Manuka (*Leptospermum scoparium*) as a Remediation Species for Biosolids Amended Land

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Abstract

The application of biowastes such as biosolids to land is a viable means of re-cycling valuable nutrients in an otherwise useless waste product. With this practice comes the risk of introducing contaminants such as heavy metals and pathogenic microorganisms into the soil system, posing a risk to humans and animals. A land-management system is needed to mitigate any potential risk. One such system finding application at many locations around the world is phytoremediation, the use of plants to remove, degrade or render harmless environmental contaminants. The plant species *Leptospermum scoparium* (Manuka) is a hardy native New Zealand plant resistant to heavy metals, with the ability to grow on degraded and erosion prone sites, and encourage re-vegetation in zones of impaired soil quality. In addition, manuka products have been shown to have antiseptic and antimicrobial properties. The aim of this research was to investigate the potential use of manuka in the remediation of contaminated land, through the use of manuka's antiseptic properties to aid in pathogen control, and on the extraction or stabilisation of heavy metals in soil.

Experiments were carried out using water extracts of manuka, as well as soil from underneath manuka stands, to test for antimicrobial activity of manuka against a number of pathogenic bacteria potentially found in biosolids. Results found that the presence of prepared manuka-water extracts significantly reduced the growth of the five bacterial strains tested, in some cases exhibiting complete die off. However, in-situ effects of antimicrobial ability in soil from underneath manuka were not observed. Further research using whole plants, and different plant components would be useful.

Research was also conducted to investigate the effect of manuka growth on Zn and Cu bioavailability in soil. Three plant species, (manuka, *Coprosma robusta* and rye grass), were grown for six months and one year in Zn - and Cu - spiked soil to assess their effect on metal availability. Results clearly showed that the three plant species investigated differ in their ability to uptake and accumulate both metals, but have no apparent effect on HM bioavailbility over a one year time period.

The experiments in this research were able to closely evaluate the potential of manuka as a remediation species for biosolids-amended land. Results indicate that further research into the potential use of manuka in this way is warranted, particularly with respect to manuka's ability to manage levels of pathogenic soil microorganisms. In addition, manuka components are economically valuable, and in future, biosolids disposal systems may be able to combine with that of manuka production to produce a sustainable disposal system with potential for economic return. The solution may be to develop manuka plantations on otherwise unuseable land, where biosolids application can help recondition soil and enhance manuka growth, whilst manuka acts as a means of 'treating' both the bacterial and inorganic contaminants in the biosolids. Continual leaf fall or rotational cropping and mulching of manuka biomass would aid in the attenuation of introduced bacteria, and manuka roots may help stabilise metals. In an age where waste reduction and contaminant control are top priority, remediation systems such as this may represent an economically, socially and environmentally acceptable solution.

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1. Research Overview

Contaminated soil, or land that is currently unusable can be remediated and re-conditioned by the used of organic amendments (biowastes) such as biosolids. This is also considered a beneficial means of recycling of this otherwise unwanted waste product. However, application of waste to land comes with its own set of problems. There is potential to introduce additional contaminants such as heavy metals (HM) and pathogenic microorganisms into the environment. For this reason, recycling of biosolids to land is restricted and highly regulated. In order to make land application more desirable, some of these barriers to use need to be lifted, but in order to do so, the subsequent risks needs to be minimised. Biosolids is most commonly applied to croplands and grasslands, however research has shown that application to forestry and plantations provides valuable nutrients to plants, as well as reducing potential contact by people or contamination of food crops. Manuka is already widely used in land restoration in New Zealand due to its hardy nature; it was therefore considered that there is potential to combine the use of manuka and biosolids for land reclamation. In addition, if manuka's antimicrobial components have an impact on pathogens in sewage sludge this may reduce the need for such intense treatment of sludge and/or allow for the shortening of exclusion periods. This would improve the economic viability and general appeal of recycling wastes in this way. The aim of the current research is to investigate the potential of manuka as a remediation species, focusing on its effect on HM and pathogenic bacteria that may transfer to soil from biosolids application to land.

2. Literature Review

2.1. Introduction

In recent times, people have begun to realise that natural resources are "exhaustible" and that conservation steps need to be taken to prevent their over-exploitation and misuse (Henderson-Sellers, 1987). More and more often we hear of circumstances where human action has threatened natural resources and environmental quality, whether it be soil, air or water. Soil is an extremely vital natural resource, especially in New Zealand where a large percentage of our productivity is based in agriculture. Billions of dollars are made yearly from exports of meat, fruit and vegetables, wool, wood, nuts, dairy products and leathers (Statistics NZ, 2005). Intensive land use has lead to C loss and nutrient depletion in regions world-wide, which can lead to loss of production unless these losses are countered. One potential means of enhancing soil productivity is the application of biosolids (treated sewage sludge) to land.

