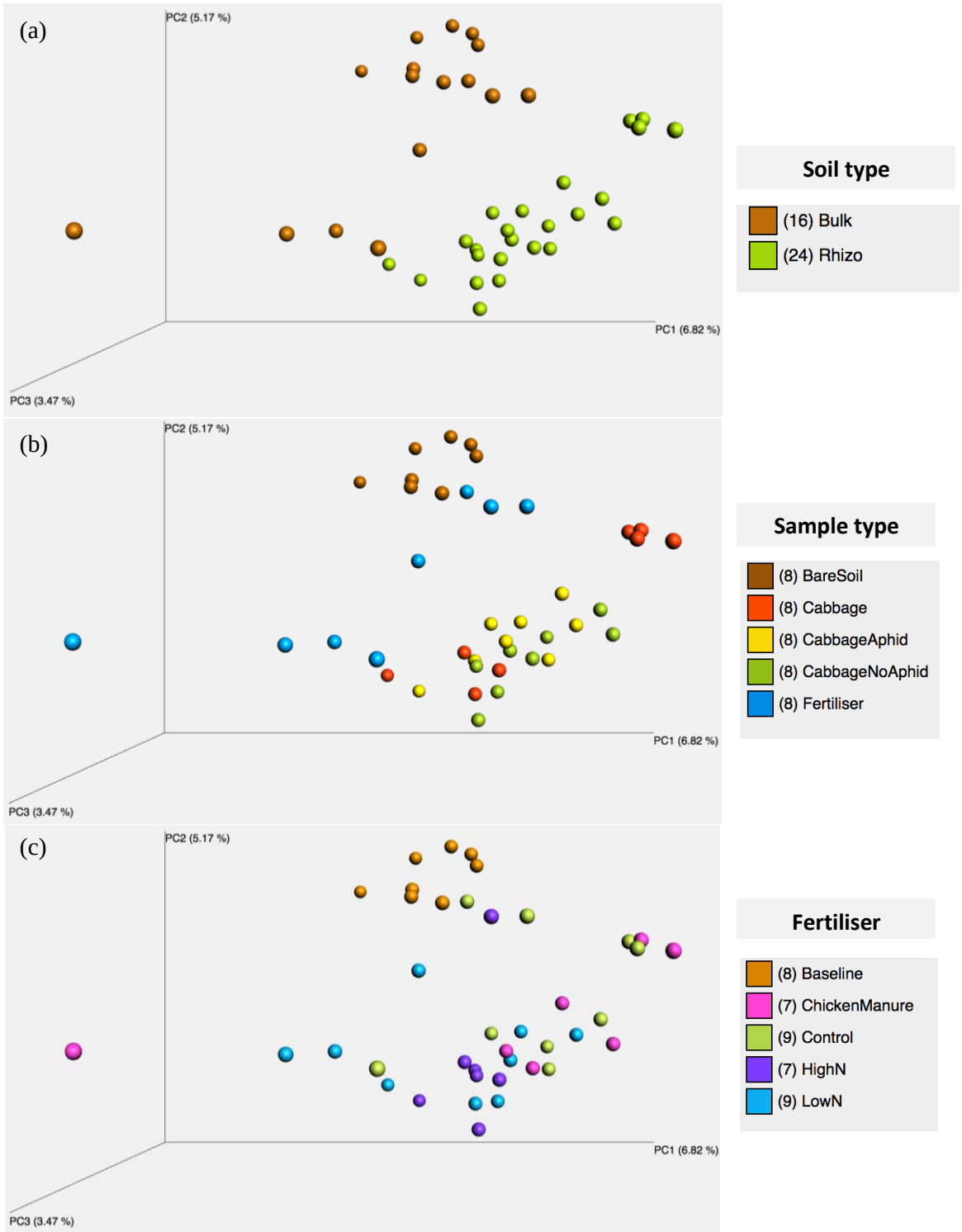


Figure 18 PCoA plots constructed using unweighted UniFrac distances with samples grouped by (a) soil type, (b) sample type and (c) fertiliser. (N.B. the bulk and rhizosphere soil types were sampled at different times.) PC1: 6.82%; PC2: 5.17% and PC3: 3.47%.

The unweighted UniFrac PCoA plots showed clear distinctions between samples grouped by sample type but not by fertiliser. The bulk soil samples (baseline and fertiliser) diverge from the

rhizosphere samples (although note that bulk and rhizosphere samples were collected at different times). The chicken manure +Fertiliser sample (S10) stands out as an outlier, suggesting that this community is dissimilar to the other +Fertiliser samples. PCoA plots comparing the effects of fertilisers and aphid herbivory on 12 week-old rhizospheres and the effect of cabbage age are given in **Appendix B**. They both show a distinct grouping of synthetic fertilisers away from control and CM soils, but there is little support for an effect of cabbage age or aphid herbivory.

Detrended correspondence analysis (DCA) was performed using UniFrac distances and was indicative of divergence in the bacterial community composition samples grouped by sample type (**Figure 19**). The DCA analysis again indicated a distinct grouping of the samples according to soil type, with clear separation of the rhizosphere and bulk soil samples (although note that these were collected at different times). The main areas of overlap occurred between the aphid and no aphid samples, which were taken from the rhizospheres of cabbages harvested at the same time (12 weeks), which appear to diverge from the 9-week cabbage rhizosphere samples. This indicates, therefore, that the rhizosphere community changes during plant development.

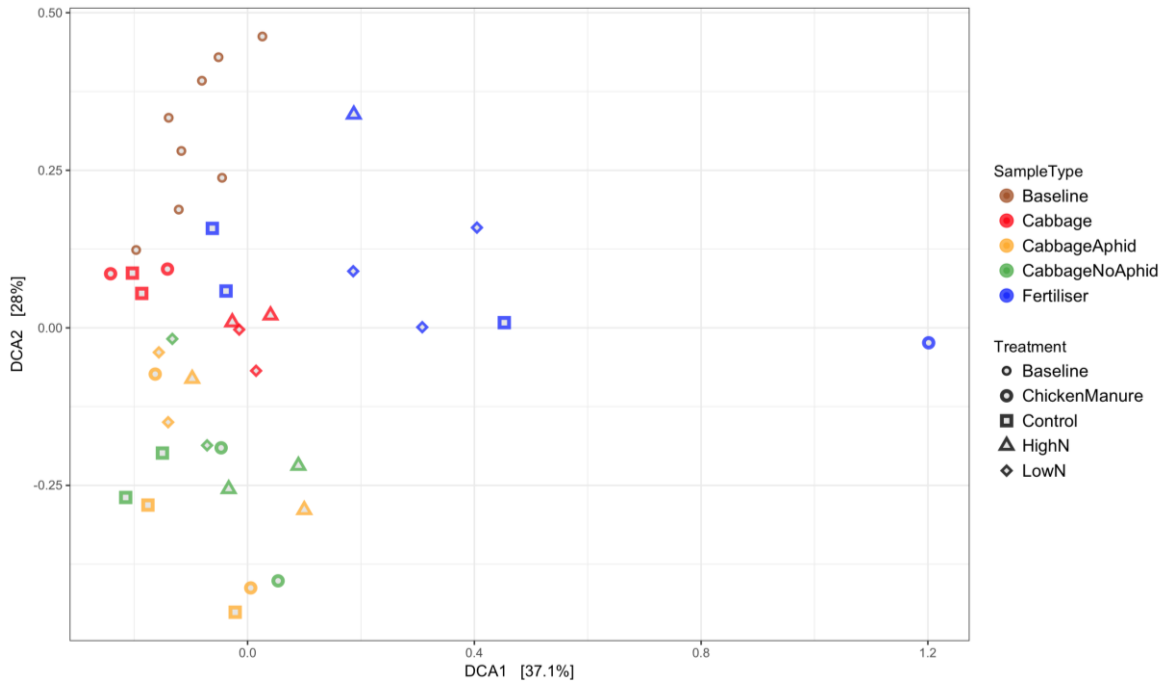


Figure 19 Detrended correspondence analysis (DCA) of beta diversity for all soil samples (*n*=40). Note the outlying S10 sample (CM bulk soil). Axes DCA1: 37.1%; DCA2: 28%.

(ii) Constrained ordination

The DCA plot indicated that linear ordination methods should be since the first DCA axis was less than 3 (**Figure 19**). The chosen method of constrained (linear) ordination was canonical analysis of principal coordinates (CAP) using unweighted UniFrac distances (**Figure 20**). When using sample type as the predicting factor, the rhizosphere samples clustered together in the CAP plot, while the bulk soil samples form two distinct groups comprising pre- and post-fertilisation samples.

When performing the analysis with fertiliser treatment as the grouping factor, the CAP plot showed the synthetically fertilised soils (low N and high N) diverging away from the control and organically fertilised (chicken manure) soils. When applying Bray-Curtis distances, the CAP plot also supports the clustering of High and Low N groups, but indicates a more distinct separation of chicken manure-treated soils from the other treatments (**Appendix B: Figure 46**). In both CAP plots it is interesting to note that the Low N and Chicken Manure treatment groups do not overlap.

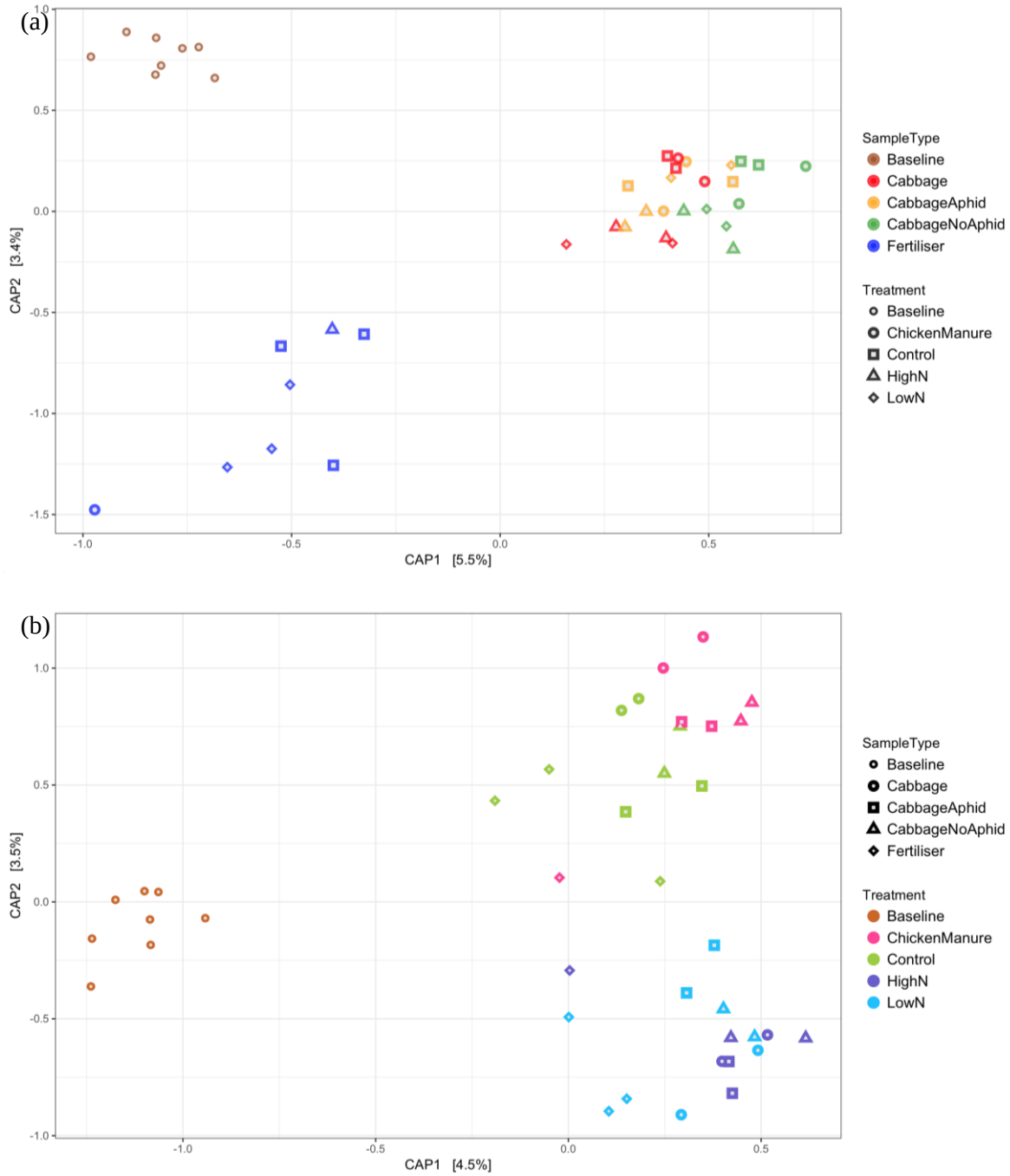


Figure 20 Canonical analysis of principal coordinates (CAP) plots created using **unweighted UniFrac** measures grouped by (a) sample type and (b) fertiliser treatment. PERMANOVA with 9999 permutations: (a) Sample type: p =0.0001, $F_{4, 35}$ =1.4271, SS = 0.5802); (b) Fertiliser treatment: p = 0.0001, $F_{4, 35}$ = 1.3089, SS = 0.5384).

UPGMA trees were constructed using UniFrac distances, assigning branch colours according to time-point (**Figure 21**). They support the trends observed in the ordination plots, with the cabbage samples grouping together by age (red branches = 9 weeks; green/yellow=12 weeks).

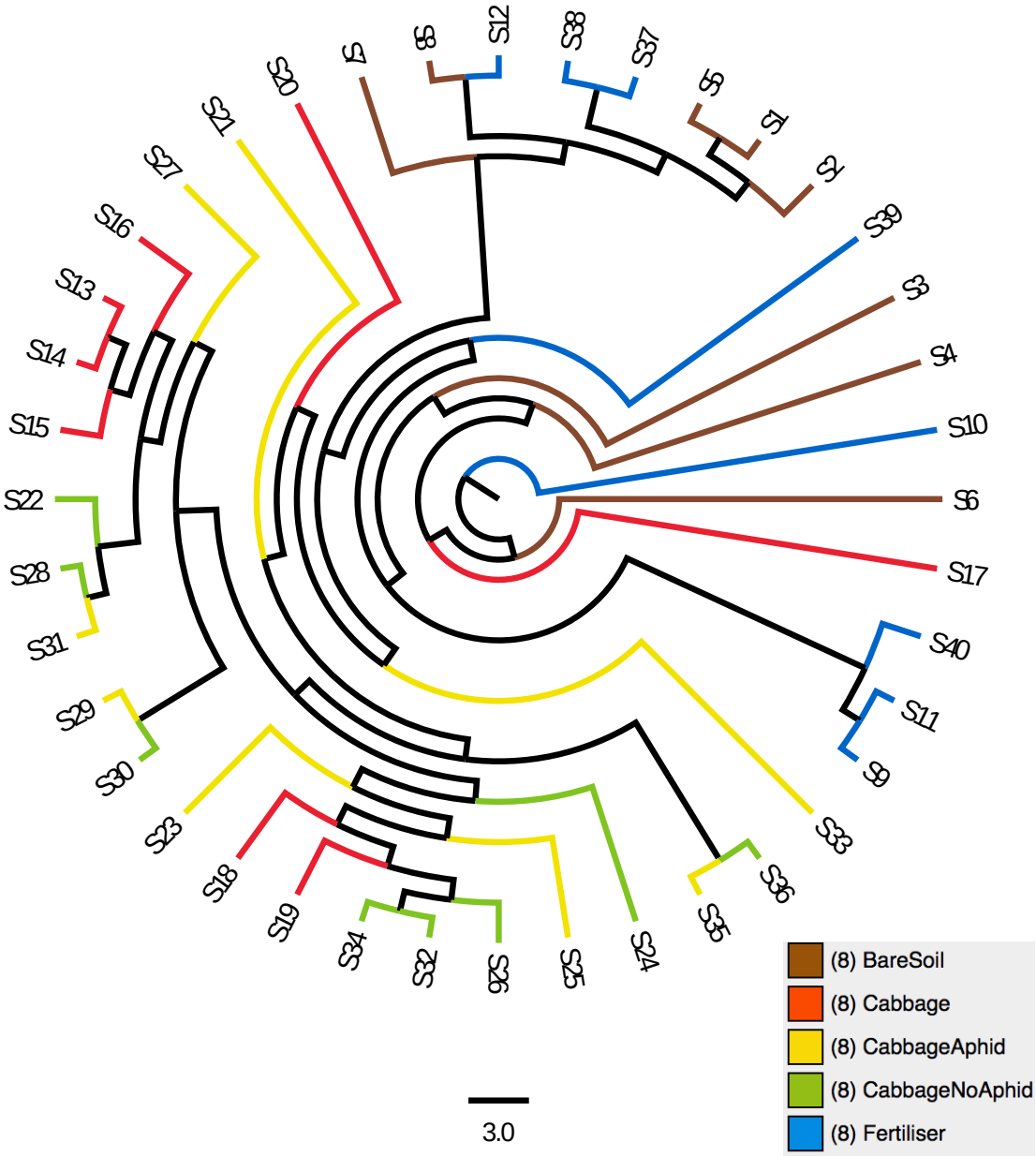


Figure 21 Beta diversity of soil samples group by sample type, depicted using UPGMA (unweighted pair-group method with arithmetic mean) hierarchical clustering created using the unweighted UniFrac distance matrix and visualised using Fig Tree v1.4.2, radial and rectangular tree layout. There is a clear separation of early bulk soil samples (brown and blue branches) and later rhizosphere soils (yellow and green branches). (Note the chicken manure fertiliser sample (S10) branching off from the others.)

(iii) Statistical analysis

Permutational multivariate analysis of variance (PERMANOVA, also known as Adonis) (Anderson, 2001) was performed using the *adonis* function in the *vegan* package in R to test for significant differences in beta diversity. ADONIS is a nonparametric method to determine the significance of grouping variables in determining distances within a distance matrix. The null hypothesis tested by PERMANOVA is that "the centroids of the groups, as defined in the space of the chosen resemblance measure, are equivalent for all groups" (Anderson and Walsh, 2013). The tests were performed for each grouping factor (cabbage age, herbivory and fertiliser treatment) using both weighted and unweighted UniFrac distances, in addition to Bray-Curtis distances. Cabbage age, fertiliser treatment and their interaction were all found to have a significant effect on community composition according to all distance metrics used, with the exception of unweighted UniFrac for which no significant interaction was detected (**Table 12**). There was no significant effect of aphid presence on beta diversity (**Table 13**).

Table 12 Results of permutational multivariate analysis of variance (PERMANOVA) analysis of dissimilarities for bacterial OTU community structure in relation to cabbage age, fertiliser treatment and their interaction using UniFrac and Bray-Curtis distances.

Diversity metric	Statistic	Cabbage age (9 vs 12wk no aphids)	Fertiliser	Cabbage age * Fertiliser
Unweighted UniFrac	Df	1, 15	3, 15	3, 15
	SS	0.1112	0.3446	0.2851
	MS	0.1112	0.1149	0.0950
	F-value	1.2374	1.2779	1.0574
	R^2	0.0762	0.2360	0.1953
	p-value	**0.026 ***	**0.001 *****	0.17
Weighted UniFrac	Df	1, 15	3, 15	3, 15
	SS	0.0603	0.0687	0.0682
	MS	0.0603	0.0229	0.0227
	F-value	5.8009	2.2007	2.1841
	R^2	0.2152	0.2449	0.2431
	p-value	**0.001 *****	**0.016 ***	**0.011 ***
Bray-Curtis	Df	1, 15	3, 15	3, 15
	SS	0.08361	0.1602	0.1311
	MS	0.08361	0.0534	0.0437
	F-value	2.5576	1.6331	1.3365
	R^2	0.13139	0.2517	0.2060
	p-value	**0.001 *****	**0.001 *****	**0.033 ***

Df = degrees of freedom; SS = sum of squares; MS= mean sum of squares; F-value = F value by permutation; R^2 = % variation explained. Asterisks indicate statistical significance ($p < 0.05$); *p*-values are based on 999 permutations (i.e. the lowest possible *p*-value is 0.001).

Table 13 Results of permutational multivariate analysis of variance (PERMANOVA) analysis of dissimilarities for bacterial OTU community structure of 12 week cabbage rhizospheres in relation to herbivory (+/- aphids), fertiliser treatment and their interaction using UniFrac and Bray-Curtis distances.

Diversity metric	Statistic	Herbivory (+/-aphids)	Fertiliser	Herbivory * Fertiliser
Unweighted UniFrac	Df	1, 15	3, 15	3, 15
	SS	0.0945	0.3232	0.2755
	MS	0.0945	0.1077	0.0918
	F-value	1.0128	1.1541	0.9838
	R^2	0.0657	0.2244	0.1913
	p-value	0.335	**0.001**	0.678
Weighted UniFrac	Df	1, 15	3, 15	3, 15
	SS	0.0095	0.0804	0.0324
	MS	0.0095	0.0268	0.0108
	F-value	0.7984	2.2632	0.9134
	R^2	0.0436	0.3705	0.1495
	p-value	0.609	**0.005 ****	0.587
Bray-Curtis	Df	1, 15	3, 15	3, 15
	SS	0.0339	0.1752	0.0986
	MS	0.0339	0.0584	0.0329
	F-value	0.9365	1.6137	0.9088
	R^2	0.0567	0.2933	0.1652
	p-value	0.601	**0.001 *****	0.789

Df = degrees of freedom; SS = sum of squares; MS= mean sum of squares; F-value = F value by permutation; R^2 = % variation explained. Asterisks indicate statistical significance ($p < 0.05$); *p*-values are based on 999 permutations (i.e. the lowest possible *p*-value is 0.001).

A permutation test for homogeneity of multivariate dispersions (PERMDISP) (Anderson, 2006) was subsequently used to test for multivariate homogeneity of dispersions for each of the groups which yielded significant PERMANOVA results (**Table 14**). The null hypothesis for PERMDISP assumes that "the average within-group dispersion (measured by the average distance to group centroid and as defined in the space of the chosen resemblance measure), is equivalent among the groups" (Anderson and Walsh, 2013). A non-significant PERMDISP result indicates that any significant PERMANOVA results can be confidently assumed to be attributed to differences in their centroids (i.e. the central location of a group of samples within the distance matrix). If the PERMDISP result is significant, then it is possible that a significant PERMANOVA result was generated due to unequal variation in the dispersion of the communities, rather than structural/compositional contrasts in their communities (Erwin *et al*, 2012). This test was performed using the betadisper function in the vegan package in R.

Table 14 Permutation test for homogeneity of multivariate dispersions (PERMDISP) of bacterial communities grouped by cabbage age, aphid herbivory and fertiliser treatment (999 permutations).

Grouping	Distance measure	D.F.	F-value	*p*-value
Fertiliser (+/- aphids 12 weeks)	Unweighted UniFrac	3, 12	0.182	0.904
Fertiliser (+/- aphids 12 weeks)	Weighted UniFrac	3, 12	0.642	0.617
Fertiliser (+/- aphids 12 weeks)	Bray Curtis	3,12	0.418	0.743
Herbivory (+/- aphids 12 weeks)	Unweighted UniFrac	1, 14	2.003	0.154
Herbivory (+/- aphids 12 weeks)	Weighted UniFrac	1, 14	0.126	0.739
Herbivory (+/- aphids 12 weeks)	Bray Curtis	1,14	0.028	0.869
Fertiliser (9 & 12 weeks no aphids)	Unweighted UniFrac	3, 12	1.020	0.404
Fertiliser (9 & 12 weeks no aphids)	Weighted UniFrac	3, 12	2.393	0.094
Fertiliser (9 & 12 weeks no aphids)	Bray Curtis	3,12	1.469	0.272
Cabbage age (9 & 12 weeks no aphids)	Unweighted UniFrac	1, 14	0.543	0.5
Cabbage age (9 & 12 weeks no aphids)	Weighted UniFrac	1, 14	0.001	0.983
Cabbage age (9 & 12 weeks no aphids)	Bray Curtis	1,14	0.031	0.862

The PERMDISP results (**Table 14**) indicated that the group dispersions of beta-diversity calculated with the UniFrac (unweighted and weighted) and Bray Curtis distances were not significantly different between any of the explanatory variables (cabbage age, herbivory, fertiliser treatment), thus any significant differences obtained using this distance measure in the PERMANOVA test can be attributed to differences in their centroid (as indicated by the PERMANOVA results).

(iv) Identification of taxa with differential abundances between groups

Statistical tests were performed using the DESeq2 package (Love *et al*, 2014) in order to identify the key taxonomic groups driving the divergences in soil microbial communities between treatment and time-point groups observed in the α and β diversity analyses. Additional results of the DESeq2 analysis, as well as figures created using STAMP, are provided in **Appendix B**.

3.3.5 Fertiliser-associated bacteria

All three fertiliser treatments were associated with increased abundance of members of the *Flavobacteriaceae* family and the class TM7-1. There were several other OTUs found to be significantly increased in fertiliser-amended soils relative to controls, as discussed below.

Chicken Manure

Rhizosphere soils in the chicken manure treatment group were significantly different from controls in plants harvested at 12 weeks (DESeq2 Benjamini-Hochberg corrected *p*-value<0.05, **Appendix B**), but not 9 weeks. OTUs which were significantly enriched in the organically fertilised soils included several members of the phylum TM7 and the families *Cytophagaceae*, *Flavobacteriaceae*, *Halomonadaceae* and *Xanthomonadaceae* (DESeq2 Benjamini-Hochberg corrected *p*-value<0.05, **Appendix B**). These included species in the genera *Algoriphagus*, *Adhaeribacter*, *Leadbetterella*, *Pontibacter*, *Crocinitomix*, *Capnocytophaga*, *Flavobacterium*, *Bacillus*, *Planctomyces*, *Thiobacillus*, *Steroidobacter*, *Dokdonella*, *Luteimonas*, *Lysobacter*, *Pseudoxanthomonas*, *Thermomonas*, *Chthoniobacter* and *Opitutus*; and orders *Sphingobacteriales* and *KD8-87* (**Figure 22**, **Appendix B**).

Synthetic fertiliser

The soil communities of the Low N treatment were significantly different from control soils for cabbages at 9 but not 12 weeks old. Low N rhizosphere soils of 9 week-old cabbages had increased abundances of OTUs assigned to the genera *Chitinophaga*, *Porphyromonas*, *Algoriphagus*, *Adhaeribacter*, *Fluviicola*, *Aequorivita*, *Flavobacterium*, *Granulicatella*, *Selenomonas*, *Leptotrichia*, *Janthinobacterium*, *Cardiobacterium*, *Arenimonas*, *Lysobacter*, *Luteolibacter* and *Sphingopyxis*; and the species *Prevotella nanceiensis*, *Veillonella dispar*, *Sphingomonas wittichii*, *Sphingopyxis alaskensis*, *Pseudoxanthomonas mexicana* and *Prosthecobacter debontii* (**Figure 23**). Also increased were several members of the phylum *TM7* and the families *Sphingomonadaceae*, *Verrucomicrobiaceae*, *Oxalobacteraceae* and *Xanthomonadaceae* (DESeq2 Benjamini-Hochberg corrected *p*-value<0.05, **Appendix B**).

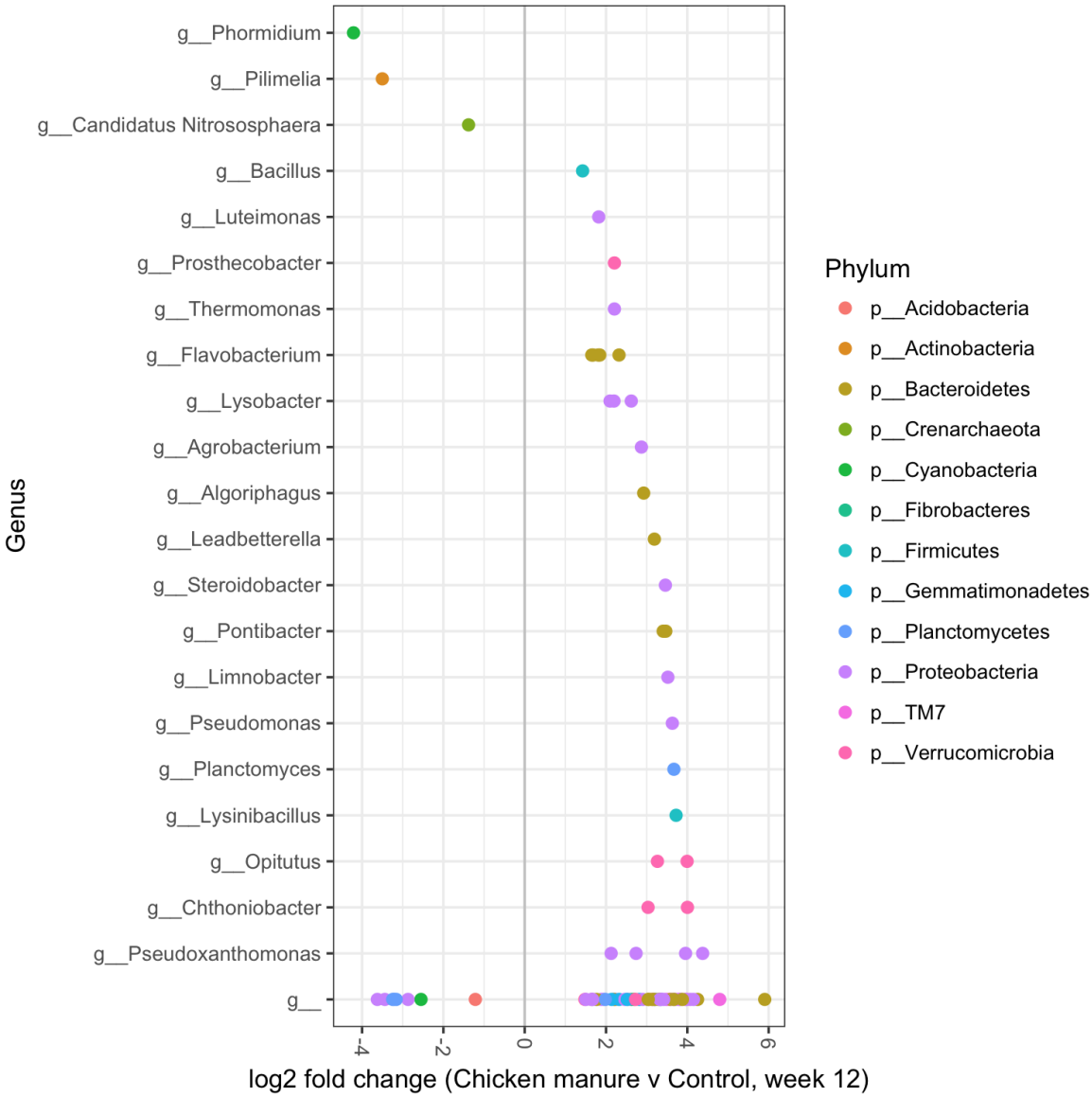


Figure 22 DESeq2 analysis results indicating the fold-change of bacterial genera in rhizosphere soil bacterial communities of chicken manure cabbages relative to control cabbages at 12 weeks. g__ represents taxa unclassified at the genus level.

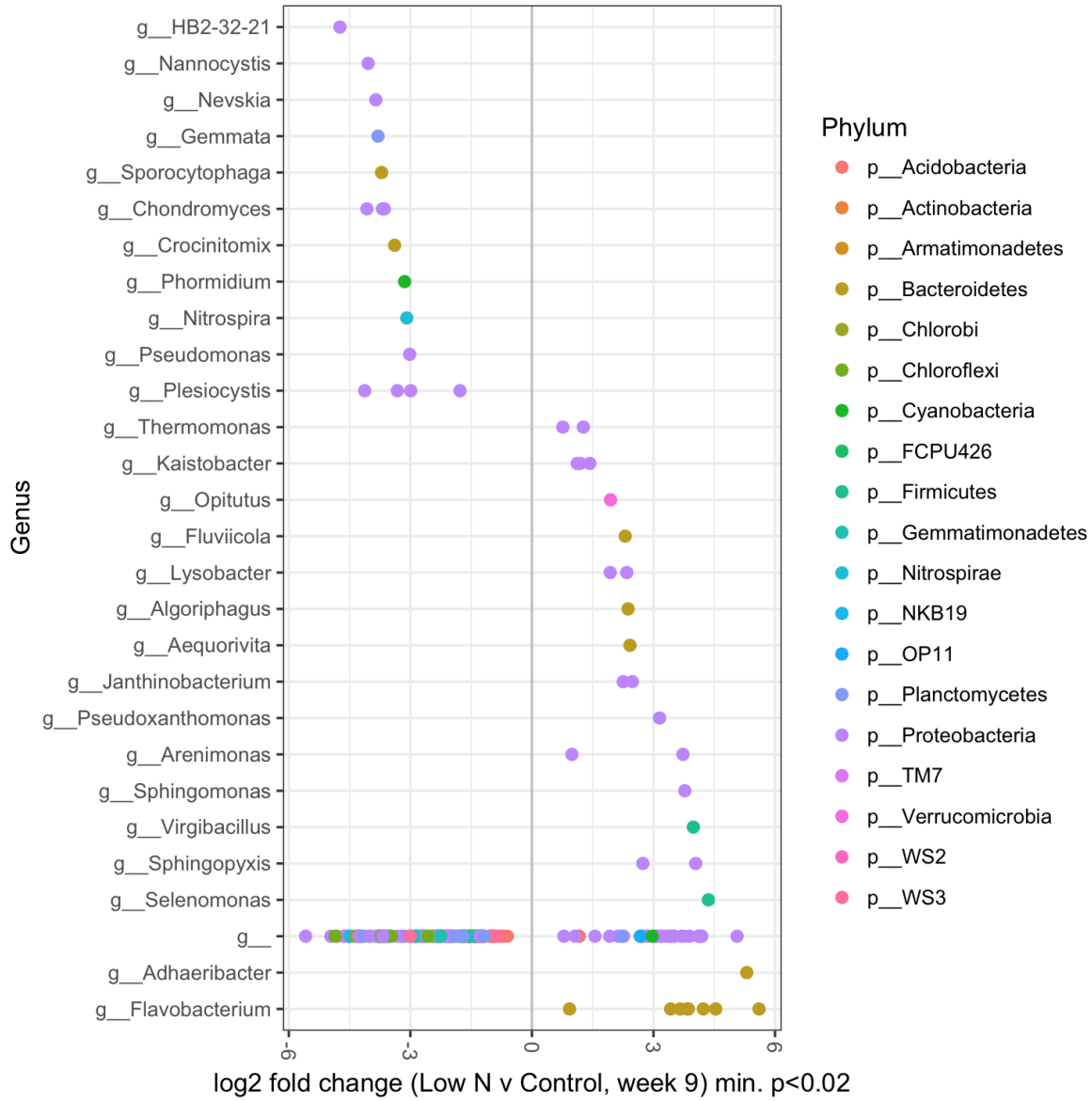


Figure 23 DESeq2 analysis results indicating the fold-change of bacterial genera in rhizosphere soil bacterial communities of Low N cabbages relative to control cabbages at 9 weeks. g__ represents taxa unclassified at the genus level.

The High N-treatment affected rhizosphere soil communities at both 9 and 12 weeks. Relative to controls, the soils of 9 week-old High N cabbages were enriched in OTUs belonging to the genera *Chitinophaga*, *Algoriphagus*, *Adhaeribacter*, *Flavobacterium*, *Granulicatella*, *Streptococcus*, *Mycoplana*, *Kaistobacter*, *Sphingopyxis*, *Limnobacter*, *Janthinobacterium*, *Arenimonas* and *Lysobacter*; and species *Brevundimonas diminuta* and *Sphingopyxis alaskensis* (DESeq2 Benjamini-Hochberg corrected *p*-value<0.05, **Appendix B**). Several of these OTUs were assigned to the phylum *TM7* and the families *Flavobacteriaceae*, *Sphingomonadaceae* and *Xanthomonadaceae*.

At 12 weeks, the High N treated soils were enriched in several OTU's of unassigned species belonging to the phyla *Gemmatimonadetes*; *Planctomycetes* and *TM7*; orders *Myxococcales*, *Sphingobacteriales*, *Sphingomonadaceae*; and the families *Chitinophagaceae*, *Verrucomicrobiaceae* and *Xanthomonadaceae* (DESeq2 corrected $p<0.05$, **Appendix B**). Also elevated in the High N soils of 12 week-old cabbages were OTUs belonging to the genera *Algoriphagus*, *Arenimonas*, *Chthoniobacter*, *Devosia*, *Dokdonella*, many *Flavobacterium*, *Fluviicola*, *Luteimonas*, *Luteolibacter*, *Lysobacter*, *Microbacterium*, *Opitutus*, *Pedobater*, *Planctomyces*, many *Pseudomonas*, *Sphingopyxis* and *Thermomonas*; and the species *Methylotenera mobilis*, *Pseudoxanthomonas mexicana* and *Prosthecobacter debontii* (**Figure 24**). Thus, both low and high N soils (relative to controls) were enriched in *Flavobacterium*, *Arenimonas*, *Sphingopyxis*, *Lysobacter* and *Thermomonas* (**Appendix B**: **Figure 59**).

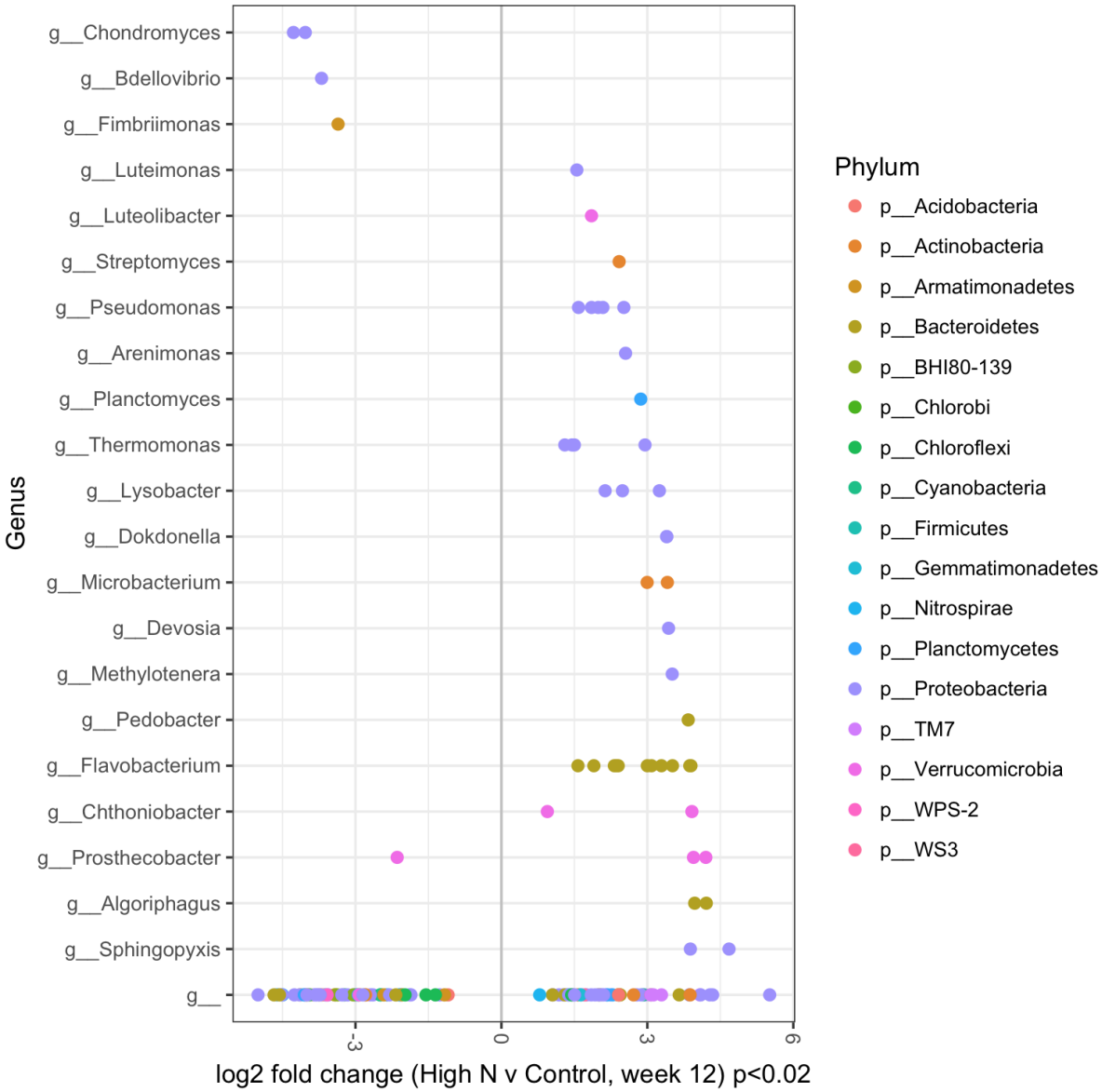


Figure 24 DESeq2 analysis results indicating the fold-change of bacterial genera in rhizosphere communities of High N cabbages relative to controls at 12 weeks. g__ represents taxa unclassified at the genus level.

An OTU belonging to the family *Nitrosomonadaceae* was also significantly more abundant in soils 9week-old HN plants relative to controls (DESeq2 Benjamini-Hochberg corrected *p*-value<0.05, **Appendix B**). It was also noted that the rhizospheres of synthetically fertilised plants harvested in week 9 had considerably lower abundances of *Nitrospira* (**Appendix B**). This genus contains species nitrifying bacteria which are important contributors towards emissions of the greenhouse gas nitrous oxide (N_2O) from soils.

3.3.6 Plant growth effects on the Rhizosphere soil community

The soil microbial communities of 9 and 12 week-old plants differed significantly in all fertiliser treatments, with the greatest age-related effects being detected in control and chicken manure-treated plants. In comparison to older (12week) plants, the 9week Control plants were enriched in many OTUs belonging to the phyla *Acidobacteria*, *Actinobacteria*, *Bacteroidetes*, *Chloroflexi*, *Planctomycetes* and *Proteobacteria* (DESeq2 Benjamini-Hochberg corrected *p*-value<0.05, **Appendix B**). These included species assigned to the genera *Lentzea*, *Agromyces*, *Microbacterium*, *Promicromonospora*, *Saccharopolyspora*, *Crocinitomixi*, *Flavobacterium*, *Caldilinea*, *Nannocystis*, *Plesiocystis*, *HB2-32-21* and *Perlucidibaca* (**Figure 25**). Older control plants had a higher abundance of OTUs from the phyla *Bacteroidetes*, *Firmicutes*, *Proteobacteria* and *TM7*; genera *Flavobacterium*, *Magnetospirillum* and *Sphingobium*; and the species *Veillonella dispar* (DESeq2 Benjamini-Hochberg corrected *p*-value<0.05, **Appendix B**).

Relative to 12week-old plants, younger plants in the Chicken Manure treatment were enriched in many OTUs assigned to the phyla *Actinobacteria*, *Chloroflexi*, *Planctomycetes* and *Proteobacteria*; including members of the genera *Iamia*, *Nocardia*, *Rubrobacter*, *Crocinitomix*, *Planctomyces*, *Plesiocystis*, *HB2-32-21* and the species *Nevskia ramose* (**Figure 25**, **Appendix B**). In comparison, 12 week-old organically fertilised plants had greater abundances of OTUs from the phyla *Bacteroidetes*, *Gemmatimonadetes*, *Proteobacteria*, *TM7* and *Verrucomicrobia* (DESeq2 Benjamini-Hochberg corrected *p*-value<0.05, **Appendix B**). At the genus level, these older plants were enriched in *Fimbriimonas*, *Algoriphagus*, *Leadbetterella*, *Flavobacterium*, *Asticcacaulis*, *Novosphingobium*, *Thiobacillus*, *Cellvibrio*, *Dokdonella*, *Luteimonas*, *Pseudoxanthomonas*, *Thermomonas*, *Opitutus*; and the species *Sphingomonas wittichii*, *Sphingopyxis alaskensis* and *Pseudoxanthomonas mexicana* (**Appendix B**).

In Low N synthetically fertilised plants, younger individuals had rhizosphere communities enriched in several members of the phyla *Proteobacteria*, and species of the genera *Adhaeribacter*, *Phormidium*, *Leptotrichia* and *Lysobacter* (**Figure 26**). The rhizospheres of older Low N plants became enriched in several OTUs assigned to the phyla *Bacteroidetes*, *Chloroflexi*, *Planctomycetes* and *Proteobacteria* (DESeq2 Benjamini-Hochberg corrected *p*-value<0.05, **Appendix B**).

Members of the genera *Flavobacterium*, *Gemmata*, *Pedomicrobium*, *Lysobacter* and *Thermomonas* were also more abundant in 12 week-old LN plants (**Figure 26**).

In comparison to older plants, High N plants at 9 weeks were more abundant in OTUs mainly belonging to the phyla *Actinobacteria* and *TM7*, including species of the genera *Lentzea* and *Granulicatella* (DESeq2 Benjamini-Hochberg corrected $p<005$, **Appendix B**). The OTUs enriched in older HN plants were predominantly members of the phyla *Bacteroidetes*, *Proteobacteria*, *TM7* and *Verrucomicrobia*; and at the genus level included *Fimbriimonas*, *Algoriphagus*, *Cytophaga*, *Fluviicola*, *Pedobacter*, *Thermomonas*, *Opitutus*, *Pseudomonas* and *Lysobacter* (**Figure 26**). Species identified as being more prevalent in 12week-old HN plants were *Sphingomonas wittichii*, *Methylotenera mobilis*, *Pseudomonas stutzeri*, *Pseudoxanthomonas mexicana* and *Prosthecobacter debontii* (**Appendix B**).

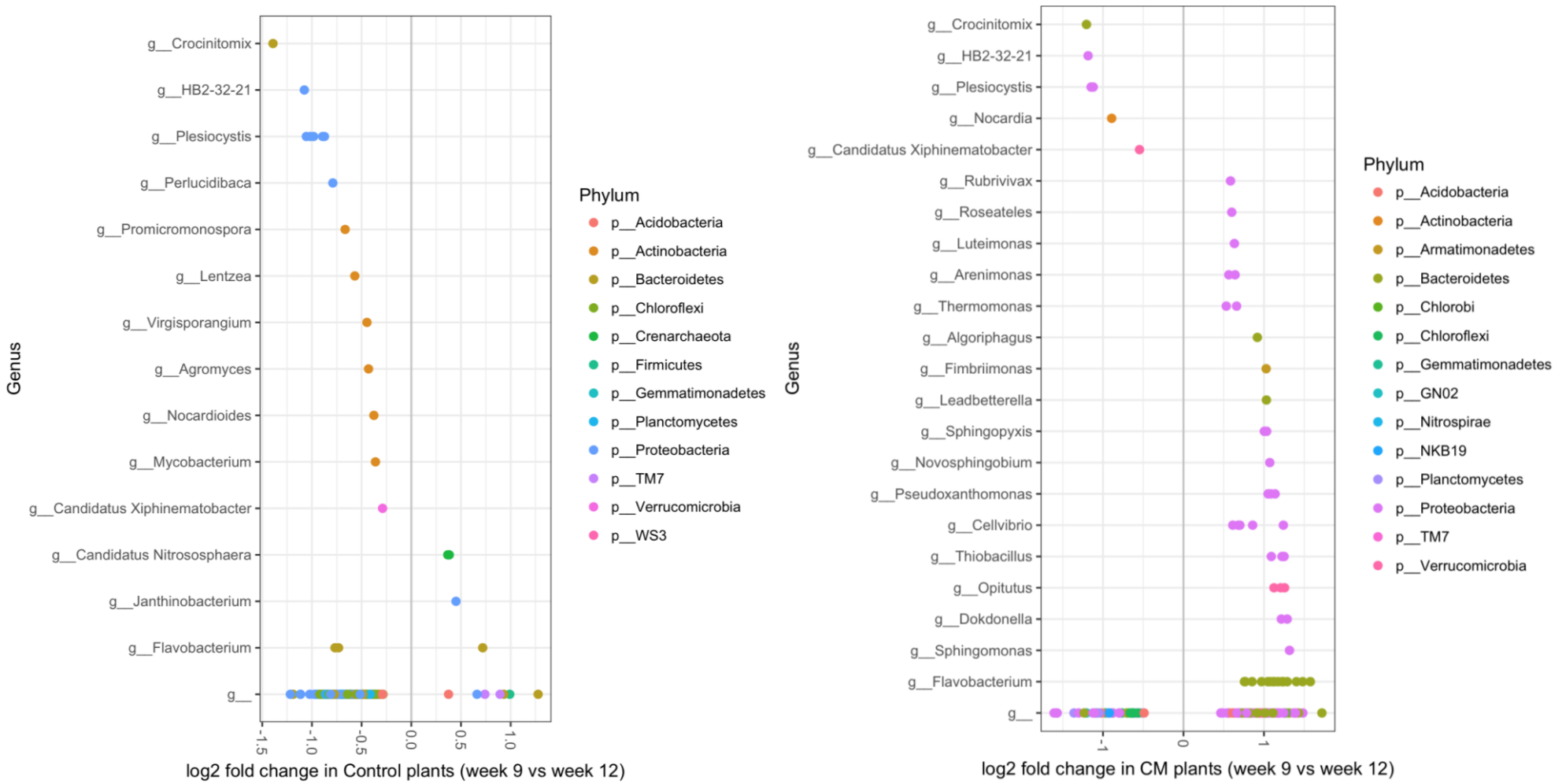


Figure 25 DESeq2 analysis results indicating the fold-change of bacterial genera in rhizosphere communities of 9 week-old plants relative to 12 week-old plants in the control (left) and chicken manure (right) treatment groups (Benjamini-Hochberg corrected *p*-value<0.05). g__ represents taxa unclassified at the genus level.

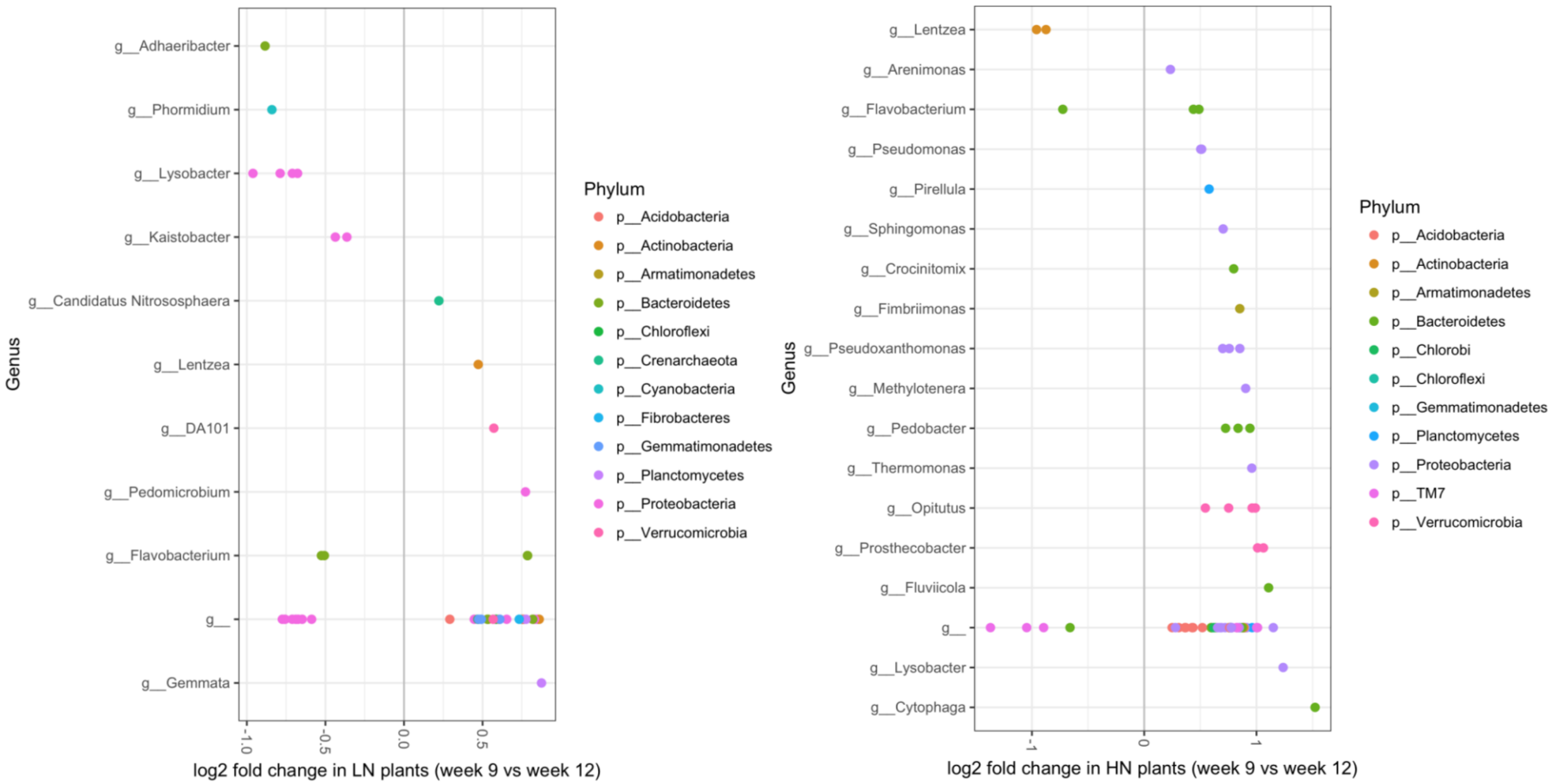


Figure 26 DESeq2 analysis results indicating the fold-change of bacterial genera between rhizosphere communities of 9 week-old plants relative to 12 week-old plants in the Low N (left) and High N (right) treatment groups (Benjamini-Hochberg corrected *p*-value<0.05). g__ represents taxa unclassified at the genus level.

In comparison to bulk soils, the rhizosphere exhibited greater species diversity and richness than the bulk soil. At the phylum level, the rhizosphere soil was enriched in *Acidobacteria* (Kruskal-Wallis $p = 0.021$), *Armatimonadetes* (Kruskal-Wallis $p = 0.029$), *GAL15* (Kruskal-Wallis $p = 0.034$), *GN02* (Kruskal-Wallis $p < 0.0001$) and *OP11* (Kruskal-Wallis $p = 0.025$). Several species of *Thiobacillus*, *Opitutus* and *Pseudoxanthomonas* were positively correlated with rhizosphere soils. The sulphur-oxidising bacteria *Thiobacillus* was almost exclusively found in rhizosphere samples, and absent from the majority of bulk soil samples (**Figure 27**). It appeared to be positively associated with the growth of *Brassica oleracea*, as it formed a larger proportion of the microbial community in 12 week-old cabbages relative to the 9 week-old plants.

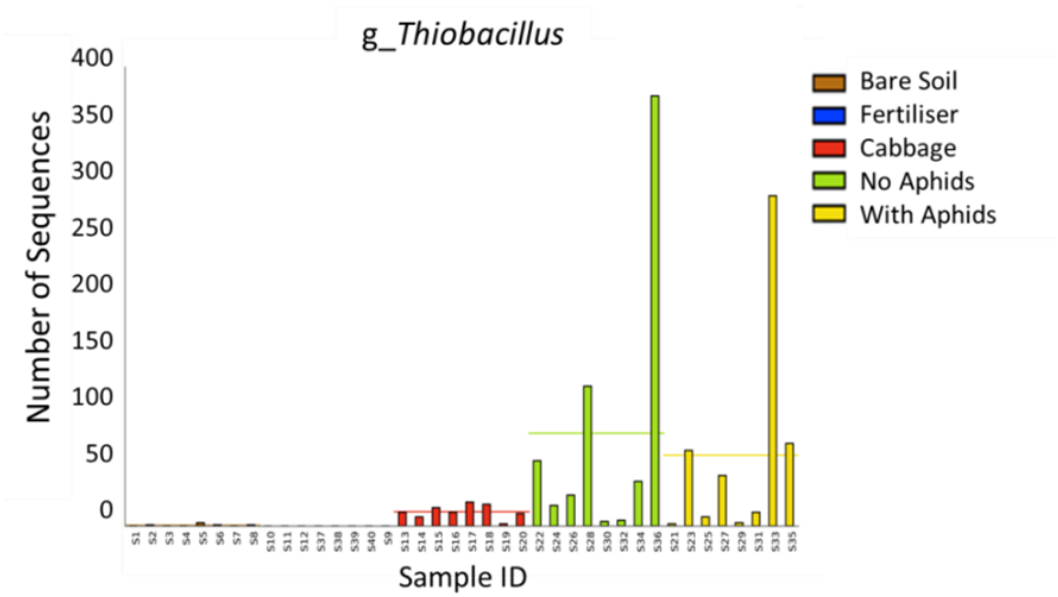


Figure 27 Comparison of the abundance of *Thiobacillus* sequences detected in each sample. They were almost exclusively found in rhizosphere soil samples (cabbage rhizosphere at 9-weeks (red), 12-weeks without aphids (green) and 12-weeks with aphids (yellow)).

There were no significant differences in relative abundances of bacteria detected between cabbages with or without aphids.

3.4 Discussion

This study aimed to identify shifts in the soil microbial community in response to fertilisers, cabbage growth and aphid herbivory. Overall, the rhizosphere community was found to differ significantly according to fertiliser inputs and, to a lesser extent, cabbage age, but no effect of aphid herbivory was found.

3.4.1 General soil microbial community structure

Overall, the dominant phyla detected by the 16S rRNA sequencing of soil samples in this study were in accordance with the common finding that soil communities are dominated by *Proteobacteria, Acidobacteria, Actinobacteria* and *Bacteroidetes* (Bulgarelli *et al*, 2013, Fierer *et al*, 2012, Fierer *et al*, 2009). The most abundant genus across all soil samples was an unclassified acidobacteria from the order *iii1-15* which has been reported to be dominant in other soils worldwide (Barnard *et al*, 2013, Wang *et al*, 2016b).

3.4.2 Fertilisers and cabbage development shape microbial diversity

Although no statistically significant differences were found, there were some interesting trends appearing in the alpha-diversity results. Firstly, the synthetically fertilized rhizosphere soils had an overall lower α-diversity in plants from both age cohorts. This is in line with other reports that the application of mineral fertilisers reduces the richness and increases the evenness of soil bacterial populations (Hartmann *et al*, 2015, Ding *et al*, 2016). α-diversity was also found to be lower in the rhizospheres of older (12 week) plants in comparison to younger (9 week) plants, but again this was not statistically supported. There is conflicting evidence in the literature regarding the correlation between plant development and rhizosphere populations. Some studies have shown that bacterial alpha-diversity (richness) in the rhizosphere declines with plant age (Liljeroth & Bååth, 1988; Chaparro *et* al, 2014; Shi *et al*, 2015) with the bacterial composition of the rhizosphere gradually converging with bulk soil communities as the plant reaches senescence (Micallef *et al*, 2009). However, other studies report that root communities are robust and unaffected by the different phases of plant development (Dombrowski *et al*, 2016).

The rhizospheres of aphid-infested plants were found to have a slightly (but not significantly) lower α-diversity comparison to uninfested plants of the same age (12 weeks). This trend could indicate a possible effect of the insects on the soil microbial community, perhaps mediated through the induced changes in the chemistry and root exudates of the host plant. This would corroborate previous findings which have indicated that aphid herbivory reduces rhizosphere bacterial abundance, potentially due to the negative effect of aphids on rhizodeposit production and

declining the allocation of photoassimilates to roots (Vestergård *et al*, 2004). However, these aphid effects on bacterial abundance can reverse at later stages of plant growth, as demonstrated by Vestergård *et al* (2004) and Lee *et al* (2012).

The PCoA plots constructed using unweighted UniFrac distances indicated a clear distinction between the microbial communities of rhizosphere and bulk soil communities, however the effects of cabbage age and fertiliser treatment seemed to have less influence. The constrained ordination (CAP) method again showed no divergence between rhizosphere samples from 9 and 12 week-old plants, or between aphid-infested and uninfested 12-week old plants. However, the CAP plots constrained to fertiliser treatments were suggestive of a significant effect, with the synthetically fertilized soils appearing to diverge away from the control and organically fertilised soil samples. The PERMANOVA results supported this finding, as cabbage age and fertiliser (and their interaction) were found to have a significant effect on beta diversity. There was no effect of herbivory on beta diversity detected.

