Effects of copper sprays on microbial communities in kiwifruit orchard soils

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Abstract

There has been a significant increase in the use of antimicrobial copper sprays on kiwifruit orchards in the Western Bay of Plenty (WBOP) since the first outbreak of the bacterial vine disease *Pseudomonas syringae* pv. *actinidiae* (Psa-V) in 2010. Studies have revealed that an accumulation of copper in soil may adversely affect soil microorganisms, which are pivotal in essential soil functions such as nutrient assimilation.

The aim of this study was to determine whether or not the use of copper sprays on WBOP kiwifruit orchards has had detrimental effects on soil microorganisms and if this has resulted in changes to the structure of soil microbiological communities.

Soil was sampled from ten WBOP kiwifruit orchards with varying levels of copper but with similar physicochemical properties. These included five sets of two adjacent orchards, of which one was organic and the other conventional, and for which data was available on soil copper levels for 2011.

DNA extracted from each soil was amplified with PCR and sequenced using Ion Torrent Sequencing technology. The resulting bacterial and archaeal sequences were binned into operational taxonomic units (OTUs) and the phylogeny of each was determined using the Ribosomal Database Project (RDP) Classifier. Soils were also analysed for a number of physicochemical properties including total, bioavailable and free ionic copper.

Non-metric Multidimensional Scaling (NMDS) and Multi-Response Permutation Procedures (MRPP) were used to examine dissimilarities between microbial communities in relation to soil copper levels, management practices and other physicochemical variables. Indicator species analyses were conducted on OTU abundance data to determine if particular OTUs were indicative of soils with the lowest and highest copper levels. Community data were also compared using a number of measures of diversity. Differences in physicochemical parameters between soils were analysed for significance using ANOVA and t-tests.

Results revealed that levels of total copper in soils have increased since 2011, with significant differences measured between each pair of orchards. Significant

differences in soil microbial communities were also revealed, with pH identified as the main driver of community composition within and between orchards and differences between orchards were also largely explained by management practices. Relative abundances of Archaea were significantly higher in conventional orchards and in soils with the highest levels of copper. However, overall community dissimilarities were not found to be related to soil copper levels. Indicator species analysis revealed that soils with < 30 mg kg^{-1} of total copper had a greater abundance of *Actinobacteria*, whilst soils with > 60 mg kg^{-1} had a higher representation of *Chlamydiae*, *Chloroflexi*, and *Thaumarchaeota*.

The conclusions of this research are that current copper levels in the study orchard soils have not significantly influenced soil microbial community composition and associated functions. However, the high number of influential variables confounded attempts at identifying differences due to copper alone. In addition, the lack of knowledge on the specific functions of individual taxa, make it very difficult to reveal the long-term implications of even subtle differences in community composition as a result of copper use.

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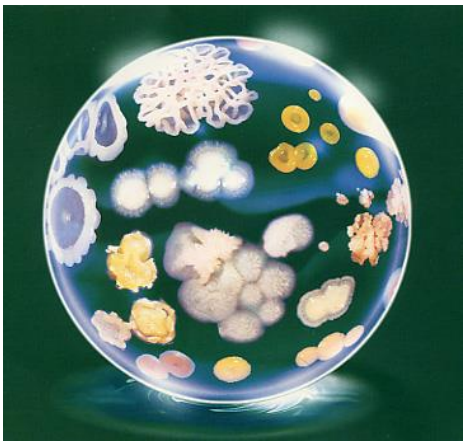

A biological universe in a gram of soil

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1 Introduction

1.1 Background to study

The bacterial disease of kiwifruit *Pseudomonas syringae* pv. *actinidiae* (Psa-V) was first positively identified in New Zealand in a Te Puke kiwifruit orchard in November 2010. Since then Kiwifruit Vine Health (KVH), the organisation whose directive is to minimise the impact of Psa-V on the NZ kiwifruit industry, have recommended spray applications of various copper formulations which have been shown to be effective at controlling the disease (Vanneste *et al.*, 2011). Prior to the Psa-V outbreak the use of copper was limited with some orchardists applying a copper sulphate spray in the autumn to promote leaf drop and as a protectant after pruning. However, since 2011 the rate of copper spray applications has increased dramatically. As evidenced by Zespri spray diaries a number of kiwifruit orchards in the Te Puke region have received up to and exceeding 20 copper sprays a year as part of a Psa-V management regime. However, many of these newer copper formulations such as copper oxide, copper hydroxide and copper oxychloride contain fewer soluble copper ions than copper sulphate and are therefore effective at much lower concentrations (Mistry, 2012). 'Bio-Gro' orchardists growing for the organic market are currently permitted to use 6 kg ha^{-1} $year^{-1}$ of copper (Cu), 3 kg more than the maximum allowed prior to Psa-V and 2 kg less than the 8 kg ha^{-1} $year^{-1}$ currently permitted for conventional growers. There are currently no alternatives for copper approved for organic orchards, however many organic growers have opted not use copper sprays out of concerns around potential detrimental effects to soil and the wider environment.

The antimicrobial properties of copper make it a very effective bactericide and fungicide and it has traditionally been applied to various crops for controlling plant diseases. Bordeaux mixture, which is a mixture of copper sulphate and lime, has been used for the control of fungal diseases in grapes and other crops since 1885 (Alloway, 2013). However, it is now widely accepted that significant contamination of agricultural soils can occur as a result of long-term use. Numerous scientific studies have been carried out on copper contaminated soils worldwide. Some studies have identified horticultural soils with levels exceeding 1,000 mg Cu kg^{-1} and up to 3,000 mg Cu kg^{-1} as was found in a >100 year old Brazilian vineyard (Alloway, 2013). Typical background soil copper

concentrations range between 2 and 50 mg Cu kg^{-1} (Alloway, 2013). In the Champagne region of France where Bordeaux mixture is widely used, a single application to a vineyard will add 3 - 5 kg Cu ha^{-1} and between three and ten applications may be made in a year (Alloway, 2013). A study by Besnard *et al*. (1999) found 100 - 1500 mg Cu kg^{-1} in Champagne vineyard soils compared with 5 - 30 mg Cu kg^{-1} in nearby arable soils.

Guinto *et al.* (2012) revealed a small but significant increase in copper levels in twelve kiwifruit orchard topsoils in the Western Bay of Plenty over a three year period between 2009 and 2012. Average copper concentrations increased from 35mg kg^{-1} to 39mg kg^{-1} over that time. A more recent study was carried out to determine how much soil copper levels in Te Puke orchards have increased between 2010 and 2014 since the increased use of protectant copper sprays (Dean & Miller, 2015). It was found that levels of copper in soil increased in response to increasing levels of copper applied, with the largest increase in a conventionally managed orchard (11 mg Cu kg^{-1} soil yr^{-1}) with a mean increase of 6.3 mg kg^{-1} soil $year^{-1}$. The concentration of copper in soil for one orchard had already reached the NZWWA (2003) guideline limit of 100 mg kg^{-1}, up from 55 mg kg^{-1} in 2010. Copper inputs ranged from 0.8 to 4.3 kg ha^{-1} yr^{-1} with an average of 1.8 kg ha^{-1} yr^{-1}, although these values may not account for copper sulphate applications which are used to promote leaf fall rather than to protect against Psa-V.

High rates of copper application to conventionally managed kiwifruit orchards in New Zealand have in some cases caused phytotoxic effects to vines as well as fruit staining (Max & Clark, 2012; Parker & Scarrow, 2011). As the tolerance to copper is much less for microbes than plants due to less well-developed homeostatic defence mechanisms (Alloway, 2008), it is likely that the adverse effects to soil microorganisms will be significant.

1.2 The Soil Environment

Soil is a complex ecosystem consisting of a physical matrix of soil particles and organic matter which contains a large diverse biomass which both influences and is subject to complex biogeochemical processes. This biologically, chemically,

and physically diverse environment forms the foundation of terrestrial ecosystems (Dominati *et al*., 2010). In recent years the links between biotic and abiotic soil components, which together greatly influence ecosystem functioning, have been more widely recognised (Naveed *et al*., 2014).

1.2.1 Physical and Chemical properties

The physical structure of soil is made up of a mineral fraction comprising weathered parent material of different size fractions, organic matter, water and gases. The mineral fractions are classed as sand silt or clay depending on the size of the individual particles and the size of these particles determines the relative surface area of each soil fraction (Table 1.1).

Table 1.1: Particle size, number and surface area per gram of and silt and clay, assuming spherical particles and maximum diameter within each size range (From Atlas and Bartha, 1997).

Soil component	Diameter (mm)	No. particles/g	Surface area (cm^2/g)
Sand	0.05 - 2.00	90	11
Silt	0.002 - 0.05	5.78×10^6	454
Clay	≤ 0.002	9.03×10^{10}	8,000,000

The texture of a soil may be classified according to the relative percentage of each size class. The United States Department of Agriculture (USDA) soil textural triangle is commonly used as a guide to determine soil texture (Figure 1.1). Soil texture influences plant rooting, water holding capacity, and the water to air ratio inside soil aggregates which may provide habitat for soil dwelling organisms (Fortuna, 2012) (Figure 1.2). Soil aggregates are formed as mineral particles, organic matter and root hairs are bound together by fungal hyphae and polysaccharides. Metals such as iron (Fe) and aluminium (Al) also stabilise soil aggregates through the formation of organic complexes (Amezketa, 1999). As fine root hairs are often present within soil aggregates, these aggregates form part of the rhizosphere, the zone influenced by plant roots (Sylvia *et al*., 2005). A single soil aggregate had been likened to an entire functioning ecosystem (Beare *et al*., 1997, Coleman *et al*., 2004).

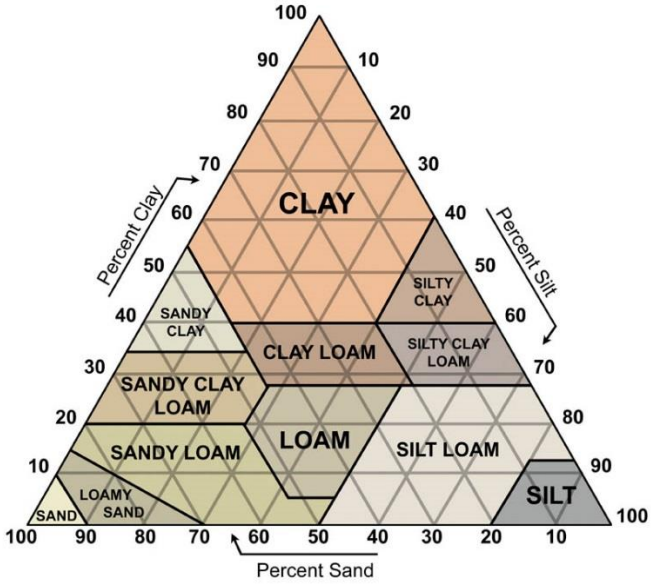


Figure 1.1: USDA Soil Textural Triangle (Soil Survey staff, USDA, 1975)

Figure 1.2: A soil aggregate or ped is a naturally formed assemblage of sand, silt, clay, organic matter, root hairs, microorganisms and their secretions, and resulting pores. (From: Fortuna, 2012)

1.2.2 Microorganisms in Soil

It has been estimated that a gram of soil may harbour over one billion individual microorganisms (Torsvik & Øvreås, 2002). The bacteria, archaea, fungi, protozoa and algae make up 90% of the total soil biomass (Liesack *et al*., 1997). In terms of

biomass, abundance, and function, bacteria/archaea and fungi are the most important microorganisms (Richards, 1994; Wollum, 1999).

1.2.2.1 *Microbial interactions and functions*

A number of interactions between microorganisms and other biota take place in soil. These can be broadly categorised as microorganism-microorganism, microorganism-plant and microorganism-'other' organism interactions (van Elsas *et al*., 2007). Interactions between microorganisms and soil invertebrates include direct predator-prey associations, and indirect associations which result in competition for resources and habitat formation and modification (Scheu *et al*., 2005). Microfauna such as amoebae, nematodes and protozoa predate bacteria and fungi and this balances growth and distribution whilst facilitating nutrient cycling through the release of ammonium (NH_4^+) (Clarholm, 1994; Irshad *et al*., 2011). Earthworms maintain soil structure and break down organic matter which also promotes the activity of microorganisms (Naveed *et al*., 2014).

Specific plant functional groups have been shown to greatly influence the abundance, composition and microbially driven processes of soil microorganisms. For example legumes which are associated with the nitrogen-fixing bacteria *Rhizobia*, and grasses which have a high root biomass and comparatively high amount of root exudates (Strecker *et al*., 2015; Roscher *et al*., 2012; Eisenhauer *et al*., 2010). The distribution of plant pathogens is also dependent on the presence of the host species. Plants therefore partially drive selective pressures on microbial communities, influencing community composition and diversity (Atlas & Bartha, 1997).

The ability of a soil to support plant growth is largely dependent on microorganisms which play a key role in decomposition and mineralisation processes (Loreau *et al*., 2001; Blaine-Metting, 1993). These processes drive nutrient cycling and therefore greatly influence soil fertility and subsequent plant growth. Plants also influence soil microbial communities by modifying light and temperature aboveground, and belowground within the rhizosphere where organic carbon from plant material and root exudates provide nutrients and alter soil pH (Atlas & Bartha, 1997). Higher plant diversity is also associated with higher microbial biomass (Zak *et al*, 2003; Eisenhauer *et al*., 2010). This is because a

greater diversity of plants provides a wider range of biochemical compounds which may be utilised by microorganisms (Grayston *et al.*, 1998) and there is less temporal variability in carbon supply (Milcu *et al*., 2010). Different plant species growing in the same soil may therefore support distinct microbial communities in the rhizosphere (Ibekwe & Kennedy, 1998; Marschner *et al*., 2001). These communities may also be similar when plants are grown in different soil types (Grayston *et al*., 1998; Miethling *et al*., 2000).

Microorganisms are responsible for catalysing a wide range of physiological processes in soil. Geochemical cycling is driven by microorganisms in which they derive metabolic energy. These processes result in nutrient assimilation, contaminant detoxification and the maintenance of conditions favourable to other biota (Madsen, 2005) (Figure 1.2).

Table 1.2: Examples of physiological processes catalysed by microorganisms in soil. Modified from Madsen (2005)

Process	Nature of Process
Carbon cycle	
C respiration	Oxidation of organic C to CO_2
Cellulose decomposition	Depolymerization, respiration
Aerobic CH_4 oxidation	CH_4 becomes CO_2
Nitrogen cycle	
N_2 fixation	N2 gas becomes NH3
NH4 + oxidation	NH3 becomes NO2 – , NO3 –
Denitrification	NO3 – is used as an electron acceptor and converted to N2 gas
Biodegradation	
Synthetic organic compounds	Decomposition, CO_2 formation
Petroleum hydrocarbons	Decomposition, CO_2 formation
Fuel additives (MTBE)	Decomposition, CO_2 formation
Nitroaromatics	Decomposition, CO_2 formation
Pharmaceuticals, personal care products	Decomposition
Chlorinated solvents	Compounds are dechlorinated through respiration in anaerobic habitats
Other elements	
H_2 oxidation	H2 is oxidized to H+, electrons reduce other substances

Soil bacteria which benefit plant health are often referred to as plant growth promoting rhizobacteria (PGPR) and are involved in: synthesis of plant available compounds, facilitation of nutrient uptake from soil, and minimising or preventing plant diseases (Hayat *et al*., 2010). These organisms may be either symbiotic such as *Rhizobium* spp., or *Azorhizobium* spp. or free-living such as *Azospirillum* spp., *Klebsiella* spp. and *Pseudomonas* spp., all of which are involved in nitrogen-fixing (Hayat *et al*., 2010). Strains belonging to the genus of *Azospirilum*, *Azotobacter*, *Klebsiella*, *Bacillus*, *Beijerinckia*, *Burkholderia*, *Enterobacter*, *Erwinia*, *Flavobacterium*, *Microbacterium*, *Pseudomonas*, *Rhizobium* and *Serratia* have been reported as having a pivotal role in phosphate solubilisation (Sturz & Nowak, 2000; Sudhakar *et al*., 2000; Mehnaz & Lazarovits, 2006). Many of these PGPRs have been used for some time to inoculate soils in order to enhance the nitrogen and phosphorous status (Bhattacharyya & Jha, 2012). As more plant-available nutrients are retained in the soil due to PGPR processes, there is less need for fertiliser applications (Hayat *et al*., 2010). A field study on the use of *Bacillus vietnamiensis* as an inoculant in rice plants resulted in greatly increased yield and a saving of 25 - 30 kg ha^{-1} of nitrogen fertiliser (Van *et al*., 2000).

Many soil bacteria produce antifungal and antibacterial compounds, plant growth regulators, and siderophores which are high affinity iron chelating compounds (Pandey & Kumar, 1989).

A number of rhizobacteria produce compounds that are toxic to a wide range of pathogenic organisms including some insects (e.g., cotton aphids), viruses (e.g., tomato mottle virus), and fungi (e.g., *Fusarium*) (Banerjee *et al*., 2006). Many of these strains have been commercialised and are widely utilised in agriculture where either plants or soil are inoculated with the bacteria. Examples of these include *Bacilus subtilus*, *B. amyloliquefaciens Paenibacillus* spp. *Pseudomonas fluorescens*, *Enterobacteriaceae*, *Azotobacter*, and *Azospirillum* (Hayat *et al*., 2010).

Certain types of rhizobacteria synthesise phytohormones, which are often referred to as plant growth regulators (PGRs), which although present in extremely low concentrations, have the ability to influence and regulate plant physiological processes (Dobbelaere *et al*., 2003). These PGRs have been classified into five

groups: auxins, gibberellins, cytokinins, ethylene and abscisic acid (Zahir *et al*., 2004).

Contaminant degrading bacteria are also of particular interest in the field of bioremediation. Specific bacteria such as *Ralstonia metallidurans* are able to tolerate high levels of metals in soil and are therefore used to remediate metal contaminated sites (Goris *et al*., 2001). Others such as *Streptomycetes acidiscabies* E13 and *Pseudomonas tolaasii* ACC23 produce siderophores which may protect plants against the inhibitory effects of nickel and cadmium respectively (Dimkpa *et al*., 2008; Dell'Amico *et al*., 2008).

1.2.2.2 ***Diversity and distribution***

Bacteria exhibit very high levels of local phylogenetic diversity where a single soil sample may contain tens of thousands of different phylotypes (Fierer, 2008). Schloss & Handelsman (2006) estimated that single gram of Alaskan soil would reveal over 480,000 unique bacteria based on more than 3% divergence in 16S rRNA gene sequences. Noguez *et al*, (2005) likened the distribution of bacteria in a 1g sample to that of vertebrates over one continent.

Diversity is a term used to describe the richness and evenness of a population. Whittaker (1972) proposed three kinds of diversity: Alpha – the number of species per plot; Beta – a ratio of total number of species to the average number of species; and Gamma – the total number of species across sites (McCune & Grace, 2002). In microbiology alpha diversity or richness is a measure of the total number of species in a given sample. In microbiological terms this relates to individual operational taxonomic units (OTUs). Evenness is a measure of the proportional abundances of OTUs in a sample. Microbial communities in soil tend to exhibit high levels of richness and evenness (Fierer, 2008). When the cumulative number of unique OTUs are plotted with sample size, a rarefaction curve is produced (Figure 1.3). The degree of linearity of the curve is proportional to the evenness of the sample population. If a curve is linear it also indicates that a sample contains a large number of rare OTUs and to elucidate the entire microbiome in terms of species richness would require a large sampling effort (Fierer, 2008).

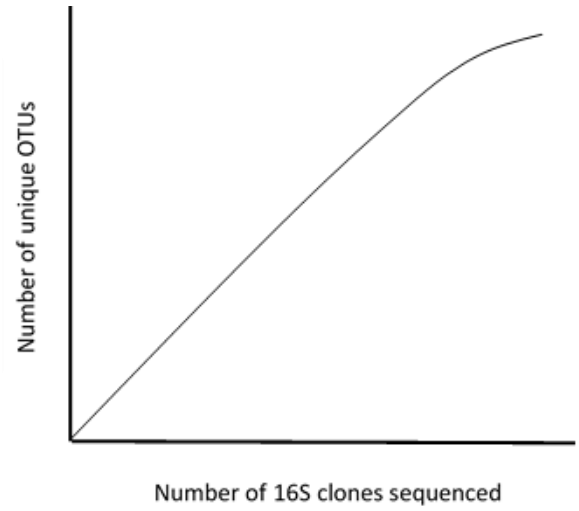


Figure 1.3: Example of a rarefaction curve where the number of unique OTUs observed is proportional to the number of clones sequenced

The Dutch microbiologist Baas Becking was one of the first scientists to attempt to explain the distribution of microorganisms over a large spatial scale. The often quoted phrase "*everything is everywhere, but the environment selects*" was coined by Baas Becking in 1934 as he presumed that there are no limitations to the dispersal of microorganisms and that their distribution is solely determined by environmental factors. The inspiration for Baas Becking's work came from the work of Martinus Beijerinck, the first professor of microbiology at Delft University in The Netherlands (de Wit & Bouvier, 2006).

It wasn't until the advent of modern sophisticated microbiological and molecular techniques in the mid-1990s that the biogeography of microorganisms began to be investigated in more detail. Techniques such as DNA fingerprinting were developed from earlier pioneering work by Carl Woese, who advanced the field of phylogenetic taxonomy in the 1970s by targeting 16S ribosomal RNA (rRNA). In the 1980s and 90s Norm Pace and colleagues at the University of Colorado used PCR to explore rRNA gene sequences and developed techniques by which to extract DNA directly from environmental samples (Pace *et al*., 1985, 1991). The amount of information gained from the use of 16srRNA techniques and genetic sequencing to reveal specific microorganisms from environmental samples has allowed the construction of comprehensive gene libraries. DeLong, along with Pace and colleagues, worked with marine samples and developed techniques using oligonucleotide probes which enabled the identification of different

phylogenetic groups without the need for culturing (DeLong *et al.*, 1989). The genetic information gained from these studies led to the construction of gene libraries initially based on marine microorganisms (DeLong *et al*., 1993). There are now comprehensive and increasingly expanding gene libraries which allow for the comparison of different organisms sampled from the environment. It is the comparison of specific microbial taxa obtained from environmental samples that is enabling a better understanding of diversity and allowing the study of microbial biogeography to advance. The advancement in sequencing techniques has also allowed greater resolution of environmental samples and the identification of previously unknown rare taxa, referred to as the "rare biosphere" (Sogin *et al*., 2006).

A study by Fierer and Jackson (2006) focussed on the concept of the biogeography of soil bacterial communities. This was possibly the first attempt at determining how entire soil bacterial communities are structured across large spatial scales. This was made possible by advances in DNA fingerprinting methods that allowed a high throughput of samples without the need for culturing. Soil bacterial diversity and richness and overall community composition was found to vary across ecosystem types and by far the best predictor of this variation was found to be pH (Figure 1.4). Unlike the previous studies on extremophiles, the conclusions of this study were that soil microbial biogeography is primarily controlled by environmental factors and is largely independent of geographic distance. They also suggested that microbial biogeography differs fundamentally from that of "macro" organisms for that reason.

A later study by Chu *et al*. (2010) on the diversity of soil bacteria in the Arctic also concluded that in terms of soil bacterial communities, the environmental variable pH is an important predictor of community-level differences. They also suggested that microbial biogeography differs fundamentally from that of macroorganisms and cannot be predicted by latitudinal gradients where genetic similarities correlate with geographic proximity to neighbouring areas, as is the case with macroorganisms.

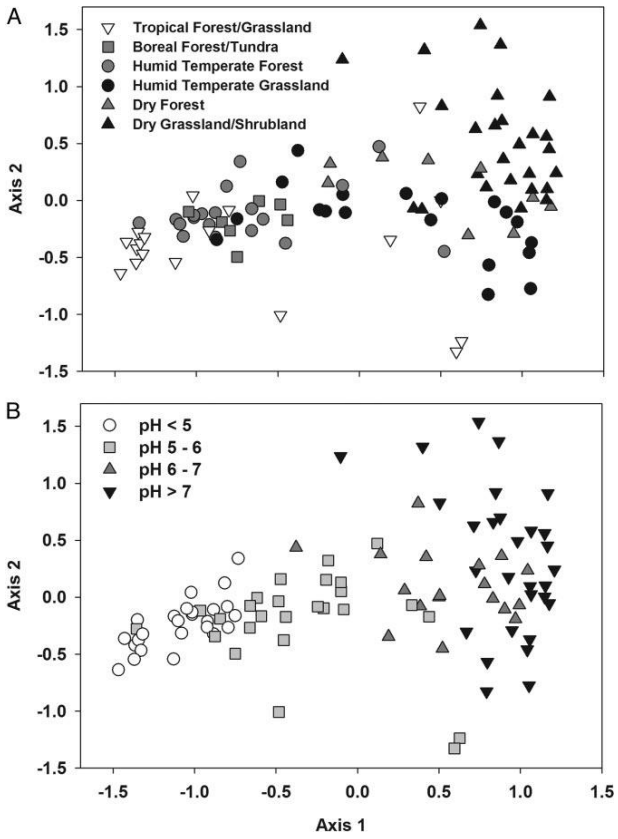


Figure 1.4: NMDS plot of soil bacterial communities overlaid with general ecosystem type and soil pH (From Fierer and Jackson, 2006)

Further evidence to support earlier suggestions that temporal factors play an important role in the distribution of microorganisms was provided in a paper by Bahl *et al*. (2011). In that study, cyanobacteria were sampled from soils of 19 hot and cold deserts around the globe. It was found that regionally distinct populations were present and that there was strong selection for hot or cold deserts. However, global distributions were limited by barriers to long-distance dispersal and predated the onset of contemporary aridity, indicating that regional genepools have been maintained over geological timescales.

A comprehensive study of British soils by Griffiths *et al*. (2011) identified distinct soil biomes each with microbial communities whose composition were influenced by a number of environmental variables including soil chemistry (especially pH), aboveground features and climatic variables. There was also a strong relationship between community dissimilarity and environmental dissimilarity and distance (Figure 1.5).

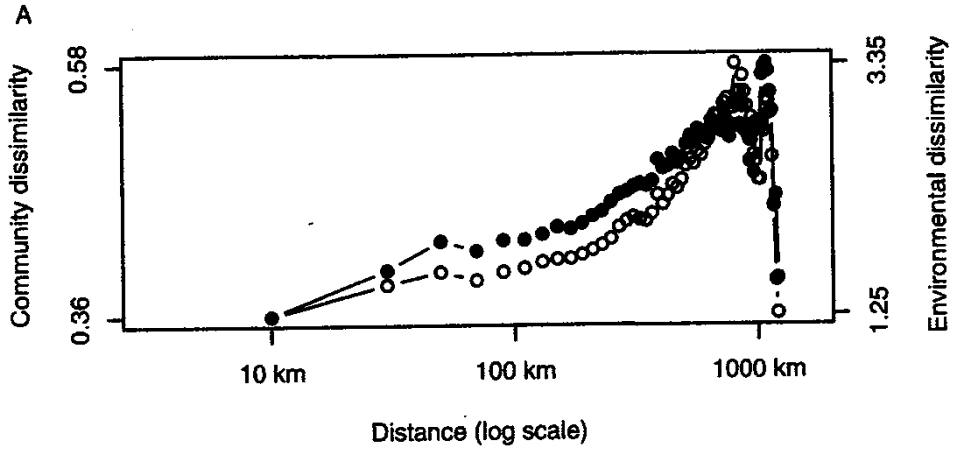


Figure 1.5: Relationships between community, spatial and environmental distances. The mean variance in community (filled circles) and environmental (open circles) dissimilarities with space (From: Griffiths *et al.*, 2011).

They also found that bacterial and plant communities were closely related, indicating that processes governing the biogeographic patterns of each may not be dissimilar (Figure 1.6).

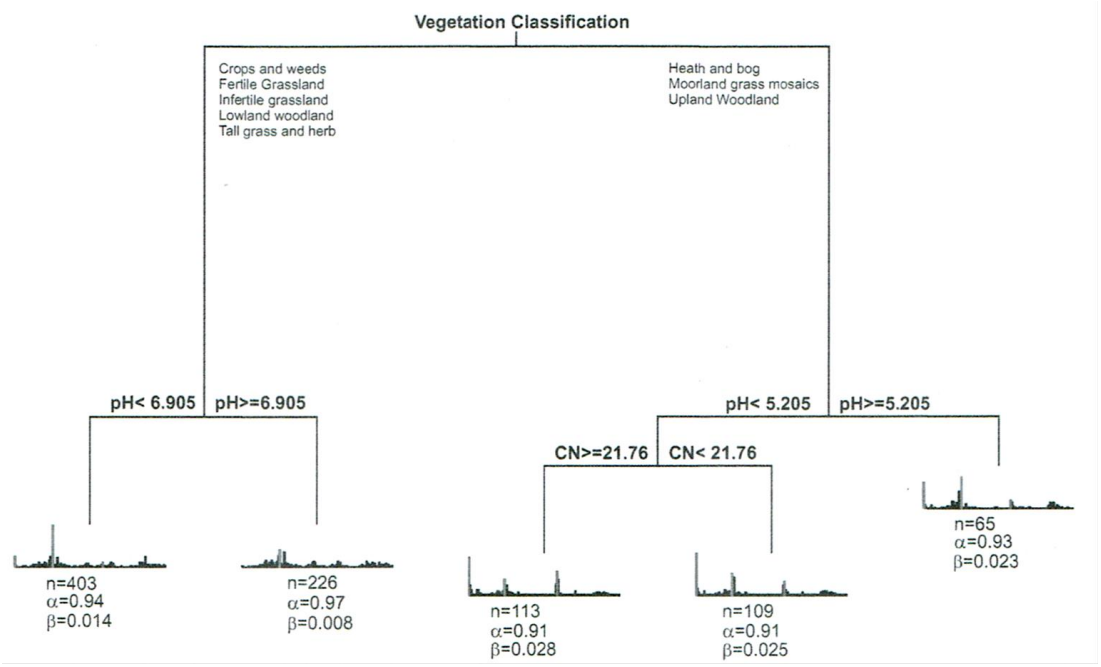


Figure 1.6: Multivariate regression tree summarizing community-environment relationships. Vegetation classification accounts for much of the 50% of the variability in genotypes that can be explained by environmental factors. (From: Griffiths *et al.*, 2011).