Disposal or recycling of biowastes (organic wastes) such as biosolids to land has become common practice in many countries, for practical and economic reasons (Albihn, 2001; Antoniadis and Alloway, 2002; Maiaomiao *et al.*, 2009). Recycling of biowaste onto land can help to avoid environmentally harmful waste management strategies, such as land filling or incineration (Veeken and Hamelers, 2002), and it has become a viable means of promoting recycling of nutrients and minimising waste (Bright, 2003; Alvarenga *et al.*, 2009). There is a move worldwide to reduce the quantity of biodegradable wastes sent to landfill. If organic wastes are kept out of landfills there is potential to recycle nutrients and produce less pollution in the form of methane and leachate (Alvarenga *et al.*, 2009). In addition to recycling potential, organic wastes such as biosolids and composts are increasingly used in land rehabilitation because they can improve the physical, chemical and biochemical properties of soil, reducing the need for inorganic fertilizers (Alvarenga *et al.*, 2009; Maiaomiao *et al.*, 2009). Nutrients in biosolids pose less of a threat than those in regular fertilisers, mainly because in sludge they are stabilized and mineralization of N is a slow process (NZWWA, 2003). Application of composted organic waste to agricultural soils

represents one of the most cost-effective methods for treatment and final disposal of these by-products (Maiaomiao *et al.*, 2009). These wastes contain nitrogen, potassium, phosphorus and organic matter that improve plant growth and other soil properties (Cameron *et al.*, 1997; Warman, 2005; Maiaomiao *et al.*, 2009). They have been shown to improve soil structure, increase soil aggregate stability, increase water storage capacity, provide aeration, improve porosity, improve ecological activity and in some cases prevent soil erosion (Yeates, 1995; Cameron *et al.*, 1997; Franco-Hernandez, 2003; NZWWA, 2003; Speir *et al.*, 2004; Casado-Vela, 2006). Addition of organic matter through biowaste amendment also adds basic cations and increases the cation exchange capacity (Shuman, 1999). The primary focus of this chapter is organic wastes and byproducts that contain biosolids and sewage sludge. It is because of the outlined benefits to soil that biosolids application has been used as a means of rehabilitating unusable land (Henry, 1994; Cameron *et al.*, 1997; Navas, 1998; Speir *et al.*, 2003a; Speir *et al.*, 2003b; Qiang *et al.*, 2004), particularly in regions where water deficit, erosion, intense human activity and degradation of soil have become huge problems (Navas 1998; Navas 1999; Casado-Vela 2006). Biosolids have been used in this way for over 40 years throughout Canada, the US and Europe (Warman, 2005). However, biosolids application to land is not without its disadvantages. As well as recycling nutrients and organic material, biosolids can introduce chemical and biological contaminants into the soil system. The main contaminants of concern are; organic pollutants (industrial and household chemicals as well as pharmaceuticals), excess nutrients such as phosphorus and nitrogen, heavy metals and pathogenic organisms (Smith, 1996). This has meant that many communities are opposed to the idea of waste application to land, and this is most likely why in New Zealand land application is not commonly practised. In Europe, disposal of biosolids to agriculture is one of the most commonly practiced disposal means, with 4.5 million tonnes applied to land in 2005 (Schmidt *et al.*, 2006). In the USA, in 2003 approximately 40% of sludge produced was applied to land (NZWWA, 2003). In New Zealand the first guidelines for disposal of sewage sludge to land were generated by the Department of Health in 1973 (NZWWA, 2003), however, the practise has never been commonly used. If managed properly, any adverse effects of land application are likely to be minimal, although this has not prevented scepticism. While a significant amount of research has focused on both the potential positive and negative effects of land application, little has investigated

potential means to minimise the adverse effects, or to remediate contamination caused by biosolids application to soil. In the research described in this thesis, an in-depth investigation has been conducted into the potential use of the native New Zealand shrub manuka (*Leptospermum scoparium)* as a remediation species for land that may be contaminated due to biosolids application, with particular reference to heavy metal and pathogenic contaminates. Manuka is known for the antiseptic properties of its honey and oil, and it is a useful regeneration species, tolerant of climate and topographic factors and good at preventing soil erosion. Hence, manuka may be a good candidate for remediation of contaminated land. The following sections outline heavy metals (HM) and pathogenic contaminants in soil and current thinking on phytoremediation, tying together the use of biosolids and manuka in land restoration initiatives.

2.2. Biosolids application to Land

2.2.1. What are biosolids?

Sewage sludge or "biosolids" is essentially a nutrient-rich product derived from the treatment of municipal wastewater (Warman, 2005). The process of its production can be seen diagrammatically in Fig. 2.1. Biosolids is generally the solid or semi-solid material derived from bacterial break down of municipal wastewater (in particular raw sewage and grey-water). What does not settle out as sludge is discharged from the treatment process as sewage effluent. After this initial step sludge is generally only 1-10% solid, but it is often dewatered in a second stage of treatment producing a semi-solid product.