3.4.3 Fertiliser-associated bacteria

All three (synthetic and organic) fertiliser treatments resulted in elevated abundance of several OTUs assigned to the phyla *Xanthomonadaceae* and candidate division *TM7*; family *Flavobacteriaceae*; and genera *Algoriphagus*, *Adhaeribacter*, *Arenimonas*, *Lysobacter* and *Thermomonas* when compared to control soils from plants of the same age. Bacteria in the candidate division *TM7* have been associated with biochar application (Xu *et al*, 2014), benzene and toluene degradation (Luo *et al*, 2009, Xie *et al*, 2011), as well as nitrification (Hanada *et al*, 2014). Nitrate concentrations have been shown to positively correlate with the abundance of *TM7* in soils under pepper (*Capsicum annum* L.) cultivation (Eo and Park, 2016). Several flavobacteria are known to have denitrifying properties (Pichinoty *et al*, 1976, Horn *et al*, 2005). Dentirification rates have been shown to respond positively to fertiliser additions in several studies (Bremner, 1997, Mulvaney *et al*, 1997), raising concerns that these farming regimes contribute to greenhouse gas (NO and N_2O) emissions. *Adhaeribacter* abundance has been shown to be substantially enhanced in organically fertilised soils and is thought to play an important role in the degradation of composts (Calleja-Cervantes *et al*, 2015). Sun *et al* (2014) reported that the abundance of *Adhaeribacter* was positively correlated with urease activity, and *Thermomonas* was positively correlated with saccharase activity.

Fertiliser additions promoted the abundance of several members of the family *Xanthomonadaceae*, such as *Lysobacter*, *Thermomonas* and *Arenimonas*. *Xanthomonadales* have been reported to respond positively to synthetic and organic fertilisers in other studies, with the impact penetrating into the subsoil (>0.2m) (Li *et al*, 2014a). Species belonging to the *Xanthomonadaceae* family

have been found to respond positively to lignin additions (Goldfarb *et al*, 2011) which may explain the increased dominance of this group in 12 week-old plant rhizosphere in comparison to 9 week-old plants. Other studies have found that *Xanthomonadaceae* increase substantially following long-term fertilisation (Campbell *et al*, 2010), and this may be beneficial for plants since certain members of this family have been reported to contribute towards the suppression of bacterial pathogens (Wu *et al*, 2014). An increase in *Lysobacter* abundance, for instance, may be beneficial for the plant since several members of this genus have been found to have antibiotic and antifungal properties which may serve as biological control agents of plant pathogens (Li *et al*, 2008, Hayward *et al*, 2010, Rosenzweig *et al*, 2012, Postma *et al*, 2008). Examples of plant diseases inhibited by *Lysobacter* species include leaf blight in rice (Ji *et al*, 2008) and damping-off in cucumber (Folman *et al*, 2004). *Lysobacter* and *Thermomonas* bacteria were reported to be dominant in soils contaminated with toxic 2,4,6-Trinitrotoluene (TNT) (George *et al*, 2008). *Thermomonas* bacteria have been shown to have genes for cyanide metabolism, and given that ammonia can result from cyanide degradation it has been proposed that they may also be capable of denitrification (Wang *et al*, 2015). Allyl cyanide is one of the products of glucosinolate (sinigrin) hydrolysis (Rungapamestry *et al* 2006), and so it may be postulated that the enhanced growth of cabbages stimulated by the addition of fertilisers may have resulted in greater levels of allyl cyanide in the soil, thereby resulting in the proliferation of cyanide-degrading bacteria such as *Thermomonas*. *Arenimonas* populations have previously been reported to respond positively to long-term organic-inorganic (manure/straw combined with NPK) fertilisation (Li *et al*, 2017), and their abundance is reported to positively correlate with N rate (Ling *et al*, 2017).

Plant N uptake has a strong impact on the composition of microbial communities, with greater N-uptake resulting in lower bacterial diversity (Bell *et al*, 2015). It is expected, therefore, that the form of N (organic or inorganic) and its availability for assimilation by plant roots can play an important role in the shaping of rhizosphere communities. This was supported by our results, which indicated a number of bacteria to differ significantly in abundance between organically and synthetically fertilised plants.

(i) Organic fertiliser

The organic fertiliser treatment was found to have a significant effect on bacterial abundances in the rhizosphere soils of 12 week-old (but not 9 week) cabbages. Relative to controls, the OTUs which exhibited the greatest increase in soils amended with poultry pellets were assigned to the order *Sphingobacteriales*; families *Cytophagaceae* and *Halomonadaceae*; genus *Steroidobacter*; genera *Adhaeribacter*, *Pseudoxanthomonas* and *Leadbetterella*; and the family *Rhodospirillaceae*. Bacteria within the family *Sphingobacteriales* have been shown be respond positively to biochar amendment (Xu *et al*, 2014) and are reported to perform roles in carbon cycling and organic matter decomposition (White *et al*, 1996). Conversely, Campbell *et al* (2010) found a negative effect of long-term organic amendments on the abundance of *Sphingobacteriales* OTUs, while another study

reported that *Sphingobacteriales* were negatively associated with mineral N-rates (Ling *et al*, 2017). *Cytophagaceae* are known to degrade complex carbohydrates (McBride *et al*., 2014) and have previously been associated with compost (Ye *et al*, 2016). *Halomonadaceae* have also been linked to compost amendments (Ye *et al*, 2016, Silva *et al*, 2016), with some species exhibiting tolerance to high temperatures and salt concentrations (Vreeland, 1992). *Steroidobacter* has been reported to be prevalent in the litter horizon of a forest soil (Baldrian *et al*, 2012) which again may indicate a role in organic matter decomposition. Pig manure has similarly been shown previously to promote soil populations of *Pseudoxanthomonas* and *Adhaeribacter* (Ding *et al*, 2014) and these may, therefore, be common responses to a variety of animal-derived organic fertiliser treatments. Species of *Pseudoxanthomonas* are capable of organic matter degradation (Kim *et al*, 2008b), and have previously been isolated from cotton waste composts (Weon *et al*, 2006) and fermented cow manure (Giannattasio *et al*, 2013).

Oceanospiralles were more abundant in CM soils. This was a curious result since these bacteria are more commonly associated with oil spills and marine environments (Cao *et al*, 2014). At the species level, the boron-tolerant bacteria *Lysinibacillus boronitolerans* was enriched in the CM soils. This may have been caused by increased boron levels in the soil as a result of the chicken manure additions, which have previously been linked to the type of bedding material used in poultry broiler houses (Bolan *et al*, 2010). Similarly, *Algoriphagus* (which was enriched in all fertiliser treated soils) is also reported to be tolerant of high levels of boron (Kabu and Akosman, 2013).

An interesting finding was that OTUs assigned to the sulphur-oxidising genus *Thiobacillus* (family *Hydrogenophilaceae*) were almost exclusively found in rhizosphere samples, particularly in the 12-week old CM cabbages (**Figure 27**). The High N soil had a greater abundance of two OTUs assigned to the genus *Limnobacter* which is another genus of thiosulphate-oxidising bacteria (Spring *et al*., 2001, Lu *et al*., 2011). This may have been caused by the influence of glucosinolates-products in the cabbage rhizosphere, supporting the theory that root-derived glucosinolates shape the soil microbial community in the rhizosphere of Brassica plants. *Thiobacillus* species are sulphur-oxidising bacteria that grow in a wide range of conditions (optimum pH <2-8 and temperature 20-50°C), deriving energy via the oxidation of one or more sulphur compounds including sulphides, thiosulphate and thiocyanate (Kelly and Wood, 2000). *Thiobacillus thioparus* bacteria possess an enzyme that can breakdown thiocyanate - a common compound found in glucosinolates (Katayama *et al*., 1998). Both *Thiobacillus* and *Janthinobacterium* (enriched in synthetically fertilised soils) also have denitrifying properties (Navarro-Noya *et al*., 2010). The *Thiobacillus* genus is also known for its ability to solubilise phosphorus, a valuable attribute given the importance of this nutrient in plant growth (Shen *et al*, 2011) and the steady depletion of rock-organic phosphate resources, which are the main origin of

P-fertilisers (Hunter *et al*, 2014). Indeed, the inoculation of soil with *Thiobacillus* in the soil has been shown to increase phosphorus availability (Boulif *et al*, 2016, Jazaeri *et al*, 2016) thus demonstrating its potential as a biofertiliser. The increase in *Limnobacter* may be less favourable, since one of its species, *L. thiooxidans*, has been reported to inhibit plant growth (Blom *et al*, 2011).

CM soils (week 12) had diminished prevalence of bacteria in the order *Cytophagales* and the genera *Phormidium* and *Candidatus Nitrososphaera*. *Candidatus Nitrososphaera* is an ammonia-oxidising archaea which has previously been found to be significantly higher in agricultural soils relative to those from non-agricultural sites (Zhalnina *et al*, 2013). The contradictory finding in the CM soils in our study may be attributed to the negative correlation between *Candidatus Nitrososphaera* with ammonium (NH^+_4) - the main form on N in manures - and soil organic matter (SOM), as reported by Zhalnina *et al* (2013). A reduction in ammonia-oxidising microorganisms may be favourable in environmental terms since they are major contributors towards nitrous oxide (N_2O) emissions from agricultural soils, which represents one of the most potent greenhouse gases (Stieglmeier *et al*, 2014).

(ii) Synthetic fertiliser

Both Low N (9 weeks) and High N (9 and 12 weeks) cabbage rhizospheres were enriched in OTUs from the families *Chitinophagaceae, Caulobacteraceae, Xanthomonadaceae* and *Sphingomonadaceae*, and genera *Arenimonas*, *Flavobacterium* and *Sphingopyxis*. The family *Chitinophagaceae*, which are members of the order *Saprospirales*, are named after their ability to degrade chitin and other complex polymeric organic matter (Glavina Del Rio *et al*., 2010). It would be expected, therefore, that that *Chitinophagaceae* bacteria may be positively associated with organic inputs, rather than mineral fertilisers. However, another study produced similar results to ours, finding the most influential OTUs exhibiting a positive response to high N mineral fertilisers included *Arenimonas*, *Sphingomonas* and unclassified *Chitinophagaceae* and *Xanthomonadaceae* (Li *et al*, 2017).

Flavobacterium bacteria perform heterotrophic denitrification (Wang *et al*, 2016a) and their abundance has previously been shown increase in chemically fertilised, but not organically managed, soils (Lavecchia *et al*, 2015). *Sphingopyxis* are members of the *Sphingomonadaceae* family which has been shown to be promoted by synthetic NPK fertilisers (Eo and Park, 2016), having positive correlations with total N and available P (Ding *et al*, 2016). Bell *et al* (2011) reported that *Sphingomonadaceae* and *Caulobacteraceae* exhibited the greatest ^{15}N-uptake in hydrocarbon-contaminated soils treated with ^{15}N-labelled mono-ammonium phosphate (MAP). This indicated that these bacteria exhibit a positive growth response to N additions, in addition to their well-documented ability to degrade hydrocarbon pollutants (Yang *et al*, 2014).

Other bacterial taxa with large fold increases in Low N soils at 9 weeks (relative to controls) included the genera *Selenomonas*, *Leptotrichia*, *Adhaeribacter*; and the families *F16* and

Cytophagaceae. Also increased were several members of the families *Verrucomicrobiaceae* and *Oxalobacteraceae*. *Selenomonas* are anaerobic bacteria commonly found in ruminant animals, and their enrichment in the LN treatment was surprising given that they are more commonly associated with manures (Udikovic-Kolic *et al*, 2014). The enrichment of *Leptotrichia* was also curious, since these bacteria are known to have sugar metabolizing properties (Thompson and Pikis, 2012) and are more commonly associated with the human oral microbiome and periodontal (gum) disease (Wang *et al*, 2013). Several studies support the finding that *Verrucomicrobia* bacteria are positively correlated with mineral fertilisers and N availability (Ding *et al*, 2016, Pan *et al*, 2014).

At the species level, the Low N (9 weeks) soils were enriched in *Prevotella nanceiensis*, *Veillonella dispar*, *Sphingomonas wittichii*, *Sphingopyxis alaskensis*, *Pseudoxanthomonas mexicana* and *Prosthecobacter debontii*. *Prevotella* use peptides and ammonia as nitrogen sources (Purushe *et al*, 2010). The anaerobic species *P. paludivivens* has been isolated from plant residues taken from a flooded rice-field soil and it is believed to play important role in the decomposition of cellulose from plant cell walls (Ueki *et al*, 2007). However, like *Leptotrichia*, *Prevotella* bacteria are more commonly associated with the human oral microbiome (Bik *et* al, 2010). *Sphingomonas* species are known to have bioremedial properties and are able to degrade organic pollutants such as crude oil (White *et al*, 1996, Al-Saleh and Hassan, 2016). The RW1 strain of *S. wittchii* has xenobiotic degrading properties, and is able to metabolise herbicide compounds (Keum *et al*, 2008).

In comparison to controls, the Low N and High N (week 9) soils had reduced abundance of the nitrifying bacteria of the phylum *Nitrospirae*, which agrees with previous reports of negative correlations between synthetic NPK fertilisers and *Nitrospirae* populations (Eo and Park, 2016). It has emerged that certain members of *Nitrospira* are capable of carrying out both the steps involved in nitrification: ammonium oxidation (ammonium to nitrite) and nitrite oxidation (nitrite to nitrate) (Daims *et al*, 2015). This result concurs with a 35 year-long study which showed that *Nitrospira* mean abundance was significantly lower in soils under mineral fertiliser management in comparison to control and organically managed soils (Ding *et al*, 2016). The decline in nitrifying bacteria may also be linked to the increased growth of synthetically fertilised cabbages (see **Chapter Two** results), as the glucosinolate hydrolysis products of Brassicaeae plants are known to have inhibitory effects on nitrification and nitrifying bacteria communities (Brown and Morra, 2009, Bending and Lincoln, 2000). Although not the case in this study, there are reports of other nitrifying bacteria being negatively affected by fertiliser additions, such as *Crenarchaeota* (Wang *et al*, 2016b). A reduction in the abundance of nitrifying bacteria could help reduce N-leaching from agricultural soils, thereby lessening the risk of environmental damage by eutrophication. Conversely, the HN soils were also enriched in a member of the family *Nitrosomonadaceae*, which contains several ammonia-oxidising bacteria and has previously been reported to respond positively to fertiliser additions (Han *et al*, 2017, Li *et al*, 2014a).

The majority of OTUs enriched in High N soils (9 weeks) were members of the phyla *Bacteroidetes* (classes *Flavobacteriia* and *Sphingobacteriia*), *Proteobacteria* (classes *Alphaproteobacteria* and *Gammaproteobacteria*) and *TM7*. These included OTUs assigned to the families *Sphingomonadaceae* (genera *Kaisobacter* and *Sphingopyxis*) and *Xanthomonadaceae* (genera *Arenimonas* and *Lysobacter*). The greatest increase occurred in an OTU assigned to the family *Erythrobacteriaceae*, which has been reported to increase in soils treated with pyrogenic organic matter (Whitman *et al*, 2016). *Bacteroidetes* are widely found to be more abundant in inorganically fertilised soils (Pan *et* al, 2014; Li *et al*, 2017; Wang *et al*, 2017).

The order *Myxococcales*, genus *Algoriphagus* and species *Prosthecobacter debontii* were also enriched in the HN soils of 12week-old plants. The abundance of *Myxococcales* bacteria has been reported to have strong positive associations with total N as well as soil organic carbon (Li *et al*, 2017). These High N treated soils were also enriched in several OTU's of unassigned species belonging to the families *Chitinophagaceae*, *Micrococcaceae*, *Verrucomicrobiaceae* and *Xanthomonadaceae*. Phosphorous fertilisation had previously been linked to increases in the abundance of *Micrococcaceae* (Wang *et al*, 2016b).

The rhizosphere communities of LN and HN plants from both age cohorts were diminished in numerous OTUs assigned to the phyla *Acidobacteria*, *Gemmatimonadetes* and *Planctomycetes*, the orders *Cytophagales* and *Myxococcales* (particularly the family *Haliangiaceae* and genus *Chondromyces*), and the class *Anaerolineae*. The 9 week-old LN rhizospheres were also depleted in several OTUs identified as members of the phylum *Armatimonadetes*. It has been proposed that *Acidobacteria* are oligotrophic, preferring resource-poor (low C mineralisation) environments with a low pH (Fierer *et al*, 2007). Furthermore, rape (*Brassica napus* var. *emerald*) plants grown phosphorous-deficient conditions have been shown to acidify soils, resulting in an increase in abundance *Acidobacteria* (Hedley *et al*, 1983). The negative association between NPK fertilisers and *Acidobacteria* abundance is supported by the findings from a number of studies which examined the effect of N or fertiliser soils from the lettuce rhizosphere (Li *et al*, 2016); long-term fertilised Arctic tundra soils (Campbell *et al*, 2010); a rice-wheat cropping system (Zhao *et al*, 2014) and soils from an agricultural field and a grassland both of which received long-term fertilisation with ammonium nitrate (NH_4NO_3) (Ramirez *et al*, 2010). In contrast, Ding *et al* (2016) reported a positive association between the *Acidobacteria* and mineral fertiliser inputs. However, their study did find that the abundance of *Haliangium* (family *Haliangiaceae*) bacteria was notably higher in soils treated with inorganic fertiliser and manure (combined) in comparison to those which received the inorganic alone. This supports, to some extent, the results of this study in which *Haliangiaceae* prevalence appeared to respond negatively to synthetic fertilisers.

3.4.4 Rhizosphere community responses to plant growth

Plant age had a significant effect on the composition of rhizospheric bacterial communities. Younger (9 week) plants were found to host more diverse microbiomes in their rhizospheres in comparison to older (12 week) plants. Younger plants were richer in bacteria of the phyla *Acidobacteria, Actinobacteria* (class *Thermoleophilia* and order *Actinomycetales*), *Bacteroidetes* (family *Cytophagaceae*, genus *Crocinitomix*), *Chloroflexi* (class *Anaerolineae*, orders *SBR1031* and *Thermomicrobia*), *Planctomycetes* (order *MVS-107*); and classes *Alphaproteobacteria* (order *Rhodospiralles*), *Deltaproteobacteria* (orders *Bdellovibrionales* and *Myxococcales* (genus *Plesiocystis*)) and *Gammaproteobacteria* (order *Alteromonadales* (genus *HB2-32*-21) and *Xanthomonadales* (family *Sinobacteraceae*)) in the phylum *Proteobacteria*. The greater abundance of *Proteobacteria* in 9week-old plants concurs with the theory that plants rhizospheres are initially colonised by fast-growing copiotrophic bacteria, often referred to as r-strategists (such as Proteobacteria and Bacteroidetes), which prefer nutrient-rich environments with high organic C-availability (Murphy *et al*, 2016; Fierer *et al*, 2007). *Actinobacteria* have also been shown to be more abundant in nutrient-rich soils, having strong positive correlations with N-availability (Bell *et al*, 2015). Several of the bacteria which were more numerous in the rhizospheres of younger plants are associated with carbon-rich environments. For instance, *Anaerolineae* and *HB2-32-21* are commonly detected in environments contaminated with polycyclic aromatic hydrocarbons (PAHs), suggesting that these bacteria may be involved in bioremediation (Akbari and Ghoshal, 2015; Obi *et al*, 2017). *SBR1031* (of the class *Anaerolineae*) populations were found to be increased in soils after the burning of crop residues (Jiménez-Bueno *et al*, 2016). *Acidobacteria* have previously been classed as oligotrophic owing to the negative correlations of some members of this phylum with soil organic C, P and N (Naether *et al*, 2012; Fierer *et al*, 2007), although this is not the case for all acidobacterial subgroups and it seems that their abundance is more strongly determined by soil pH rather than C availability (Jones *et al*, 2009b).

As plants grow, the availability of nutrients in the soil is depleted which can lead to more oligotrophic-dominated soil communities. Older (12week-old) plants were associated with increases in the abundance of OTUs from the phyla *Bacteroidetes* (families *Chitinophagaceae, Cytophagaceae* and *Flavobacteriaceae* (order *Sphingobacteriales*; genera *Flavobacterium* and *Algoriphagus*); *Gemmatimonadetes, TM7, Proteobacteria* (families *Rhodospirillaceae, Sphingomonadaceae* (species *Sphingomonas wittichii*), *Xanthomonadaceae* (genera *Thermomonas* and *Lysobacter* and species *Pseudoxanthomonas mexicana*), and order *Myxococcales*); and *Verrucomicrobia* (genera *Opitutus*). Chaparro *et al* (2014) found that the rhizospheres of *Arabidopsis* plants at the bolting/flowering stages exhibited increases in *Bacteroidetes, Cyanobacteria*, and significant decline in the abundance of *Acidobacteria* and *Actinobacteria* in comparison to the seedling/vegetative growth stages. They also reported that members of the

Cytophagaceae and *Sphingobacteriales* were significantly more abundant in the later stages of plant development, which concurs with our findings. *TM7* and *Sphingomonadaceae* bacteria have both been previously been reported to be associated with the senescing stage of plant growth (İnceoğlu *et al*, 2011, Pfeiffer *et al*, 2017). Li *et al* (2014b) reported that the families *Xanthomonadaceae*, *Sphingomonadaceae* and *Flavobacteriaceae* were enriched in the rhizosphere of maize plants. They also found a relationship between the rhizosphere community composition and the plant growth stage, with *Chitinophaga* (family *Chitinophagaceae*) being one of the more dominant bacteria during the later growth stage. Similarly, de Campos *et al* (2013) found that *Xanthomonadaceae* and *Flavobacteriaceae* dominated the bacterial community of canola (*Brassica napus* L. var *oleifera*) rhizospheres at the flowering stage. In nature *Flavobacterium* are known to mineralise organic substrates (e.g. carbohydrates, amino acids and proteins) and degrade organic matter and other organisms (bacteria, fungi and insects) using a variety of enzymes (Bernadet *et al*, 2006; Kolton *et al*, 2016). Previous studies show that *Algoriphagus* populations are significantly reduced in response to ethylene (ET) treatment (Carvalhais *et al*, 2014). Certain *Thermomonas* species have nitrite-reducing abilities (Kim *et al*, 2006) and are commonly found in hydrocarbon-contaminated soils (Akbari and Ghoshal, 2015; Kaplan and Kitts, 2004). *Opitutaceae* are commonly found in rhizosphere environments, such as those of rice (Breidenbach *et al*, 2016), maize (Correa-Galeote *et al*., 2016) and cucumbers plants (Tian and Gao, 2014).

These results indicate that the rhizosphere community changes significantly during the development of *B. oleracea*. Plant growth stage has been shown to have a significant effect on the soil microbial community in a number of other plants, such as potato (*Solanum tuberosum*) (Pfeiffer *et al*, 2017) and maize (*Zea mays* L.) (Cavaglieri *et al*, 2009). Rhizodeposition of carbon-rich compounds (e.g. sloughed-off root border cells, mucilage, organic acids), which serve as a significant energy source for microbial growth, declines significantly with plant age (Nguyen, 2003, Chaparro *et al*, 2013). This can result in the microbial community of the rhizosphere and bulk soil converging as the plant ages (Micallef *et al*, 2009). The influence of rhizodeposition on soil microbial communities can be affected by N inputs. A study by Ge *et al* (2017) found that increasing N fertilization rates resulted in a reduction in the bacterial incorporation of root-derived ^{13}C from rice plants despite the increase in rhizodeposition rates. Conversely, AM fungi and actinomycetes showed a positive response in ^{13}C uptake with increased N inputs.

Although there was no effect of aphid herbivory on the rhizosphere microbial community detected in this study, there have been other reports supporting such an interaction. For instance, aphid infestations have been shown to correlate with increased abundance of the beneficial PGPR strain *Bacillus subtilis* GB03, and a reduction in the prevalence of the pathogenic *Ralstonia solanacearum* SL1931 (Lee *et al*, 2012). *Bacillus subtilis* GB03 produces the volatile organic compound (VOC) 2,3-butanediol which can induce ISR (induced systemic resistance) in the model plant *Arabidopsis thaliana* against the pathogenic *Erwinia carotovora* (Ryu *et al*, 2005). It is

hypothesized that above-ground herbivores, including phloem-feeders such as aphids and whiteflies, can promote plant defence against pathogens by inducing plant immune responses and possibly by enhancing root exudation to recruit beneficial PGPR (Lee *et al*, 2012, Yang *et al*, 2011). The abundance of rare microbes in the soil has been shown to influence aphid and plant fitness, with a reduction in population of rare microbes being associated with greater aphid body size and plant biomass (Hol *et al*, 2010).

3.4.5 Study limitations and Future work

This study was limited by the small sample sizes used for 16S rRNA sequencing. Optimally, a minimum of 3 samples would have been used for each treatment in each sampling stage. However, this was limited by the number of samples that could be pooled in a single sequencing run, in addition to financial constraints. This was compounded somewhat by the fact that the fertiliser sampling stage had only one replicate for the chicken manure and high N treatments due to sub-standard quality of the second DNA samples. This may have been due to human error, but the high organic content of the poultry litter may have also reduced the efficiency of its DNA extraction procedure. In an effort to improve the reliability of the 16S rRNA sequencing results in spite of these small sample sizes, DNA was extracted from pooled soil samples from 5 pots for each treatment at each time-point. The costs of NGS services have rapidly fallen over the past decade, and continue to do so, thereby reducing this limitation for future studies. The use of DNA as opposed to RNA also means that we cannot differentiate between active and inactive bacteria. Furthermore, the type of nucleic acid used in microbial community analysis can significantly affect the outcome of the results, as demonstrated by (Kim *et al*, 2013). RNA extractions are more time-constrained than for DNA and require samples to be processed rapidly.

The comparison of bulk and rhizosphere soil communities would be enhanced by taking simultaneous samples for both throughout the course of the experiment. This would provide a greater insight into the influence of the plant on the soil microbial community and would indicate whether the cabbage rhizosphere and bulk communities converge over time, as suggested by other studies.

Chapter 4: *Thiobacillus thioparus* as a PGPR to enhance glucosinolate production in *B. oleracea*

4.1 Introduction

4.1.1 Glucosinolates

Glucosinolates (GLS) are a class of sulphur- and nitrogen-containing secondary metabolites characteristic of cruciferous vegetables (Capparales). Glucosinolates have been shown to confer several desirable properties, such as herbivore deterrence, cancer chemoprotection, antifungal activity, and strong flavour (Kiddle *et al*, 2001, Mazzola *et al*, 2001). GLS compounds are classed as *β*-thioglucoside *N*-hydroxysulphates (also known as (Z)-(or cis)-N-hydroximinosulphate esters or *S*-glucopyranosyl thiohydroximates), which all share a common basic structure consisting of three moieties: (i) a variable aglycone side chain (R), (ii) *β*-D-glucopyranose moiety and (iii) a sulphonated aldoxime moiety (Fahey *et al*, 2001, Wittstock and Halkier, 2002). The sulphur (S) group is often balanced by a (potassium) cation, and the side chain (R) is derived from one of three amino acid precursors: methionine, tryptophan or phenylalanine (Verkerk *et al*, 2009, Ishida *et al*, 2014).

To date, 16 families (including the families Brassicaceae, Capparaceae and Caricaseae) of glucosinolate-producing plants have been identified, including several commercially important Brassica crops such as cabbage, broccoli, cauliflower and Brussels sprouts (Verkerk *et al*, 2009, Miao *et al*, 2013, Fahey *et al*, 2001, Bressan *et al*, 2009). There are known to be over 130 different GLS in nature, with a single plant species producing up to 23 different GLS (Fahey *et al*, 2001, Verkerk *et al*, 2009, Hanschen *et al*, 2015). GLS can be broadly split into three groups based on the amino acid from which they are derived: aliphatic, aromatic or indole (**Figure 28**) (Miao *et al*, 2013, Kiddle *et al*, 2001). Aliphatic GLS have the precursor amino acids alanine (Ala), leucine (Leu), isoleucine (Ile), methionine (Met) or valine (Val); aromatic GLS are derived from phenylalanine (Phe) or tyrosine (Tyr); and indole GLS are formed solely from tryptophan (Trp) (Halkier and Gershenzon, 2006). The majority of the R groups of these GLS are modified from their amino acid precursors, usually with one or more additional methylene moieties (Halkier and Gershenzon, 2006). Aliphatic GLS are the most common form, accounting for approximately 50% of all known GLS structures (Clarke, 2010).

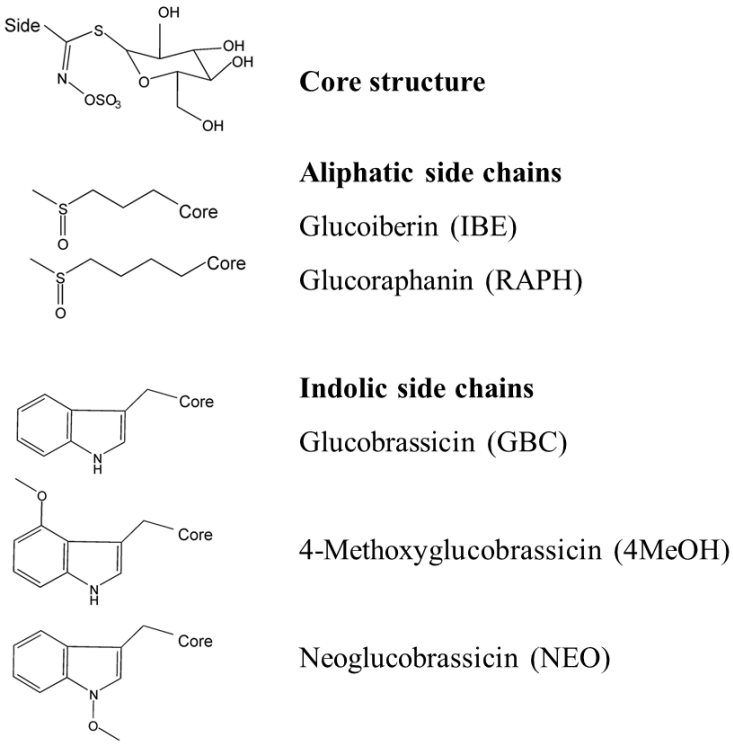


Figure 28 Chemical structures of some glucosinolates in *Brassica oleracea* (modified from Beekwilder *et al* (2008)).

4.1.2 Within-plant variation in glucosinolate profiles

In addition to interspecies differences, the concentration and composition of glucosinolates within a plant species varies considerably between genetic varieties, tissue types and developmental stages (Rask *et al*, 2000, Petersen *et al*, 2002, Verkerk *et al*, 2009, Wentzell and Kliebenstein, 2008). Glucosinolates can occur in all parts of the plants (seed, leaves, roots and flowers) at significantly different concentrations (Brown *et al*, 2003). The highest GLS concentrations tend to occur in plant reproductive organs (e.g. seeds, flowers and florets), where they can be up to 40 times greater than that of vegetative tissues (Clarke, 2010). The GLS content of *Brassica* seeds can be up to 10% dry weight, which is considerably higher than that of the plants as a whole (approximately 1% dry weight) (Fahey *et al*, 2001, Brown *et al*, 2003). The total GLS content of a plant tends to increase over time, with GLS accumulating in both roots and shoots throughout its development until the onset of senescence, usually peaking at the flowering stage (Malik *et al*, 2010, Petersen *et al*, 2002). Leaf age is another important predictor of GLS content. Older, fully expanded, senescing leaves generally have lower GLS levels in comparison to younger, developing foliage (Porter *et al*, 1991, Reifenrath and Müller, 2007).

Plant breeding can be used to create plants with specific GLS profiles (concentrations and compositions) which have desirable properties, such as resistance to herbivores, health benefits and flavour (Verkerk *et al*, 2009). However, the effectiveness of these techniques may be hampered by certain environmental variables which are critical in determining GLS production. Indeed, GLS

production can be influenced by a variety of environmental and physiological factors, including soil fertility, climate and soil moisture levels (Verkerk *et al*, 2009, Ishida *et al*, 2014). Under drought conditions, *Brassica* cultivars and wild varieties of Capperales have been found to exhibit increased accumulation of GLS in shoots and roots, particularly when the drought occurs at later growth stages (Radovich *et al*, 2005, Jensen *et al*, 1996, Tong *et al*, 2014). Conversely, Khan *et al* (2010) reported that GLS concentrations of drought-stressed broccoli (*Brassica oleracea* L. var. *italica* Plenck) plants was reduced in comparison to well-watered plants, although this trend was reversed when S supply to the plant was increased. This highlights the complexity of the relationship between GLS production and abiotic factors. Furthermore, the extent of environmental influence on GLS profiles varies with different types of GLS. For instance, the amount and variety of indole GLS are more strongly affected by environmental factors than aliphatic or aromatic GLS (Verkerk *et al*, 2009).

4.1.3 Health benefits of Glucosinolates

The focus of glucosinolate research has shifted in recent years from their toxic, anti-herbivory properties towards the associated health benefits (Schonhof *et al*, 2007). GLS have been found to have anti-carcinogenic properties, with consumption of Brassica vegetables being linked to reduced risk of colorectal (Verkerk *et al*, 2009, Seow *et al*, 2002), lung (Wang *et al*, 2004), stomach (Hansson *et al*, 1993) and prostate (Kirsh *et al*, 2007) cancer. The chemo-preventive properties of GLS have been attributed to the induction of phase II detoxification enzymes, such as quinone reductase, by certain glucosinolate hydrolysis products (Halkier and Gershenzon, 2006). Sulforaphane, the hydrolysis product of glucoraphanin (RAPH, 4-methylsufinylbutyl glucosinolate), has been identified as a possible treatment for *Helicobacter pylori*-related gastritis and the associated risk of stomach cancer, owing to its bactericidal properties (Fahey *et al*, 2002). Plant breeding programmes have been established which aim to enhance these health benefits of crucifer plants by increasing their GLS concentration and improving the retention of these phytochemicals during food processing (Hennig *et al*, 2014). However, these compounds confer a bitter, unpleasant flavour which may compromise consumer quality standards.

4.1.4 Sulphur availability and GLS production

Sulphur (S) is widely considered to be the fourth major plant nutrient, after nitrogen (N), potassium (K) and phosphorus (P) (Anandham *et al*, 2011, Vidyalakshmi and Srida, 2007, Kertesz and Mirleau, 2004). S is required for the production of certain amino acids, such as cysteine and methionine, which are precursors to all plant S-containing metabolites (Anandham *et al*, 2011). Until recently, S availability was rarely an issue in crop production. However, following the

imposition of S pollution regulations, such as the Convention for the Long-Range Transboundary Air pollutants (CLRTAP) (commonly referred to as the Gothenburg protocol) created by the United Nations Economic Commission (UNECE) in 1979, atmospheric S deposition has declined substantially in Europe, by 60% between 1980 and 1997 and by 22% from 2000 to 2014 (Allen and Shachar-Hill, 2009, EMEP, 2016, EMEP, 1999). Subsequently, the incidence of S-deficiency in crops has risen which could have important implications for glucosinolate production in Brassicas, given that GLS can account for as much as 30% of the total S content in certain plant organs (Falk *et al*, 2007). Indeed, S-deficient soils have been associated with reduced aliphatic GLS concentrations and S fertilisation has been shown to produce 50-fold increases in total GLS content in some plant species (Zhao *et al*, 1994, Falk *et al*, 2007). Methionine-derived GLS (aliphatic) tend to exhibit stronger positive responses to S fertilisation than those formed from tryptophan (indole GLS) (Falk *et al*, 2007). Kim *et al* (2002) reported that increased S-fertiliser additions caused vegetable turnip rape (*Brassica rapa* L.) to have significantly higher total GLS. Similarly, elevating S supply resulted in increased total GLS concentrations in broccoli florets, with the highest level of S fertilisation (1000 mg S plant^{-1}) resulting in a ten-fold increase in glucoraphanin (RAPH) and four-fold increase in glucobrassicin (GBC) concentrations in comparison to those receiving the lowest S supply (Krumbein *et al*, 2001).

Consequently, S fertilisation can result in the increased performance of specialist crucifer herbivores, as Yusuf and Collins (1998) demonstrated by showing that plants grown under high sulphur fertiliser regimes were more susceptible to infestations of the crucifer specialist aphid *Brevicoryne brassicae* than those receiving lower S inputs. Furthermore, the uptake of glucosinolates by the aphids actually led to declines in foliar glucosinolate levels of sulphur-treated plants. The crucifer specialist *Plutella xylostella* (L.) also has improved performance in response to increased S supply up to a point (Marazzi and Städler, 2004). It is not known, however, whether this holds true for non-specialist, generalist aphid species such as *Myzus persicae* (Falk *et al*, 2007).

4.1.5 Microbial-mediated aphid deterrence

Microbial-induced changes in glucosinolate profiles have been demonstrated using a strain of *Enterobacter radicincitans* on *Arabidopsis thaliana* (Brock *et al*, 2013). This may have consequences for herbivory, as demonstrated by the inoculation of calabrese plants (*Brassica oleracea*) with various species of *Bacillus* (*B. cereus*, *B. subtilis* and *B. amyloliquefaciens*), which inhibited the performance of the specialist cabbage aphid (*B. brassicae*) (Gadhave and Gange, 2016). *B. amyloliquefaciens* has also been shown to enhance the tolerance of bell pepper (*Capsicum annuum*) to *M. persicae* herbivory without impacting on yield (Herman *et al*, 2008), thus representing a potential viable microbiological biocontrol agent against these crop pests.

4.1.6 Sulphur oxidising bacteria

Approximately 95% of soil S is present in organic forms which cannot be absorbed by plants (Allen and Shachar-Hill, 2009). In order to be biologically available, organic S must be converted into inorganic forms (usually sulphates) by microbial-mediated sulphur-oxidation processes (Anandham *et al*, 2011, Tourna *et al*, 2014). Inoculating soils with S-oxidising bacteria (SOB) has been shown to increase the yield of many crop plants, including: sugarcane, canola, groundnut, yam bean (Grayston and Germida, 1990, Scherer, 2001, Anandham *et al*, 2007, Anandham *et al*, 2008, Stamford *et al*, 2008).

Three sulphur-oxidising bacterial pathways have been identified: (i) the *Parracoccus pantotrophus* sulphur oxidation pathway (typical of facultative chemolithotrophic *Alphaproteobacteria*); (ii) the branched thiosulphate oxidation pathway (characteristic of photolithotrophic SOB); and (iii) the tetrathionate (S_4) intermediate pathway involving polythionates (mainly associated with obligate chemolithotrophic *Thiobacillus* and *Acidobacillus* spp.) (Kelly *et al*, 1997, Friedrich *et al*, 2005, Hensen *et al*, 2006). Alkaline soils are typically poor in sulphur-oxidising bacteria (Anandham *et al*, 2011). The inoculation of alkaline soils with SOB can enhance the availability of other nutrients, including P, Fe, Mn, Zn, Ca, Cu, Na and SO_4 (Ansori and Gholami, 2015, Anandham *et al*, 2014, Grayston and Germida, 1991, Tourna *et al*, 2014). Thiosulphate-oxidising bacteria possess a number of additional PGP properties, such as ACC deaminase activity which can promote root growth (Anandham *et al*, 2011).

4.1.7 *Thiobacillus thioparus*

The sulphur-oxidising bacteria *Thiobacillus thioparus* was first isolated by Beijerinck in 1904, who discovered that the bacteria was able to convert tetrathionate into sulphate and sulphur (Kelly *et al*, 1997). *T. thioparus* is an obligate chemolithoautotroph, and the acidity incurred by the oxidation of inorganic sulphur compounds can promote the mobilisation (solubilisation) of other nutrients such as zinc, iron and manganese (Vidyalakshmi *et al*, 2009). *Thiobacillus* species can significantly enhance mobilisation of inorganic phosphates, thereby increasing P-uptake by plants (Jazaeri *et al*, 2016). Thiobaccili have been investigated for their potential role in various industrial and agricultural applications. These include the development of biotrickling filters (using *T. thioparus* biofilms) for the removal of gaseous sulphur pollutants (e.g. hydrogen sulphide) produced by industrial processes (e.g. waste water treatment plants). Its use as a biofertiliser has been experimented with *Melissa oficinalis* (Afkhami-Fathabad *et al*, 2014) and groundnut (Anandham *et al*, 2007) with promising results of increased yield.

The enzyme thiocyanate hydrolase was isolated from *T. thioparus* (strain THI 115) by Katayama *et al* (1992). *T. thioparus* uses this enzyme in order to derive energy from thiocyanate sources, including GLS hydrolysis products, by breaking them down into carbonyl sulphide and ammonia. Another enzyme common to SOB which use the *Parracoccus pantotrophus* sulphur oxidation pathway is the thiosulphate-oxidising multi-enzyme complex, referred to as SoxXAYZB (Petri *et al*, 2001). The *soxB* gene of this enzyme system has been shown to be ubiquitous among all thiosulphate-oxidising SOB (Meyer *et al*, 2007).

Aims

While the previous two chapters explored the effects of varying nitrogen inputs on soil-cabbage-aphid relations, this chapter instead focuses on the importance of sulphur (S) availability in the production of glucosinolates (GLS) in *Brassica oleracea*. It was hypothesised that the augmentation of the sulphur-oxidising bacteria (SOB) population in the soil would have cascading effects on higher trophic levels by influencing plant defences and, subsequently, population dynamics of the generalist pest *Myzus persicae* on the host plant. In order to test this hypothesis, the sulphur-oxidising *Thiobacillus thioparus* was tested for its potential as a PGPR using a variety of techniques. Two lines of inoculation methods (normal and sterile soil) were used, and their efficacy assessed at the three trophic levels. Firstly, the abundance of SOB in the rhizosphere was quantified using molecular methods (quantitative PCR); secondly, the foliar GLS concentrations of the cabbages were measured via HPLC analysis; and, finally, the performance of the green peach aphid *Myzus persicae* on the plants was compared. It was predicted that the SOB inoculation would be more successful in sterile soil than in "normal" (non-sterile) soil. Elemental sulphur was used as a benchmark for comparison, and it was hypothesised that S-treated plants would exhibited the highest GLS concentrations and lowest aphid populations.

4.2 Materials and Methods

4.2.1 Soil preparation

Soil was collected from a farm near Ipsden, Oxfordshire, UK (51°32'59.559" N, 1°05'8.43" W) as described previously (Chapter 2). The soil was collected using a shovel from the perimeter of the field as it had recently been sown with crops. The soil was transported back to the laboratory at the University of Southampton, where it was stored in bags in the dark at 4°C until further processing. The soil was spread out to air-dry before being sieved (2mm) to remove stones, plant matter and small invertebrates. It was then potted up into plastic pots (10cm diameter, ~400g soil pot^{-1}) and watered to approximately 40% WHC using distilled water (dH_2O). The water holding capacity (WHC) of the soil was determined volumetrically to be 59.91 ml $100g^{-1}$ for fresh soil (73.35 ml water was held by 100g oven-dried soil at 100% WHC). To achieve ~ 40% WHC, 12.96mL of distilled water was added per 100g soil.

4.2.2 Soil sterilisation

One of the greatest challenges in achieving a successful PGPR inoculant is ensuring that the bacteria can persist in the field and compete with other resident soil microbes. As this was an early-stage experiment, the magnitude of this problem was reduced by using "sterile" soil for half of the treatment groups. This was achieved by twice autoclaving bagged portions of soil (121°C, 2100 mBar for 15 minutes). Sterility (or near-sterility) was confirmed by plating a dilution series onto Nutrient Agar plates which were incubated at 30°C for 7 days to confirm no bacterial growth. DNA extraction was also performed to confirm <3ng/µl of DNA was retrieved from the autoclaved soil. This soil will be hereafter referred to as "sterile" soil, although to achieve complete sterility requires more powerful, rigorous methods such as gamma radiation. Soil which was not autoclaved will be referred to as "normal" soil. Sterile soil was potted up into bleach sterilised 4"-diameter plant pots to minimise contamination, and kept in trays in the greenhouse. It was then watered regularly with dH2O.

4.2.3 Soil inoculation

A variety of inoculation methods were used to compare their success in terms of survival of the inoculum and plant-growth promoting properties. The inoculation methods used were seed soaking, soil drenching, and pre-incubation in sterile soil (for either 7 or 14 days). Control plants received no inoculum (dH_2O only), and a sulphur fertilisation treatment was included for comparison of the efficacy of the bacterial inoculation.

4.2.3.1 Inoculum preparation

The *Thiobacillus thioparus* (Beijerinck 1904) culture used for inoculation was the DSM 505 type strain (also referred to as ATCC 8158, CIP 104484, NCIB 8370) purchased from the German Collection of Microorganisms and Cell Cultures (Leibniz Institute DSMZ, Germany). The 16S gene of this strain (Genbank HM173629.1) was found to have 95% sequence similarity with that of the OTU identified in the cabbage rhizosphere in Chapter 3 (Greengenes 13_5 OTU: 683573, Genbank FM212997.1). The full alignment of the two sequences, determined using the EMBOSS Matcher (http://www.ebi.ac.uk), is provided in **Appendix C** (**Figure 63**).

For long-term storage, the strain was maintained in 50% glycerol at -80 °C. Broth cultures of *T. thioparus* were grown in 500ml of sterile DSMZ #36 (*T. thioparus*) liquid media in 500ml conical flasks which were incubated in an orbital shaker (120rpm) at 30°C (Boretska *et al*, 2013, Tóth *et al*, 2015). The media recipe contained $(NH_4)_2SO_4$ (0.1 g), K_2HPO_4 (4 g), KH_2PO_4 (4 g), $MgSO_4$ x $7H_2O$ (0.1 g), $CaCl_2$ (0.1 g), $FeCl_3$ x $6H_2O$ (0.02 g), $MnSO_4$ x H_2O (0.02 g), $Na_2S_2O_3$ x $5H_2O$ (10g), dissolved in 1L distilled H_2O. Bromocresol purple (0.008g) was added as a pH indicator and the pH of the solution was adjusted to pH6.6 using 10 mol l^{-1} NaOH, before adding 12g agar (for solid media) and autoclaving at 121°C. Cultures were used at the mid-log growth phase (approx. 2.5 – 3 $OD_{600nm,}$ c. 15 days), and bacterial cells were harvested by centrifuging 45ml of the culture in a falcon tube at 4230 x *g* at room temperature for 20 minutes. The resulting pellet was then washed twice in equal volume of sterile PBS with a 15 minute centrifugation (4230 x *g* at 4°C) between each wash to recover the pellet. Finally, the pellet was re-suspended in 5ml sterile distilled water. Inoculation concentration was determined spectrophotometrically by measuring optical density at 600nm (OD_{600nm}) and also by plating serial dilutions onto *Thiobacillus thioparus* (DSMZ #36) media and counting the number of colony forming units (CFU) after an incubation period.

4.2.4 Plant and soil inoculation

Seeds were sterilised aseptically in a laminar flow hood by placing seeds (n = approx. 10) in a sterile Eppendorf tube, washed with 500µl 70% ethanol (EtOH), and mixed well. After 5 minutes, the EtOH was removed and replaced with 500µl 50% bleach for an incubation time of approximately 10 minutes. The seeds were then washed 4 times in sterile dH_2O to remove the bleach solution, dried on filter papers in petri dishes and were finally plated out onto Nutrient Agar to verify sterility and stored in the dark at 30°C.

A total of 24 (±1) plants were used for each of the treatments described below, with the exception of HSN (*n*=20), D14 (*n*=15) and HSS (*n*=18) which were not fully replicated owing to logistical issues. Each of the treatments used are described below.

(i) Seed inoculation

Surface sterilised seeds (*n* = approx. 30) were transferred aseptically from the nutrient agar plates to falcon tubes containing *T. thioparus* inoculum re-suspended in 7mL of distilled water. The tubes were shaken at 180 rpm at 30°C for approximately 2 hours (Naveed *et al*, 2014). Seeds were then placed (aseptically) onto filter paper to dry in a laminar flow cabinet for 1 hour. Two seeds were sown into each pot containing approximately 400g (unsterilized) soil.

(ii) Rhizosphere inoculation

The prepared bacterial inoculum (in 5ml sterile dH_2O, described above) was applied to the rhizosphere using a 1ml sterile syringe which was inserted at five points surrounding the seedling (**Figure 29**).

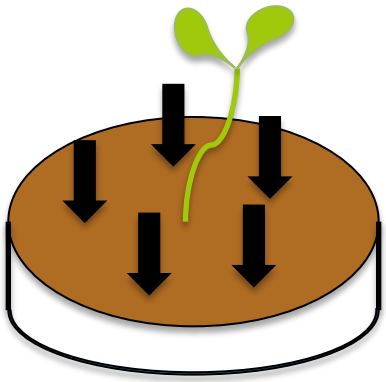


Figure 29 The inoculation points used for the rhizosphere inoculation (indicated by the black arrows) of seedlings.

(iii) Pre-incubation with sterile soil

Sterile 250ml Duran flasks were filled with 50g sterile soil and inoculated with the *T. thioparus* inoculum to give a final cell concentration of $1x10^8$ CFU g^{-1} dry wt soil. The flasks were incubated in the dark at 30°C for either 7 or 14 days. Once the pre-incubation period was complete, 4g of this inoculated soil was incorporated into the potted soil by mixing using a spatula to obtain an approximate 1% w/w inoculum concentration.

(iv) Sulphur fertilisation

The amount of elemental sulphur was based on rates used in published studies on the effects of sulphur fertilisation (Ngezimana, 2013, Ostrowska *et al*, 2008). 0.16g of sulphur powder ("Yellow Sulphur" Vitax Ltd., UK) was added to per pot soil at the time of sowing.

The treatment abbreviations are provided in **Table 15**.

Table 15 Abbreviations used for each of treatments. Additionally, suffixes are added in the Results section to denote the growth period before harvest (8 or 12 weeks) and to indicate plants which were infested with *M. persicae* aphids for 2 weeks prior to harvesting (+A).

Abbreviation	Soil type	Treatment
NC	Normal	Control
Seed	Normal	Seeds soaked in *T. thioparus* culture
Rhizo	Normal	*T. thioparus* inoculant applied to soil (root zone)
HSN	Normal	Sulphur fertiliser (0.16g pot^{-1})
SC	Sterilised	Control
D7	Sterilised	7 days pre-incubation (30°C) of sterile soil with *T. thioparus*
D14	Sterilised	14 days pre-incubation (30°C) of sterile soil with *T. thioparus*
HSS	Sterilised	Sulphur fertiliser (0.16g pot^{-1})

4.2.5 *T. thioparus* enumeration

To verify the success of the inoculum preparation, the bacterial enumeration of the inoculum (at the time of inoculation) was determined by CFU count methods (**Figure 30**). For the 7-day and 14-day inoculated soils, 10g of soil was diluted in 100ml of sterile dH_2O in a Stomacher® bag, which was pulsified in a Pulsifier for 30 seconds and then a dilution series ($x10^{-1}$ to 10^{-6}) was prepared using sterile PBS. 100µl of each dilution was plated out in triplicate on *T. thioparus* agar, and incubated at 30°C. Colony forming units (CFUs) were counted after 7 days of incubation, and the log concentration was calculated using the formula $ml^{-1} = \frac{no.\ of\ CFUs \times dilution\ factor}{volume\ plated\ (ml)}$.

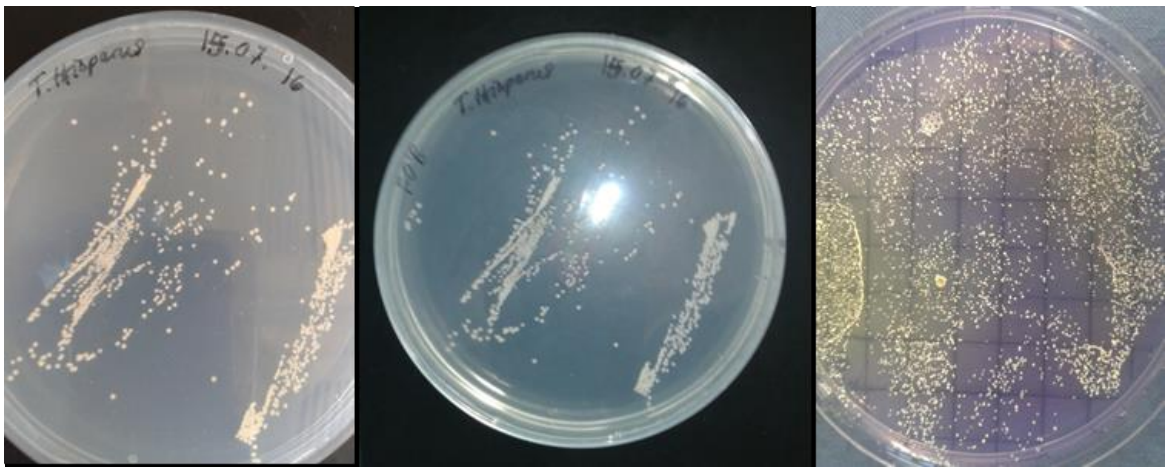


Figure 30 *Thiobacillus thioparus* cultures grown on selective agar media.

4.2.6 Glasshouse experiment

The glasshouse experiment was conducted at the University of Southampton from November 2014 until April 2015. Four surface-sterilised *Brassica oleracea* L. var. *capitata* seeds were sown into each of the soil-filled pots, and following germination, were thinned to one plant per pot. The

plants were grown in a glasshouse for 6-10 weeks under 16h light, 24°C (±2°C) and 60% relative humidity. At the end of the sixth or eighth week they were moved to the insectary for the 2-week aphid infestation period. To allow for post-harvest processing, the plants were sown in groups at staggered sowing dates. The plants were either harvested at week 8 or 12 in order to test for the durability of the treatment effects, and also to see whether the effects of enhanced SOB populations in the soil altered with plant growth.

The success of the inoculation methods after harvesting was checked by diluting 15g of rhizosphere soil in 150ml sterile dH_2O, which was pulsified for 15seconds and serially diluted in dH_2O. The dilutions were plated out in triplicate onto *T. thioparus*-selective agar plates.

4.2.7 Plant growth assessment

The cabbages were grown in a glasshouse and regularly randomized for the position under the light racks, and watered with distilled water. The number of leaves and stem height was measured for each plant. The N-analysis of freeze-dried plant samples was performed by Forest Research (Centre for Ecosystems, Society and Biosecurity).

4.2.8 Aphid herbivory

Aphid herbivory was examined by using a small paintbrush to introduce five mixed instar apterous adult *Myzus persicae* to half of the plants (randomly selected) from each treatment group. The plants were placed within Perspex cages, and each plant (including those without aphids) was enclosed in an air-permeable perforated (<1 mm) transparent polyethylene bag secured to the pot with an elastic band in order to prevent spread of aphids to neighbouring plants (**Figure 31**). After 14 days, the total aphid population was counted by removing each individual using a paintbrush immediately before harvesting the plants for further analysis.

Figure 31 Experimental set-up for the glasshouse phase (left) and infestation of *B. oleracea* with *Myzus persicae* aphids (right).

4.2.9 Harvesting plants for glucosinolate analysis

In order to examine the effect of plant growth and temporal effects of the *T. thioparus* and S-fertilisation, the cabbages were harvested at two different ages. Approximately half of the plants within each treatment were harvested at 8 weeks, and the rest at 12 weeks. The experiment was staggered (i.e. separate sowing dates) to allow for the processing time of each plant (e.g. counting aphids, flash-freezing plants). Unfortunately, some of the treatments (HSN, HSS and D14) were not fully replicated owing to unforeseen logistical issues, which occurred during the mid-point of the experiment. This was compounded by several samples being unsuccessful in the HPLC analysis. However, with the exception of the D14 12-week sample groups, all treatments had a minimum of 3 replicates each (**Table 16**).

The cabbages were cut at the base of the stem and the aboveground biomass (fresh weight) was quickly weighed before flash-freezing the plant. The flash-freezing was performed by wrapping the plant in aluminium foil (labelled on the opaque side) folded up into a parcel. Two holes were pierced at either end of the foil parcel to allow draining of liquid nitrogen. Using forceps the parcel was then immersed in liquid nitrogen (contained in a Dewar flask) for 5 seconds and repeated holding the parcel from the other end. These were stored at -80°C until freeze-drying. The three youngest leaves of each plant were freeze-dried (Edwards 'Modulyo' freeze-drier) for 48 hours or until the samples reached a constant weight. The freeze-dried samples were ground using a grinding mill (Retsch), and 50-100mg of the ground powder was weighed out and aliquoted in 2ml Eppendorf tubes.

Table 16 Treatments applied to *B. oleracea*, with details of the age of the plant at harvest and the number of plants per treatment (*n*).

Soil treatment	Plant treatment	Code	Replicates (*n*)	
			8 weeks	*12 weeks*
Normal soil	Control	NC	5	5 (+1*)
	Control with aphids	NC + A	7	6
	Seed inoculation	Seed	6	6
	Seed inoculation with aphids	Seed + A	6	6
	Rhizosphere inoculation	Rhizo	7	4 (+2*)
	Rhizosphere inoculation with aphids	Rhizo + A	4	6
	Sulphur fertilisation	HSN	4	6
	Sulphur fertilisation with aphids	HSN + A	4	6
Sterilised soil	Control	SC	3	6
	Control with aphids	SC + A	8	7
	7-day incubation	D7	4	7
	7-day incubation with aphids	D7 + A	4	8
	14-day incubation	D14	6	1
	14-day incubation with aphids	D14 + A	6	2
	Sulphur fertilisation	HSS	3	6
	Sulphur fertilisation with aphids	HSS + A	3	6

*Bracketed numbers represent additional samples that failed to yield successful HPLC results in the GLS analysis.

4.2.10 Glucosinolate analysis

Chromatographic separation of GLS from the freeze-dried cabbage samples was performed using HPLC methods at the German Centre for Integrative Biodiversity Research (iDiv), Leipzig, Germany. The methods for GLS extraction were provided by Nicole van Dam (2011, personal communication), which had been adapted from Graser *et al* (2000). In brief, GLS were extracted from the freeze-dried, ground cabbage samples by boiling briefly at ~90°C with 70% methanol (MeOH) solution (using boiling chips), before being placed in an ultrasonic bath for 15 minutes and centrifuged (4500 rpm) for 10 minutes. The supernatant from each tube was sequentially added to DEAE-Sephadex ® A25 (Sigma-Aldrich) prepared columns (EC, 1990). DEAE-Sephadex ® A25 is a weak anion exchanger containing diethylaminoethyl. Each column was rinsed with 70% MeOH, MilliQ water and finally 20mM NaOAc buffer. Eppendorf tubes were placed beneath the columns to collect the eluted solutions (**Figure 32**), and 20µl sulfatase solution was added to the columns and subsequently flushed again with 50µ NaOAc buffer. Sulfatase catalyses the hydrolysis of glucosinolates, resulting in breakdown products called desulphoglucosinolates (Hanson *et al*, 2004). The columns were left to stand overnight and the following day the resulting desulphoglucosinolates were eluted from the columns with 2x 0.75 ml MilliQ water. The tubes were frozen and placed in a freeze-dryer overnight. The residue was re-

dissolved in 1ml of MilliQ water and vortexed. The samples were stored at 4°C until HPLC analysis.

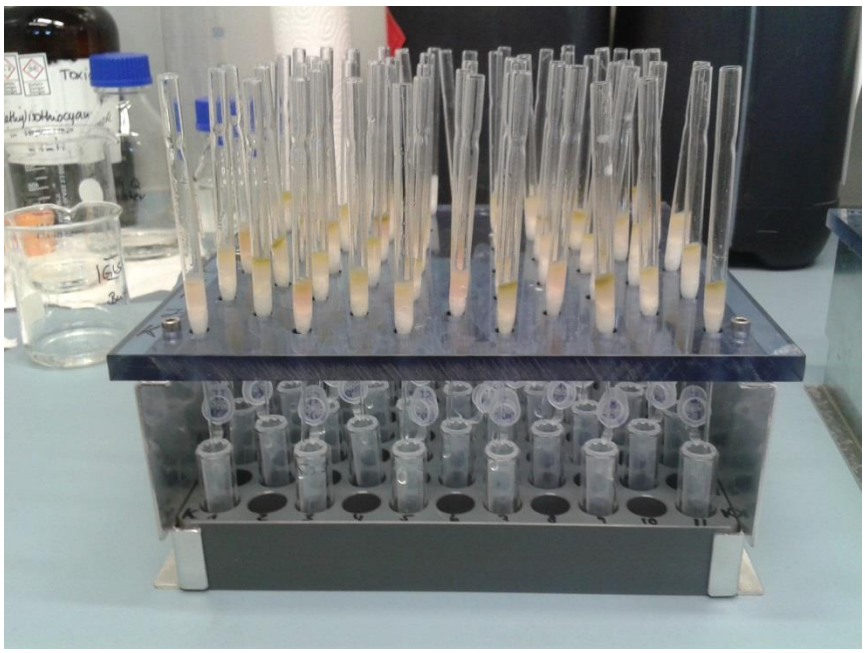


Figure 32 DEAE-Sephadex ® A25 prepared columns for glucosinolate extractions (German Centre for Integrative Biodiversity Research (iDiv)).