The findings of this study were somewhat mirrored by the results of a study of biogeographic patterns of soil archaea in flooded and non-flooded Chinese paddy field soils (Zheng *et al*., 2013). The conclusions of this study were that both environmental and spatial variables such as pH, sampling depth, and latitude/longitude were the main drivers of archaeal distribution and that these drivers may be similar to those that influence macroorganism communities.

1.2.3 Copper in the Environment

1.2.3.1 *Biogeochemistry*

Copper is a naturally occurring trace metal in soil and the background concentration in uncontaminated soil is generally related to the geology of the parent material, with levels ranging between 1 to 140 mg Cu kg^{-1} worldwide (Gadd, 2005; Romić *et al*., 2014). It is also a trace element that is essential in a number of biochemical processes in living organisms (Hooda, 2010).

Copper may be present in soil in a number of chemical forms. The principle forms of copper are the cuprous (Cu^{+}) and cupric (Cu^{2+}) cations although the Cu^{+} ion is unstable in typical agricultural soils and will readily oxidise to the more stable Cu^{2+} ion (Cornelis, 2005). Copper will bind with varying affinities to soil constituents generally in the following order: Mn oxides > organic matter > Fe oxides > clay minerals (Alloway, 2013).

Copper speciation and subsequent bioavailability and toxicity is influenced by a number of environmental and biotic factors which govern a range of chemical processes including redox reactions, complexation, dissolution/precipitation, and adsorption/desorption including those that are microbially mediated (Romić *et al*., 2014; Gadd, 2005). In most soils precipitation of Cu is limited and concentration is mainly controlled by sorption processes (Alloway, 2013). The mobility of copper in soil depends on the metal retention capacity of the soil which is a factor of the surface electrochemical properties of aluminosilicates, oxides, clay particles and organic matter. These mineral components will form complexes with copper and limit mobility and bioavailability in soil solution (Evangelou, 1998). The most important sink for copper in soil is organic matter with which it forms very strong, stable complexes and thus restricts the mobility, bioavailability and

toxicity of the cupric ion (Cu^{2+}) (McBride, 1994). Organic matter greatly influences the cation exchange capacity (CEC) of a soil due to the large number of negatively charged organic substances such as proteins, sugars and humic matter (Hooda, 2010). The Cu^{2+} ion is also bound more tightly to organic matter than any other divalent transition metal. In soil pore water Cu^{2+} binds to dissolved organic matter (DOM) (Alloway, 2013). The stability constants (log *K*) for divalent cations in soil with varying pH are shown below. Where the higher the number, the more stable the metal-organic complex.

pH 3.5 – Cu (5.8) > Fe (5.1) > Ni (3.5) > Pb (3.1) > Co (2.2) > Ca (2.0) > Zn (1.7) > Mn (1.5) > Mg (1.2)

pH 5 - Cu (8.7) > Pb (6.1) Fe (5.8) > Ni (4.1) > Mn (3.8)> Co (3.7) > Ca (2.9) > Zn (2.3) > Mg (2.1)

(From: Stevenson & Ardakani, 1972).

It is evident from the above sequences that the greater the pH of a soil, the more stable the metal ion complexes are. Therefore, changes to the pH of a soil will likely influence the bioavailablity of Cu^{2+} to soil biota. A positive correlation between pH and retention of copper in soil has been found in a number of studies (Tyler & McBride, 1982; Gupta & Aten, 1993) as has the decrease in the bioavailability of copper with an increase in organic matter and CEC (Lejon *et al*., 2008; McBride, 1981; Vulkan *et al*., 2000; Weng *et al*., 2002). Other studies have shown a decrease in plant tissue Cu concentrations with an increase in soil pH (Alva *et al*., 1993; Lexmond, 1980).

At the root/soil interface or rhizosphere, biochemical processes can greatly influence the speciation of copper in soil. Root-induced alkalisation of the soil may prevent copper exposure to the plant by reducing copper solubility and promoting the complexation of the Cu^{2+} ion (Bravin *et al*., 2012; Youssef & Chino, 1989). Root exudates may also directly bind free cupric ions, thereby reducing the bioavailability of copper to the plant (Bruus Pedersen *et al*., 2000).

The mobility of copper through the soil profile and potential leaching into deeper horizons and groundwater is dependent on the dissolved metal concentration in soil solution. This is dependent on solid-liquid partitioning and is usually quantified as the ratio of copper in the solid phase to that in the soil solution phase

and quantified as K_d. The free Cu^{2+} ion is considered to be that which is bioavailable, and its concentration in soil solution is dependent on the total metal pool in soil, K_d and speciation in solution (Alloway, 2013). The total metal loading and degree of saturation of sorption sites will also determine the amount of free copper in soil solution, and subsequent mobility. More pervious, coarser textured soils will also allow for greater migration of copper down the soil profile (Hooda, 2010). Although copper is readily adsorbed to organic matter in soil, mobility can be enhanced by an increase in the amount of dissolved organic matter (DOM) in soil solution. For example, an increase in DOM as a result of biosolids application to soil has been shown to increase copper mobility (Ashworth & Alloway, 2007; Heemsbergen *et al*., 2009). Plant growth will also result in a reduction in the activity of the free Cu^{2+} in soil solution due to complexation with dissolved organic carbon (Degryse *et al*., 2009; Römkens *et al*., 1999). Other land management practices that affect the organic matter status or pH of a soil will also influence the mobility and bioavailability of copper.

1.2.4 Copper toxicity

1.2.4.1 *Effects on plants*

Plants utilise copper in the production of key enzymes and proteins, which are involved in carbohydrate metabolism, reproduction, cell wall metabolism and water relations (Marschner, 1995). However, at high enough concentrations copper may become phytotoxic. This may come about through exposure to and uptake of copper through plant roots (Alva *et al.*, 1999) or from cuticular penetration of copper deposits into plant tissues (Orbović *et al*., 2007). Although uptake by plants is controlled by complex homeostatic mechanisms, which minimise detrimental effects (Clemens, 2001) and therefore direct toxicity to mature horticultural crops via soil exposure is relatively rare (Merrington *et al.*, 2002).

Elevated soil copper concentrations have also been shown to detrimentally affect plant/mycorrhizal associations thereby indirectly affecting the health of the plant by disrupting nutrient pathways (Georieva *et al*., 2002).

1.2.4.2 *Effects on microbial functions*

Some bacterial groups, including *Pseudomonas* and some species of symbiotic N_2-fixing *Rhizobium* are particularly sensitive indicators of copper pollution (Berg *et al.*, 2012; Alloway, 2010; Giller, 1998). McBride *et al.* (1981) found that copper in grape vineyard soils reduced the rate of ammonification, a process mediated by bacteria. The activity of soil microorganisms is particularly relevant at the soil/root interface, the rhizosphere. It is here that interactions between plant roots, microorganisms and soil constituents take place (Bowen and Rovira, 1999). Changes in the microbial community composition in the rhizosphere will affect the overall function of the entire soil biotic community and soil rhizosphere/plant interactions by disrupting key biogeochemical processes such as the degradation of organic matter and nutrient assimilation (Kunhikrishnan, 2011). A study by Kandeler *et al*. (1996) concluded that heavy metal pollution severely decreases the functional diversity of soil microbial communities and impairs specific pathways of nutrient cycling. The enzymes arylsulfatase and phosphatase which are involved in sulphur and phosphorous cycling were particularly affected.

Microorganisms also have the ability to degrade pesticides and herbicides such as atrazine (Dewey *et al*., 2012), glyphosate (Kim *et al.*, 2011) and DDT (Gaw *et al.*, 2006) and detrimental effects to these specific organisms may disrupt the associated beneficial processes.

In addition to the loss of beneficial functional traits, the microbial balance may also be altered in favour of pests and disease-causing organisms (Berg *et al*., 2010).

1.2.4.3 *Effects to microbial communities in soil*

Significant changes in the microbial community structure in soils can arise as a result of copper contamination and associated toxicity to microorganisms. Altered composition of microbial communities as a result of long-term copper inputs to growing systems has been highlighted in a number of papers where copper-tolerant strains dominate (Berg *et al.*, 2012; Mertens *et al.*, 2010; Viti *et al*., 2008; Zhou *et al*., 2011). The phenomenon of pollution- induced community tolerance (PICT) arises where microbial communities exposed to a toxic chemical select for

microbes resistant to that chemical (Wakelin *et al.*, 2014). Navel *et al.* (2010) observed variations in microbial communities which were significantly correlated with the concentration of copper in soil and also with compost amendments. The soils with the most bioavailable copper had an enrichment of *Actinobacteria*. These results concurred with those of Lejon *et al.* (2008) in which organic matter status was shown to control the effects of copper on microbial communities. That particular study looked at the impact of copper in soil with variable organic status on soil microbial communities. The results showed that copper contamination resulted in shifts in bacterial-ARISA profiles. The bands in the region 350 to 450 bp were cloned and sequenced and the data showed that 57% of these clones belonged to *Actinobacteria*, 27% to *Gemmatimonadetes*, 13% to *Proteobacteria*, and 3% to the *Fibrobacteres* class. In a study carried out on copper-polluted citrus groves in Florida, sequencing of partial 16S rRNA gene fragments revealed that the more polluted soils were dominated by *γ-Proteobacteria*, *Acidobacteria*, *Firmicutes*, and *β-Proteobacteria* (Zhou *et al*., 2011).

Naveed *et al.* (2014) highlighted a loss of soil biodiversity and functions along a Cu gradient, where bacterial richness and diversity as well as dehydrogenase activity decreased sharply at levels above 175 mg kg^{-1} soil.

Of particular relevance and significance in terms of human health, researchers have found that soil copper exposure results in PICT with co-selection for antibiotic resistant bacteria. In some cases this has resulted in environmental reservoirs of bacteria resistant to clinically important antibiotics such as vancomycin, which are used to treat multi-resistant staphylococcal infections (MRSA) (Berg *et al*., 2010; Fernández-Calviño & Bååth, 2013).

Copper has not only been shown to be highly toxic to microorganisms (hence its bactericidal properties) but also to other soil fauna such as earthworms and springtails (e.g., Berg *et al.*, 2012; de Boer *et al*., 2012; Dewey *et al.*, 2012; Giller *et al.*, 1998; Merrington *et al.*, 2002; Zhou *et al.*, 2011). As a number of interactions take place between microorganisms and macro- and mesofauna in soil, toxicity to these organisms can result in effects on microbial community structure and functioning (Scheu *et al.*, 2002). The use of foliar copper sprays has also shown to negatively impact non-target beneficial organisms in the canopy such as ladybirds (Lo & Blank, 1992a&b).

A large number of studies have revealed that effects on plant, and soil health in terms of macro-, meso- and microbiota correlate well with bioavailable copper (i.e., easily extractable and free ionic Cu^{2+}) but not necessarily with total copper (e.g., Merrington *et al.*, 2002; Sauvé *et al.*, 1996). A number of factors are known to control copper speciation, bioavailability, distribution and impact on soil communities. The most influential being soil organic status, pH and soil texture (Brun *et* al., 1998; Lejon *et al*., 2008; Navel, 2010).

Although the toxicity of copper to soil microorganisms has been well documented, much of the supporting evidence has arisen from laboratory studies or greenhouse and field trials where soils have been spiked. Many authors now acknowledge that further studies of toxicity need to be carried out *in situ* in 'real-world' conditions.

1.3 **Regulatory guidelines**

Various guidelines have been prepared for a range of soil contaminants which set upper limits based on risks to human health and ecological receptors. Although New Zealand does not currently have one set of guidelines for 'safe' or 'acceptable' levels of copper in agricultural soils the Guidelines for the Safe Application of Biosolids to Land (2003) are the most generally cited which give an upper limit of total copper in soil of 100 mg kg^{-1} (NZWWA, 2003) and this value is based on the level in soil deemed to have the lowest observed effect on ecological receptors. The Health and Environmental Guidelines for Selected Timber Treatment Chemicals (Ministry for the Environment & Ministry of Health, 1997) give a limit of 40 mg kg^{-1} (total copper) and this is considered the lowest value that achieves protection of human and plant health, although the value derived specifically for the protection of plant health is 130 mg kg^{-1}. Another set of guidelines have been developed for Australia and New Zealand which are based on the critical load of copper in soil deemed to be toxic, which is > 60 mg Cu kg^{-1} (ANZECC, 1992). In the Netherlands the soil target value for copper has been set at 36 mg kg^{-1} based on the ''assumption of negligible risks to the ecosystem'' and where mandatory remediation is required if levels exceed 190 mg kg^{-1} (Ministry of Housing, Spatial Planning and Environment, 2000). In fact the use of copper in horticultural production systems has been banned in the

Netherlands (Mackie *et al.*, 2012). The values derived for copper limits in these guidelines generally relate to agricultural soils and the protection of plant and human health. In terms of soil biota however it has been shown that earthworms and microorganisms are much less tolerant of elevated copper levels (Mackie *et al.*, 2013; Naveed *et al.*, 2014; Van Zwieten, 2004). In which case, key ecological functions such as aeration and nutrient assimilation may become compromised before any direct effects to plants are observed.

There is much debate around the relevance of total copper values in the identification of risk to ecological receptors as the relationship between total copper and bioavailable is not necessarily linear. The bioavailable fraction is considered to be that which is able to directly come into contact with and cause effect to biological entities. However, total copper values are a measure of the total pool of copper in soil which may be easily monitored over time and directly relate to total agrichemical inputs.

1.4 Review of methods

There are a number of environmental and edaphic factors that will influence the presence and distribution of microbes in soil. In order to determine the potential effects of copper on bacterial/archaeal communities, a range of these determining factors need to be measured and evaluated for each sampling site. A sampling plan designed to minimise the variability of these factors between sites will allow for better comparisons of microbial communities which may be altered due to copper inputs. An overview and explanation of the theory behind the methods used in this study to measure a range of these variables are outlined below.

1.4.1 Soil sampling

The uppermost 0-10 cm zone of the soil profile is considered the most ‘biologically active’ and is where the majority of microbially mediated organic matter breakdown and nutrient mineralisation occurs (Murphy, 2014). It is also the zone in which most of the total load of copper in soil occurs where it has a strong affinity for organic matter. It is for this reason that this zone is most often

sampled in studies pertaining to the impacts of copper on microbial communities and processes.

The location in an orchard from which a soil sample is taken is also important. Kiwifruit orchards are subject to frequent vehicular movements in the alleyways between vine rows which may lead to localised soil compaction. Many vine rows have applications of herbicides, fertilisers and other organic amendments such as compost. It is therefore preferable to sample these locations separately so that these variables may be considered independently.

1.4.2 Physical and chemical analyses

1.4.2.1 *Temperature*

The distribution and activity of soil microorganisms is largely mediated by soil temperature which changes seasonally and in response to edaphic factors such as ground cover and soil type (Standing and Killham, 2007). The biochemical activity of Mesophylic microbial communities tends to double with every 10ºC rise in temperature between 0ºC and 30ºC /35ºC and this is referred to as the Q10 relationship (Standing and Killham, 2007). Therefore seasonal and diurnal temperature fluctuations will mediate microbial processes.

1.4.2.2 *Particle Size, bulk density, porosity and water-filled pore space*

The bulk density (BD) of a soil gives an indication of the degree of compaction and porosity. A soil with a high BD value will have reduced aeration and capacity for water and nutrient movement. Porosity is portion of the soil volume occupied by pore spaces. Studies have shown that organic orchard soils tend to have a lower bulk density than conventionally managed orchard soils with correspondingly higher levels of organic matter (Swezey *et al.*, 1998; Glover *et al.*, 2000). Soil compaction can also arise when there is a decline in bioturbation associated with a decline in the activities of earthworms and plant roots (Naveed *et al*., 2014).

Bacteria exist in water-filled micropores (< 10 μm) and their spatial distribution is dependent on the movement of water in soil (Naveed *et al.*, 2014). This in turn is

controlled by the bulk density and particle size distribution (Chau *et al.*, 2011). It has been found that although smaller particle size fractions of soil may harbour a higher number of bacteria, coarser textured soils may have higher species diversity and richness due to more numerous micro-habitats in isolated water films (Chau *et al.*, 2011). Water filled pore space (WFPS) is the relative proportion of water to total pore space and has been found to be a good indicator of aerobic microbial activity where maximum respiration rates have been found to occur at a WFPS of 60% (Linn & Doran, 1984; Huang *et al.*, 1995).

1.4.2.3 ***Soil Moisture***

Soil moisture content is a determining factor in the aeration-dependent activity of soil microorganisms (Skopp *et al.*, 1990). A low soil moisture content may also restrict substrate supply to microorganisms resulting in reduced activity (Stark and Firestone, 1995) and cause spatial isolation of microorganisms thus influencing microbial distribution (Treves *et al.*, 2003). Water is a transport medium for dissolved ions, nutrients, gases and heat and microorganisms themselves (Standing & Killham, 2007). The water content of a soil expressed as a percentage of the water holding capacity (WHC) may be used to determine if conditions are favourable for aerobic microbial activity where 50% to 70% WHC are considered optimal (Atlas & Bartha, 1997).

1.4.2.4 ***Organic Matter***

Soil organic matter (SOM) provides the substrate and energy source for heterotrophic soil microorganisms (Standing & Killham, 2007). The SOM content of soil will therefore directly influence the microbial community composition of the soil. SOM is also one of the most reactive soil constituents with copper where it forms strong complexes in soil (Lejon *et al.*, 2008; Dumestre *et al.*, 1999; Singh, 1971). Therefore the amount of organic matter in soil will partly determine its bioavailability.

Brockett *et al.* (2012) found that soil moisture and organic matter were most closely related to microbial communities in a study across seven biogeoclimatic zones in western Canada.

1.4.2.5 ***Soil pH and EC***

Soil pH has been shown to be one of the most important influencing factors in the spatial distribution of microorganisms in soil (Fierer & Jackson, 2006; Griffiths *et al.*, 2011). It not only affects microorganisms and enzymatic processes directly, but also by influencing the dissociation and solubility of molecules which may have indirect effects (Atlas & Bartha, 1997). The pH of a soil will strongly influence the mobility and bioavailability of copper by influencing adsorption and desorption processes (Lopez-Periago *et al*., 2008; Sauvé *et al.*, 1995; Cavallaro & McBride, 1980). pH in particular has been shown to have the largest influence on metal toxicity to soil microorganisms (Kunito *et al*., 1999; Giller *et al*., 1998). The relationship between $CaCl_2$ extractable copper (Cu_{CaCl2}) and total extractable copper (Cu_T) is also strongly influenced by soil pH (Fan *et al.*, 2011; Brun *et al*., 1998).

Electrical conductivity (EC) may be used as a measure of the level of soluble salts in a soil. Soluble salts (e.g., Na, K, Ca, Mg, NH_4, Cl, HCO_3, $SO4^{2-}$), may increase in soils due to drought, saline irrigation water or accumulation from long-term fertiliser inputs (Jones, 2001). This may lead to effects on plant growth, soil microbial biomass, microbial processes such as litter decomposition, nitrification and denitrification and enzyme activities (Skopp *et al.*, 1990; Rietz & Haynes, 2003; Wichern *et al.*, 2006).

The use of a salt solution such as $CaCl_2$ or potassium chloride (KCl) may be used in preference to water for measurements of pH, as it will counteract the effect of soil salinity and allow for accuracy and consistency in values, especially in sandy soils or soils with a low cation exchange capacity (CEC) (Jones, 2001).

1.4.2.6 *Active Carbon*

The permanganate oxidizable carbon (POXC) method (Weil, 2003) has been shown to be a good measure of the labile soil C pool and to be strongly correlated with particulate organic C (POC), microbial biomass C (MBC), and soil organic C (SOC) but with a greater sensitivity to changes in environmental or management variation (Culman *et al.*, 2012). This method is also a relatively rapid and inexpensive means by which to assess changes in the labile soil C pool.

1.4.2.7 *Copper*

Total copper (Cu_T) is a measure of the entire recoverable fraction of copper in soil including strongly bound and complexed as well as labile and biologically available forms. The total fraction of copper in a soil may not all be bioavailable, however it is a useful and easily comparable measure by which to monitor changes in the amount of copper in soils over time. Particularly in the assessment of the *potential* hazard to soils as well as by providing a measure by which to compare a soil with quality standards (Romić *et al.*, 2014). For the sites sampled in this study, values of Cu_T can also be compared with previous data from other studies (e.g., Benge, 2011) to determine any increases over time.

The soluble and potentially biologically available fraction of copper in soil has been determined in numerous studies by the use of weak extractants such as EDTA, NH_4OAc, DTPA, and $CaCl_2$. A number of studies have shown good correlations between $CaCl_2$ extractable copper (Cu_{CaCl2}) and biological effects such as reduced enzyme activity (Dewey *et al*., 2012; Wang *et al*., 2009), plant effects (Brun *et al.*, 1998), soil C mineralisation rate (Wang *et al*., 2009) and microbial biomass C (Merrington *et al.*, 2002). This unbuffered salt solution also has a comparable ionic strength (0.3M) to the total salt concentration of many soils, therefore the concentration of copper in soil extracts will correlate with differences in soil pH (Jones, 2001). Cu_{CaCl2} has also been significantly correlated with other soil properties such as organic matter content, cation exchange capacity (CEC) and carbonate content (Romić *et al.*, 2014).

Divalent free copper (Cu^{2+}) has been shown in recent years to be a better predictor of direct toxicity to soil microorganisms, invertebrates and plants than other measures such as Cu_T and Cu_{CaCl_2} (Zhou *et al.*, 2011). A number of studies have shown that copper toxicity to microorganisms is a direct function of free Cu^{2+} concentration (Menkissoglu, 1991; Cabral, 1994; Dumestre, 1999; Vulkan *et al.*, 2000; Hasman *et al.*, 2009) although other studies have found that some copper complexes may also cause effect to certain bacteria (Brandt *et al.*, 2006; Maderova *et al.*, 2011; McLean *et al.*, 2013). The measurement of Cu^{2+} in soil solution may be carried out in a number of ways including the use of a Cu^{2+} specific electrode, diffusive gradients in thin films (DGT) and whole cell biosensors or determined using chemical speciation models such as the Windermere Humic Aqueous Model (WHAM) or free ion activity model (FIAM).

1.4.3 Microbial analyses

There are a wide range of microbiological, biochemical and genetic approaches to studying microbial communities in soil. These range from traditional direct culturing methods that identify individual taxa through to metagenomics which have enabled the characterisation of the taxonomic structure of microbial communities and the identification of functional genes, and metaproteomics and metatranscriptomics which can reveal the functions actually expressed by these communities *in situ*. Other techniques for assessing the overall effect of perturbations to microbial communities may involve the measurement of biochemical parameters such as enzymes and phospholipid fatty acids (PLFAs).

1.4.3.1 ***Enzyme analysis***

Dehydrogenases are a good measure of soil microbial activity (Taylor *et al.*, 2002; Mills *et al.*, 2006) as they are a key component of intact cells and provide a direct measure of the total oxidative activities of microorganisms in the initial stages of organic matter breakdown (Dick, 1997). They have also been shown to be good indicators of copper contamination in soil (Wyszkowska *et al.*, 2005; Jeyakumar, 2010). For these reasons they have been used as a measure of ecotoxicology in a

number of studies. The method of Casida *et al.* (1965) is based on the theory that the reduction of 2,3,5-triphenyltetrazolium chloride to the red-coloured triphenyl formazan takes place within microbial cells during respiration. This end product can be measured spectrophotometrically and it provides an indirect measure of metabolically active microbes in soil (Praveen & Tarafdar, 2003).

1.4.3.2 ***Community analysis***

Many studies have found a direct correlation between metal contaminants in soils and microbial community structure e.g., (Frostegård *et al.*, 2011; Wakelin *et al.*, 2014; Li *et al.*, 2015). The 16S ribosomal rRNA gene (16S rRNA) is highly conserved between different species of bacteria and archaea (Weisburg *et al.*, 1991) and therefore phylogenetic studies that target this gene are often used to elucidate microbial communities in environmental samples (Coenye & Vandamme, 2003). Also, bacteria have been found to be more sensitive to metal contamination than fungi (Doelman, 1985; Hiroki, 1992). For this reason, techniques that specifically target the 16S rRNA gene may be used to provide a useful indication of changes to soil microbial communities in response to metal inputs.

A study by Smit *et al*. (2006) found distinct differences in microbial community structure between copper contaminated and non-contaminated soils by using a high throughput DNA fingerprinting method. The diversity was also found to be lower in the contaminated soil. Ribosomal RNA intergenic spacer analysis (RISA) has been used successfully in a number of studies to compare soil microbial communities subject to different environmental perturbations (Ranjard *et al.*, 2001). Other biochemical and molecular methods such as PLFA, denaturing gradient gel electrophoresis (DGGE) fingerprint analysis and terminal restriction fragment length polymorphisms (T-RFLPs) of PCR-amplified 16S ribosomal DNA have been widely used to detect changes in microbial community composition in soils contaminated with metals (Frey *et al.*, 2006; Lejon *et al.*, 2008; Wang *et al.*, 2008; Mackie *et al.*, 2013; Li *et al.*, 2015).

1.4.3.3 *Next generation sequencing*

Metagenomics is a culture-independent genomic analysis that has enabled the characterisation of the taxonomic structure of entire microbial communities. Recent metagenomic techniques involving high throughput sequencing have been used for both microbial community structure and function analyses. These techniques which target the 16S rRNA gene allow the determination of the complete DNA sequence of a multitude of individual operational taxonomic unit (OTU) genomes concurrently. Next generation sequencing, whilst providing more definitive taxonomic classification than other techniques, is much less time-consuming and labour intensive and in recent years has also become much more cost-effective. Ion Torrent sequencing (ITS) uses semi-conductor technology in which the addition of individual nucleotides during DNA synthesis are registered by the release of hydrogen ions. Individual reads are then assigned to specific taxa (Salipante *et al.*, 2014).

1.4.3.4 *Functional profiling of microbial communities*

In order to realise the potential implications of changes in microbial communities, the specific functions of certain groups or individual OTUs needs to be determined. Phylogenetic profiling using 16S rRNA marker genes has enabled the elucidation of microbial communities and the identification of individual bacteria or archaea. However, these techniques cannot predict the metabolic and functional capabilities of individual microbial taxa and communities (Langille *et al.*, 2013). Deep metagenomic sequencing of microbial communities can produce metabolic and functional profiles of a large number of individual OTUs in entire communities, however the cost of these analyses are generally prohibitive (Langille *et al.*, 2013). Predictive functional profiling of microbial communities can now be carried out by using a computational approach which compares 16S rRNA marker gene sequences with a database of reference genomes for which functional capabilities are known. The software programme phylogenetic investigation of communities by reconstruction of unobserved states (PICRUSt) has demonstrated that phylogeny and function are sufficiently linked to allow the prediction of function from phylogenetic data (Langille *et al.*, 2013).

1.5 Study Aims and Objectives

The overall aim of this study was to determine whether or not the use of copper sprays on kiwifruit orchards has had detrimental effects on soil microorganisms and if this has resulted in changes to the structure of soil microbiological communities and therefore what the implications of this may be in terms of long-term orchard sustainability.

Specific objectives were as follows:

1. To determine if soil copper levels have increased as a result of the increased use of copper sprays as an orchard management tool in response to Psa-V.
2. To identify potential differences in soil microbial communities between conventionally and organically managed orchards.
3. To identify potential differences in soil microbial communities between orchards that use or do not use copper sprays.
4. To elucidate the entire soil microbiome for each orchard block in order to determine if copper sprays may result in changes to microbial communities.
5. To identify if specific groups of bacteria or archaea are over- or under-represented in soils receiving inputs of copper.
6. To determine if copper may reduce the abundance of certain groups of bacteria or archaea associated with specific beneficial functional traits.
7. To identify which fraction of copper (total, $CaCl_2$-extractable or Cu^{2+}) is most closely associated with effects to soil microbiological communities.

1.6 Hypothesis

The use of copper sprays in Western Bay of Plenty kiwifruit orchards has resulted in compositional changes in soil microbial communities which may have negative implications for long-term sustainable production.

1.7 Study Sites

Ten kiwifruit orchards within the Te Puke region in the Western Bay of Plenty were selected for the study. Five groups of two adjacent orchards – one organic and one conventional – were chosen based on location, soil type and kiwifruit variety (Figure 1.7). The location of orchard blocks and orchard images are displayed in Appendix A.

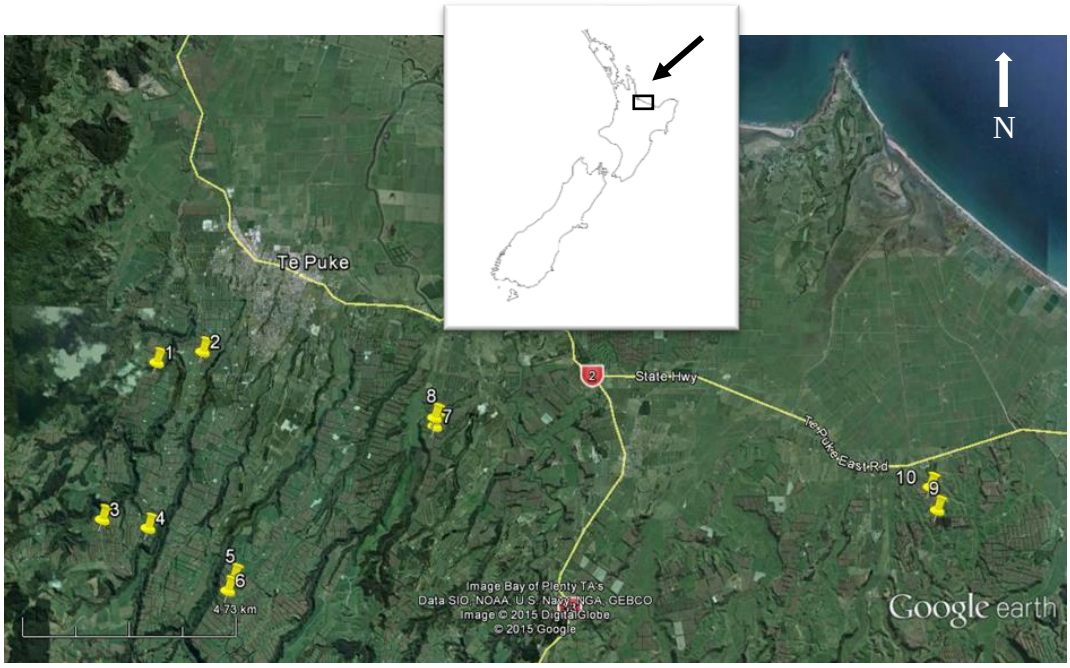


Figure 1.7: Locations of orchard study sites

Soils from these orchards had been analysed in 2011 for a range of soil physical and chemical parameters, including metals, by the Agricultural Research Group on Sustainability (ARGOS). The study was carried out prior to the spread of Psa-V across the region and the subsequent widespread use of protective copper sprays. Based on this data, only orchards growing the Hayward (green) variety of fruit were selected as soil copper levels were found to be higher in these orchards. It would also potentially limit the variability of soil characteristics due to different management practices for green and gold varieties.