Most sewage treatment plants consist of two stages of treatment, primary treatment and secondary treatment. Primary treatment, sometimes referred to as mechanical treatment, involves screening and/or solid settlement of wastewater, whereby large objects and grits are removed from the system. This is then followed by secondary treatment, which usually involves a process of biological breakdown using bacteria and protozoa that consume and bind biodegradable organics, and other less

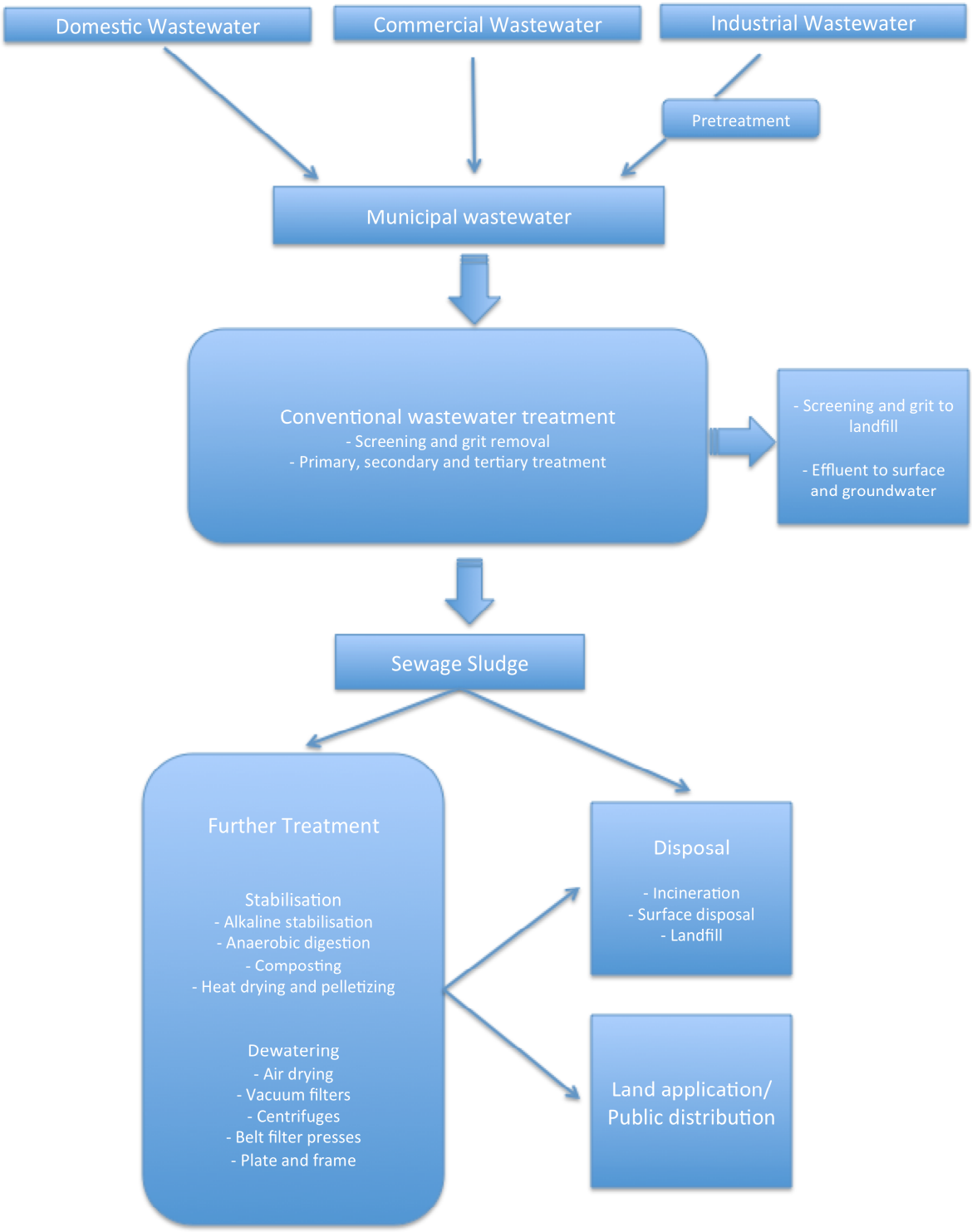


Figure 2.1 *The standard process of sewage sludge production (after Pepper et al., 2006)*

soluble particles, into floc particles. This biological floc is then settled out and removed (as sludge); leaving water that is relatively "clean" and can be disposed of into waterways. Many modern treatment plants will include a tertiary treatment step in sewage processing, to further improve the quality of effluent. This can be carried out in a number of ways, such as via sand filtration, lagooning, bacterial nutrient removal and chemical precipitation, using flocculating agents such as Al and Fe. After these steps, the accumulated sludge can be treated again prior to its disposal; the main purpose of this is to reduce the amount of organic matter and numbers of pathogenic organisms present (Pepper, 2006). The three most common ways of doing this are anaerobic digestion, aerobic digestion and composting.

2.2.2. Methods of biosolids disposal

Early methods of sewage disposal involved direct pumping into oceans and waterways, however, this is no longer considered acceptable in most countries. In some regions disposal in this way does still occur, however, most now treat waste via secondary and tertiary treatment prior to discharge (Cameron *et al.*, 1997). Even though methods have improved, there are still major threats to the environment caused by sewage disposal, and society has yet to come up with a fully acceptable means of discarding of it (Cameron *et al.*, 1997).

Modern disposal practices include disposal to landfill, incineration and land application. Landfill disposal of sewage sludge has been deemed undesirable for two main reasons. Firstly, the excessive quantities of nutrients and contaminants present in waste permeate the underlying soil, potentially ending up in groundwater and making their way to other water supplies. Secondly, large amounts of methane, carbon dioxide, and other harmful greenhouse gases are produced as a result of microbial decomposition of sludge dumped in such large quantities (Cameron *et al.*, 1997; NZWWA, 2003). Landfilling is also becoming less economical with increasing costs and a growing shortage of suitable space for new landfills (NZWWA, 2003). Incineration is also highly undesirable due to the volume of harmful emissions released into the atmosphere

during the burning process, as well as large quantities of toxic elements left behind in ash (Cameron *et al.*, 1997).