The desulphoglucosinolates were separated on an HPLC system (UltiMate 3000 Dionex, Thermo Scientific) equipped with a UV-diode array detector (DAD) and a reverse phase C-18 column (Acclaim ™ 300 C18 LC column, 4.6 x 150mm, particle size 3µm, particle distance 300A, Thermo Scientific). Sinigrin was used at five concentrations as an external standard and detection was performed by monitoring the wavelength at 229nm and 272nm. A binary system flow acetonitrile-water gradient was established with a run time of 45 minutes, a flow rate of 0.75 ml min^{-1}, and a column temperature of 40°C. The two solvents A (water) and B (acetonitrile) were injected at the following rates: 0-1minute, 98% A and 2% B; 1-35 min, 35% B and 65% A; 35-40min 2% B and 98% A. The injection volume was 10 µL, and 50 µL for repeats (samples which failed on the first run). Chromatogram analyses were performed using the EZChrom Elite 3.2.1 software. All assessed values are based on peak area at a monitoring wavelength of 254 nm.

A total of 167 samples were successfully analysed for GLS content, with an additional 5 samples failing to yield normal HPLC readings. Instances where no GLS was detected in the HPLC (*n.d.* (not detected)) were changed to 0 in order to calculate the mean and standard error for sample groups.

4.2.11 PCR and qPCR of the *soxB* gene

Rhizosphere soil samples were taken from each plant at the time of harvesting. DNA was extracted from a 0.25g subsample using the PowerSoil kit according to the manufacturer's instructions (see Chapter 3 methods). To corroborate the results, DNA was also extracted from colonies grown from soil dilutions plated out onto *Thiobacillus* agar where possible. DNA quality was checked by 1.5% agarose gel electrophoresis, and the quantity was measured photometrically at 260 and 280 nm using NanoDrop. DNA samples of sufficient quality and quantity were then diluted to a concentration of 5ng μl^{-1} for use in quantitative PCR (qPCR).

Quantitative PCR reactions were carried out using the iCycler iQ™ 5 MultiColor Real-Time PCR Detection System (Bio-Rad). The qPCR reaction mix was prepared using 10µl of 2xPrecisionPLUS qPCR MasterMix premixed with SYBR®green (PrimerDesign), 0.5 µl of each primer (10 µM), 4 µl nuclease-free water and 5 µl of sample DNA (5ng μl^{-1}), giving a final volume of 20 µl per reaction. The reaction mix was pipetted into Microseal 96-well skirted optical PCR plates with transparent adhesive seals and centrifuged to remove air-bubbles and to ensure that the mixture was at the bottom of the well. 16S rDNA was chosen as the housekeeping/reference gene, and soxB was the gene of interest (GOI). The quantitative PCR reaction was set to a three-step programme: (i) an initial hold of 95°C for 2 min (enzyme activation step); followed by (ii) 50 cycles of 95°C for 15s and 60°C for 1min (data collection and real-time analysis step); and finally (iii) a temperature gradient from 60 to 95°C at increments of 0.5°C after cycle 2 for 30sec (melt curve phase). Positive controls (DSM505 type strain *T. thioparus* DNA) and negative controls (nuclease-free water) were included in each qPCR run. Each sample was run in duplicate.

The amplification of the 16S rRNA gene was achieved using the forward primer 5'-CCTACGGGAGGCAGCAG -3' (Muyzer *et al*, 1993) and reverse primer 5'-ATTACCGCGGCTGCTGG - 3' (Muyzer *et al*, 1993), which amplify a 194-bp section of the 16S rRNA gene. In order to quantify the abundance of the *soxB* gene, primers were designed to amplify a 511bp fragment of the partial *soxB* gene for thiosulfate-oxidizing enzyme (thiosulfohydrolase *SoxB*) in *Thiobacillus thioparus* strain DSM 505 (GenBank: AJ294326.1). The forward primer was soxB 215F (5'- CAGGTGTTCAAGCCCTATGTC-3') and the reverse primer was soxB 311R (3'- GCTCCAGTCAGGGACCATGTAG-5').

To generate a standard curve, a dilution series (10^{-1} to 10^{-4}) was created using the positive control (DSM505 type strain *T. thioparus*) DNA. The standard curve was used to calculate the efficiency of the qPCR amplifications using the formula:

$$Efficiency = \left(10^{(-1 \div m)} - 1\right) \times 100\%$$

where *m* is the gradient of the slope obtained from linear regression obtained when the logarithm of the initial template concentration is plotted against the C_q value. If the efficiency was below 50%, the qPCR was repeated.

Standards and samples were assessed in duplicate, and the relative quantity of *soxB* genes in each sample were quantified in arbitrary units (AU) using the relative quantitation method using the standard curve and normalizing to the reference (16S) gene. The relative abundance of 16S and *soxB* genes in each experimental sample were calculated using the formulae proposed by Livak & Schmittgen (2001):

$$Relative\ quantity\ of\ target\ (GOI)\ =\ 10^{((C_q - b) \div m)}$$

where *b* is the y-intercept and *m* is the gradient of the standard curve-generated linear regression, and C_q represents the average threshold cycle (Illumina, 2010). As the number of copies in the standard curve samples was not known, the quantity is given in arbitrary units (AU). The *soxB* gene quantification was then normalised to the reference gene by dividing by the quantity of *16S*:

$$soxB\ ratio\ =\ soxB\ quantity / 16S\ quantity$$

4.2.12 Statistical analysis

Samples which had multiple *n.a.*'s (i.e. not detected) from the HPLC results were removed from the data set prior to analysis, otherwise instances of *n.a.*'s were converted to 0 into the dataset. To investigate differences in the plant leaf concentrations of the GLS based on their chemical structure, the GLS were grouped into aliphatic and indole GLS. D14 samples from the 12week, sterile soil treatment group were excluded from the analyses as there were insufficient replicates. Shapiro-Wilks and Bartlett tests were conducted to test whether the data followed a normal distribution and was homoscedastic (equal variance). Data which did not follow a normal distribution were transformed (natural logarithm and, in one case, 1/square root) in order to satisfy the assumptions of the statistical tests used. The *soxB* quantifications (ratios relative to 16S), indole GLS and (in some cases) aphid counts were natural-log (ln) transformed in order to obtain a normal distribution. When testing for effects of aphid abundance, aphid-infested samples which had a final population of <10 aphids were excluded from the analysis as they constituted unsuccessful infestations. One-way ANOVA tests were used to identify potential treatment effects on *soxB* abundance and GLS concentrations (aliphatic, indole and total). Any significant results were followed up with a Tukey's post-hoc pairwise comparison test. For heteroscedastic data, a Welch's ANOVA was conducted and Kruskal-Wallis tests for data which did not follow a normal distribution. In these instances, Dunn's test was used for post-hoc pairwise comparisons. Spearman's rank correlation tests were performed using the *corr.test* function in R. All statistical tests were performed in R© version 3.3.0 (http://www.R-project.org).

4.3 Results

The average primer efficiencies in the qPCR experiments were 61.88% for the 16S primers and 72.01% for the *soxB* primers. This was suboptimal (ideally it would be 90-100%), however, there was a high correlation of the linear regression as indicated by the r^2 values (98.95% for 16S and 98.06% for *soxB*) and it was considered to be sufficient for rough estimations of *soxB* abundance. A full list of the efficiencies and r^2 values is supplied in **Appendix C**.

4.3.1 Soil sterilisation and plant age effects on SOB populations

Soil sterility and plant age appeared to have considerable influence on the abundance of sulphur-oxidising bacteria (SOB) in the cabbage rhizosphere according to the quantification of the *soxB* gene. A three-way ANOVA was performed comparing *soxB* ratios of control plant soils factoring in plant age, soil sterility (SC or NC) and aphid presence (Y/N). SOB populations were significantly different according to soil sterility ($F_{3, 38}$=18.195, p<0.001) and plant age ($F_{1, 38}$=5.294, p=0.027). Aphid presence did not appear to have a significant effect on *soxB* ratios ($F_{1, 38}$=0.358, p>0.05). The average *soxB* abundance was significantly higher in the rhizosphere soil of sterile soil control (SC) plants in comparison to NC plants, and 8-weeks rather than 12-weeks (**Table 17**).

Table 17 Mean (±SE) rhizosphere *soxB* abundance (arbitrary units) of control cabbages grouped according to harvest time-point and *M. persicae* presence. Different letters indicate significantly different values between treatments as determined by one-way ANOVA.

Age	**Treatment**	***n***	***soxB*:16S ratio (SE)**
8 weeks	NC	5	0.00020 ^{ab} (0.00006)
	NC+A	7	0.00054 ^{abc} (0.00026)
	SC	3	0.00525 ^{cd} (0.00292)
	SC+A	8	0.00239 ^{d} (0.00118)
12 weeks	NC	6	0.00005 ^{a} (0.00001)
	NC +A	6	0.00012 ^{a} (0.00004)
	SC	7	0.00107 ^{bcd} (0.00036)
	SC +A	7	0.00189 ^{cd} (0.00053)
ANOVA	$F_{7, 41}$		9.7564
	P		**<0.0001**

soxB*:* Ln-transformed for ANOVA only (raw data given in above table).

4.3.2 Treatment effects on SOB abundance

The *T. thioparus* inoculation methods yielded varying results, with no single method being uniformly successful. The full set of qPCR results are given in **Appendix C**. Overall, pre-incubation of *T. thioparus* in sterile soil (D7 and D14) proved to be the most successful inoculation method, with the rhizospheres of these plants yielding up to 6.38 log CFU ml^{-1} g^{-1} of soil at the time of harvesting. This corroborated the *soxB* quantification results, with D7 having a maximum *soxB*:16S ratio of 0.07 and D14 a maximum of 0.02, which was considerably higher than that of sterile controls, which had a maximum of 0.011. When comparing plants grown in sterile soils, the SOB population was consistently highest in the rhizospheres of plants in the HSS treatment and lowest in the SC treatment across all aphid and harvest age scenarios (**Figure 33**).

In the absence of aphids, the rhizosphere SOB populations in D7 and D14 treatments were comparable to that of SC plants at the 8-week harvest. However, when plants were exposed to aphids over the fortnight prior to harvesting at 8 weeks, the *soxB* abundance in D7 rhizosphere soils was more than ten-fold that of control (SC) plants (Tukey HSD: *p*-adjusted = 0.049). This elevation of SOB in D7 soils was also apparent after 12 weeks growth, although it was not significantly different from the *soxB* abundance in the rhizospheres of controls (Tukey HSD $p>0.05$, **Figure 33**).

In "normal", non-sterile soils, the sulphur and *T. thioparus* treatments generally had a comparatively weak effect on the abundance of SOB populations in the rhizosphere. The seed inoculation method proved the least successful. In fact, in several instances the *soxB* abundance in the rhizospheres of seed-inoculated plants was actually lower in comparison to control plants (**Figure 33**). The only treatment to produce a substantial increase in *soxB* abundance in non-sterile soil relative to controls was the rhizosphere (Rhizo) inoculation method. These plants yielded a maximum of 3.90 log CFU ml^{-1} g^{-1} soil and a maximum *soxB*:16S ratio of 18.306, which was almost ten-fold the maximum detected in the rhizospheres of control plants (NC) at 1.877. According to SOB population assessments, the Rhizo treatment was most successful in 8 week-old cabbages (both with and without aphids). Again, the SOB inoculation appeared far more successful in plants exposed to aphid herbivory, particularly in aphid-infested 8 week-old plants where the quantified *soxB* abundance in Rhizo soils was significantly larger than that enumerated in the rhizospheres of control (NC) and seed-inoculated (Seed) plants (Tukey's post-hoc test $p<0.05$, **Figure 33**).

Plant age appeared to be negatively correlated with rhizosphere *soxB* populations within each treatment, except for SC and HSN, which exhibited no significant difference in soxB between 8 and 12 week-old plants.

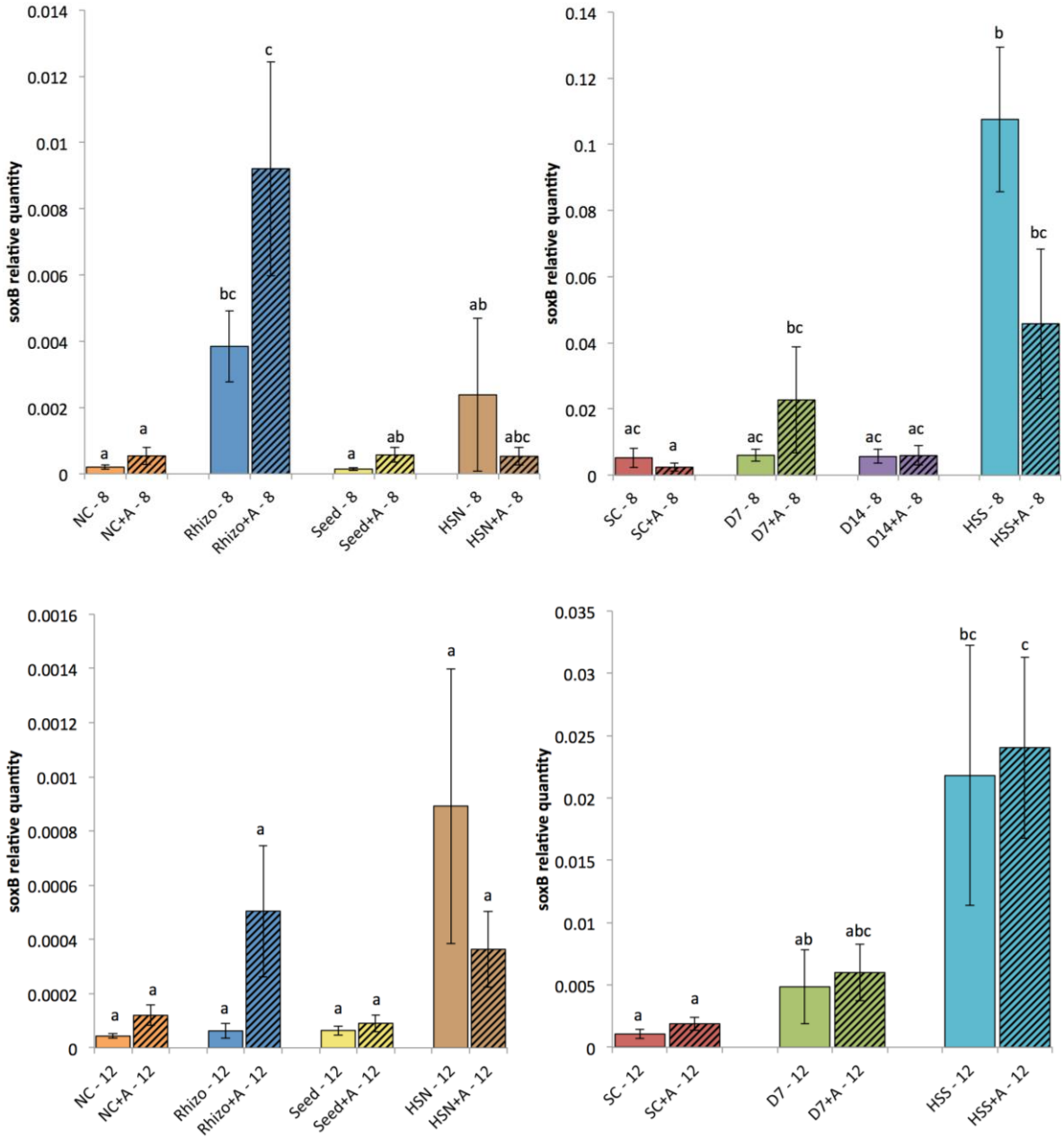


Figure 33 Relative abundance of *soxB* detected in *B. oleracea* rhizosphere in each treatment (normalised to 16S) at the 8-week (top) and 12-week (bottom) harvests, with (hatched bars) and without (open bars) aphids. Means (±SE) of *soxB* (given as a ratio relative to 16S quantification) are shown. Different letters indicate significant differences between treatments (within the same harvest age and soil sterility) according to Tukey HSD (using log-transformed data). Note the scales of the x-axis differ according to soil sterility (non-sterile and sterile treatments) and plant age (8 and 12 weeks).

4.3.3 SOB abundance and foliar GLS concentration

In total, 9 glucosinolates were detected in *B. oleracea* leaves: progoitrin (PRO), sinigrin (SIN), gluoiberin (IBE), glucorapharin (RAPH), gluconapin (GNA), glucobrassicin (GBC), neoglucobrassicin (NEO), 4-hyroxyglucobrassicin (4OH) and 4-methoxyglucobrassicin (4MeOH). These were grouped according to their chemical structure into aliphatic and indole GLS (**Table 18**). The individual GLSs will be referred to throughout this chapter using the abbreviations given in **Table 18**. The most dominant aliphatic GLS were sinigrin and glucoiberin, whilst the most abundant indole glucosinolates were glucobrassicin and 4-methoxyglucobrassicin.

Table 18 Glucosinolates detected in *B. oleracea* leaves and their molecular formulae.

Glucosinolate group	Common name & abbreviation		Side chain	Molecular formula
Aliphatic	progoitrin	PRO	2-hydroxy-3-butenyl	$C_{11}H_{19}NO_{10}S_2$
	sinigrin	SIN	2-propenyl	$C_{10}H_{16}KNO_9S_2$
	glucoiberin	IBE	3-methylsulfinylpropyl	$C_{11}H_{20}NO_{10}S_3^-$
	glucoraphanin	RAPH	4-methylsulfinylbutyl	$C_{12}H_{23}NO_{10}S_3$
	gluconapin	GNA	3-butenyl	$C_{11}H_{18}NO_9S_2$
Indole	glucobrassicin	GBC	Indol-3-ylmethyl	$C_{16}H_{20}N_2O_9S_2$
	neoglucobrassicin	NEO	1-methoxyindol-3-ylmethyl	$C_{17}H_{22}N_2O_{10}S_2$
	4-hyroxyglucobrassicin	4OH	4-hydroxyindol-3-ylmethyl	$C_{16}H_20N_2O_{10}S_2$
	4-methoxyglucobrassicin	4MeOH	4-methoxyindol-3-ylmethyl	$C_{17}H_{22}N_2O_{10}S_2$

The sterilisation of soil by autoclaving (twice) did not seem to affect GLS production in cabbage leaves, as there were no significant differences in either aliphatic, indole or total GLS concentrations detected between control plants (of the same harvest age and aphid treatment) grown in sterile (SC) and normal (NC) soil (**Table 19**). A significant interaction between cabbage age and total GLS concentration was detected in control plants from both soil treatments (SC and NC) (two-way ANOVA: $F_{3, 41}$=3.7330, p = 0.0184), with the older plants generally having higher GLS content. Control cabbages grown in sterilised soils (SC) in the absence of aphids showed a particularly strong increase in total aliphatic GLS concentrations (IBE, SIN, PRO, GNA and RAPH) with age (**Table 19**). It was therefore deemed appropriate to separate the two plant age cohorts when performing statistical analyses.

Table 19 Mean (±SE) foliar glucosinolate content (μmol mg^{-1} dry weight) of control cabbages grouped according to harvest time-point and *M. persicae* presence. Different letters indicate significantly different values between treatments as determined by one-way ANOVA, with the exception of Total Indole, for which a Kruskal-Wallis test was performed.

Age	Treatment	*n*	Total Aliphatic	Total Indole	Total GLS
8 weeks	NC	5	6.633^{bc} (0.243)	2.936^{ab} (1.123)	9.568^{b} (1.011)
	NC+A	7	2.864^{a} (0.352)	1.312^{ab} (0.168)	4.176^{a} (0.408)
	SC	3	2.702^{ab} (0.992)	4.051^{ab} (1.527)	6.753^{ab} (0.745)
	SC+A	8	3.073^{a} (0.440)	3.224^{a} (0.932)	6.298^{ab} (0.656)
12 weeks	NC	6	5.456^{ac} (1.100)	1.367^{ab} (0.398)	6.823^{ab} (1.362)
	NC +A	6	6.107^{bc} (0.545)	0.835^{b} (0.151)	6.942^{ab} (0.658)
	SC	7	7.886^{c} (0.897)	1.860^{ab} (0.524)	9.747^{b} (1.313)
	SC +A	7	6.751^{c} (0.625)	1.735^{ab} (0.370)	8.486^{b} (0.781)
ANOVA	$F_{7, 41}$		8.6905	(χ^2) 15.994	4.1466
	P		**<0.0001**	**0.02517**	**0.001571**

Indole: Kruskal-Wallis test with Dunn's post-hoc test (Benjamini-Hochberg adjusted *p*) using dunnTest function in FSA package in R.

Overall, the enhancement of soil SOB populations, through either the addition of elemental S or inoculation with *T. thioparus*, appeared to broadly result in greater GLS levels in *B. oleracea*, in particular those belonging to the aliphatic class. After 8 weeks of growth in sterile soils, plants in the D14 and HSS treatments produced considerably higher foliar aliphatic GLS concentrations relative to sterile controls (**Table 20**). However, statistically there was little support for a significant treatment-specific relationship between SOB populations (as determined by *soxB* quantification) and GLS concentration in experimental plants. Only aphid-colonised 12 week-old plants in the "sterile" treatments (SC, D7 and HSS) collectively showed signs of enhanced SOB abundance being positively correlated with GLS production, with significant correlations being detected between *soxB* abundance and aliphatic (Spearman's rank correlation: S=358, $p<0.001$, *rho* = 0.7675325), indole (Spearman's rank correlation: S= 4732, $p<0.001$, *rho* = 0.6935) and total GLS (Spearman's rank correlation: S=720, $p=0.014$, *rho* = 0.5325). This was predominantly caused by the HSS plants having significantly higher aliphatic GLS concentrations than all other treatments (**Table 20**).

In the non-sterile treatments, the rhizosphere inoculated plants had significantly higher GLS content in the aphid-infested 8week-old cohort (**Table 21**). The seed inoculation, which appeared

to be largely unsuccessful according to the SOB quantification results (**Figure 33**), exhibited significantly lower aliphatic concentrations (8weeks no aphids) and higher indole levels (12 weeks no aphids; 8 weeks with aphids; and 12 weeks with aphids) (**Table 21**).

Table 20 Treatment variations in concentration (μmol mg^{-1} dry weight) of indole and aliphatic glucosinolates under sterile soil conditions. (Results denoted with * used one-way tests to allow for unequal variance).

Treatment		**SC**	**D7**	**D14**	**HSS**	***d.f.***	***F***	***P***	***Tukey HSD***
8 wk no aphids	Aliphatic	2.702	3.330	4.210	3.940	*3,12*	*0.457*	*0.717*	*n.s.d.*
	Indole	4.051	2.219	5.588	2.097	*3,12*	*5.643*	***0.012***	D14 - D7, HSS
12 wk no aphids	Aliphatic	7.886	9.171	*n.a.*	11.182	*2,17*	*1.109*	*0.379**	*n.s.d.*
	Indole	1.860	2.629	*n.a.*	2.311	*2,17*	*2.036*	*0.161*	*n.s.d.*
8 wk, + aphids	Aliphatic	3.074	4.373	4.157	4.048	*3,17*	*0.595*	*0.627*	*n.s.d.*
	Indole	3.224	2.662	1.407	1.210	*3,17*	*2.567*	*0.089*	*n.s.d.*
12 wk + aphids	Aliphatic	6.751	7.852	*n.a.*	11.888	*2,18*	*14.539*	***<0.001***	HSS - SC, D7
	Indole	1.735	1.586	*n.a.*	2.220	*2,18*	*0.874*	*0.434*	*n.s.d.*

Table 21 Treatment variations in concentration (μmol mg^{-1} dry weight) of indole and aliphatic glucosinolates under normal (non-sterile) soil conditions. (Results denoted with * used one-way tests for unequal variance).

Treatment		**NC**	**Rhizo**	**Seed**	**HSN**	***d.f.***	***F***	***P***	**Tukey's HSD**
8 wk no aphids	Aliphatic	6.633	5.951	3.513	5.368		*7.981*	***0.008****	Seed - NC
	Indole	2.936	2.269	1.199	1.472	*3,18*	*2.703*	*0.076*	*n.s.d.*
12 wk no aphids	Aliphatic	5.456	6.751	6.665	8.405		*0.871*	*0.487**	*n.s.d.*
	Indole	1.367	1.494	3.787	1.378	*3,20*	*6.819*	***0.002***	Seed - NC, Rhizo, HSN
8 wk + aphids	Aliphatic	2.864	5.460	3.324	4.742	*3,17*	*4.191*	***0.022***	Rhizo - NC
	Indole	1.312	2.372	3.530	0.778	*3,17*	*8.20*	***0.001***	Seed - NC, HSN
12 wk + aphids	Aliphatic	6.107	5.241	6.499	9.148	*3,20*	*6.535*	***0.003***	HSN - NC, Rhizo, Seed
	Indole	0.835	0.510	3.785	1.620	*3,20*	*35.270*	***<0.001***	HSN - NC, Rhizo, Seed Seed - NC, Rhizo

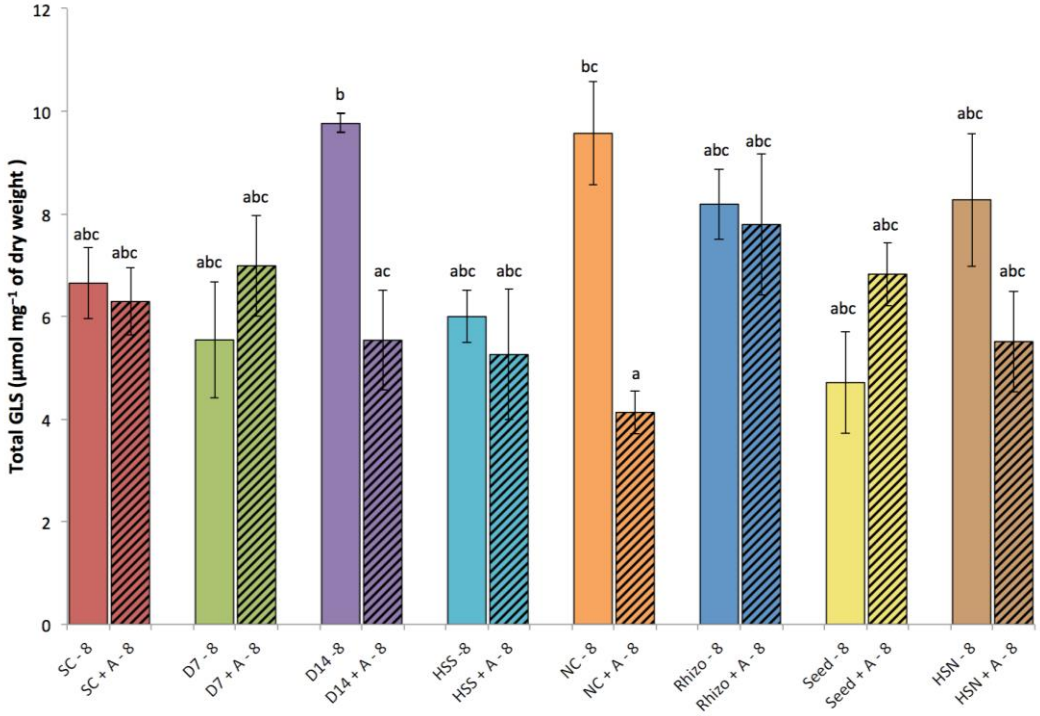


Figure 34 Mean total glucosinolate concentrations of 8-week old cabbages grown under different treatments (µmol mg^{-1} dry weight). Letters indicate significant differences; hatched bars indicate aphid-infested plants.

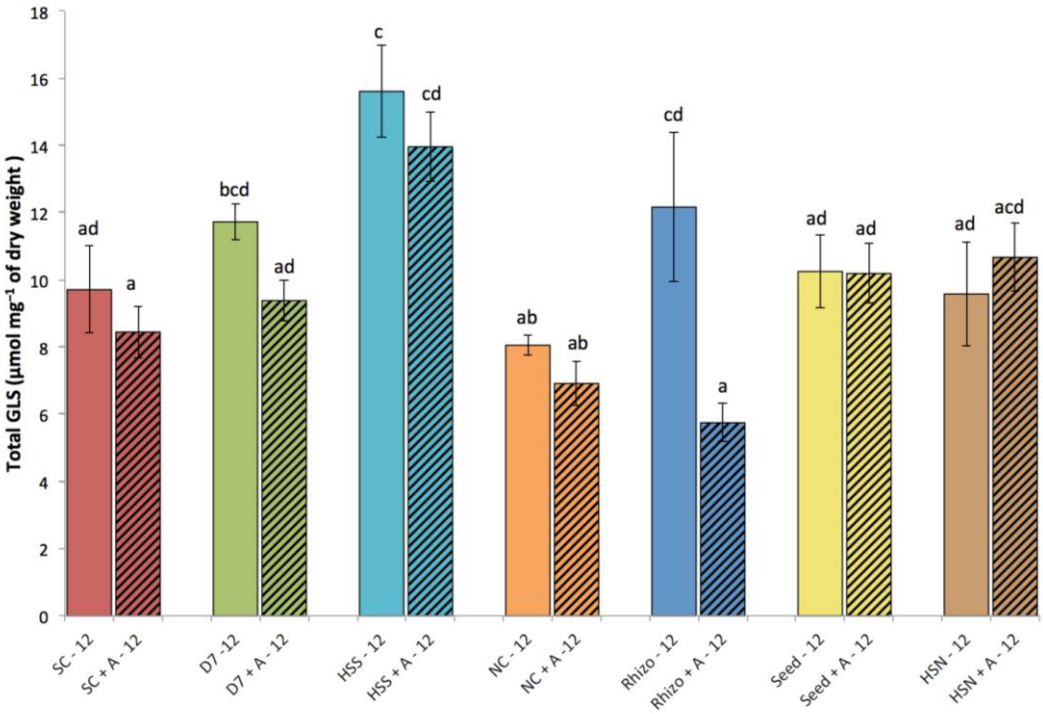


Figure 35 Mean total glucosinolate concentrations of 12-week old cabbages grown under different treatments (µmol mg^{-1} dry weight). Letters indicate significant differences; hatched bars indicate aphid-infested plants.

The impact of sulphur additions on total GLS in plants grown in sterile soil seemed to strengthen over time, as HSS plants displayed the greatest increase in total GLS levels in comparison to all treatments when grown for an additional 4 weeks. In the absence of aphids, 12 week-old HSS plants had on average 123.5% higher total GLS concentrations than the 8 week-old plants. In the aphid-infested plants, this disparity widened to a 168.3% rise in total GLS concentrations in 12-week plants relative to their younger equivalents. Indeed, at 8 weeks the total GLS content of HSS plants was either no different or slightly lower than controls (SC), however, at 12 weeks it was considerably higher than SC plants (**Figure 34** and **Figure 35**).

4.3.3.1 Aliphatic GLS

Examination of individual aliphatic GLS revealed a significant difference in GNA levels between sterile treatments in 8 week, undamaged plants (ANOVA: $F_{3, 12} = 4.712$, $p = 0.0214$), with D14 plants having significantly higher concentrations than SC plants, in which this aliphatic GLS was not detected at all (Tukey HSD: $p=0.0135$). In 12 week-old, aphid-damaged plants, IBE (one-way ANOVA: $F_{2, 18}=14.41$, $p=0.0002$) and PRO (one-way ANOVA: $F_{2, 18}=12.99$, $p=0.0003$) concentrations were significantly higher in HSS plants than all other treatments (Tukey HSD $p<0.05$). Sinigrin (SIN) levels were also significantly higher in these HSS plants than controls (ANOVA: $F_{2, 18} = 3.74$, $p = 0.0438$; Tukey HSD $p=0.0394$). Paradoxically, when comparing the quantified *soxB* abundance in HSS soils with aliphatic concentrations, a negative association was detected (Spearman's: $p = 0.0091$, S = 20849, *rho* = -0.5676). In HSS plants, the individual aliphatic GLS responsible for this relationship were PRO (Spearman's: $p = 0.0086$, S = 2088.9, *rho* = -0.5706), GNA (Spearman's: $p = 0.0085$, S = 2090.1, *rho* = -0.5715363), RAPH (Spearman's: $p = 0.0300$, S = 1975.7, *rho* = -0.4855), and IBE (Spearman's: $p = 0.0395$, S = 1946, *rho* = -0.4637).

In non-sterile (normal) soils, the *T. thioparus* and sulphur-treated plants consistently had higher total aliphatic GLS concentrations, with a couple of exceptions. Seed inoculated plants had significantly lower total aliphatic GLS levels than NC plants at 8 weeks without aphids, and the Rhizo plants had the lowest total GLS levels in 12-week aphid-infested plants, although not statistically different from controls (**Figure 35**). However, the aphid-infested 8-week cohort of rhizosphere-inoculated plants had significantly higher aliphatic GLS levels relative to controls (**Table 21**). Similarly, sulphur additions (HSN) resulted in significantly higher aliphatic GLS levels than all other treatments in the 12-week, aphid-infested group only (**Table 21**). At the individual glucosinolate level, one-way ANOVAs revealed that the 8-week (no aphids) seed-inoculated plants had significantly lower concentrations of IBE ($F_{3, 18} = 5.701$, $p = 0.0063$), PRO ($F_{3, 18} = 4.87$, $p = 0.0119$) and RAPH ($F_{3, 18} = 5.069$, $p = 0.0102$) than other plant treatments, namely NC and Rhizo. The aphid-infested Rhizo and HSN plants had significantly higher concentrations of IBE (ANOVA $F_{3, 17} =10.08$, $p = 0.0005$) and RAPH (Kruskal-Wallis: $p = 0.0033$, $\chi^2=13.699$) than controls at 8 weeks. In 12-week aphid-infested "normal" plants, a significant

increase in IBE ($F_{3,\ 20}$ = 4.895, p = 0.0104), PRO ($F_{3,\ 20}$=3.686, p = 0.0292) and SIN ($F_{3,\ 20}$ = 5.812, p = 0.0050) were observed in HSN plants relative to Rhizo, and, in the case of SIN, control plants (Tukey's HSD p<0.05).

4.3.3.2 Indole GLS

When all the samples (normal and sterile, aphids and no aphids) were pooled together, a positive correlation was detected between *soxB* abundance and indole GLS concentrations (Spearman's correlation: p = 0.0095, S = 680580, *rho* = 0.19674775). On closer inspection, it appeared that this was largely due to increases in GBC levels (Spearman's correlation: p = 0.02564, S =703740, *rho* = 0.1702), which was the only significant correlation detected between *soxB* and an individual GLS.

A comparison of the different SOB-enhancing treatments, however, revealed mixed effects on foliar indole GLS concentrations. In both sterile and normal soils, elemental sulphur amendments seemed to strikingly inhibit indole GLS production in the leaves of 8 week-old plants, but slightly increase it in 12 week-old plants (**Table 20** and **Table 21**). In some instances, the enlargement of soil SOB populations seemed to promote indole GLS production in cabbage leaves. The total indole concentration of 8-week old D14 plants (no aphids), for instance, was more than double that of D7 and HSS plants, and 25% higher than controls. The leaves of these D14 plants also had an overall total GLS concentration that was significantly higher than all other treatments (Tukey pairwise comparison: p<0.05, **Table 20**). Further analysis revealed that the *soxB* abundance in the rhizospheres of D14 plants was positively correlated with indole GLS concentrations (Spearman's: p = 0.0213, S = 226, *rho* = 0.5964). This trend was also evident in SC plants (Spearman's: p = 0.0424, S = 1532, *rho* = 0.4108). In normal soils, seed soaking (the least successful inoculation method in terms of rhizosphere *soxB* abundance) yielded significantly higher indole concentrations than all other treatments in all instances apart from 8 weeks without aphids (**Table 21**). However, in contrast to the other treatments, the correlation between *soxB* and indole GLS in Seed plants was found to be negative (Spearman's: p = 0.0116, S = 3476, *rho* = -0.5113).

Promoting SOB populations by pre-incubation of sterile soil with *T. thioparus* (D7 and D14 treatments) appeared to significantly enhance 4MeOH levels in 8 week-old, undamaged plants (one-way ANOVA $F_{3,\ 12}$=20.17, p <0.001). D14 plants in this group had significantly higher 4MeOH levels than all other treatments, whilst levels in D7 plants were significantly higher than controls only (Tukey's HSD p<0.05). The HSS treatment also produced substantially higher 4MeOH levels in 12 week-old plants (without aphids) relative to controls (Kruskal-Wallis p=0.0221, χ^2=7.6211). Conversely, further analysis revealed a negative correlation of *soxB* abundance and the foliar levels of 4OH in HSS plants (Spearman's: p = 0.0017, S = 2203.3, *rho* = -0.6567). Furthermore, when aphids were introduced to the plants which were subsequently harvested at 8 weeks, 4MeOH was significantly reduced in D14 plants in comparison to all other

treatments, and the concentrations of this indole GLS in D7 plants were also significantly lower than controls (SC). This may be a result of increased allocation of plant resources towards aliphatic GLS production. The only indole to differ between treatments in 12 week, aphid-infested sterile plants was 4OH (Kruskal-Wallis p=0.01891, χ^2=7.9362) with concentrations in D7 plants being more than 11-fold that of sterile controls.

In all but one (8 weeks, no aphids) of the aphid/harvest scenarios, the seed inoculation treatment yielded the highest total indole GLS concentration. At 12 weeks, both with and without aphid herbivory, seed-inoculated plants had significantly higher GBC (one-way ANOVAs: no aphids: F=6.104, p=0.0040; with aphids: F=8.7038, p=0.0335), 4MeOH (no aphids: F=5.132, p=0.0086; with aphids: F=10.793, p=0.0129) and NEO (no aphids: F = 7.9267, p=0.0476; with aphids: F=11.087, p=0.0113) concentrations than at least one other treatment. In undamaged 8 week-old plants, the Rhizo inoculation yielded significantly higher GBC concentrations (Kruskal-Wallis p=0.0138, χ^2=7.0602), which were more than 26 times higher than the mean concentration in NC plants.

4.3.4 GLS content and Aphid herbivory

4.3.4.1 Control plants

In the case of cabbages grown in non-sterile soil, *M. persicae* herbivory was associated with significantly lower aliphatic and total GLS concentrations of 8 week-old control (NC+A) plants in comparison to undamaged controls (**Table 21** and **Figure 35**). The mean aliphatic concentration of infested 8 week NC plants was 56.8% lower than their undamaged equivalents (one-way ANOVA*:* $F_{1, 10}$ = 64.85, p <0.001), whilst total GLS concentrations were 55.3% lower in aphid-infested plants (ANOVA p>0.05). Incidentally, these NC+A plants also exhibited the highest aphid populations of all the treatment groups, with up to 567 aphids on a single plant (mean=243). Conversely, at the 12-week harvest, NC+A plants had considerably (11.9%), although not quite statistically significant, higher aliphatic GLS content in comparison to aphid-free NC plants (Kruskal-Wallis p<0.05).

Aphid infestation of sterile control plants did not seem to cause any significant alterations in aliphatic GLS content when harvested at either 8 or 12 weeks (**Table 20**). Sterile control (SC) cabbages subjected to aphid herbivory had on average a slightly (13.8%) higher aliphatic GLS content after 8 weeks than their undamaged counterparts, although this was not statistically significant (one-way ANOVA: $F_{1, 9}$ = 0.162, p=0.697). The aphids seemed to have an opposite, although similarly small, impact on aliphatic GLS concentrations in older (12 week) cabbages which had slightly lower (14.4%), but, again not statistically different, aliphatic GLS concentrations when exposed to aphid herbivory (one-way ANOVA: $F_{1, 12}$= 1.079, p=0.319).

A more consistent effect of aphid herbivory was observed in relation to indole GLS, with the phloem-feeding insects being negatively associated with total indole GLS concentration of both NC and SC plants (**Table 20** and **Table 21**). However, neither 8 nor 12 week-old SC plants exhibited significant differences in their indole GLS concentration between aphid-infested and undamaged plants (one-way ANOVA p>0.0.5), although it appeared to be consistently lower in damaged plants. Similarly, when grown in normal soil, 8 week-old NC leaves exposed to aphids had substantially diminished indole GLS concentrations in comparison to undamaged plants, although this trend did not attain statistical significance (natural logarithm-transformed data, one-way ANOVA: $F_{1, 10}$= 4.32, p=0.0644). A similar pattern was observed in NC cabbages harvested at 12 weeks, but again the difference was not significant (one-way ANOVA: $F_{1, 10}$ = 1.563, p=0.240).

4.3.4.2 Sulphur- and SOB-treated plants

Overall, aphid herbivory was not correlated with the total GLS concentration of plants in all sterile treatments and ages (ANOVA p>0.05), although there were indications of a weak negative relationship (**Table 22**). The only instances of significant declines in total GLS of aphid-attacked plants occurred at 8 weeks in NC (ANOVA p = 0.0001, $F_{7, 35}$ = 4.414; Tukey's HSD: p = 0.0028) and D14 plants (ANOVA p = 0.0107, $F_{7, 32}$ = 3.215, Tukey's HSD: p = 0.0067). This coincided with the 8 week-old D14 plants having significantly larger aphid populations than on SC and HSS plants (Tukey's pairwise comparison p<0.05). There were anomalies, however, as aphid populations were associated with increased total GLS in D7 (8 week) and HSS (12 week) plants, which respectively had 26.8% and 4.6% higher total GLS concentrations than undamaged plants (**Table 20**).

Aliphatic GLS concentrations had a significant negative correlation with the number of aphids when all treatments were grouped as one (**Table 22**). The individual GLS which accounted for this decline were identified as IBE (Spearman's: p = 0.0242, S = 102990, *rho* = -0.2536), PRO (Spearman's: p = 0.0029, S = 109330, *rho* = -0.3307), RAPH (Spearman's: p = 0.0043, S = 108270, *rho* = -0.3178) and GNA (Spearman's: p = 0.0182, S = 103950, *rho* = -0.2652).

Contrastingly, indole GLS concentrations were positively correlated with aphid populations (**Table 22**). Specifically, the levels of NEO (Spearman's rank correlation: p = 0.0062, S =57072, *rho* = 0.3054) and 4OH (Spearman's rank correlation: p = 0.0288, S =61938, *rho* = 0.2461) were both positively associated with the number of aphids colonising plants. When subdividing the plants into "normal" and "sterile" groups, the number of aphids populating a "normal" cabbage did not correlate with either total GLS or aliphatic GLS, however, the positive association of aphid

herbivory with indole GLS levels was evident (**Table 22**). In "sterile" plants, however, there were no significant correlations between indole GLS and aphid abundance (**Table 22**).

Table 22 Spearman's rank correlation test results comparing the number of *M. persicae* aphids on infested *B. oleracea* plants in normal and sterile treatments. Significant results are highlighted in **bold**.

Factor	**Sterile + aphids**			**Normal + aphids**			***All treatments + aphids***		
	S	*p-value*	*rho*	S	*p-value*	*rho*	S	*p-value*	*rho*
soxB	5492.7	0.182	0.231	11344.0	0.694	-0.064	81898.0	0.978	0.003
Aliphatic	8168.2	0.409	-0.144	13223.0	0.135	-0.240	102060.0	**0.032**	-0.242
Indole	6088.8	0.399	0.147	5627.3	**0.002**	0.472	56311.0	**0.005**	0.315
Total GLS	8694.3	0.209	-0.218	10249.0	0.813	0.039	91125.0	0.338	-0.109

4.3.5 Aphids and SOB abundance

Soil sterilisation appeared to have little influence on aphid population growth, as there was no statistical difference in final aphid population counts between sterile (SC) and normal (NC) control plants. The age of the plant, however, did seem to affect *M. persicae* population growth during the 14-day colonisation period (**Figure 36**). Aphid populations were generally larger on younger (8 week) plants than older (12 week) plants, regardless of treatment (one-way ANOVA: $F_{1, 77} = 6.342$, $p = 0.0134$). However, when comparing aphid populations within treatments, effect of plant age was of borderline significance in both D7 (one-way ANOVA $F_{1, 10} = 5.041$, $p = 0.0514$) and SC ($F_{1, 10} = 3.162$, $p = 0.0571$). Rhizo plants were the only group to show a strong effect of age on aphid abundance (one-way ANOVA $F_{1, 7} = 7.671$, $p = 0.0277$), with those harvested at 12 weeks hosting significantly smaller aphid populations than 8 week-old plants. Furthermore, there were two exceptions to this trend as both SC and Seed plants had larger aphid populations on 12-week plants.

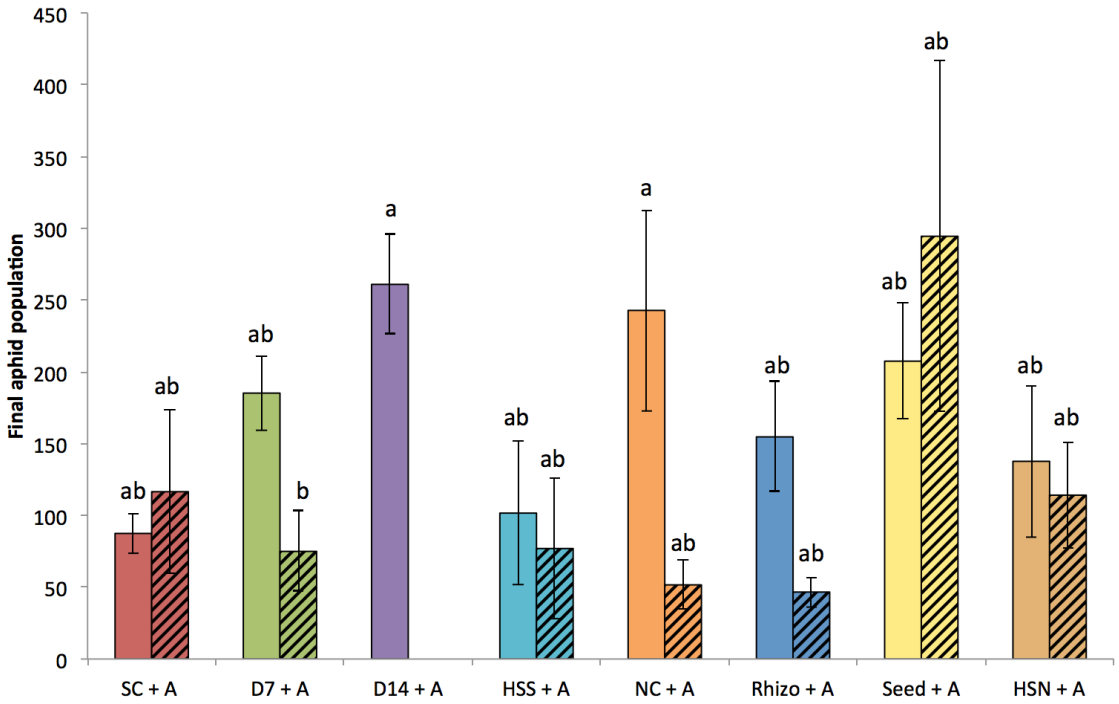


Figure 36 Aphid populations on plants grown under different sterile and non-sterile treatments for 8 weeks (open bars) and 12 weeks (hatched bars) (mean ±S.E.). (Normal (nonsterile) soil treatment codes: **NC:** normal control; **Seed**: seed *T. thioparus* inoculation; **Rhizo:** rhizosphere *T. thioparus* inoculation; **HSN**: sulphur fertiliser treatment.)

The number of aphids populating a plant after the 14-day colonisation period did not correspond significantly with *soxB* abundance in either normal (Spearman's rank correlation: $p = 0.694$, S = 11344, *rho* = -0.0642) or sterile (Spearman's rank correlation: $p = 0.1824$, S = 5492.7, *rho* = 0.2307) soils. Nonetheless, plants which were inoculated using what was generally regarded as the least successful method - seed inoculation - had larger aphid populations than the other SOB-enhancing "normal" treatments at both harvest ages. SOB populations in the rhizospheres of aphid-attacked NC, Rhizo, D7 and D14 plants were larger than those of their aphid-free counterparts, both at 8 and 12 weeks (**Figure 33**). Contrastingly, in both normal and sterile conditions, soils that received elemental S additions had lower *soxB* abundances when aphids were present in comparison to the soils of aphid-free plants. This trend was most evident in sterile soils, with the quantified *soxB* abundance in aphid-infested HSS plant rhizospheres at both harvest time-points being less than half of that estimated for undamaged plants (**Figure 33**).

At 8 weeks, each of the SOB-enhanced sterile soil treatments (D7, D14 and HSS) yielded higher aphid counts than control plants. However, after 12 weeks, the mean abundance of aphids on both D7 and HSS plants was roughly two-thirds the size of that of control (SC) plants, possibly indicating a time-lag in the beneficial effects of enhanced SOB populations. At both time-points, Rhizo plants supported smaller aphid populations than controls (NC), whilst HSN plants had lower

aphid abundance relative to control plants at 8 weeks only, having more than double the NC aphid population at 12 weeks (**Table 23** and **Table 24**). However, a two-way ANOVA found no significant interaction between aphid population size and *soxB* abundance.

Table 23 Plant and aphid metrics for infested **8 week**-old cabbages (mean per treatment).

Treatment	Stem height (cm)	No. of leaves	Fresh aboveground biomass (g)	No. of Aphids	Aphids/ cabbage fresh wt (g^{-1})	Total N (%)	Total C (%)
NC	3.700	12.857	*n.a.*	242.857	*n.a.*	3.461	37.033
Rhizo	4.050	13.500	21.198	154.750	7.300	2.281	39.692
Seed	3.200	11.333	11.733	207.500	17.685	3.877	36.612
HSN	3.600	10.000	10.320	137.750	13.348	1.710	38.163
SC	4.225	10.625	13.302	87.500	6.578	3.857	40.596
D7	3.775	10.750	16.633	185.250	11.137	3.335	39.700
D14	4.000	9.833	14.415	261.000	18.107	3.481	40.703
HSS	4.100	10.000	14.144	101.667	7.188	4.515	38.417

Table 24 Plant and aphid metrics for infested **12 week**-old cabbages (mean per treatment).

Treatment	Stem height (cm)	No. of leaves	Fresh aboveground biomass (g)	No. of Aphids	Aphids/ cabbage fresh wt (g^{-1})	Total N (%)	Total C (%)
NC	4.833	17.500	37.595	51.667	1.374	1.305	41.157
Rhizo	4.867	17.667	33.132	46.667	1.408	1.650	40.952
Seed	4.967	18.333	33.128	294.500	8.890	1.195	41.779
HSN	4.100	15.333	19.813	114.167	5.762	2.052	41.572
SC	5.443	14.286	28.353	116.571	4.111	2.052	41.719
D7	5.438	15.375	33.125	75.000	2.264	1.790	41.411
D14	6.000	13.500	24.277	45.500	1.874	1.536	41.455
HSS	5.117	14.000	33.212	77.000	2.318	1.064	40.699

At 12 weeks, cabbages grown in sterilised soil were taller and had fewer leaves, but exhibited *M. persicae* infestation levels which were on average more than double that of control plants grown in normal soil. A similar pattern was observed for cabbages harvested at 8 weeks, except that the number of aphids on NC plants was almost triple the average number on SC plants. When all samples were pooled together, *soxB* abundance was negatively correlated with fresh aboveground biomass (Spearmen's: $p = 0.0036$, S= 885690, *rho* = -0.2271). The sulphur-fertilised soils (HSS and HSN) did not exhibit this relationship, whereas the *T. thioparus*-inoculated soils (normal and sterile) did (Spearman's: $p = 0.00396$, S= 125110, *rho* = -0.3130). On further inspection, it was revealed that this was true for non-sterile treatments (Rhizo and Seed) only (Spearman's: $p = 0.003157$, S = 21715, *rho* = -0.4305). There was no significant correlation between *soxB*

abundance and plant biomass in the *T. thioparus* inoculations (D7 and D14) in sterile soil (Spearman's: p =0.2731, S = 10802, *rho* =-0.1820).

4.4 Part II: Sulphur-Nitrogen ratios and plant-aphid performance

4.4.1 Economic threshold of N fertiliser

The economic injury level (EIL) and economic threshold (ET) are two interlinking methods used to measure the detrimental impacts of pests on crop yields. The EIL refers to either the minimum abundance of pests, or the minimum level of damage to crops, which is economically equivalent to the cost of implementing sufficient control measures (Leslie, 2009, Pedigo *et al*, 1986). The ET can be defined as the threshold of pest damage at which management action should be taken in order to avoid reaching the EIL during the crop growth period (Leslie, 2009). As discussed in Chapter 2, the amount of N applied to plants can have important implications for the level of aphid infestations due to its effect on plant quality. Consequently, fertilisation levels are intrinsically linked to EIL and ET as they can affect the attractiveness and susceptibility of crops to insect herbivores.

Several investigations into the balance between N-fertilisation rates and the economic costs of aphid infestations have shown that increased N-inputs to crops are only beneficial up to a point, beyond which they are offset by the corresponding detrimental effects entailed by rising aphid populations (Mahdavi-Arab *et al*, 2014). Hosseini *et al* (2010) examined the relationship between varying N-fertilisation rates, cucumber yield and cotton aphid (*Aphis gossypii*) populations through mesocosm experiments. They found that the yield of aphid-infested plants declined linearly with increasing N fertilisation (lowest yield at highest N input), which contrasted to the positive correlation observed between yield and N input that was observed when aphids were absent. Davies *et al* (2004) reported similar results of *A. gossypii* and varying N levels on chrysanthemum plants, proposing the explanation that the aphids may have a negative impact on photosynthesis in the leaves of plants receiving higher levels of N fertilisers. These results conflict in part with those of Sauge *et al* (2010), who tested five levels of N fertilisation on the infestation rates of aphids (*M. persicae*) on peach (*Prunus persicae* (L.)). They showed that aphid abundance was positively correlated with the three intermediate N levels over time, but was reduced on plants in both the lowest and highest N dosage treatments. This was a surprising result given that plants in the highest N fertilisation treatment had the highest plant biomass and leaf N content. Clearly there are complex and system-specific interactions occurring. Considering the scarcity of published research on the EIL of *M. persicae* on *B. oleracea* L. var. *capitata* in relation to different N levels, this is an area of research which warrants further investigation, with previous studies having looked at just two fertiliser application rates (Kalule and Wright, 2002).

4.4.2 Glucosinolate production and nitrogen-sulphur dynamics

The amount of available nitrogen (N) and sulphur (S) in the soil has been shown to influence glucosinolate content of several plants, with the increases in the two nutrients appearing to elicit opposing responses in plant glucosinolate levels. Although there are some conflicting reports of the effects of N on glucosinolate concentrations (Staley *et al*, 2010, Øvsthus *et al*, 2015), elevated N additions have frequently been shown to suppress GLS production (Chen *et al*, 2004, Chun *et al*, 2015 (In Press)), whereas increasing S availability results in higher GLS levels (Zhao *et al*, 1993, Zhao *et al*, 1994) as demonstrated in Part I of this chapter. It has been demonstrated in both broccoli and horseradish plants, that low N and high S applications (N:S ratio ranging between 7:1 and 10:1) result in higher GLS levels than those receiving optimal N and low S amendments (Schonhof *et al*, 2007, Rosen *et al*, 2005, Krumbein *et al*, 2001). The balance of these nutrients is also important in determining crop yield, with an N:S ratio of approximately 4:1 being optimal for achieving higher cauliflower yields (Čekey *et al*, 2014).

The mechanism behind this relationship is believed to be related to the limited ability of plants to utilise N under S-deficient conditions, in addition to the inhibition of S-absorption by the presence of an adequate N-supply (Schonhof *et al*, 2007). The reduction of sulphate to methionine (via cysteine) in plants is regulated by N (Koprivova *et al*, 2000). This was demonstrated in a study by Kim *et al* (2002), which concluded that S-uptake in turnip rape was supressed with increasing N inputs, which in turn hampered GLS production. This relationship between GLS production and N and S levels may hold significant prospects for producers of cruciferous crops, as fertilisation regimes may be tailored to maximise the health-promoting properties of their crops by reducing N inputs. Furthermore, this could help reduce the detrimental environmental impacts of fertiliser-N leachates from fields.

In turn, it may be hypothesised that this elevated glucosinolate content would lead to reductions in herbivory by generalist insects. The southern armyworm (*Spodoptera eridania*) and the small white butterfly (*Pieris rapae*), both exhibited faster growth rates on black mustard (*Brassica nigra*) plants when grown under deficient S and sufficient N conditions, which also had lower concentrations of the glucosinolate (sinigrin) hydrolysis product allyl isothiocyanate (Wolfson, 1982). Thus, the proportions of N and S added to crops can be manipulated to enhance this constitutive defence.

Aims

In this final experiment, I aimed to draw together some of the earlier findings regarding the effects of N supply on plant-aphid dynamics, with those seen in Part I of this chapter relating to S and SOB effects. Firstly, I investigated the effect of varying N-inputs on cabbage growth and aphid populations with a view to estimating the amount of N at which the EIL is attained. Secondly, I tested different combinations of N and S inputs at a range of concentrations. Lastly, I sought to

find out if equivalent effects could be achieved using *T. thioparus* inoculants in place of the S additions. Due to pressing time limitations, the results are not as reliable as one would hope. However, they offer a useful insight into possible relationships between *B. oleracea* performance and *M. persicae* population dynamics in relation to N and S supply.

4.5 Methods

4.5.1 Nitrogen rate economic threshold

To gauge an estimate of the EIL in *B. oleracea* in response to aphids and N fertilisation, I used the crude measurement of aphid:biomass ratios. Three different rates of nitrogen application were used: 0.16g total N litre^{-1} soil, 0.32g total N litre^{-1} soil and 0.60g total N litre^{-1} soil (equating to 0.06g, 0.12g and 0.24g total N pot^{-1}; or 0.31g, 0.62g, and 1.24g Chempak pot^{-1}). This was prepared as an aqueous solution containing the required amount of the synthetic fertiliser Chempak in 20ml of distilled water. The N solution was administered to the plants after seedling establishment (3-5cm tall) using a sterile syringe which was inserted into 4-5 points of the soil surrounding the seedling. Control plants received 20ml distilled water. All pots were kept in individual saucers to prevent mixing of leachates, and were watered with distilled water regularly.

4.5.2 Sulphur:Nitrogen rates and aphid herbivory

Soil and seeds were sterilised as before (**Chapter 4: Part I**). Elemental sulphur fertiliser (Vitax Ltd) was applied at 0.16g pot^{-1}. As the sulphur powder was hydrophobic, it was applied in its dry form, mixed into the soil using a spatula, and 20ml distilled water was subsequently added via syringe. The three N:S ratios used were 0:1, 1:1 and 10:1, as shown in **Table 25**. Controls received distilled water only.

Table 25 Ratios and quantities of N, S and *T. thioparus*-inoculated soil applied to each plant.

Treatment	Chempak (g)	Total N (g)	Total S (g)	Total S *T. thioparus*-soil (g)
Control	0	0	0	n.a.
0:1 N:S	0	0	0.160	n.a.
1:1 N:S	0.66	0.128	0.128	n.a.
10:1 N:S	0.74	0.145	0.015	n.a.
***T.t.* + no N**	0	0	n.a.	4
***T.t.* + medium N**	0.66	0.128	n.a.	4
***T. t.* + high N**	0.74	0.145	n.a.	4

4.5.3 *Thiobacillus thioparus* soil inoculation

Following on from its success in previous experiments, sterile soil pre-incubated with *T. thioparus* inoculum was chosen as the preferred inoculation method in this experiment. The preparation of the inoculum was identical to that described in the earlier part of this chapter. The inoculated soil was incubated for up to 14 days, before being applied to the pots at 1% w/w rate (4g pot^{-1}) and thoroughly mixed with a spatula before transplanting the seedling into the pot. A 1g subsample of the inoculated soil was serially diluted and plated out for quantification by CFU counts. The methods were the same as those described in the first section of this chapter (**Chapter 4: Part I**).

4.5.4 Aphid herbivory and Plant growth

Plants were transferred from the glasshouse to the insectary. In the N-fertilisation treatment group, plants were taken to the insectary six weeks after fertilisation (two weeks prior to harvesting), and five mixed instar *M. persicae* adults were introduced to all experimental plants using a fine paintbrush. The aphids were subsequently allowed to feed, colonize and reproduce for 14 days at which point the plants were harvested. The final number of aphids on each plant were counted using a destructive method in which leaves were sequentially removed and the aphids picked off individually using a paintbrush. In the N:S and *T. thioparus* + N treatment groups, half of the plants were randomly selected for aphid infestation 30 days after treatments were administered. The infestation period and final aphids population counts followed the same procedures as for the N-fertilisation groups. The other (uninfested) half of the N:S and *T. thioparus* + N plants were used for CFU counts using serial dilutions of the rhizosphere soil to enumerate the SOB population.

Plant biometric data (number of leaves, stem height, fresh and dry weight of the above-ground biomass (dry and fresh weights)) was recorded for all plants at both harvest ages. Each treatment had a minimum of three sample replicates ($n = 3$), except for the *T. thioparus* + High N treatment which suffered several plant deaths ($n = 4$), and so only 2 samples were available (for both aphids and CFU count).