2 Methods

2.1 Field Procedure and Sampling

2.1.1 Sampling design

Three blocks within each orchard were selected so that the ages of vines in each block were all similar (20 to 30 years). Each block included 20 sampling sites - 10 within vine rows and 10 adjacent inter-row/alleyway. Within rows and alleyways were sampled separately so as to determine the potential effects on microbial communities of herbicides, organic amendments, plant root exudates, soil compaction etc. in addition to copper applications. The sampling sites were selected so as to cover and be representative of the entire block whilst omitting edges and areas with young or missing vines as this could potentially lead to greater variability in physical and biological soil characteristics. Sampling was carried out on a systematic basis across each block in that sample sites were located at regular intervals.

Each within row sampling site was located 40 cm from the base of a vine and the adjacent alleyway site located at right angles to the within row site and between vehicle tracks.

2.1.2 Data collection

A pre-designed field data sheet was used to record measurements and observations at each site (Appendix B). The location, of each block and individual sampling site was marked with a hand-held global positioning system (GPS) and photographed for future reference e.g., (Figure 2.1). GPS was also used to determine the aspect and altitude of each block.

Figure 2.1: Typical photograph of a sampling site showing vegetation cover within the vine row and alleyway

Measurements of soil temperature were made with a soil temperature probe (EMGA, P300W) at three locations within each block.

A brief description of vegetative cover was made for each block which included the percentage cover and dominant species present. The presence/absence of herbicide strips or use of organic amendments was also noted and a brief qualitative list of soil macrofauna present at the time of sampling was compiled.

2.1.3 Soil sampling

At each sampling site the top layer of vegetation was scraped away and a garden trowel was used to sub-sample the top 10cm of soil. The soil was then placed into a sterile, zip lock plastic bag labelled with the date, orchard name, block identifier, and position. Each bag would ultimately contain a composite of each of the 10 vine row or alleyway sub-samples. In order to prevent cross-contamination the trowel was sterilised with 70% ethanol and air-dried between sampling. Immediately after sampling each block, the composite soil samples were placed on ice in a cool box.

Ten undisturbed soil cores (10 cm depth) were also sampled randomly from each of three vine rows and three inter row sites across each block for bulk density measurements. These were extracted with a Daiki corer (Daiki Rika Kogyo Co., Ltd., Japan).

2.1.4 Soil preparation

After transportation to the laboratory, composite soil samples were immediately homogenised and sieved through a 2 mm sieve which was sterilised with 70% ethanol and dried prior to and after sieving each sample. A small sub-sample from each composite sample was transferred into a 30 ml sterile plastic vial and stored frozen at -20ºC for subsequent DNA extraction and sequencing. A portion of each composite sample was air dried at 32 ºC in a drying cabinet and the remainder stored at 4 ºC. Soils to be analysed for 'total' and $CaCl_2$-extractable copper were oven-dried in a Binder forced convection drying oven and ground with a mortar and pestle to ensure homogeneity.

2.2 Physical and chemical analyses

2.2.1 Bulk density and porosity

Soil cores were dried for 24 hrs at 105 ºC and weighed. The bulk density of each soil core was determined by dividing the oven-dry soil weight (g) by the volume of the core (100 cm^3).

Soil porosity was calculated as follows:

Porosity % = 100 – (bulk density / particle density x 100)

Where particle density = oven dry soil wt (g) / volume of soil (100 cm^3).

2.2.2 Particle size

The Bouyoucous hydrometer method (Bouyoucous, 1962 & Gee & Bauder, 1986) was used to determine the particle size of each composite soil sample. Briefly,

50 - 100 g of soil was pre-treated with H_2O_2 to remove organic matter and then dispersed in 10% sodium hexametaphosphate solution and left to settle overnight. The soil solution was then transferred to a soil dispersion cup and mixed for 1 min with a mechanical mixer. The mixture was then transferred to a 1000 ml sedimentation cylinder and soil particles were brought into suspension with a plunger and again left to settle. Readings were taken from a hydrometer suspended in solution after 40 sec and 2 hr time intervals to determine the settling times of clay and silt from which % sand was also determined. The calculated percentage of sand, silt and clay was then used to classify the soil based on the USDA textural triangle.

2.2.3 Soil moisture and water-filled pore space

Soil moisture was determined by calculating the difference between field-moist and oven-dry (105ºC) soil as follows:

$$\text{Soil moisture} = \frac{\text{field moist soil wt (g)} - \text{oven dry soil wt (g)}}{\text{field moist soil wt (g)}} \times 100$$

Water-filled pore space was calculated as follows:

$$\text{WFPS} = \frac{100 \times (\text{gravimetric moisture content (g g}^{-1}) \times \text{bulk density})}{\text{particle density (=2.65 g cm}^{2})}$$

2.2.4 Organic matter and active carbon

The Loss on Ignition (LOI) method was used to determine soil organic matter (SOM). Approximately 5 g of oven dry soil was accurately weighed into a pre-weighed crucible and placed into a microwave furnace (CEM Phoenix) set at 375 ºC for 3 hrs. SOM was calculated as follows:

$$\text{SOM} = \frac{\text{oven dry soil wt (g)} - \text{soil wt after ignition (g)}}{\text{oven dry soil wt (g)}} \times 100$$

The permanganate oxidizable carbon method based on Weil *et al*. (2003) was used to determine the active or biologically available carbon content of each soil.

2.5 g of air-dry soil was weighed into polypropylene 50-mL screw-top centrifuge tubes. To each tube, 18 mL of deionized water and 2 ml of 0.2 M Potassium permanganate stock solution were added and tubes were shaken for 2 min at 240 rpm on an orbital shaker. Tubes were removed from the shaker and left to settle for exactly 10 min. After 10 min, 0.5 mL of the supernatant was transferred into a second 50-mL centrifuge tube and mixed with 49.5 mL of deionized water.

Sample absorbance was read with a UV/vis spectrophotometer with the wavelength set at 550 nm. Permanganate oxidizable C was determined by the following formula:

POXC (mg kg soil) = [0.02 mol L^{-1} – (a+b * ABS)] * (9000 mg C mol^{-1})(0.02 L solution wt^{-1})

Where: 0.02 mol L^{-1} = concentration of the initial $KMnO_4$ solution

a = intercept and b = slope of the standard curve

ABS = absorbance of the unknown soil sample

9000 mg = amount of C oxidized by 1 mole of MnO_4 changing from Mn^{7+} to Mn^{4+}

0.02 L = volume of $KMnO_4$ solution reacted, and

wt = mass of soil (kg)

2.2.5 Soil pH and EC

Soil pH and EC were determined with a stainless steel electrode and meter (IQ Scientific Instruments). EC and $pH_{(water)}$ measurements were taken in a 1:5 mixture of air-dry soil and deionised (DI) water that had been shaken for 2 hrs on an orbital shaker. $pH_{(CaCl2)}$ measurements were measured following the same procedure but in a 1 : 2.5 mixture of air-dry soil and 0.01 M $CaCl_2$.

Both electrodes were calibrated with buffers of known pH and EC values prior to measurements.

2.2.6 Copper

2.2.6.1 *Total Cu*

For $Cu_{(T)}$ analysis pre-dried and ground soils were extracted for 30 mins at 95ºC with *aqua regia* (1 g soil in 4 ml 50% HNO_3 and 10 ml 20% HCL) and made up to 100 ml with DI water. 40ml of sample was centrifuged at 4,000 rpm and a 20 ml aliquot diluted 1:5 for chloride ion adjustment and filtered with a 0.45 micron cellulose filter into a 15ml Falcon tube. Samples were acidified with conc. HNO_3 (2 % by vol.) and stored at 4 ºC until analysis. Total copper and other metals (Li^7, B^{10}, Na^{23}, Mg^{24}, Al^{27}, S^{34}, K^{39}, Ca^{43}, V^{51}, Cr^{52}, Fe^{54}, Mn^{55}, Co^{59}, Ni^{60}, Cu^{65}, Zn^{68}, As^{75}, Se^{82}, Sr^{88}, Ag^{109}, Cd^{111}, In^{115}, Ba^{137}, Tl^{205}, Pb^{207}, Bi^{209} and U^{238}) were determined by inductively coupled plasma mass spectrometry (ICP-MS) ELAN® DRC II (Perkin Elmer Inc., Münster, Germany). The ICP analysis was carried out by technical staff at the University of Waikato.

2.2.6.2 *$CaCl_2$-extractable Cu*

For $Cu_{(CaCl2)}$ analysis oven-dried and ground soils were extracted by shaking 5 g soil in 50 ml 0.01 M $CaCl_2$ for 2 hrs on an orbital shaker. Extracts were then filtered through a 0.45 micron cellulose filter into a 15 ml Falcon tube, acidified with conc. HNO_3 (2 % by vol.) and stored at 4 ºC until analysis. $Cu_{(CaCl2)}$ and other metals were determined by ICP-MS as above.

2.2.6.3 *Cu^{2+} ions*

Free Cu^{2+} ions were determined in soil solution extracts with a cupric ion selective electrode (CuISE) (Orion Cupric ionplus® Sure-Flow® Solid State Combination 9629BNWP) coupled with a benchtop pH/Ion meter (2100 - Eutech Instruments) according to the methods of Sauvé *et al.*, 1995, Avdeef *et al.*, 1995 and Luo, 2004. Extracts were obtained by shaking 10 g of oven-dry soil in 20 ml 0.01 M $CaCl_2$ in 50ml polypropylene screw-top centrifuge tubes laid horizontally on an orbital shaker set at 200 rpm for 16 hours. Samples were then centrifuged for 10 minutes at 2,500 rpm and the supernatant filtered through Whatman No.1 filter paper prior to Cu^{2+} determination.

The copper ISE used in Cu^{2+} analysis was polished with aluminium oxide powder, soaked for 10 min in 0.025 M H_2SO_4 and rinsed thoroughly in DI water each day

prior to analysis (as recommended by Blaedel and Dinwiddie, 1975) and soaked in DI water when not in use. The ISE outer filling solution was changed regularly.

Approximately 10ml of sample was transferred into a polystyrene sample cup to which 100 µl 10 ml^{-1} of KNO_3 ionic strength adjustor (ISA) was added. The sample cup with magnetic stirrer was placed on a magnetic stirrer plate and set to stir gently at a constant rate. The magnetic plate was insulated with a cardboard mat so as to prevent potential heat transfer due to friction. The Cu ISE was then placed into the solution and a paper bag placed over the sample and ISE to obtain constant, reduced lighting conditions. Once the millivolt reading was stable (< 0.3 mV min^{-1}) readings were recorded. As temperature influences the release of Cu^{2+} in solution, solutions were measured at the same temperature as calibration standard solutions.

ISE calibration standards were made by first preparing a standard stock solution comprising 10.272 g KNO_3, 2.203 ml ethylenediamine (EN) and 2.420 g $Cu(NO_3)_2.3H_2O$ made up to 1 L with DI water. The stock solution was then apportioned to five 200 ml glass bottles and the pH of each solution adjusted with 10% HNO_3 or 1 M KOH to achieve nominal pH values of 4, 5, 6, 7 and 9.

The actual pH of each standard solution was measured with a calibrated glass pH electrode (Metrohm) at the time of Cu ISE measurement. Calculations of pCu^{2+} for each standard were made by entering the pH values into stoichiometric equations obtained from Luo (2004) which are based on stability constants for EN taken from Avdeef *et al.* (1983) (Appendix D). A standard calibration curve was plotted by entering the calculated pCu^{2+} value against the electrode potential reading (mV) for each standard. A new standard calibration curve was made for each set of measurements. Calibration curves were considered to be acceptable when the slope of the calibration was within 95% of the theoretical Nernst value of -29.58 at 25 ºC. Values of pCu^{2+} for samples were obtained by using the regression equation produced for each calibration curve. Calculations of Cu^{2+} (mg kg^{-1} soil) for each sample were made based on the formula $pCu^{2+} = - \log [Cu^{2+}]$ and the weight of soil.

2.2.7 Dehydrogenase

Dehydrogenase was measured in each soil sample according to the method of Casida *et al*. (1964). 15 g of air-dried soil was thoroughly mixed with 0.35 g of $CaCO_3$ and 6 g of this mixture was weighed into 50 ml polypropylene centrifuge tubes in duplicate. 1 ml of 3% aqueous triphenyltetrazolium chloride (TTC) and 3.5 ml of DI water were added to each tube and mixed. A blank was included with each sample set (i.e., 1 ml of 3% aqueous TTC and 4 ml of DI water). Samples were incubated in the dark at 37° for 24 hours. After 24 hours 10 ml of methanol was added to each tube and shaken for 1 min at 300 rpm on an orbital shaker in the dark. The suspension was filtered through 150 mm filter paper into a 50 ml centrifuge tube ensuring the complete removal of the entire triphenylformazan (TPF) product from the filter paper. The filtrate was then diluted to 40 ml with methanol. The colour intensity was measured with a spectrophotometer set at 485 nm and a 1 cm cuvette with the blank sample used to zero the spectrophotometer.

The amount of TPF produced was calculated using the standard equations from Pepper & Gerba (2004) where:

$$x\ (\mu g\ ml^{-1}) = \frac{A - 0.00629}{0.0415} \qquad \text{DHA}\ (\mu g\ \text{TPF}\ g^{-1}\ \text{soil}) = \frac{x\ *\ (40\ ml)}{6\ g}$$

2.3 Microbial community analyses

2.3.1 DNA extraction and preparation

DNA was extracted with the PowerSoil® DNA Isolation Kit (MoBio Laboratories, Carlsbad, CA, USA) according to manufacturer instructions. The extraction procedure is detailed in Appendix E. Extracted DNA was quantified and checked for purity using a NanoDrop® ND-1000 UV-Vis spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA). DNA was stored frozen at -20ºC until further use.

Due to the high concentration of DNA recovered from soil as indicated by UV-Vis spectroscopy, extracts were diluted 1:5 with sterile Milli-Q water before quantification with a Qubit® fluorometer (Invitrogen by Life Technologies) prior to PCR. This assay is highly selective for double-stranded DNA (dsDNA). 2 µL

of template DNA was added to 98 μL of dilution buffer in 500 μl thin-walled PCR tubes, and dsDNA concentration was measured relative to Qubit® DNA standards (one high and one low).

2.3.2 PCR amplification

The V4 region of the bacterial 16S rRNA gene was amplified with reverse primer 806r and barcoded forward primer 515f with the following sequences:

Forward:

A adaptor + *library key* + **IonXpress barcode** + Barcode adaptor + *515f primer*
(5'CCATCTCATCCCTGCGTGTCTCCGAC*TCAG***XXXXXXXXXX**GAT*GTGCCAGC MGCCGCGGTAA* '3)

Reverse:

P1 + *806r primer*

(5'CCACTACGCCTCCGCTTTCCTCTCTATGGGCAGTCGGTGAT*GGACTA CHVGGGTWTCTAAT* '3)

These primers have been shown to target a wide diversity of bacteria without strong bias towards particular groups (Bates *et al*., 2011). Each PCR reaction mixture contained 2 μL of template DNA (1 ng total), 11.88 μL UV-sterilized MilliQ water, 1 μL bovine serum albumin (BSA) (0.4 mM), 3 μL dNTPs (2 mM), 3 μL 10 X buffer, 3 μL $MgCl_2$ (50 mM), 0.12 μL Platinum® Taq DNA Polymerase (Life Technologies) (5 U μl^{-1}) and 0.5 μL of each primer (10 mM). The PCR master mix was treated with ethidium monoazide (EMA) at a ratio of 1:100 prior to the addition of Taq, forward primers and template DNA. This step was performed so as to prevent the amplification of potential contaminant dsDNA present in the master mix reagents (Rueckert and Morgan, 2007).

Thermocycling conditions were set at 94ºC for 3 min for the initial denaturation followed by 30 cycles of 45 seconds at 94ºC, 1 min at 50ºC and 1.5 min at 72ºC for denaturation, annealing and extension/elongation, with a final elongation phase at 72ºC for 10 min. Samples were then held at 4ºC indefinitely. PCR reactions were run in triplicate and PCR products were later pooled.

2.3.3 Amplicon visualisation

Gel electrophoresis was used to visualise and analyse the quality and yield of PCR products. Agarose gel (0.3 g) was prepared in 1 X TAE buffer solution (40 mM Tris, 20 mM acetic acid, and 1mM EDTA) with 1 µL of SYBR® Safe DNA gel stain (Life Technologies). The gel chamber was filled with 1 X TAE buffer solution and 5 µL of PCR product and negative/positive controls were mixed with 2.5 X loading dye and loaded into wells. A 1 kb ladder was loaded into the first and last well. The gel was run for 30 min at 60V. Preparation of gel and sample runs were undertaken in reduced lighting conditions. Gels were visualised under UV light on an Alpha Innotech AlphaImager Imaging System and the size of DNA fragments were estimated by comparison with molecular weight markers in the 1 kb ladder.

2.3.4 Amplicon clean-up and preparation for sequencing

PCR amplicons were cleaned with Solid Phase Reversible Immobilization (SPRI) paramagnetic bead-based technology. SPRI®select was used with left-side size selection to exclude fragments below the size range for target amplicons of 380–410 base pairs whilst at the same time removing primer dimers. 30 µL of PCR product was mixed with 21 µL of SPRI®select and placed on a magnetic rack to allow the target DNA to bind to the magnetic beads and to separate from solution. The supernatant containing contaminants and excluded fragments was extracted, and DNA bound to SPRI beads was washed with 85% ethanol, dried and eluted with 20 µl 1 X TE (10mM Tris and 1mM EDTA). SPRI beads were removed from the eluent containing DNA on a magnetic rack and eluted DNA transferred into a new tube. SPRI-cleaned PCR products were quantitated with a Qubit® dsDNA High Sensitivity assay kit, and DNA was diluted to 300 pg μL^{-1} with 1 X TE based on Qubit concentration values. A final equimolar library was prepared by pooling 5 µl of each of the 300 pg μL^{-1} products into one tube which was then diluted to 26 pM with 1 X TE (1.22 ng of DNA in 200 µL).

2.3.5 Ion Torrent sequencing

Sequencing of prepared DNA was carried out at the Waikato DNA Sequencing Unit using the Ion Torrent PGM Sequencer following manufacturer protocols.

2.3.6 Data processing

Sequence processing and the generation of an OTU abundance table were carried out by Dr Charles Lee using Waikato DNA Sequencing Unit's standardised protocols for 16S rRNA gene PCR amplicons developed for Ion Torrent PGM. Briefly, sequence data were quality-trimmed based on length and homopolymer counts using Mothur 1.17.0. (Schloss *et al*., 2009). The resulting high quality sequences were dereplicated, ranked by abundance, binned into operational taxonomic units (OTUs), and checked for PCR chimeras using UPARSE (Edgar 2013). The abundance of each OTU in all samples was calculated and stored in an Excel spreadsheet for downstream analyses. Representative sequences of each OTU were analysed using the Ribosomal Database Project (RDP) Classifier to analyse and determine phylogeny to provide automatic taxonomic assignments. Classification at each taxonomic level was assigned a percentage confidence score, where a higher number indicates a higher level of certainty that the taxonomic assignment is accurate.

OTU abundance data were converted into presence/absence as well as percentage relative abundance values based on the total number of sequence reads in each sample. Abundant and rare OTUs in the relative abundance OTU dataset were then segregated based on threshold selection criteria of $\geq$ 0.1% and $\leq$ 0.1% abundance across all site samples, respectively. The rare OTU dataset was analysed separately as is thought that rare species may be better indicators of ecosystem stress than more common species (Poos and Jackson, 2012) and are also considered to be essential for understanding changes in community composition over time (Shade *et al.,* 2014). Comparisons of OTU relative abundance across sites at the phylum level were based on a $\geq$ 70% confidence in classification at that level. OTUs for which classification at the phylum level was less than 70% confident were re-classified as 'other'. Rare species (defined as

occurring in < 5% of samples as recommended by McCune and Grace [2002]) were excluded from each of the four datasets prior to multivariate analyses.

2.4 Statistical analyses

2.4.1 Physiochemical data

Statistical analyses were carried out with the software programmes Statistica 64 version 12 by Statsoft Inc. Data were log (x + 1) transformed prior to analyses where necessary to compress high values and spread low values into a range within the same order of magnitude. Statistically significant differences between sampling sites in terms of physical and chemical parameters were determined by one-way ANOVA and paired t-tests. In order to satisfy the assumptions of ANOVA normality and homoscedasticity of the data were checked with the Shapiro-Wilk and Brown-Forsythe tests respectively prior to analyses. Non-parametric data were analysed with the Kruskal-Wallis test.

2.4.2 Microbial community data

The statistical programme PC-ORD Multivariate Analysis of Ecological Data (v6, McCune & Mefford, 1999) was used to analyse community data. This software has been specifically designed for multivariate analyses of ecological communities.

In order to visualise groupings of sites based on community composition using presence/absence and relative abundance data, non-metric multidimensional scaling (NMDS) joint plots (Kruskal, 1964; Mather, 1976) were constructed based on dissimilarity matrices. Prior to analysis, data for sites O3B1VR and O7B1VR were removed from each dataset based on the observation that these sites had significantly lower values for OTU richness and total sequence reads, which was probably more related to DNA quality than field conditions. The community data matrix (primary matrix) was constructed using Sørensen (Bray Curtis) distance measures. The Sørensen distance measurement is widely used as it consistently distinguishes between ecologically distinct groups (McCune and Grace, 2002).

Relative Euclidean distance measures were used to construct the secondary matrix of environmental variables. Because ranked distances are used in NMDS, assumptions of normality or linearity among variables are not required (McCune & Grace, 2002). The final ordinations were constructed based on 250 runs each of real and randomised data using a random starting configuration. Ordinations were preferentially constructed in 2 dimensions for ease of visualisation and interpretation. However, 3 dimensional plots were constructed where recommended due to high stress values if differentiation between groups could be easily visualised. The final stress and instability of ordinations were based on the Monte Carlo test using 250 randomised runs. Stress values reported in PC-ORD are percentage values (i.e., 100 times that which are usually reported). The Monte Carlo test was used to determine if NMDS had extracted stronger axes than expected by chance (McCune & Grace, 2002). Points on the ordination were overlaid with categorical variables as grouping variables (orchard, position, management and herbicide use). A coefficient of determination (r^2) was used to represent the quality of data reduction and an indication of how the variance represented was distributed among the primary axes. Environmental variables were plotted as vectors on the joint plots if r^2 values were greater than 0.4.

Multi-response permutation procedures (MRPP) were used to test the hypothesis of no difference between groups based on Sørensen (Bray Curtis) distance measures. MRPP does not require the assumption of multivariate normality or homogeneity of variances and has therefore been recommended for community data (McCune & Grace, 2002). Groups were defined by orchard, soil Cu_T, and Cu_{CaCl2} concentration range, position, management type and herbicide use. Pair-wise *post hoc* tests were performed to distinguish groups where there was more than one group for each variable (i.e. orchard and Cu_T). In MRPP a test statistic (T) is used to describe the separation between groups where the more negative the number, the stronger the separation. The p value is used to evaluate the likelihood of an observed difference being due to chance where the value is calculated as the proportion of randomized runs with stress ≤ observed stress. The effect size that is independent of sample size is provided by the chance-corrected within-group agreement value (A). This value describes within group homogeneity compared to the random expectation. The value of A determines the degree of heterogeneity within groups. An A value of 0 indicates that heterogeneity within groups is that

expected by chance and a value of 1 indicates that all items within groups are identical. Values above 0.1 indicate a relatively high degree of separation and above 0.3, very high (McCune & Grace, 2002).

A series of scatterplots were constructed to identify relationships between OTU richness, diversity, total number of rRNA sequence reads and measured environmental variables. One way ANOVA and t-tests were used to determine the significance of differences between orchards and sites grouped by similar physicochemical properties.

Indicator species analyses (Dufrêne & Legendre, 1997) was then performed as it is able to determine the strength of association between individual OTUs and environmental variables and to detect and describe the value of different OTUs in indicating particular environmental conditions (McCune & Grace, 2002). Indicator values were used to determine the degree to which a particular OTU is associated with a particular group where a maximum value of 100 for a particular OTU corresponds to perfect indication for that group. Monte Carlo tests based on 1,000 randomisations were conducted on each observed indicator value (*IV*) to obtain *p*-values which would determine the significance of differences between groups.

2.5 Quality Control

2.5.1 Sampling, soil preparation and storage

All equipment used in the collection of soil samples was sterilised with 70% ethanol prior to sampling and between samples to prevent cross-contamination. New, sterile vials were used for the storage of soil reserved for DNA extraction and sequencing. Samples for DNA extraction and sequencing were stored at -20ºC and those for enzyme analysis were stored for less than a week at 4ºC.

2.5.2 Analytical

2.5.2.1 *Physical and Chemical*

Only analytical grade reagents and Type 2 deionised (DI) water were used in the preparation of reagents used for analysis. Weighing balances and pipettes had been calibrated and glassware such as volumetric flasks were of type A. All glassware had been washed in laboratory grade detergent (Decon™) and rinsed thoroughly in DI water. Samples sent for ICP-MS analysis were stored in new Falcon tubes. Standard protocols for chemical and physical soil analyses were followed. All samples were run in duplicate.

2.5.2.2 *Microbiological*

Standard PC1 and PC2 protocols were followed during DNA extraction, PCR and sample preparation. All sample and reagent containment tubes, pipettes and tips were ethanol and UV sterilised prior to use. PCR cabinets, bio-containment cabinets, centrifuges and all other equipment were cleaned with ethanol and UV sterilised prior and after use. Reagents and Type 1 Milli-Q water used in the preparation of master mixes and dilution of samples were also UV sterilised. All laboratory equipment had been calibrated and standards with known values were used to calibrate DNA quantification units. Samples for PCR were run in triplicate and later pooled for analysis.

3 Results

3.1 Field Data

3.1.1 Vegetation cover

The vegetation cover in orchards varied considerably between conventional and organic orchards and this was mostly due to the use of herbicides in the vine rows of the conventional orchards (Figure 3.1, Table 3.1, Appendix C). The organic orchards had a very high percentage cover of vegetation (95%) although in orchard 4 there was evidence that a coconut-based organic moss spray had been used and the percentage vegetation cover was less (75 - 90%).

Figure 3.1: Vegetation cover typical of orchards under organic (left) and conventional (right) management regimes (note the herbicide strip at right)

The floral composition and overall diversity of the orchards were similar although, the survey of flora present was brief and not exhaustive (Table 3.2). A number of orchards had a much higher prevalence of nitrogen-fixing species such as clover and oxalis.

Table 3.1: Physical and biotic observations and measurements for each sampling site.

Orchard	Type	Blk. #	Mean soil temp. (°C)	Altitude (masl)	Herbicide strip?	% veg. cover vr	% veg. cover aw	Macrofauna present in or on soil
1	Organic	1	17.1	113	no	95	95	Numerous earthworms, springtails, amphipods, spiders, field crickets, centipedes, slugs
		2	17.8	102	no	95	95	Numerous earthworms, springtails, ants, native bees
		3	17.4	102	no	95	95	Numerous earthworms, springtails, amphipods, native earwig, centipedes, slugs, leafhoppers
2	Conv.	1	16.1	78	yes	20	45	A few earthworms and some beetle larvae
		2	16.5	84	yes	15	20	Beetle larvae, a few earthworms (only in alleyways)
	Conv.	3	16.7	87	yes	20	40	Very few earthworms
3	Conv.	1	16.1	187	yes	10	95	Earthworms, *Symphyla sp.*, scarab beetles, beetle larvae, amphipods, spiders, ants, slugs
		2	16.7	189	yes	20	95	Earthworms, *Symphyla sp.*, scarab beetles, beetle larvae, amphipods, spiders, ants, slugs
		3	16.1	197	yes	10	95	Earthworms, *Symphyla sp.*, scarab beetles, beetle larvae, amphipods, spiders, ants, slugs
4	Organic	1	16.4	196	no*	90	90	A few earthworms
		2	16.1	195	no*	85	85	A few earthworms
		3	16.2	192	no*	75	75	Nothing obvious
5	Conv.	1	16.3	192	yes	10	65	Very few. Scarab beetles, *Symphyla*, few earthworms (more abundant in alleyways)
		2	16.2	190	yes	10	65	Very few. Beetle larvae and earthworms
		3	16.8	189	yes	10	65	Very little, occasional earthworm
6	Organic	1	16.4	177	no	95	90	Very few, *Symphyla*, amphipods, tiny centipede,
		2	16.2	184	no	95	95	Amphipods, beetle larvae, millipedes, juvenile earthworms, leaf hoppers
		3	16.6	186	no	95	95	Amphipods, adult & juvenile earthworms, springtails, numerous millipedes in vine rows
7	Conv.	1	17.1	62	yes	15	75	Very few, occasional earthworm, *Symphyla* and beetle larvae, more earthworms in alleyways
		2	17.4	62	yes	15	75	A few earthworms in alleyways only
		3	17.3	60	yes	5	50	A few earthworms in alleyways only
8	Organic	1	19.1	66	no	95	95	Lots, large and juvenile earthworms, amphipods, *Symphyla*, centipedes, beetle larvae, slugs, earwigs, springtails, mites, leaf hoppers spiders
		2	19.2	64	no	95	95	As block 1 but much less numerous
		3	19.4	66	no	95	95	As block 1 but less numerous
9	Conv.	1	16.5	28	yes**	85	90	A few beetle larvae, earthworms, centipedes
		2	16.4	37	yes**	85	85	A few beetle larvae, slugs and centipedes. Earthworms mostly in alleyways
		3	16.3	36	yes**	65	80	As blocks 1 and 2
10	Organic	1	16.9	30	no	95	95	Numerous amphipods, earthworms, springtails, millipedes, leafhoppers, spiders
		2	17.0	30	no	95	95	As block 1
		3	17.0	30	no	95	85	As block 1

* Organic moss spray (coconut-based). ** Only around vines

Table 3.2: Description of flora present in each orchard

Orchard	Type	Groundcover composition
1	Organic	Dense sward of long grass with some buttercup, dock, oxalis and sheep sorrel.
2	Conv.	Sparse cover of grass, creeping mallow, black nightshade, fleabane, wild strawberry, dock, sedges, *Euphorbia* sp., lawn daisy and fumitory predominantly in alleyways.
3	Conv.	Buttercup, grass, dock, wild strawberry and oxalis in alleyways with only *Tradescantia* and *Euphorbia* sp. in vinerows.
4	Organic	Grass, buttercup, clover, field daisy, sedges, dock, chickweed throughout.
5	Conv.	Grass, *Tradescantia* (Blk 1 only), dock, oxalis, and violets mostly in alleyways with liverwort in rows.
6	Organic	Grass, buttercup, dock, violets, foxgloves, geranium, mint and clover with sedges and arum lilies around vines.
7	Conv.	Grass in alleyways, *Euphorbia* sp. in vine rows
8	Organic	Dense sward of grass, clover, buttercup, dock, creeping mallow, *Euphorbia* sp., chickweed and oxalis.
9	Conv.	Dense sward of grass, sedges, wild strawberry, black nightshade, dock, creeping mallow and chickweed in non-sprayed areas.
10	Organic	Grass, buttercup, dock, arum lilies, Japanese honeysuckle

3.1.2 Soil Macrofauna

A qualitative summary of the macro- and mesofauna identified in each orchard block is presented in Table 3.1. Overall the organic orchards seemed to have a greater number of species and individual taxa than the conventional orchards, although for orchard 4 (organic) presence and visual abundance was low while orchard 3 (conventional) presence and visual abundance was relatively high. The presence and visual abundance of each taxon was noticeably reduced in soils where total copper levels exceeded 50 mg kg^{-1}. In these soils the only taxa identified were earthworms, beetles, and *Symphyla* sp. with an absence of springtails, chilopods, molluscs and amphipods.