One means of avoiding these potentially harmful waste management strategies is application to land (Veeken and Hamelers, 2002). This is becoming more common in many countries due to economic and practical reasons (Albihn, 2001). Biosolids can improve soil fertility and quality (as mentioned previously), essentially a way of beneficial recycling rather than disposal (Bright, 2003). Biosolids has, in some instances, been used as a means of promoting the remediation of contaminated land and the rehabilitation of degraded sites. Some researchers have shown that application of biosolids can decrease the solubility of metals in mine tailings whilst improving fertility (Veiga, 2002; Rate, 2004; Mercuri, 2005). Organic amendments, such as biosolids, applied to soil are frequently used to remediate metal-contaminated soil because they are thought to add active surface area that can help adsorb metals (Shuman, 1999). Although there are clear benefits to this practise, biosolids can also contain an array of contaminants and therefore application to land requires adequate management to avoid soil contamination.

2.2.3. Contaminants in biosolids

2.2.3.1. Pathogenic microorganisms

Pathogenic organisms can end up in soil by a number of mechanisms, including the addition of organic wastes (such as biosolids) to land through the process of land-based disposal. There are numerous literature reviews available on the subject of pathogens in sewage waste (waste water and biosolids) (Horswell and Aislabie 2006; Pepper 2006; Sindhu and Toze 2009), in the following part of this chapter a brief review of the subject will be presented.

Wastewater contains an array of different pathogenic organisms, from viruses to protozoa (NZWWA 2003; Pepper 2006; Venglovsky 2006). Pathogens are present in

raw, untreated sewage because they are discharged in the excreta of infected humans and animals (Carrington, 2001; Horswell and Aislabie, 2006). Depending on the number of people infected at any one time, as well as the presence or absence of hospitals and abattoirs, pathogen numbers in sewage changes dramatically over time and between locations (Sindhu and Toze, 2009). Like other contaminants, pathogenic organisms and viruses are adsorbed or trapped within biological floc in wastewater and subsequently removed in sludge (Pepper, 2006; Sindhu and Toze, 2009). As a result, there are concerns surrounding the transport of pathogenic organisms to soil and their survival after biosolids application, and the potential risk for contamination of food crops and surface and groundwaters (Horswell *et al.*, 2009). A hygienically safe end product from sewage treatment is needed before application to land is viable (Albihn and Vinneras, 2007). After initial sewage treatment, sludge can be classed into two categories according to its pathogen content, class A and class B. "Class A biosolids" is sludge that has had sufficient treatment either by aerobic digestion, air drying, anaerobic digestion, composting, irradiation, pasteurization or lime stabilisation, to reduce pathogen numbers to below detection limit (Franco-Hernandez 2003; Pepper 2006; Horswell, Hewitt et al. 2009). The following is a brief overview of each of these methods for pathogen removal (after Pepper *et al*. 2006):

- **Aerobic digestion:** Biosolids are agitated with air, maintaining aerobic conditions for between 40 days at 20°C and 60 days at 15°C
- **Air drying:** Biosolids are air dried for a minimum of three months on sand beds or unpaved basins
- **Anearobic digestion:** Biosolids are treated in the absence of air for a specific mean cell resistence time of between 15 days at 35°C and 60 days at 20°C
- **Composting:** By using tradional composting methods (within-vessel, static aerated pile or windrow) the temperature of the biosolids is raised to above 40°C, and maintained for five days, with at least four hours at temperatures above 55°C
- **Lime stabilisation:** Enough quick-lime is added to the biosolids to raise the pH to 12.

Although it is accepted that pathogen numbers will reduce during sludge processing it is unlikely that they can be completely eliminated (Horswell *et al.*, 2009). In terms of pathogen content there is no restriction on the land-based disposal of class A biosolids in most countries (no detectable pathogens). "Class B biosolids" are treated to a lesser degree and may still contain detectable amounts of pathogens, up to 10^5 cfu per g of dry biosolids (NZWWA, 2003). Any higher than 10^5 cfu g^{-1} dry biosolids is not considered suitable for land use, and the use of class B biosolids is restricted to minimize potential exposure. Restrictions include where it can be applied, subsequent land use, length of time between application and the security of the land (i.e. degree of access by people and livestock) (Pepper, 2006). Sufficient time must be given for a further reduction in pathogens numbers before the land can be used for cropping or public access (NZWWA, 2003).

Among the thousands of organisms found in biosolids, some are pathogenic to humans and other animals. There are bacterial species, including *Salmonella* sp, *E. coli*, *Campylobacter* and *Shigella* sp, some of which can infect humans via oral consumption, producing symptoms such as nausea, headache, diarrhoea, fever, muscle ache and abdominal pain (Pepper, 2006). Viruses can also be found in biosolids, including enteroviruses, calciviruses, hepatitis A and E and adenovirus, which is the most common and persistent in wastewater (Pepper, 2006). Also present may be helminths (e.g. roundworms) and protozoa, including *Cryptosporidium* and *Giardia,* as well as infectious fungi and yeasts. A summary of the likely species to be found in biosolids is presented in Table 2.1 It has been stated that the most important pathogens in terms of human health are those spread by the faecal-oral route such as *Campylobacter sp*. and *Salmonella sp*. (Horswell and Aislabie, 2006). Some species of *Salmonella*, *Campylobacter* and spore-forming bacteria (such as *Clostridium*) are known to be resistant to high temperatures, and thus may survive the treatment process (Wagner *et al.*, 2008).