4.5.5 Preliminary Results

4.5.5.1 *Economic threshold of N-fertilisation rates*

The High N treatment (1.24g Chempak pot^{-1}) resulted in several plant deaths, suggesting that this level of N may be toxic to young *B. oleracea* plants. This was also the case when used in combination with *T. thioparus*-inoculated soil. It was also observed that during the first few weeks of growth, plants in the highest N treatment were considerably smaller than other plants, which is in agreement with Scheirs and De Bruyn (2004). At the final harvest (6 weeks after

transplantation), however, there was little difference in the fresh biomass of Medium and High N plants (**Figure 37**).

The preliminary results indicate that biomass increased with increasing N rates up to the point of the High N treatment, however the effect was not significant (one-way ANOVA $p>0.05$). There was also no significant effect on aphid numbers (one-way ANOVA $p>0.05$).

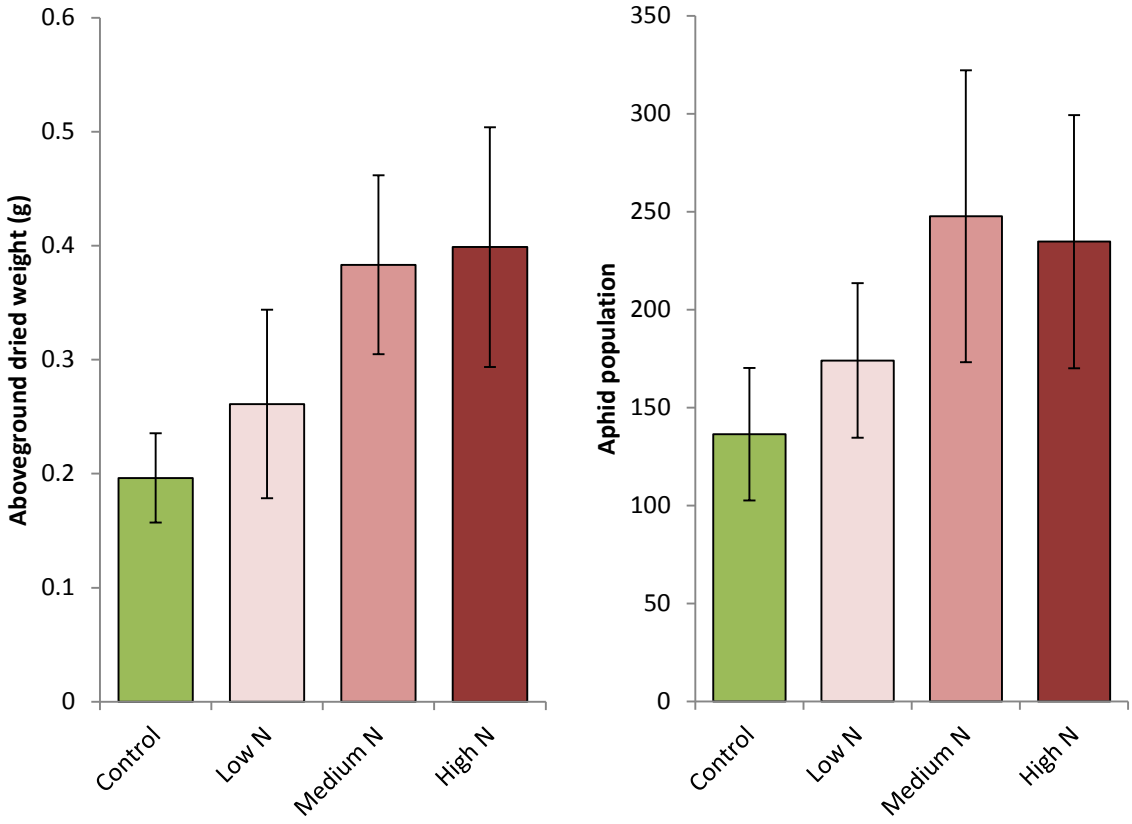


Figure 37 The effect of different N fertilisation rates on *B. oleracea* dry weight and the number of *M. persicae* (mean ±SE). Plants were infested at 6 weeks post-treatment, and harvested at 8 weeks. (Control and Medium N: $n=10$; Low and High N: $n=9$.)

4.5.5.2 N:S effects on plant yield and aphid abundance

The *T. thioparus* culture used for the soil inoculations had an average of 7.23 log CFUs ml^{-1} (±0.06 SE) for the 7 day incubation and 4.93 log CFUs ml^{-1} (±0.05 SE) for the 14-day incubated soil. See **Appendix C** for further data. However, the plating out of soil dilutions taken from the plant rhizospheres after harvesting yielded little or no growth of *T. thioparus*, indicating that the inoculation was unsuccessful on this occasion. The reasons for this were unclear, given the positive test result obtained from the inoculum CFU count at the time of inoculation. Ideally, (q)PCR would have been used to verify the reliability of these results; however, unfortunately this was not feasible owing to time constraints.

Plant yield (aboveground fresh biomass) varied significantly between the *T. thioparus*+N treatments (one-way analysis of means (not assuming equal variance F=91.156, p<0.001), with the aphid-infested *T. thioparus* + medium N plants having significantly lower mean biomass in comparison to controls and N:S 0:1 plants (Kruskal-Wallis χ^2=25.2095, p=0.02) (**Figure 38**). Aphid numbers did not differ in abundance between treatments (ANOVA p>0.05, **Figure 39**).

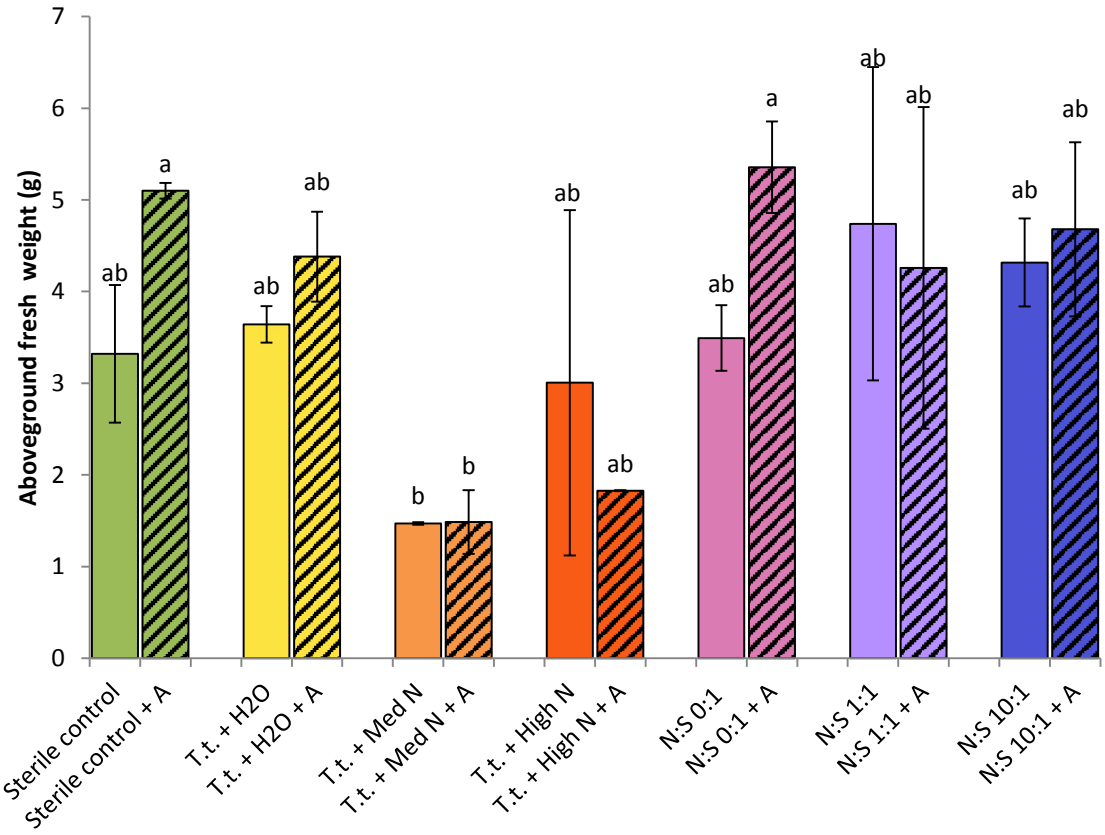


Figure 38 The fresh weight biomass of *B. oleracea* plants under different treatments after 4 weeks (open bars) and a further 2 weeks with aphids (hatched bars) (mean ±SE). (n=3 or 4, except T.t.+HighN, T.t.+HighN+A and N:S-1:1 n=2.)

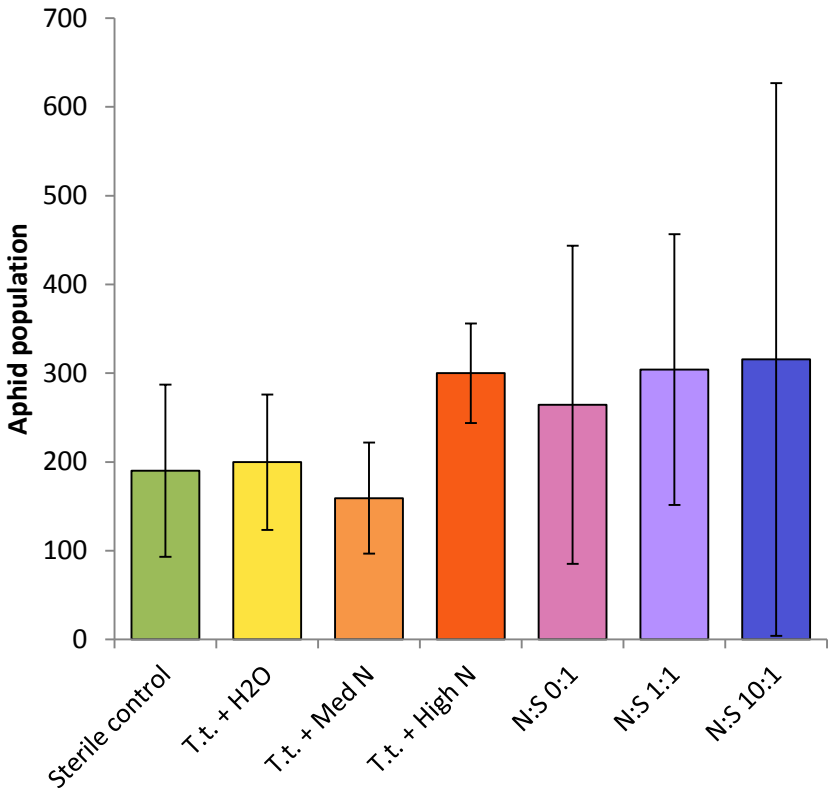


Figure 39 Aphid abundance after 2 weeks colonisation period on cabbages under differed *T. thioparus* and N treatments, and N:S treatments (mean ±SE). Cabbages were infested at 4 weeks post-treatment, and harvested at 6 weeks. (*n* =3, except for Sterile control, T.t. + High N and N:S 10:1 where *n* = 2 owing to plant deaths/unsuccessful aphid inoculations (resulting in large error margin for N:S 10:1); and T.t. + H_2O (*n* = 4).).

4.6 Discussion

The reduction of soil microbial diversity, achieved by repeated autoclaving, resulted in significantly higher SOB populations in the rhizosphere of control *Brassica oleracea* plants. This supports previous findings of 16S rRNA sequencing investigations (see Chapter 3) which indicated that *B. oleracea* plants support and enhance the abundance of SOB, such as members of the *Thiobacillus* genus, in their rhizosphere community. This may be a result of active recruitment by the plant, for example by the breakdown of GLS-containing roots, and the release of rhizodeposits and other root exudations, which promote the growth of SOB. The reduction in soil biodiversity in "sterile" soils could facilitate the formation of cabbage-selected microbial communities; whereas in "normal" soils, the diversity of resident microbiota may impede the ability of plant to shape the rhizosphere community for its own benefit.

According to enumeration of the SOB population in rhizosphere soils by molecular methods (qPCR), the inoculation of soil with *T. thioparus* was generally found to be more successful in sterilised soils than in "normal" (non-sterile) soils. This was as expected since the eradication of the vast majority of the soil's naturally resident microbial population by exposure to high temperatures would considerably reduce the level of competition for resources, thereby enabling the inoculum to proliferate more freely. The *T. thioparus* inoculations tended not to enhance SOB populations (or GLS concentrations) to the extent that sulphur additions did. However, the injection of *T. thioparus* inoculum into the rhizosphere of seedlings ("Rhizo" treatment) did appear to have a strong impact on SOB populations after 8 weeks of plant growth. In some instances the levels of *soxB*, and also GLS, detected in the Rhizo treatments exceeded those of elemental sulphur-amended (HSN) plants. However, the effects of the rhizosphere inoculations were far less evident after an additional 4 weeks. This suggests that although it may be effective in the short term, this inoculation method may require a second (or more) application during the plant growth period to improve the persistence of the inoculation.

The highest *soxB* levels, and thus interpreted as the largest SOB populations, were detected in the rhizosphere of plants grown in HSS soils (sulphur-fertilised sterile soils). This result was curious given that the sterile nature of the environment (twice-autoclaved soil and surface-sterilised seeds). One possibility is that the bacteria originated from within the plant seed. Endophytic bacteria are endosybiotic microbes which colonize and co-exist with plant tissues (Kloepper & Beauchamp, 1992). They have been reported to occur in the seeds of many plant species following surface-sterilisation (Taški-Ajduković and Vasić, 2005, Kaga *et al*, 2009, Šmerda *et al*, 2005, Truyens *et al*, 2015). These bacteria can internally colonise the plant, and subsequently be transmitted to the soil via the roots. Studies have demonstrated that these endophytic bacteria can confer benefits to the plant, for instance tolerance of metal contaminants, as found to be exhibited by *Nicotiana tabacum* seeds in response to exposure to cadmium (Mastretta *et al*, 2009). Pleban *et al* (1995)

isolated endophytic bacteria from surface disinfected seeds of cauliflower (*Brassica oleracea* L. cv. '202/A'), and a member of the *Thiobacillus* genus has previously been identified as one of the endophytic bacteria isolated from surface sterilised maize seeds (Liu *et al*, 2012). It is, therefore, possible that sulphur-oxidising bacteria were present within the surface-sterilised cabbage seeds and subsequently led to their proliferated in the sulphur-fertilised soil during the plant growth period. This would require further testing for endophytic bacteria, by grinding surface-sterilised seeds and plating out the contents on selective agar.

The positive influence of SOB-promoting treatments on total GLS concentration in cabbages was apparent in 12 week-old plants only. Given that SOB populations, as determined by qPCR of *soxB* gene, were generally larger in the rhizospheres of treated plants at 8 weeks, this may indicate a time-lag effect of the SOB population on plant chemistry. When splitting the GLS into classes (indole and aliphatic), the abundance of SOB tended to have a slight negative correlation with aliphatic GLS, and a small, but positive, relationship with indole GLS. The abundance of *soxB* in sulphur-amended sterile soils in particular was negatively correlated with aliphatic GLS content, which was also the case in seed-inoculated plants. However, given that the overall *soxB* levels in the rhizospheres of seed-inoculated plants were negligible, this result may be less insightful. This concurs with reports of a negative effect of S fertilisation on aliphatic GLS production in broccoli plants (Aires *et al*, 2006), but contradicts other reports that methionine-derived (aliphatic) GLS have exhibit stronger positive responses to S fertilisation than those formed from tryptophan (indole GLS) (Falk *et al*, 2007).

In contrast, indole GLS production appeared to be promoted by increasing the SOB population, when comparing all samples as one, irrespective of treatment, harvest time point, aphid presence or soil sterility. This bodes well for the prospect of using SOB inoculums as pest control methods, since indole GLS have been shown to exert an anti-feedant role on insect herbivores such as *M. persicae* (Kim *et al*, 2008a). GBC was the main indole GLS responsible for this trend, which corroborates with the findings of Krumbein *et al* (2001) who demonstrated an increase in GBC levels following the S-fertilisation of broccoli. Furthermore, there could be valuable health benefits of this trait since the breakdown product of GBC, indole-3-carbinol (I3C), has been shown to confer anti-cancer effects (Fares, 2014).

Contrary to previous reports of the positive effects of S fertilisation on yield, the prevalence of SOB in *T. thioparus* inoculated non-sterile soils was negatively related to cabbage biomass (fresh weight). This was not observed in sterile soils inoculated with the SOB, and demonstrates, therefore, the confounding effects which can occur when applying bacterial inoculants in natural soils in comparison to sterile substrates, as so often used in studies of this nature. It may be that

co-inoculation with another PGPR species, such as N-fixing bacteria, could recover the loss of yield while maintaining the positive effects on GLS production.

Irrespective of plant age, soil sterility and treatment, aphid-infested plants had a lower foliar indole GLS concentration than undamaged cabbages, particularly those grown in "normal" soil. This concurs with previous studies which report a reduction in GLS content following insect attack (Kim and Jander, 2007). In contrast, when taking the number of aphids on the plant into consideration, there was an overall negative relationship between *M. persicae* populations and aliphatic GLS, and a positive correlation between aphid abundance and indole GLS concentrations of plants grown in non-sterile soil. This corroborates previous findings of Kim and Jander (2007) which showed that aphid feeding on Arabidopsis plants induced the production of indole GLS, namely 4-methoxyindol-3-ylmethylglucosinolate (4MeOH). In the results presented here, there was no overall increase in 4MeOH (4-methoxyindol-3-ylmethyl) production in aphid-infested plants, however the indole GLS 4OH and NEO both exhibited significant positive associations with aphid abundance.

Aphid population growth appeared to be slightly inhibited by the *T. thioparus* and S treatments on some occasions where they had smaller populations than control plants (D7, and HSS 12 weeks; Rhizo 8 and 12 weeks, HSN 8 weeks). However, *soxB* abundance did not correlate either positively or negatively with aphid populations. Previous work has shown that specialist herbivores perform better on plants receiving S supplements, however there is a lack of knowledge on the effects on generalist species. A study which investigated the growth-promoting effects of *Bacillus amyloliquefaciens* on *Arabidopsis* found that the bacteria transcriptionally induced S-uptake by the plant, which resulted in higher GLS and greater protection against the generalist herbivore *Spodoptera exigua* (Aziz *et al*, 2016). Yusuf and Collins (1998) showed that plants grown under high sulphur fertiliser regimes exhibited increased susceptibility to infestations of the crucifer specialist aphid *Brevicoryne brassicae*. It may be expected, therefore, that generalist species such as *M. persicae* may show the opposite response and have lower reproduction rates in plants with higher rhizosphere SOB populations. This was not evident in the results, however, possibly indicating that the rates of S or inoculum applied to the soils were insufficient to induce noticeable effects on aphid performance.

The preliminary investigation into the effects of varying N and S availability on plant growth and aphid performance produced inconclusive results. The results from the N fertiliser experiment indicate that increasing N-input had no significant benefits in terms of cabbage yield. The plants were harvested at 8 weeks, and it is possible that stronger effects may emerge later on in plant development. There was a lack of significant treatment effects on aphid abundance in all combinations of N/S/*T. thioparus*, which may be indicative of insufficient differences in the effect of these treatments on plant chemistry. However, given the small sample size, we cannot be

entirely confident in these results and it may be worth repeating this experiment given the widespread reports of significant implications of N:S ratios in the literature.

The *T. thioparus*-inoculated plants (in Part II experiment) had significantly greater biomass in comparison to plants which also received N fertilisers. This seems to be caused by a stunting effect of high N supply on plant growth, rather than a promotional effect of the SOB, however, since the biomass of *T. thioparus* plants was comparable to that of the controls. The *T. thioparus* treatments failed to produce positive results in the CFU count, which suggests that the inoculation was unsuccessful, and I would again advise a repeat of this experiment using greater sample numbers and a modified *T. thioparus* inoculation method. Nevertheless, it does appear that there may have been some effect occurring since the addition of N with *T. thioparus* resulted in significantly smaller plants (in terms of above-ground biomass) in comparison to those receiving *T. thioparus* only. This is in contrast to the effects of N-additions in the previous experiment, where the same N intermediate N treatment (medium N) resulted in slightly increased biomass. However, the plants in the two experiments were harvested at different stages (N experiment: 8 weeks, N:S/*T. thioparus* experiment: 4 weeks without aphids; 6 weeks with aphids) which may be contributing to these differences. The N:S plants did not show such pronounced signs of supressed growth, with their biomass being highly similar to that of control plants. This may, therefore, suggest that the addition of S counteracts the negative effect of increased N inputs on cabbage growth.

4.6.1 Study limitations and Future work

The results in Part I are promising, however it is evident from the large variation in the success of the SOB enrichments that the inoculation methods require some modifications. It could be recommended that the seed inoculation is re-designed, perhaps to include polymer additives, such as gum arabic, to aid the survival of the inoculum on the seed coat. The comparison of a combination of *T. thioparus* and S as a treatment against S-only plants may generate interesting results, and repeated inoculations over the plant growth period may also be a more effective approach. Owing to logistical issues, sample numbers were not even across treatments and this weakens the statistical veracity of the results. Finally, measurements of the phloem and root GLS concentrations may provide further insights into the interactions, particularly given that they are likely to have greater impacts on aphids and the rhizosphere respectively, in comparison to foliar GLS levels.

As mentioned, the experiments in Part II were restricted by time pressures. This experiment requires a larger sampling pool and duration. The design of this study could also be made more elaborate by including GLS measurements, to measure the response of individual GLS are to the adjustments in nutrient supply as these may confer significant responses in aphid performance.

Indeed, Cole (1997) proposed that the optimum GLS profiles for deterring both specialist and generalist aphids was characterised by low levels on SIN and PRO, but high concentrations of GNA and 4-pentylglucosiolate. If repeated, I would also recommend firstly improving the *T. thioparus* inoculation methods, which for an unknown reason did not appear to have been successful on this occasion.

Chapter 5: General Discussion

The overarching aim of this thesis was to gain a better understanding of the role of the soil microbiome in relation to plant growth and aphid herbivory. This was achieved using lab- and glasshouse-based (mesocosm) experiments, which utilised natural field soil in combination with different fertiliser treatments applied at field levels to allow results to be relevant to farming systems. The interdisciplinary studies used both investigative and manipulative experimental approaches, employing a mixture of bioinformatics, molecular and ecological techniques. Firstly, I investigated the response of the soil microbial community to fertilisers, *B. oleracea* growth and aphid herbivory. This was carried out using high-throughput sequencing of the 16S rRNA gene and subsequent detailed explorative and interpretive analysis of the results. Secondly, a key finding of the 16S rRNA sequencing study - the proliferation of the *Thiobacillus* population in the rhizosphere of *B. oleracea* growth - was chosen as the basis for a more controlled approach to investigating soil-plant-insect dynamics. This involved the development of inoculation techniques and manipulation of the soil community by sterilisation, with the aim of testing the potential of this sulphur-oxidising bacteria to influence plant growth and chemistry in such a way that aphid herbivory may be controlled to some extent. Finally, I took components from the previous two chapters (N and S fertilisation) to conduct a preliminary study investigating their synergistic effects on aphid-cabbage dynamics.

5.1 Aphid and plant responses to fertilisers

In Chapter Two, it was shown that the addition of organic (chicken manure) and synthetic (NPK) fertiliser additions resulted in significantly increased plant growth and nutrition. Plant biomass appeared to be enhanced in both treatments after 12 weeks, however, only the synthetically fertilised cabbages exhibited this trend when exposed to aphid herbivory. This may indicate that synthetic fertilisers enhance the ability of *B. oleracea* to withstand aphid attack.

As predicted, foliar N content of *B. oleracea* was positively correlated with N concentration of the synthetic fertilisers added. There was no evidence that the foliar N levels of organically fertilised plants differed from those which received an equal dose of N in the form of NPK mineral fertiliser. This concurs with the findings of Liu *et al* (2014) who also reported that the type of fertiliser, organic or mineral, did not affect the N content of plants when applied at equal N rates. They also drew this conclusion from the results of a pot experiment, and so it is possible that the same effect is not reproduced in the field. Indeed, field studies have reported that the mineral (nitrate) content of organically fertilised plants was significantly (in some cases more than 50%) lower than in plants receiving mineral fertilisers, although the yields were similar (Lairon *et al*, 1984). This is

supported by the hypothesis that the slow-release of minerals from organic fertilisers results in lower plant N concentrations relative to synthetically fertilised plants. Studies have shown that plant N uptake is greater when it is available in inorganic, rather than organic, forms. A breakthrough study by Jones *et al* (2013) demonstrated for the first time that plants can assimilate N compounds in their organic forms (e.g. amino acids) *in situ*, thereby disproving the conventional theory that plants were restricted to inorganic N uptake only. However, the plants (wheat) were found to be stronger competitors against microbes for inorganic N (NH_4^+) in comparison to organic N (glutamate). The inconsistency in these results may also be attributed to a variety of other factors, such as plant genotype, which influence plant N uptake (Mazahar *et al*, 2015).

This study found that the abundance of *Myzus persicae* on cabbages was not affected by any of the fertiliser treatments. However, there were signs of the synthetic fertiliser treatments having associations with shorter aphid developmental times and faster reproduction rates in comparison to the control and organic treatments. This would support previous reports that aphids favour plants with higher N content, and that the use of synthetic fertilisers results in increased pressure on crops from pests such as the aphid *Rhopalosiphum maidis* (Morales *et al*, 2001). The lack of a significant response in my experiment may be due to the insufficient N rates, as the maximum rate I applied was equivalent to 136 kg ha^{-1}, whereas the study by Morales *et al* (2001) used a rate of 425 kg ha^{-1}.

5.2 Brassica-growth and fertiliser inputs shape the soil microbiome

In Chapter Three, I described the use of 16S rRNA NGS to investigate the response of soil microbial communities to the fertilisers, *B. oleracea* growth and aphid herbivory. The results demonstrate that these soil bacterial communities showed a stronger response to the growth of *Brassica oleracea* plants and fertiliser treatments than they did to aphid herbivory. Although rhizosphere and bulk soil samples were collected at different times, it was interesting to note that the rhizosphere communities were more diverse than bulk soils, both in terms of species richness and abundance (α-diversity). Plants are known to impact on soil microbial communities through various means, such as the release of carbon-rich root exudates and their effect on soil pH (see Hinsinger *et al* (2003)).

The fertiliser treatments yielded several significant effects on the composition of the rhizosphere communities. The chicken manure-treated soils had higher, although not significantly so, alpha-diversity metrics (observed species and Chao1) than the synthetically fertilised soils. This supports the widely-held theory that organic fertilisers promote bacterial diversity (Hartmann *et al*, 2015). The chicken manure amendments induced several changes in the OTU abundance of bacteria taxa including members of the families *Cytophagaceae* and *Halomonadaceae*. This may be explained by the inherently diverse microbial community associated with poultry manure, which despite the

sterilisation process of commercial pelleted manure, may still remain in a viable but non-culturable (VBNC) state, thereby evading detection in lab-testing of commercial products.

The composition (β-diversity) of the cabbage-associated soil microbiome was distinctly different to that of the bulk soil, although these samples were not taken simultaneously. At the phylum level, one of the most noticeable differences in the composition of bulk and rhizosphere communities was the proportional augmentation of Acidobacteria in the rhizosphere, which suggests a possible acidifying effect of *B. oleracea* roots on the surrounding soil. Root-mediated changes in soil pH are thought to be caused by the release of hydrogen cations (H^+) or anions (OH^-) ions, a plant mechanism that is believed to have evolved as a way to optimise cation-anion exchange between roots and the soil environment (Riley and Barber, 1969). Mechanisms behind the soil-acidifying effects of roots have been extensively studied. One example of environmentally induced soil acidification by roots is the plant stress response to P-deficiency. This was demonstrated in greater purple lupin (*Lupinus pilosus* L.) plants grown under P-limited conditions, which were found to respond to this abiotic stress by increasing the release of H^+ ions via the enhanced activity of H^+-ATPase and elevated root exudation of citrate (Ligaba *et al*, 2004b). However, this does not appear to be a uniform response across the plant kingdom, as citrate (organic ion) production has been shown to be unaffected by P-deficiency in several species such as oilseed rape (*B. napus* L.) (Ligaba *et al*, 2004a), wheat (Delhaize *et al*, 1993) and soybean (Yang *et al*, 2000). The interactions between *Brassica* roots and soil microbial communities under P-deficient conditions are reviewed by Hunter *et al* (2014) . They give a detailed discourse on the effects of *Brassica* root exudates (foremost malate and citrate) on soils, remarking on the importance of root exudates in shaping the composition and activity of soil microbial communities.

The generalist aphid *Myzus persicae* appeared to exert little effect on the rhizosphere bacterial community, although, as commented on earlier in regard to the absence of pronounced treatment-effects on aphid abundance, it is possible that effects may emerge only over a longer period than that tested in this study. Soil microbe-plant-insect interactions have received little attention in the literature until relatively recently. A complex multi-trophic study by Bennett *et al* (2016) looked at the effect of the soil arbuscular mycorrhizal fungi (AMF) on *Solanum* species under attack by the potato aphid *Macrosiphum euphorbiae* with reference to the aphid endosymbiont *Hamiltonella defensa*. They tested whether the AMF affected the performance of the parasitoid wasp *Aphidus ervi*, finding that it was enhanced on AMF plants. Further evidence of fungi-associated enhanced plant defence against aphid herbivory via the increased attraction of parasitoids has been demonstrated using bean plants (*Vicia faba*) and pea aphids (*Acythosiphon pisum*) (Babikova *et al*, 2013). The explanation for these phenomena require further investigation, however it seems to be regulated in part by changes in plant volatile production.

5.3 Promoting the abundance of sulphur-oxidising bacteria in the soil and consequences for glucosinolate production and aphid herbivory in *B. oleracea*

The work described in Chapter Four used the same soil-plant-insect model system as the previous experiments, but with the focus shifting from nitrogen to sulphur. It aimed to test the potential for augmented populations of sulphur-oxidising bacteria (SOB) in the soil to have positive effects on plant growth and the production of chemical defence compounds, and its implications for aphid herbivory. This was achieved using *Thiobacillus thioparus* as the inoculant, elemental sulphur as a benchmark for comparison, and relative quantification of the *soxB* gene by qPCR as a measure of the SOB rhizosphere population. Given that thiocyanate is a component of several glucosinolate breakdown products (Cole, 1976), and that sulphur is a vital component of GLS (Aghajanzadeh *et al*, 2014), there is a strong case for the possibility of mutualistic association between *Thiobacillus* spp. and *Brassica* plants. Furthermore, this is supported by the results from my 16S rRNA NGS experiment, in which *Thiobacillus* was shown to have a significantly higher relative abundance in the cabbage rhizosphere, an effect which seemed to strengthen over time.

A variety of inoculation methods were tested (see Appendix C), using both sterile and non-sterile soil. As expected, the sterile soil had a stronger success rate in terms of inoculation strength (quantified by the abundance of the *soxB* gene) and duration (comparing SOB populations at 8 and 12 weeks after inoculation). Sterile soils amended with elemental S, which was used for comparison, outperformed the *T. thioparus* treatments in terms of SOB enumeration. In "normal" (non-sterile) soils, however, soils which received the *T. thioparus* inoculum via injection into the root zone (Rhizo treatment) exhibited *soxB* levels which surpassed those of the S-treated soils. This was true after 8 weeks only, however, which indicated inferiority in the longevity of the inoculum in comparison to the sulphur fertiliser treatment. It would be interesting to see whether administering a second inoculation during the growth of the cabbage would improve these results.

Interestingly, the Rhizo treatment exhibited higher SOB populations under aphid-infested cabbages. This trend was also true for all *T. thioparus* inoculated soils, whereas the S-fertilised soils tended to have smaller SOB populations in the presence of aphids. This suggests a distinct difference in the effects of these two treatments on the dynamics of SOB populations with respect to aphid herbivory. In contrast, the seed inoculation seemed largely unsuccessful in terms of its effects on the SOB community. This may be remedied by re-designing the methods, perhaps incorporating a substance (e.g. polysaccharides) to aid adhesion of the bacteria to the seed coat.

The HPLC results showed SOB-enrichment of sterile soils resulted in significantly increased total GLS concentrations in the leaves of 12 week-old *B. oleracea* plants. A more detailed analysis of the results revealed that the SOB population was positively correlated with indole GLS

concentrations, but was negatively associated with aliphatic GLS. Aphid-infestation also exhibited opposing relationships to the two classes of GLS, but in the reverse, i.e. aphid presence was positively correlated with aliphatic GLS, but negatively with indole GLS. However, when accounting for aphid numbers (as opposed to presence or absence), the correlations were switched (i.e. a negative relationship between aphid population size and aliphatic GLS, and a positive correlation with indole GLS). There is support in the literature for the inducement of higher indole GLS production in response to aphid herbivory (Kim and Jander, 2007). An enhancement of indole GLS production in response to SOB may, therefore, be regarded as a promotion of plant defences. This requires further investigation in order to tease out the direction of the effects (top-down versus bottom-up). Nevertheless, it is a promising result which shows potential for the development of a PGPR inoculant with both bio-pesticide and bio-fertiliser traits. Thus, the results may be interpreted as showing that enhancement of the SOB population can lead to reduced aphid abundance by increasing indole GLS, with which the insects were negatively associated. This requires further testing and increased sample pools to enable a conclusion to be confidently made.

A possible caveat of using SOB as PGPR however, was the observation in this investigation that increased abundances of SOB in natural (non-sterile) soils was associated with a reduction in plant yield (fresh biomass). This relationship was not observed in the sterile treatments, which demonstrates one of the pitfalls of glasshouse trials of this nature, in that positive results observed under controlled conditions frequently fail to be replicated in the field. Yet, this may be outweighed by the value of increased GLS content of the plant, particularly when taking into consideration the health-benefits (anti-carcinogenic) associated with indole GLS. Another cautionary note is that the acidifying effects of SOB may be detrimental to the environment, and it is not recommended that this inoculant is used in plants with low tolerance to acidic conditions. Incidentally, it may serve as a beneficial application for ericaceous plants which thrive in acidic soils.

The preliminary investigation into the effect of varying N and S fertilisation rates on cabbage-aphid dynamics was suggestive of several trends. Firstly, the highest N treatment appeared to stunt the growth of *B. oleracea*, particularly in the early stages of plant development. This was evident both in the N-only plants, and when combined with *T. thioparus*-inoculated soils. The addition of S fertiliser appeared to compensate for this effect, as the N:S treated plants were no different from controls in terms of biomass. However, they did appear more susceptible to *M. persicae* colonisation, which may be attributed to the enhanced N-status of these plants.

5.4 Study limitations & Future work

There are several areas of this work which were hampered by the limitations of time and resources. The plant growth and the aphid infestation periods were potentially too short to indicate the full impacts of the different treatments. Plant development is known to be an important factor in both rhizosphere microbial community dynamics and GLS production. Also, an extended growth period would be more representative of farming systems, as *B. oleracea* are normally harvested 70-120 days after sowing (Maynard and Hochmuth, 2007). This would require either scaling up the size of pots used in order to accommodate larger plants, or transitioning the study into a field experiment.

The *M. persicae* measurements could also be limiting the interpretation of these experiments. The MRGR and r_i measurements were not made in the final experiments owing to the poor success rate of these methods in the first experiments. This resulted in part from the design of the clip cages, which were not always successful in isolating the aphids, and caused some damage to the leaves. A more lightweight cage which is less damaging to the plant would be preferable. Also, as I mentioned earlier, the infestation period was generally insufficient for assessing aphid fecundity parameters (r_m) and may need extending.

The 16S rRNA NGS study was limited by the number of samples (40) that could be processed in a single sequencing run, without compromising the quality and number of reads obtained. If there were no financial constraints, it would have been preferable to run a minimum of three samples per treatment at each time-point. This was exacerbated by the loss of two samples at the fertiliser time-point (High N and chicken manure) due to their failure to meet quality control standards. This was attributed to human error, although in the case of the chicken manure sample, high humic content may have inhibited the DNA extraction. Additionally, it would be interesting to monitor the bulk (non-rhizosphere) soil in conjunction with the rhizosphere sampling time-points as it is likely to have been altered on a temporal scale, albeit probably not to the extent of the changes induced by the rhizosphere effect. In retrospect, the use of tap water to water plants was inappropriate given that I was investigating soil microbial communities and the water may have introduced contaminating microbes to the system.

In the final set of experiments I used qPCR of the *soxB* gene as a method of enumerating the SOB population in the rhizosphere. To calculate the abundance I used a relative quantification approach whereby DNA obtained from a pure culture of *T. thioparus* was used as the standard, from which the amount of *soxB* in each experimental sample was quantified in relative, rather than absolute, terms using arbitrary units. A potentially more robust and accurate approach would be to clone the target gene sequence (*soxB*) into plasmids, as this would enable the calculation of *soxB* gene copy numbers in each sample, thus providing absolute quantitation of the SOB population rather than a relative enumeration. However, this is a highly laborious technique and even when this approach is

used, there are still a number of potential biases associated with real-time qPCR which can significantly affect the quantification of copy numbers (Lee *et al*, 2006, Brankatschk *et al*, 2012).

In my experiment, I measured GLS concentrations of the leaves. However, foliar GLS levels have been shown to be unrepresentative of those in the phloem. To further investigate the impact of SOB on plant-insect dynamics, the effects on phloem concentrations should be studied, perhaps also looking into root GLS levels as well. It may be that the SOB population has differential effects on these two plant components. It would also be intriguing to test whether there are any soil SOB effects on plant volatile production and chemo-signalling. If so, this could warrant further investigation into natural predator (e.g. parasitoid wasps) responses to the inoculations.

The tested *T. thioparus* inoculation methods produced inconsistent results, indicating that the procedure requires improvement. Possible adjustments which may improve the effectiveness of the inoculation include the combination of the inoculum with polymers in the seed coating approach and pelletizing the bacteria with a dried substrate (e.g. sterile soil) as demonstrated by Anandham *et al* (2007). Following the refinement of inoculation methods, I would also propose testing SOB enhancement on other plant species, especially other cruciferous crops.

The preliminary investigation into the role of N and S availability in plant-aphid dynamics was strongly limited by time. I believe this is an area of research worth pursuing, given the support in the literature for the role of these nutrients in GLS production and insect herbivory. Awad *et al* (2011) conducted a field experiment which compared the effect of adding *T. thiooxidans* with differing N-dosages on onion growth, reporting that the combining the SOB with the fertiliser resulted in greater onion yield and bulb weight. This could be taken further by testing the efficacy of a dual inoculation of SOB and N-fixing bacteria. This was shown to have promotional effects on a cultivar of groundnut (*Archis hypogeae* L.), using a combination of *Thiobacillus* strains obtained from drainage water and *Rhizobium* (Anandham *et al*, 2007). The combined inoculation was found to have synergistic effects, resulting in a significant increase in the number and dry weight of nodule, plant biomass, and root and shoot lengths. However, the effects were only found to be significant in pot trials (greenhouse) using sterilised soil and not in the field, thus demonstrating once again the difficulty in performing successful inoculations in natural soils as opposed to sterile ones. However, Awad *et al* (2011) successfully achieved higher onion yields in the field using a co-inoculant of *T. thiooxidans* with the N-fixing bacteria *Azotobacter chroococcum* and *Azospirillum lipoferum*. In this case, though, the inoculants were combined with organic manure before application to the field, which may have confounded their results as the controls did not receive any manure.

Following on from these investigations into the soil-cabbage-aphid system with reference to N and S availability, it would be logical to next explore the role of phosphorus. P is commonly regarded

as the fourth most important nutrient for plant growth. Since production of acidic compounds by *T. thioparus* is known to result in P-solubilisation, it would be interesting to monitor its effect on P-concentrations in inoculated plants. This has been studied in wheat (*Triticum aestivum* cv. Tetra) by Babana *et al* (2016), in which a combination of *T. thioparus* and Tilemsi rock phosphate was shown to result in significantly higher grain and straw yields, which were similar to those of plants treated with ammonium phosphate (100 kg ha^{-1}) . Another known PGP property of *T. thioparus* is bioremediation, either via chromium (VI) reduction (Donati *et al*, 2003), or the enhanced oxidation of sulphide minerals (Groudev *et al*, 2001). Volatile production by bacteria have been shown to influence plant-pathogen dynamics (e.g. *Bacillus subtilis* GB03 with *Arabidopsis thaliana* and *Erwinia carotovora*) via ISR (induced systemic resistance), and this could be another avenue of research worth testing in *T. thioparus* (Ryu *et al*, 2005).

5.5 Final conclusions

In this thesis I have reported several insightful findings regarding soil-cabbage-aphid interactions. The main conclusions are:

- Synthetic fertiliser applications significantly altered the nutrient status of *B. oleracea* plants (foliar N concentration), but their effects on aphid populations and the rhizosphere community were negligible.
- The organic fertiliser treatment, pelleted poultry manure, yielded plants with N concentrations comparable to those of cabbages receiving an equivalent N dose via mineral fertilisers, and was the only fertiliser treatment to substantially alter the soil bacterial community.
- *B. oleracea* growth and development led to distinct changes in the diversity (species richness and abundance) and composition of the soil microbiome.
- The sulphur-oxidising bacteria (SOB) *Thiobacillus thioparus* was more abundant following the transplantation of cabbages into the soil, and appeared to proliferate in response to the growth and development of the plant.
- The enlargement of SOB populations, either by inoculation with *T. thioparus* cultures or S amendments, was successful in both sterile and non-sterile soils. However, inoculations in non-sterile soils were less durable.
- Enhancing the SOB community resulted in significantly increased foliar indole GLS concentrations, whereas aliphatic GLS appeared to be negatively affected. There were no detectable effects of SOB populations on aphid populations.
- Increased aphid abundance was associated with higher indole GLS but lower aliphatic GLS concentrations.

These results demonstrate the dynamic nature of soil communities, and the strong influence of plants in shaping them. To my knowledge, this is the first study to examine the microbial community of *B. oleracea* var. L *capitata* rhizospheres in such depth, and also to demonstrate the potential for exploiting these interactions with soil microbes to enhance GLS production. This finding has the potential to contribute towards the development of novel and environmentally compatible approaches to enhancing crop production. Soil plant-growth promoting bacteria offer a natural and effective alternative to synthetic fertilisers and pesticides. Here, I have shown that the positive association between *B. oleracea* and the SOB *T. thioparus* may be exploited to enhance both human health-benefiting and aphid-deterring attributes in cabbages.

Appendices

Appendix A

Table 26 Plant and aphid measurements under organic and synthetic fertiliser regimes.

		***B. oleracea* measurements**				***M. persicae* measurements**			
Treatment	**Sample**	Fresh weight (g)	Dry Weight (g)	Stem Height (cm)	No of Leaves	Nymphs	Apterous adults	Alate adults	Mean adult weight (g)
CM	1,2	34.736	3.3145	4.9	13	181	57	0	0.000675
CM	1,4	32.41	3.4397	5.5	11	160	65	1	0.000414
CM	1,6	29.852	2.5723	7	12	254	71	1	0.000473
CM	5,3	34.4	3.1065	5.7	16	66	32	0	0.000281
CM	5,4	19.253	1.6812	5.6	15	224	47	1	0.000461
Control	1,4	25.97	2.6584	6.2	13	222	61	1	0.000497
Control	2,1	27.096	2.5201	5.2	13	135	32	0	0.000421
Control	2,5	27.9445	2.2495	6.2	15	149	36	1	0.000459
Control	4,3	25.168	2.6349	4.7	12	158	37	0	0.000471
Control	5,2	19.062	1.8239	5.7	12	43	13	0	0.000228
High N	1,1	52.318	3.9844	5.8	16	155	95	1	0.000518
High N	1,4	45.151	2.9377	7.2	14	177	83	0	0.0006
High N	1,5	45.138	2.9982	6.5	13	167	44	0	0.000718
High N	2,1	44.436	2.7127	6.1	15	212	59	0	0.000628
High N	4,1	47	4.0409	6.9	13	271	73	1	0.000477
Low N	2,1	44.357	3.8181	5.5	15	45	30	0	0.000247
Low N	2,4	38.048	3.2393	5.4	13	80	30	0	0.00051
Low N	4,4	41.066	3.4311	8.4	14	91	27	0	0.000388
Low N	4,5	33.2643	2.5122	6.5	13	357	56	0	0.00057
Low N	5,2	40.43	2.6576	5.8	14	157	27	1	0.000491

Table 27 MRGR results for caged *M. persicae* individuals on *B. oleracea* under different fertiliser treatments.

Plant treatment	Initial weight (W_1) (mg)	Final weight (W_2) (mg)	Intervening Time (days)	MRGR mg,mg^{-1},day^{-1}
Chicken manure (1) pot 5	50.0000	90.0000	7	0.0840
Chicken manure (5) pot 4	73.3333	95.0000	10	0.0259
Chicken manure (3) pot 5	13.3333	120.0000	10	0.2197
Chicken manure (3) pot 4	23.3333	60.0000	10	0.0944
Chicken manure (1) pot 2	46.6667	80.0000	10	0.0539
Chicken manure (2) pot 3	23.3333	130.0000	9	0.1909
Chicken manure (4) pot 5	40.0000	80.0000	6	0.1155
Chicken manure (1) pot 4	26.6667	110.0000	9	0.1575
Chicken manure (4) pot 6	56.6667	130.0000	9	0.0923
Chicken manure (2) pot 2	43.3333	110.0000	9	0.1035
Control (4) pot 3	60.0000	80.0000	7	0.0411
Control (4) pot 5	10.0000	20.0000	10	0.0693
Control (2) pot 1	96.6667	175.0000	7	0.0848
Control (2) pot 6	50.0000	140.0000	7	0.1471
Control (3) pot 4	13.3333	55.0000	10	0.1417
Control (1) pot 6	23.3333	30.0000	5	0.0503
Control (2) pot 5	56.6667	120.0000	9	0.0834
Control (1) pot 3	33.3333	40.0000	9	0.0203
Control (5) pot 6	33.3333	80.0000	9	0.0973
Control (5)pot 5	36.6667	50.0000	9	0.0345
High N (1) pot 1	16.6667	165.0000	10	0.2293
High N (1) pot 2	15.0000	280.0000	10	0.2927
High N (3) pot 2	73.3333	270.0000	10	0.1303
High N (3) pot 6	20.0000	150.0000	10	0.2015
High N (4) pot 6	50.0000	230.0000	10	0.1526
High N (5) pot 5	46.6667	180.0000	9	0.1500
High N (3) pot 1	56.6667	170.0000	9	0.1221
High N (5) pot 1	56.6667	140.0000	9	0.1005
High N (2) pot 5	60.0000	345.0000	9	0.1944
High N (1) pot 5	33.3333	120.0000	9	0.1423
Low N (1) pot 6	40.0000	115.0000	7	0.1509
Low N (2) pot 3	35.0000	270.0000	10	0.2043
Low N (3) pot 5	50.0000	140.0000	10	0.1030
Low N (1) pot 4	86.6667	220.0000	10	0.0932
Low N (3) pot 4	33.3333	140.0000	10	0.1435
Low N (4) pot 4	55.0000	180.0000	9	0.1317
Low N (3) pot 2	33.3333	120.0000	2	0.6405
Low N (5) pot 6	63.3333	140.0000	9	0.0881
Low N (5) pot 3	22.5000	150.0000	9	0.2108
Low N (1) pot 5	46.6667	250.0000	9	0.1865

Appendix B

QIIME scripts used for 16S rRNA sequencing analysis

pick_open_reference_otus.py

assign_taxonomy.py

make_otu_table.py

filter_alignment.py

make_phylogeny.py

Remove singletons, chloroplast, and mitochondria OTUs:

filter_otus_from_otu_table.py

Rarefy to 198,288 sequences (=minimum number of seqs/sample):

single_rarefaction.py

Alpha Diversity

multiple_rarefactions.py

alpha_diversity.py

collate_alpha.py

make_rarefaction_plots.py

compare_alpha_diversity.py

Beta Diversity

jackknifed_beta_diversity.py

make_bootstrapped_tree.py

beta_diversity_through_plots.py

Use unweighted_unifrac_dm.txt file output of beta_diversity_through_plots.py for compare_catgeories.py functions(Adonis, ANOSIM etc.) after installing *permute* and *optparse* libraries in R:

ADONIS:

(using weighted and unweighted UniFrac distances)

compare_categories.py --method adonis

Calculate permutation test for homogeneity of multivariate dispersions (PERMDISP):

compare_categories.py --method permdisp

Make UPGMA tree:

beta_diversity.py

Appendix B

DESeq2 analysis example:

differential_abundance.py -i unrare_cabbage_age.biom -o diff_abundance_9v12wks_Unrare_FERTILISER_Con-CM.txt -m qiime_meta_9vs12week.txt -a DESeq2_nbinom -c Fertiliser -x Control -y ChickenManure -d

Convert to spf file for STAMP analyses [In Picrust:]**:**

biom_to_stamp.py

checkHierarchy.py

To remove the problematic Clostridium sequences:

1) Use QIIME to convert biom to txt file:

biom convert -i otu_table_no_chloroplasts_or_mitochondria_rare198288.biom -o otu_table_no_chloroplasts_or_mitochondria_rare198288_EXCEL.txt --to-tsv --header-key taxonomy

2) Open in Excel to remove problematic sequences (rows 5155 and 75177) & convert back to biom:

biom convert -i otu_table_no_chloroplasts_or_mitochondria_rare198288_EXCEL.txt -o otu_table_no_chloroplasts_or_mitochondria_rare198288_Clostridium_removed.biom --to-json --table-type="OTU table" --process-obs-metadata taxonomy

3) Then copy the output file to Picrust folder and convert back to spf (as above) and check hierarchy again:

biom_to_stamp.py -m taxonomy otu_table_no_chloroplasts_or_mitochondria_rare198288_Clostridium_removed.biom > otu_table_no_chloroplasts_or_mitochondria_rare198288_Clostridium_removed_STAMP.spf

checkHierarchy.py otu_table_no_chloroplasts_or_mitochondria_rare198288_Clostridium_removed_STAMP.spf

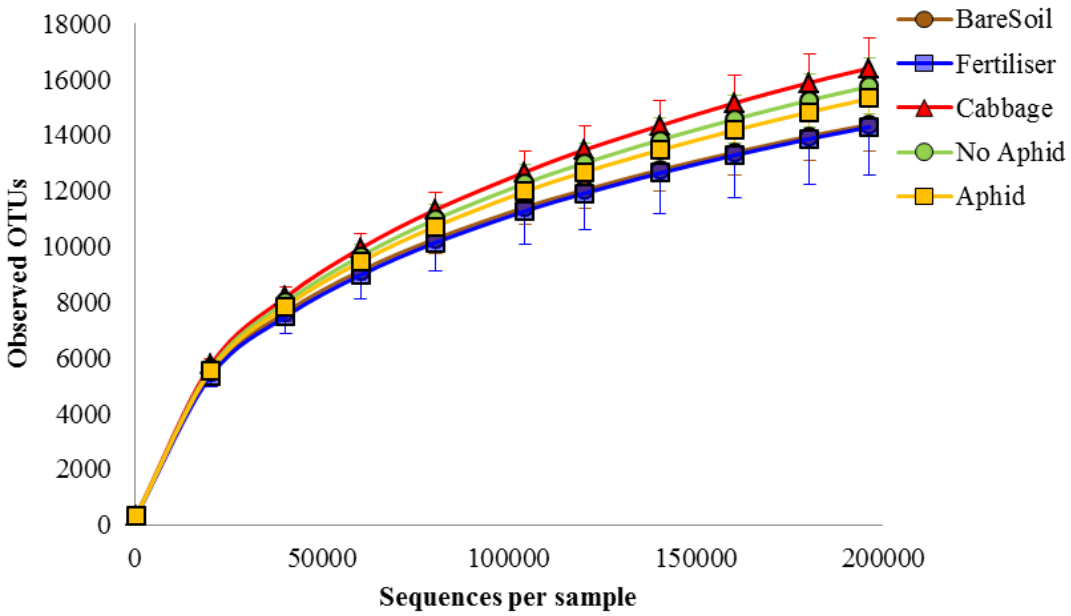


Figure 40 Rarefaction curve of the average observed OTUs (species) in bulk (Bare Soil and Fertiliser) and rhizosphere (Cabbage, No Aphid, Aphid) soil bacterial communities.

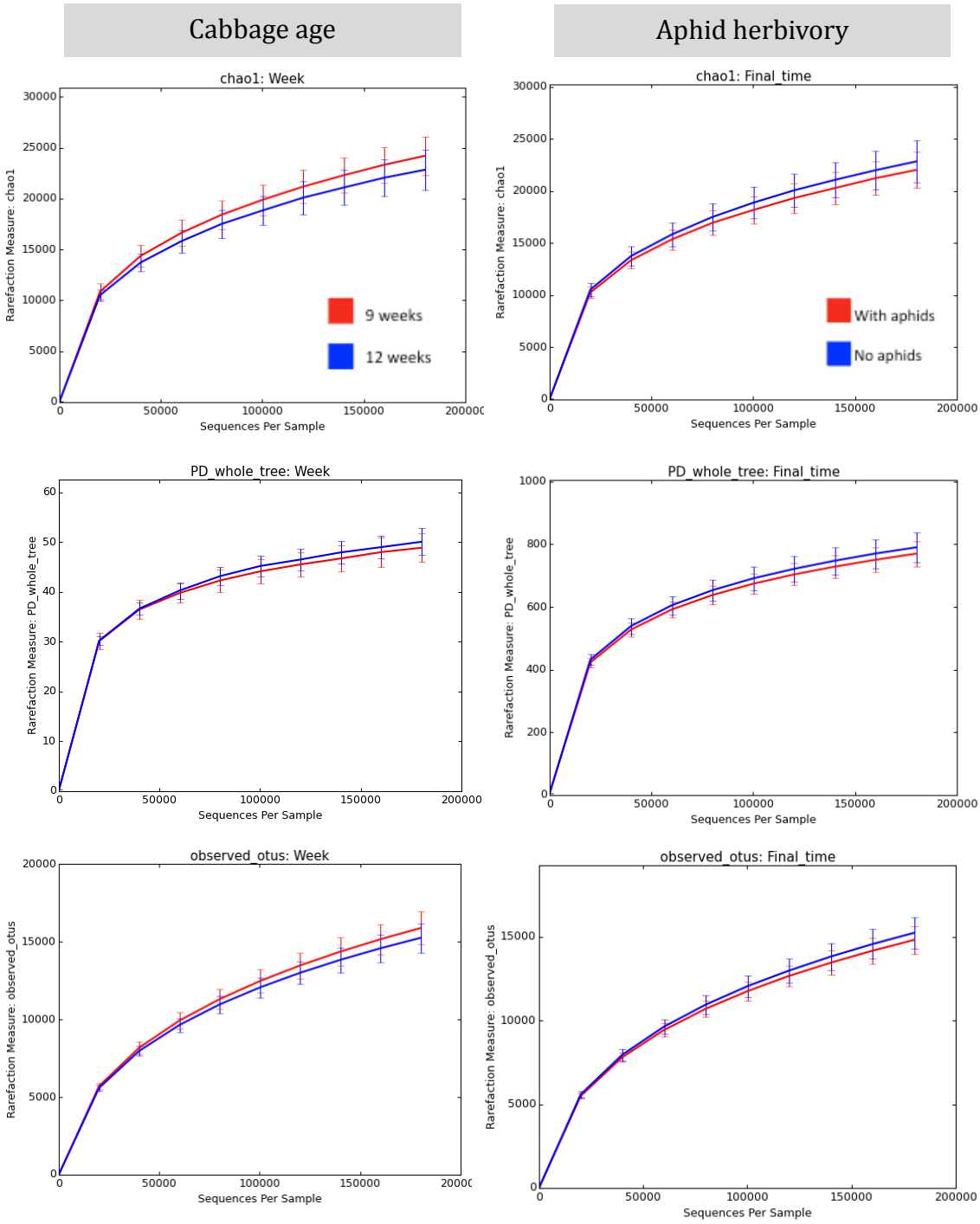


Figure 41 Rarefaction curves showing the alpha diversity comparing 9 and 12 week-old cabbages (left), and aphid-infested and aphid-free (12 week-old) plants (right).

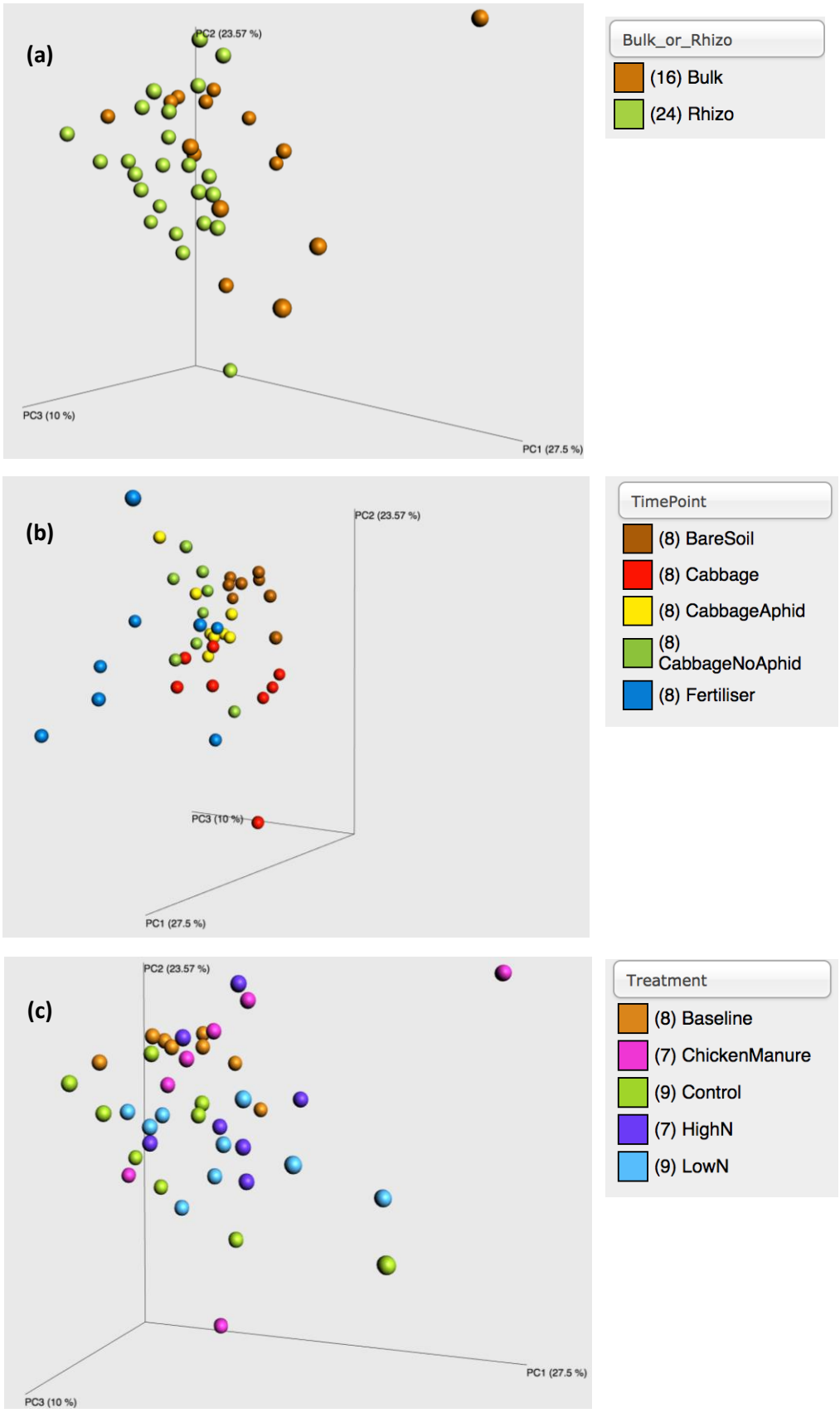


Figure 42 PCoA of jackknifed beta diversity coloured by (a) soil type, (b) sample type and (c) fertiliser treatment using matrices constructed from weighted UniFrac distances.