3.2 Soil Physicochemical Properties

Values for a range of measured physicochemical parameters are presented in Table 3.3 and Appendix G.

3.2.1 Soil Organic Matter and Active Carbon

Overall the organic orchard soils had significantly higher levels of organic matter than the conventionally-managed orchards ($p < 0.001$) (Figure 3.2, Table 3.3). Orchards 1 and 6 had the highest levels ($\bar{x}$ = 17%) and orchard 9 had the lowest ($\bar{x}$ = 9%). On average the vine rows had significantly lower organic matter than the alleyways ($p < 0.001$), with the exception of orchards 1 and 6.

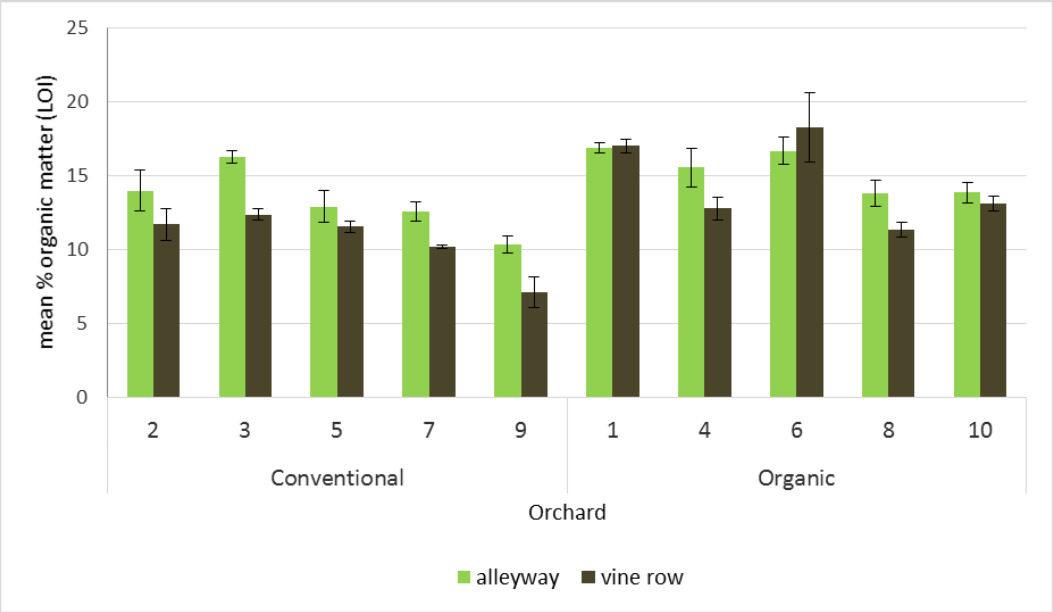


Figure 3.2: Mean % organic matter (loss on ignition) for each orchard. Error bars denote ± SD.

Table 3.3: Summary of physical and chemical soil properties for each orchard (values are mean values for three blocks)

Orchard	Management regime	Bulk Density (g cm^{-3})	WFPS	pH H_2O	pH $CaCl_2$	EC	% Moisture	% OM (LOI)	DHA (µg TPF g^{-1} soil 24 hr^{-1})	Active C (mg kg^{-1})	Cu_T (mg kg^{-1})	Cu_{CaCl2} (mg kg^{-1})	pCu^{2+}
1	Organic	0.66	52	6.55	6.38	121	34.64	16.92	225	1070	21	0.09	< 19
2	Conventional	0.75	54	6.88	6.66	143	28.88	12.83	157	843	68	0.25	15.2
3	Conventional	0.74	55	6.41	6.24	165	30.19	14.30	151	844	43	0.17	17.6
4	Organic	0.73	57	6.40	5.97	124	32.18	14.15	205	755	60	0.19	18.0
5	Conventional	0.76	51	6.34	6.15	126	28.24	12.21	107	789	48	0.14	17.7
6	Organic	0.65	48	6.28	6.03	147	32.09	17.48	205	1065	29	0.11	16.9
7	Conventional	0.78	54	6.16	5.74	140	27.22	11.38	106	740	59	0.18	17.2
8	Organic	0.73	46	6.62	6.34	118	24.99	12.55	202	924	42	0.56	< 19
9	Conventional	0.85	61	6.73	6.33	122	26.82	8.73	149	691	23	0.17	< 19
10	Organic	0.74	57	6.84	6.50	139	32.39	13.47	166	855	31	0.16	< 19

The levels of biologically active carbon, measured as permanganate-oxidisable carbon (POXC) are also higher in soils from organic orchards (Figure 3.3) and are well correlated with that of organic matter measured as % loss on ignition (R^2 = 0.556) (Figure 3.4).

A relationship between levels of organic matter and POXC, and the three different measures of copper in soil could not be established through correlation analysis.

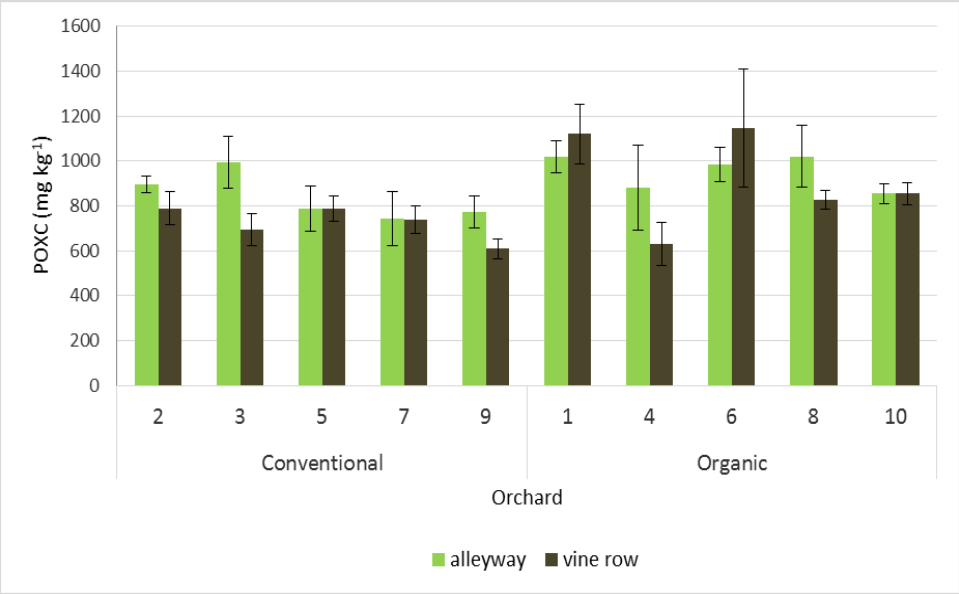


Figure 3.3: Mean permanganate-oxidisable carbon (POXC) for each orchard. Error bars denote ± SD.

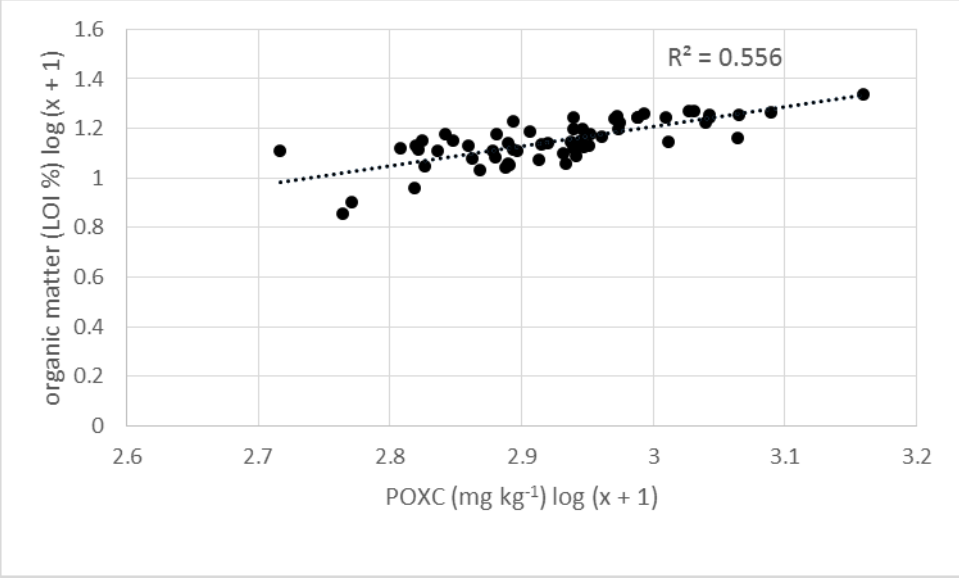


Figure 3.4: Relationship between organic matter (LOI) and biologically active carbon (POXC).

3.2.2 Bulk Density and Porosity

The bulk density and porosity of the study orchard soils were very similar, ranging between 0.66 - 0.90 g cm^{-3} and 66 - 77% respectively (Figure 3.5) Although, on average the bulk density was significantly lower ($p < 0.05$) and the porosity significantly higher ($p < 0.05$) in the organic orchards. The soils within vine rows of the conventionally managed orchards were significantly more compacted than the alleyways ($p < 0.05$), whereas in two of the organic orchards the alleyways were more compacted than the vine rows. Overall, the bulk density and porosity values for the top 10 cm of soil in all orchards indicate well-aerated soils with high levels of organic matter. For these orchards there is a very good correlation between bulk density and organic matter in the soil (Figure 3.7). This may explain the lower bulk density values for the organic orchard soils which also have higher levels of organic matter.

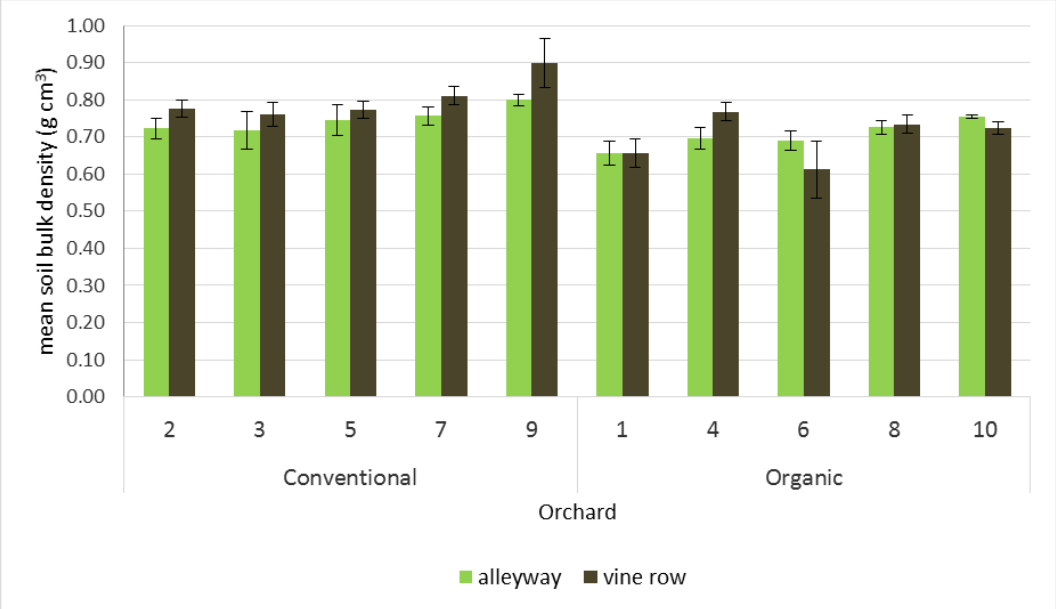


Figure 3.5: Mean bulk density of each orchard soil. Error bars denote ± SD.

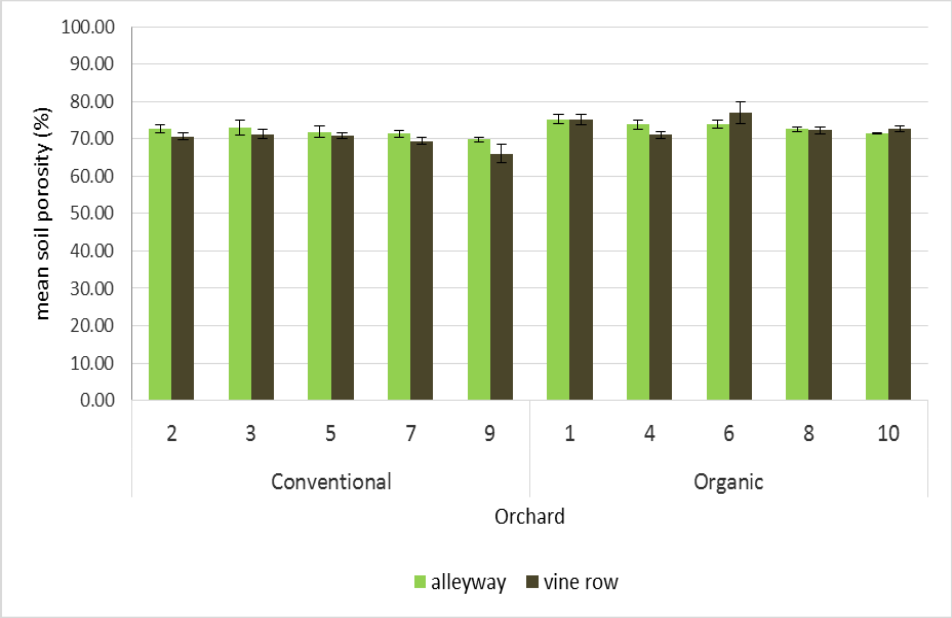


Figure 3.6: Mean porosity of each orchard soil. Error bars denote ± SD.

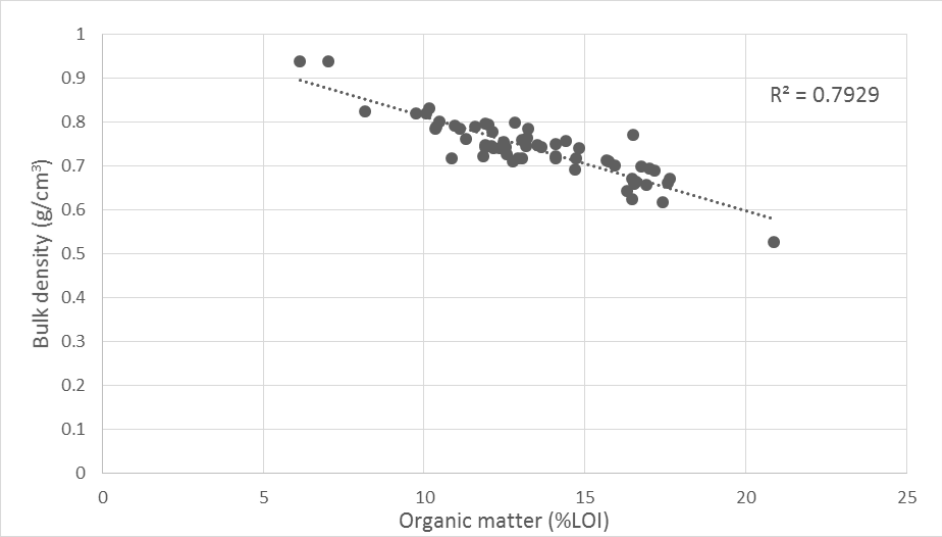


Figure 3.7: Relationship between soil bulk density and organic matter for 10 orchards.

3.2.3 Soil moisture and water-filled pore space

Soil moisture values for each site ranged from 21.6% to 36.3%. Orchard 8 had the driest soils ($\bar{x}$ = 25% soil moisture) and orchard 1 the wettest ($\bar{x}$ = 32% soil moisture). The water-filled pore space (WFPS) of soils ranged between 39% for the vine rows of block 2 within orchard 6 and 70% for the alleyways of block 3 in orchard 9 (Figure 3.8). Forty-eight of the soil samples (80%) had a WFPS value

within 10% of the optimum value of 60%. All of the soil samples from orchard 8 had a less than optimal range of 42% – 48%.

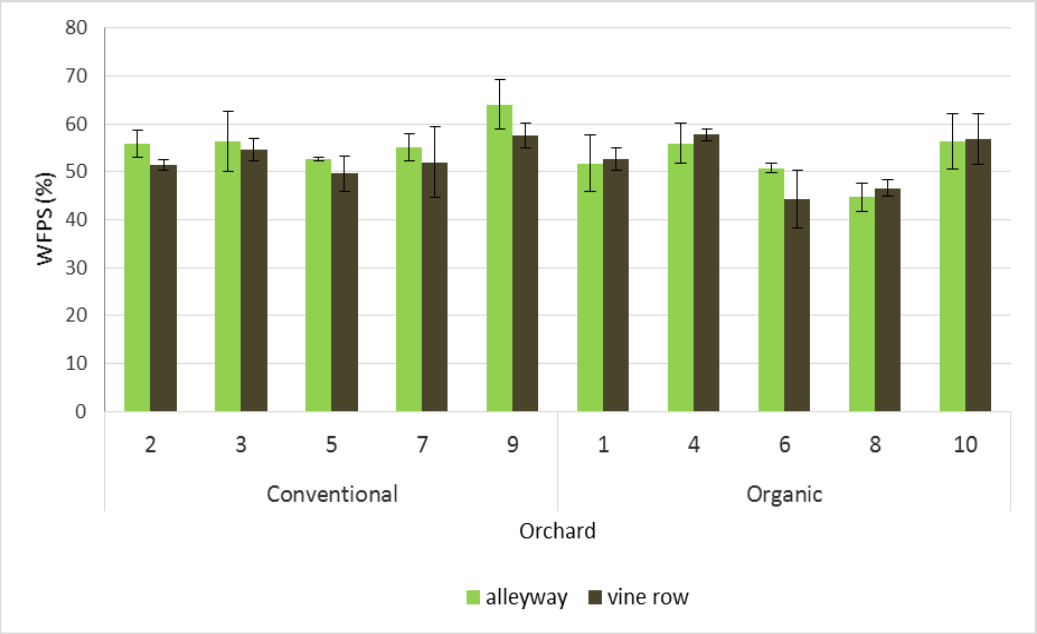


Figure 3.8: Mean water-filled pore space of orchard soils. Error bars denote ± SD.

3.2.4 Particle size and soil texture

The texture of all of the soils in the study were classified as either loams or sandy loams (Appendix F). The proportion of clay minerals in each soil were very similar in the range of 6 – 15%. Orchard 4 soils (loam) had the least percentage sand (41 - 49%) while those of orchard 9 (sandy loam) were the sandiest (60 – 62%).

3.2.5 Soil pH and EC

Most of the soils had a pH_{water} within the optimum range for kiwifruit soils of 6.2 – 6.5 (Rahman *et al.*, 2011) (Figure 3.9). Orchard 7 soils had the lowest pH values (pH_{water} = 6.16, pH_{CaCl_2} = 5.74) and orchard 2 the highest (pH_{water} = 6.88, pH_{CaCl_2} = 6.66). The pH readings of soils extracted with water were consistently higher than readings from $CaCl_2$ extracts. Correlation analyses did not reveal any relationship between the pH of soils and total $CaCl_2$-extractable or pCu^{2+}. There were however significant differences in pH between paired orchards, although differences were not related to the overall management practices employed.

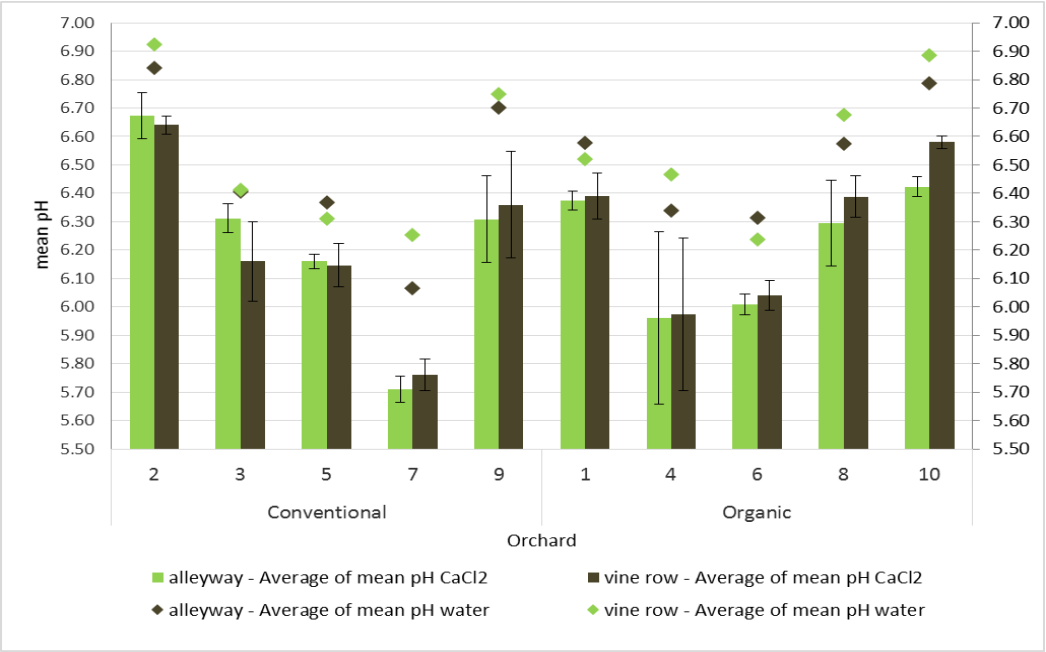


Figure 3.9: Mean pH of orchard soils. Error bars denote ± SD.

The EC values for all soils were all within a similar range and relatively low (Figure 3.10). At these levels the soils are considered non-saline and in which microbial processes such as respiration, decomposition and nitrification are not adversely affected (Smith & Doran, 1996). The vine rows of blocks 2 and 3 within orchard 8 had the lowest EC values of 86 µs cm^{-1} and the highest values were measured in soil from the alleyway of block 3 in orchard 7 (197 µs cm^{-1}).

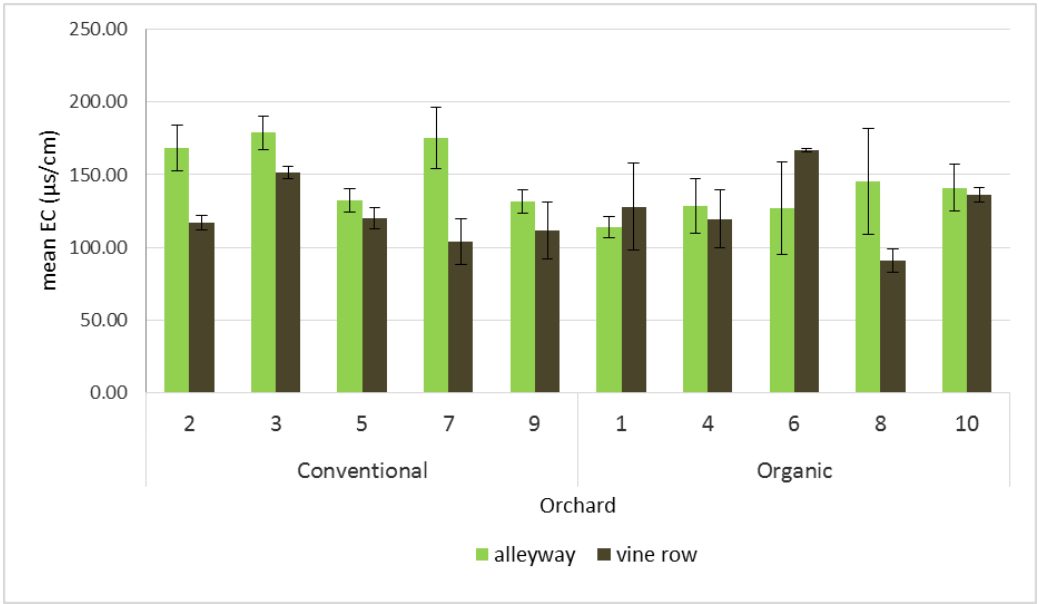
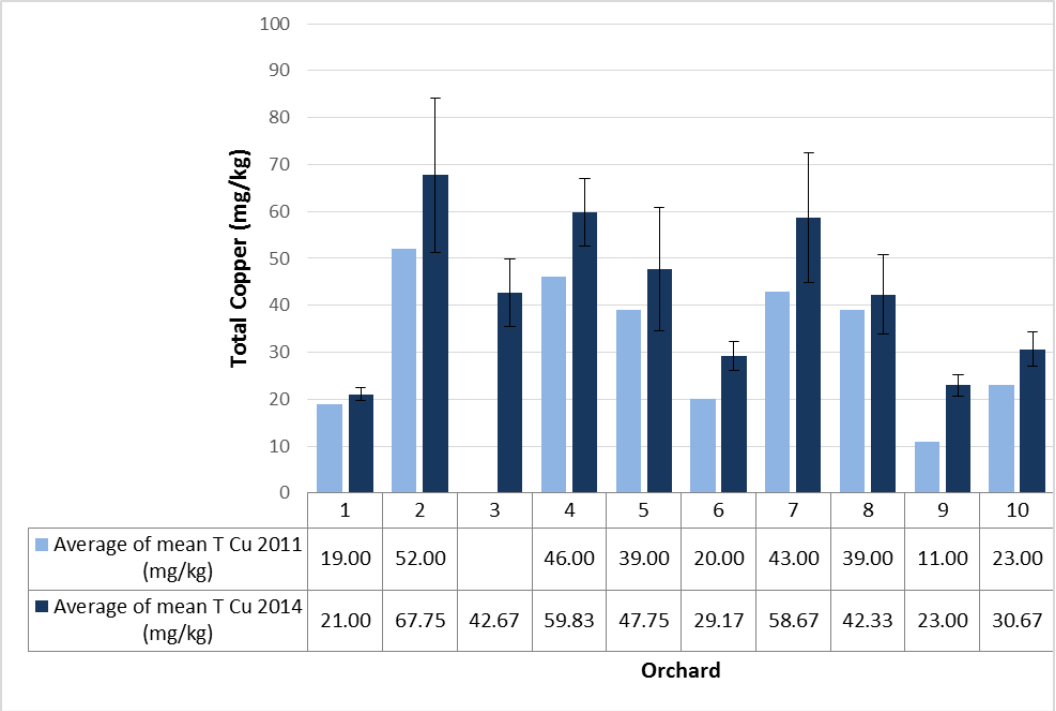


Figure 3.10: Mean electrical conductivity (EC) of orchards soils. Error bars denote ± SD.

3.2.6 Copper

Levels of total recoverable copper in study orchard soils appear to have increased since 2011 (Figure 3.11 & Table 3.4). Although, 2011 values are for the top 0 - 15 cm of soil and may therefore be slightly less than that would be expected for the top 0 - 10 cm. There was however only a slight difference in 2011 and 2014 values for orchards 1 and 8 for which no copper was applied. The levels of copper in orchard 8 ($\bar{x}$ = 42.33) are relatively high considering copper is no longer used and may be the result of past land use. This value exceeds the soil target value of 40 mg kg^{-1} as stated in the Health and Environmental Guidelines for Selected Timber Treatment Chemicals (1997).



	1	2	3	4	5	6	7	8	9	10
Average of mean T Cu 2011 (mg/kg)	19.00	52.00		46.00	39.00	20.00	43.00	39.00	11.00	23.00
Average of mean T Cu 2014 (mg/kg)	21.00	67.75	42.67	59.83	47.75	29.17	58.67	42.33	23.00	30.67

Figure 3.11: Total copper in orchard soils 2011 and 2014. Figures for 2014 are the mean concentration for vine rows and alleyways across three blocks. Error bars denote ± SD. Only mean data is available for 9 orchards sampled in 2011.