Some organisms have a long survival time in the environment, and some have been shown to multiply subsequent to the completion of wastewater treatment (Albihn, 2001). A number of researchers have found apparent re-growth (or re-colonisation) of pathogenic bacteria in biosolids some months after reporting complete die-off (Vasseur

et al., 1996; Zaleski *et al.*, 2005; Eamens and Waldron, 2006). Eamens *et al*. (2006) found that levels of spiked *E.coli*, *Salmonella* and *Clostridium* were still above background levels more than six months after addition, which is longer than the recommended restraint periods for some land uses (e.g. forestry) (NZWWA, 2003). The mechanisms for potential spread of pathogens are wind, water, crop harvesting, insects, birds or mammals, which can all transport pathogens long distances (Albihn, 2001). The pathogenic microorganisms that are most often subject to regulation are *Salmonella* sp, enteric viruses, helminth ova and cysts and oocytes of protozoa (NZWWA, 2003). Previously, it had been thought that these pathogenic microorganisms rapidly die-off after land application due to natural attenutation and processes such as filtering, adsorption and competition with indigenous organisms. However, pathogens such as *E. coli* 0157:H7, which can cause severe infections in humans, have been found to be capable of surviving in soil for very long periods of time, potentially passing on infections from livestock to humans (Avery *et al.*, 2004). Some research has noted that pathogenic bacteria and virus numbers are reduced to minimum levels within 2-3 months after application (Cameron *et al.*, 1997), but results are varied within the literature and depend on a variety of factors including soil type and environmental conditions (Horswel *et al*., 2007). An experiment conducted by Avery *et al*. (2004) looked into the persistence of *E. coli* 0157:H7 in soils amended with a variety of organic wastes. They found that, especially in the surface of amended soils, *E. coli* were harboured within the top 5cm of soil and survival was above the infectious dose for humans for eight weeks. Destructive sampling at eight weeks showed evidence of pathogen movement right through the soil cores, as well as their presence on vegetation. In New Zealand the recommended exclusion period for grade B biosolids is at least one year for salad crops and fruit that will not be peeled, and at least 6 months for public areas (i.e., golf courses, public parks), forests and pasture (NZWWA, 2003). Based on Avery *et al*. (2004) it cannot be concluded if pathogen numbers would have been at undetectable levels by six months. It has also been suggested that biosolids should be incorporated into soil as opposed to surface applied (NZWWA, 2003), as this will potentially reduce contact of waste by humans and animals, and may inhibit pathogen survival in soil (Avery *et al.*, 2004). However, in many studies it has been demonstrated that soil incorporation of spiked biosolids can enhance pathogen survival,

Table 2.1 *Pathogenic bacteria that may be found in biosolids (after Horswell and Aislabie et al., 2006)*

Organism	Disease and Symptom
Escherichia coli	Gastroenteritis (diarrhoea), Indicator organism.
Salmonella sp.	Salmonellosis (gastroenteritis)
Shigella	Shigellosis, causes bacillary dysentery and diarrhoea
Leptospira sp.	Leptospirosis, causing a type of jaundice
Legionella	Pneumonia, often called legionaires disease
Yersinia enterolitica	Acute gastroenteritis including abdominal pain and diarrhoea
Clostridium sp.	An endospore forming, thermotolerant organism causing gas gangrene, tetanus, botulism
Listeria monocytogenes	Listeriosis, can grow at low temperatures,infections in pregnant woman can cause miscarriage
Campylobacter sp.	Campylobacteriosis, diarrhoea and vomiting
staphylococcus	Boils, septicemia or infection. Associated with mucus membranes.
Streptococcus	Include: Strep throat, meningitis, bacterial pneumonia, necrotizing fasciitis, endocarditis. Also normal flora of intestinal tract of animals

presumably due to protection within the soil matrix (Avery *et al.*, 2004; Hutchison *et al.*, 2004; Eamens and Waldron, 2006; Albihn and Vinneras, 2007). The rate of pathogen die-off during the period of constraint is affected by many factors including season, sunlight, soil temperature, soil moisture, soil type, pH and indigenous microbial population (through microbial competition and predation) (Carrington, 2001). Colder weather is found to increase pathogen persistence in the environment (Pepper, 2006; Albihn and Vinneras, 2007), as is increased moisture (Horswell and Aislabie, 2006; Pepper, 2006) and a near neutral soil pH (Pepper, 2006). Sunlight helps to reduce pathogen numbers, and an abundant natural microbial population reduces pathogens through competition and predation (Horswell and Aislabie, 2006; Pepper, 2006). The main problem with applying periods of constraint to allow for microorganism decay is that the factors that control die-off cannot be controlled or predicted (Carrington, 2001), and every site and situation will be different.