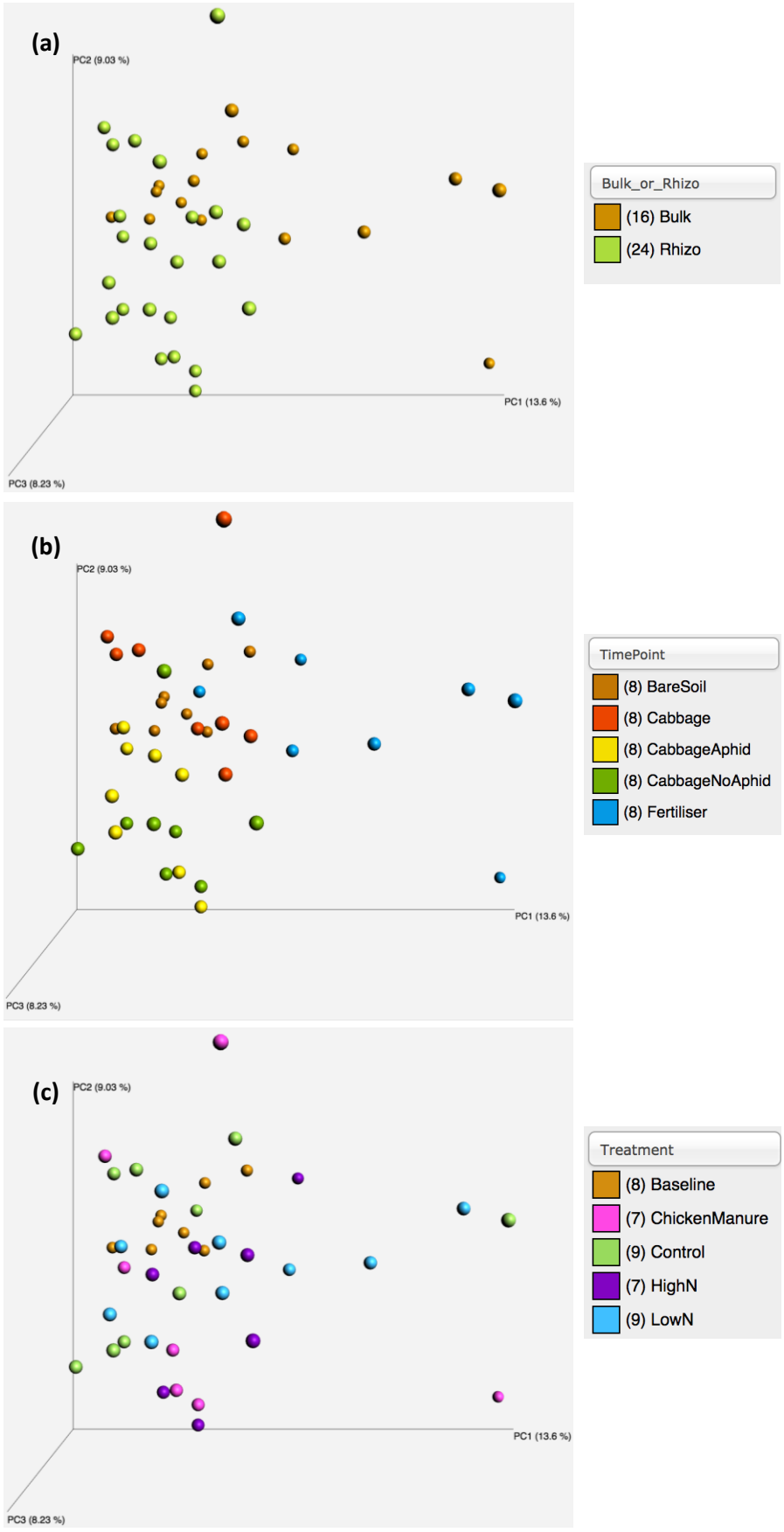


Figure 43 PCoA of jackknifed beta diversity coloured by (a) soil type, (b) sample type and (c) fertiliser treatment using matrices constructed from Bray-Curtis distances.

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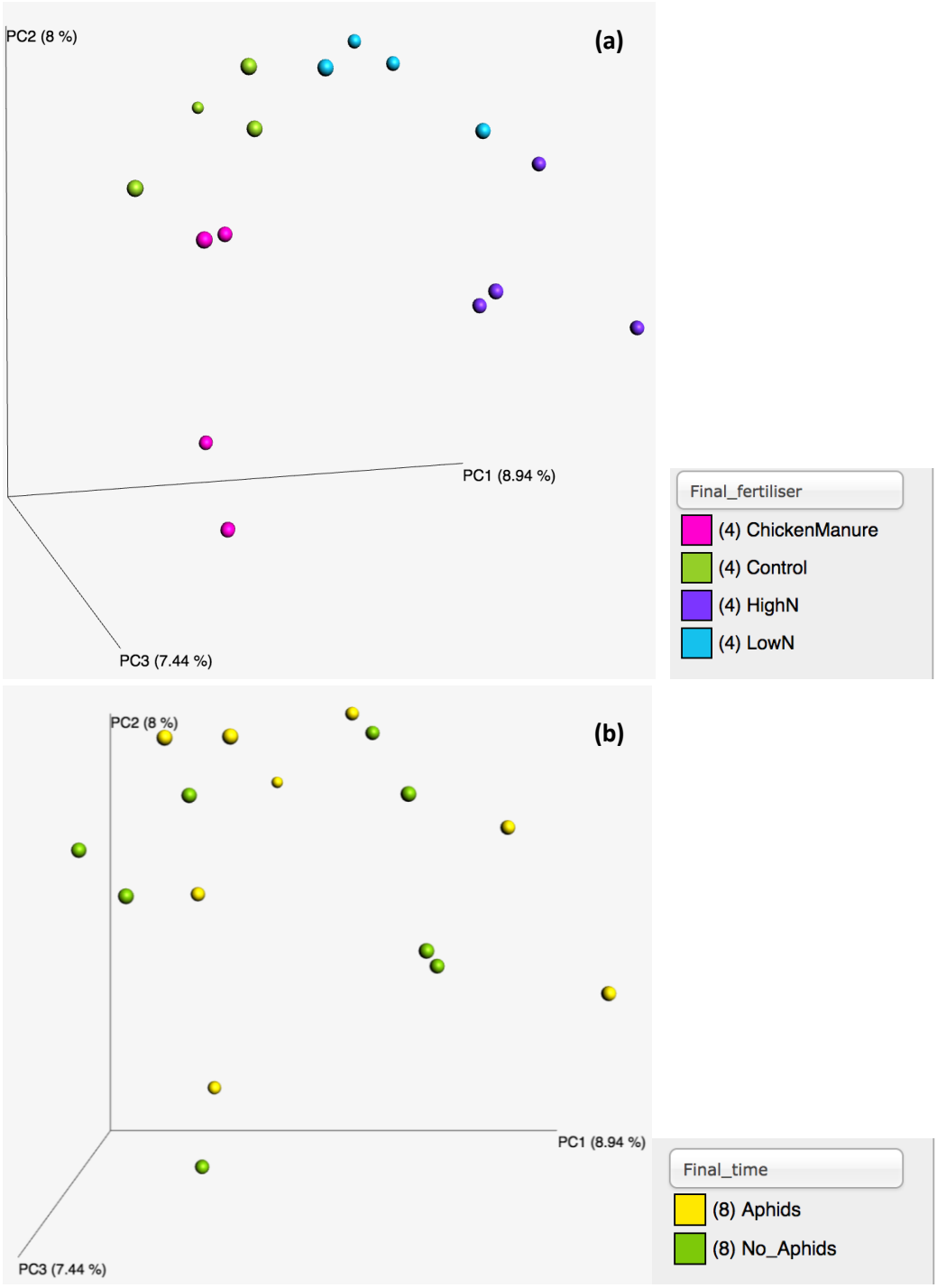


Figure 44 PCoA plots of beta-diversity for 12week-old cabbage rhizosphere samples only, coloured by (a) fertiliser and (b) aphid treatments, using unweighted UniFrac distances.

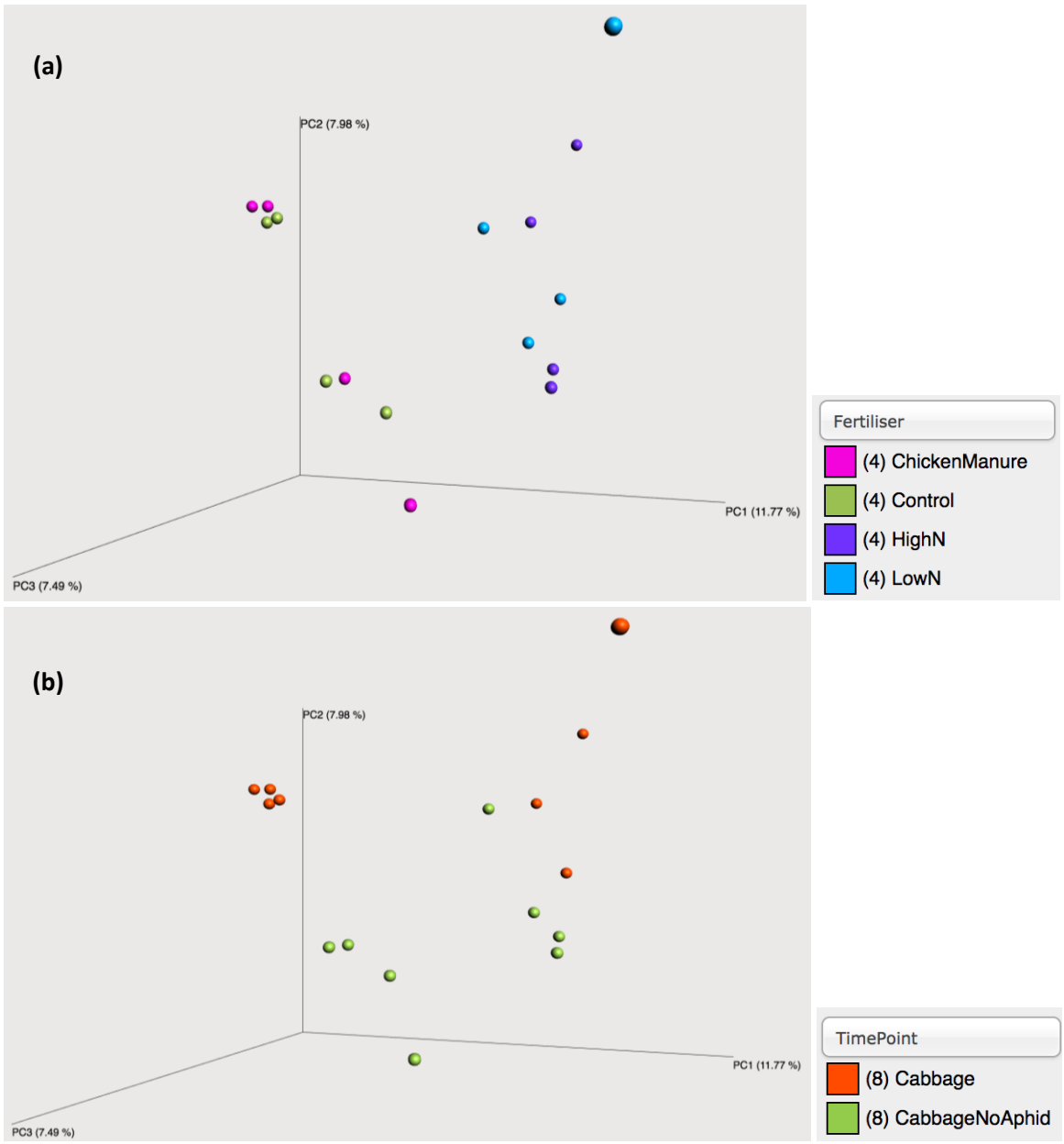


Figure 45 PCoA plots of beta-diversity for 9 and 12week-old cabbage rhizosphere (no aphids) samples only, coloured by (a) fertiliser and (b) cabbage age, using unweighted UniFrac distances.

Appendix B

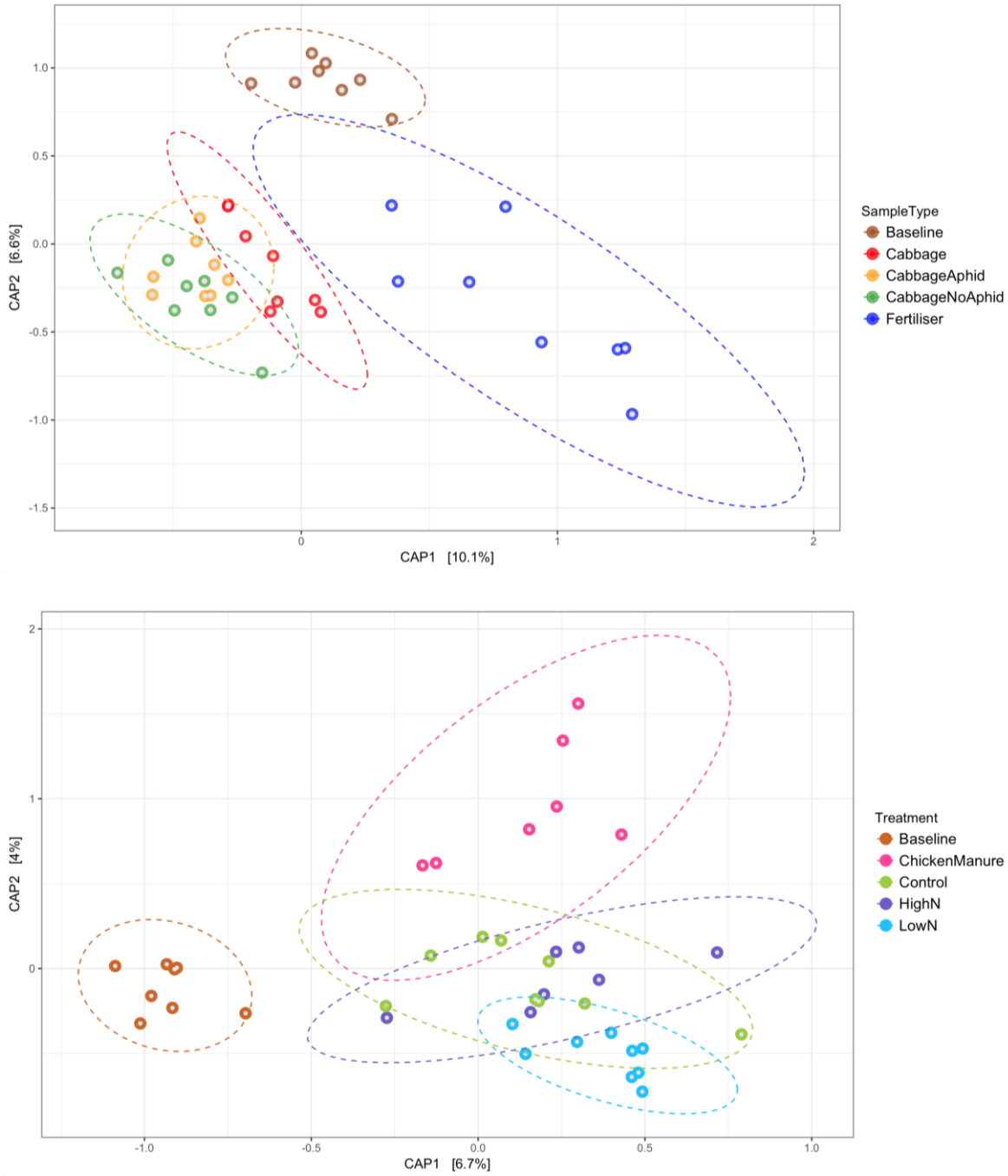


Figure 46 Canonical analysis of principal coordinates (CAP) plot based on **Bray-Curtis** distances by (a) sample type and (b) fertiliser treatment. Sample type PERMANOVA with 9999 permutations: p =0.0002, $F_{4, 35}$ = 2.5567, SS = 0.46195). Treatment PERMANOVA with 9,999 permutations p = 0.0002, $F_{4, 35}$ = 1.6151, SS = 0.31834). Ellipses are based on a multivariate t-distribution and 0.95 confidence interval.

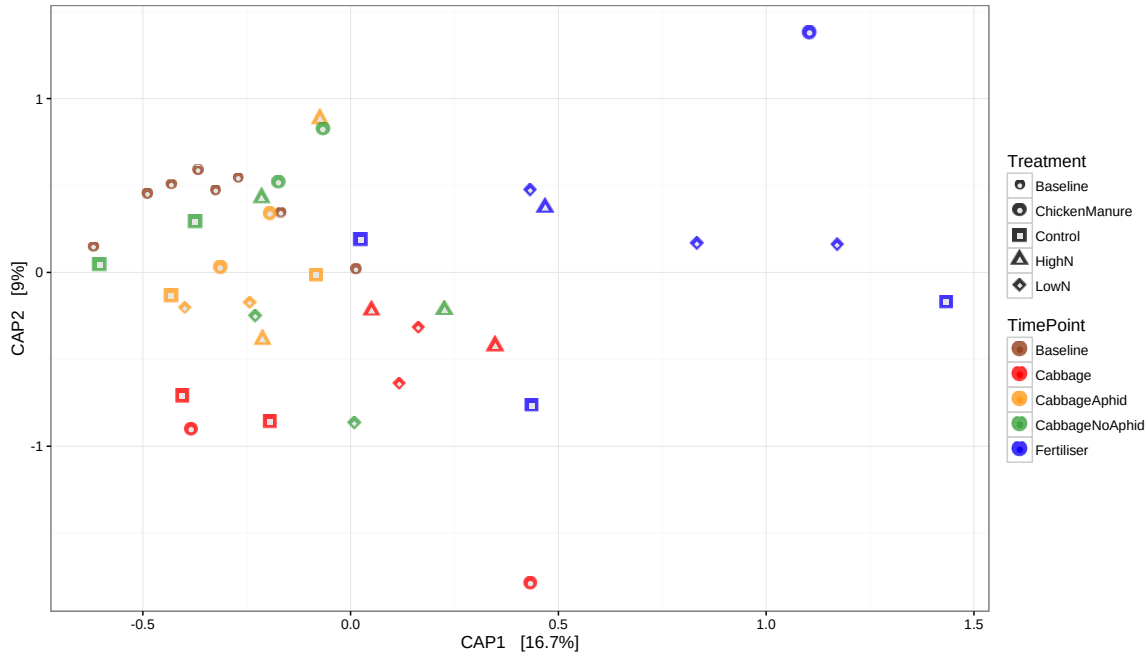


Figure 47 Canonical analysis of principal coordinates (CAP) plot using weighted UniFrac measures grouped by sample type.

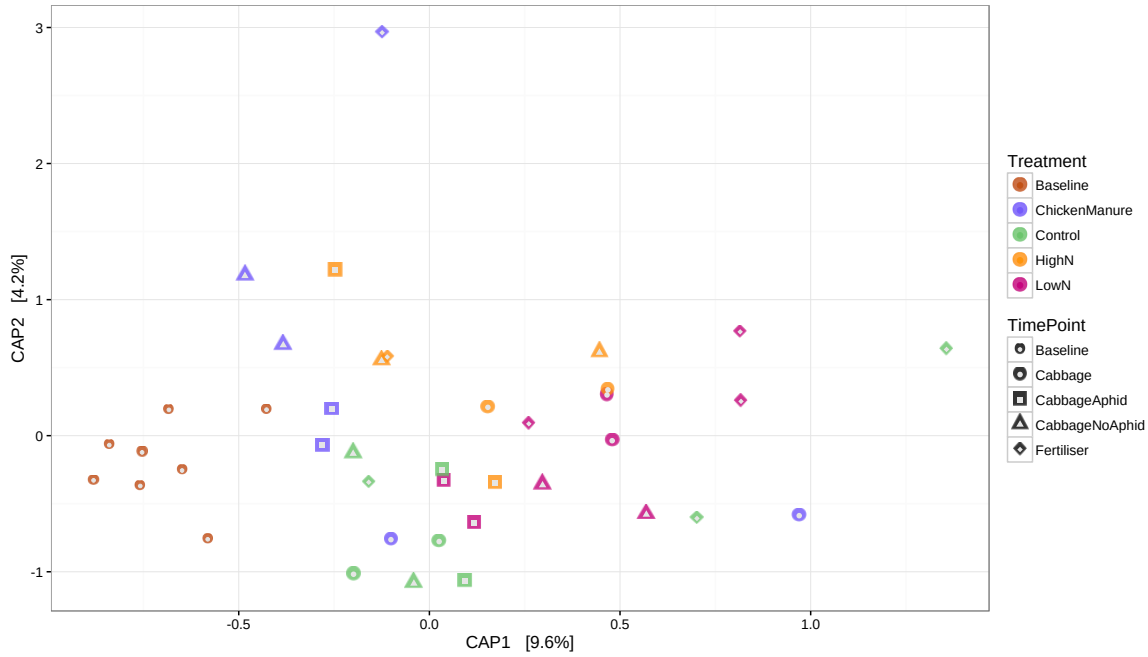


Figure 48 Canonical analysis of principal coordinates (CAP) plot using weighted UniFrac measures grouped by fertiliser treatment.

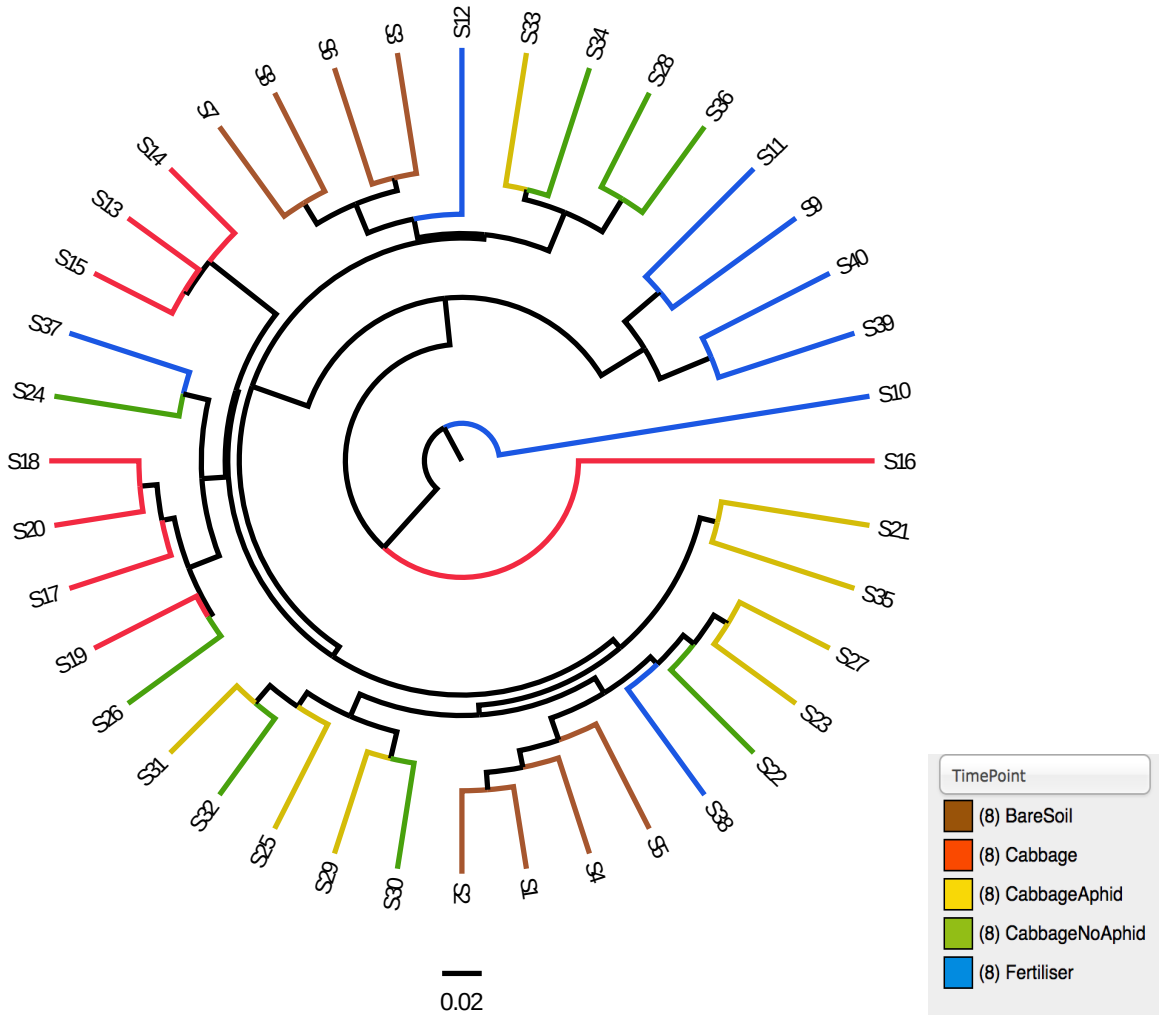


Figure 49 UPGMA trees constructed from weighted UniFrac distance hierarchical clustering created using weighted beta diversity matrix with branches coloured by sample type. Visualised using Fig Tree v1.4.2, radial and rectangular tree layouts. (Note, again, the chicken manure fertiliser sample (S10) and cabbage sample (S16) branching off from the others.)

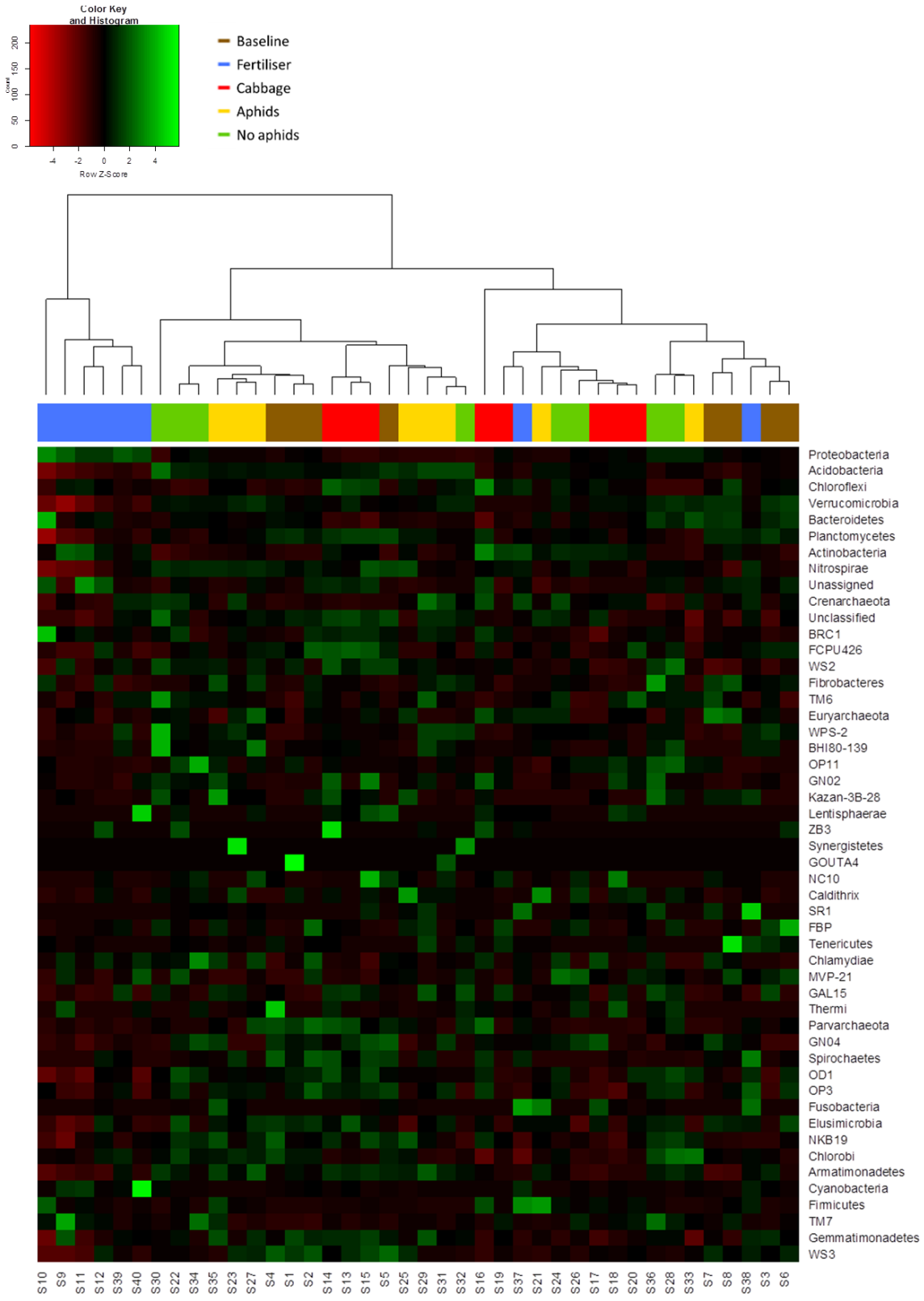


Figure 50 Hierarchically clustered heat map of the relative abundance of bacteria at the phylum level (grouped by sample type) with UPGMA (average neighbour) dendrogram. Note the consistent clustering of the 12-week cabbage rhizosphere samples (+/- aphids), whereas the bulk soil samples (baseline and fertiliser) appear more widely dispersed.

Appendix B

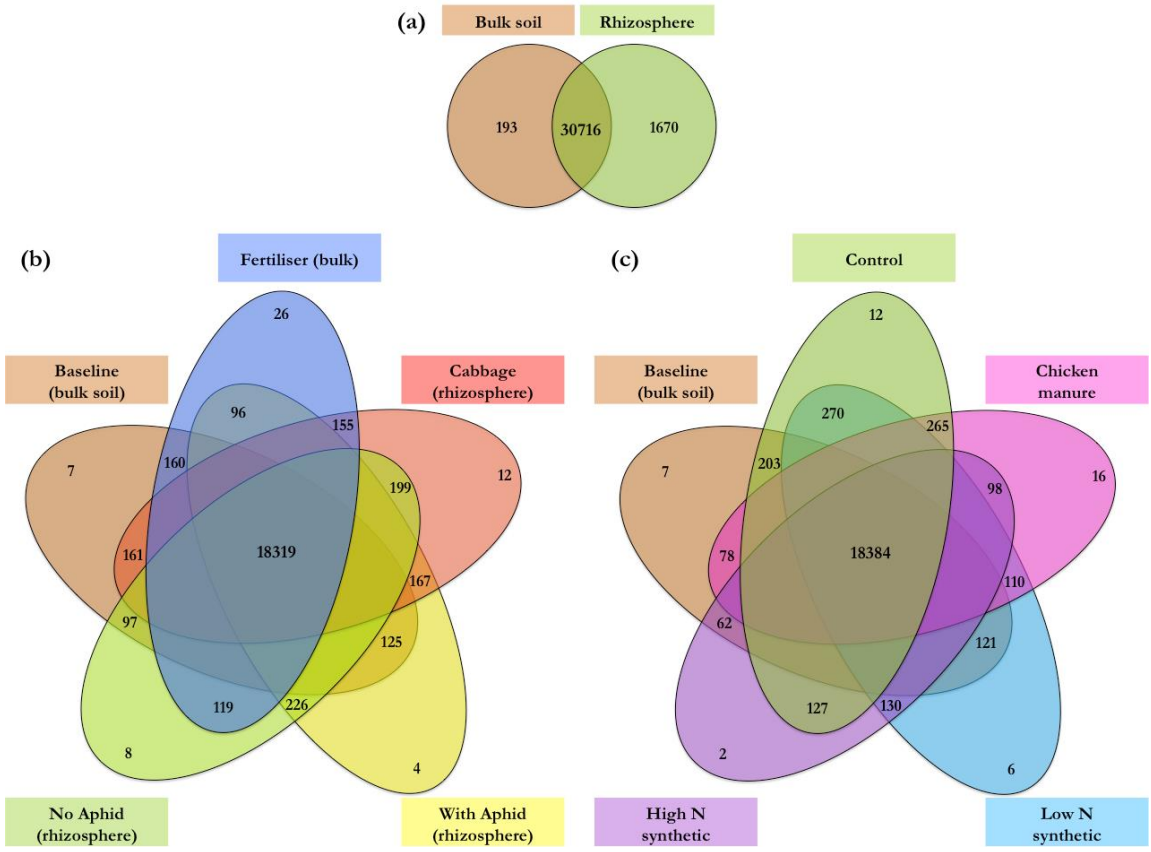


Figure 51 Venn diagrams indicating the number of shared and unique OTUs in samples according to (a) soil type, (b) time-point and (c) treatment (using OTU table restricted to OTUs occurring in a minimum of 10% samples).

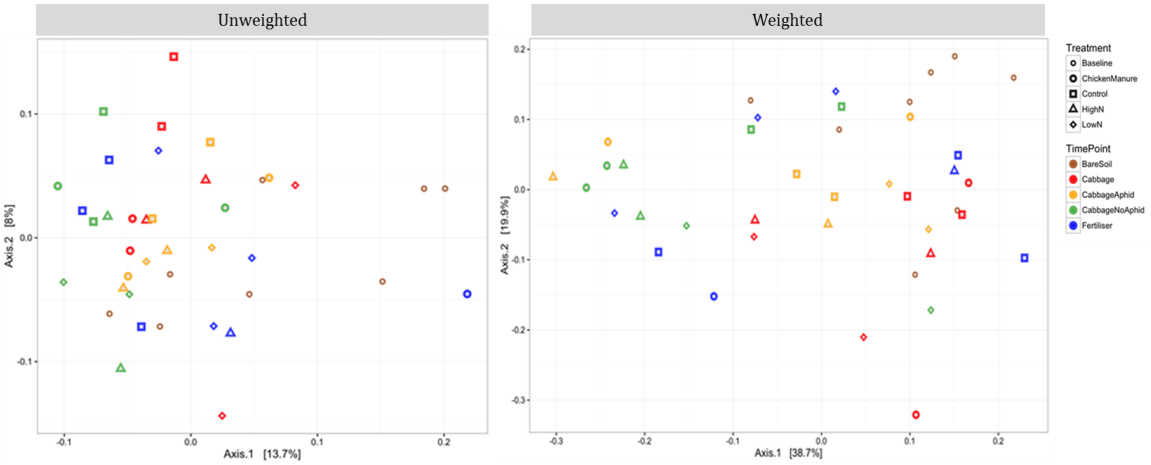


Figure 52 PCoA plots indicating sample types and treatments, constructed using weighted and unweighted UniFrac distances.

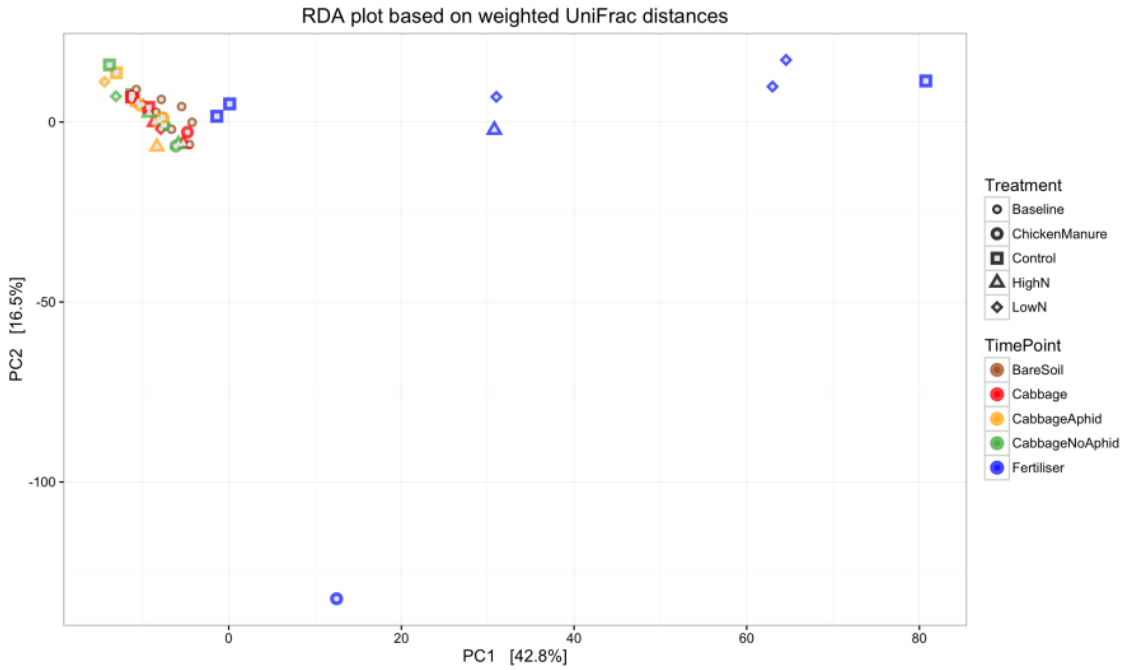


Figure 53 Distance-based redundancy analysis (RDA) plot based on weighted UniFrac distances.

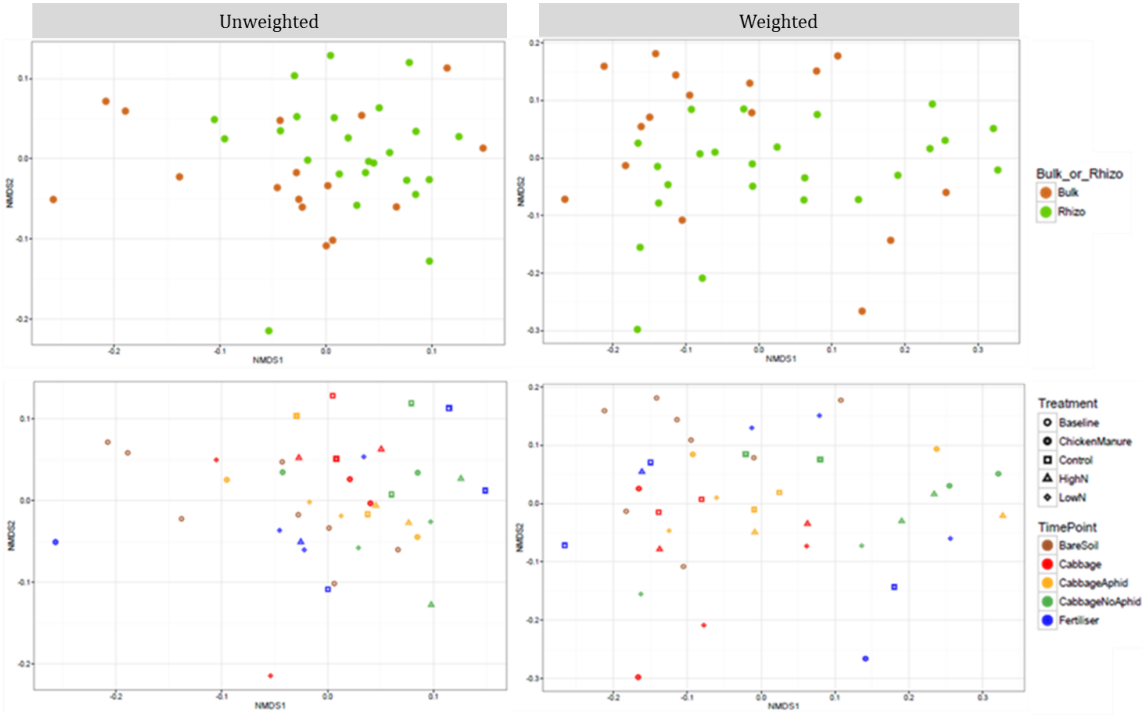


Figure 54 NMDS plot using weighted and unweighted UniFrac distances. (Weighted NMDS stress = 0.1142888 with 2 dimensions; and Unweighted NMDS stress = 0.1792568 with 3 dimensions).

Appendix B

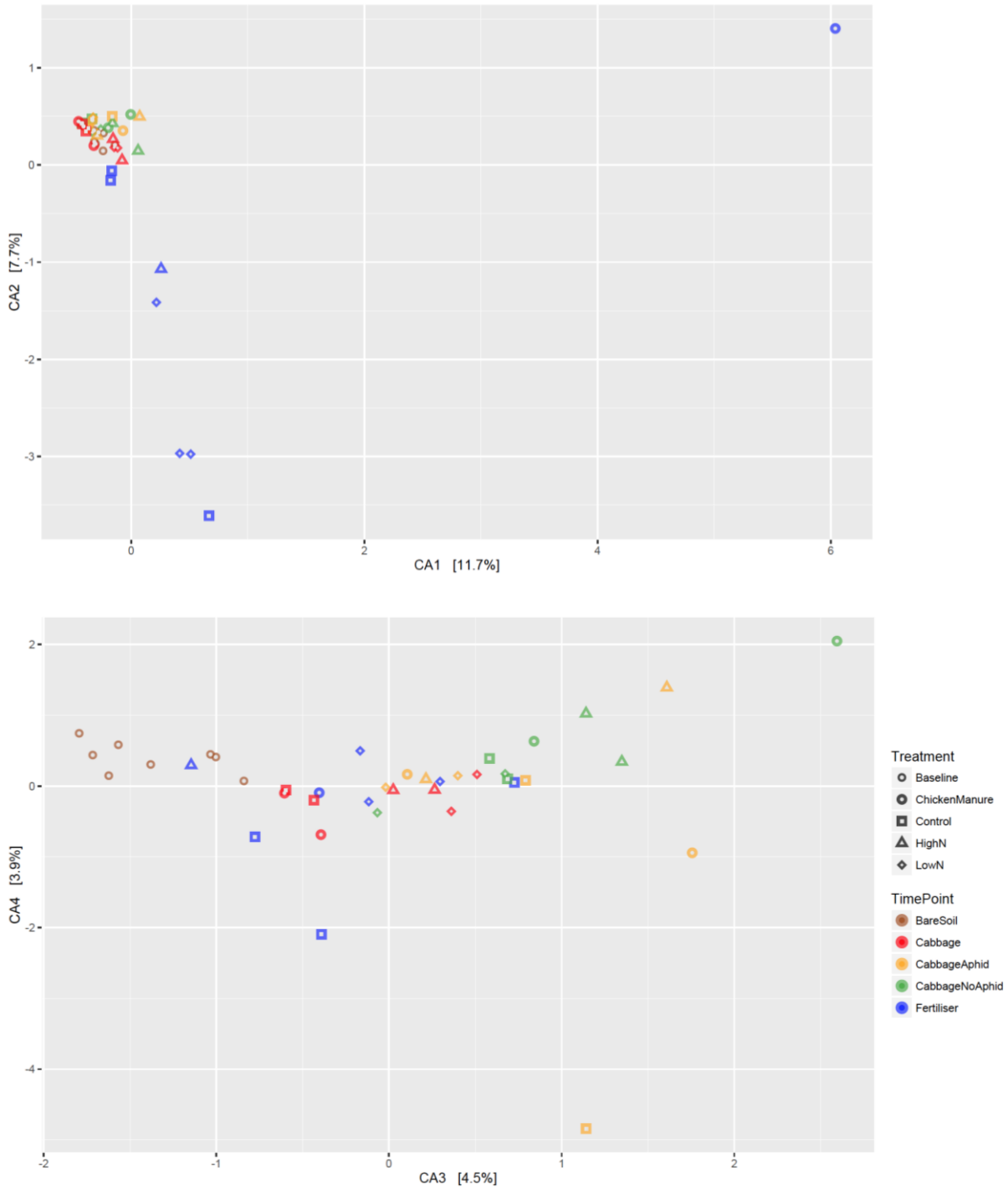


Figure 55 CCA ordination plot of axes 1 & 2 (top) and 3 & 4 (bottom). Axes CA1 (11.7%); CA2 (7.7%); CA3 (4.5%); CA4 (3.9%).

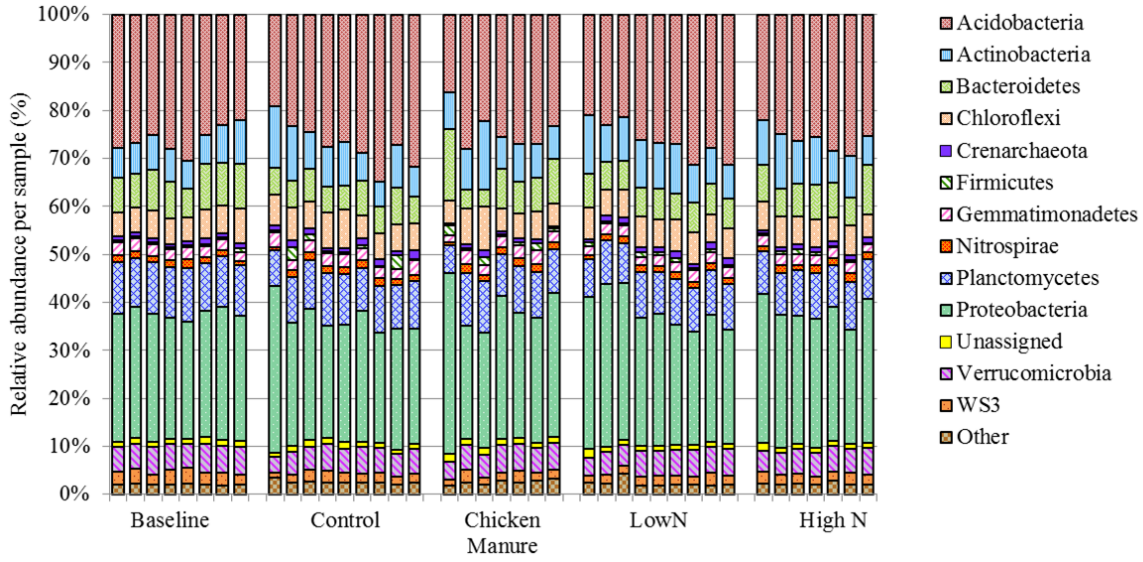


Figure 56 Relative abundance of the top ten phyla in each soil sample grouped by treatment. The remaining phyla are grouped under "Other".

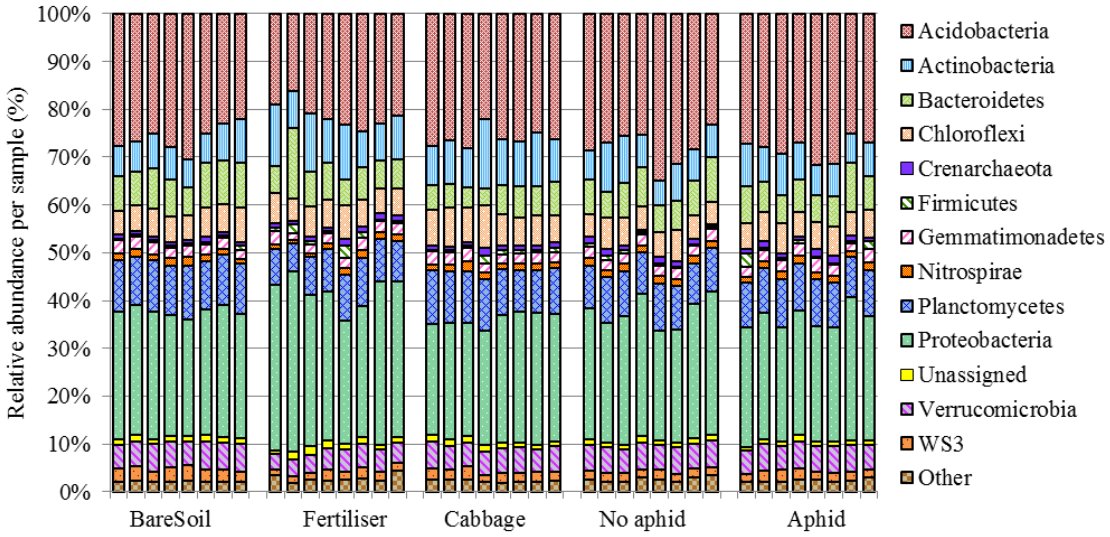


Figure 57 Relative abundance of the top ten phyla grouped by sample type. The remaining phyla are grouped under "Other".

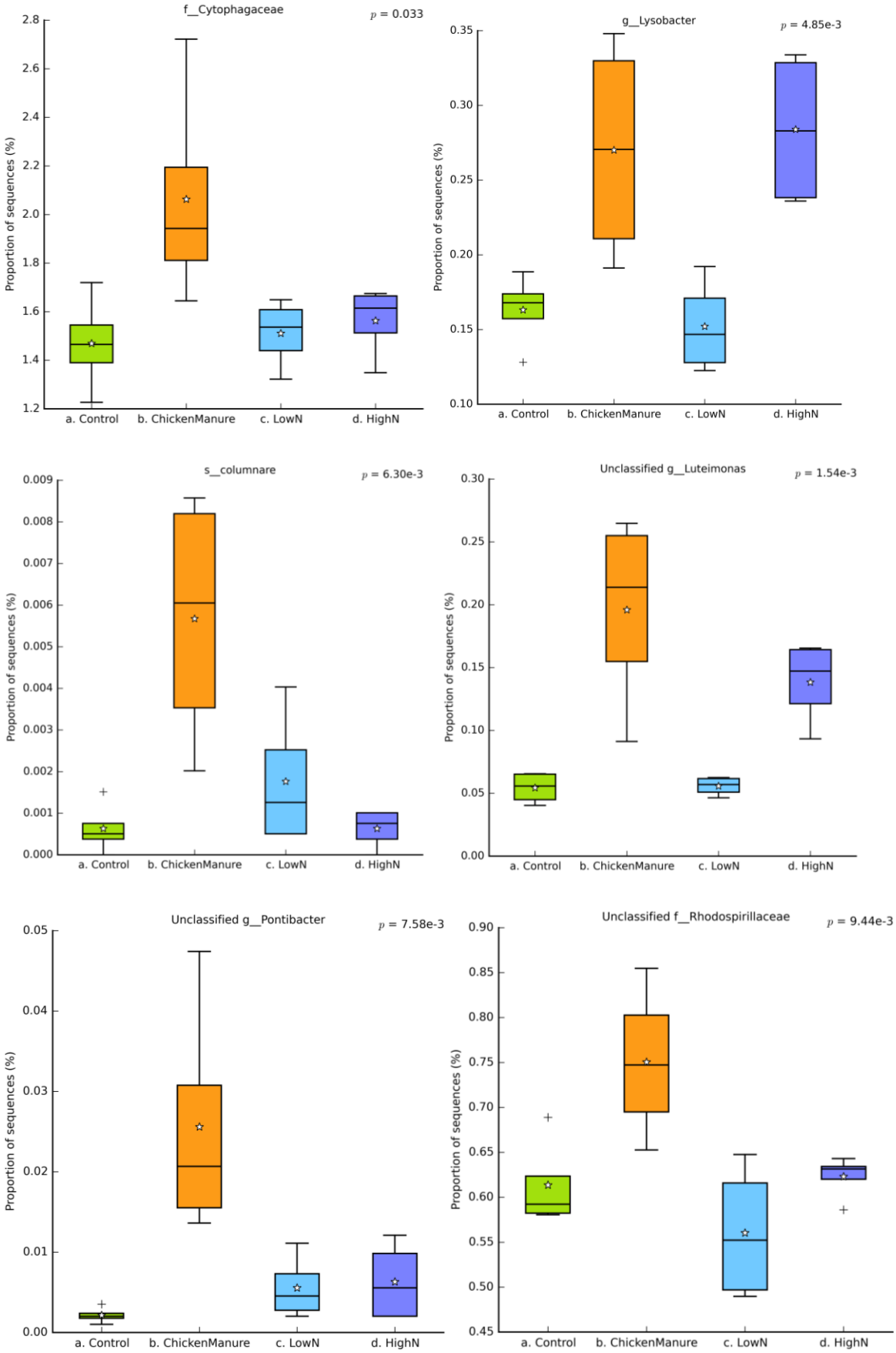


Figure 58 OTU's enriched in organically fertilised soils at 12 weeks (*Cytophagaceae*, *Lysobacter*, *Flavobacterium columnare*, *Luteimonas*, *Pontibacter* and *Rhodospirillaceae*).

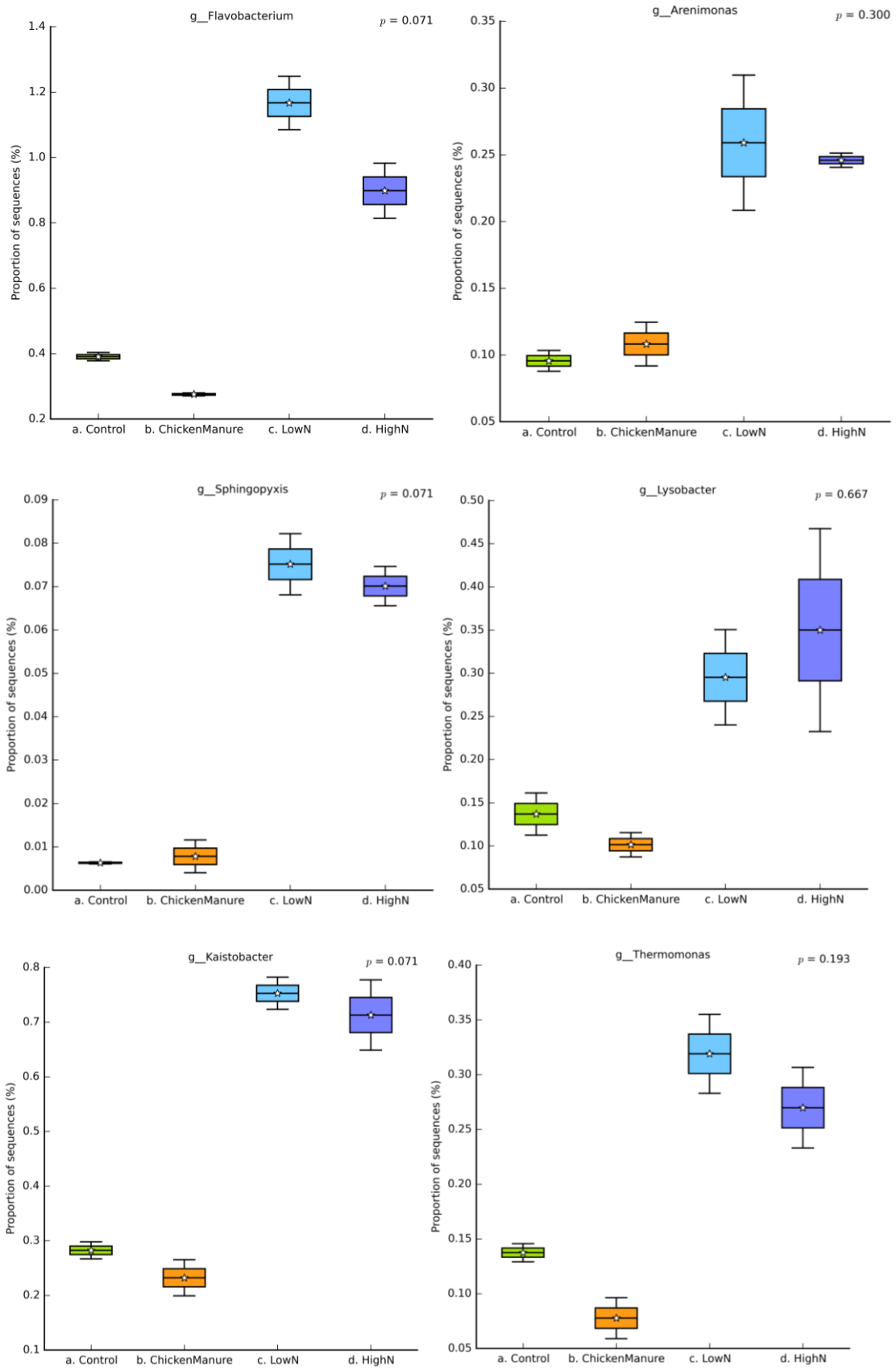


Figure 59 OTU's enriched in synthetically fertilised soils of 9 week-old cabbage rhizospheres, assigned to the genera (a) *Flavobacterium*; (b) *Arenimonas*; (c) *Sphingobacterium*; (d) *Lysobacter*; (e) *Kaisobacter*; (f) *Thermomonas*.

Appendix B

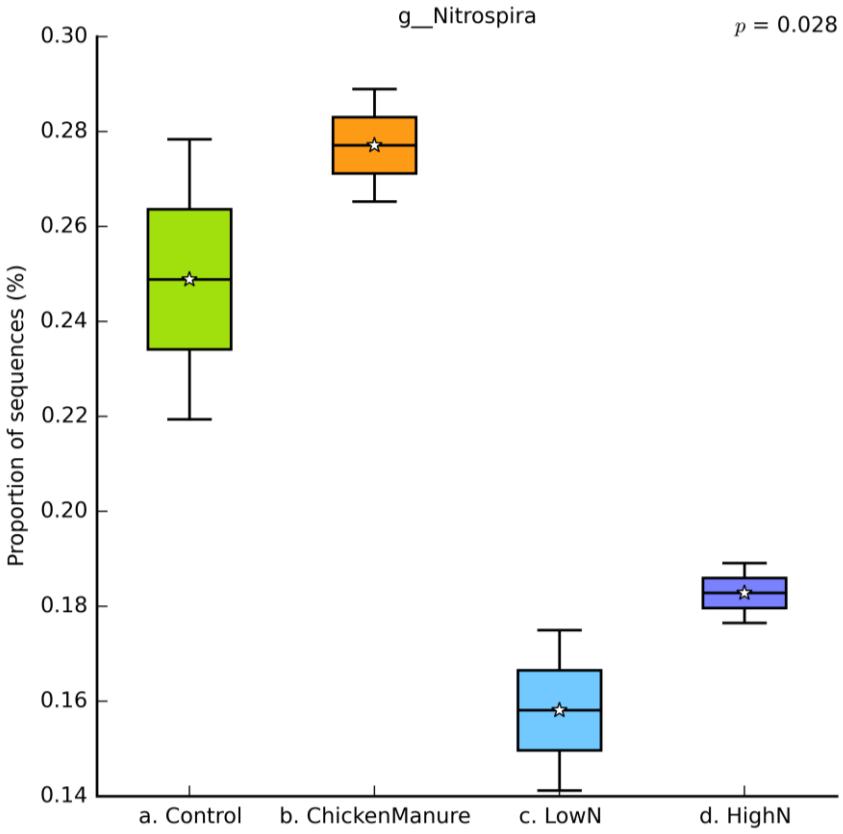


Figure 60 The abundance of *Nitrospira* in soils under different fertiliser treatments at 9 weeks.

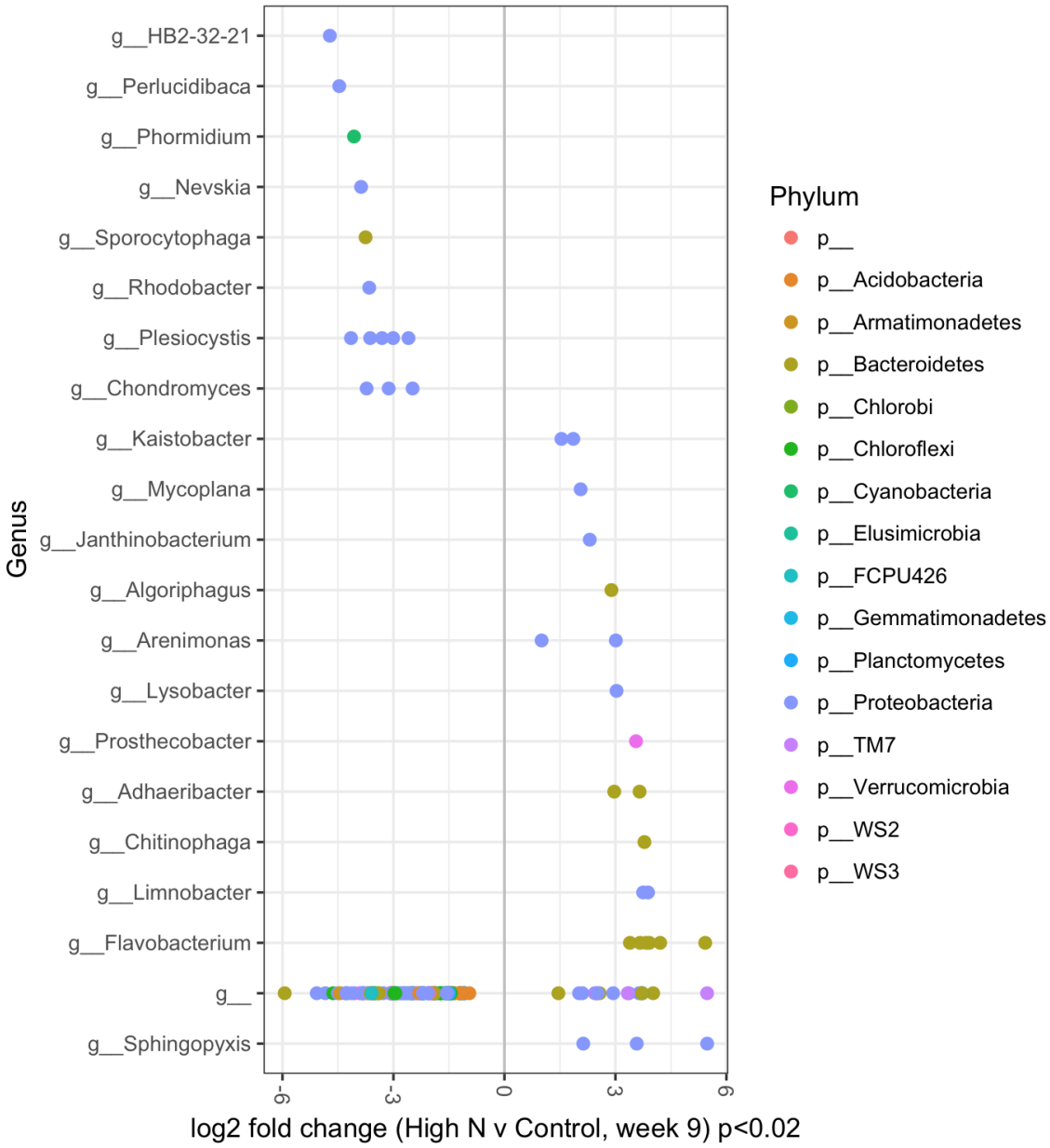


Figure 61 DESeq2 analysis results indicating the fold-change of bacterial genera between rhizosphere soil bacterial communities of High N and control cabbages at 9 week.