Six of the ten orchards in the study have soil copper levels exceeding the Health and Environmental Guidelines for Selected Timber Treatment Chemicals for total copper in soil. The highest mean level of copper was found in orchard 2 (68 mg kg^{-1}) in which the vine row of block 2 had a concentration of 91 mg kg^{-1}. This is a conventionally managed orchard in which copper has been applied

frequently (according to spray diary records) and the level of copper is well in excess of the ANZECC (1992) Guidelines and Health and Environmental Guidelines for Selected Timber Treatment Chemicals (1997) of 60 and 40 mg kg^{-1} respectively and close to the NZWWA (2003) guideline target value of 100 mg kg^{-1}. The alleyway sample from block 3 of orchard 4 had a concentration of 70 mg kg^{-1} Cu_T even though this orchard is subject to an organic management regime, albeit one that includes copper sprays (Table 3.4).

Table 3.4: Copper levels in study orchard soils 2014 (vr = vine row, aw = alleyway). Lower pCu^{2+} values indicate higher free Cu^{2+} activity in soil solution where pCu^{2+} = - log $[Cu^{2+}]$.

Orchard	Management Regime	Copper applied	Blk #	Cu_T (mg kg^{-1})		Cu_{CaCl_2} (mg kg^{-1})		pCu^{2+}	
				vr	aw	vr	aw	vr	aw
1	Organic	No	1	23	22	0.09	0.10	< 19	< 19
			2	22	20	0.08	0.07	< 19	< 19
			3	20	20	0.10	0.11	< 19	< 19
2	Conventional	Yes	1	71	70	0.27	0.32	16.0	15.3
			2	91	77	0.26	0.26	16.3	15.4
			3	44	55	0.43	0.44	14.7	13.5
3	Conventional	Yes	1	44	53	0.19	0.22	17.7	17.0
			2	37	44	0.17	0.20	18.0	< 19
			3	33	47	0.11	0.16	17.8	< 19
4	Organic	Yes	1	67	58	0.18	0.18	< 19	< 19
			2	58	56	0.20	0.20	18.1	18.0
			3	51	70	0.19	0.19	18.1	17.7
5	Conventional	Yes	1	57	39	0.22	0.11	17.7	17.5
			2	51	40	0.12	0.12	< 19	18.0
			3	68	33	0.15	0.11	< 19	< 19
6	Organic	Yes	1	30	27	0.12	0.10	16.7	17.2
			2	35	27	0.09	0.09	15.8	17.5
			3	29	29	0.18	0.13	17.0	17.1
7	Conventional	Yes	1	73	47	0.16	015	17.6	17.3
			2	71	46	0.18	0.17	16.5	17.9
			3	71	46	0.21	0.20	17.2	16.8
8	Organic	No	1	44	41	0.16	0.62	< 19	< 19
			2	50	30	0.95	0.50	< 19	< 19
			3	53	38	0.51	0.66	< 19	< 19
9	Conventional	Yes	1	19	24	0.19	0.24	< 19	< 19
			2	24	25	0.17	0.15	< 19	< 19
			3	23	25	0.07	0.19	< 19	< 19
10	Organic	Yes	1	35	29	0.20	0.25	< 19	< 19
			2	33	27	0.17	0.18	< 19	< 19
			3	35	27	0.10	0.08	< 19	< 19

Mean soil Cu_T values for each pair of conventional and organic orchards were significantly different as determined by pair-wise t-tests ($p < 0.05$). Soil sampled within vine rows generally had a higher mean concentration of Cu_T but a lower concentration of Cu_{CaCl2} *cf.* alleyways (Figure 3.12 & Figure 3.13).

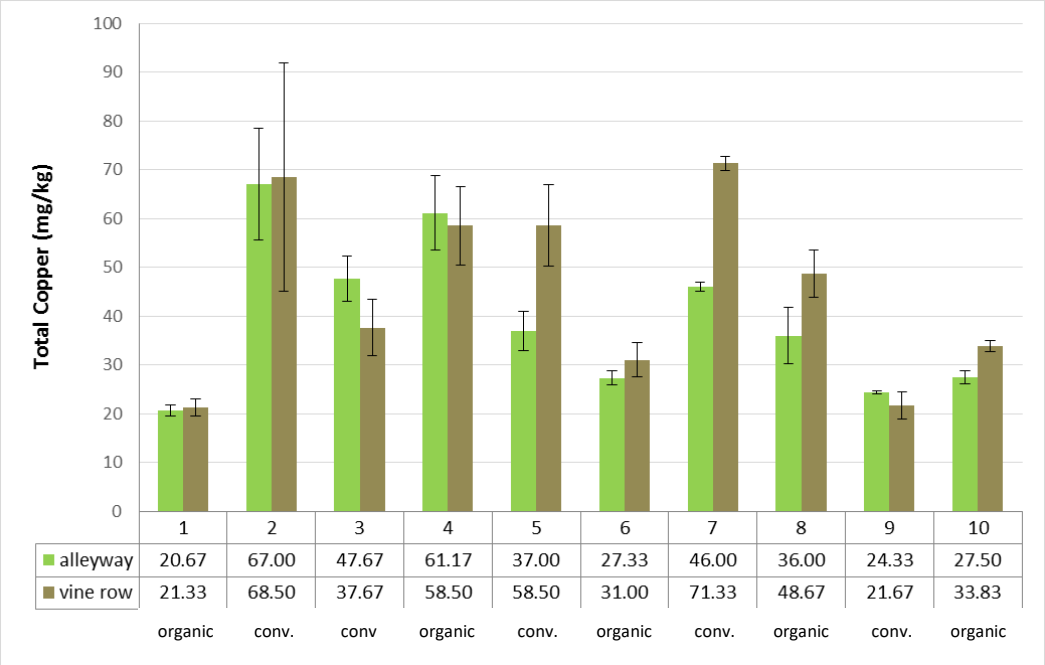


Figure 3.12: Total recoverable copper levels in vine rows and alleyways for each study orchard. Error bars denote means ± SD of three blocks per orchard.

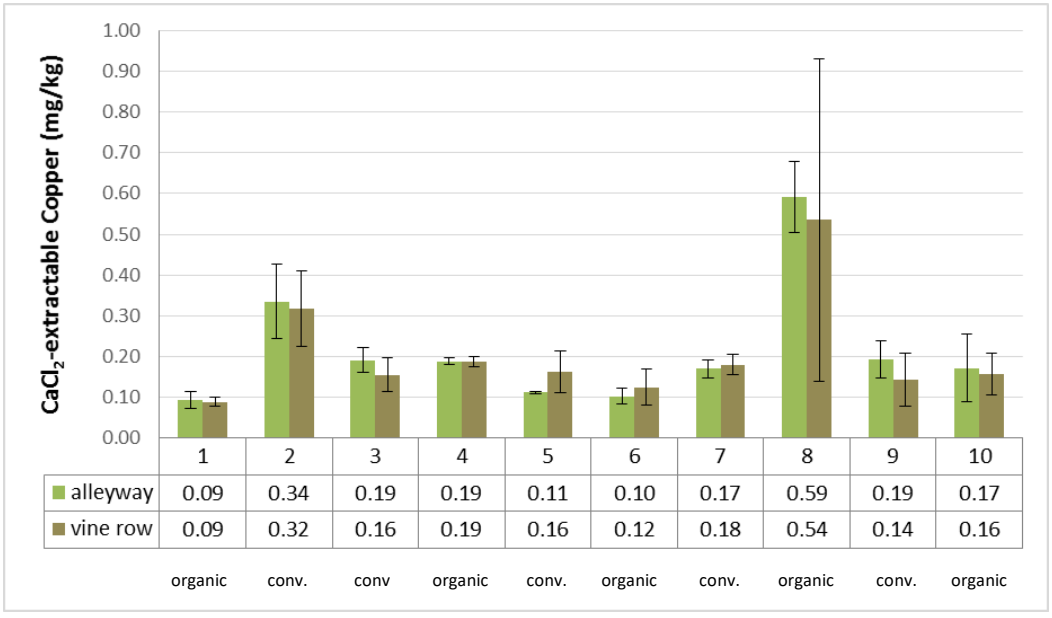


Figure 3.13: $CaCl_2$-extractable copper levels in vine rows and alleyways for each study orchard. Bars denote means ± SD of three blocks per orchard.

The soils with the highest levels of Cu_{CaCl2} were sampled from the organically managed orchard 8, in which copper sprays have not been used for some time and in which compost amendments are frequently applied. The soils within this orchard, compared to the adjacent conventional orchard 7 are lower in Cu_T but higher in Cu_{CaCl2}. More consistent results were observed for orchard 2 where both Cu_T and Cu_{CaCl2} in soils are significantly higher ($p < 0.05$) than those in the adjacent organic orchard 1. For all other orchards, differences in the levels of Cu_{CaCl2} were much less pronounced and insignificant.

Levels of free cupric ions in soil solution were very low for all orchards (Table 3.4). The pCu^{2+} values for soil samples from four orchards and seven sites within another three orchards were below the range of calibration of the cupric ISE and could not be determined. The highest free Cu^{2+} levels were identified in soils from orchard 2 (pCu^{2+} = 13.5 – 16.3) although these values do not correlate with Cu_T or Cu_{CaCl2}. The vine row of block 2 in orchard 2 had the highest Cu_T value of 90 mg kg^{-1} with a pCu^{2+} value of 16.3, whereas block 3 had the highest Cu^{2+} level (pCu^{2+} = 13.5), with a Cu_T value of 44 mg kg^{-1}.

There is a tenuous relationship between Cu_T and Cu_{CaCl2} for these orchard soils (Figure 3.14). This indicates that the bioavailability of copper to soil organisms may be dependent on a range of biogeochemical factors which may differ according to location. When results were analysed by location within the orchards (i.e., vine rows or alleyways) the relationship became slightly more linear (Figure 3.15). There was little or no relationship between Cu_T and Cu_{CaCl2} for orchard 8 and data for this orchard was excluded from these correlation analyses.

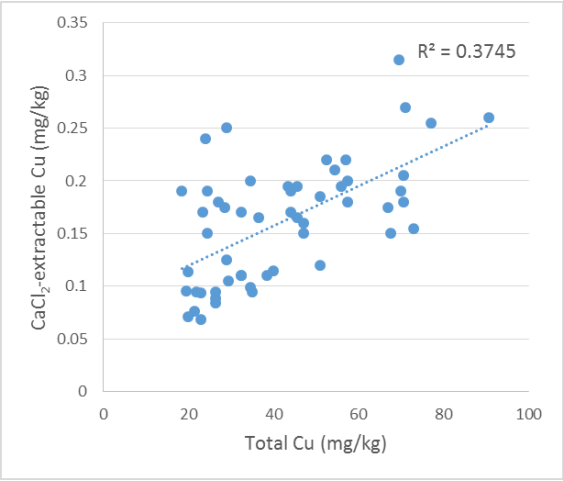


Figure 3.14: Scatterplot showing overall relationship between Cu_T and Cu_{CaCl2} for each block and location across 9 orchards

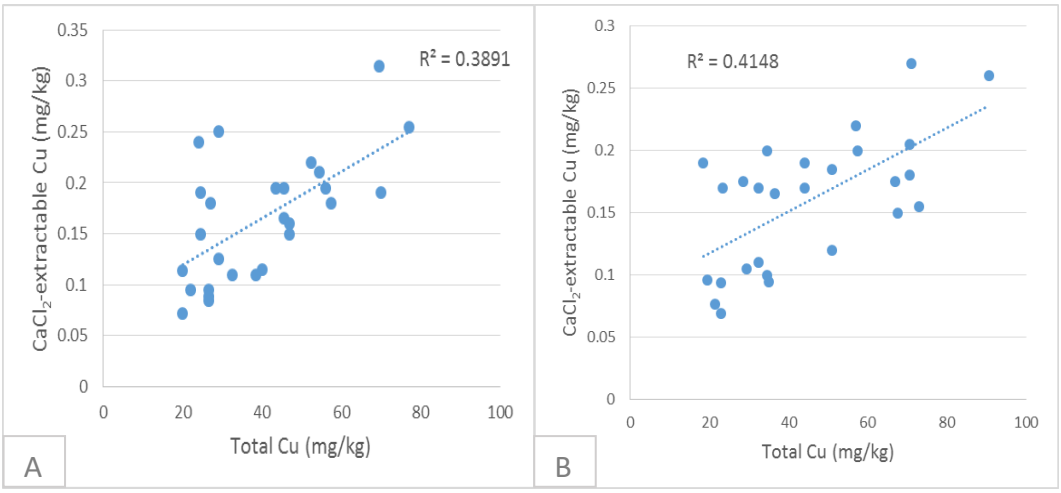


Figure 3.15: Scatterplots showing relationship between Cu_T and Cu_{CaCl2} for each block by location across 9 orchards (A = alleyways, B = vine rows)

The level of Cu_{CaCl2} ranged from 0.2% to 2% of the value of Cu_T. However, correlation analyses of levels of bioavailable (Cu_{CaCl2}) copper as a percentage of total copper (Cu_T) and the variables pH, organic matter (LOI) or POXC did not reveal any obvious relationships. There was also no relationship between pCu^{2+} and pH values for each orchard where pCu^{2+} was within a measurable range.

3.2.7 Dehydrogenase

Overall there was significantly more dehydrogenase activity (DHA) in the organic orchard soils compared with the conventional orchard soils ($p < 0.001$) (Figure 3.16). In the conventionally managed orchards, levels of DHA were lower in the vine rows ($p = 0.005$), whereas for organic orchards, the differences between orchard positions were neither consistent nor statistically significant.

Correlation analyses of log (x+1) transformed data showed little or no relationship between dehydrogenase levels and Cu_T or Cu_{CaCl2} across all orchards, irrespective of management regime or position within the orchard.

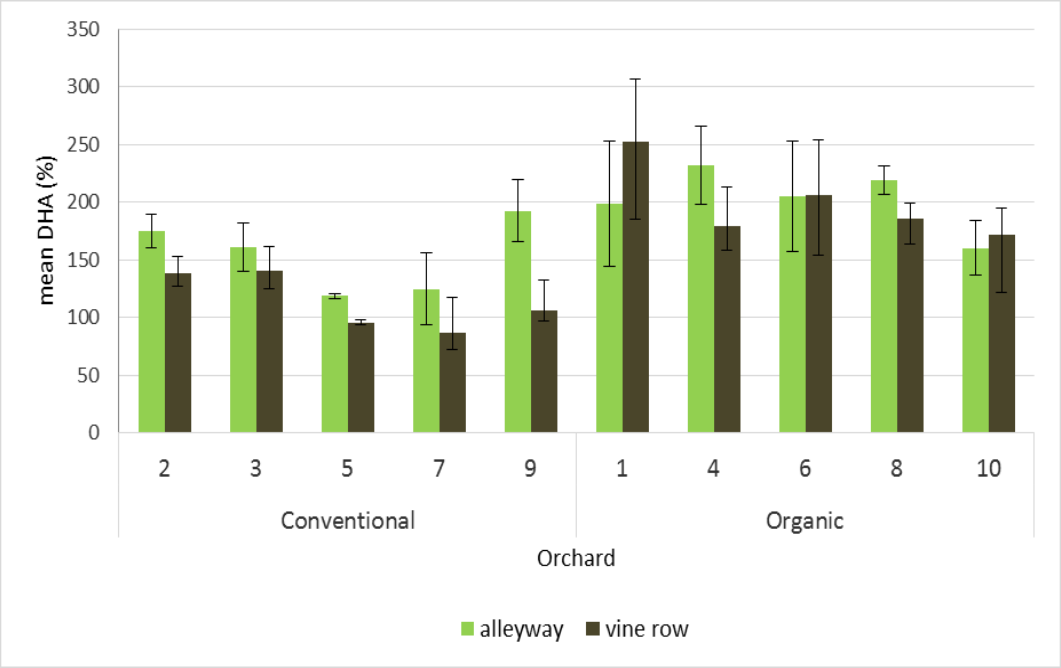


Figure 3.16: Differences in dehydrogenase activity between orchards. Bars denote means ± SD of three blocks per orchard.

3.3 Microbial community analyses

3.3.1 PCR

Good DNA yields were obtained from the Powersoil® DNA extractions as quantified by Nanodrop®. Sample concentrations ranged between 39 ng μL^{-1} and 138 ng μL^{-1} and the 260/280 ratio for each sample was in the range of 1.8 - 2.0 which indicated a high level of purity. Gel electrophoresis revealed prominent visible bands for each sample (Appendix H), although some smearing and primer dimers were also evident. The initial dilution of sample 13 (orchard 3, block 1, vine row) did not amplify and was re-run with a fresh dilution, which did amplify. Qubit quantification of DNA in pooled PCR products after SPRI cleaning, storage and immediately prior to sequencing, revealed DNA concentrations ranging from 0.631 ng μL^{-1} to 45.2 ng μL^{-1} (Appendix H).

3.3.2 Diversity

Sequencing generated a total of 11,583 unique rRNA gene sequences from 60 samples. Across all sites 41 distinct phyla, 94 classes, 156 orders, 53 families and 832 genera were represented. Species area (rarefaction) curves (Figure 3.17) indicated that sample sizes were adequate for the purposes of capturing all OTUs present across all sampling locations.

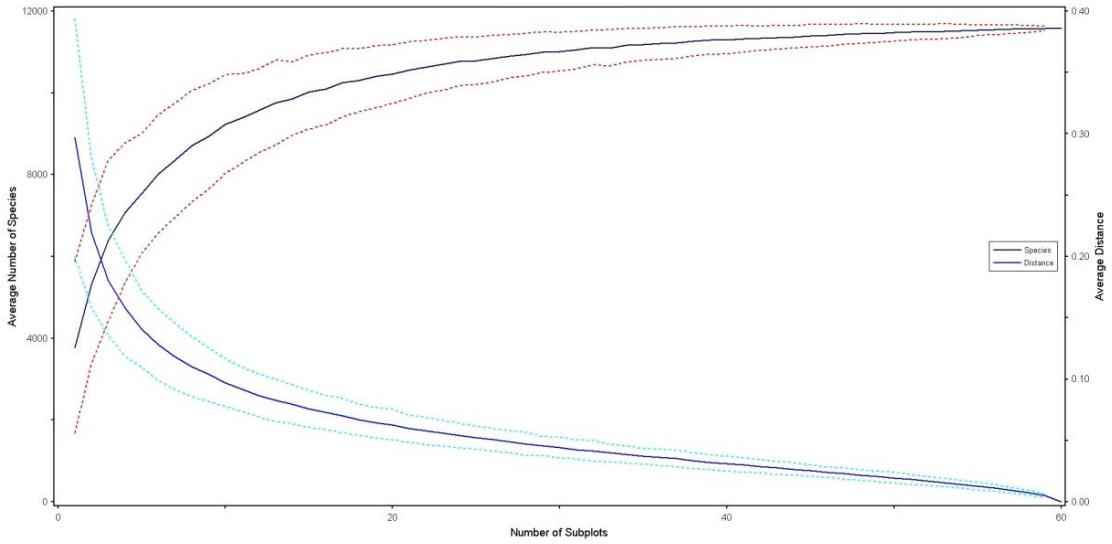


Figure 3.17: Rarefaction curves of total number of OTUs by number of samples (black line) and average Sorensen distance by number of samples (blue line).

The sample from the alleyways of block 1 in orchard 5 displayed the highest total OTU richness with a total of 7,772 OTUs and also the highest abundance of individual OTUs with a total of 182,973 rRNA gene sequences (Table 3.5). This orchard is subject to a conventional management regime. The two samples from the vine rows of blocks 1 in orchards 3 and 7 had the lowest OTU richness with 840 and 1,185 OTUs and an overall abundance of 1,674 and 2,704 sequences respectively. However, it is possible that the DNA in these particular samples may have degraded over time. Therefore, data for these two sample sites were excluded from subsequent analyses.

Table 3.5: Numbers of individual OTUs and rRNA gene sequences identified in each soil. (vr = vine row, aw = alleyway).

Orchard	Management Regime	Copper applied	Blk #	No. OTUs		No. rRNA gene sequences	
				vr	aw	vr	aw
1	Organic	No	1	3,685	3,475	27,581	21,581
			2	3,160	3,363	17,820	22,077
			3	3,822	3,326	23,961	20,496
2	Conventional	Yes	1	3,970	3,759	24,335	20,046
			2	4,424	4,001	35,066	25,969
			3	3,691	3,612	19,655	20,088
3	Conventional	Yes	1	840	3,498	1,674	22,157
			2	3,582	3,704	19,922	20,413
			3	6,361	3,105	117,163	16,686
4	Organic	Yes	1	3,785	3,516	24,782	21,664
			2	3,152	3,766	16,860	24,995
			3	3,855	3,214	25,076	18,612
5	Conventional	Yes	1	4,014	7,772	30,293	182,973
			2	4,121	4,109	28,341	27,877
			3	3,893	3,910	23,506	28,230
6	Organic	Yes	1	6,238	3,854	128,684	28,187
			2	3,438	7,146	20,925	157,112
			3	3,745	3,120	23,426	16,396
7	Conventional	Yes	1	1,185	2,851	2,704	14,216
			2	3,333	3,179	17,260	19,337
			3	3,375	3,446	21,564	21,602
8	Organic	No	1	3,785	3,397	28,296	21,165
			2	3,037	3,907	16,086	26,562
			3	3,569	3,663	25,261	23,905
9	Conventional	Yes	1	4,326	4,025	30,824	28,668
			2	3,861	3,762	26,280	22,016
			3	3,912	4,625	25,011	42,513
10	Organic	Yes	1	2,863	2,659	16,444	13,204
			2	3,843	3,445	26,818	21,982
			3	4,116	4,198	38,829	38,134

There was a high degree of similarity in measures of phylotype diversity and evenness between sample sites with Shannon's diversity scores (*H'*) ranging from 6.88 to 7.45 (Figure 3.18) and Pielou's evenness scores ranging from 0.82 to 0.88. For nine out of ten orchards OTU diversity was on average higher in the soils sampled from vinerows, although the differences between vine rows and alleyway sites were not statistically significant. There was a high degree of variance in OTU diversity for vine row sites in orchard 5, ranging from 2863 to 7772.

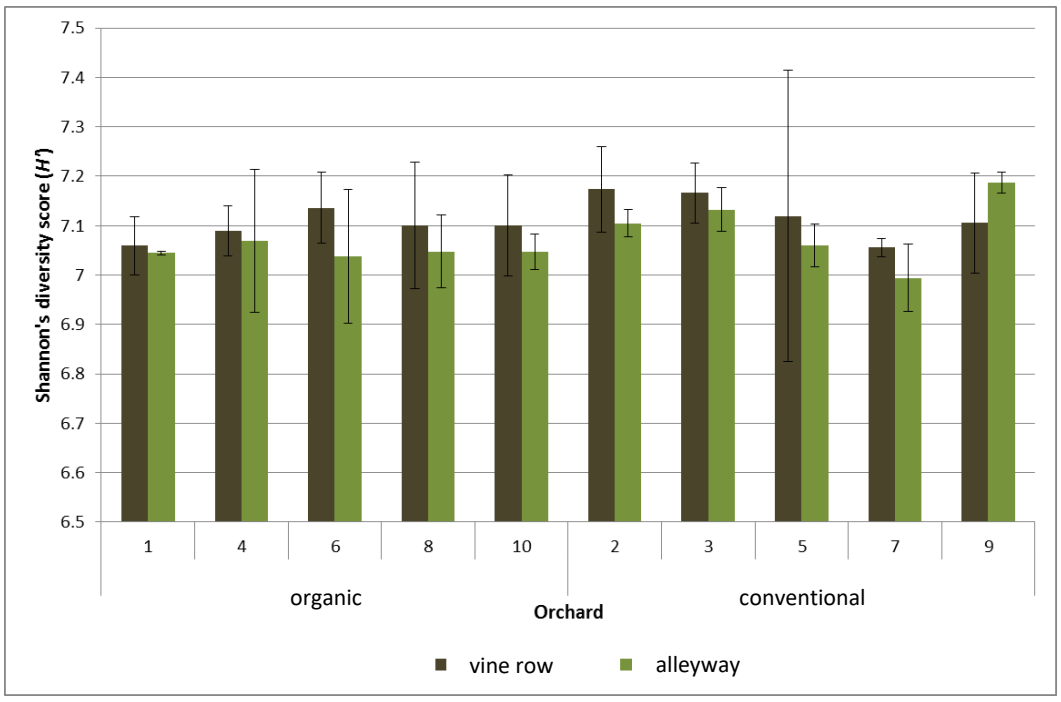


Figure 3.18: Shannon's diversity measures (*H'*) for samples from vine rows and alleyways within each orchard grouped by management. Bars denote means ± SD of three blocks per orchard.

Correlation analyses did not reveal relationships between OTU richness and total number of rRNA sequences per sample and all other physicochemical parameters including Cu_T and Cu_{CaCl_2} (data not shown). Neither were there relationships between individual phylotype diversity (Shannon's *H*') or Pielou's evenness scores (*J*) and all other physicochemical parameters, including copper levels and pH. However, when sites were grouped by soil Cu_T levels (Table 3.6) overall differences between Shannon's diversity indices as determined by t-tests were highly significant ($p < 0.001$). Although, the high degree of significance was mainly attributed to a greater phylotype diversity within group 4. In addition, differences in means between groups 1 and 5 with the lowest and highest Cu_T levels respectively were not significant (7.085 *cf.* 7.082; $p < 0.09$). This result together with the results of correlation analyses may indicate that the differences between these groups are due to variables other than Cu_T alone. Results of one-way ANOVA determined no significant differences in species richness, Shannon diversity and Pielou's evenness between orchards or between sites grouped according to the total concentration of copper in the soil (data not shown). However, conventional orchards had close to significantly higher species richness scores than organic orchards as determined by t-tests (3,676 *cf.* 4,075; $p = 0.055$).

Table 3.6: Groups to which orchard sites were assigned based on soil Cu_T concentration

Group	Cu_T (mg kg^{-1})	Sites
1	0 - 30	O1 B1 AW, O1 B1 VR, O1 B2 AW, O1 B2 VR, O1 B3 AW, O1 B3 VR, , O6 B1 AW, O6 B1 VR, O6 B2 AW, O6 B3 AW, O6 B3 VR, O8 B2 AW, O9 B1 AW, O9 B1 VR, O9 B2 AW, O9 B2 VR, O9 B3 AW, O9 B3 VR O10 B1 AW, O10 B2 AW, O10 B3 AW
2	30 – 40	O3 B2 VR, O3 B3 VR, O5 B1 AW, O5 B3 AW, O6 B2 VR, O8 B3 AW, O10 B1 VR, O10 B2 VR, O10 B3 VR
3	40 – 50	O2 B3 VR, O3 B1 VR, O3 B2 AW, O3 B3 AW, O5 B2 AW, O7 B1 AW, O7 B2 AW, O7 B3 AW, O8 B1 AW, O8 B1 VR, O8 B2 VR
4	50 – 60	O2 B3 AW, O3 B1 AW, O4 B1 AW, O4 B2 AW, O4 B2 VR, O4 B3 VR, O5 B1 VR, O5 B2 VR, O8 B3 VR
5	> 60	O2 B1 AW, O2 B1 VR, O2 B2 AW, O2 B2 VR, O4 B1 VR, O4 B3 AW, O5 B3 VR, O7 B1 VR, O7 B2 VR, O7 B3 VR

3.3.3 Community composition

Bacterial OTUs were by far the most dominant across all samples corresponding to 98.7% of the total unique rRNA gene sequences. Of these, the most abundant individual OTU is from the genus *Nitrospira* which occurs in all sites ranging from 0.45% to 2.84% ($\bar{x}$ = 1.69%) of the relative percentage abundance in all samples. The next most abundant individual OTUs are from the genus *Bacillales* and *Rhodoplanes*, averaging 0.91% relative abundance in each site sample. There were no significant differences in the relative abundance of these three most abundant OTUs between sites grouped by total soil copper levels (Figure 3.19).

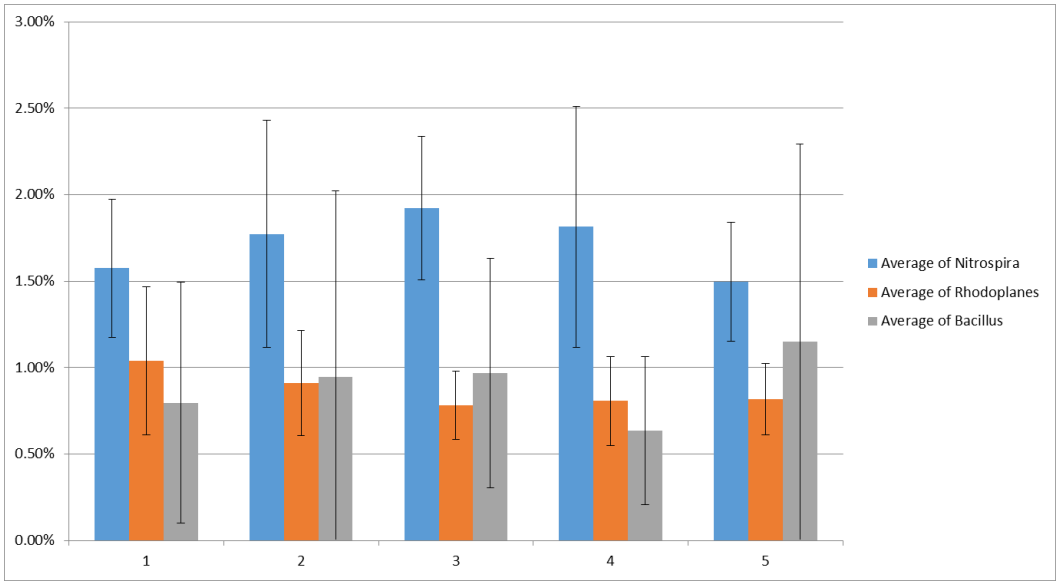


Figure 3.19: Mean percentage relative abundance of the 3 most abundant taxa in soils grouped according to total soil copper levels. Error bars denote ± SD.

Archaeal sequences accounted for 1.3% of the total unique rRNA gene sequences. Of these the most abundant OTUs are from the phylum *Thaumarchaeota*. Most of these were identified with 100% confidence as belonging to the families of *Nitrososphaera* and *Nitrosopumilus*. *Nitrososphaera* was present in all site samples, although there was a significantly higher mean percentage relative abundance of these taxa for conventional orchards, (1.0% *cf*. 0.4% for organic orchards, $p < 0.01$) and also for sites with the highest levels of Cu_T (0.9% for group 5 *cf*. 0.5% for group 1, $p < 0.05$). *Nitrosopumilus* was only identified in 50 out of 60 samples. The ten samples in which *Nitrosopumilus* was absent were all from organic orchards, including all samples from orchard 8. There was a greater mean percentage relative abundance of *Nitrosopumilus* in conventional orchards (1.1% for conventional *cf*. 0.4% for organic, $p < 0.01$). There was however no difference in percentage relative abundance of these taxa between sites grouped by levels of soil Cu_T.