2.2.3.2. Heavy Metals

What are heavy metals

The term heavy metal is often used loosely and lacks clear definition. There are a considerable number of definitions, and many have suggested that the term is in fact meaningless (McLaren, 2005). Some say heavy metals are metals of atomic weight above sodium (Bennett, 1986; Lewis, 1997), where others have said they are elements with a density greater than 5 g cm^{-3} (Dungan and Frankenberger, 2002). One definition that may be of value states heavy metals are a group of elements between copper and lead on the periodic table of elements having atomic weights between 63 and 200 and specific gravities greater than 4.0. (Ghosh and Singh, 2005) This includes many of the elements that are frequently called heavy metals such as zinc (Zn), copper (Cu), cadmium (Cd) and lead (Pb), as well as many that are not often thought of as being (or may not be) metals, such as arsenic (As) and selenium (Se). However, this definition leaves out elements that are usually included when talking about heavy metals such as iron (Fe), mercury (Hg), chromium (Cr) and nickel (Ni). For the benefit of this paper, the term heavy metals will be used for all elements that in the literature have been

discussed in association with contamination of soil, and which have been termed heavy metals by the authors.

There is agreement that many heavy metals are trace elements in the environment, and most are toxic at high concentrations. Trace elements are elements that are needed to sustain life but only in very minute quantities, found in matter in low concentrations of just a few mg/kg or less (McLaren, 2005). It has been proposed that a better term for describing heavy metals would be "toxic trace elements" (McLaren, 2005). This may be the way people perceive them in the future.

Sources of heavy metals in soil

Metals occur naturally in all soils, waters and living organisms (Wood, 1995). Although small amounts of most heavy metals (trace elements) are needed to sustain life, large amounts inhibit many of the biological processes in soil including enzyme activity. Heavy metals are toxic to most organisms when present at excessive concentrations (Giller *et al.*, 1998), primarily due to their protein binding capacity (Speir *et al.*, 2002). Although trace elements in soils originate from both natural and anthropogenic sources (Robinson *et al.*, 2006), it is generally accepted that almost all heavy metal contamination occurs through direct human action. This can be via; metal mining operations (Giller *et al.*, 1998; Moreno *et al.*, 2001), atmospheric deposition (Gianfreda and Bollag, 1996; Dungan and Frankenberger, 2002), corrosion of galvanised power towers (Jacquat *et al.*, 2009), animal manure and effluent (Speir *et al.*, 1999c; Bolan *et al.*, 2003), sewage sludge application (Giller *et al.*, 1998; Kunito *et al.*, 2001; Speir, 2008), copper/chromium/arsenic (CCA) timber treatment plants, and leaching from CCA treated wood (Bardgett *et al.*, 1994; Speir *et al.*, 1999a; Bolan *et al.*, 2006). In addition, some organic and mineral fertilizers have been found to contain significant quantities of heavy metal impurities (Gianfreda and Bollag, 1996; Moreno *et al.*, 2001; Dungan and Frankenberger, 2002; Bolan *et al.*, 2006)**.**

Sources of heavy metals in biosolids

Heavy metals (HMs) are one of the major contaminants of concern when discussing organic waste disposal on land (Singh, 2008). In municipal solid waste and composts such as organic waste from kitchens and gardens, elevated levels of many heavy metals have been observed. Heavy metal contamination of these types of biowastes comes directly from the components added (Veeken and Hamelers, 2002). For biowaste products derived from sewage, heavy metals end up in wastewater from a number of sources, including cosmetics, detergents, corrosion of pipes (i.e., copper plumbing pipes), paints, foodstuffs, pesticides, treated wood and many more. These contaminants are subsequently filtered out into the biological floc portion to remove them from effluent, and thus HM are concentrated in the solid portion of sewage sludge.

Heavy metal bioavailability in soil

Heavy metal (HM) pollution is of great concern because once metals have entered a soil system they cannot be bio-degraded (Dungan and Frankenberger, 2002), they are strongly absorbed to soil components, and have a very long resistance time in the environment (Wood, 1995; Smith, 2009). Due to their persistence in soil, investigations into toxicity of HM do not focus on degradation but on the form or speciation present. Many studies focus on total concentrations of a HM to determine toxicity, but the impact of HM on the soil environment cannot be predicted by total metal concentrations and is more related to the soluble or mobile concentrations in soil (Tessier *et al.*, 1979; Robinson *et al.*, 2006; Maiaomiao *et al.*, 2009).

Heavy metals in soil can be present in a number of forms, and the form determines its "bioavailability", (availability to living organisms). As bioavailability has been closely related to toxicity to organisms and plants (Fujii and Kaneko, 2009) many have suggested that regulatory limits for HM in soil should be based on bioavailable concentrations and not their total amount (Castaldi *et al.*, 2009a). Current regulatory limits for soil HM concentrations in NZ can be seen in Table 2.2. (based on NZWWA, 2003). These limits are based on total metals and do not take into account bioavailable

concentrations. The forms of HM in soil include; soluble, exchangeable, bound to carbonates, bound to iron and manganese oxides, bound to organic matter, or in residual minerals (Tessier *et al.*, 1979). Methods have been developed to investigate the bioavailability of HM fractions present in a soil sample through sequential extraction using different reagents. The following is an explanation of these different fractions as outlined by Tessier (1979)

- **Soluble and Exchangeable:** Water soluble and exchangeable metals are considered to be the most directly available to plants**.**
- **Bound to carbonates:** Trace metal concentration that is associated with sediment carbonates and is heavily influenced by soil pH. This fraction is considered to be loosely bound.
- **Bound to iron and manganese oxides:** Iron and manganese oxides exist as a sort of cement between or coating soil particles. Trace metals bind to these oxides, but the relationship is thermodynamically unstable.
- **Bound to organic matter:** Trace metals bound to living organisms, detritus, and organic coatings on minerals. Natural organic matter such as humic and fulvic acids complex and peptize HM, these can be released when organic matter is degraded.
- **Residual:** Trace metals are considered to be residual after all other fractions have been removed. The residual fraction describes any trace metals contained within the crystal structure of primary and secondary minerals. In nature these HM are not expected to be released into solution and are not considered to have any bioavailability.