Table 28 OTUs enriched in the rhizospheres of **Chicken manure**-treated **12 week**-old cabbages (relative to controls). DESeq2 threshold: Benjamini-Hochberg corrected *p*-value <0.03, log2 fold change 1.2.

OTU	Taxonomy	Log2 fold change	*p*-value (corrected)
New.ReferenceOTU1136	p_Bac; c_Bacteroidia; o_Bacteroidales; f_VC21_Bac22; g_; s_	2.352	0.0065
1143479	p_Bac; c_Cytophagia; o_Cytophagales; f_Cyclobacteriaceae; g_Algoriphagus; NA	2.369	0.0021
549553	p_Bac; c_Cytophagia; o_Cytophagales; f_Cytophagaceae; g_; s_	2.898	<0.0001
New.ReferenceOTU724	p_Bac; c_Cytophagia; o_Cytophagales; f_Cytophagaceae; g_; s_	2.669	0.0006
New.ReferenceOTU2144	p_Bac; c_Cytophagia; o_Cytophagales; f_Cytophagaceae; g_; s_	1.972	0.0188
New.CleanUp.ReferenceOTU232825	p_Bac; c_Cytophagia; o_Cytophagales; f_Cytophagaceae; g_; s_	2.138	0.0217
New.ReferenceOTU2312	p_Bac; c_Cytophagia; o_Cytophagales; f_Cytophagaceae; g_; s_	1.761	0.0292
913174	p_Bac; c_Cytophagia; o_Cytophagales; f_Cytophagaceae; g_Leadbetterella; s_	2.553	0.0024
New.CleanUp.ReferenceOTU16904	p_Bac; c_Cytophagia; o_Cytophagales; f_Cytophagaceae; g_Pontibacter; s_	2.191	0.0177
New.CleanUp.ReferenceOTU197368	p_Bac; c_Flavobacteriia; o_Flavobacteriales; f_Cryomorphaceae; g_Crocinitomix; s_	2.284	0.0109
102122	p_Bac; c_Flavobacteriia; o_Flavobacteriales; f_Cryomorphaceae; g_Crocinitomix; s_	2.144	0.0217
New.CleanUp.ReferenceOTU172123	p_Bac; c_Flavobacteriia; o_Flavobacteriales; f_Flavobacteriaceae; g_; s_	2.119	0.0259
92636	p_Bac; c_Flavobacteriia; o_Flavobacteriales; f_Flavobacteriaceae; g_Capnocytophaga; s_	2.271	0.0120
574686	p_Bac; c_Flavobacteriia; o_Flavobacteriales; f_Flavobacteriaceae; g_Flavobacterium; s_	1.501	0.0149
New.ReferenceOTU253	p_Bac; c_Sphingobacteriia; o_Sphingobacteriales; f_; g_; s_	4.371	<0.0001
1006099	p_Bac; c_Sphingobacteriia; o_Sphingobacteriales; f_; g_; s_	4.487	<0.0001
New.CleanUp.ReferenceOTU17462	p_Bac; c_Sphingobacteriia; o_Sphingobacteriales; f_; g_; s_	3.284	<0.0001
New.CleanUp.ReferenceOTU207341	p_Bac; c_Sphingobacteriia; o_Sphingobacteriales; f_; g_; s_	2.348	0.0068
1081489	p_Fibrobacteres; c_Fibrobacteria; o_258ds10; f_; g_; s_	2.132	0.0009
801579	p_Firmicutes; c_Bacilli; o_Bacillales; f_Planococcaceae; g_Lysinibacillus; s_boronitolerans	2.216	0.0120
New.ReferenceOTU434	p_Gem; c_Gemmatimonadetes; o_KD8-87; f_; g_; s_	2.174	0.0029
New.ReferenceOTU1057	p_Gem; c_Gemmatimonadetes; o_KD8-87; f_; g_; s_	2.307	0.0033
4359270	p_Gem; c_Gemmatimonadetes; o_KD8-87; f_; g_; s_	1.844	0.0120
New.ReferenceOTU960	p_Planctomycetes; c_OM190; o_CL500-15; f_; g_; s_	2.302	0.0120
New.CleanUp.ReferenceOTU261125	p_Planctomycetes; c_Planctomycetia; o_Planctomycetales; f_Planctomycetaceae; g_Planctomyces; s_	2.361	0.0036

4361041	p_Pro; c_Alphaproteobacteria; o_BD7-3; f_; g_; s_	2.090	0.0259
New.CleanUp.ReferenceOTU293903	p_Pro; c_Alphaproteobacteria; o_Rhizobiales; f_Phyllobacteriaceae; g_; s_	2.477	0.0031
New.CleanUp.ReferenceOTU78909	p_Pro; c_Alphaproteobacteria; o_Rhodospirillales; f_Rhodospirillaceae; g_; s_	2.531	0.0014
New.CleanUp.ReferenceOTU199028	p_Pro; c_Alphaproteobacteria; o_Rhodospirillales; f_Rhodospirillaceae; g_; s_	2.054	0.0293
New.CleanUp.ReferenceOTU259522	p_Pro; c_Alphaproteobacteria; o_Sphingomonadales; f_Erythrobacteraceae; g_; s_	2.431	0.0033
New.CleanUp.ReferenceOTU105773	p_Pro; c_Alphaproteobacteria; o_Sphingomonadales; f_Erythrobacteraceae; g_; s_	2.212	0.0120
New.CleanUp.ReferenceOTU206107	p_Pro; c_Betaproteobacteria; o_Burkholderiales; f_Alcaligenaceae; g_; s_	2.413	0.0031
New.CleanUp.ReferenceOTU222874	p_Pro; c_Betaproteobacteria; o_Burkholderiales; f_Alcaligenaceae; g_; s_	2.392	0.0033
New.ReferenceOTU458	p_Pro; c_Deltaproteobacteria; o_Myxococcales; f_; g_; s_	2.094	0.0217
805785	p_Pro; c_Gammaproteobacteria; o_Oceanospirillales; f_Halomonadaceae; g_; s_	2.845	0.0001
4461200	p_Pro; c_Gammaproteobacteria; o_Oceanospirillales; f_Halomonadaceae; g_; s_	2.576	0.0014
New.CleanUp.ReferenceOTU47305	p_Pro; c_Gammaproteobacteria; o_Oceanospirillales; f_Halomonadaceae; g_; s_	2.072	0.0253
551472	p_Pro; c_Gammaproteobacteria; o_Xanthomonadales; f_Sinobacteraceae; g_Steroidobacter; s_	2.772	0.0003
New.CleanUp.ReferenceOTU302471	p_Pro; c_Gammaproteobacteria; o_Xanthomonadales; f_Xanthomonadaceae; g_; s_	1.892	0.0014
750541	p_Pro; c_Gammaproteobacteria; o_Xanthomonadales; f_Xanthomonadaceae; g_; s_	2.296	0.0048
4008562	p_Pro; c_Gammaproteobacteria; o_Xanthomonadales; f_Xanthomonadaceae; g_; s_	2.167	0.0050
824502	p_Pro; c_Gammaproteobacteria; o_Xanthomonadales; f_Xanthomonadaceae; g_; s_	2.322	0.0097
New.CleanUp.ReferenceOTU207407	p_Pro; c_Gammaproteobacteria; o_Xanthomonadales; f_Xanthomonadaceae; g_; s_	2.175	0.0127
New.ReferenceOTU2575	p_Pro; c_Gammaproteobacteria; o_Xanthomonadales; f_Xanthomonadaceae; g_; s_	1.600	0.0133
New.CleanUp.ReferenceOTU24991	p_Pro; c_Gammaproteobacteria; o_Xanthomonadales; f_Xanthomonadaceae; g_Dokdonella; s_	2.122	0.0109
New.ReferenceOTU95	p_Pro; c_Gammaproteobacteria; o_Xanthomonadales; f_Xanthomonadaceae; g_Dokdonella; s_	1.969	0.0293
1052930	p_Pro; c_Gammaproteobacteria; o_Xanthomonadales; f_Xanthomonadaceae; g_Luteimonas; s_	1.633	0.0008
3333615	p_Pro; c_Gammaproteobacteria; o_Xanthomonadales; f_Xanthomonadaceae; g_Lysobacter; s_	1.935	0.0217
266510	p_Pro; c_Gammaproteobacteria; o_Xanthomonadales; f_Xanthomonadaceae; g_Pseudoxanthomonas; NA	2.701	0.0008
New.CleanUp.ReferenceOTU184941	p_Pro; c_Gammaproteobacteria; o_Xanthomonadales; f_Xanthomonadaceae; g_Pseudoxanthomonas; NA	2.412	0.0036
New.ReferenceOTU2677	p_Pro; c_Gammaproteobacteria; o_Xanthomonadales; f_Xanthomonadaceae; g_Pseudoxanthomonas; NA	2.040	0.0090
New.ReferenceOTU610	p_Pro; c_Gammaproteobacteria; o_Xanthomonadales; f_Xanthomonadaceae; g_Thermomonas; NA	1.712	0.0181
New.CleanUp.ReferenceOTU314415	p_TM7; c_; o_; f_; g_; s_	2.743	0.0008
New.CleanUp.ReferenceOTU22058	p_TM7; c_; o_; f_; g_; s_	2.233	0.0120

New.CleanUp.ReferenceOTU104445	p_TM7; c_TM7-1; o_; f_; g_; s_	2.762	0.0007
New.CleanUp.ReferenceOTU262703	p_TM7; c_TM7-1; o_; f_; g_; s_	2.412	0.0041
New.CleanUp.ReferenceOTU289691	p_TM7; c_TM7-1; o_; f_; g_; s_	2.120	0.0188
New.ReferenceOTU570	p_Ver; c_[Pedosphaerae]; o_[Pedosphaerales]; f_auto67_4W; g_; s_	1.432	0.0071
New.CleanUp.ReferenceOTU45992	p_Ver; c_[Spartobacteria]; o_[Chthoniobacterales]; f_[Chthoniobacteraceae]; g_Chthoniobacter; s_	2.266	0.0041
New.CleanUp.ReferenceOTU65212	p_Ver; c_[Spartobacteria]; o_[Chthoniobacterales]; f_[Chthoniobacteraceae]; g_Chthoniobacter; s_	2.064	0.0177
New.CleanUp.ReferenceOTU196676	p_Ver; c_Opitutae; o_Opitutales; f_Opitutaceae; g_Opitutus; s_	2.475	0.0018

Taxonomic levels: **p** = phylum; **c** = class; **o** = order; **f** = family; **g** = genus; **s** = species (blank = unassigned); Phylum abbreviations: **Bac:** Bacteroidetes, **Gem**: Gemmatimonadetes, **Pla**: Planctomycetes, **Pro**: Proteobacteria, **Ver**: Verrucomicrobia.

Table 29 OTUs enriched in the rhizospheres of **Low N**-treated **9 week**-old cabbages (relative to controls). DESeq2 threshold: Benjamini-Hochberg corrected *p*-value <0.03, log2 fold change 1.2.

OTU	**Taxonomy**	**Log2 fold change**	***p*-value (corrected)**
324677	p_Bac; c_[Saprospirae]; o_[Saprospirales]; f_Chitinophagaceae; g_Chitinophaga; s_	2.584	0.0115
561537	p_Bac; c_Bacteroidia; o_Bacteroidales; f_Porphyromonadaceae; g_Porphyromonas; s_	2.935	0.0043
New.ReferenceOTU808	p_Bac; c_Cytophagia; o_Cytophagales; f_Cyclobacteriaceae; g_Algoriphagus; NA	2.035	0.0043
New.ReferenceOTU841	p_Bac; c_Cytophagia; o_Cytophagales; f_Cytophagaceae; g_; s_	3.113	0.0013
New.ReferenceOTU1493	p_Bac; c_Cytophagia; o_Cytophagales; f_Cytophagaceae; g_Adhaeribacter; s_	3.556	<0.0001
92131	p_Bac; c_Flavobacteriia; o_Flavobacteriales; f_Cryomorphaceae; g_Fluviicola; s_	2.015	0.0064
New.ReferenceOTU310	p_Bac; c_Flavobacteriia; o_Flavobacteriales; f_Flavobacteriaceae; g_Aequorivita; s_	2.070	0.0110
4471717	p_Bac; c_Flavobacteriia; o_Flavobacteriales; f_Flavobacteriaceae; g_Flavobacterium; s_	3.752	<0.0001
New.CleanUp.ReferenceOTU235757	p_Bac; c_Flavobacteriia; o_Flavobacteriales; f_Flavobacteriaceae; g_Flavobacterium; s_	3.632	<0.0001
674655	p_Bac; c_Flavobacteriia; o_Flavobacteriales; f_Flavobacteriaceae; g_Flavobacterium; s_	3.885	<0.0001
New.ReferenceOTU2530	p_Bac; c_Flavobacteriia; o_Flavobacteriales; f_Flavobacteriaceae; g_Flavobacterium; s_	2.883	0.0016
New.ReferenceOTU348	p_Bac; c_Flavobacteriia; o_Flavobacteriales; f_Flavobacteriaceae; g_Flavobacterium; s_	2.534	0.0074
New.CleanUp.ReferenceOTU310941	p_Bac; c_Flavobacteriia; o_Flavobacteriales; f_Flavobacteriaceae; g_Flavobacterium; s_	2.449	0.0130

1049387	p_Bac; c_Flavobacteriia; o_Flavobacteriales; f_Flavobacteriaceae; g_Flavobacterium; s_	2.386	0.0224
4299136	p_Chloroflexi; c_Thermomicrobia; o_JG30-KF-CM45; f_; g_; s_	2.287	0.0035
1118729	p_Cyanobacteria; c_ML635J-21; o_; f_; g_; s_	2.262	0.0269
New.ReferenceOTU363	p_Firmicutes; c_Bacilli; o_Lactobacillales; f_Carnobacteriaceae; g_Granulicatella; s_	2.584	0.0153
4451561	p_Firmicutes; c_Clostridia; o_Clostridiales; f_Veillonellaceae; g_Selenomonas; NA	4.636	<0.0001
New.ReferenceOTU1707	p_Fusobacteria; c_Fusobacteriia; o_Fusobacteriales; f_Leptotrichiaceae; g_Leptotrichia; s_	3.992	<0.0001
New.ReferenceOTU1467	p_Gemmatimonadetes; c_Gemm-3; o_; f_; g_; s_	2.763	0.0085
New.CleanUp.ReferenceOTU212207	p_OP11; c_; o_; f_; g_; s_	2.230	0.0087
1143479	p_Planctomycetes; c_Planctomycetia; o_Pirellulales; f_Pirellulaceae; g_; s_	2.854	0.0060
582973	p_Pro; c_Alphaproteobacteria; o_BD7-3; f_; g_; s_	2.572	0.0137
New.CleanUp.ReferenceOTU237938	p_Pro; c_Alphaproteobacteria; o_Caulobacterales; f_Caulobacteraceae; g_; s_	3.377	0.0002
570086	p_Pro; c_Alphaproteobacteria; o_Rhizobiales; f_; g_; s_	2.725	0.0064
New.CleanUp.ReferenceOTU172513	p_Pro; c_Alphaproteobacteria; o_Sphingomonadales; f_Erythrobacteraceae; g_; s_	2.746	0.0027
New.CleanUp.ReferenceOTU215757	p_Pro; c_Alphaproteobacteria; o_Sphingomonadales; f_Erythrobacteraceae; g_; s_	1.860	0.0128
New.CleanUp.ReferenceOTU3069	p_Pro; c_Alphaproteobacteria; o_Sphingomonadales; f_Sphingomonadaceae; g_; s_	3.256	<0.0001
New.ReferenceOTU1163	p_Pro; c_Alphaproteobacteria; o_Sphingomonadales; f_Sphingomonadaceae; g_; s_	2.875	<0.0001
New.ReferenceOTU2179	p_Pro; c_Alphaproteobacteria; o_Sphingomonadales; f_Sphingomonadaceae; g_; s_	2.886	<0.0001
New.ReferenceOTU2168	p_Pro; c_Alphaproteobacteria; o_Sphingomonadales; f_Sphingomonadaceae; g_; s_	1.721	0.0048
925323	p_Pro; c_Alphaproteobacteria; o_Sphingomonadales; f_Sphingomonadaceae; g_Sphingomonas; s_wittichii	2.615	0.0071
New.ReferenceOTU1312	p_Pro; c_Alphaproteobacteria; o_Sphingomonadales; f_Sphingomonadaceae; g_Sphingopyxis; s_	2.811	0.0006
594013	p_Pro; c_Alphaproteobacteria; o_Sphingomonadales; f_Sphingomonadaceae; g_Sphingopyxis; s_alaskensis	2.440	<0.0001
2474239	p_Pro; c_Betaproteobacteria; o_Burkholderiales; f_Oxalobacteraceae; g_; s_	2.245	0.0103
New.CleanUp.ReferenceOTU106133	p_Pro; c_Betaproteobacteria; o_Burkholderiales; f_Oxalobacteraceae; g_Janthinobacterium; s_	1.968	0.0264
New.ReferenceOTU2050	p_Pro; c_Deltaproteobacteria; o_Myxococcales; f_; g_; s_	2.536	0.0145
949789	p_Pro; c_Gammaproteobacteria; o_Cardiobacteriales; f_Cardiobacteriaceae; g_Cardiobacterium; s_	2.981	0.0035
3333615	p_Pro; c_Gammaproteobacteria; o_Legionellales; f_; g_; s_	2.661	0.0115
New.CleanUp.ReferenceOTU243552	p_Pro; c_Gammaproteobacteria; o_Xanthomonadales; f_Xanthomonadaceae; g_; s_	3.063	0.0001
New.CleanUp.ReferenceOTU189229	p_Pro; c_Gammaproteobacteria; o_Xanthomonadales; f_Xanthomonadaceae; g_; s_	2.847	0.0001
2938351	p_Pro; c_Gammaproteobacteria; o_Xanthomonadales; f_Xanthomonadaceae; g_; s_	2.329	0.0057

New.CleanUp.ReferenceOTU220559	p_Pro; c_Gammaproteobacteria; o_Xanthomonadales; f_Xanthomonadaceae; g_; s_	2.314	0.0187
593908	p_Pro; c_Gammaproteobacteria; o_Xanthomonadales; f_Xanthomonadaceae; g_; s_	2.315	0.0224
New.ReferenceOTU760	p_Pro; c_Gammaproteobacteria; o_Xanthomonadales; f_Xanthomonadaceae; g_; s_	1.914	0.0224
New.ReferenceOTU1135	p_Pro; c_Gammaproteobacteria; o_Xanthomonadales; f_Xanthomonadaceae; g_; s_	2.329	0.0291
New.CleanUp.ReferenceOTU263039	p_Pro; c_Gammaproteobacteria; o_Xanthomonadales; f_Xanthomonadaceae; g_Arenimonas; s_	3.055	<0.0001
4371273	p_Pro; c_Gammaproteobacteria; o_Xanthomonadales; f_Xanthomonadaceae; g_Lysobacter; s_	1.942	0.0170
New.ReferenceOTU2186	p_Pro; c_Gammaproteobacteria; o_Xanthomonadales; f_Xanthomonadaceae; g_Lysobacter; s_	1.670	0.0287
New.CleanUp.ReferenceOTU134102	p_Pro; c_Gammaproteobacteria; o_Xanthomonadales; f_Xanthomonadaceae; g_Pseudoxanthomonas; s_mexicana	2.887	0.0002
226596	p_TM7; c_TM7-3; o_; f_; g_; s_	2.557	0.0046
747857	p_TM7; c_TM7-3; o_CW040; f_F16; g_; s_	3.268	0.0009
342427	p_TM7; c_TM7-3; o_EW055; f_; g_; s_	2.790	0.0029
New.ReferenceOTU1271	p_TM7; c_TM7-3; o_EW055; f_; g_; s_	2.330	0.0209
813047	p_TM7; NA; NA; NA; NA; NA	2.566	0.0189
New.CleanUp.ReferenceOTU191540	p_Ver; c_Verrucomicrobiae; o_Verrucomicrobiales; f_Verrucomicrobiaceae; g_Luteolibacter; s_	2.750	0.0089
898309	p_Ver; c_Verrucomicrobiae; o_Verrucomicrobiales; f_Verrucomicrobiaceae; g_Prosthecobacter; s_debontii	3.055	0.0016
New.CleanUp.ReferenceOTU145307	p_Ver; c_Verrucomicrobiae; o_Verrucomicrobiales; f_Verrucomicrobiaceae; g_Prosthecobacter; s_debontii	2.477	0.0259

Taxonomic levels: **p** = phylum; **c** = class; **o** = order; **f** = family; **g** = genus; **s** = species (blank = unassigned); Phylum abbreviations: **Bac:** Bacteroidetes, **Pla**: Planctomycetes, **Pro**: Proteobacteria, **Ver**: Verrucomicrobia.

Table 30 OTUs enriched in the rhizospheres of High N-treated 9 week-old cabbages (relative to controls). DESeq2 threshold: Benjamini-Hochberg corrected *p*-value <0.03, log2 fold change 1.2.

OTU	Taxonomy	log2 fold change	*p*-value (corrected)
1143479	p_Bac; c_Cytophagia; o_Cytophagales; f_Cyclobacteriaceae; g_Algoriphagus; NA	2.519	0.0001
New.ReferenceOTU1812	p_Bac; c_Cytophagia; o_Cytophagales; f_Cytophagaceae; g_; s_	2.589	0.0155
New.CleanUp.ReferenceOTU256384	p_Bac; c_Cytophagia; o_Cytophagales; f_Cytophagaceae; g_Adhaeribacter; s_	2.415	0.0141
4471717	p_Bac; c_Cytophagia; o_Cytophagales; f_Cytophagaceae; g_Adhaeribacter; s_	2.339	0.0214
324677	p_Bac; c_Flavobacteriia; o_Flavobacteriales; f_Flavobacteriaceae; g_Flavobacterium; s_	3.771	<0.0001
New.ReferenceOTU1306	p_Bac; c_Flavobacteriia; o_Flavobacteriales; f_Flavobacteriaceae; g_Flavobacterium; s_	3.841	<0.0001
New.CleanUp.ReferenceOTU215757	p_Bac; c_Flavobacteriia; o_Flavobacteriales; f_Flavobacteriaceae; g_Flavobacterium; s_	3.804	<0.0001
New.ReferenceOTU363	p_Bac; c_Flavobacteriia; o_Flavobacteriales; f_Flavobacteriaceae; g_Flavobacterium; s_	3.645	<0.0001
New.CleanUp.ReferenceOTU79365	p_Bac; c_Sphingobacteriia; o_Sphingobacteriales; f_Sphingobacteriaceae; g_; s_	2.572	0.0190
949789	p_Firmicutes; c_Bacilli; o_Lactobacillales; f_Carnobacteriaceae; g_Granulicatella; s_	2.448	0.0282
New.CleanUp.ReferenceOTU248133	p_Pro; c_Alphaproteobacteria; o_Caulobacterales; f_Caulobacteraceae; g_Brevundimonas; s_diminuta	2.871	0.0065
360547	p_Pro; c_Alphaproteobacteria; o_Rhizobiales; f_Phyllobacteriaceae; g_; s_	3.024	0.0022
860929	p_Pro; c_Alphaproteobacteria; o_Sphingomonadales; f_Erythrobacteraceae; g_; s_	5.130	<0.0001
New.ReferenceOTU310	p_Pro; c_Alphaproteobacteria; o_Sphingomonadales; f_Sphingomonadaceae; g_; s_	3.659	<0.0001
New.ReferenceOTU808	p_Pro; c_Alphaproteobacteria; o_Sphingomonadales; f_Sphingomonadaceae; g_; s_	3.017	<0.0001
New.ReferenceOTU841	p_Pro; c_Alphaproteobacteria; o_Sphingomonadales; f_Sphingomonadaceae; g_; s_	2.697	<0.0001
1127882	p_Pro; c_Alphaproteobacteria; o_Sphingomonadales; f_Sphingomonadaceae; g_Sphingopyxis; s_	4.058	<0.0001
4393056	p_Pro; c_Alphaproteobacteria; o_Sphingomonadales; f_Sphingomonadaceae; g_Sphingopyxis; s_	2.415	0.0205
674655	p_Pro; c_Alphaproteobacteria; o_Sphingomonadales; f_Sphingomonadaceae; g_Sphingopyxis; s_alaskensis	1.905	0.0033
832166	p_Pro; c_Betaproteobacteria; o_Methylophilales; f_Methylophilaceae; g_; s_	2.837	0.0025
New.ReferenceOTU645	p_Pro; c_Betaproteobacteria; o_Nitrosomonadales; f_Nitrosomonadaceae; g_; s_	2.027	0.0288
New.ReferenceOTU2530	p_Pro; c_Gammaproteobacteria; o_Xanthomonadales; f_Xanthomonadaceae; g_; s_	2.898	0.0003
New.ReferenceOTU348	p_Pro; c_Gammaproteobacteria; o_Xanthomonadales; f_Xanthomonadaceae; g_; s_	2.514	0.0009
New.CleanUp.ReferenceOTU235757	p_Pro; c_Gammaproteobacteria; o_Xanthomonadales; f_Xanthomonadaceae; g_Arenimonas; s_	2.453	0.0019

3333615	p_Pro; c_Gammaproteobacteria; o_Xanthomonadales; f_Xanthomonadaceae; g_Lysobacter; s_	2.608	0.0003
New.ReferenceOTU1734	p_Pro; c_Gammaproteobacteria; o_Xanthomonadales; f_Xanthomonadaceae; g_Lysobacter; s_	2.355	0.0022
New.ReferenceOTU540	p_Pro; c_Gammaproteobacteria; o_Xanthomonadales; f_Xanthomonadaceae; g_Lysobacter; s_	2.047	0.0096
New.CleanUp.ReferenceOTU318461	p_TM7; c_TM7-1; o_; f_; g_; s_	4.686	<0.0001
New.CleanUp.ReferenceOTU269779	p_TM7; c_TM7-1; o_; f_; g_; s_	4.140	<0.0001
316001	p_TM7; c_TM7-1; o_; f_; g_; s_	4.132	<0.0001
279572	p_TM7; c_TM7-1; o_; f_; g_; s_	3.216	0.0001
New.ReferenceOTU167	p_TM7; c_TM7-3; o_; f_; g_; s_	2.937	0.0006
4398174	p_TM7; c_TM7-3; o_; f_; g_; s_	2.921	0.0016
New.ReferenceOTU2811	p_TM7; c_TM7-3; o_; f_; g_; s_	2.343	0.0209
New.ReferenceOTU1707	p_TM7; c_TM7-3; o_EW055; f_; g_; s_	3.536	<0.0001

Taxonomic levels: **p** = phylum; **c** = class; **o** = order; **f** = family; **g** = genus; **s** = species (blank = unassigned); Phylum abbreviations: **Bac:** Bacteroidetes, **Fir**: Firmicutes, **Pro**: Proteobacteria.

Table 31 OTUs enriched in the rhizospheres of **High N**-treated **12 week**-old cabbages (relative to controls). DESeq2 threshold: Benjamini-Hochberg corrected *p*-value <0.03, log2 fold change 1.2.

OTU	**Taxonomy**	**Log2 fold change**	***p*-value (corrected)**
4384238	p_Acidobacteria; c_Solibacteres; o_Solibacterales; f_PAUC26f; g_; s_	1.760	0.0245
New.ReferenceOTU197	p_Act; c_Acidimicrobiia; o_Acidimicrobiales; f_C111; g_; s_	1.937	0.0143
261123	p_Act; c_Actinobacteria; o_Actinomycetales; f_Microbacteriaceae; g_Microbacterium; s_	2.261	0.0026
525029	p_Act; c_Actinobacteria; o_Actinomycetales; f_Microbacteriaceae; g_Microbacterium; s_	2.068	0.0067
191973	p_Act; c_Actinobacteria; o_Actinomycetales; f_Micrococcaceae; g_; s_	2.192	0.0088
204714	p_Act; c_Actinobacteria; o_Actinomycetales; f_Micrococcaceae; NA; NA	1.284	0.0195
New.CleanUp.ReferenceOTU32181	p_Armatimonadetes; c_[Fimbriimonadia]; o_[Fimbriimonadales]; f_; g_; s_	1.945	0.0014
New.ReferenceOTU2758	p_Armatimonadetes; c_[Fimbriimonadia]; o_[Fimbriimonadales]; f_; g_; s_	1.470	0.0040
3334351	p_Bac; c_[Saprospirae]; o_[Saprospirales]; f_Chitinophagaceae; g_; s_	2.068	0.0001

New.ReferenceOTU985	p_Bac; c_[Saprospirae]; o_[Saprospirales]; f_Chitinophagaceae; g_; s_	2.770	0.0003
3555664	p_Bac; c_[Saprospirae]; o_[Saprospirales]; f_Chitinophagaceae; g_; s_	2.265	0.0048
New.CleanUp.ReferenceOTU75840	p_Bac; c_[Saprospirae]; o_[Saprospirales]; f_Chitinophagaceae; g_; s_	1.810	0.0255
1143479	p_Bac; c_Cytophagia; o_Cytophagales; f_Cyclobacteriaceae; g_Algoriphagus; NA	3.565	<0.0001
New.CleanUp.ReferenceOTU147562	p_Bac; c_Cytophagia; o_Cytophagales; f_Cyclobacteriaceae; g_Algoriphagus; NA	2.120	0.0093
New.ReferenceOTU2784	p_Bac; c_Cytophagia; o_Cytophagales; f_Cytophagaceae; g_; s_	1.361	0.0117
New.ReferenceOTU2144	p_Bac; c_Cytophagia; o_Cytophagales; f_Cytophagaceae; g_; s_	1.803	0.0255
2972179	p_Bac; c_Flavobacteriia; o_Flavobacteriales; f_Cryomorphaceae; g_; s_	1.884	0.0296
563671	p_Bac; c_Flavobacteriia; o_Flavobacteriales; f_Cryomorphaceae; g_Fluviicola; s_	2.478	0.0020
570086	p_Bac; c_Flavobacteriia; o_Flavobacteriales; f_Cryomorphaceae; g_Fluviicola; s_	2.193	0.0034
New.CleanUp.ReferenceOTU233367	p_Bac; c_Flavobacteriia; o_Flavobacteriales; f_Cryomorphaceae; g_Fluviicola; s_	2.102	0.0154
New.CleanUp.ReferenceOTU122374	p_Bac; c_Flavobacteriia; o_Flavobacteriales; f_Cryomorphaceae; g_Fluviicola; s_	2.022	0.0238
807522	p_Bac; c_Flavobacteriia; o_Flavobacteriales; f_Flavobacteriaceae; g_; s_	3.637	<0.0001
New.CleanUp.ReferenceOTU93054	p_Bac; c_Flavobacteriia; o_Flavobacteriales; f_Flavobacteriaceae; g_; s_	2.751	0.0003
617834	p_Bac; c_Flavobacteriia; o_Flavobacteriales; f_Flavobacteriaceae; g_; s_	2.022	0.0222
New.ReferenceOTU1306	p_Bac; c_Flavobacteriia; o_Flavobacteriales; f_Flavobacteriaceae; g_Flavobacterium; s_	2.914	<0.0001
New.ReferenceOTU363	p_Bac; c_Flavobacteriia; o_Flavobacteriales; f_Flavobacteriaceae; g_Flavobacterium; s_	3.127	<0.0001
324677	p_Bac; c_Flavobacteriia; o_Flavobacteriales; f_Flavobacteriaceae; g_Flavobacterium; s_	2.662	<0.0001
574686	p_Bac; c_Flavobacteriia; o_Flavobacteriales; f_Flavobacteriaceae; g_Flavobacterium; s_	2.044	<0.0001
New.ReferenceOTU1948	p_Bac; c_Flavobacteriia; o_Flavobacteriales; f_Flavobacteriaceae; g_Flavobacterium; s_	2.025	0.0003
New.ReferenceOTU760	p_Bac; c_Flavobacteriia; o_Flavobacteriales; f_Flavobacteriaceae; g_Flavobacterium; s_	2.367	0.0018
New.CleanUp.ReferenceOTU206227	p_Bac; c_Flavobacteriia; o_Flavobacteriales; f_Flavobacteriaceae; g_Flavobacterium; s_	2.246	0.0081
4324048	p_Bac; c_Flavobacteriia; o_Flavobacteriales; f_Flavobacteriaceae; g_Flavobacterium; s_	2.018	0.0187
New.ReferenceOTU1927	p_Bac; c_Flavobacteriia; o_Flavobacteriales; f_Flavobacteriaceae; g_Flavobacterium; s_	1.615	0.0196
New.CleanUp.ReferenceOTU162268	p_Bac; c_Flavobacteriia; o_Flavobacteriales; f_Flavobacteriaceae; g_Flavobacterium; s_	1.912	0.0296
591337	p_Bac; c_Sphingobacteriia; o_Sphingobacteriales; f_; g_; s_	2.074	0.0087
1087471	p_Bac; c_Sphingobacteriia; o_Sphingobacteriales; f_; g_; s_	1.578	0.0172
4325369	p_Bac; c_Sphingobacteriia; o_Sphingobacteriales; f_; g_; s_	1.929	0.0222
New.ReferenceOTU400	p_Bac; c_Sphingobacteriia; o_Sphingobacteriales; f_Sphingobacteriaceae; g_; s_	1.759	0.0187
New.ReferenceOTU814	p_Bac; c_Sphingobacteriia; o_Sphingobacteriales; f_Sphingobacteriaceae; g_Pedobacter; s_	2.246	0.0040

New.CleanUp.ReferenceOTU160649	p_Bac; c_Sphingobacteriia; o_Sphingobacteriales; f_Sphingobacteriaceae; g_Pedobacter; s_	1.907	0.0272
790190	p_Chlorobi; c_OPB56; o_; f_; g_; s_	1.862	0.0187
4349218	p_Chloroflexi; c_Anaerolineae; o_Caldilineales; f_Caldilineaceae; g_; s_	1.674	0.0284
New.CleanUp.ReferenceOTU113625	p_Chloroflexi; c_Anaerolineae; o_SBR1031; f_A4b; g_; s_	1.819	0.0075
New.CleanUp.ReferenceOTU96818	p_Cyanobacteria; c_ML635J-21; o_; f_; g_; s_	2.260	0.0061
4371273	p_Cyanobacteria; c_ML635J-21; o_; f_; g_; s_	2.115	0.0066
New.ReferenceOTU2849	p_Gemmatimonadetes; c_Gemm-1; o_; f_; g_; s_	1.466	0.0092
New.ReferenceOTU1875	p_Gemmatimonadetes; c_Gemm-1; o_; f_; g_; s_	1.288	0.0136
New.ReferenceOTU719	p_Gemmatimonadetes; c_Gemm-2; o_; f_; g_; s_	1.701	0.0296
New.ReferenceOTU1163	p_OP11; c_; o_; f_; g_; s_	1.996	0.0061
New.ReferenceOTU1651	p_Planctomycetes; c_OM190; o_CL500-15; f_; g_; s_	1.662	0.0271
New.CleanUp.ReferenceOTU140855	p_Planctomycetes; c_Planctomycetia; o_Gemmatales; f_Gemmataceae; g_; s_	1.949	0.0222
New.CleanUp.ReferenceOTU193773	p_Planctomycetes; c_Planctomycetia; o_Pirellulales; f_Pirellulaceae; g_; s_	1.795	0.0066
New.CleanUp.ReferenceOTU180057	p_Planctomycetes; c_Planctomycetia; o_Planctomycetales; f_Planctomycetaceae; g_Planctomyces; s_	1.969	0.0103
New.CleanUp.ReferenceOTU212012	p_Pro; c_Alphaproteobacteria; o_BD7-3; f_; g_; s_	2.482	0.0014
New.CleanUp.ReferenceOTU261069	p_Pro; c_Alphaproteobacteria; o_BD7-3; f_; g_; s_	2.032	0.0198
New.CleanUp.ReferenceOTU310941	p_Pro; c_Alphaproteobacteria; o_Caulobacterales; f_Caulobacteraceae; g_; s_	2.026	0.0071
573545	p_Pro; c_Alphaproteobacteria; o_Rhizobiales; f_; g_; s_	2.466	0.0019
1119668	p_Pro; c_Alphaproteobacteria; o_Rhizobiales; f_Hyphomicrobiaceae; g_Devosia; s_	2.565	0.0001
New.ReferenceOTU1965	p_Pro; c_Alphaproteobacteria; o_Rhodobacterales; f_Hyphomonadaceae; g_; s_	1.609	0.0065
4312153	p_Pro; c_Alphaproteobacteria; o_Sphingomonadales; f_Sphingomonadaceae; g_; s_	2.395	0.0036
New.ReferenceOTU808	p_Pro; c_Alphaproteobacteria; o_Sphingomonadales; f_Sphingomonadaceae; g_; s_	1.436	0.0252
4393056	p_Pro; c_Alphaproteobacteria; o_Sphingomonadales; f_Sphingomonadaceae; g_Sphingopyxis; s_	2.827	<0.0001
New.CleanUp.ReferenceOTU61731	p_Pro; c_Alphaproteobacteria; o_Sphingomonadales; f_Sphingomonadaceae; g_Sphingopyxis; s_	2.274	0.0026
1127882	p_Pro; c_Alphaproteobacteria; o_Sphingomonadales; f_Sphingomonadaceae; g_Sphingopyxis; s_	1.991	0.0200
614860	p_Pro; c_Betaproteobacteria; o_; f_; g_; s_	1.676	0.0294
547327	p_Pro; c_Betaproteobacteria; o_Burkholderiales; f_Comamonadaceae; g_; s_	1.862	0.0148
539184	p_Pro; c_Betaproteobacteria; o_Methylophilales; f_Methylophilaceae; g_Methylotenera; s_mobilis	2.072	0.0166
New.ReferenceOTU1222	p_Pro; c_Betaproteobacteria; o_MND1; f_; g_; s_	1.810	0.0002
1025830	p_Pro; c_Deltaproteobacteria; o_Myxococcales; f_; g_; s_	2.930	<0.0001

New.CleanUp.ReferenceOTU152067	p_Pro; c_Deltaproteobacteria; o_Myxococcales; f_; g_; s_	2.234	0.0061
New.ReferenceOTU1726	p_Pro; c_Deltaproteobacteria; o_Myxococcales; f_; g_; s_	1.933	0.0248
4322538	p_Pro; c_Gammaproteobacteria; o_Pseudomonadales; f_Pseudomonadaceae; g_; s_	1.978	0.0288
961783	p_Pro; c_Gammaproteobacteria; o_Pseudomonadales; f_Pseudomonadaceae; g_Pseudomonas; s_	1.716	0.0001
New.ReferenceOTU1144	p_Pro; c_Gammaproteobacteria; o_Pseudomonadales; f_Pseudomonadaceae; g_Pseudomonas; s_	1.862	0.0003
764682	p_Pro; c_Gammaproteobacteria; o_Pseudomonadales; f_Pseudomonadaceae; g_Pseudomonas; s_	2.453	0.0006
769643	p_Pro; c_Gammaproteobacteria; o_Pseudomonadales; f_Pseudomonadaceae; g_Pseudomonas; s_	1.793	0.0006
New.CleanUp.ReferenceOTU218157	p_Pro; c_Gammaproteobacteria; o_Pseudomonadales; f_Pseudomonadaceae; g_Pseudomonas; s_	2.485	0.0023
New.ReferenceOTU1447	p_Pro; c_Gammaproteobacteria; o_Pseudomonadales; f_Pseudomonadaceae; g_Pseudomonas; s_	1.985	0.0054
New.CleanUp.ReferenceOTU186820	p_Pro; c_Gammaproteobacteria; o_Pseudomonadales; f_Pseudomonadaceae; g_Pseudomonas; s_	1.803	0.0174
New.CleanUp.ReferenceOTU278335	p_Pro; c_Gammaproteobacteria; o_Pseudomonadales; f_Pseudomonadaceae; g_Pseudomonas; s_	2.068	0.0187
646549	p_Pro; c_Gammaproteobacteria; o_Pseudomonadales; f_Pseudomonadaceae; g_Pseudomonas; s_	1.421	0.0216
New.CleanUp.ReferenceOTU8478	p_Pro; c_Gammaproteobacteria; o_Pseudomonadales; f_Pseudomonadaceae; g_Pseudomonas; s_	1.681	0.0217
New.CleanUp.ReferenceOTU135833	p_Pro; c_Gammaproteobacteria; o_Pseudomonadales; f_Pseudomonadaceae; g_Pseudomonas; s_	2.025	0.0233
New.ReferenceOTU2808	p_Pro; c_Gammaproteobacteria; o_Thiotrichales; f_Piscirickettsiaceae; g_; s_	1.953	0.0011
New.ReferenceOTU225	p_Pro; c_Gammaproteobacteria; o_Xanthomonadales; f_Sinobacteraceae; g_; s_	1.761	0.0053
New.ReferenceOTU1770	p_Pro; c_Gammaproteobacteria; o_Xanthomonadales; f_Sinobacteraceae; g_; s_	1.679	0.0149
New.ReferenceOTU2530	p_Pro; c_Gammaproteobacteria; o_Xanthomonadales; f_Xanthomonadaceae; g_; s_	2.360	<0.0001
New.CleanUp.ReferenceOTU220559	p_Pro; c_Gammaproteobacteria; o_Xanthomonadales; f_Xanthomonadaceae; g_; s_	2.238	0.0011
New.CleanUp.ReferenceOTU302471	p_Pro; c_Gammaproteobacteria; o_Xanthomonadales; f_Xanthomonadaceae; g_; s_	1.680	0.0040
New.ReferenceOTU2432	p_Pro; c_Gammaproteobacteria; o_Xanthomonadales; f_Xanthomonadaceae; g_; s_	1.613	0.0060
696181	p_Pro; c_Gammaproteobacteria; o_Xanthomonadales; f_Xanthomonadaceae; g_; s_	1.754	0.0145
New.CleanUp.ReferenceOTU133373	p_Pro; c_Gammaproteobacteria; o_Xanthomonadales; f_Xanthomonadaceae; g_; s_	1.775	0.0149
New.CleanUp.ReferenceOTU134102	p_Pro; c_Gammaproteobacteria; o_Xanthomonadales; f_Xanthomonadaceae; g_; s_	1.671	0.0156
593908	p_Pro; c_Gammaproteobacteria; o_Xanthomonadales; f_Xanthomonadaceae; g_; s_	1.698	0.0217
New.CleanUp.ReferenceOTU235757	p_Pro; c_Gammaproteobacteria; o_Xanthomonadales; f_Xanthomonadaceae; g_Arenimonas; s_	2.127	0.0007
New.CleanUp.ReferenceOTU24991	p_Pro; c_Gammaproteobacteria; o_Xanthomonadales; f_Xanthomonadaceae; g_Dokdonella; s_	2.333	0.0015
New.ReferenceOTU95	p_Pro; c_Gammaproteobacteria; o_Xanthomonadales; f_Xanthomonadaceae; g_Dokdonella; s_	2.224	0.0039
1052930	p_Pro; c_Gammaproteobacteria; o_Xanthomonadales; f_Xanthomonadaceae; g_Luteimonas; s_	1.456	0.0026
142320	p_Pro; c_Gammaproteobacteria; o_Xanthomonadales; f_Xanthomonadaceae; g_Lysobacter; s_	2.672	<0.0001

3561138	p_Pro; c_Gammaproteobacteria; o_Xanthomonadales; f_Xanthomonadaceae; g_Lysobacter; s_	1.974	0.0057
114573	p_Pro; c_Gammaproteobacteria; o_Xanthomonadales; f_Xanthomonadaceae; g_Pseudoxanthomonas; s_mexicana	2.616	0.0003
1049387	p_Pro; c_Gammaproteobacteria; o_Xanthomonadales; f_Xanthomonadaceae; g_Pseudoxanthomonas; s_mexicana	2.219	0.0066
New.ReferenceOTU610	p_Pro; c_Gammaproteobacteria; o_Xanthomonadales; f_Xanthomonadaceae; g_Thermomonas; NA	2.374	<0.0001
1004022	p_Pro; c_Gammaproteobacteria; o_Xanthomonadales; f_Xanthomonadaceae; g_Thermomonas; s_	1.334	0.0088
New.ReferenceOTU230	p_Pro; c_Gammaproteobacteria; o_Xanthomonadales; f_Xanthomonadaceae; g_Thermomonas; s_	1.366	0.0093
947308	p_Pro; c_Gammaproteobacteria; o_Xanthomonadales; f_Xanthomonadaceae; g_Thermomonas; s_	1.206	0.0149
New.CleanUp.ReferenceOTU233368	p_Pro; c_Gammaproteobacteria; o_Xanthomonadales; f_Xanthomonadaceae; NA; NA	1.708	0.0166
663641	p_TM7; c_; o_; f_; g_; s_	2.019	0.0174
New.CleanUp.ReferenceOTU170442	p_TM7; c_TM7-1; o_; f_; g_; s_	2.550	0.0014
New.ReferenceOTU167	p_TM7; c_TM7-3; o_; f_; g_; s_	3.088	<0.0001
New.CleanUp.ReferenceOTU107206	p_TM7; c_TM7-3; o_; f_; g_; s_	2.470	0.0019
New.CleanUp.ReferenceOTU28944	p_TM7; c_TM7-3; o_; f_; g_; s_	2.437	0.0020
New.CleanUp.ReferenceOTU263079	p_TM7; c_TM7-3; o_; f_; g_; s_	1.991	0.0143
New.ReferenceOTU1707	p_TM7; c_TM7-3; o_EW055; f_; g_; s_	2.570	0.0015
New.CleanUp.ReferenceOTU276037	p_TM7; c_TM7-3; o_EW055; f_; g_; s_	2.124	0.0124
572735	p_TM7; c_TM7-3; o_I025; f_; g_; s_	1.989	0.0268
New.CleanUp.ReferenceOTU43498	p_TM7; NA; NA; NA; NA; NA	2.348	0.0032
248066	p_Ver; c_[Pedosphaerae]; o_[Pedosphaerales]; f_; g_; s_	1.375	0.0074
New.CleanUp.ReferenceOTU36020	p_Ver; c_[Spartobacteria]; o_[Chthoniobacterales]; f_[Chthoniobacteraceae]; g_; s_	2.139	0.0097
New.CleanUp.ReferenceOTU45992	p_Ver; c_[Spartobacteria]; o_[Chthoniobacterales]; f_[Chthoniobacteraceae]; g_Chthoniobacter; s_	2.208	0.0034
New.CleanUp.ReferenceOTU310346	p_Ver; c_Opitutae; o_Opitutales; f_Opitutaceae; g_Opitutus; s_	1.771	0.0257
909170	p_Ver; c_Verrucomicrobiae; o_Verrucomicrobiales; f_Verrucomicrobiaceae; g_Luteolibacter; s_	1.599	0.0089
New.ReferenceOTU1135	p_Ver; c_Verrucomicrobiae; o_Verrucomicrobiales; f_Verrucomicrobiaceae; g_Prosthecobacter; s_debontii	3.131	<0.0001
4451561	p_Ver; c_Verrucomicrobiae; o_Verrucomicrobiales; f_Verrucomicrobiaceae; g_Prosthecobacter; s_debontii	3.028	<0.0001
New.ReferenceOTU2084	p_WS3; c_PRR-12; o_; f_; g_; s_	1.535	0.0142
808847	p_WS3; c_PRR-12; o_LD1-PA13; f_; g_; s_	1.379	0.0249

Taxonomic levels: **p** = phylum; **c** = class; **o** = order; **f** = family; **g** = genus; **s** = species (blank = unassigned); Phylum abbreviations: **Act**: Actinobacteria, **Bac:** Bacteroidetes, **Pro**: Proteobacteria, **Ver**: Verrucomicrobia.

Table 32 OTUs with significantly higher abundance in 9 week-old control cabbages relative to 12 week-old control plants (DESeq2 threshold fold change = 1.2; Benjamini-Hochberg corrected p<0.02).

OTU	Taxonomy	log2 fold change	*p*-value (corrected)
4339223	p_Aci; c_Acidobacteria-5; o_; f_; g_; s_	1.706	0.0146
New.ReferenceOTU1926	p_Aci; c_Acidobacteria-6; o_CCU21; f_; g_; s_	2.495	0.0013
New.ReferenceOTU323	p_Aci; c_Acidobacteria-6; o_CCU21; f_; g_; s_	1.727	0.0014
New.ReferenceOTU1397	p_Aci; c_Acidobacteria-6; o_CCU21; f_; g_; s_	1.766	0.0018
637937	p_Aci; c_Acidobacteria-6; o_CCU21; f_; g_; s_	1.390	0.0058
2068089	p_Aci; c_Acidobacteria-6; o_CCU21; f_; g_; s_	1.755	0.0149
1864542	p_Aci; c_Acidobacteria-6; o_iii1-15; f_RB40; g_; s_	1.583	0.0091
790420	p_Act; c_Acidimicrobiia; o_Acidimicrobiales; f_AKIW874; g_; s_	1.499	0.0112
696618	p_Act; c_Acidimicrobiia; o_Acidimicrobiales; f_koll13; g_; s_	2.031	0.0023
125565	p_Act; c_Acidimicrobiia; o_Acidimicrobiales; f_koll13; g_; s_	1.728	0.0139
547806	p_Act; c_Actinobacteria; o_Actinomycetales; f_Actinosynnemataceae; g_Lentzea; NA	1.734	0.0107
4479606	p_Act; c_Actinobacteria; o_Actinomycetales; f_Microbacteriaceae; g_Agromyces; s_	1.348	0.0156
814239	p_Act; c_Actinobacteria; o_Actinomycetales; f_Microbacteriaceae; g_Microbacterium; NA	2.071	0.0109
1108876	p_Act; c_Actinobacteria; o_Actinomycetales; f_Nocardioidaceae; g_; s_	1.392	0.0134
New.ReferenceOTU2686	p_Act; c_Actinobacteria; o_Actinomycetales; f_Promicromonosporaceae; g_Promicromonospora; s_	2.044	0.0043
268513	p_Act; c_Actinobacteria; o_Actinomycetales; f_Pseudonocardiaceae; g_Saccharopolyspora; s_	2.817	0.0013
1022861	p_Act; c_Thermoleophilia; o_Gaiellales; f_Gaiellaceae; g_; s_	1.466	0.0155
New.ReferenceOTU818	p_Act; c_Thermoleophilia; o_Solirubrobacterales; f_; g_; s_	2.771	0.0004
546371	p_Act; c_Thermoleophilia; o_Solirubrobacterales; f_; g_; s_	1.441	0.0153
1129425	p_Act; c_Thermoleophilia; o_Solirubrobacterales; f_Solirubrobacteraceae; g_; s_	1.578	0.0065
New.ReferenceOTU2290	p_Bac; c_Cytophagia; o_Cytophagales; f_Cytophagaceae; g_; s_	3.539	<0.0001
159655	p_Bac; c_Cytophagia; o_Cytophagales; f_Cytophagaceae; g_; s_	2.513	<0.0001
1008533	p_Bac; c_Cytophagia; o_Cytophagales; f_Cytophagaceae; g_; s_	2.167	<0.0001
266789	p_Bac; c_Cytophagia; o_Cytophagales; f_Cytophagaceae; g_; s_	2.107	0.0001
New.ReferenceOTU787	p_Bac; c_Cytophagia; o_Cytophagales; f_Cytophagaceae; g_; s_	2.632	0.0002

142247	p_Bac; c_Cytophagia; o_Cytophagales; f_Cytophagaceae; g_; s_	2.172	0.0013
New.ReferenceOTU2289	p_Bac; c_Cytophagia; o_Cytophagales; f_Cytophagaceae; g_; s_	2.391	0.0087
533113	p_Bac; c_Cytophagia; o_Cytophagales; f_Cytophagaceae; g_; s_	2.475	0.0093
New.ReferenceOTU688	p_Bac; c_Cytophagia; o_Cytophagales; f_Cytophagaceae; g_; s_	2.231	0.0118
New.ReferenceOTU1625	p_Bac; c_Cytophagia; o_Cytophagales; f_Cytophagaceae; g_; s_	1.509	0.0139
New.ReferenceOTU1249	p_Bac; c_Flavobacteriia; o_Flavobacteriales; f_Cryomorphaceae; g_Crocinitomix; s_	4.074	<0.0001
4156020	p_Bac; c_Flavobacteriia; o_Flavobacteriales; f_Cryomorphaceae; g_Crocinitomix; s_	2.220	0.0199
574686	p_Bac; c_Flavobacteriia; o_Flavobacteriales; f_Flavobacteriaceae; g_Flavobacterium; s_	2.224	0.0001
New.ReferenceOTU1948	p_Bac; c_Flavobacteriia; o_Flavobacteriales; f_Flavobacteriaceae; g_Flavobacterium; s_	2.334	0.0001
New.ReferenceOTU691	p_Chloroflexi; c_Anaerolineae; o_Caldilineales; f_Caldilineaceae; g_Caldilinea; s_	2.193	0.0109
New.ReferenceOTU2194	p_Chloroflexi; c_Anaerolineae; o_CFB-26; f_; g_; s_	1.871	0.0012
New.ReferenceOTU1860	p_Chloroflexi; c_Anaerolineae; o_H39; f_; g_; s_	2.634	0.0031
4390206	p_Chloroflexi; c_Anaerolineae; o_SBR1031; f_A4b; g_; s_	2.267	<0.0001
New.ReferenceOTU346	p_Chloroflexi; c_Anaerolineae; o_SBR1031; f_A4b; g_; s_	2.311	0.0002
New.ReferenceOTU616	p_Chloroflexi; c_Anaerolineae; o_SBR1031; f_A4b; g_; s_	2.684	0.0010
New.ReferenceOTU1865	p_Chloroflexi; c_Anaerolineae; o_SBR1031; f_A4b; g_; s_	1.790	0.0012
New.ReferenceOTU2364	p_Chloroflexi; c_Anaerolineae; o_SBR1031; f_A4b; g_; s_	1.959	0.0023
185950	p_Chloroflexi; c_Anaerolineae; o_SBR1031; f_A4b; g_; s_	1.671	0.0025
New.ReferenceOTU377	p_Chloroflexi; c_Anaerolineae; o_SBR1031; f_A4b; g_; s_	1.716	0.0055
New.ReferenceOTU2093	p_Chloroflexi; c_Anaerolineae; o_SBR1031; f_A4b; g_; s_	1.848	0.0055
New.ReferenceOTU2482	p_Chloroflexi; c_Anaerolineae; o_SBR1031; f_A4b; g_; s_	2.020	0.0065
114049	p_Chloroflexi; c_Anaerolineae; o_SBR1031; f_A4b; g_; s_	1.786	0.0065
207355	p_Chloroflexi; c_Anaerolineae; o_SBR1031; f_A4b; g_; s_	1.480	0.0066
1106540	p_Chloroflexi; c_Anaerolineae; o_SBR1031; f_A4b; g_; s_	1.692	0.0071
811434	p_Chloroflexi; c_Anaerolineae; o_SBR1031; f_A4b; g_; s_	2.525	0.0081
249472	p_Chloroflexi; c_Anaerolineae; o_SBR1031; f_A4b; g_; s_	1.532	0.0087
New.ReferenceOTU2776	p_Chloroflexi; c_Anaerolineae; o_SBR1031; f_A4b; g_; s_	1.986	0.0100
New.ReferenceOTU1435	p_Chloroflexi; c_Anaerolineae; o_SBR1031; f_A4b; g_; s_	2.188	0.0131
New.ReferenceOTU1710	p_Chloroflexi; c_Anaerolineae; o_SBR1031; f_A4b; g_; s_	1.513	0.0131

1114553	p_Chloroflexi; c_Anaerolineae; o_SBR1031; f_A4b; g_; s_	2.341	0.0143
New.ReferenceOTU1663	p_Chloroflexi; c_Anaerolineae; o_SBR1031; f_A4b; g_; s_	1.603	0.0152
New.ReferenceOTU2007	p_Chloroflexi; c_Anaerolineae; o_SBR1031; f_A4b; g_; s_	1.689	0.0188
New.ReferenceOTU1331	p_Chloroflexi; c_Anaerolineae; o_SBR1031; f_oc28; g_; s_	1.911	0.0109
New.ReferenceOTU8	p_Chloroflexi; c_Anaerolineae; o_SBR1031; f_oc28; g_; s_	1.613	0.0125
New.ReferenceOTU1113	p_Chloroflexi; c_Anaerolineae; o_SBR1031; f_SJA-101; g_; s_	1.857	0.0002
New.ReferenceOTU1572	p_Chloroflexi; c_Anaerolineae; o_SBR1031; f_SJA-101; g_; s_	2.102	0.0012
New.ReferenceOTU894	p_Chloroflexi; c_C0119; o_; f_; g_; s_	2.121	0.0144
New.ReferenceOTU799	p_Chloroflexi; c_Chloroflexi; o_[Roseiflexales]; f_[Kouleothrixaceae]; g_; s_	1.552	0.0087
New.ReferenceOTU1364	p_Chloroflexi; c_Chloroflexi; o_[Roseiflexales]; f_[Kouleothrixaceae]; g_; s_	1.695	0.0146
New.ReferenceOTU835	p_Chloroflexi; c_Gitt-GS-136; o_; f_; g_; s_	1.850	0.0012
New.ReferenceOTU2652	p_Chloroflexi; c_S085; o_; f_; g_; s_	2.773	0.0027
New.ReferenceOTU2782	p_Chloroflexi; c_S085; o_; f_; g_; s_	2.116	0.0153
549954	p_Chloroflexi; c_Thermomicrobia; o_JG30-KF-CM45; f_; g_; s_	1.577	0.0020
247875	p_Chloroflexi; c_Thermomicrobia; o_JG30-KF-CM45; f_; g_; s_	1.919	0.0079
New.ReferenceOTU1505	p_FCPU426; c_; o_; f_; g_; s_	2.369	0.0002
New.ReferenceOTU2810	p_Gemmatimonadetes; c_Gemm-1; o_; f_; g_; s_	3.327	<0.0001
New.ReferenceOTU2579	p_Gemmatimonadetes; c_Gemm-1; o_; f_; g_; s_	2.606	0.0041
2536925	p_Gemmatimonadetes; c_Gemmatimonadetes; o_KD8-87; f_; g_; s_	2.323	0.0054
New.ReferenceOTU1994	p_Gemmatimonadetes; c_Gemmatimonadetes; o_KD8-87; f_; g_; s_	2.071	0.0146
New.ReferenceOTU1427	p_Planctomycetes; c_C6; o_MVS-107; f_; g_; s_	2.665	<0.0001
New.ReferenceOTU550	p_Planctomycetes; c_C6; o_MVS-107; f_; g_; s_	2.852	<0.0001
New.ReferenceOTU2040	p_Planctomycetes; c_C6; o_MVS-107; f_; g_; s_	2.325	<0.0001
New.ReferenceOTU1080	p_Planctomycetes; c_C6; o_MVS-107; f_; g_; s_	2.399	0.0007
New.ReferenceOTU299	p_Planctomycetes; c_C6; o_MVS-107; f_; g_; s_	2.809	0.0009
New.ReferenceOTU1590	p_Planctomycetes; c_C6; o_MVS-107; f_; g_; s_	1.937	0.0020
New.ReferenceOTU1903	p_Planctomycetes; c_C6; o_MVS-107; f_; g_; s_	1.905	0.0030
New.ReferenceOTU99	p_Planctomycetes; c_C6; o_MVS-107; f_; g_; s_	2.643	0.0041
New.ReferenceOTU855	p_Planctomycetes; c_C6; o_MVS-107; f_; g_; s_	2.189	0.0064

New.ReferenceOTU1968	p_Planctomycetes; c_C6; o_MVS-107; f_; g_; s_	2.108	0.0065
New.CleanUp.ReferenceOTU170892	p_Planctomycetes; c_C6; o_MVS-107; f_; g_; s_	2.606	0.0067
New.CleanUp.ReferenceOTU67965	p_Planctomycetes; c_C6; o_MVS-107; f_; g_; s_	2.476	0.0113
New.ReferenceOTU577	p_Planctomycetes; c_OM190; o_CL500-15; f_; g_; s_	2.486	0.0001
New.ReferenceOTU1323	p_Planctomycetes; c_OM190; o_CL500-15; f_; g_; s_	1.448	0.0156
New.ReferenceOTU1864	p_Planctomycetes; c_OM190; o_CL500-15; f_; g_; s_	1.422	0.0156
742935	p_Planctomycetes; c_OM190; o_CL500-15; f_; g_; s_	1.973	0.0168
800292	p_Pro; c_Alphaproteobacteria; o_Rhizobiales; f_; g_; s_	2.176	0.0013
575885	p_Pro; c_Alphaproteobacteria; o_Rhizobiales; f_; g_; s_	1.607	0.0186
New.ReferenceOTU1233	p_Pro; c_Alphaproteobacteria; o_Rhodospirillales; f_; g_; s_	1.536	0.0194
614860	p_Pro; c_Betaproteobacteria; o_; f_; g_; s_	2.326	0.0139
509402	p_Pro; c_Betaproteobacteria; o_MND1; f_; g_; s_	2.316	0.0005
New.ReferenceOTU1285	p_Pro; c_Betaproteobacteria; o_MND1; f_; g_; s_	1.520	0.0149
New.ReferenceOTU2568	p_Pro; c_Deltaproteobacteria; o_; f_; g_; s_	2.463	0.0075
New.ReferenceOTU271	p_Pro; c_Deltaproteobacteria; o_; f_; g_; s_	2.027	0.0122
New.CleanUp.ReferenceOTU120765	p_Pro; c_Deltaproteobacteria; o_Bdellovibrionales; f_Bacteriovoracaceae; g_; s_	2.441	0.0156
New.CleanUp.ReferenceOTU47285	p_Pro; c_Deltaproteobacteria; o_Myxococcales; f_; g_; s_	3.310	0.0001
4358255	p_Pro; c_Deltaproteobacteria; o_Myxococcales; f_; g_; s_	2.106	0.0001
New.CleanUp.ReferenceOTU122362	p_Pro; c_Deltaproteobacteria; o_Myxococcales; f_; g_; s_	3.078	0.0005
New.ReferenceOTU1795	p_Pro; c_Deltaproteobacteria; o_Myxococcales; f_; g_; s_	1.683	0.0010
New.ReferenceOTU438	p_Pro; c_Deltaproteobacteria; o_Myxococcales; f_; g_; s_	2.431	0.0022
832626	p_Pro; c_Deltaproteobacteria; o_Myxococcales; f_; g_; s_	1.880	0.0024
New.CleanUp.ReferenceOTU165359	p_Pro; c_Deltaproteobacteria; o_Myxococcales; f_; g_; s_	2.270	0.0065
New.CleanUp.ReferenceOTU1448	p_Pro; c_Deltaproteobacteria; o_Myxococcales; f_; g_; s_	2.601	0.0065
113261	p_Pro; c_Deltaproteobacteria; o_Myxococcales; f_; g_; s_	1.899	0.0071
New.ReferenceOTU330	p_Pro; c_Deltaproteobacteria; o_Myxococcales; f_; g_; s_	1.944	0.0094
2362284	p_Pro; c_Deltaproteobacteria; o_Myxococcales; f_; g_; s_	1.356	0.0109
816438	p_Pro; c_Deltaproteobacteria; o_Myxococcales; f_; g_; s_	2.486	0.0111
New.ReferenceOTU2373	p_Pro; c_Deltaproteobacteria; o_Myxococcales; f_; g_; s_	2.065	0.0135

New.ReferenceOTU56	p_Pro; c_Deltaproteobacteria; o_Myxococcales; f_; g_; s_	2.070	0.0146
819038	p_Pro; c_Deltaproteobacteria; o_Myxococcales; f_Haliangiaceae; g_; s_	3.592	0.0000
4347970	p_Pro; c_Deltaproteobacteria; o_Myxococcales; f_Haliangiaceae; g_; s_	2.936	0.0002
New.CleanUp.ReferenceOTU117810	p_Pro; c_Deltaproteobacteria; o_Myxococcales; f_Haliangiaceae; g_; s_	2.019	0.0153
New.ReferenceOTU2825	p_Pro; c_Deltaproteobacteria; o_Myxococcales; f_Nannocystaceae; g_Nannocystis; s_	2.270	0.0131
New.ReferenceOTU824	p_Pro; c_Deltaproteobacteria; o_Myxococcales; f_Nannocystaceae; g_Plesiocystis; s_	3.147	<0.0001
New.ReferenceOTU1021	p_Pro; c_Deltaproteobacteria; o_Myxococcales; f_Nannocystaceae; g_Plesiocystis; s_	3.042	<0.0001
135973	p_Pro; c_Deltaproteobacteria; o_Myxococcales; f_Nannocystaceae; g_Plesiocystis; s_	2.955	0.0006
327106	p_Pro; c_Deltaproteobacteria; o_Myxococcales; f_Nannocystaceae; g_Plesiocystis; s_	2.713	0.0056
New.CleanUp.ReferenceOTU120596	p_Pro; c_Deltaproteobacteria; o_Myxococcales; f_Nannocystaceae; g_Plesiocystis; s_	2.672	0.0065
New.CleanUp.ReferenceOTU104522	p_Pro; c_Deltaproteobacteria; o_Myxococcales; f_Nannocystaceae; g_Plesiocystis; s_	2.431	0.0135
New.CleanUp.ReferenceOTU64728	p_Pro; c_Deltaproteobacteria; o_Myxococcales; f_Nannocystaceae; g_Plesiocystis; s_	2.314	0.0184
New.CleanUp.ReferenceOTU221801	p_Pro; c_Deltaproteobacteria; o_Myxococcales; f_OM27; g_; s_	2.893	0.0013
New.CleanUp.ReferenceOTU198032	p_Pro; c_Deltaproteobacteria; o_Myxococcales; NA; NA; NA	2.464	0.0030
New.ReferenceOTU2269	p_Pro; c_Deltaproteobacteria; o_NB1-j; f_MND4; g_; s_	2.396	0.0003
852722	p_Pro; c_Deltaproteobacteria; o_Spirobacillales; f_; g_; s_	2.486	0.0131
763169	p_Pro; c_Gammaproteobacteria; o_; f_; g_; s_	1.915	0.0040
New.CleanUp.ReferenceOTU313341	p_Pro; c_Gammaproteobacteria; o_; f_; g_; s_	2.058	0.0093
New.ReferenceOTU1235	p_Pro; c_Gammaproteobacteria; o_; f_; g_; s_	1.554	0.0138
727795	p_Pro; c_Gammaproteobacteria; o_Alteromonadales; f_Alteromonadaceae; g_HB2-32-21; s_	3.205	0.0003
848718	p_Pro; c_Gammaproteobacteria; o_Pseudomonadales; f_Moraxellaceae; g_Perlucidibaca; s_	2.413	0.0107
New.ReferenceOTU2808	p_Pro; c_Gammaproteobacteria; o_Thiotrichales; f_Piscirickettsiaceae; g_; s_	1.816	0.0100
New.ReferenceOTU225	p_Pro; c_Gammaproteobacteria; o_Xanthomonadales; f_Sinobacteraceae; g_; s_	2.280	0.0001
436590	p_Pro; c_Gammaproteobacteria; o_Xanthomonadales; f_Sinobacteraceae; g_; s_	2.401	0.0043
1107631	p_Pro; c_Gammaproteobacteria; o_Xanthomonadales; f_Sinobacteraceae; g_; s_	2.169	0.0043
New.ReferenceOTU1770	p_Pro; c_Gammaproteobacteria; o_Xanthomonadales; f_Sinobacteraceae; g_; s_	2.318	0.0065
New.ReferenceOTU870	p_Pro; c_Gammaproteobacteria; o_Xanthomonadales; f_Xanthomonadaceae; g_; s_	1.644	0.0122
New.CleanUp.ReferenceOTU132031	p_TM7; c_TM7-1; o_; f_; g_; s_	2.662	0.0087
New.CleanUp.ReferenceOTU96698	p_Verrucomicrobia; c_[Spartobacteria]; o_[Chthoniobacterales]; f_[Chthoniobacteraceae]; g_; s_	2.485	0.0155

Appendix B

Table 33 OTUs with significantly lower abundance in 9week-old control cabbages relative to 12week-old controls (DESeq2 threshold fold change = 1.2; Benjamini-Hochberg corrected $p<0.02$).