In terms of the total number of OTUs within the domain Archaea, conventional orchards had a significantly higher mean percentage relative abundance than organic orchards (1.8% *cf*. 0.8%, $p < 0.01$). Samples from soils with the highest levels of Cu_T (> 60 mg kg^{-1}) also had a significantly higher mean percentage relative abundance of archaeal sequences compared with samples from sites with the lowest levels of Cu_T (1.7% *cf*. 1.1%, $p < 0.01$).

The most dominant phyla in terms of relative abundance based on a ≥ 70% certainty threshold are the *Proteobacteria*, which account for on average 30% of the total, followed by *Actinobacteria* (15%), *Bacterioidetes* (11%), and *Acidobacteria* (9%) (Figure 3.20). In terms of the relative abundances of each phyla, all sites were reasonably comparable. However the relative abundance of *Actinobacteria* ranged fairly widely from 5% to 24% ($\bar{x}$ = 30%). OTUs within the phylum *Firmucutes* also ranged fairly widely across sites with a relative abundance of 0.5% to 8% ($\bar{x}$ = 2%). The wide range in values for *Proteobacteria* (18% - 42%; $\bar{x}$ = 30%) were mostly due to the distinct profiles of sites O3 B1 VR and O10 B1 VR which have a relative under- and over-abundance of OTUs within this phylum. Within each class of the *Proteobacteria* the *α-Proteobacteria* and *δ-Proteobacteria* are the most represented making up 33% and 32% of the total number of individual OTUs. In terms of the relative abundance of individual gene

sequences, the *α-Proteobacteria* are the most numerous making up 42% of the total, followed by *ß-Proteobacteria* (25%) *γ-Proteobacteria* and *δ-Proteobacteria* (18% & 16% respectively).

A comparison of the mean percentage relative abundance of OTUs grouped on the basis of lowest (group 1) or highest (group 5) soil copper levels revealed significant differences between the phyla *Chlamydiae*, *Chloroflexi, Actinobacteria* and *Thaumarchaeota* (Table 3.7). The differences however, although significant, were subtle.

Table 3.7: Significant differences in percentage relative abundance of phyla observed for sites grouped by total soil copper levels.

Phylum	mean % relative abundance		% difference	*p* value
	Group 1 0-30 mg kg^{-1}	Group 5 > 60 mg kg^{-1}		
Chlamydiae	1.96	3.15	1.20	0.0006
Chloroflexi	0.66	0.91	0.25	0.003
Actinobacteria	17.49	15.11	2.38	0.02
Thaumarchaeota	0.66	1.15	0.49	0.03

There were no statistically significant differences between groups 1 and 5 in terms of the relative abundances of either *Pseudomonas* or *Rhizobiales* which have been shown to be particularly sensitive indicators of copper pollution (Berg *et al.*, 2012; Alloway, 2010; Giller, 1998).

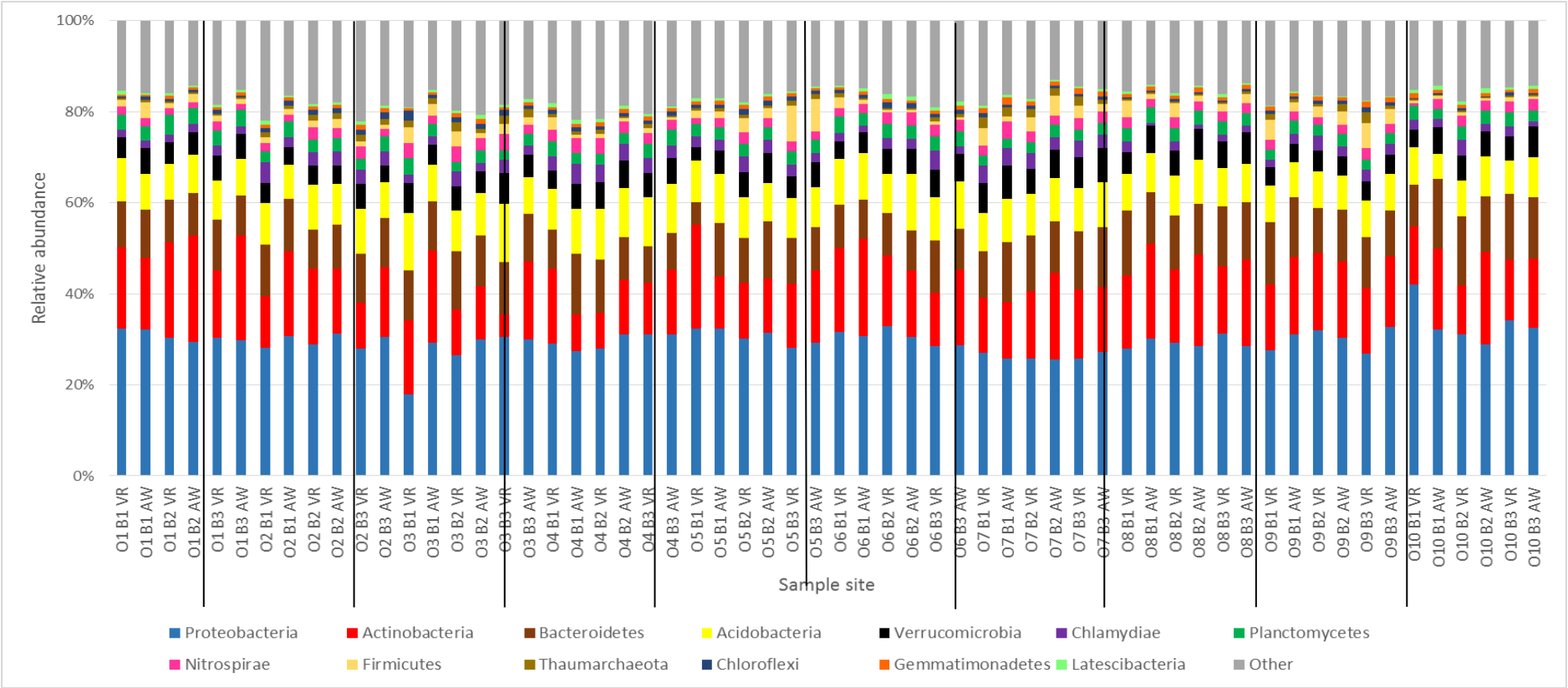


Figure 3.20: Relative % abundance of each phyla at each sample site (O = Orchard, B = orchard block, AW = alleyway, VR = vine row). Classification is based on a ≥ 70% degree of certainty in assignment at the phylum level. Phyla grouped as 'other' include *Aquificae*, *Armatimonadetes*, BRC1, candidate division WPS-1 and 2, *Candidatus Saccharibacteria*, cyanobacteria/chloroplast, *Diapherotrites*, *Euryarchaeota*, *Hydrogenedentes*, *Microgenomates*, *Pacearchaeota*, *Parcubacteria*, *Parcubacteria* and *Woesearchaeota*.

3.3.4 Non-metric multidimensional scaling (NMDS)

A total of 1,151 OTUs that occurred in fewer than 5% of sites were removed from the primary matrix prior to analyses. Sites O3 B1 VR and O7 B1 VR were also omitted from analyses as they were significant outliers due to the comparatively low species richness and total number of sequences which may have been due to DNA degradation. NMDS of community dissimilarity between orchards based on presence/absence data for all OTUs resulted in a 2 dimensional ordination with a stress value of 21.48 (p = 0.004) (Figure 3.21). A 3 dimensional solution was recommended (stress = 13.54) however, this would have been more difficult to report.

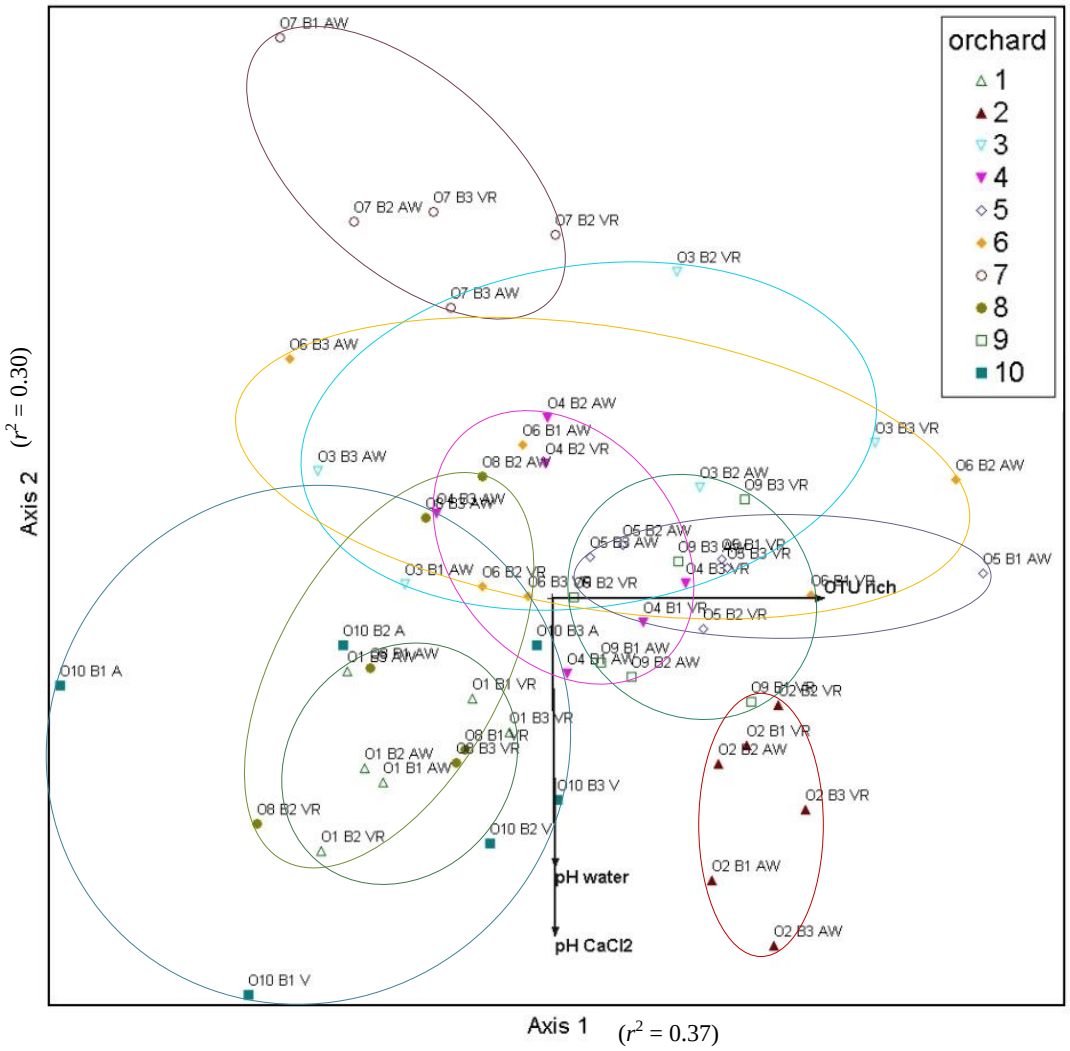


Figure 3.21: NMDS ordination of bacterial communities within each sampling site based on presence/absence data and grouped by orchard. Distance between points is proportional to dissimilarity. Stress = 21.48. Vectors for environmental variables are shown at $r^2 > 0.4$

The paired orchards 1 and 2, and 7 and 8 are highly distinct, indicating a high degree of dissimilarity in bacterial community composition between these orchards. Orchards 9 and 10 also form distinct clusters but to a lesser degree and there is much higher dissimilarity between individual sites within orchard 10. The dissimilarity of sites between orchards 1 and 2, and 9 and 10 can be mostly attributed to OTU richness. Whereas the pH_{CaCl_2} and pH_{water} vectors (r^2 = 0.545 and 0.416), which show higher levels of influence in site separation explain the dissimilarity between orchards 9 and 10. All other explanatory variables had r^2 values below the cut-off threshold of 0.4 including all forms of copper for which r^2 values were below 0.1.

The NMDS ordination based on % relative abundance data for all OTUs, which is more indicative of actual dissimilarity between bacterial communities at each site, highlighted distinct groups that were more aligned with pH than OTU richness (Figure 3.22).

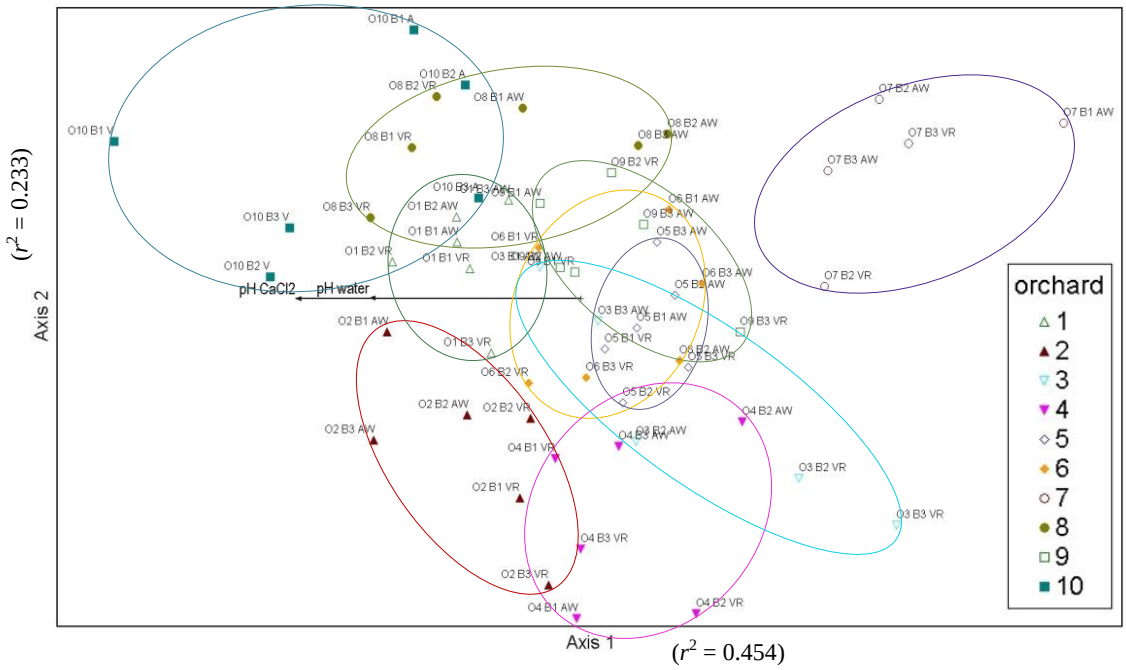


Figure 3.22: NMDS ordination of bacterial communities within each sampling site based on % relative abundance data and grouped by orchard. Distance between points is proportional to dissimilarity. Stress = 21.27. Vectors for environmental variables are shown at $r^2 > 0.4$.

This ordination again revealed that the bacterial community assemblages within the paired orchards 1 and 2, 7 and 8, and 9 and 10 are distinct. For orchards 7 and 8, and 9 and 10 the differences are again mostly correlated with pH (r^2 = 0.601 and 0.445). However, the dissimilarity between communities within orchards 1 and 2 is more aligned with axis 2 with a weak correlation with cadmium (r^2 = 0.323). Cu_T was only very weakly correlated with community dissimilarity along Axis 2 (r^2 = 0.237) and there was no correlation with Cu_{CaCl_2}.

NMDS of community dissimilarity between all sites based on % relative abundance data of all OTUs for each of 5 groups according to soil Cu_T concentration did not reveal distinct clusters (Figure 3.23). Sites were assigned to one of the following groups:

1 = 0-30, 2 = 30-40, 3 = 40-50, 4 = 50-60, 5 > 60 mg Cu_T kg^{-1}

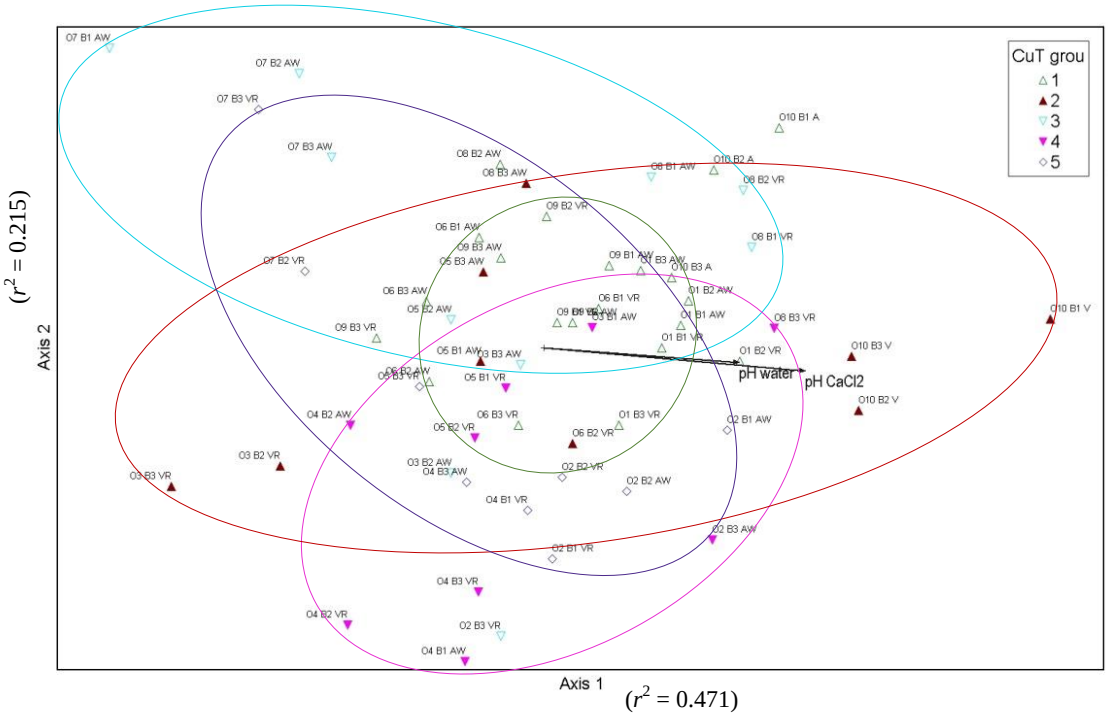


Figure 3.23: NMDS ordination of bacterial communities within each sampling site based on % relative abundance data and grouped by Cu_T concentration. Distance between points is proportional to dissimilarity. Stress = 21.32. Vectors for environmental variables are shown at r^2 > 0.4.

pH (water and $CaCl_2$ – extractable) was again the main explanatory variable for site separation with this ordination (r^2 = 0.41 and 0.55 respectively) although sites grouped by Cu_T were not aligned with this variable.

NMDS of alleyway sites revealed separation between clusters grouped by Cu_T, with sites with the highest (group 5) and lowest (group 1) levels of total copper forming distinct clusters (Figure 3.24). There was also reasonable separation between groups 4 & 5 (> 50 mg Cu_T kg^{-1}) and 1 & 2 (0 - 40 mg Cu_T kg^{-1}).

Again, pH (water and $CaCl_2$ – extractable) was shown to be the environmental parameter that is most strongly correlated with community separation (r^2 = 0.772 and 0.682 respectively). Total copper was only weakly correlated (r^2 = 0.247), with a range of other soil parameters seemingly having a higher degree of influence over site separation.

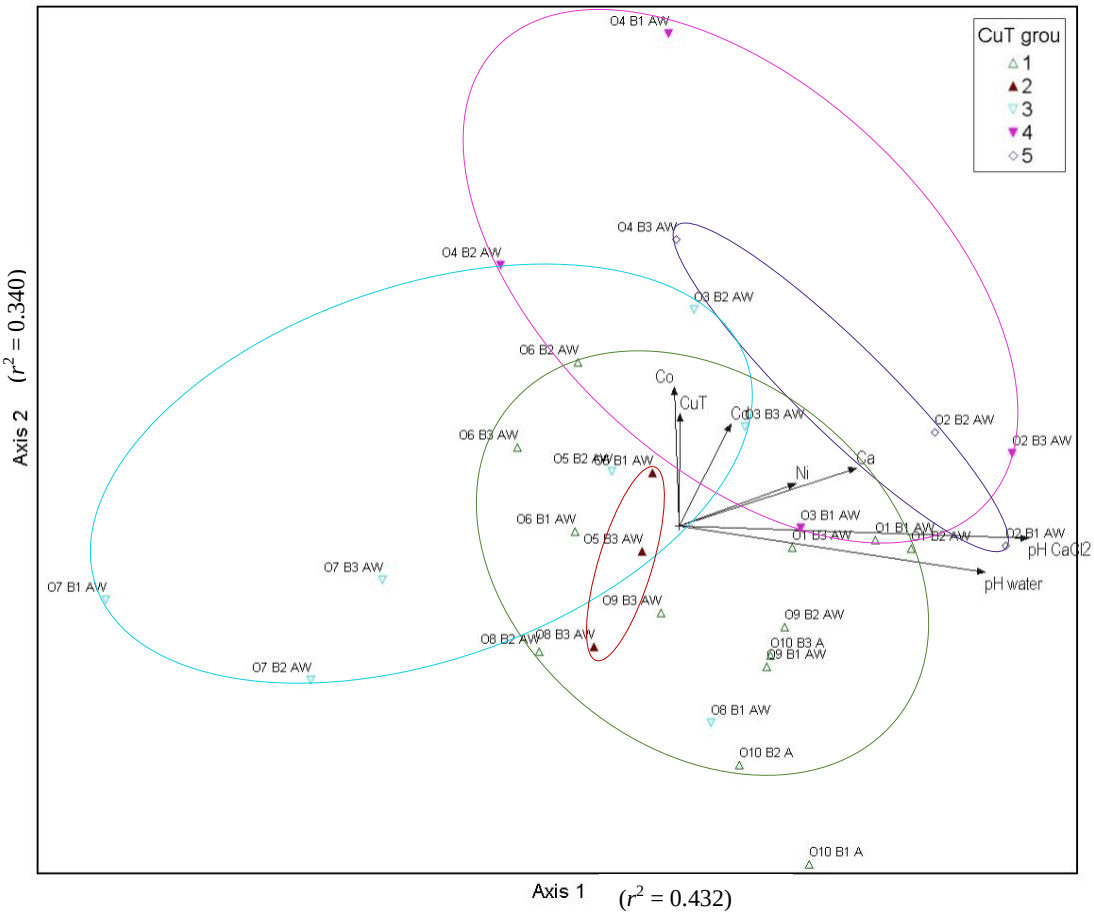


Figure 3.24: NMDS ordination of bacterial communities within alleyway sites based on % relative abundance data and grouped by Cu_T concentration. Distance between points is proportional to dissimilarity. Stress = 17.41. Vectors for environmental variables are shown at r^2 > 0.24.

The 3D NMDS ordination based on % relative abundance data for all OTUs and grouped by management practice, revealed highly distinct clusters (Figure 3.25).

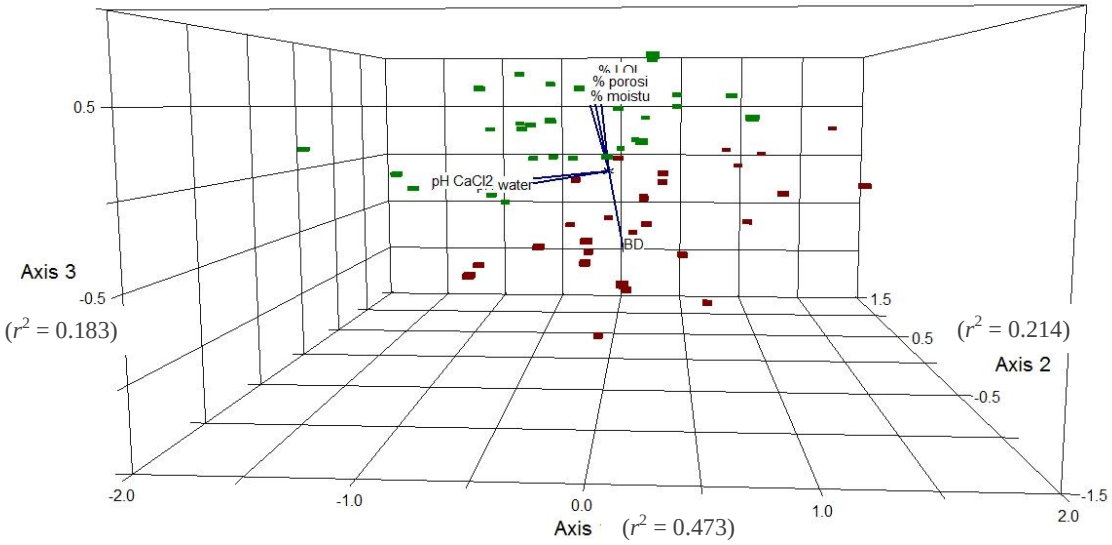


Figure 3.25: NMDS of % relative abundance data grouped by management practice (red squares = conventional; green squares = organic). Stress = 12.41. Explanatory vectors shown at $r^2 > 0.4$.

The explanatory variables organic matter (% LOI), % porosity, bulk density and % moisture mostly accounted for the divisions between groups along axis 3 (r^2 = 0.57, 0.51, 0.52 and 0.42 respectively) with pH mostly accounting for the dissimilarity along axis 1. The strong negative correlation between higher organic matter and porosity with a lower bulk density as determined previously is evidenced here and indicates that these factors, along with pH and moisture influence bacterial community composition across these study sites. The 3D NMDS plot of sites grouped by herbicide use showed a good degree of separation between treatments (Figure 3.26). Again, the groupings are aligned with the explanatory variables organic matter, porosity and bulk density along Axis 3 and pH along Axis 1.

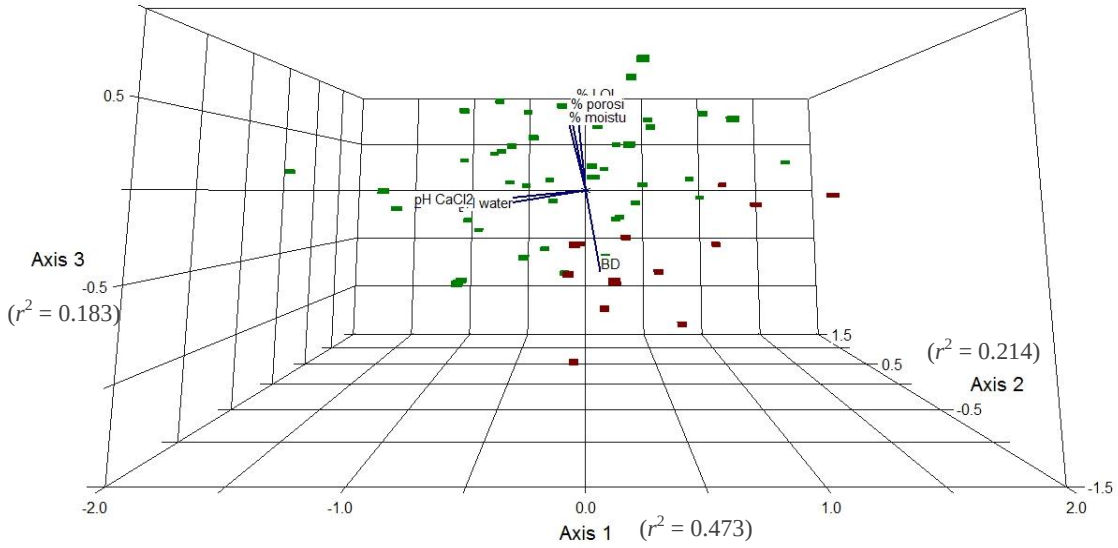


Figure 3.26: NMDS of % relative abundance data grouped by glyphosate use (red squares = glyphosate; green squares = none). Stress = 12.41. Explanatory vectors shown at $r^2 > 0.5$.

NMDS of both rare and abundant OTUs constituting < 0.1% and > 0.1% relative abundance across all samples revealed very similar cluster patterns to ordinations based on total relative abundance for all OTUs when grouped by management, herbicide use, position, and levels of copper. However, differences were observed between ordinations of rare OTUs and total OTUs when grouped by orchard. Much greater separation of sites between paired orchards was observed for the ordination of rare OTUs (Figure 3.27). The main explanatory variables for which r^2 values were above 0.4, in addition to pH_{CaCl2} (0.47) and pH_{water} (0.43), included calcium (0.5), organic matter (0.5), and bulk density (0.41). There was a very low degree of dissimilarity between rare OTUs in samples sites in orchards 2 and 5 compared with other orchards. For orchard 5 the degree of dissimilarity is similar to that revealed in the NMDS of total OTUs. However, upon comparison of these two ordinations, it appears that rare OTUs in orchard 7 must be less dissimilar across sampling sites than abundant OTUs. In contrast, the degree of dissimilarity of between rare OTUs in orchard 1 sampling sites is greater than that for abundant species.