Table 2.2. *New Zealand soil limit or ceiling concentrations*

(after NZWWA, 2003)

Metal	mg kg^{-1} soil (dry weight)
Arsenic (As)	20
Cadmium (Cd)	1
Chromium (Cr)	600
Copper (Cu)	100
Lead (Pb)	300
Mercury (Hg)	1
Nickel (Ni)	60
Zinc (Zn)	300

The solubility of a metal depends on many soil physico-chemical and biological properties (Robinson *et al.*, 2006; Castaldi *et al.*, 2009a; b). In particular, soil pH, total carbon content, dissolved organic matter (DOC) and clay content (Santibanez *et al.*, 2008; Smith, 2009; Yadav *et al.*, 2009). Heavy metals have a high affinity for humic acids, clays and organic matter in soil (Ghosh and Singh, 2005; Smith, 2009), forming stable complexes with humic substances and clay, and generally show limited bioavailability (Shuman, 1999; Smith, 2009). Confirming this, studies have shown that application of organic amendments to soil can stabilise the form of HM and reduce uptake by plants (Yadav *et al.*, 2009). Soil pH significantly effects HM bioavailability, with acidic soils exhibiting higher metal availability and promoting HM uptake by plants. Conversely, lower transfer rates of HMs to plant tissue is observed in high pH soils (Smith, 2009). Some clays carry permanent negative charge, however, for organic matter and other clay minerals the charge can be pH dependent. At normal pH, the predominant charge on variably charged colloids is net negative (Robinson *et al.*, 2006). Most heavy metal ions carry a positive charge and can therefore be retained by negative binding sites in the soil matrix (Robinson *et al.*, 2006). In a study by Wang and Zhang, (2009) pH was found to have a significant negative influence on the concentration of HMs associated with the exchangeable fraction, with increased pH exhibiting a reduced concentration of exchangeable metals in soil solution. The same study also noted that an increased concentration of soil OM reduces the availability of Cu, attributed to the formation of stable Cu-organic complexes (Wang *et al.*, 2009). Temperature has also been shown to effect HM availability, with one experiment showing that metals were more available to plants at 25°C than they were at 15°C (Antoniadis and Alloway, 2002).

Different types of waste exhibit a different distribution of HM bioavailability. It has been shown that the distribution of heavy metals in raw biosolids amended soils is different from that in compost-amended soils (Maiaomiao *et al.*, 2009). In some cases increased metal bioavailability in the presence of amended organic substrates (biosolids) has been observed due to the added amounts of dissolved organic matter/carbon (DOC) (Zhou and Wong, 2001; Santibanez *et al.*, 2008), defined as organic compounds produced during decomposition, or soluble organic ligands of either low-molecular weight organic acids or soluble humic acids (Shuman, 1999). Increased availability has

been related to the formation of complexes between metals and DOC released from biosolids, which in turn prevents sorption of metals to the soil (Zhou and Wong, 2001). These same authors noted that sorption decreased further when existing soil pH was increased and that the decrease in sorption promoted by sewage sludge DOM was greater than that by compost DOM (Zhou and Wong, 2001). The observed relationship between sorption and DOM is also significant to plant uptake, as the derived HM-DOC complexes can be absorbed by plant roots (Santibanez *et al.*, 2008). Therefore, fresh organic wastes that are high in water-soluble organic compounds can increase the solubility of metals (Santibanez *et al.*, 2008), whilst those that are more stabilized (composted or aged) contain a higher proportion of humified organic matter, binding and decreasing available HM (Santibanez *et al.*, 2008). Movement of HM through the soil profile is thought to be minimal, with HMs applied with sludge accumulating in the surface layers of soil, not moving much past the "zone of sludge incorporation" (Emmerich, 1982; Chang, 1984). However, there has also been evidence to the contrary suggesting that there is a risk of HM movement in soils treated with sewage sludge, and that this is again related to DOC (Antoniadis and Alloway, 2002). In situations of high application rates, biosolid colloids can be significant contaminant carriers, posing a risk for soil and groundwater contamination (Karathanasis *et al.*, 2005). Mobile biosolid colloids may exhibit greater ability to sorb metals than the surrounding soil, facilitating metal movement (Karathanasis *et al.*, 2005). This apparent effect differs between types of amendment as well as metal type. Karathanasis *et al*. (2005) found that biowaste colloids of anaerobically-digested biosolids transported twice as much metal as poultry manure waste and four times as much as lime-stabilized biosolids.