OTU	Taxonomy	log2 fold change	*p*-value (corrected)
New.ReferenceOTU360	p_Bac; c_[Saprospirae]; o_[Saprospirales]; f_Chitinophagaceae; g_; s_	-2.661	0.0055
1118729	p_Bac; c_Cytophagia; o_Cytophagales; f_Cytophagaceae; g_; s_	-3.578	<0.0001
324677	p_Bac; c_Flavobacteriia; o_Flavobacteriales; f_Flavobacteriaceae; g_Flavobacterium; s_	-2.016	0.0089
859868	p_Firmicutes; c_Clostridia; o_Clostridiales; f_Gracilibacteraceae; g_; s_	-2.841	0.0022
765240	p_Firmicutes; c_Clostridia; o_Clostridiales; f_Ruminococcaceae; g_; s_	-2.861	0.0043
342427	p_Firmicutes; c_Clostridia; o_Clostridiales; f_Veillonellaceae; g_Veillonella; s_dispar	-2.456	0.0194
New.CleanUp.ReferenceOTU191480	p_Gemmatimonadetes; c_Gemm-3; o_; f_; g_; s_	-2.116	0.0406
259049	p_Pro; c_Alphaproteobacteria; o_Rhodospirillales; f_Rhodospirillaceae; g_Magnetospirillum; s_	-3.300	0.0005
New.CleanUp.ReferenceOTU126211	p_Pro; c_Alphaproteobacteria; o_Rhodospirillales; f_Rhodospirillaceae; g_Magnetospirillum; s_	-2.851	0.0045
276270	p_Pro; c_Alphaproteobacteria; o_Rhodospirillales; f_Rhodospirillaceae; g_Magnetospirillum; s_	-2.752	0.0066
2474239	p_Pro; c_Alphaproteobacteria; o_Sphingomonadales; f_Erythrobacteraceae; g_; s_	-1.862	0.0079
4336568	p_Pro; c_Alphaproteobacteria; o_Sphingomonadales; f_Sphingomonadaceae; g_Sphingobium; s_	-2.689	0.0067
822419	p_Pro; c_Betaproteobacteria; o_Burkholderiales; f_Oxalobacteraceae; NA; NA	-1.407	0.0291
4333969	p_Pro; c_Betaproteobacteria; o_Rhodocyclales; f_Rhodocyclaceae; NA; NA	-2.314	0.0336
500250	p_Pro; c_Deltaproteobacteria; o_Myxococcales; f_OM27; g_; s_	-2.242	0.0383
New.CleanUp.ReferenceOTU150915	p_TM7; c_TM7-1; o_; f_; g_; s_	-2.199	0.0469
New.ReferenceOTU1537	p_TM7; c_TM7-1; o_; f_; g_; s_	-2.075	0.0052
New.CleanUp.ReferenceOTU147333	p_TM7; c_TM7-3; o_EW055; f_; g_; s_	-2.556	0.0066

Table 34 OTUs with significantly higher abundance in 9week-old **Chicken Manure** cabbages relative to 12 week-old CM plants (DESeq2 threshold fold change = 1.2; Benjamini-Hochberg corrected $p<0.05$).

OTU	Taxonomy	log2 fold change	*p*-value (corrected)
3793255	p_Aci; c_Acidobacteria-6; o_CCU21; f_; g_; s_	2.707	0.0057
1116539	p_Aci; c_Sva0725; o_Sva0725; f_; g_; s_	2.902	0.0096
New.CleanUp.ReferenceOTU275127	p_Act; c_Acidimicrobiia; o_Acidimicrobiales; f_EB1017; g_; s_	2.322	0.0402
4093080	p_Act; c_Acidimicrobiia; o_Acidimicrobiales; f_Iamiaceae; g_Iamia; s_	2.603	0.0237
260552	p_Act; c_Actinobacteria; o_Actinomycetales; f_; g_; s_	2.565	0.0317
40439	p_Act; c_Actinobacteria; o_Actinomycetales; f_Nocardiaceae; g_Nocardia; s_	2.443	0.0140
New.CleanUp.ReferenceOTU10775	p_Act; c_Actinobacteria; o_Actinomycetales; f_Streptomycetaceae; NA; NA	2.378	0.0244
511366	p_Act; c_Rubrobacteria; o_Rubrobacterales; f_Rubrobacteraceae; g_Rubrobacter; s_	2.326	0.0339
New.ReferenceOTU597	p_Act; c_Thermoleophilia; o_Solirubrobacterales; f_; g_; s_	2.569	0.0317
New.ReferenceOTU508	p_Act; c_Thermoleophilia; o_Solirubrobacterales; f_; g_; s_	2.267	0.0317
New.ReferenceOTU2290	p_Bacteroidetes; c_Cytophagia; o_Cytophagales; f_Cytophagaceae; g_; s_	2.031	0.0268
90078	p_Bacteroidetes; c_Cytophagia; o_Cytophagales; f_Flammeovirgaceae; g_; s_	3.010	0.0049
New.ReferenceOTU1249	p_Bacteroidetes; c_Flavobacteriia; o_Flavobacteriales; f_Cryomorphaceae; g_Crocinitomix; s_	3.347	0.0000
3087743	p_Chloroflexi; c_Anaerolineae; o_Caldilineales; f_Caldilineaceae; g_; s_	2.679	0.0028
New.ReferenceOTU2194	p_Chloroflexi; c_Anaerolineae; o_CFB-26; f_; g_; s_	1.695	0.0313
New.ReferenceOTU1680	p_Chloroflexi; c_Anaerolineae; o_GCA004; f_; g_; s_	1.873	0.0434
New.ReferenceOTU2007	p_Chloroflexi; c_Anaerolineae; o_SBR1031; f_A4b; g_; s_	1.902	0.0398
333120	p_Chloroflexi; c_Anaerolineae; o_SBR1031; f_oc28; g_; s_	2.845	0.0174
New.ReferenceOTU1113	p_Chloroflexi; c_Anaerolineae; o_SBR1031; f_SJA-101; g_; s_	2.053	0.0032
New.ReferenceOTU1572	p_Chloroflexi; c_Anaerolineae; o_SBR1031; f_SJA-101; g_; s_	1.823	0.0317
New.CleanUp.ReferenceOTU224946	p_Chloroflexi; c_C0119; o_; f_; g_; s_	2.265	0.0344
New.ReferenceOTU799	p_Chloroflexi; c_Chloroflexi; o_[Roseiflexales]; f_[Kouleothrixaceae]; g_; s_	1.663	0.0375
New.CleanUp.ReferenceOTU202001	p_Chloroflexi; c_Chloroflexi; o_Herpetosiphonales; f_; g_; s_	2.888	0.0036
New.CleanUp.ReferenceOTU207794	p_Chloroflexi; c_Chloroflexi; o_Herpetosiphonales; f_; g_; s_	2.372	0.0372
559563	p_Chloroflexi; c_Chloroflexi; o_Herpetosiphonales; f_; g_; s_	2.111	0.0418

New.ReferenceOTU276	p_Chloroflexi; c_Thermomicrobia; o_JG30-KF-CM45; f_; g_; s_	2.470	0.0042
New.CleanUp.ReferenceOTU176867	p_Chloroflexi; c_Thermomicrobia; o_JG30-KF-CM45; f_; g_; s_	2.261	0.0453
New.CleanUp.ReferenceOTU121878	p_Chloroflexi; c_Thermomicrobia; o_JG30-KF-CM45; f_; g_; s_	2.197	0.0103
New.ReferenceOTU201	p_Chloroflexi; c_Thermomicrobia; o_JG30-KF-CM45; f_; g_; s_	1.942	0.0317
549954	p_Chloroflexi; c_Thermomicrobia; o_JG30-KF-CM45; f_; g_; s_	1.830	0.0239
2772794	p_Firmicutes; c_Clostridia; o_Clostridiales; f_; g_; s_	2.276	0.0458
New.CleanUp.ReferenceOTU89122	p_GN02; c_BB34; o_; f_; g_; s_	3.099	0.0013
New.CleanUp.ReferenceOTU9290	p_GN02; c_BB34; o_; f_; g_; s_	2.469	0.0477
328957	p_Nitrospirae; c_Nitrospira; o_Nitrospirales; f_0319-6A21; g_; s_	1.852	0.0320
New.ReferenceOTU365	p_NKB19; c_; o_; f_; g_; s_	2.505	0.0114
New.ReferenceOTU1427	p_Planctomycetes; c_C6; o_MVS-107; f_; g_; s_	2.310	0.0030
New.ReferenceOTU2011	p_Planctomycetes; c_OM190; o_agg27; f_; g_; s_	1.550	0.0471
New.ReferenceOTU2736	p_Planctomycetes; c_Planctomycetia; o_Gemmatales; f_Isosphaeraceae; g_; s_	2.191	0.0382
801268	p_Planctomycetes; c_Planctomycetia; o_Gemmatales; f_Isosphaeraceae; g_; s_	1.706	0.0411
New.ReferenceOTU1617	p_Planctomycetes; c_Planctomycetia; o_Pirellulales; f_Pirellulaceae; g_; s_	2.282	0.0434
783003	p_Planctomycetes; c_Planctomycetia; o_Planctomycetales; f_Planctomycetaceae; g_Planctomyces; s_	2.198	0.0362
New.CleanUp.ReferenceOTU219783	p_Planctomycetes; c_vadinHA49; o_DH61; f_; g_; s_	3.304	0.0010
881637	p_Planctomycetes; c_vadinHA49; o_PHOS-HE93; f_; g_; s_	2.761	0.0070
767403	p_Pro; c_Alphaproteobacteria; o_Rhizobiales; f_Methylocystaceae; g_; s_	2.521	0.0304
New.ReferenceOTU1703	p_Pro; c_Alphaproteobacteria; o_Rhodospirillales; f_; g_; s_	2.409	0.0304
1106016	p_Pro; c_Alphaproteobacteria; o_Rhodospirillales; f_; g_; s_	1.894	0.0105
New.ReferenceOTU496	p_Pro; c_Alphaproteobacteria; o_Rhodospirillales; f_; g_; s_	1.767	0.0336
New.CleanUp.ReferenceOTU303628	p_Pro; c_Alphaproteobacteria; o_Rickettsiales; f_; g_; s_	3.869	<0.0001
New.CleanUp.ReferenceOTU301842	p_Pro; c_Betaproteobacteria; o_Procabacteriales; f_Procabacteriaceae; g_; s_	3.170	0.0023
New.ReferenceOTU271	p_Pro; c_Deltaproteobacteria; o_; f_; g_; s_	2.245	0.0177
819455	p_Pro; c_Deltaproteobacteria; o_; f_; g_; s_	2.209	0.0413
New.CleanUp.ReferenceOTU4464	p_Pro; c_Deltaproteobacteria; o_Bdellovibrionales; f_Bacteriovoracaceae; g_; s_	3.851	<0.0001
New.CleanUp.ReferenceOTU120765	p_Pro; c_Deltaproteobacteria; o_Bdellovibrionales; f_Bacteriovoracaceae; g_; s_	2.754	0.0176
New.CleanUp.ReferenceOTU246964	p_Pro; c_Deltaproteobacteria; o_MIZ46; f_; g_; s_	2.532	0.0359

New.CleanUp.ReferenceOTU47285	p_Pro; c_Deltaproteobacteria; o_Myxococcales; f_; g_; s_	3.946	<0.0001
New.CleanUp.ReferenceOTU116651	p_Pro; c_Deltaproteobacteria; o_Myxococcales; f_; g_; s_	2.662	0.0239
816438	p_Pro; c_Deltaproteobacteria; o_Myxococcales; f_; g_; s_	2.611	0.0228
New.CleanUp.ReferenceOTU27961	p_Pro; c_Deltaproteobacteria; o_Myxococcales; f_; g_; s_	2.579	0.0038
New.CleanUp.ReferenceOTU200098	p_Pro; c_Deltaproteobacteria; o_Myxococcales; f_; g_; s_	2.458	0.0456
New.CleanUp.ReferenceOTU1448	p_Pro; c_Deltaproteobacteria; o_Myxococcales; f_; g_; s_	2.431	0.0410
New.ReferenceOTU1021	p_Pro; c_Deltaproteobacteria; o_Myxococcales; f_Nannocystaceae; g_Plesiocystis; s_	3.194	<0.0001
New.ReferenceOTU824	p_Pro; c_Deltaproteobacteria; o_Myxococcales; f_Nannocystaceae; g_Plesiocystis; s_	3.155	<0.0001
New.ReferenceOTU157	p_Pro; c_Deltaproteobacteria; o_Myxococcales; f_OM27; g_; s_	2.449	0.0079
New.CleanUp.ReferenceOTU29976	p_Pro; c_Deltaproteobacteria; o_Myxococcales; f_Polyangiaceae; g_; s_	2.323	0.0289
New.CleanUp.ReferenceOTU38773	p_Pro; c_Deltaproteobacteria; o_NB1-j; NA; NA; NA	2.134	0.0367
727795	p_Pro; c_Gammaproteobacteria; o_Alteromonadales; f_Alteromonadaceae; g_HB2-32-21; s_	2.885	0.0087
552580	p_Pro; c_Gammaproteobacteria; o_Alteromonadales; f_Alteromonadaceae; g_HB2-32-21; s_	2.496	0.0402
New.CleanUp.ReferenceOTU94822	p_Pro; c_Gammaproteobacteria; o_PYR10d3; f_; g_; s_	2.788	0.0173
822205	p_Pro; c_Gammaproteobacteria; o_Xanthomonadales; f_Sinobacteraceae; g_; s_	2.323	0.0265
104155	p_Pro; c_Gammaproteobacteria; o_Xanthomonadales; f_Sinobacteraceae; g_Nevskia; s_ramosa	2.877	0.0025
New.CleanUp.ReferenceOTU276075	p_Pro; c_Gammaproteobacteria; o_Xanthomonadales; f_Sinobacteraceae; g_Nevskia; s_ramosa	2.367	0.0382

Table 35 OTUs with significantly lower abundance in 9week-old **Chicken Manure** cabbages relative to 12 week-old CM plants (DESeq2 threshold fold change = 1.2; Benjamini-Hochberg corrected $p<0.05$).

OTU	Taxonomy	log2 fold change	p-value(corrected)
New.CleanUp.ReferenceOTU48915	p_Armatimonadetes; c_[Fimbriimonadia]; o_[Fimbriimonadales]; f_[Fimbriimonadaceae]; g_Fimbriimonas; s_	-2.650	0.0041
New.ReferenceOTU1533	p_Bac; c_[Saprospirae]; o_[Saprospirales]; f_Chitinophagaceae; g_; s_	-3.425	0.0009
3334351	p_Bac; c_[Saprospirae]; o_[Saprospirales]; f_Chitinophagaceae; g_; s_	-2.938	0.0015
4480003	p_Bac; c_[Saprospirae]; o_[Saprospirales]; f_Chitinophagaceae; g_; s_	-2.628	0.0038
New.ReferenceOTU700	p_Bac; c_[Saprospirae]; o_[Saprospirales]; f_Chitinophagaceae; g_; s_	-2.588	0.0079
New.CleanUp.ReferenceOTU133416	p_Bac; c_[Saprospirae]; o_[Saprospirales]; f_Chitinophagaceae; g_; s_	-2.513	0.0453

New.ReferenceOTU360	p_Bac; c_[Saprospirae]; o_[Saprospirales]; f_Chitinophagaceae; g_; s_	-2.440	0.0372
1066654	p_Bac; c_[Saprospirae]; o_[Saprospirales]; f_Chitinophagaceae; g_; s_	-2.439	0.0325
New.CleanUp.ReferenceOTU197883	p_Bac; c_[Saprospirae]; o_[Saprospirales]; f_Chitinophagaceae; g_; s_	-2.077	0.0402
2476144	p_Bac; c_[Saprospirae]; o_[Saprospirales]; f_Chitinophagaceae; NA; NA	-3.175	0.0013
New.ReferenceOTU2346	p_Bac; c_[Saprospirae]; o_[Saprospirales]; f_Saprospiraceae; g_; s_	-2.767	0.0001
New.CleanUp.ReferenceOTU169105	p_Bac; c_[Saprospirae]; o_[Saprospirales]; f_Saprospiraceae; g_; s_	-2.549	0.0339
New.CleanUp.ReferenceOTU47708	p_Bac; c_[Saprospirae]; o_[Saprospirales]; f_Saprospiraceae; g_; s_	-1.897	0.0477
New.ReferenceOTU1659	p_Bac; c_[Saprospirae]; o_[Saprospirales]; f_Saprospiraceae; g_; s_	-1.803	0.0244
1143479	p_Bac; c_Cytophagia; o_Cytophagales; f_Cyclobacteriaceae; g_Algoriphagus; NA	-2.420	0.0024
New.ReferenceOTU2144	p_Bac; c_Cytophagia; o_Cytophagales; f_Cytophagaceae; g_; s_	-4.188	<0.0001
New.ReferenceOTU2312	p_Bac; c_Cytophagia; o_Cytophagales; f_Cytophagaceae; g_; s_	-3.438	<0.0001
1118729	p_Bac; c_Cytophagia; o_Cytophagales; f_Cytophagaceae; g_; s_	-3.314	0.0011
549553	p_Bac; c_Cytophagia; o_Cytophagales; f_Cytophagaceae; g_; s_	-2.359	0.0049
1108632	p_Bac; c_Cytophagia; o_Cytophagales; f_Cytophagaceae; g_; s_	-1.886	0.0104
913174	p_Bac; c_Cytophagia; o_Cytophagales; f_Cytophagaceae; g_Leadbetterella; s_	-2.625	0.0034
New.ReferenceOTU369	p_Bac; c_Flavobacteriia; o_Flavobacteriales; f_Flavobacteriaceae; g_Flavobacterium; NA	-2.994	<0.0001
747857	p_Bac; c_Flavobacteriia; o_Flavobacteriales; f_Flavobacteriaceae; g_Flavobacterium; s_	-3.854	<0.0001
New.ReferenceOTU2105	p_Bac; c_Flavobacteriia; o_Flavobacteriales; f_Flavobacteriaceae; g_Flavobacterium; s_	-3.676	<0.0001
New.CleanUp.ReferenceOTU201518	p_Bac; c_Flavobacteriia; o_Flavobacteriales; f_Flavobacteriaceae; g_Flavobacterium; s_	-3.249	0.0014
New.ReferenceOTU1306	p_Bac; c_Flavobacteriia; o_Flavobacteriales; f_Flavobacteriaceae; g_Flavobacterium; s_	-3.225	<0.0001
324677	p_Bac; c_Flavobacteriia; o_Flavobacteriales; f_Flavobacteriaceae; g_Flavobacterium; s_	-3.123	<0.0001
New.ReferenceOTU333	p_Bac; c_Flavobacteriia; o_Flavobacteriales; f_Flavobacteriaceae; g_Flavobacterium; s_	-3.111	0.0003
New.CleanUp.ReferenceOTU181551	p_Bac; c_Flavobacteriia; o_Flavobacteriales; f_Flavobacteriaceae; g_Flavobacterium; s_	-2.969	0.0054
960076	p_Bac; c_Flavobacteriia; o_Flavobacteriales; f_Flavobacteriaceae; g_Flavobacterium; s_	-2.894	<0.0001
New.ReferenceOTU2772	p_Bac; c_Flavobacteriia; o_Flavobacteriales; f_Flavobacteriaceae; g_Flavobacterium; s_	-2.848	0.0094
New.ReferenceOTU1990	p_Bac; c_Flavobacteriia; o_Flavobacteriales; f_Flavobacteriaceae; g_Flavobacterium; s_	-2.784	0.0031
New.ReferenceOTU2853	p_Bac; c_Flavobacteriia; o_Flavobacteriales; f_Flavobacteriaceae; g_Flavobacterium; s_	-2.773	0.0001
New.CleanUp.ReferenceOTU170378	p_Bac; c_Flavobacteriia; o_Flavobacteriales; f_Flavobacteriaceae; g_Flavobacterium; s_	-2.401	0.0265
1055322	p_Bac; c_Flavobacteriia; o_Flavobacteriales; f_Flavobacteriaceae; g_Flavobacterium; s_	-2.241	0.0034

509372	p_Bac; c_Flavobacteriia; o_Flavobacteriales; f_Flavobacteriaceae; g_Flavobacterium; s_	-2.071	0.0477
New.ReferenceOTU453	p_Bac; c_Flavobacteriia; o_Flavobacteriales; f_Flavobacteriaceae; g_Flavobacterium; s_	-2.005	0.0213
922724	p_Bac; c_Flavobacteriia; o_Flavobacteriales; f_Flavobacteriaceae; g_Flavobacterium; s_	-1.962	0.0453
1144682	p_Bac; c_Sphingobacteriia; o_Sphingobacteriales; f_; g_; s_	-2.290	0.0034
New.ReferenceOTU2367	p_Bac; c_Sphingobacteriia; o_Sphingobacteriales; f_; g_; s_	-2.285	0.0304
New.ReferenceOTU253	p_Bac; c_Sphingobacteriia; o_Sphingobacteriales; f_; g_; s_	-2.069	0.0325
New.ReferenceOTU478	p_Bac; c_Sphingobacteriia; o_Sphingobacteriales; f_; g_; s_	-2.050	0.0248
4396611	p_Bac; c_Sphingobacteriia; o_Sphingobacteriales; f_Sphingobacteriaceae; g_; s_	-3.078	0.0049
790190	p_Chlorobi; c_OPB56; o_; f_; g_; s_	-3.205	0.0004
New.CleanUp.ReferenceOTU100999	p_Gemmatimonadetes; c_Gemm-3; o_; f_; g_; s_	-2.538	0.0025
New.CleanUp.ReferenceOTU306621	p_Gemmatimonadetes; c_Gemm-5; o_; f_; g_; s_	-2.114	0.0477
New.CleanUp.ReferenceOTU163575	p_Gemmatimonadetes; c_Gemm-5; o_; f_; g_; s_	-2.019	0.0402
1104970	p_Gemmatimonadetes; c_Gemmatimonadetes; o_; f_; g_; s_	-2.180	0.0113
New.ReferenceOTU2131	p_Gemmatimonadetes; c_Gemmatimonadetes; o_Gemmatimonadales; f_; g_; s_	-2.463	0.0042
New.ReferenceOTU1057	p_Gemmatimonadetes; c_Gemmatimonadetes; o_KD8-87; f_; g_; s_	-2.566	0.0177
New.ReferenceOTU960	p_Planctomycetes; c_OM190; o_CL500-15; f_; g_; s_	-2.503	0.0040
New.CleanUp.ReferenceOTU179021	p_Pro; c_Alphaproteobacteria; o_; f_; g_; s_	-2.559	0.0419
4361041	p_Pro; c_Alphaproteobacteria; o_BD7-3; f_; g_; s_	-2.837	0.0097
New.ReferenceOTU2430	p_Pro; c_Alphaproteobacteria; o_BD7-3; f_; g_; s_	-2.430	0.0237
1105085	p_Pro; c_Alphaproteobacteria; o_Caulobacterales; f_Caulobacteraceae; g_Asticcacaulis; NA	-3.517	0.0005
2474239	p_Pro; c_Alphaproteobacteria; o_Sphingomonadales; f_Erythrobacteraceae; g_; s_	-3.115	<0.0001
New.ReferenceOTU841	p_Pro; c_Alphaproteobacteria; o_Sphingomonadales; f_Sphingomonadaceae; g_; s_	-3.871	<0.0001
New.ReferenceOTU310	p_Pro; c_Alphaproteobacteria; o_Sphingomonadales; f_Sphingomonadaceae; g_; s_	-3.478	<0.0001
New.ReferenceOTU808	p_Pro; c_Alphaproteobacteria; o_Sphingomonadales; f_Sphingomonadaceae; g_; s_	-3.236	<0.0001
722895	p_Pro; c_Alphaproteobacteria; o_Sphingomonadales; f_Sphingomonadaceae; g_Novosphingobium; s_	-2.615	0.0140
87167	p_Pro; c_Alphaproteobacteria; o_Sphingomonadales; f_Sphingomonadaceae; g_Sphingomonas; s_wittichii	-3.459	<0.0001
1127882	p_Pro; c_Alphaproteobacteria; o_Sphingomonadales; f_Sphingomonadaceae; g_Sphingopyxis; s_	-2.624	0.0022
674655	p_Pro; c_Alphaproteobacteria; o_Sphingomonadales; f_Sphingomonadaceae; g_Sphingopyxis; s_alaskensis	-2.685	0.0031
New.ReferenceOTU1646	p_Pro; c_Betaproteobacteria; NA; NA; NA; NA	-2.683	0.0009

New.ReferenceOTU729	p_Pro; c_Betaproteobacteria; o_Burkholderiales; f_Comamonadaceae; g_; s_	-2.836	0.0038
791738	p_Pro; c_Betaproteobacteria; o_Burkholderiales; f_Comamonadaceae; g_; s_	-2.629	0.0049
New.CleanUp.ReferenceOTU288692	p_Pro; c_Betaproteobacteria; o_Burkholderiales; f_Comamonadaceae; g_; s_	-2.482	0.0239
New.ReferenceOTU919	p_Pro; c_Betaproteobacteria; o_Burkholderiales; f_Comamonadaceae; g_; s_	-1.959	0.0324
New.CleanUp.ReferenceOTU169953	p_Pro; c_Betaproteobacteria; o_Burkholderiales; f_Comamonadaceae; NA; NA	-2.549	0.0239
New.ReferenceOTU757	p_Pro; c_Betaproteobacteria; o_Burkholderiales; f_Comamonadaceae; NA; NA	-1.885	0.0260
New.ReferenceOTU1102	p_Pro; c_Betaproteobacteria; o_Burkholderiales; f_Comamonadaceae; NA; NA	-1.749	0.0440
683573	p_Pro; c_Betaproteobacteria; o_Hydrogenophilales; f_Hydrogenophilaceae; g_Thiobacillus; s_	-3.285	<0.0001
850808	p_Pro; c_Betaproteobacteria; o_Hydrogenophilales; f_Hydrogenophilaceae; g_Thiobacillus; s_	-3.061	0.0010
New.ReferenceOTU2625	p_Pro; c_Betaproteobacteria; o_Hydrogenophilales; f_Hydrogenophilaceae; g_Thiobacillus; s_	-2.849	0.0005
New.ReferenceOTU1863	p_Pro; c_Betaproteobacteria; o_Methylophilales; f_Methylophilaceae; g_; s_	-3.110	0.0010
832166	p_Pro; c_Betaproteobacteria; o_Methylophilales; f_Methylophilaceae; g_; s_	-2.580	0.0167
643182	p_Pro; c_Betaproteobacteria; o_Thiobacterales; f_; g_; s_	-2.454	0.0476
New.CleanUp.ReferenceOTU76182	p_Pro; c_Deltaproteobacteria; o_Bdellovibrionales; f_Bacteriovoracaceae; g_; s_	-2.490	0.0289
New.ReferenceOTU516	p_Pro; c_Deltaproteobacteria; o_Myxococcales; f_; g_; s_	-3.216	0.0018
4405719	p_Pro; c_Deltaproteobacteria; o_Myxococcales; f_; g_; s_	-2.907	0.0023
New.CleanUp.ReferenceOTU5788	p_Pro; c_Deltaproteobacteria; o_Myxococcales; f_; g_; s_	-2.666	0.0265
New.CleanUp.ReferenceOTU100865	p_Pro; c_Deltaproteobacteria; o_Myxococcales; f_; g_; s_	-2.613	0.0317
New.ReferenceOTU1969	p_Pro; c_Deltaproteobacteria; o_Myxococcales; f_; g_; s_	-2.566	0.0265
New.CleanUp.ReferenceOTU203013	p_Pro; c_Deltaproteobacteria; o_Myxococcales; f_; g_; s_	-2.230	0.0115
New.CleanUp.ReferenceOTU32971	p_Pro; c_Deltaproteobacteria; o_Myxococcales; f_; g_; s_	-1.788	0.0477
New.ReferenceOTU1199	p_Pro; c_Deltaproteobacteria; o_Myxococcales; f_Haliangiaceae; g_; s_	-2.048	0.0477
500250	p_Pro; c_Deltaproteobacteria; o_Myxococcales; f_OM27; g_; s_	-3.758	0.0001
741010	p_Pro; c_Gammaproteobacteria; o_Alteromonadales; f_Alteromonadaceae; g_Cellvibrio; s_	-2.853	0.0103
222753	p_Pro; c_Gammaproteobacteria; o_Alteromonadales; f_Alteromonadaceae; g_Cellvibrio; s_	-2.237	0.0034
225453	p_Pro; c_Gammaproteobacteria; o_Alteromonadales; f_Alteromonadaceae; g_Cellvibrio; s_	-1.835	0.0263
3323643	p_Pro; c_Gammaproteobacteria; o_Xanthomonadales; f_Xanthomonadaceae; g_; s_	-3.853	0.0000
New.CleanUp.ReferenceOTU234497	p_Pro; c_Gammaproteobacteria; o_Xanthomonadales; f_Xanthomonadaceae; g_; s_	-3.016	0.0049
New.CleanUp.ReferenceOTU216416	p_Pro; c_Gammaproteobacteria; o_Xanthomonadales; f_Xanthomonadaceae; g_; s_	-2.697	0.0210

New.ReferenceOTU348	p_Pro; c_Gammaproteobacteria; o_Xanthomonadales; f_Xanthomonadaceae; g_; s_	-2.629	0.0027
696181	p_Pro; c_Gammaproteobacteria; o_Xanthomonadales; f_Xanthomonadaceae; g_; s_	-2.299	0.0247
New.ReferenceOTU2530	p_Pro; c_Gammaproteobacteria; o_Xanthomonadales; f_Xanthomonadaceae; g_; s_	-2.289	0.0390
New.ReferenceOTU1061	p_Pro; c_Gammaproteobacteria; o_Xanthomonadales; f_Xanthomonadaceae; g_; s_	-1.814	0.0287
New.CleanUp.ReferenceOTU24991	p_Pro; c_Gammaproteobacteria; o_Xanthomonadales; f_Xanthomonadaceae; g_Dokdonella; s_	-3.372	<0.0001
New.ReferenceOTU95	p_Pro; c_Gammaproteobacteria; o_Xanthomonadales; f_Xanthomonadaceae; g_Dokdonella; s_	-3.142	0.0003
1052930	p_Pro; c_Gammaproteobacteria; o_Xanthomonadales; f_Xanthomonadaceae; g_Luteimonas; s_	-1.649	0.0287
146193	p_Pro; c_Gammaproteobacteria; o_Xanthomonadales; f_Xanthomonadaceae; g_Pseudoxanthomonas; NA	-2.947	0.0005
New.ReferenceOTU2677	p_Pro; c_Gammaproteobacteria; o_Xanthomonadales; f_Xanthomonadaceae; g_Pseudoxanthomonas; NA	-2.713	0.0009
266510	p_Pro; c_Gammaproteobacteria; o_Xanthomonadales; f_Xanthomonadaceae; g_Pseudoxanthomonas; NA	-2.453	0.0433
New.CleanUp.ReferenceOTU184941	p_Pro; c_Gammaproteobacteria; o_Xanthomonadales; f_Xanthomonadaceae; g_Pseudoxanthomonas; NA	-2.404	0.0458
1049387	p_Pro; c_Gammaproteobacteria; o_Xanthomonadales; f_Xanthomonadaceae; g_Pseudoxanthomonas; s_mexicana	-2.572	0.0289
1004022	p_Pro; c_Gammaproteobacteria; o_Xanthomonadales; f_Xanthomonadaceae; g_Thermomonas; s_	-1.748	0.0242
New.CleanUp.ReferenceOTU289691	p_TM7; c_TM7-1; o_; f_; g_; s_	-3.224	0.0018
New.ReferenceOTU1537	p_TM7; c_TM7-1; o_; f_; g_; s_	-3.129	0.0005
New.CleanUp.ReferenceOTU141870	p_TM7; c_TM7-1; o_; f_; g_; s_	-2.947	0.0013
New.CleanUp.ReferenceOTU58769	p_TM7; c_TM7-1; o_; f_; g_; s_	-2.847	0.0009
New.CleanUp.ReferenceOTU40167	p_TM7; c_TM7-1; o_; f_; g_; s_	-2.433	0.0289
New.CleanUp.ReferenceOTU197235	p_TM7; c_TM7-1; o_; f_; g_; s_	-2.362	0.0269
New.CleanUp.ReferenceOTU154259	p_TM7; c_TM7-3; o_EW055; f_; g_; s_	-2.716	0.0267
New.ReferenceOTU1707	p_TM7; c_TM7-3; o_EW055; f_; g_; s_	-2.664	0.0081
4308576	p_Verrucomicrobia; c_[Pedosphaerae]; o_[Pedosphaerales]; f_Ellin517; g_; s_	-2.433	0.0244
New.ReferenceOTU961	p_Verrucomicrobia; c_[Pedosphaerae]; o_[Pedosphaerales]; f_R4-41B; g_; s_	-2.402	0.0237
New.CleanUp.ReferenceOTU146257	p_Verrucomicrobia; c_Opitutae; o_Opitutales; f_Opitutaceae; g_Opitutus; s_	-2.964	0.0049
New.ReferenceOTU1806	p_Verrucomicrobia; c_Opitutae; o_Opitutales; f_Opitutaceae; g_Opitutus; s_	-2.935	0.0001
New.CleanUp.ReferenceOTU268260	p_Verrucomicrobia; c_Opitutae; o_Opitutales; f_Opitutaceae; g_Opitutus; s_	-2.774	0.0155
255112	p_Verrucomicrobia; c_Verrucomicrobiae; o_Verrucomicrobiales; f_Verrucomicrobiaceae; g_; s_	-2.462	0.0010
New.ReferenceOTU2123	p_Verrucomicrobia; c_Verrucomicrobiae; o_Verrucomicrobiales; f_Verrucomicrobiaceae; g_; s_	-2.061	0.0163

Appendix B

Table 36 OTUs with significantly higher abundance in 9week-old **Low N** cabbages relative to 12 week-old LN plants (DESeq2 threshold fold change = 1.2; Benjamini-Hochberg corrected $p<0.05$).

OTU	Taxonomy	log2 fold change	*p*-value (corrected)
4471717	p_Bac; c_Cytophagia; o_Cytophagales; f_Cytophagaceae; g_Adhaeribacter; s_	2.649	0.0186
4322321	p_Cyanobacteria; c_Oscillatoriophycideae; o_Oscillatoriales; f_Phormidiaceae; g_Phormidium; s_	2.682	0.0347
561537	p_Firmicutes; c_Clostridia; o_Clostridiales; f_Veillonellaceae; g_Selenomonas; NA	4.224	<0.0001
92131	p_Fusobacteria; c_Fusobacteriia; o_Fusobacteriales; f_Leptotrichiaceae; g_Leptotrichia; s_	3.397	0.0041
860929	p_Pro; c_Alphaproteobacteria; o_Sphingomonadales; f_Erythrobacteraceae; g_; s_	2.261	0.0440
New.ReferenceOTU310	p_Pro; c_Alphaproteobacteria; o_Sphingomonadales; f_Sphingomonadaceae; g_; s_	2.057	0.0184
New.ReferenceOTU808	p_Pro; c_Alphaproteobacteria; o_Sphingomonadales; f_Sphingomonadaceae; g_; s_	1.983	0.0173
New.ReferenceOTU841	p_Pro; c_Alphaproteobacteria; o_Sphingomonadales; f_Sphingomonadaceae; g_; s_	1.685	0.0417
New.ReferenceOTU348	p_Pro; c_Gammaproteobacteria; o_Xanthomonadales; f_Xanthomonadaceae; g_; s_	1.946	0.0246
3323643	p_Pro; c_Gammaproteobacteria; o_Xanthomonadales; f_Xanthomonadaceae; g_; s_	1.862	0.0246
New.ReferenceOTU1734	p_Pro; c_Gammaproteobacteria; o_Xanthomonadales; f_Xanthomonadaceae; g_Lysobacter; s_	2.796	0.0025
New.ReferenceOTU540	p_Pro; c_Gammaproteobacteria; o_Xanthomonadales; f_Xanthomonadaceae; g_Lysobacter; s_	2.308	0.0233
4299136	p_TM7; c_TM7-3; o_CW040; f_F16; g_; s_	2.828	0.0265

Table 37 OTUs with significantly lower abundance in 9week-old Low N cabbages relative to 12 week-old LN plants (DESeq2 threshold fold change = 1.2; Benjamini-Hochberg corrected $p<0.05$

OTU	Taxonomy	log2 fold change	*p*-value (corrected)
4336218	p_Actinobacteria; c_Acidimicrobiia; o_Acidimicrobiales; f_EB1017; g_; s_	-2.607	0.0265
New.CleanUp.ReferenceOTU100831	p_Bac; c_Flavobacteriia; o_Flavobacteriales; f_Flavobacteriaceae; g_Flavobacterium; s_	-2.437	0.0440
New.CleanUp.ReferenceOTU35655	p_Bac; c_Flavobacteriia; o_Flavobacteriales; f_Flavobacteriaceae; g_Flavobacterium; s_	-2.429	0.0426
New.ReferenceOTU13	p_Bac; c_Sphingobacteriia; o_Sphingobacteriales; f_; g_; s_	-2.634	0.0426
New.ReferenceOTU110	p_Chloroflexi; c_Anaerolineae; o_H39; f_; g_; s_	-2.780	0.0265
New.ReferenceOTU2047	p_Chloroflexi; c_TK10; o_B07_WMSP1; f_FFCH4570; g_; s_	-2.248	0.0426
New.ReferenceOTU960	p_Planctomycetes; c_OM190; o_CL500-15; f_; g_; s_	-2.487	0.0173
New.ReferenceOTU763	p_Planctomycetes; c_Planctomycetia; o_Gemmatales; f_Gemmataceae; g_; s_	-2.227	0.0246
248146	p_Planctomycetes; c_Planctomycetia; o_Gemmatales; f_Gemmataceae; g_Gemmata; s_	-2.680	0.0246
New.ReferenceOTU1596	p_Pro; c_Alphaproteobacteria; o_Rhizobiales; f_Hyphomicrobiaceae; g_Pedomicrobium; s_	-2.267	0.0246
4475022	p_Pro; c_Deltaproteobacteria; o_Myxococcales; f_; g_; s_	-3.459	0.0035
New.CleanUp.ReferenceOTU90825	p_Pro; c_Deltaproteobacteria; o_Myxococcales; f_; g_; s_	-2.992	0.0186
258814	p_Pro; c_Deltaproteobacteria; o_Myxococcales; f_; g_; s_	-2.810	0.0281
New.ReferenceOTU1074	p_Pro; c_Deltaproteobacteria; o_Myxococcales; f_; g_; s_	-2.553	0.0426
114170	p_Pro; c_Deltaproteobacteria; o_Myxococcales; f_; g_; s_	-1.890	0.0265
751138	p_Pro; c_Gammaproteobacteria; o_Xanthomonadales; f_Xanthomonadaceae; g_Lysobacter; s_	-2.883	0.0063
541979	p_Pro; c_Gammaproteobacteria; o_Xanthomonadales; f_Xanthomonadaceae; g_Thermomonas; s_	-2.602	0.0186
1001960	p_Verrucomicrobia; c_[Pedosphaerae]; o_[Pedosphaerales]; f_; g_; s_	-3.050	0.0146

Taxonomic levels: **p** = phylum; **c** = class; **o** = order; **f** = family; **g** = genus; **s** = species (blank = unassigned); Phylum abbreviations: **Act**: Actinobacteria, **Bac:** Bacteroidetes, **Pro**: Proteobacteria.

Table 38 OTUs with significantly higher abundance in 9week-old **High N** cabbages relative to 12 week-old HN plants (DESeq2 threshold fold change = 1.2; Benjamini-Hochberg corrected $p<0.05$).

OTU	Taxonomy	log2 fold change	*p*-value (corrected)
547806	p_Act; c_Actinobacteria; o_Actinomycetales; f_Actinosynnemataceae; g_Lentzea; NA	3.119	0.0000
New.CleanUp.ReferenceOTU71712	p_Act; c_Actinobacteria; o_Actinomycetales; f_Actinosynnemataceae; g_Lentzea; NA	2.539	0.0418
949789	p_Firmicutes; c_Bacilli; o_Lactobacillales; f_Carnobacteriaceae; g_Granulicatella; s_	2.777	0.0175
New.CleanUp.ReferenceOTU318461	p_TM7; c_TM7-1; o_; f_; g_; s_	3.733	0.0000
316001	p_TM7; c_TM7-1; o_; f_; g_; s_	3.698	0.0001
New.CleanUp.ReferenceOTU269779	p_TM7; c_TM7-1; o_; f_; g_; s_	3.188	0.0010
279572	p_TM7; c_TM7-1; o_; f_; g_; s_	2.913	0.0020
New.CleanUp.ReferenceOTU168583	p_TM7; c_TM7-1; o_; f_; g_; s_	2.486	0.0175

Taxonomic levels: **p** = phylum; **c** = class; **o** = order; **f** = family; **g** = genus; **s** = species (blank = unassigned); Phylum abbreviations: **Act**: Actinobacteria

Table 39 OTUs with significantly lower abundance in 9week-old **High N** cabbages relative to 12 week-old HN plants (DESeq2 threshold fold change = 1.2; Benjamini-Hochberg corrected $p<0.05$).

OTU	Taxonomy	log2 fold change	p-value (corrected)
1119329	p_Aci; c_Acidobacteria-6; o_iii1-15; f_; g_; s_	-2.245	0.0118
New.ReferenceOTU197	p_Act; c_Acidimicrobiia; o_Acidimicrobiales; f_C111; g_; s_	-2.654	0.0241
1005605	p_Act; c_Actinobacteria; o_Actinomycetales; f_; g_; s_	-2.594	0.0311
New.ReferenceOTU1620	p_Armatimonadetes; c_[Fimbriimonadia]; o_[Fimbriimonadales]; f_[Fimbriimonadaceae]; g_Fimbriimonas; s_	-2.478	0.0239
New.CleanUp.ReferenceOTU68597	p_Bac; c_[Saprospirae]; o_[Saprospirales]; f_Chitinophagaceae; g_; s_	-2.733	0.0222
1143479	p_Bac; c_Cytophagia; o_Cytophagales; f_Cyclobacteriaceae; g_Algoriphagus; NA	-2.000	0.0207
113298	p_Bac; c_Cytophagia; o_Cytophagales; f_Cytophagaceae; g_; s_	-2.642	0.0290
1118729	p_Bac; c_Cytophagia; o_Cytophagales; f_Cytophagaceae; g_; s_	-2.227	0.0321
1087462	p_Bac; c_Cytophagia; o_Cytophagales; f_Cytophagaceae; g_Cytophaga; s_	-4.221	<0.0001
570086	p_Bac; c_Flavobacteriia; o_Flavobacteriales; f_Cryomorphaceae; g_Fluviicola; s_	-3.168	<0.0001
New.CleanUp.ReferenceOTU93054	p_Bac; c_Flavobacteriia; o_Flavobacteriales; f_Flavobacteriaceae; g_; s_	-2.557	0.0241
New.ReferenceOTU1650	p_Bac; c_Sphingobacteriia; o_Sphingobacteriales; f_Sphingobacteriaceae; g_Pedobacter; s_	-2.723	0.0002
New.ReferenceOTU1193	p_Bac; c_Sphingobacteriia; o_Sphingobacteriales; f_Sphingobacteriaceae; g_Pedobacter; s_	-2.495	0.0387
976441	p_Bac; c_Sphingobacteriia; o_Sphingobacteriales; f_Sphingobacteriaceae; g_Pedobacter; s_	-2.139	0.0451
New.ReferenceOTU1760	p_Chloroflexi; c_Anaerolineae; o_H39; f_; g_; s_	-2.259	0.0175
New.ReferenceOTU2131	p_Gemmatimonadetes; c_Gemmatimonadetes; o_Gemmatimonadales; f_; g_; s_	-2.507	0.0172
New.CleanUp.ReferenceOTU115362	p_Planctomycetes; c_Planctomycetia; o_Pirellulales; f_Pirellulaceae; g_; s_	-2.776	0.0059
New.CleanUp.ReferenceOTU182991	p_Pro; c_Alphaproteobacteria; o_Rhodospirillales; f_Rhodospirillaceae; g_; s_	-2.592	0.0186
New.ReferenceOTU2733	p_Pro; c_Alphaproteobacteria; o_Rhodospirillales; f_Rhodospirillaceae; g_; s_	-2.014	0.0387
87167	p_Pro; c_Alphaproteobacteria; o_Sphingomonadales; f_Sphingomonadaceae; g_Sphingomonas; s_wittichii	-2.054	0.0450
New.ReferenceOTU2176	p_Pro; c_Betaproteobacteria; o_Methylophilales; f_Methylophilaceae; g_Methylotenera; s_mobilis	-2.707	0.0222
New.CleanUp.ReferenceOTU152067	p_Pro; c_Deltaproteobacteria; o_Myxococcales; f_; g_; s_	-2.670	0.0239
New.CleanUp.ReferenceOTU292557	p_Pro; c_Deltaproteobacteria; o_Spirobacillales; f_; g_; s_	-2.564	0.0356

New.ReferenceOTU1060	p_Pro; c_Gammaproteobacteria; o_Pseudomonadales; f_Pseudomonadaceae; g_Pseudomonas; NA	-4.945	<0.0001
928406	p_Pro; c_Gammaproteobacteria; o_Pseudomonadales; f_Pseudomonadaceae; g_Pseudomonas; s_stutzeri	-2.963	0.0106
New.ReferenceOTU1708	p_Pro; c_Gammaproteobacteria; o_Xanthomonadales; f_Xanthomonadaceae; g_; s_	-3.271	0.0001
4008562	p_Pro; c_Gammaproteobacteria; o_Xanthomonadales; f_Xanthomonadaceae; g_; s_	-2.643	0.0004
751138	p_Pro; c_Gammaproteobacteria; o_Xanthomonadales; f_Xanthomonadaceae; g_Lysobacter; s_	-3.489	0.0002
146193	p_Pro; c_Gammaproteobacteria; o_Xanthomonadales; f_Xanthomonadaceae; g_Pseudoxanthomonas; NA	-2.505	0.0175
1049387	p_Pro; c_Gammaproteobacteria; o_Xanthomonadales; f_Xanthomonadaceae; g_Pseudoxanthomonas; s_mexicana	-3.038	0.0002
114573	p_Pro; c_Gammaproteobacteria; o_Xanthomonadales; f_Xanthomonadaceae; g_Pseudoxanthomonas; s_mexicana	-2.609	0.0095
541979	p_Pro; c_Gammaproteobacteria; o_Xanthomonadales; f_Xanthomonadaceae; g_Thermomonas; s_	-2.781	0.0095
New.CleanUp.ReferenceOTU170442	p_TM7; c_TM7-1; o_; f_; g_; s_	-2.889	0.0095
New.CleanUp.ReferenceOTU28944	p_TM7; c_TM7-3; o_; f_; g_; s_	-2.513	0.0449
New.CleanUp.ReferenceOTU58782	p_TM7; c_TM7-3; o_I025; f_; g_; s_	-2.872	0.0095
New.CleanUp.ReferenceOTU20097	p_TM7; c_TM7-3; o_I025; f_; g_; s_	-2.822	0.0187
New.CleanUp.ReferenceOTU82142	p_TM7; c_TM7-3; o_I025; f_; g_; s_	-2.563	0.0248
New.CleanUp.ReferenceOTU43498	p_TM7; NA; NA; NA; NA; NA	-2.639	0.0264
4225240	p_Ver; c_Opitutae; o_Opitutales; f_Opitutaceae; g_Opitutus; s_	-2.885	0.0095
New.CleanUp.ReferenceOTU146257	p_Ver; c_Opitutae; o_Opitutales; f_Opitutaceae; g_Opitutus; s_	-2.788	0.0111
New.CleanUp.ReferenceOTU310346	p_Ver; c_Opitutae; o_Opitutales; f_Opitutaceae; g_Opitutus; s_	-2.223	0.0480
4451561	p_Ver; c_Verrucomicrobiae; o_Verrucomicrobiales; f_Verrucomicrobiaceae; g_Prosthecobacter; s_debontii	-3.038	0.0002
New.ReferenceOTU1135	p_Ver; c_Verrucomicrobiae; o_Verrucomicrobiales; f_Verrucomicrobiaceae; g_Prosthecobacter; s_debontii	-2.894	<0.0001
New.ReferenceOTU673	p_Ver; c_Verrucomicrobiae; o_Verrucomicrobiales; f_Verrucomicrobiaceae; g_Prosthecobacter; s_debontii	-2.571	0.0311

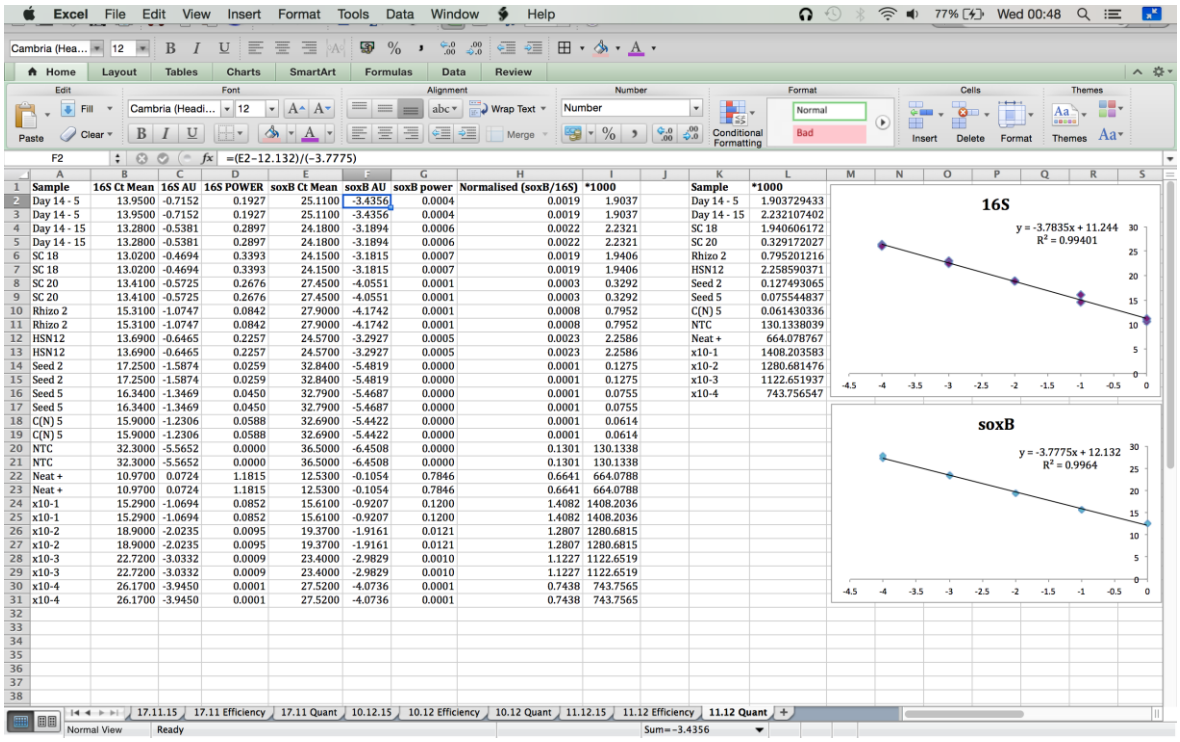
Taxonomic levels: **p** = phylum; **c** = class; **o** = order; **f** = family; **g** = genus; **s** = species (blank = unassigned); Phylum abbreviations: **Act**: Actinobacteria, **Bac:** Bacteroidetes, **Pro**: Proteobacteria, **Ver**: Verrucomicrobia

Appendix C

Table 40 Efficiencies and r^2 values of qPCR experiments performed to quantify the abundance of SOB populations.

qPCR date	16S		soxB	
	Efficiency	**R^2**	**Efficiency**	**R^2**
17.08.15 (b)	59.73	99.54	72.67	96.26
18.08.15	71.44	98.87	81.37	93.57
15.09.15	63.97	99.98	76.74	98.60
17.09.15	65.05	99.27	78.73	96.32
18.09.15	57.64	99.43	71.97	97.37
21.09.15 (a)	54.65	98.88	69.38	98.82
21.09.15 (b)	54.53	98.85	67.32	99.08
23.09.15	60.45	99.53	76.40	99.36
12.10.15.	50.45	98.42	58.99	98.87
13.10.15	60.22	98.51	64.65	98.94
21.10.15	71.62	99.22	77.65	97.98
22.10.15	51.53	98.83	61.53	98.28
23.10.15	51.23	98.34	76.51	98.44
26.10.15	71.51	99.25	69.16	98.60
27.10.15	49.96	94.94	68.14	98.56
28.10.15	67.83	99.52	70.51	98.90
30.10.15	56.89	98.53	92.28	96.53
5.11.15			67.94	98.11
6.11.15	66.30	99.29	65.22	98.15
17.11.15	51.19	99.70	62.87	97.06
10.12.15	79.49	99.68	70.26	99.80
11.12.15	83.78	99.40	83.96	99.64
S.D.	***2.13***	***0.22***	***1.69***	***0.30***
AVERAGE	**61.88**	**98.95**	**72.01**	**98.06**

Appendix C



(a)

	A	B	C	D	E	F	G	H	I
F2						=(E2-12.132)/(-3.7775)			
1	**Sample**	**16S Ct Mean**	**16S AU**	**16S POWER**	**soxB Ct Mean**	**soxB AU**	**soxB power**	**Normalised (soxB/16S)**	***1000**
2	Day 14 - 5	13.9500	-0.7152	0.1927	25.1100	-3.4356	0.0004	0.0019	1.9037

(b)

	A	B	C	D	E	F	G	H	I
G2							=POWER(10,F2)		
1	**Sample**	**16S Ct Mean**	**16S AU**	**16S POWER**	**soxB Ct Mean**	**soxB AU**	**soxB power**	**Normalised (soxB/16S)**	***1000**
2	Day 14 - 5	13.9500	-0.7152	0.1927	25.1100	-3.4356	0.0004	0.0019	1.9037

(c)

	A	B	C	D	E	F	G	H	I
H2								=G2/D2	
1	**Sample**	**16S Ct Mean**	**16S AU**	**16S POWER**	**soxB Ct Mean**	**soxB AU**	**soxB power**	**Normalised (soxB/16S)**	***1000**
2	Day 14 - 5	13.9500	-0.7152	0.1927	25.1100	-3.4356	0.0004	0.0019	1.9037

Figure 62 Example of calculations used to quantify the relative abundance of SOB populations using qPCR data. (a) Arbitrary Units (AU) = $(\mathrm{C}_t\ mean - b) \div m$; (b) quantity = $10^{((\mathrm{C}_t\ mean - b) \div m)}$; and (c) normalisation to housekeeping gene = $soxB\ quantity \div 16S\ quantity$.