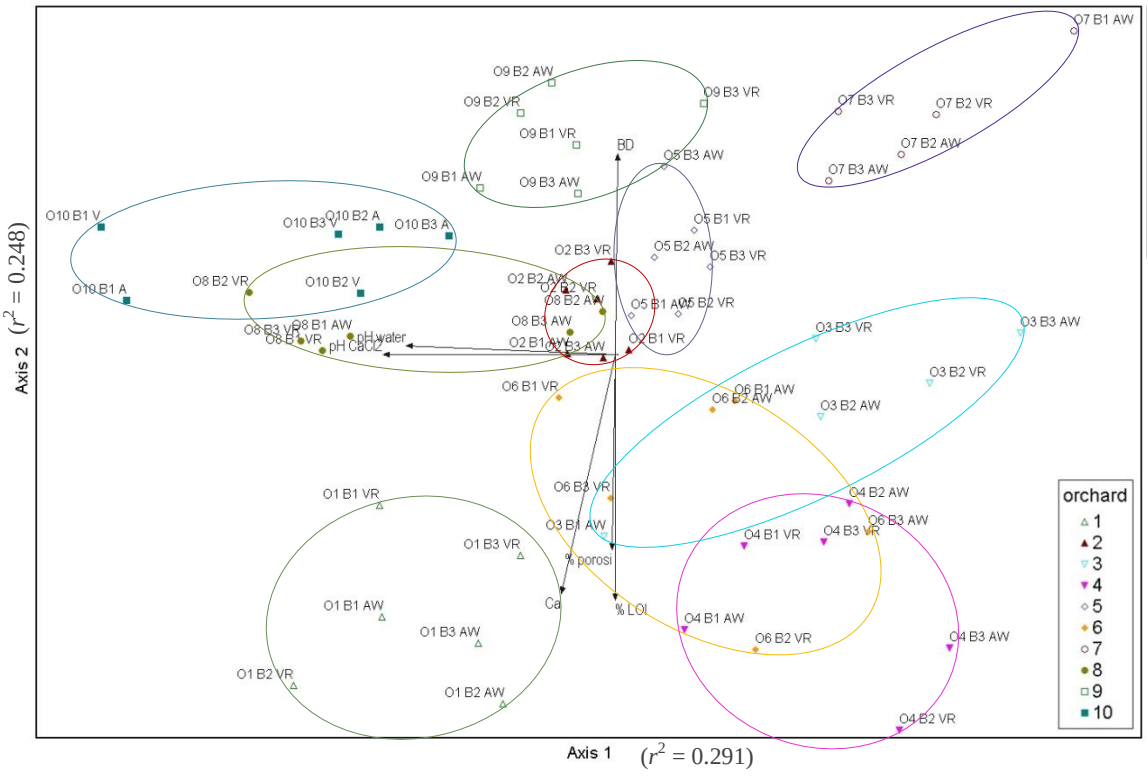


Figure 3.27: NMDS ordination of bacterial communities within each sampling site based on % relative abundance data of rare OTUs and grouped by orchard. Distance between points is proportional to dissimilarity. Stress = 23.65. Vectors for environmental variables are shown at $r^2 > 0.4$.

3.3.5 Multi-response permutation procedures (MRPP)

MRPP using relative abundance data for all OTUs revealed a very highly significant degree of separation between all orchards (T = -20.97, A = 0.724, p = 0). The A value indicates that there is a very high degree of OTU similarity within groups. Pair-wise MRPP analysis of sites between adjacent, paired orchards (Table 3.8) indicated that bacterial communities were all highly dissimilar (p = < 0.01). Interestingly, MRPP revealed significant differences in bacterial communities between the paired orchards 5 and 6, and 3 and 4 for which NMDS did not reveal distinct clusters.

Table 3.8: MRPP pairwise comparisons of paired orchards based on OTU relative abundance data for all OTUs.

Orchards compared	Test stat. (*T*)	*A*	*p*
1 and 2	-6.54	0.43	0.0004
3 and 4	-4.36	0.30	0.0011
5 and 6	-5.14	0.31	0.0006
7 and 8	-5.14	0.37	0.0019
9 and 10	-6.57	0.38	0.0005

The degree of dissimilarity between sites grouped by Cu_T was tested with MRPP pair-wise comparisons which identified significant differences between groups 1 and 5 (p = 0.002), 1 and 4 (p = 0.005), and 1 and 3 (p = 0.04) (Table 3.9). The test (T) statistic for each pairwise comparison indicated a reasonably low degree of dissimilarity between groups and the low A values indicated a high level of heterogeneity within groups. However it is apparent that group 1 which encompasses sites with the lowest levels of Cu_T are the most distinct in terms of OTU community assemblages.

Table 3.9: MRPP pairwise comparisons of sites grouped by Cu_T and based on OTU relative abundance data.

Cu_T groups compared	Test stat. (*T*)	*A*	*p*
1 and 5	-4.20	0.095	0.002
1 and 4	-3.54	0.077	0.005
1 and 3	-2.08	0.041	0.038

Pairwise comparisons of alleyway sites only and grouped by Cu_T only revealed significant community dissimilarity between groups 2 and 5 (p = 0.04, A = 0.21) although the degree of separation was not very strong (T = -1.92). Dissimilarities in community composition between all other alleyway sites grouped by this variable were not significant.

When groups were compared according to management practice, the degree of dissimilarity between groups was very high (T = -14.733, A = 0.16, p = 0), as indicated by the NMDS ordination. However, the degree of similarity within management groups was much lower due to the high degree of separation between sites within each grouping category. MRPP of sites grouped by glyphosate use showed significant group dissimilarity (p < 0.0001) as indicated by the T statistic

(-8.32), although *A* (0.09) was very close to zero indicating heterogeneity within groups is almost that expected by chance.

MRPP using relative abundance data for rare OTUs again revealed a very similar and degree of separation between all orchards (T = -20.67, A = 0.694, p = 0) and also between paired orchards as revealed by pair-wise MRPP analysis (Table 3.10).

Table 3.10: MRPP pairwise comparisons of paired orchards based on OTU relative abundance data for rare OTUs.

Orchards compared	Test stat. (*T*)	*A*	*p*
1 and 2	-6.62	0.44	0.0004
3 and 4	-4.88	0.26	0.0009
5 and 6	-6.00	0.26	0.0003
7 and 8	-5.53	0.41	0.001
9 and 10	-6.11	0.30	0.0003

MRRP of rare OTU relative abundance data again revealed significant differentiation between sites grouped by levels of Cu_T in soil (Table 3.11).

Table 3.11: MRPP pairwise comparisons of sites grouped by Cu_T and based on OTU relative abundance data for rare OTUs.

Cu_T groups compared	Test stat. (*T*)	*A*	*p*
1 and 5	-3.45	0.07	0.005
1 and 4	-2.24	0.05	0.03
1 and 3	-2.65	0.05	0.01

The degree of dissimilarity between OTU assemblages of rare species between group 1 and groups 3, 4 and 5 was very comparable with that observed for all OTU relative abundance data. Also, the degree of dissimilarity of rare OTUs based on pair-wise comparisons of all other groups was again, not significant.

3.3.6 Indicator species analysis

Indicator species analysis of sites grouped by soil copper levels (Table 3.6) revealed 743 significant indicator OTUs. Significant ($p < 0.05$) observed maximum indicator (IV_{max}) values ranged from 19% to 63%, where a value of

100% implies a perfect indication for an individual OTU that is always and exclusively associated with a particular group. Of the 743 indicator OTUs, the degree of confidence in phylum affiliation for 243 taxa was less than 70% and these were subsequently grouped as 'other'. Of the 743 indicator OTUs, 196 (26%) were affiliated to group 5 (> 60 mg Cu_T kg^{-1}). A total of 67 OTUs out of 196 were classified as 'other' based on a low degree of confidence in phylum classification. Of the 129 indicator OTUs in group 5 for which the degree of confidence in phylum affiliation was > 70%, 34 (27%) belonged to the *Proteobacteria*, of which the *Delta Alpha and Gammaproteobacteria* were evenly distributed (Figure 3.28).

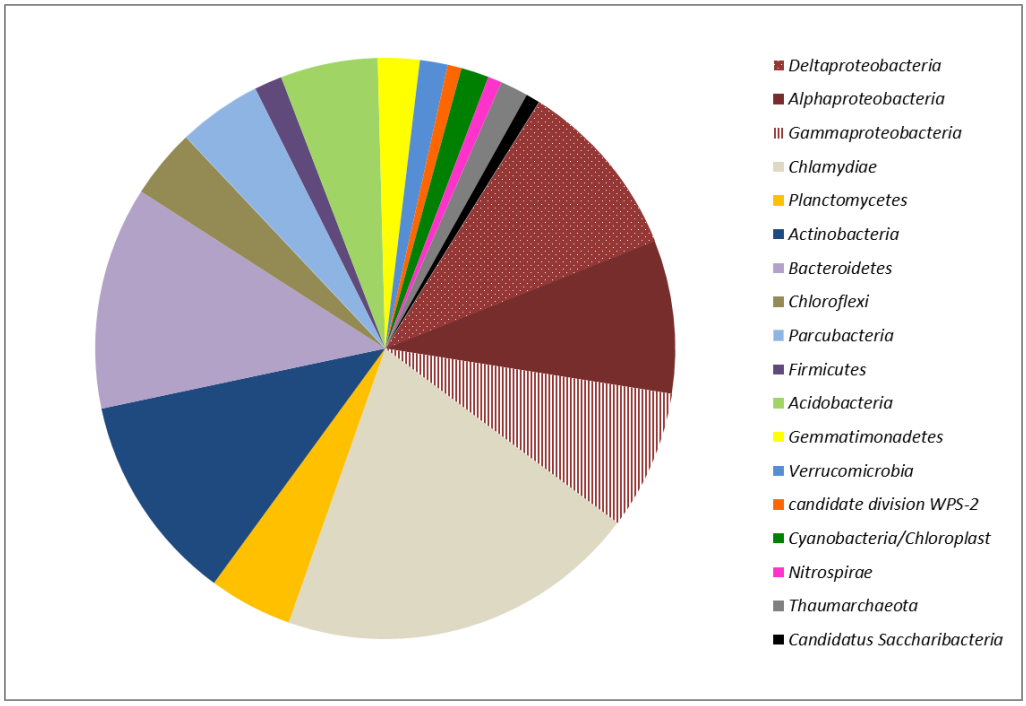


Figure 3.28: Phylum-level affiliations (> 70% confidence) of indicator OTUs associated with orchard sites exceeding > 60 mg Cu_T kg^{-1} soil.

The next most abundant taxa were affiliated with *Chlamydiae* (20%), *Bacteroidetes* (12%) and *Actinobacteria* (12%). The two indicator OTUs with the highest indicator values (> 50%) in group 5 were identified as belonging to the phylum *Acidobacteria* within the order Gp10 and the species *Propionibacterineae nocardioidaceae*.

A total of 96 indicator OTUs were affiliated with group 1 (0 - 30 mg Cu_T kg^{-1}). Of these, 26 (27%) were classified as 'other' due to a low degree of confidence in phylum classification. Of the 70 indicator OTUs in group 1 for which the degree of confidence in phylum affiliation was > 70%, 29 (43%) belonged to the *Actinobacteria*. *Proteobacteria* were the next most represented phyla of which *Alphaproteobacteria* made up 12% of the total followed by *Gammaproteobacteria* (6%) and *Deltaproteobacteria* (3%). A number of other taxa were affiliated with *Bacteroidetes* (13%) and *Planktomycetes* (10%) (Figure 3.29).

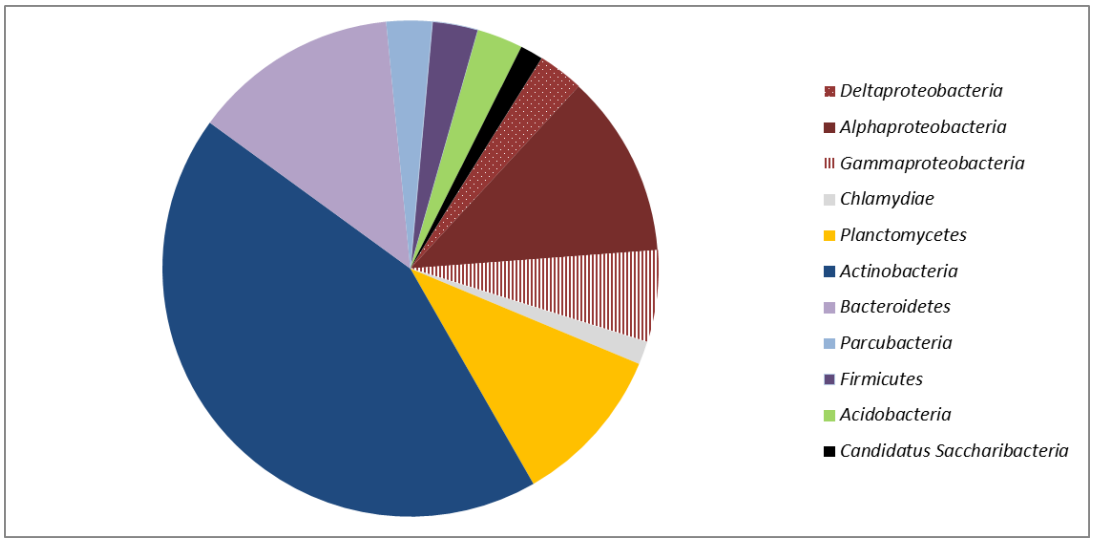


Figure 3.29: Phylum-level affiliations (> 70% confidence) of indicator OTUs associated with orchard sites with 0 - 30 mg Cu_T kg^{-1} soil.

The OTU with the highest observed indicator value (46%) for group 1 was *Pseudofulvimonas*, a member of the order *Xanthomonadales* within the *Gammaproteobacteria* class. The other two OTUs with indicator values above 40% were affiliated with the *Actinobacteria*, one of which was classified as the species *Gaiella*. A total of 26 individual OTUs within the order *Chlamydiales* have a significant degree of association with group 5, compared with only one in group 1 and these are mostly affiliated to the families *Parachlamidiaceae* and *Simkaniaceae*.

Indicator OTUs found in group 5 sites but not in group 1 sites include those within the phyla *Nitrospirae*, *Thaumarchaeota*, *Cyanobacteria*, Candidate division WPS-2, *Verrucomicrobia*, *Gemmatimonadetes*, and *Chloroflexi*.

4 Discussion

4.1 Soil physicochemical properties

Soil copper levels did not necessarily relate to the total amount and frequency of copper applied since 2012. Orchard 8 had relatively high levels of total copper and also the highest levels of $CaCl_2$-extractable copper. This is regardless of the fact that copper has not been applied to this orchard for a number of years and applications of compost and other bio-amendments are made frequently. Although, the pH of soils in this orchard are much higher than soils in the adjacent conventional orchard which are relatively low, and this may explain the much lower pCu^{2+} measurements even though the $CaCl_2$-extractable copper levels were the highest measured. Due to very low pCu^{2+} in soil extracts and technical difficulties in obtaining accurate data, significant differences in pCu^{2+} across all sites and subsequent effects on bacterial community composition could not be determined. Other methods by which to determine levels of free ionic copper in soil solution may have provided more useful information. The technique of diffusive gradients in thin films (DGT) may have been a more appropriate method to use as it provides an *in situ* measure of bioavailable copper that is potentially toxic to microorganisms (Davison & Zhang, 1994). Differences in community composition and other measures of diversity and activity could potentially have been ascribed to Cu^{2+} in soil solution if more accurate data on this most bioavailable form of copper had been obtained.

Dehydrogenase activity (DHA) was found to be significantly lower in the conventional orchards where levels of activity in soils from vine rows were also consistently lower than in soils from alleyways. Vine rows within the conventionally managed orchards are subject to frequent glyphosate applications, unlike the organic orchards. Glyphosate applications to soil have been shown to reduce dehydrogenase activity by up to 48% (Wolińska & Stępniewska, 2012; Zabaloy *et al*., 2008). Higher levels of organic matter in soil, as measured for the organic orchards in this study, have also been shown to significantly enhance DHA in soil (Fließbach *et al.*, 2007). A study by Sebiomo *et al*. (2013) attributed a reduction in DHA to a resulting reduction in organic matter as a result of herbicide use rather than direct toxicity. This is because DHA is a product of the oxidation of organic matter by microorganisms (Dick, 1997). This could in part

explain the marked differences between conventional and organic orchards and also vine rows, which receive glyphosate applications, and alleyways, which do not. The lack of correlation between measured soil copper levels and DHA may have been due to the large number of variables which made it difficult to observe a direct cause and effect. It may also be that levels of copper in soil were below that which would result in a reduction in DHA, especially the most bioavailable free ionic form.

The soil particle sizes of all orchards were similar with clay minerals comprising between 6 and 15% of the total. Organic orchards tended to have higher levels of soil organic matter, labile carbon and porosity, whilst conventional orchard soils generally had a higher bulk density, which was most likely attributed to the lower soil organic matter and possibly reduced bioturbation associated with a decline in the activities of earthworms and plant roots (Naveed *et al*., 2014). The findings of this study concur with those of Swezey *et al.* (1998) & Glover *et al.*(2000) who found that organic orchard soils tend to have a lower bulk density than conventionally managed orchard soils with correspondingly higher levels of organic matter. The correspondingly higher levels of aeration and capacity for water and nutrient movement in organic orchard soils may explain much of the differences in enzyme activity and bacterial community composition.

4.2 **Bacterial OTU richness and diversity**

There were no significant differences in species richness, Shannon's diversity and Pielou's evenness scores between orchards or between sites grouped according to the total concentration of copper in the soil. The observed differences in OTU richness between organic and conventional orchards could potentially be related to differences in management practices such as compost and biological amendments or the presence and diversity of plant, fungal or invertebrate species. Studies have shown that the bacterial community composition of soils is largely influenced by groundcover and organic amendments (Bonilla *et al*., 2012; Piao *et al*., 2008; Yao *et al*., 2005). Above-ground variables such as groundcover may also influence below-ground bacterial communities in a number of ways. Communities within orchard soils to which herbicide applications are made were shown to be distinct from those that had a cover of vegetation. The response of

communities to this treatment may be a direct response to toxicity or indirectly in response to a number of variables including a reduction in symbiotic plant host species, and a subsequent change in organic matter, soil compaction and aeration, temperature, moisture and light levels.

4.3 Community composition

The community composition of bacterial OTUs sequenced from each site at the level of phylum were not obviously dissimilar. The relative abundances of OTUs within each phylum for all sites were within a similar range and there were no significant differences in the most abundant individual taxa between sites grouped according to total soil copper levels. However, at the level of phylum significant differences between sites were observed for the phyla *Chlamydiae*, *Chloroflexi*, *Actinobacteria* and *Thaumarchaeota*, with a higher representation of *Chlamydiae*, *Chloroflexi*, and *Thaumarchaeota* in sites with the highest levels of copper compared with those with the lowest levels. Conversely, the relative abundances of OTUs within the phylum *Actinobacteria* were significantly greater in soils with the lowest levels of copper.

An OTU within the genus *Nitrospira* was the most abundant taxa identified in all site samples with no difference in relative abundance between sites with the lowest and highest levels of Cu_T or between organic or conventional orchards. These bacteria play a key role in the nitrogen cycle in the process of oxidation of nitrite to nitrate (Koch *et al*., 2015). A completely nitrifying bacterium from the genus *Nitrospira* for which the genome encodes pathways for ammonia and nitrite oxidation, has also recently been cultured (Daims *et al*., 2015). This suggests that *Nitrospira* may be a key component in the biogeochemical cycling of nitrogen.

The relative abundances of OTUs within the domain Archaea, although only comprising 1.3% of the total, were significantly higher in conventional orchards and also in orchards with the highest levels of Cu_T. OTU sequences from the families *Nitrososphaera* and *Nitrosopumilus* were the most dominant archaea found. These archaea have been shown to be chemolithoautotrophic ammonia-oxidizers which may play a significant role in nitrogen and carbon cycling (Zhalnina *et al*., 2014). There is an indication from the results of this study that

these archaea may be less sensitive to soil conditions typical of conventional orchards and better able to adapt to an increase in soil copper than bacteria, and perhaps fill a niche normally occupied by more copper-sensitive bacterial taxa. The essential process of nitrogen assimilation would, however remain unaltered. Kandler & König (1998) concluded that the membranes of archaea are less permeable to ions that those of bacteria and are adapted well to extreme conditions. A study by Chao-Rong and Qi-Chun (2011) found that there was no response of archaeal communities to copper pollution and concluded that they are perhaps more tolerant than bacteria and fungi. A study carried out by Mertens *et al*. (2010) concluded that the structure of ammonia-oxidising bacterial and archaeal soil communities changed in response to long-term copper contamination. However, the changes were associated with increased tolerance of specific taxa and not an overall loss of functional stability.

This study revealed no significant differences in the relative abundances of *Pseudomonas* or *Rhizobiales* between soils with the highest and lowest levels of $Cu_{T,}$ even though these taxa have been shown to be particularly sensitive indicators of copper pollution (Berg *et al.*, 2012; Alloway, 2010; Giller, 1998).

4.4 NMDS and MRPP

NMDS and MRPP revealed significant differences in bacterial communities between paired orchards. Each pair of orchards included one conventionally-managed and one organic. Soil copper levels were generally higher in the conventional orchards with the exception of orchard 9 for which levels were lower than the adjacent organic orchard in which copper was frequently applied.

NMDS of OTU relative abundance data clustered by different grouping variables revealed that pH was most strongly aligned with cluster separation. $pH_{(CaCl_2)}$ was always a stronger predictor than $pH_{(water)}$. This is most likely due to the fact that the weak $CaCl_2$ extractant simulates salts normally present in soil water, and therefore enables a very good estimation of soil pH *in situ* (Kissel & Vendrell, 2004). These findings are in agreement with numerous studies that have shown pH to be the main driving factor in the spatial distribution of bacterial communities (e.g., Fierer & Jackson, 2006; Griffiths *et al.*, 2011). The differences

in pH between paired orchards are most likely to be attributed to orchard management practices rather than natural variation as there is little spatial separation between each.

When MRPP was performed on relative abundance data for all OTUs and rare OTUs grouped by soil copper levels (Cu_T), dissimilarities between groups 1 (Cu_T = 0–30 mg kg^{-1}) and 5 (Cu_T = >60 mg kg^{-1}) were significant. Significant differences between group 1 and groups 3 (40–50 mg kg^{-1}) and 4 (50–60 mg kg^{-1}) were also revealed. However, MRPP of relative abundance data for all OTUs from alleyway sites and grouped by Cu_T, only revealed significant differences between groups 2 and 5 and not groups 1 and 5. Alleyway sites are not subject to management practices that are unique to conventional and organic orchards, such as compost amendments and herbicide and other chemical and biological applications. The lack of dissimilarity in bacterial OTU assemblages between alleyway sites with the highest and lowest levels of total copper may indicate that the significant differences observed between all sites within groups 1 and 5 may be due to variables other than copper alone. NMDS vectors, aside from pH for which r^2 values were higher than that for Cu_T (r^2 = 0.247), included those associated with calcium, cadmium, cobalt and nickel.

NMDS and MRRP of rare OTU abundance data was comparable with that performed on total abundance data. This indicates that the contributions of abundant and rare OTUs to differences between communities are similar.

MRPP of OTU assemblages for sites grouped by either conventional or organic management practices revealed two significantly distinct clusters. Although pH was again the environmental variable that explained much of the difference in OTU relative abundance between sites, dissimilarity between sites was mostly attributed to organic matter (% LOI), % porosity, bulk density and % moisture. Sites grouped by herbicide use within vine rows also revealed distinct clusters and again, the same variables indicated in community dissimilarity in organic and conventional orchards aligned with group separation. These findings concur with those of Brockett *et al.* (2012) where organic matter and soil moisture were found to closely correlate with microbial community composition across seven biogeoclimatic zones in western Canada.

The degree of variability between sites in terms of groundcover and also management practices such as foliar sprays and organic or bio-amendments have confounded attempts at determining the specific effects of copper. As these variables could not be quantified it was not possible to determine which if any were potentially influential in altering soil bacterial community composition.

4.5 Indicator species

Study soils with the lowest levels of total copper (0 – 30 mg kg^{-1}) had a higher prevalence of bacterial OTUs within the phylum *Actinobacteria* compared with the soils exceeding 60 mg kg^{-1}. These findings are contrary to a number of studies including those of Navel *et al.* (2010) and Lejon *et al.* (2008) who found that *Actinobacteria* enrichment in soil correlated with bioavailable copper. Although, these findings do concur with those of de Boer *et al*. (2012) and Naveed *et al*. (2014), where long-term copper contamination was shown to adversely affect *Actinobacteria* communities. Although those two studies had contrasting results for *Gammaproteobacteria*, with each revealing either a positive or negative correlation with soil copper concentrations. In the present study, *Gammaproteobacteria* did not seem to be adversely affected by increasing soil copper levels. In agreement with the present study, Naveed *et al*. (2014) also found that the relative abundances of *Chlamydiae*, *Gemmatimonadetes*, and *Nitrospirae* increased with increasing soil copper concentrations. The results of this study revealed that indicator OTUs from the phylum *Nitrospirae* were associated with soils with the highest levels of copper but were not indicative of soils with the lowest levels of copper. Archaeal OTUs within the Phylum *Thaumarchaeota* were also indicators of the highest levels of soil copper as to be expected based on previous community analyses of these taxa.

In a study carried out on copper-polluted citrus groves in Florida, sequencing of partial 16S rRNA gene fragments revealed that the more polluted soils were dominated by *Gammaproteobacteria*, *Acidobacteria*, *Firmicutes*, and *Betaproteobacteria* (Zhou *et al*., 2011). The authors suggested that these taxa may have greater tolerances to environmental stresses, although the levels of copper in the study soils were mostly within the range of 160-250 mg kg^{-1}, much higher than levels measured in this study (< 91 mg kg^{-1}).

5 Conclusions

Although the results of this study revealed distinct soil bacterial communities within each of the study orchards, the differences between sites cannot be attributed to soil copper levels as the sole determining factor. Other factors including the overall management practices employed and the application of herbicides to vine rows showed a high degree of influence on the bacterial community composition of orchard soils.

The composition of bacterial communities across all sites was mostly influenced by soil pH. These results support the conclusions of previous studies that this variable is one of the most important influencing factors in the spatial distribution of microorganisms in soil. pH has also been shown to be a strong predictor of metal toxicity to soil microbes, where a lower soil pH may lead to a higher bioavailable fraction of metals in soil (Lopez-Periago *et* al., 2008; Sauvé *et al.*, 1995; Cavallaro & McBride, 1980). However, this study found no relationship between bioavailable ($CaCl_2$-extractable & pCu^{2+}) copper and bacterial community composition, although free ionic copper (pCu^{2+}) which has been shown to be the form most toxic to microorganisms could not be measured accurately as levels in soil extracts were very low (pCu^{2+} = 13 to < 19).

Although soil copper levels in a number of orchards were relatively high, direct and obvious effects on bacterial diversity and community composition were not observed. There are a number of potential explanations why this is the case. Sorption of copper is regulated by many soil components including clay minerals, organic content and humic substances, as well as CEC and pH (Morley and Gadd, 1995). It has also been postulated that because bacteria are often present in biofilms, or trapped within microaggregates, they may be somewhat protected from exposure to toxic metals, including free ionic forms in pore water (Almås *et al*., 2005; Giller, 2009).

All of the conventional orchards, which generally had higher levels of soil copper, were also subject to frequent glyphosate and other agrichemical applications. Complex interactions between different agrichemicals take place in the soil environment (Renner *et al*., 2003; Vijver *et al*., 2012) and glyphosate has been shown to act as a chelating agent with divalent and trivalent cations and this has

been shown to significantly reduce the bioavailability of copper to soil organisms (Zhou *et al*., 2012 & 2013). Studies in which earthworms were exposed to either copper-spiked soil or a combination of copper and glyphosate revealed that the application of glyphosate significantly reduced weight loss and the uptake of copper (Zhou *et al*., 2013). Various other agrichemicals, such as pesticides, fungicides, surfactants, and plant growth regulators may therefore potentially interact with copper, and this will have implications in terms of toxicity to microorganisms.

The differing variables between each pair of orchards were multiple and dissimilarities in bacterial communities were likely to be due to a combination of factors rather than a singular variable such as soil copper level. Also one variable may have an indirect effect on another e.g., copper applications may result in a decrease in soil pH and community effects may be due to the prevalence of bacteria which favour a particular pH range rather than direct copper toxicity.

The levels of total copper in these study soils are relatively low compared with those reported in the literature in a number of studies that have been carried out on the effects to microbial communities in soil. In a number of those studies where free Cu^{2+} ions or Cu_{CaCl2} were shown to be toxic to, and influence the microbial community composition of soils, the levels of total copper in soil were also high (> 100 mg kg^{-1}) (e.g., Naveed *et al*., 2014, Berg *et al*., 2012, Zhou *et al*., 2011). Dean & Miller (2015) found that levels of free Cu^{2+} in a number of soils that had been spiked with increasing concentrations of copper in the previous year increased significantly once Cu_T concentrations reached 200 mg kg^{-1}. Levels of Cu^{2+} were within a very similar range for soils with less than 100 mg kg^{-1} of Cu_T. These soils were sampled from a similar location to the soils in this study with very similar physicochemical properties. This indicates that the levels of total copper in these study orchard soils were below that which would cause noticeable effects on microbial communities.

This study did however reveal positive effects of copper on archaeal communities, where soils with the highest levels of Cu_T had significantly higher abundances of archaeal OTUs compared with soils with low Cu_T. A number of these OTUs have been shown to be associated with nitrogen assimilation. It is therefore possible that individual OTUs that perform a particular function, if reduced in number by copper pollution, may be substituted by less sensitive species or strains that

perform the same function. Where assessments of copper toxicity to microbes involve measuring parameters such as microbial biomass C or mineralisable N, these changes in community composition may be overlooked. Subtle effects on community composition could also be overlooked when assessing sequencing data if differentiation between OTUs to the level of species or strain is not possible.

The levels of copper in the study orchard soils were at relatively low levels in 2014. However, copper sprays are still recognised and recommended as one of the most effective measures at reducing the spread of Psa-V. If these sprays continue to be used at current rates, and due to the cumulative nature of copper, soil copper levels may increase to a point in the future at which adverse effects to microbial functioning will be observed. Detrimental effects to soil microorganisms could alter the balance in favour of copper tolerant species which may compromise essential soil processes such as nutrient cycling and disease suppression. There are still significant gaps in knowledge about the specific functions of bacteria and archaea, and without this knowledge it is difficult to assess the real implications of copper pollution on soil health. However, as indicated by Barea, *et al.* (1997), the maintenance of an active and diverse soil microbial community is key to maintaining soil quality and soil quality should be key to maintaining sustainable kiwifruit production systems.

6 References

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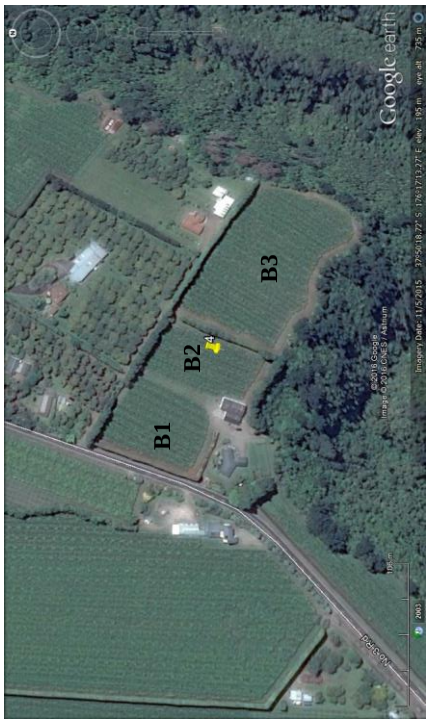
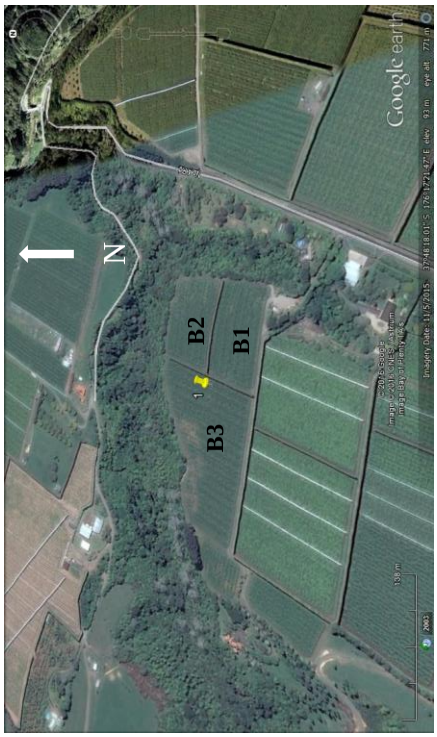
Appendix A: Locations of orchard blocks

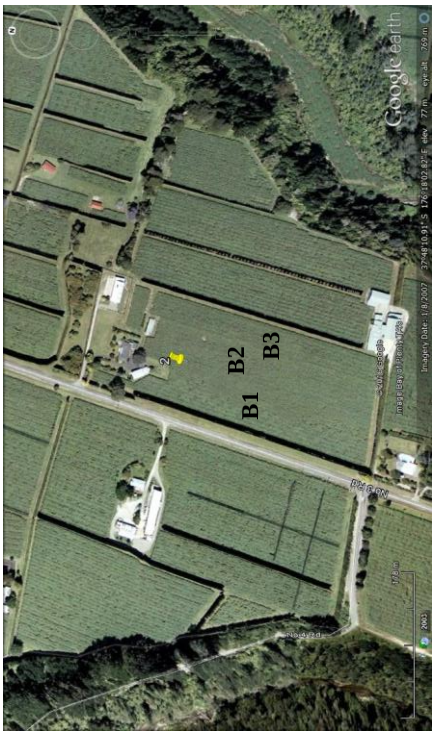
Orchard 3 – conventional



Orchard 4 – organic



Orchard 1 – organic



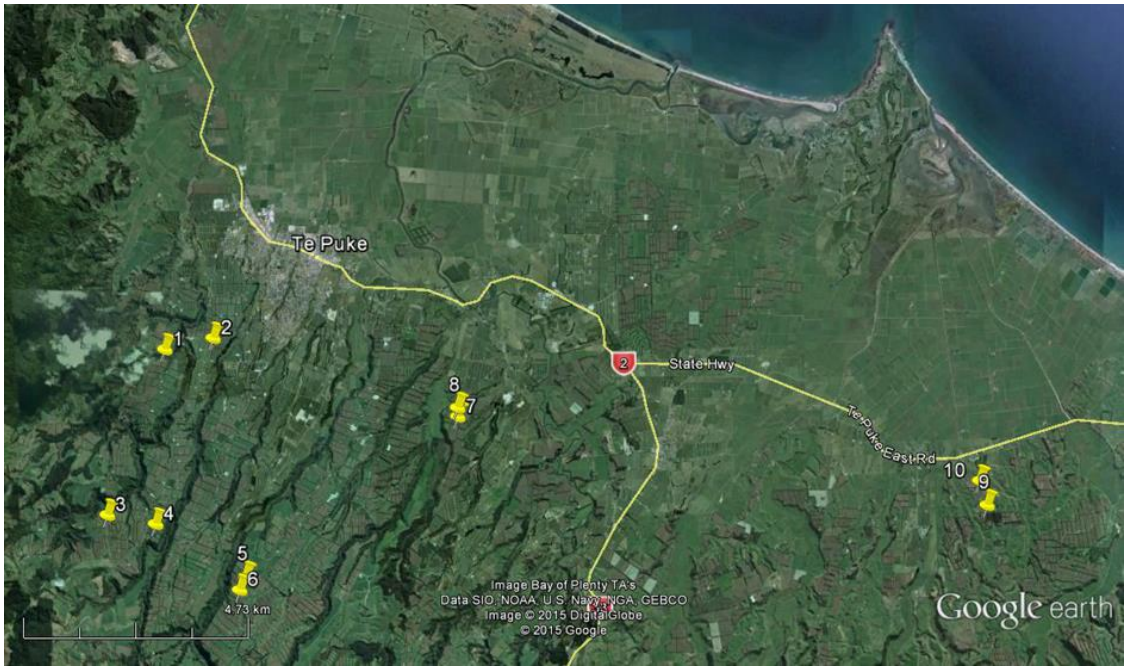
Orchard 2 - conventional

Orchard 5 – conventional

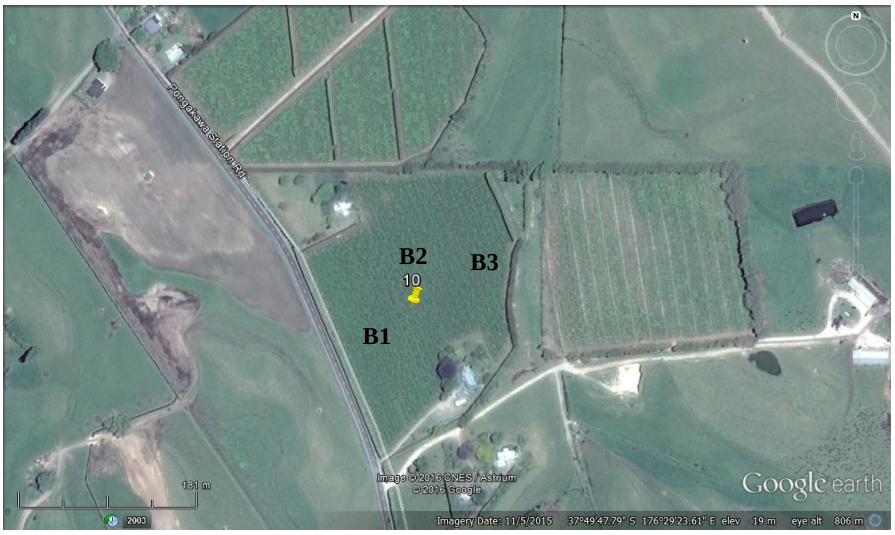
Orchard 7 – conventional and Orchard 8 – organic

Orchard 6 - organic

Orchard 9 – conventional



Locations of 10 orchards



Orchard 10 - organic

Appendix B: Orchard data sheet

Orchard Name:_____ **KPIN:**_____ **Conv./Org.:**_____ **Date:**_____

Block:_____ **Rep #:** _____ **Approx. vine age:**_____

Time:_____ **Altitude(m.a.s.l.):**_____ **Aspect:**_____ **Block photo #**_____

GPS & photo # (x10): ☐

1) **2)** **3)** **4)** **5)**

6) **7)** **8)** **9)** **10)**

Soil Temp (°C):_____

Herbicide strip?_____

% groundcover:_____ **Dominant species:**_____

Organic ammendments?_____

Litter depth (cm):_____ **Earthworms/other fauna?**_____

Copper used?_____ **Form of copper?**_____

Sample checklist (√)

Vinerow

BD samples (x3) ☐ **Comp. Sample?** ☐

Comp. micro sample? ☐

Alleyway

BD sample (x3)? ☐ **Comp. Sample?** ☐

Comp. micro sample? ☐

Notes:

Appendix C: Site images – block and groundcover views

Orchard 1

Orchard 2

Orchard 3

Orchard 4

Orchard 5

Orchard 6

Orchard 7

Orchard 8

Orchard 9

Orchard 10

Appendix D: Calculation of pCu^{2+} for ethylenediamine metal ion buffers

(Excerpt from Luo, 2004)

The pCu for ethylenediamine-Cu(II) solutions was calculated as follows. In the experiments described in Chapter 2, the Cu^{2+} buffer solutions had a total ethylenediamine concentration [en]T = 0.0303 mol L^{-1} and a total Cu(II) concentration of [Cu]T = 0.0100 mol L^{-1}. Under these conditions, with a stoichiometric excess of en over Cu(II), most of the Cu(II) is present as the $Cu(en)2^{2+}$ complex. We calculate the concentration of en not bound by Cu(II), denoted [en]' as follows:

$$[en]' = [en]T - 2[Cu]T$$

The concentration of free en at any given pH can be calculated from this value by considering the following acid-base equilibria of en

$$H^+ + en \; Hen^+$$

$$H^+ + Hen^+ \; H_2en^{2+}$$

Thus

$$[en]' = [en] + [Hen^+] + [H_2en^{2+}]$$

$$= [en] (1 + K1H [H^+] + K1H \, K_2H [H^+]2) \qquad (1)$$

which allows calculation of [en] for a given pH.

Finally, the concentration of free Cu^{2+} can be obtained by considering the formation reactions for its en complexes

$$Cu^{2+} + en \; Cu(en)^{2+}$$

$$Cu(en)^{2+} + en \; Cu(en)2^{2+}$$

Thus:

$$[Cu]T = [Cu^{2+}] + [Cu(en)^{2+}] + [Cu(en)2^{2+}]$$

$$= [Cu^{2+}] (1 + K1[en] + K1 \, K2 \, [en]2) \qquad (2)$$

Substituting [en] obtained from equation (1) into equation (2) allows calculation of $[Cu^{2+}]$ and thus pCu = -log ($[Cu^{2+}]$) for each solution. It is necessary, having obtained the result, to check the initial assumption that the $Cu(en)2^{2+}$ complex dominates over $Cu(en)^{2+}$.

Appendix E: MoBio PowerLyzer® PowerSoil® DNA Isolation Kit method

1. 0.5 g of soil was added to each PowerBead tube which contained a buffer to disperse soil particles and begin dissolving humic acids whilst protecting nucleic acids from degradation.
2. Samples were gently vortexed and Solution C1 containing SDS and other disruption agents was added to each tube.
3. Tubes were placed horizontally on a Vortex-Genie® with 24-tube adaptor and shaken at maximum speed for 10 minutes to lyse the sample DNA.
4. Tubes were centrifuged at 10,000 x g for 30 seconds at room temperature.
5. The supernatant containing sample DNA was transferred to 2 mL collection tubes and 250 μL of Solution C2 Inhibitor Removal Technology® (IRT) was added to each tube. This solution contains a reagent which precipitates non-DNA organic and inorganic material including humic substances, cell debris, and proteins. Samples were then vortexed for 5 seconds and then incubated at 4°C for 5 minutes.
6. Tubes were centrifuged for 1 min at 10,000 x g.
7. 600 μL of supernatant containing cleaned DNA was transferred into 2 mL collection tubes and 200 μL of Solution C3 IRT was added to remove additional non-DNA organic and inorganic material and samples were then briefly vortexed and incubated at 4°C for 5 minutes.
8. Tubes were centrifuged for 1 min at 10,000 x g.
9. 700 μL of supernatant was transferred into 2 mL collection tubes and 1.2 mL of Solution C4, a high concentration salt solution, was added to each tube.
10. 640 μL of supernatant was loaded onto a spin filter with silica membrane and centrifuged at 10,000 x g for 1 minute at room temperature. Flow through was discarded. This step was repeated twice. This step allowed for DNA to selectively bind to the silica membrane in the spin filter in the presence of a high salt concentration.
11. 500 μL of Solution C5, an ethanol-based wash solution, was added to tubes with spin filters to remove residual salt, humic acid, and other

contaminants, whilst allowing DNA to stay bound to the silica membrane. Tubes were centrifuged at 10,000 x g for 30 seconds at room temperature.

12. Flow through was discarded and tubes were centrifuged at room temperature for 1 minute at 10,000 x g to completely remove the wash solution.
13. Spin filters were placed in 2 mL collection tubes and eluted with 100 μL of Solution C6 (10 mM Tris) which was added to the centre of the filter membrane.
14. Tubes were centrifuged at room temperature for 30 seconds at 10,000 x g and spin filters discarded.

Appendix F: Particle sizes of orchard soils

Orchard	% Clay	% Silt	% Sand	texture
1	9	43	49	loam
1	7	35	58	sandy loam
1	6	33	62	sandy loam
2	6	42	53	sandy loam
2	7	40	53	sandy loam
2	9	41	50	loam
3	9	36	56	sandy loam
3	6	40	55	sandy loam
3	7	41	53	sandy loam
4	15	45	41	loam
4	9	42	49	loam
4	12	41	47	loam
5	10	40	50	loam
5	9	37	54	sandy loam
5	9	38	53	sandy loam
6	10	38	52	loam
6	11	39	50	loam
6	8	40	52	sandy loam
7	10	35	55	sandy loam
7	13	36	51	loam
7	10	37	53	sandy loam
8	11	40	49	loam
8	7	37	56	sandy loam
8	8	38	54	sandy loam
9	9	29	62	sandy loam
9	10	30	60	sandy loam
9	10	29	61	sandy loam
10	11	37	52	loam
10	7	40	53	sandy loam
10	10	41	49	loam

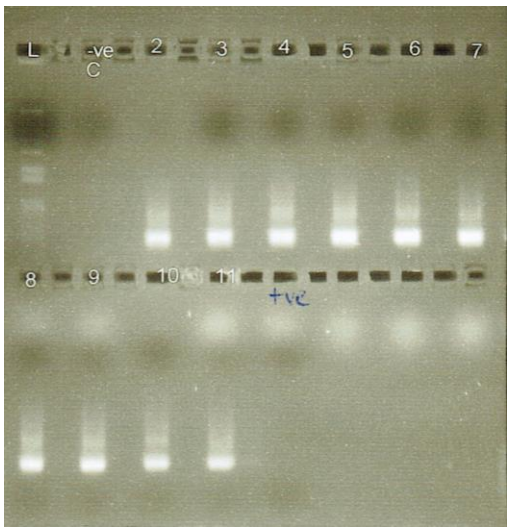
Appendix G: Summary of physicochemical and categorical data for each sample site

Orchard	management	Block	Position	Cu_T 2014 (mg/kg)	Cu_{CaCl2} (mg/kg)	pCu2+	pH water	pH CaCl2	EC (dS m^{-1})	DHA (µg TFP g^{-1} soil 24 hr^{-1})	% moisture	POXC (mg/kg)	% porosity	BD	wfps (%)	% OM (LOI)
1	Organic	1	vine row	23.00	0.09	<19	6.48	6.49	0.16	272.36	35.26	1228.22	76.71	0.62	50.78	17.43
1	Organic	1	alleyway	22.00	0.10	<19	6.58	6.35	0.12	261.19	34.17	1102.53	75.20	0.66	53.02	16.92
1	Organic	2	vine row	21.50	0.08	<19	6.57	6.35	0.11	306.41	34.85	1160.40	73.79	0.69	55.27	17.00
1	Organic	2	alleyway	20.00	0.07	<19	6.66	6.41	0.11	169.36	34.86	982.68	73.99	0.69	56.95	17.18
1	Organic	3	vine row	19.50	0.10	<19	6.53	6.34	0.11	177.42	34.39	972.61	75.13	0.66	51.70	16.52
1	Organic	3	alleyway	20.00	0.11	<19	6.50	6.37	0.12	165.39	34.34	972.63	76.44	0.62	45.34	16.47
2	Conventional	1	vine row	71.00	0.27	15.96	6.82	6.65	0.12	150.02	27.84	722.08	71.51	0.75	50.89	12.47
2	Conventional	1	alleyway	69.50	0.32	15.32	6.80	6.62	0.16	190.68	30.99	868.76	72.11	0.74	54.28	14.81
2	Conventional	2	vine row	90.50	0.26	16.29	6.84	6.61	0.12	140.76	26.80	869.18	70.69	0.78	50.80	12.14
2	Conventional	2	alleyway	77.00	0.26	15.36	6.80	6.63	0.16	169.53	28.79	883.70	72.09	0.74	54.09	12.35
2	Conventional	3	vine row	44.00	0.17	14.73	7.12	6.67	0.11	125.62	25.68	775.53	69.78	0.80	52.69	10.49
2	Conventional	3	alleyway	54.50	0.21	13.51	6.94	6.77	0.19	163.81	33.16	939.78	73.96	0.69	59.08	14.71
3	Conventional	1	vine row	44.00	0.19	17.69	6.38	6.29	0.15	143.34	27.26	774.46	69.92	0.80	54.09	12.82
3	Conventional	1	alleyway	52.50	0.22	17.00	6.47	6.29	0.17	184.93	30.55	1094.18	73.21	0.71	54.94	15.73
3	Conventional	2	vine row	36.50	0.17	18.04	6.37	6.18	0.15	154.15	28.14	661.68	71.90	0.74	52.65	12.11
3	Conventional	2	alleyway	43.50	0.20	<19	6.37	6.37	0.19	150.51	32.85	1021.12	70.94	0.77	63.10	16.51
3	Conventional	3	vine row	32.50	0.11	17.75	6.50	6.01	0.16	123.92	30.29	642.32	72.04	0.74	57.14	12.17
3	Conventional	3	alleyway	47.00	0.16	<19	6.39	6.28	0.17	147.41	32.03	869.72	74.70	0.67	51.03	16.48
4	Organic	1	vine row	67.00	0.18	<19	6.44	6.29	0.11	199.83	29.19	519.59	70.01	0.79	57.73	11.90
4	Organic	1	alleyway	57.50	0.18	<19	6.41	6.31	0.14	270.27	31.62	759.91	72.80	0.72	51.11	14.09
4	Organic	2	vine row	57.50	0.20	18.07	6.55	5.84	0.14	158.94	32.65	666.64	71.90	0.74	59.00	13.17
4	Organic	2	alleyway	56.00	0.20	18.02	6.40	5.78	0.14	217.09	34.36	781.48	73.55	0.70	57.71	15.94
4	Organic	3	vine row	51.00	0.19	18.07	6.41	5.81	0.11	177.67	31.74	703.75	71.16	0.76	56.44	13.21
4	Organic	3	alleyway	70.00	0.19	17.73	6.22	5.79	0.11	207.61	33.54	1101.25	74.95	0.66	59.04	16.60
5	Conventional	1	vine row	57.00	0.22	17.65	6.25	6.08	0.11	95.92	26.10	756.84	70.42	0.78	53.28	11.12
5	Conventional	1	alleyway	38.50	0.11	17.54	6.37	6.19	0.14	119.01	29.04	781.12	70.03	0.79	52.22	11.99
5	Conventional	2	vine row	51.00	0.12	<19	6.35	6.23	0.13	93.25	26.07	852.80	70.21	0.79	49.63	11.60
5	Conventional	2	alleyway	40.00	0.12	18.01	6.34	6.14	0.14	115.75	30.76	892.99	72.61	0.73	52.82	12.57
5	Conventional	3	vine row	67.50	0.15	<19	6.33	6.14	0.12	97.97	26.33	754.45	71.84	0.75	45.98	11.89
5	Conventional	3	alleyway	32.50	0.11	<19	6.40	6.16	0.12	120.55	31.16	693.34	72.98	0.72	52.91	14.10

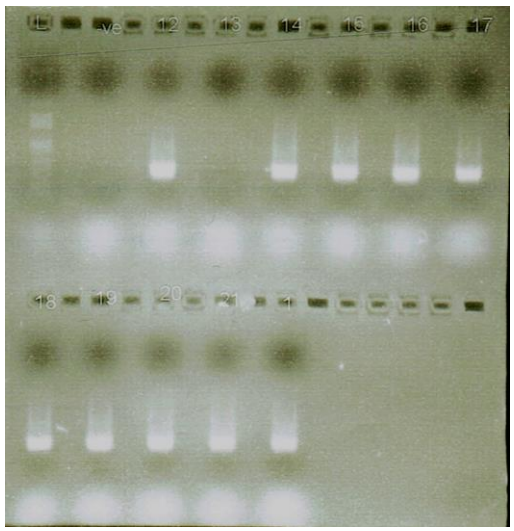
Orchard	management	Block	Position	Cu_T 2014 (mg/kg)	Cu_{CaCl2} (mg/kg)	pCu2+	pH water	pH CaCl2	EC (dS m^{-1})	DHA (µg TFP g^{-1} soil 24 hr^{-1})	% moisture	POXC (mg/kg)	% porosity	BD	wfps (%)	% OM (LOI)
6	Organic	1	vine row	29.50	0.11	16.71	6.28	6.10	0.17	264.67	30.15	1063.77	74.68	0.67	43.82	17.66
6	Organic	1	alleyway	26.50	0.10	17.17	6.31	6.05	0.15	150.09	30.23	941.35	73.17	0.71	50.31	15.69
6	Organic	2	vine row	35.00	0.09	15.82	6.24	6.03	0.17	169.58	36.29	1443.12	80.16	0.53	38.66	20.88
6	Organic	2	alleyway	26.50	0.09	17.48	6.37	6.00	0.14	233.52	32.68	1071.54	75.02	0.66	52.01	17.57
6	Organic	3	vine row	28.50	0.18	16.96	6.20	6.00	0.17	182.93	30.94	933.79	75.77	0.64	50.46	16.31
6	Organic	3	alleyway	29.00	0.13	17.06	6.27	5.98	0.09	231.88	32.23	936.77	73.66	0.70	50.11	16.76
7	Conventional	1	vine row	73.00	0.16	17.59	6.25	5.82	0.11	95.01	25.26	770.70	69.11	0.82	48.15	10.05
7	Conventional	1	alleyway	47.00	0.15	17.30	6.03	5.72	0.17	108.61	28.39	684.32	71.96	0.74	56.55	11.91
7	Conventional	2	vine row	70.50	0.18	16.48	6.10	5.75	0.12	70.00	26.79	669.65	68.63	0.83	60.45	10.16
7	Conventional	2	alleyway	45.50	0.17	17.87	6.02	5.66	0.15	105.05	28.65	659.78	71.96	0.74	51.84	12.55
7	Conventional	3	vine row	70.50	0.21	17.21	6.41	5.71	0.09	94.69	24.70	775.96	70.43	0.78	47.48	10.35
7	Conventional	3	alleyway	45.50	0.20	16.80	6.16	5.75	0.20	160.29	29.50	879.56	70.37	0.79	56.87	13.25
8	Organic	1	vine row	43.50	0.16	<19	6.64	6.40	0.10	171.95	24.98	787.87	72.76	0.72	45.16	11.85
8	Organic	1	alleyway	40.50	0.62	<19	6.61	6.46	0.19	209.27	26.16	882.61	72.99	0.72	44.63	14.74
8	Organic	2	vine row	49.50	0.95	<19	6.81	6.46	0.09	174.47	24.78	818.05	72.91	0.72	48.37	10.86
8	Organic	2	alleyway	29.50	0.50	<19	6.60	6.16	0.13	213.76	22.73	1024.52	72.97	0.72	41.80	13.04
8	Organic	3	vine row	53.00	0.51	<19	6.59	6.31	0.09	211.89	26.30	872.85	71.25	0.76	46.39	11.29
8	Organic	3	alleyway	38.00	0.66	<19	6.52	6.27	0.12	232.99	25.01	1156.92	71.81	0.75	47.71	13.51
9	Conventional	1	vine row	18.50	0.19	<19	6.82	6.51	0.09	97.34	21.58	579.56	64.64	0.94	54.81	6.14
9	Conventional	1	alleyway	24.00	0.24	<19	6.83	6.47	0.13	181.37	29.74	857.95	70.23	0.79	63.04	10.39
9	Conventional	2	vine row	23.50	0.17	<19	6.82	6.42	0.11	105.66	25.97	658.11	68.89	0.82	59.84	8.17
9	Conventional	2	alleyway	24.50	0.15	<19	6.65	6.30	0.14	222.95	30.47	726.96	70.17	0.79	59.49	10.94
9	Conventional	3	vine row	23.00	0.07	<19	6.61	6.15	0.13	114.55	23.73	589.02	64.66	0.94	57.90	7.01
9	Conventional	3	alleyway	24.50	0.19	<19	6.64	6.16	0.13	172.89	29.45	736.69	69.13	0.82	69.61	9.76
10	Organic	1	vine row	34.50	0.20	<19	6.81	6.56	0.13	190.93	31.72	830.06	72.92	0.72	55.09	12.92
10	Organic	1	alleyway	29.00	0.25	<19	6.78	6.39	0.12	181.07	31.33	864.47	71.36	0.76	53.31	13.05
10	Organic	2	vine row	32.50	0.17	<19	6.83	6.60	0.14	115.06	32.78	821.44	73.23	0.71	52.78	12.74
10	Organic	2	alleyway	27.00	0.18	<19	6.78	6.46	0.14	164.80	30.43	894.37	71.75	0.75	52.73	14.08
10	Organic	3	vine row	34.50	0.10	<19	7.02	6.59	0.14	209.13	33.48	912.21	72.00	0.74	62.88	13.64
10	Organic	3	alleyway	26.50	0.08	<19	6.80	6.43	0.16	134.77	34.57	805.12	71.45	0.76	63.02	14.40

Appendix H: Qubit quantification values and gel electrophoresis images

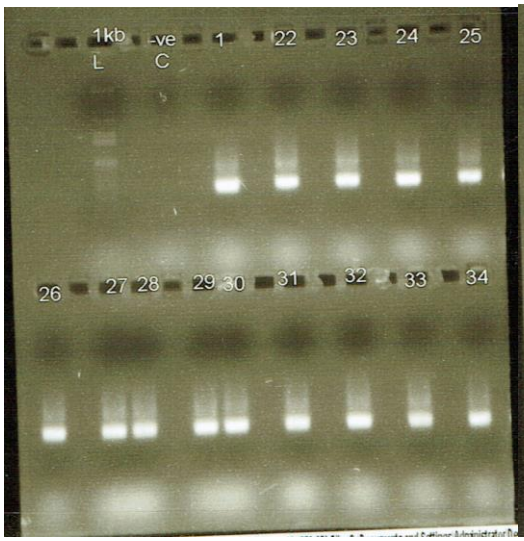
Orchard	Management Regime	Blk #	vine row		alleyway	
			Gel band #	DNA conc. (ng/µl)	Gel band #	DNA conc. (ng/µl)
1	Organic	1	**1**	38.5	**2**	31.3
		2	**3**	45.2	**4**	35.2
		3	**5**	39.4	**6**	35.8
2	Conventional	1	**7**	26.9	**8**	38.8
		2	**9**	33.6	**10**	25.8
		3	**11**	27.1	**12**	2.8
3	Conventional	1	**13**	1.02	**14**	30.9
		2	**15**	25.8	**16**	17.8
		3	**17**	0.6	**18**	21.8
4	Organic	1	**19**	16.9	**20**	27.6
		2	**21**	27.8	**22**	16.8
		3	**23**	20.7	**24**	10.2
5	Conventional	1	**25**	26.8	**26**	27.7
		2	**27**	16.1	**28**	25.3
		3	**29**	13.1	**30**	17.4
6	Organic	1	**31**	10.8	**32**	22.4
		2	**33**	16.3	**34**	16.9
		3	**35**	11.4	**36**	11.9
7	Conventional	1	**37**	3.1	**38**	11.9
		2	**39**	9.5	**40**	11.8
		3	**41**	30.6	**42**	12.1
8	Organic	1	**43**	12.2	**44**	11.6
		2	**45**	9.4	**46**	12.2
		3	**47**	8.7	**48**	8.3
9	Conventional	1	**49**	14.5	**50**	15.3
		2	**51**	20.8	**52**	14.8
		3	**53**	26.0	**54**	29.8
10	Organic	1	**55**	17.1	**56**	15.9
		2	**57**	10.4	**58**	21.8
		3	**59**	19.0	**60**	14.4



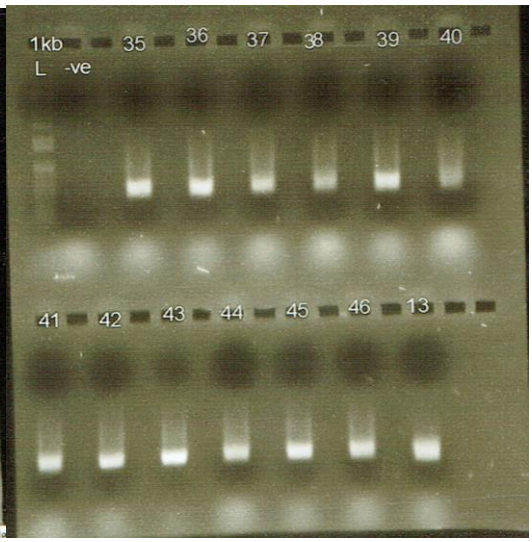
Samples 2 – 11



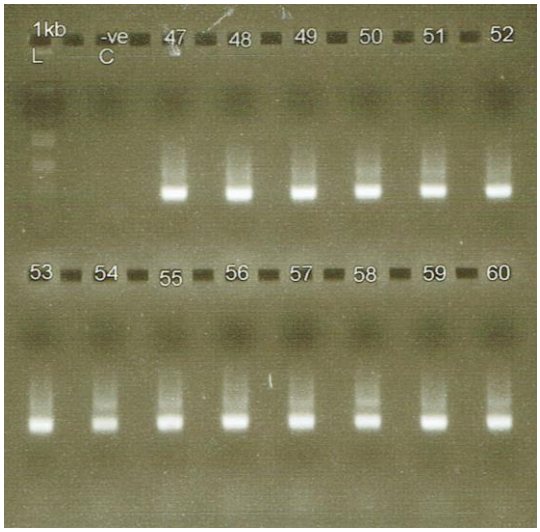
Samples 12 - 21



Samples 22 – 34 and 1 repeat



Samples 35 – 46 and 13 repeat



Samples 47 - 60