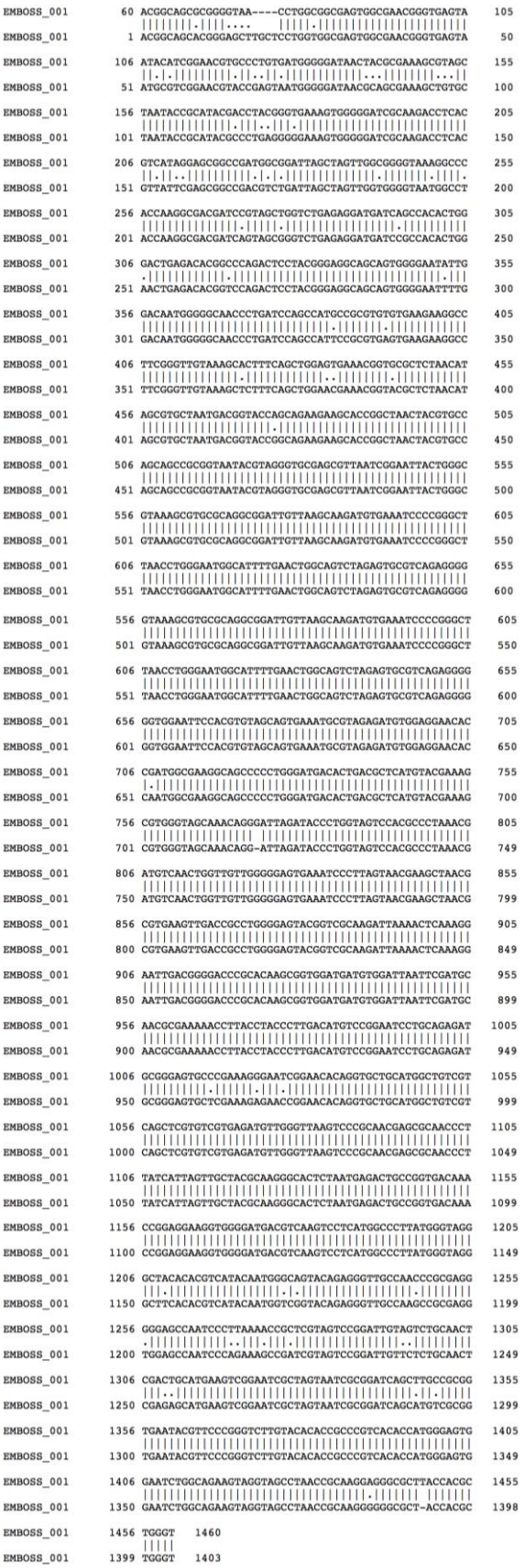


Figure 63 Sequence alignment of the 16S genes of the *Thiobacillus* OTU 683573 (Genbank accession FM212997.1) identified in the rhizosphere soil (Chapter 3) and the *T. thioparus* DSM 505 (Genbank accession HM173629.1) used for the inoculation.

Table 41 Abundance of SOB in the rhizospheres of experimental *B. oleracea* as determined by qPCR of the *soxB* gene. Means (±SE) of *soxB* (given as a ratio relative to 16S quantification) are shown. Different letters indicate significant differences between treatments (within the same harvest age and aphid treatment group) according to one-way ANOVA (using log-transformed data).

Treatment	**Mean (±SE) *soxB* AU**							
	8 weeks		*8 weeks + aphids*		*12 weeks*		*12 weeks + aphids*	
SC	0.005248	ad	0.002390	abc	0.001075	bc	0.001890	bc
D7	0.006000	cd	0.022725	cd	0.004853	cd	0.006000	cd
D14	0.005621	cd	0.005927	bcd	*na*		*na*	
HSS	0.107552	d	0.045778	d	0.054331	d	0.024045	d
NC	0.000201	ab	0.000540	a	0.000052	a	0.000120	a
Rhizo	0.003847	bcd	0.009211	cd	0.000092	ab	0.000505	ab
Seed	0.000141	a	0.000568	ab	0.000064	a	0.000091	a
HSN	0.002384	ac	0.000528	ab	0.000892	abc	0.000364	ab

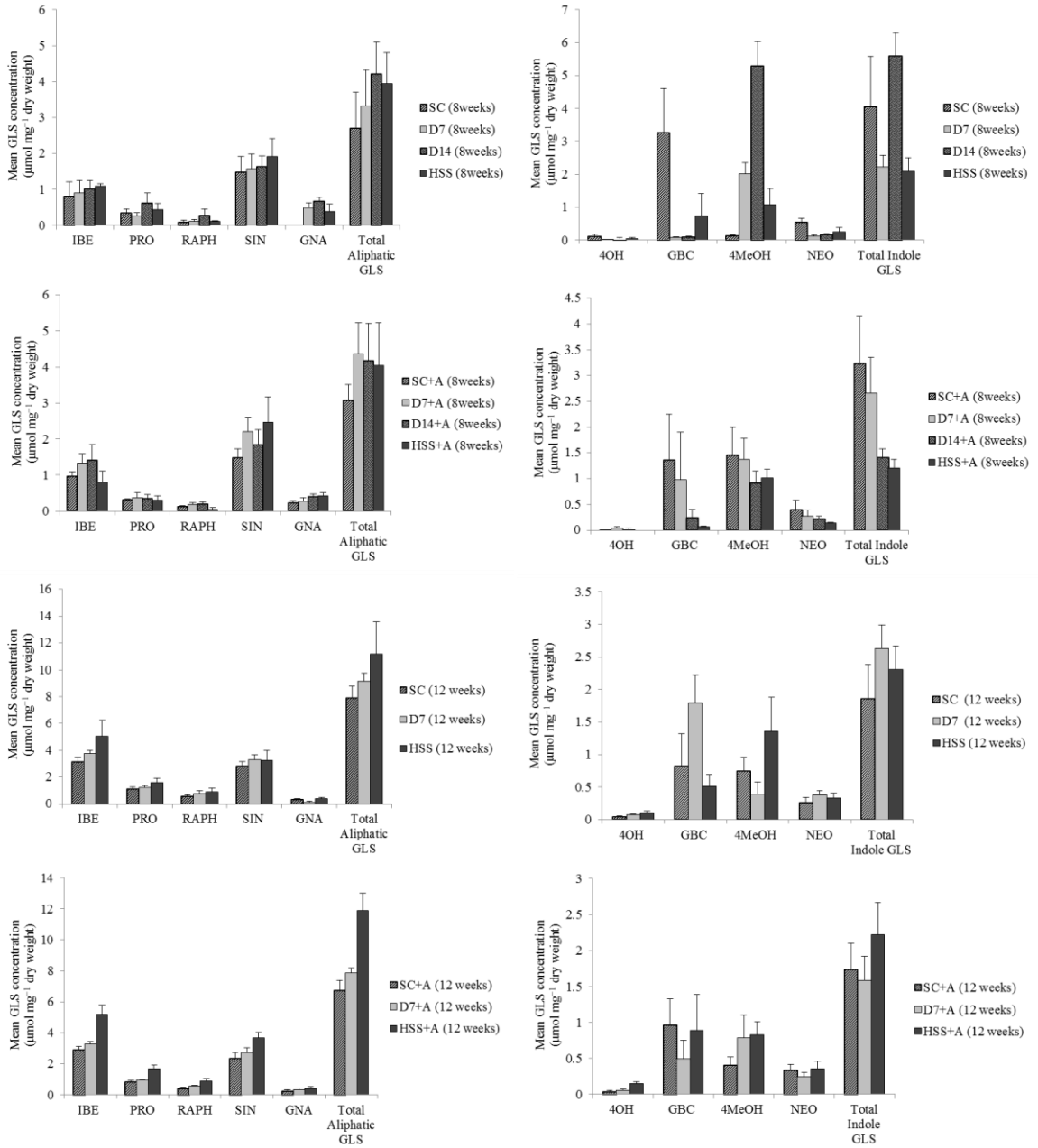


Figure 64 Mean concentrations of individual aliphatic and indole GLS (expressed as µmol mg^{-1} of dry weight) in plants grown in sterile soil, harvested at 8 and 12 weeks (excluding D14+A 12 week samples. (Sterile soil treatment abbreviations: **SC**: Sterile control; **D7**= 7-day incubation with *T. thioparus* inoculation; **D14**= 14-day incubation with *T. thioparus* inoculation; **HSS**=sulphur fertiliser treatment.)

Appendix C

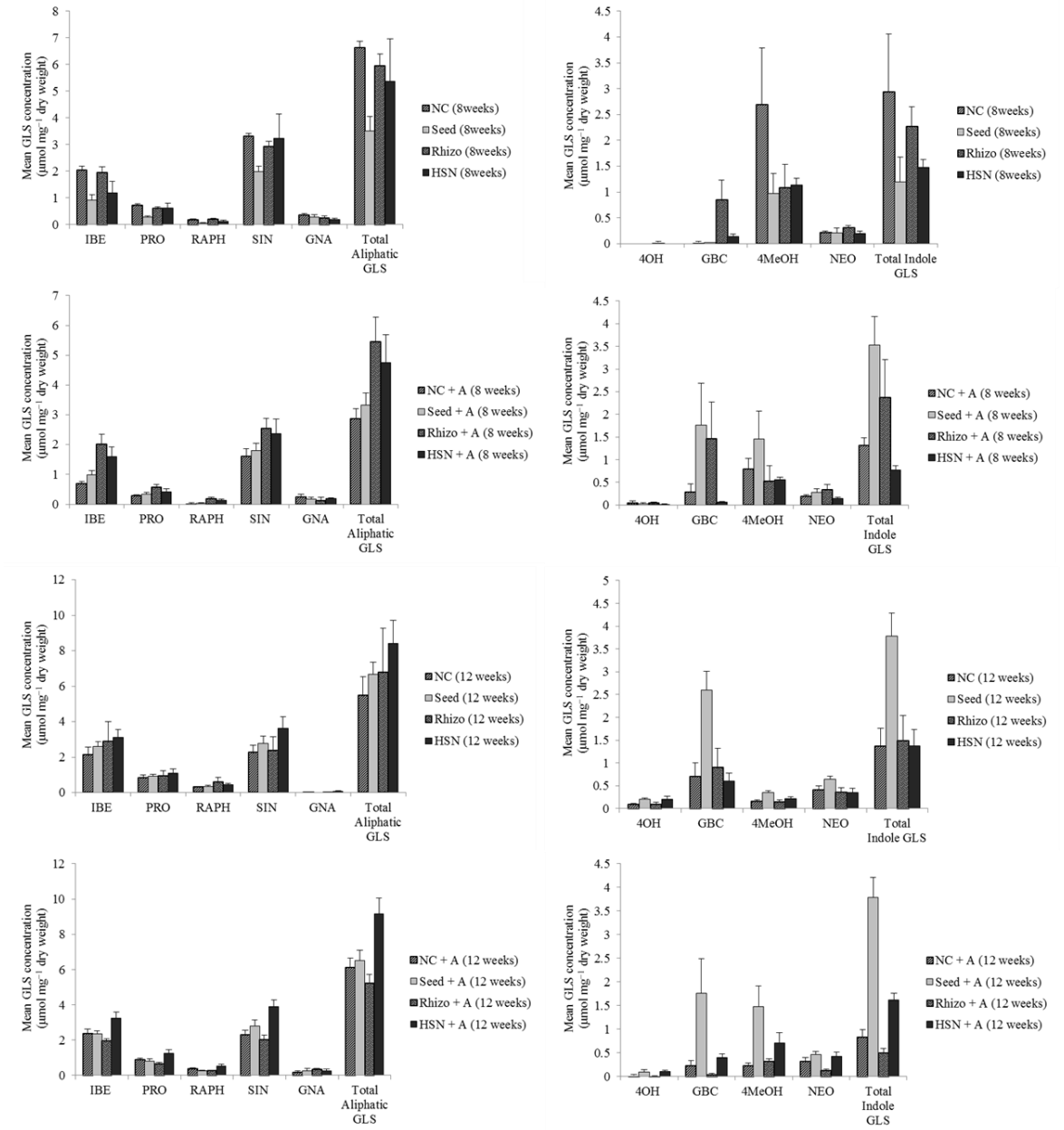


Figure 65 Mean concentrations of individual aliphatic and indole GLS (expressed as μmol mg^{-1} of dry weight) in plants grown in normal (non-sterile) soil, harvested at 8 and 12 weeks. (Normal (nonsterile) soil treatment codes: **NC:** normal control; **Seed**: seed *T. thioparus* inoculation; **Rhizo:** rhizosphere *T. thioparus* inoculation; **HSN**: sulphur fertiliser treatment.)

Table 42 Glucosinolate concentrations (μmol mg^{-1}) and soxB quantification (AU) for cabbages grown in "normal" (non-sterile) soil, harvested at 8 weeks - Control (NC) and sulphur addition (HSN) data.

Treatment	Rep	Aphids	soxB quant	IBE	PRO	RAPH	SIN	GNA	ALIPHATIC	4OH	GBC	4MeOH	NEO	INDOLE	TOTAL GLS
NC	4	384	0.0276	0.6929	0.3075	*n.d.*	1.9535	0.4452	3.3991	*n.d.*	*n.d.*	1.8002	0.2072	2.0075	5.4066
NC	7	567	0.0281	0.4715	0.2019	*n.d.*	1.8645	0.2428	2.7806	*n.d.*	0.0175	0.5602	0.2578	0.8354	3.6161
NC	11	n/a	0.0449	1.9900	0.5163	0.1408	3.1417	0.2485	6.0374	*n.d.*	0.0295	1.8231	0.1952	2.0478	8.0852
NC	6	188	0.0778	0.6636	0.4146	*n.d.*	2.7919	0.5501	4.4201	*n.d.*	0.0294	1.2516	0.2052	1.4862	5.9063
NC	8	n/a	0.0807	2.1593	0.6660	0.1414	3.3954	0.3850	6.7470	*n.d.*	0.0563	0.9351	0.1805	1.1719	7.9189
NC	3	9	0.1780	0.9072	0.3067	0.1089	1.1304	0.2554	2.7086	*n.d.*	*n.d.*	1.0679	0.0878	1.1558	3.8644
NC	10	n/a	0.2127	2.0796	0.7784	0.1826	3.6842	0.3909	7.1157	*n.d.*	0.0411	2.2572	0.2016	2.4999	9.6156
NC	9	n/a	0.2752	1.5145	0.8837	0.1456	2.9851	0.5616	6.0906	*n.d.*	0.0326	6.9890	0.3195	7.3410	13.4316
NC	12	n/a	0.3905	2.4787	0.7657	0.3152	3.3400	0.2726	7.1721	*n.d.*	*n.d.*	1.4567	0.1606	1.6173	8.7895
NC	5	91	0.6995	0.9496	0.3110	0.0953	0.8179	*n.d.*	2.1738	0.2842	1.0218	0.1570	0.1397	1.3185	3.4923
NC	1	231	0.8900	0.6943	0.3299	*n.d.*	1.7700	0.2968	3.0910	*n.d.*	*n.d.*	0.6109	0.1254	0.7362	3.8272
NC	2	230	1.8771	0.4352	0.1145	*n.d.*	0.9228	*n.d.*	1.4725	0.0367	0.9137	0.0763	0.3353	1.3252	2.7977
HSN	13	n/a	0.0014	1.5945	0.6757	0.1619	3.8909	0.2343	6.5573	*n.d.*	0.1219	1.5083	0.2372	1.8674	8.4247
HSN	11	n/a	0.1098	0.0838	0.1734	*n.d.*	0.9504	0.1225	1.3301	*n.d.*	0.0371	1.0817	0.0877	1.2066	2.5367
HSN	10	n/a	0.1275	2.0548	1.0983	0.2014	5.2437	0.2797	8.8779	*n.d.*	0.2963	0.9760	0.2903	1.5626	10.4405
HSN	7	283	0.1486	1.1377	0.2485	0.1146	1.4990	0.1838	3.1835	*n.d.*	0.0425	0.6333	0.1970	0.8728	4.0563
HSN	8	83	0.3220	2.5767	0.7224	0.2332	3.7401	0.2082	7.4806	0.0394	0.1145	0.6194	0.1839	0.9178	8.3984
HSN	9	41	0.3328	1.3258	0.4003	0.1150	2.3293	0.1729	4.3434	*n.d.*	0.0429	0.3966	0.0814	0.5209	4.8643
HSN	6	144	1.3085	1.3879	0.2926	0.1144	1.9326	0.2329	3.9602	*n.d.*	0.0584	0.5972	0.1066	0.7623	4.7225
HSN	12	n/a	9.2959	1.0311	0.5424	0.0945	2.8796	0.1583	4.7058	*n.d.*	0.0868	0.9680	0.1965	1.2512	5.9571

n.d.: not detected.

Table 43 Glucosinolate concentrations (μmol mg^{-1}) and *soxB* quantification (AU) for cabbages grown in "normal" (non-sterile) soil, harvested at 8 weeks - *T. thioparus* inoculated samples (*Rhizo and Seed)* data.

Treatment	Rep	Aphids	soxB quant	IBE	PRO	RAPH	SIN	GNA	ALIPHATIC	4OH	GBC	4MeOH	NEO	INDOLE	TOTAL GLS
Rhizo	6	-	0.2442	2.7923	0.8016	0.3299	3.5093	0.4289	7.8621	*n.d.*	0.0526	2.4810	0.3256	2.8592	10.7212
Rhizo	7	-	0.3407	1.0953	0.3941	0.0944	2.2930	0.2007	4.0776	*n.d.*	0.0374	0.7723	0.1581	0.9677	5.0453
Rhizo	5	-	2.5212	1.9515	0.7689	0.2601	3.5637	*n.d.*	6.5443	0.0537	1.7630	0.2224	0.4417	2.4271	8.9715
Rhizo	20	55	3.2811	2.2290	0.7315	0.2328	3.0530	0.0314	6.2777	0.0930	1.9220	0.1693	0.2761	2.3674	8.6452
Rhizo	3	-	4.4209	2.2945	0.4120	0.1896	2.5341	0.4622	5.8925	*n.d.*	0.0441	0.5662	0.1759	0.7862	6.6787
Rhizo	23	-	5.6219	1.9293	0.6016	0.1761	3.0252	*n.d.*	5.7321	0.1177	2.1623	0.2467	0.4608	2.8698	8.6019
Rhizo	4	-	6.4995	1.5700	0.5905	0.1586	2.4307	0.5955	5.3453	*n.d.*	0.0297	3.0973	0.2052	3.3322	8.6775
Rhizo	1	218	6.5121	2.7072	0.6566	0.2700	2.8163	0.4590	6.9091	*n.d.*	0.0485	1.5528	0.1993	1.8007	8.7098
Rhizo	22	-	7.2787	2.1251	0.6652	0.2690	3.1472	*n.d.*	6.2064	0.0536	1.8615	0.1859	0.3675	2.4149	8.6213
Rhizo	21	213	8.7463	2.0087	0.5842	0.1768	2.7341	*n.d.*	5.5038	0.0650	3.5512	0.2934	0.6823	4.5269	10.0307
Rhizo	2	133	18.3061	1.1641	0.3164	0.0883	1.5798	*n.d.*	3.1486	*n.d.*	0.3432	0.1099	0.1807	0.6338	3.7824
Seed	11	-	0.0412	1.5394	0.5091	0.1514	2.8876	0.7564	5.8439	*n.d.*	0.0285	2.9268	0.6364	3.5918	9.4356
Seed	6	129	0.0614	0.5406	0.2640	*n.d.*	1.3892	0.3258	2.5196	*n.d.*	0.0238	1.6600	0.0818	1.7656	4.2852
Seed	7	-	0.0790	0.3611	0.1803	*n.d.*	1.3763	0.2385	2.1562	*n.d.*	*n.d.*	0.6115	0.0682	0.6797	2.8359
Seed	10	-	0.0870	0.8275	0.2296	*n.d.*	1.8853	0.0983	3.0407	*n.d.*	*n.d.*	0.4924	0.0891	0.5815	3.6222
Seed	12	-	0.0954	1.5463	0.3130	0.1500	1.9590	0.2533	4.2216	*n.d.*	0.0318	0.4939	0.2176	0.7432	4.9648
Seed	4	400	0.1440	1.3093	0.2372	0.0725	1.6237	0.3390	3.5816	*n.d.*	0.0710	3.1419	0.1864	3.3993	6.9809
Seed	9	-	0.1912	0.6327	0.2612	*n.d.*	2.0067	0.2175	3.1182	*n.d.*	0.0407	0.6559	0.1422	0.8388	3.9570
Seed	3	197	0.2915	0.7606	0.2908	0.0758	1.5642	0.3558	3.0473	*n.d.*	0.0228	3.3475	0.1487	3.5190	6.5663
Seed	8	-	0.3534	0.5425	0.2500	*n.d.*	1.7675	0.1356	2.6956	*n.d.*	0.0173	0.6304	0.1121	0.7598	3.4555
Seed	1	201	0.4930	0.7136	0.3079	0.0539	1.6007	*n.d.*	2.6760	0.1398	3.2273	0.2086	0.6243	4.0603	6.7363
Seed	5	184	0.8698	1.5021	0.6143	0.0598	3.0801	*n.d.*	5.2563	0.0401	1.6989	0.2541	0.2343	2.1873	7.4436
Seed	2	134	1.5512	1.0545	0.2818	*n.d.*	1.5281	*n.d.*	2.8645	*n.d.*	5.5448	0.1595	0.3649	6.0692	8.9337

Table 44 Glucosinolate concentrations (μmol mg^{-1}) and *soxB* quantification (AU) for cabbages grown in "normal" (non-sterile) soil, harvested at 12 weeks - *Control (NC) and sulphur addition (HSN) data*.

Treatment	Rep	Aphids	soxB quant	IBE	PRO	RAPH	SIN	GNA	ALIPHATIC	4OH	GBC	4MeOH	NEO	INDOLE	TOTAL GLS
NC	13	107	0.0214	1.5505	0.4672	0.2422	1.3511	0.1972	3.8082	*n.d.*	0.0412	0.2712	0.1402	0.4527	4.2609
NC	16	85	0.0473	3.2422	1.1220	0.4989	2.8345	*n.d.*	7.6975	0.0448	0.3207	0.2313	0.4659	1.0179	8.7154
NC	17	4	0.0751	2.5211	0.8824	0.5377	1.8824	*n.d.*	5.8237	0.0546	0.3431	0.1590	0.4954	0.9975	6.8212
NC	15	52	0.1554	2.1817	0.8735	0.3569	2.2044	0.4893	6.1058	*n.d.*	0.0582	0.3951	0.1809	0.6342	6.7399
NC	18	4	0.1574	2.7793	1.0111	0.3531	2.9742	*n.d.*	7.1177	0.0626	0.5786	0.1966	0.5047	1.2800	8.3977
NC	14	58	0.2647	2.0450	0.9097	0.2473	2.5217	0.3651	6.0889	*n.d.*	0.1177	0.2079	0.1388	0.4644	6.5534
NC	19	-	0.0289	1.6840	1.0330	0.2301	2.7997	*n.d.*	5.7468	0.0790	2.0122	0.2678	0.6029	2.8830	8.6298
NC	20	-	0.0610	2.7068	1.0600	0.3843	2.9248	*n.d.*	7.0759	0.1629	0.4394	0.2150	0.5108	1.1653	8.2412
NC	21	-	0.0598	2.2417	0.9898	0.3245	2.7499	*n.d.*	6.3059	0.0586	1.1099	0.1756	0.4200	1.7055	8.0114
NC	22	-	0.0242	2.7409	0.8078	0.3829	2.2375	0.0277	6.1969	0.0837	0.2698	0.1146	0.3866	0.7710	6.9679
NC	23	-	0.0448	3.2249	1.0142	0.4311	2.6549	*n.d.*	7.3251	0.1571	0.3848	0.1843	0.5296	1.0987	8.4238
NC	24	-	0.0906	*n.d.*	0.0203	*n.d.*	0.0643	*n.d.*	0.0846	*n.d.*	0.0068	0.0160	0.0127	0.0355	0.1202
HSN	3	-	0.0016	2.7821	0.4952	0.5101	1.6743	0.2551	5.7168	*n.d.*	0.0226	0.2194	0.0542	0.2962	6.0130
HSN	5	-	0.7672	1.4489	0.4387	0.2271	1.5856	*n.d.*	3.7002	*n.d.*	0.1936	0.0573	0.1453	0.3962	4.0964
HSN	17	163	0.0781	2.1001	0.9036	0.2289	4.4566	*n.d.*	7.6892	0.2070	0.6268	0.3981	0.7141	1.7390	9.4283
HSN	18	-	3.3385	4.1675	1.5289	0.5314	4.8625	0.0785	11.1688	0.3850	0.7858	0.2463	0.4270	1.4591	12.6279
HSN	14	18	0.2716	3.5034	1.4500	0.4901	4.4044	0.4668	10.3148	*n.d.*	0.4391	1.2063	0.3051	1.9505	12.2653
HSN	1	129	0.3315	3.7333	1.1140	0.8386	2.8974	0.5360	9.1192	*n.d.*	0.0542	1.4570	0.1603	1.6715	10.7907
HSN	15	245	0.5061	3.4789	1.4962	0.4639	4.4057	0.4481	10.2928	0.0714	0.2829	0.7468	0.5115	1.5412	11.8340
HSN	19	-	0.7129	4.3104	1.5739	0.4620	5.5921	0.0774	12.0158	0.3536	1.0605	0.2701	0.5590	1.8896	13.9054
HSN	16	126	0.9644	2.2267	0.6178	0.2269	2.4838	*n.d.*	5.5552	0.1403	0.4177	0.1530	0.3047	0.8754	6.4306
HSN	20	-	0.5081	2.8279	0.8991	0.3263	3.9269	*n.d.*	7.9803	0.3415	1.0168	0.2904	0.5439	1.8511	9.8314
HSN	2	4	0.0310	4.3228	1.9552	0.8804	4.6498	0.1086	11.9167	0.1574	0.5476	0.2731	0.5455	1.3662	13.2829
HSN	4	-	0.0267	3.2661	1.7430	0.6767	4.0973	0.0674	9.8506	0.1330	0.5261	0.2335	0.4009	1.1605	11.0111

Table 45 Glucosinolate concentrations (μmol g^{-1}) and *soxB* quantification (AU) for cabbages grown in "normal" (non-sterile) soil, harvested at 12 weeks - *T. thioparus* inoculated samples (*Rhizo and Seed)* data..

Treatment	Rep	Aphids	soxB quant	IBE	PRO	RAPH	SIN	GNA	ALIPHATIC	4OH	GBC	4MeOH	NEO	INDOLE	TOTAL GLS
Rhizo	11	53	0.0307	2.3094	0.8744	0.3477	2.5161	0.5472	6.5947	*n.d.*	0.1234	0.4514	0.2014	0.7762	7.3710
Rhizo	10	66	0.0744	2.2648	0.6403	0.2765	2.3227	0.3404	5.8447	*n.d.*	0.0496	0.3596	0.1576	0.5668	6.4115
Rhizo	14	-	0.1634	*n.d.*	*n.d.*	*n.d.*	0.0831	0.0086	0.0917	*n.d.*	*n.d.*	0.0160	0.0042	0.0202	0.1118
Rhizo	14	-	0.1634	*n.d.*	*n.d.*	*n.d.*	0.0831	0.0086	0.0917	*n.d.*	*n.d.*	0.0160	0.0042	0.0202	0.1118
Rhizo	15	-	0.1353	*n.d.*	0.0229	*n.d.*	0.0672	0.0170	0.1072	*n.d.*	*n.d.*	0.0049	0.0065	0.0114	0.1185
Rhizo	16	-	0.0145	3.4948	1.2687	0.4510	3.7497	*n.d.*	8.9642	0.2593	0.5715	0.2744	0.5728	1.4187	10.3829
Rhizo	17	-	0.1073	3.1648	1.0000	0.4338	2.5572	*n.d.*	7.1558	0.0367	2.6868	0.2192	0.5081	3.4141	10.5699
Rhizo	18	-	0.0182	7.4309	2.0090	1.9290	5.1138	0.1073	16.5899	0.1066	1.4967	0.1714	0.4720	2.1401	18.7300
Rhizo	13	65	0.1801	2.0484	0.8648	0.2926	2.4543	0.3478	6.0078	0.0265	0.0645	0.4118	0.1776	0.6539	6.6617
Rhizo	9	27	0.4621	1.4304	0.4584	0.1881	1.2199	0.1699	3.4667	*n.d.*	*n.d.*	0.2648	0.0772	0.3420	3.8087
Rhizo	19	-	0.1114	3.1614	1.2492	0.6438	2.5442	*n.d.*	7.5986	0.1662	0.6561	0.1777	0.5555	1.3893	8.9879
Rhizo	12	4	0.6704	1.9416	0.5832	0.2102	2.3226	0.3826	5.4402	*n.d.*	0.0431	0.2731	0.1390	0.4552	5.8954
Rhizo	8	65	1.6109	1.7083	0.5320	0.2284	1.4164	0.2074	4.0925	*n.d.*	*n.d.*	0.1772	0.0641	0.2413	4.3339
Seed	14	220	0.0173	1.8075	0.4758	0.2069	1.6349	0.4536	4.5787	*n.d.*	0.0313	2.6229	0.3523	3.0065	7.5852
Seed	13	83	0.0285	2.3191	0.8649	0.2535	2.9307	0.5361	6.9043	*n.d.*	0.2113	2.2780	0.3303	2.8197	9.7240
Seed	18	317	0.0497	2.9471	1.3397	0.3513	4.2987	*n.d.*	8.9368	0.2446	3.6229	0.6534	0.6965	4.9728	13.9096
Seed	15	33	0.0780	2.2961	0.5409	0.2070	2.2285	0.6242	5.8967	*n.d.*	0.1371	2.4250	0.2578	2.8200	8.7166
Seed	16	248	0.1641	2.1070	0.8012	0.2513	2.8202	*n.d.*	5.9797	0.1112	3.3729	0.3532	0.5167	4.2428	10.2225
Seed	17	866	0.2074	2.7090	0.8201	0.3327	2.8377	*n.d.*	6.6994	0.2108	3.1925	0.5063	0.5824	4.2812	10.9806
Seed	19	-	0.1391	1.5053	0.4344	0.1864	1.4046	*n.d.*	3.5307	0.0887	1.1986	0.1698	0.4246	1.7930	5.3236
Seed	20	-	0.0616	2.7122	1.1238	0.3196	4.0032	*n.d.*	8.1588	0.1925	4.0852	0.4450	0.5934	5.1236	13.2824
Seed	21	-	0.0812	2.5281	1.0488	0.2378	3.7028	*n.d.*	7.5176	0.2032	2.4627	0.4298	0.6705	3.5631	11.0807
Seed	22	-	0.0421	2.6806	0.8110	0.3473	2.1992	*n.d.*	6.0381	0.2032	3.3397	0.4344	0.9206	4.6947	10.7328
Seed	23	-	0.0225	3.3491	1.0885	0.5439	2.9960	*n.d.*	7.9775	0.2832	2.0217	0.3457	0.5732	2.9406	10.9181
Seed	24	-	0.0402	2.9447	1.0317	0.4687	2.3197	*n.d.*	6.7649	0.2398	2.4606	0.2577	0.6801	3.3984	10.1632

Table 46 Glucosinolate concentrations (μmol mg^{-1}) and *soxB* quantification (AU) for cabbages grown in "sterile" (autoclaved) soil, harvested at 8 weeks - *Control (SC) and sulphur addition (HSS) data.*

Treatment	Rep	Aphids	soxB quant	IBE	PRO	RAPH	SIN	GNA	ALIPHATIC	4OH	GBC	4MeOH	NEO	INDOLE	TOTAL GLS
SC	14	38	0.2186	1.2899	0.3722	0.1665	2.3193	0.4752	4.6232	*n.d.*	0.0948	2.7332	0.1593	2.9873	7.6105
SC	16	51	0.5654	0.9925	0.4229	0.1219	2.0001	*n.d.*	3.5374	*n.d.*	1.4972	0.0879	0.3555	1.9406	5.4779
SC	13	127	0.6004	1.5179	0.4367	0.2401	2.3133	0.2359	4.7440	*n.d.*	0.0258	0.6305	0.1147	0.7710	5.5150
SC	12	40	0.7454	0.7777	0.3155	0.1345	1.4588	0.2695	2.9560	*n.d.*	0.0247	1.9210	0.1332	2.0789	5.0349
SC	15	120	1.2277	0.9970	0.2389	0.1477	1.4678	0.3208	3.1722	*n.d.*	0.0310	1.5697	0.1386	1.7393	4.9116
SC	17	-	2.1943	1.3420	0.4922	0.1594	2.2039	*n.d.*	4.1975	0.0660	2.0237	0.1355	0.5572	2.7163	6.9139
SC	21	-	2.4627	*n.d.*	0.1295	*n.d.*	0.6958	*n.d.*	0.8252	0.2303	5.9474	0.1768	0.7356	6.8598	7.6850
SC	11	115	2.4746	0.4118	0.2486	0.0544	0.9623	0.4013	2.0785	*n.d.*	0.0792	4.4482	0.1310	4.6584	6.7369
SC	19	86	3.0387	0.4971	0.1467	*n.d.*	0.4455	*n.d.*	1.0893	0.0488	7.3026	0.1551	1.6187	9.0764	10.1656
SC	18	-	11.0880	1.0610	0.3982	0.1059	1.5191	*n.d.*	3.0842	0.0173	1.8427	0.0846	0.3346	2.2620	5.3462
HSS	11	201	16.2558	0.7906	0.2737	*n.d.*	2.1504	0.5896	3.8043	*n.d.*	0.0411	1.3018	0.1395	1.4825	5.2868
HSS	13	58	30.7902	0.2648	0.1298	*n.d.*	1.4252	0.3145	2.1343	*n.d.*	0.0700	0.7341	0.1090	0.9131	3.0474
HSS	14	-	74.7655	1.1220	0.4397	0.1378	1.8105	0.7075	4.2176	*n.d.*	0.0417	1.7699	0.1163	1.9279	6.1455
HSS	12	46	90.2879	1.3367	0.5325	0.1373	3.8159	0.3845	6.2069	*n.d.*	0.0786	1.0039	0.1506	1.2331	7.4400
HSS	16	-	98.8407	0.9442	0.1604	0.0826	1.1293	*n.d.*	2.3165	0.1181	2.0987	0.1344	0.5030	2.7362	5.0527
HSS	15	58	149.0494	1.1787	0.7316	0.1201	2.8237	0.4324	5.2865	*n.d.*	0.0451	1.3439	0.1192	1.5082	6.7947
HSS	15	-	149.0494	1.1787	0.7316	0.1201	2.8237	0.4324	5.2865	*n.d.*	0.0451	1.3439	0.1192	1.5082	6.7947
HSS	12	46	334.5743	1.3367	0.5325	0.1373	3.8159	0.3845	6.2069	*n.d.*	0.0786	1.0039	0.1506	1.2331	7.4400

Table 47 Glucosinolate concentrations (µmol mg^{-1}) and *soxB* quantification (AU) for cabbages grown in "sterile" (autoclaved) soil, harvested at 8 weeks - Data for plants treated with sterile soil pre-incubated with *T. thioparus* for 7 (D7) or 14 (D14) days.

Treatment	Rep	Aphids	soxB quant	IBE	PRO	RAPH	SIN	GNA	ALIPHATIC	4OH	GBC	4MeOH	NEO	INDOLE	TOTAL GLS
D7	5	192	13.3050	0.9215	0.2918	0.1484	2.0222	*n.d.*	3.3838	0.0569	3.7419	0.2225	0.6448	4.6092	7.9930
D7	6	194	5.3157	1.6621	0.2668	0.1824	2.0368	0.4306	4.5786	0.0235	0.0626	1.4215	0.1399	1.6240	6.2026
D7	7	115	2.1345	1.8759	0.7528	0.3067	3.3414	0.4107	6.6874	0.0916	0.0778	2.1811	0.1562	2.4152	9.1026
D7	8	240	70.1448	0.8498	0.2087	0.1156	1.4064	0.2599	2.8405	0.0122	0.0308	1.6599	0.1236	1.8143	4.6547
D7	9	-	10.2199	*n.d.*	*n.d.*	*n.d.*	0.4287	0.1720	0.6008	*n.d.*	0.0296	1.5275	0.0738	1.6309	2.2316
D7	10	-	3.0292	1.4586	0.3409	0.2009	2.0856	0.5279	4.6139	0.0239	0.0607	1.5087	0.1381	1.7076	6.3215
D7	11	-	3.0903	1.3884	0.3102	0.1506	2.2668	0.8201	4.9360	*n.d.*	0.1494	2.0334	0.1821	2.3649	7.3009
D7	12	-	7.6603	0.7525	0.3999	0.1040	1.4899	0.4242	3.1704	*n.d.*	0.0734	2.9679	0.1068	3.1481	6.3185
D14	4	112	2.0278	1.5191	0.4138	0.2197	2.0755	0.4006	4.6287	*n.d.*	0.0748	1.0839	0.1421	1.3008	5.9295
D14	5	330	20.0063	0.9877	0.3298	0.1095	1.5123	n.a.	2.9392	0.1035	1.0566	0.1915	0.4691	1.7172	4.6564
D14	6	247	1.6307	1.9572	0.3041	0.2596	1.8908	0.4914	4.9032	*n.d.*	0.1164	0.5079	0.1343	0.7585	5.6617
D14	7	329	0.8650	0.8909	0.2416	0.1323	1.5874	0.5384	3.3906	0.0196	0.0726	0.9536	0.1975	1.2236	4.6142
D14	8	227	7.1802	3.1005	0.7871	0.4332	3.5965	0.5310	8.4483	0.0473	0.1008	0.9700	0.2514	1.3221	9.7704
D14	9	321	3.8509	*n.d.*	*n.d.*	*n.d.*	0.2846	0.3503	0.6349	*n.d.*	0.0371	1.7988	0.1150	1.9508	2.5857
D14	10	-	4.0611	1.0453	0.4122	0.1258	2.0106	0.7894	4.3835	*n.d.*	0.0611	5.4957	0.2049	5.7618	10.1453
D14	11	-	1.6121	0.9903	0.3079	0.1358	1.4989	0.8070	3.7398	*n.d.*	0.0532	5.5830	0.1832	5.8194	9.5592
D14	12	-	4.9737	1.1114	0.4315	0.1165	2.0579	0.9483	4.6656	*n.d.*	0.1002	4.9298	0.2108	5.2407	9.9063
D14	13	-	4.7532	2.0084	2.0487	1.1807	2.6090	0.2230	8.0698	0.2286	0.1990	1.9730	0.0628	2.2348	10.3046
D14	14	-	2.5095	0.6799	0.2913	0.0858	0.9633	0.5749	2.5952	*n.d.*	0.0478	6.7333	0.1824	6.9635	9.5587
D14	15	-	15.8188	0.2562	0.2169	*n.d.*	0.6263	0.7092	1.8086	*n.d.*	0.1135	7.0219	0.1451	7.2805	9.0892

Table 48 Glucosinolate concentrations (μmol mg^{-1}) and soxB quantification (AU) for cabbages grown in "sterile" (autoclaved) soil, harvested at 12 weeks - Control (SC) and sulphur addition (HSS) data.

Treatment	Rep	Aphids	soxB quant	IBE	PRO	RAPH	SIN	GNA	ALIPHATIC	4OH	GBC	4MeOH	NEO	INDOLE	TOTAL GLS
SC	1	1	4.1111	3.1789	0.9393	0.0008	2.0662	*n.d.*	6.1852	0.029	2.4356	0.1429	0.716	3.2944	9.4796
SC	2	4	0.1835	2.0963	0.591	0.3725	1.268	0.4815	4.8092	0.0224	0.05	0.8133	0.1164	0.9797	5.789
SC	3	150	2.0222	2.4744	0.7318	0.3679	2.1985	0.3987	6.1714	*n.d.*	0.0774	0.5212	0.0884	0.6869	6.8584
SC	4	44	0.6853	3.5233	0.9014	0.6229	2.2533	0.651	7.9519	*n.d.*	0.0729	0.7815	0.1782	1.0325	8.9844
SC	5	8	2.2711	2.9156	0.76	0.5291	1.7954	*n.d.*	6.0001	0.0823	0.6993	0.1275	0.4081	1.2349	7.235
SC	6	-	0.3773	1.3625	0.4917	0.1485	1.6166	0.2959	3.9152	*n.d.*	0.0239	0.9873	0.0932	1.1043	5.0195
SC	7	-	2.2978	3.4336	0.8756	0.4806	2.6771	0.3595	7.8265	*n.d.*	*n.d.*	0.481	0.1115	0.5925	8.419
SC	8	-	0.118	3.2091	1.0437	0.5316	2.8222	0.5484	8.155	0.0385	0.0616	1.8968	0.1692	2.1275	10.2826
SC	9	-	1.1842	2.6777	0.7691	0.4278	2.2214	0.3905	6.4866	*n.d.*	0.0675	0.7094	0.0908	0.8677	7.3543
SC	10	-	0.4311	3.6578	1.228	0.8977	2.1266	0.4193	8.3294	0.0744	0.0391	0.5522	0.1405	0.7319	9.0613
SC	20	123	10.2453	1.149	0.2694	0.1397	0.8311	*n.d.*	2.3892	*n.d.*	1.8466	0.0741	0.5693	2.49	4.8792
SC	22	409	0.9072	2.7251	0.6803	0.3927	2.3956	0.0763	6.2701	0.0565	1.8013	0.2847	0.4145	2.5004	8.7705
SC	23	200	3.0505	3.5133	1.3274	0.4827	4.4264	0.1197	9.8695	0.0761	1.5734	0.1641	0.4138	2.1513	12.0208
SC	24	-	2.4956	3.1056	1.2089	0.357	3.9356	0.1157	8.7228	0.0298	2.6495	0.2477	0.6203	3.5175	12.2402
SC	25	-	0.6196	4.4273	2.0247	0.9754	4.1784	0.1633	11.769	0.1248	2.8888	0.358	0.5663	3.8131	15.5821
HSS	1	5	48.2459	5.5714	1.7761	0.8686	3.5461	0.1625	11.9247	0.0872	1.9596	0.2412	0.6924	2.8932	14.8179
HSS	2	9	18.1686	7.7618	2.4108	1.4952	4.6751	0.5363	16.8793	0.2373	0.1481	1.264	0.2929	1.705	18.5842
HSS	3	3	15.4869	5.218	1.2047	0.9454	2.3127	0.6091	10.2898	0.0624	0.1187	0.8117	0.1456	1.076	11.3658
HSS	4	-	18.8142	9.3561	2.4257	1.937	4.2076	0.204	18.1304	0.1608	0.9152	0.2527	0.6397	1.8076	19.938
HSS	5	-	60.9445	5.8488	2.0053	1.2039	3.4848	0.1573	12.7002	0.08	0.9327	0.2078	0.4678	1.6083	14.3085
HSS	6	81	13.9872	4.6655	1.9443	0.8858	3.4859	0.5348	11.5162	0.2111	0.1147	0.8566	0.1768	1.148	12.6642
HSS	7	51	4.2728	4.3073	1.9178	0.6537	4.5501	0.6183	12.0472	0.1865	0.0993	1.3578	0.1838	1.6409	13.6881
HSS	8	-	4.0598	4.181	1.6251	0.6547	3.5609	0.6858	10.7076	0.0991	0.079	3.0269	0.2016	3.3075	14.0151
HSS	9	-	3.7115	5.3629	1.9956	0.7013	5.5664	0.6138	14.24	0.1825	0.083	2.9025	0.298	3.2835	17.5235
HSS	10	-	21.4785	5.2227	1.344	1.0182	2.591	0.6235	10.7993	0.1176	0.1402	1.1281	0.2061	1.4744	12.2737
HSS	17	313	44.1088	3.6207	0.9208	0.4858	3.5543	0.0878	8.6694	0.0852	2.8842	0.443	0.6588	3.9859	12.6553
HSS	18	-	216.9793	0.4127	0.1002	*n.d.*	*n.d.*	*n.d.*	0.5129	*n.d.*	0.9228	0.6297	0.1938	1.7464	2.2593

Table 49 Glucosinolate concentrations (μmol mg^{-1}) and *soxB* quantification (AU) for cabbages grown in "sterile" (autoclaved) soil, harvested at 8 weeks - Data for plants treated with sterile soil pre-incubated with *T. thioparus* for 7 (*D7*) or 14 (*D14*) days.

Treatment	Rep	Aphids	soxB quant	IBE	PRO	RAPH	SIN	GNA	ALIPHATIC	4OH	GBC	4MeOH	NEO	INDOLE	TOTAL GLS
D7	1	12	7.3008	3.6983	0.9104	0.7931	1.8221	*n.d.*	7.2239	*n.d.*	0.6521	0.0861	0.5043	1.2425	8.4664
D7	2	17	0.7596	3.0159	0.9248	0.6496	2.1168	0.6964	7.4036	0.0822	0.0514	0.3668	0.1209	0.5391	7.9426
D7	3	8	0.8532	2.4916	0.9178	0.5571	1.7237	0.4953	6.1854	0.0768	0.0318	0.3247	0.0970	0.4535	6.6389
D7	4	-	0.7693	3.6262	0.9417	1.0243	1.7514	0.4689	7.8124	0.0705	0.0691	1.5350	0.1357	1.7398	9.5522
D7	13	31	5.9984	3.4191	0.8459	0.6423	2.7516	0.4135	8.0724	*n.d.*	0.0747	1.0932	0.1238	1.2918	9.3642
D7	14	29	2.9784	2.8905	1.0402	0.4844	2.5984	0.5443	7.5577	*n.d.*	0.0731	2.7459	0.2021	3.0211	10.5788
D7	15	138	19.5969	3.4317	1.0159	0.4349	3.4206	0.3918	8.6948	0.0563	0.1474	1.1954	0.2134	1.5562	10.2510
D7	16	173	0.9866	3.7531	0.8828	0.4449	3.1351	0.0628	8.2786	0.1303	0.8024	0.1946	0.2429	1.2400	9.5185
D7	17	192	9.5251	3.5231	1.1116	0.4404	4.2325	0.0941	9.4017	0.1119	2.1267	0.3061	0.4573	2.8901	12.2919
D7	18	-	22.3857	4.0200	1.6813	1.9888	2.2877	0.1204	10.0982	0.0702	2.0038	0.1535	0.2451	2.4025	12.5007
D7	19	-	2.4733	3.0511	0.9487	0.3438	2.9646	*n.d.*	7.3081	0.0343	3.8440	0.2007	0.5419	4.5866	11.8947
D7	20	-	3.9164	3.1291	1.2359	0.3930	3.9090	*n.d.*	8.6670	0.1375	2.2274	0.1971	0.4001	2.8245	11.4915
D7	21	-	1.2841	3.6011	0.8697	0.4102	3.6484	*n.d.*	8.5294	0.0657	1.3876	0.1897	0.4229	2.0001	10.5295
D7	22	-	0.5266	4.2541	1.0707	0.6047	3.7589	0.1264	9.8148	0.0505	1.5109	0.2119	0.5812	2.3040	12.1188
D7	23	-	2.6176	4.5932	1.8575	0.6895	4.6626	0.1626	11.9654	0.0812	1.4842	0.2046	0.3442	2.0330	13.9984
D14	1	65	0.3958	3.1218	0.9374	0.4545	2.4781	0.0806	7.0725	0.0460	0.5545	0.1729	0.5326	1.2600	8.3324
D14	2	26	0.0365	5.4062	1.3805	0.8312	3.3756	0.6243	11.6177	0.1045	0.0698	0.9107	0.1927	1.1733	12.7910
D14	3	-	0.0096	2.5066	0.6372	0.4004	2.0578	0.3229	5.9248	*n.d.*	0.0902	0.1522	0.0722	0.3145	6.2394

Table 50 ANOVA results for treatment effects on glucosinolate concentration, *soxB* abundance and aphid population counts under normal soil conditions. (Results denoted with * were one-way tests (not assuming equal variance)).

Treatment	Measurement	Transformation	*d.f.*	*F*		Tukey's Pairwise post-hoc	
					P	Pairwise comparison	*P*
Normal 8 wk no aphids	Aliphatic	None		7.981	**0.008***	Seed-NC	*0.027*
	Indole	Natural log (ln)	3,18	2.703	0.076		
	Total GLS	None	3,18	4.174	**0.0021**	Seed-NC	*0.0018*
	soxB	Natural log (ln)		6.852	**0.013***	Seed-Rhizo	*0.034*
Normal 12 wk no aphids	Aliphatic	None		0.871	0.487*		
	Indole	None	3,20	6.819	**0.002**	Seed-HSN Seed-NC Seed-Rhizo	*0.006* *0.006* *0.010*
	Total GLS	None	3,20	0.741	0.540		
	soxB	Natural log (ln)		0.519	0.768*		
Normal 8 wk + aphids	Aliphatic	Natural log (ln)	3,17	4.191	**0.022**	Rhizo-NC	*0.026*
	Indole	Natural log (ln)	3,17	8.20	**0.001**	Seed-NC Seed-HSN	*0.012* *0.001*
	Total GLS	None	3,17	4.44	**0.018**	Rhizo-NC	*0.020*
	soxB	Natural log (ln)	3,17	7.31	**0.002**	Rhizo-HSN Rhizo-NC Seed-Rhizo	*0.021* *0.002* *0.009*
	Aphids	None	3,17	0.675	0.579		
Normal 12 wk + aphids	Aliphatic	None	3,20	6.535	**0.003**	NC-HSN Rhizo-HSN Seed-HSN	*0.019* *0.002* *0.046*
	Indole	Natural log (ln)	3,20	35.270	**<0.001**	NC-HSN Rhizo-HSN Seed-HSN Seed-NC Seed-Rhizo	*0.013* *<0.001* *0.004* *<0.001* *<0.001*
	Total GLS	None	3,20	9.361	**<0.001**	NC-HSN Rhizo-HSN Seed-NC Seed-Rhizo	0.015 0.001 0.038 0.004
	soxB	Natural log (ln)	3,20	1.891	0.1635		
	Aphids	Natural log (ln)	3,20	2.37	0.101		

Table 51 ANOVA results for treatment effects on glucosinolate concentration, *soxB* abundance and aphid population counts under sterile soil conditions. (Results denoted with * were one-way tests (not assuming equal variance)).

Treatment	Measurement	Transformation	*d.f.*	*F*	*P*	Tukey's Pairwise Post-hoc	
						Pairwise comparison	*P*
Sterile 8 wk no aphids	Aliphatic	None	3,12	0.457	0.717		
	Indole	Natural log (ln)	3,12	5.643	**0.012**	D7-D14 HSS-D14	0.022 0.027
	Total GLS	None	3,12	10.348	**0.001**	D7-D14 HSS-D14 SC-D14	0.002 0.008 0.031
	soxB	(1/square root)	3,12	5.276	**0.015**	HSS-D14 SC-HSS	0.015 0.026
Sterile 12 wk no aphids	Aliphatic	None		1.109	0.379*		
	Indole	Natural log (ln)	2,17	2.036	0.161		
	Total GLS	None		1.249	0.335*		
	soxB	Natural log (ln)	2,17	11.265	**0.001**	SC-HSS HSS-D7	0.001 0.018
Sterile 8 wk, + aphids	Aliphatic	None	3,17	0.595	0.627		
	Indole	Natural log (ln)	3,17	2.567	0.089		
	Total GLS	None	3,17	0.583	0.634		
	soxB	Natural log (ln)	3,17	6.670	**0.004**	SC-D7	0.049
	Aphids	None	3,17	9.364	**0.001**	SC-D14 HSS-D14	0.001 0.013
Sterile 12 wk + aphids	Aliphatic	Natural log (ln)	2,18	14.539	**<0.001**	SC-HSS HSS-D7	<0.001 0.002
	Indole	Natural log (ln)	2,18	0.874	0.434		
	Total GLS	None	2,18	12.930	**<0.001**	SC-HSS HSS-D7	<0.001 0.002
	soxB	Natural log (ln)	2,18	9.468	**0.002**	SC-HSS HSS-D7	0.001 0.027
	Aphids	Natural log (ln)	2,18	0.156	0.857		

Table 52 Biometrics of *B. oleracea* receiving different N-doses, and *M. persicae* population counts after 14 days colonisation (*M. persicae* initial $n = 5$).

Treatment	Rep	No. Leaves	Senesced leaves	Stem height	Aboveground biomass (g)		Total Aphids	Aphids. g^{-1}. cabbage (fresh weight)
					Fresh weight	*Dried weight*		
Control	1	4	0	2.1	0.262	0.01	47	179.3893
Control	2	7	0	2.4	0.361	0.03	22	60.9418
Control	3	11	3	6.2	4.529	0.33	315	69.5518
Control	4	12	3	5.6	4.847	0.3485	45	9.2841
Control	5	12	0	6.1	3.4192	0.1793	39	11.4062
Control	6	11	2	4.8	4.347	0.2636	80	18.4035
Control	7	11	3	4.6	2.612	0.172	289	110.6432
Control	8	12	1	5	3.97	0.3006	203	51.1335
Control	9	7	0	3.2	1.21	0.0715	172	142.1488
Control	10	13	3	5.3	3.17	0.2575	152	47.9495
Low N	1	10	4	6.4	5.357	0.4556	385	71.8686
Low N	2	9	3	3	0.72	0.06637	191	265.2778
Low N	3	13	4	6.1	10.936	0.563	302	27.6152
Low N	4	13	4	6	8.9021	0.7163	69	7.7510
Low N	5	9	0	3.3	1.6361	0.119	181	110.6289
Low N	6	10	4	4.2	1.446	0.1367	151	104.4260
Low N	7	10	0	3.7	2.244	0.1294	213	94.9198
Low N	8	7	0	3.4	0.91	0.0934	31	34.0659
Low N	9	7	1	2.2	0.76	0.0701	43	56.5789
Medium N	1	13	2	6.5	9.05	0.5667	801	88.5083
Medium N	2	8	0	4.1	2.535	0.15929	248	97.8304
Medium N	3	13		6.1	8.08	0.66	201	24.8762
Medium N	4	15	2	3.1	2.1693	0.1219	86	39.6441
Medium N	5	14	6	5.4	4.099	0.2922	149	36.3503
Medium N	6	12	5	5.2	5.13	0.3766	388	75.6335
Medium N	7	15	6	6	6.39	0.608	45	7.0423
Medium N	8	17	6	6.4	12.31	0.7655	422	34.2811
Medium N	9	13	0	4.2	3.72	0.1803	73	19.6237
Medium N	10	8	0	3	0.89	0.1014	63	70.7865
High N	1	12	3	5.7	3.735	0.21	92	24.6319
High N	2	12	2	6	4.308	0.2291	238	55.2461
High N	3	13	5	6.3	9.753	0.7467	158	16.2001
High N	4	13	3	4.1	3.921	0.2584	67	17.0875
High N	5	8	2	1.8	0.6632	0.0442	18	27.1411
High N	6	12	4	5.9	5.54	0.4268	659	118.9531
High N	7	17	6	5.7	11.36	0.9764	307	27.0246
High N	8	15	3	6.7	8.13	0.6002	352	43.2964
High N	9	8	0	2.5	1.11	0.0971	221	199.0991

Table 53 Performance of *B. oleracea* under different *T. thioparus* and N-fertiliser combinations and final count of *M. persicae* after a two-week colonisation period (initial $n = 5$).

Treatment	Rep	Above-ground biomass (g)		No. Leaves	Senesced leaves	Stem Height (cm)	Aphid count
		Fresh weight	Dried weight				
Sterile control	1	1.96	140	7	1	1.5	-
Sterile control	2	4.55	356.9	8	0	3.8	-
Sterile control	3	3.45	281.1	8	0	2.8	-
Sterile control	4	4.99	357.8	10	1	3.3	0
Sterile control	5	5.04	336.4	8	2	3.3	287
Sterile control	6	5.27	284.3	9	0	2.6	93
T.t. + H2O	1	3.26	247.9	8	0	2.1	-
T.t. + H2O	2	3.74	327.5	8	0	2.8	-
T.t. + H2O	3	3.93	341.8	8	0	3.4	-
T.t. + H2O	4	3	223.7	8	0	3	215
T.t. + H2O	5	5.04	372.5	9	2	3.6	132
T.t. + H2O	6	4.37	342	8	1	3.1	48
T.t. + 0.62g Chem	1	1.48	413.8	7	0	1.9	-
T.t. + 0.62g Chem	2	1.44	99.6	6	0	2.3	-
T.t. + 0.62g Chem	3	1.49	108.94	7	2	2	-
T.t. + 0.62g Chem	4	0.77	127.3	5	0	1.7	126
T.t. + 0.62g Chem	5	2.29	50.5	7	1	2.6	92
T.t. + 0.62g Chem	6	1.07	138.1	6	1	1	75
T.t. + 0.62g Chem	7	1.8141	66	7	2	2.1	344
T.t. + 0.74g Chem	1	4.89	127.6	7	0	2.4	-
T.t. + 0.74g Chem	2	1.12	194.9	6	0	2.2	-
T.t. + 0.74g Chem	3	5.1174	88.2	9	2	4.2	404
T.t. + 0.74g Chem	4	1.822	134.8	7	3	2.1	356
T.t. + 0.74g Chem	5	1.8319	110.8	6	1	2.6	244
N:S 0:1	1	3.89	266	8	0	2.1	-
N:S 0:1	2	3.81	292.3	8	0	1.9	-
N:S 0:1	3	2.78	231.1	8	0	2.5	-
N:S 0:1	4	5.82	404.7	9	2	3.9	107
N:S 0:1	5	5.89	343.9	9	2	3.5	0
N:S 0:1	6	3.86	209.9	8	2	3.2	64
N:S 0:1	7	5.8535	408.5	9	2	3.9	622
N:S 1:1	1	3.03	208.6	8	0	2.2	-
N:S 1:1	2	6.45	521.4	9	0	2.8	-
N:S 1:1	3	2.09	101.2	7	0	3.1	129
N:S 1:1	4	2.95	161	7	0	2.6	175
N:S 1:1	5	7.7328	509.7	10	4	3.6	608
N:S 10:1	1	3.36	242	8	0	1.9	-
N:S 10:1	2	4.86	376.6	9	0	3.3	-
N:S 10:1	3	4.73	371.2	9	0	3.2	-
N:S 10:1	4	3.06	158.8	8	3	2.7	0
N:S 10:1	5	4.63	231.2	9	3	3.4	4
N:S 10:1	6	0.24	19.1	5 *dead plant*		1.2	0 *dead*
N:S 10:1	7	6.3507	422.7	9	2	3.4	627

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