Many biological factors also influence HM bioavailability. Plant species can have an impact primarily through rhizodeposition (Cheng *et al.*, 2004), although this has not been widely investigated and will be discussed further in this chapter. A study by Fujii *et al*. (2009) looking into the effect of earthworm presence on Cu availability in soil found that the toxicity of Cu was primarily controlled by the soil aging process, with reduced toxicity observed over time due to the gradual adsorption onto soil particles. However, they also found that earthworm presence altered the bioavailable concentration of Cu, decreasing it significantly in Cu-spiked soils compared to those without earthworms (Fujii and Kaneko, 2009).

"In situ" remediation of HM contaminated soils is based on either extraction or stabilisation of the contaminants (Castaldi *et al.*, 2009a). Many studies have focussed on the use of additions or amendments to soil that immobilise the HM, but many of the associate techniques have limitations (Castaldi *et al.*, 2009a). Another means to remediate HM polluted soils is thorough the ues of plants (phytoremediation).

2.2.4. Phytoremediation

Plants have frequently been used in the remediation of heavy metal (HM) contaminated soils (phytoremediation). Phytoremediation of HM contaminated sites can be carried out in two ways; phytoextraction or phytostabilisation (Ghosh and Singh, 2005; King *et al.*, 2008; Vamerali *et al.*, 2009). Phytoextraction is primarily through accumulation of metals in plant material and subsequent removal, removing the contaminants from the site altogether (Ghosh and Singh, 2005; Craw *et al.*, 2007; King *et al.*, 2008). This is carried out by the use of plants known to be heavy metal hyperaccumulators (Reeves, 2006). Phytostabilisation works by using plants to stabilise a contaminant within the soil profile, thereby preventing its leaching and reducing the chance of contact by organisms (Ghosh and Singh, 2005; King *et al.*, 2008; Santibanez *et al.*, 2008). In the case of degraded soil, plants can be used to improve soil fertility by regaining soil structure, helping to retain soil moisture, prevent erosion and by introducing organic substrates.

2.2.4.1. Interactions between plants and heavy metals in soil

Through the establishment of metal tolerant species, plants can achieve metal stabilisation by; decreasing the amount of water in the profile (preventing leaching or water movement), providing wind shields that prevent erosion and distribution of soil to other areas, and by encouraging sorption, precipitation or complexation of contaminants within the soil profile (Ghosh and Singh, 2005; Mertens *et al.*, 2007; Brunner *et al.*, 2008; King *et al.*, 2008; Santibanez *et al.*, 2008). It has been suggested that some plants

can influence the speciation and solubility of HM in soil (Cheng *et al.*, 2004), primarily through altering rhizosphere soil properties such as pH and altering microbial community structure (Cheng *et al.*, 2004; Castaldi *et al.*, 2009a)

The degree of HM uptake into plants varies widely from species to species and also depends on the HM of concern (Yadav *et al.*, 2009). Zinc is relatively labile and therefore easily taken up into plant tissue, however, Cu is more strongly sorbed to soil colloids (soil organic matter) and plant uptake is more limited (Smith, 2009). Heavy metal uptake is also dependent on the total concentration of metal in the soil, its bioavailability and soil physico-chemical properties (Smith, 2009). Some root exudates can be in the form of DOC, which as outlined earlier can facilitate HM mobility in soil (Antoniadis and Alloway, 2002). The relationship between specific plant species and HM has not been widely assessed, and it is likely that there will be a different interaction depending on the plant species and HM of interest.

2.3. Manuka (*Leptospermum scoparium*)

Kingdom: Plantae

Division: Magnoliophyta

Class: Magnoliopsida

Order: Myrtales

Family: Myrtaceae

Genus: *Leptospermum*

Species: *L. scoparium*

Manuka (*Leptospermum scoparium)*, or New Zealand tea tree is a native New Zealand woody shrub found throughout most of the country, from lowlands to subalpine areas (manuka can occur up to 1250 m in elevation) (Perry, 1997; Perry *et al.*, 1997;

Scott *et al.*, 2000; Stephens *et al.*, 2005). Manuka is an evergreen tree, usually growing to a height of 2-5 m but occasionally up to 8 m. Manuka leaves are small and densely branching (Figure 2.2.), and the flowers are white, bowl shaped and pollinated by a range of insects including coleoptera and diptera (beetles and common flies) (Douglas *et al.*, 2001; Maddocks-Jennings *et al.*, 2005; Stephens *et al.*, 2005). Manuka is frequently mistaken for kanuka (*Kunzea ericoides),* another indigenous member of the Myrtaceae family found widely throughout New Zealand. It is distinguished from manuka by its larger size, smaller leaves, flowers and fruit (Porter and Wilkins, 1998). Three broad groupings of manuka have been defined in New Zealand (Perry, 1997), and similar "tea tree species" are found throughout Australia (Stephens *et al.*, 2005). Manuka has an attractive appearance and fragrance, and it is commonly found in private gardens as well as native bush. Many ornamental varieties and cultivars are available in plant nurseries with a range in flower colour, appearance and size (Lis-Balchin *et al.*, 1996).

Figure 2.2 *Manuka (Leptospermum scoparium) flowers and leaves*

A very versatile plant, in the past manuka has been used in teas, for food, for medicines and for its wood, and more recently for essential oils and honey production (Perry *et al.*, 1997; Stephens *et al.*, 2005). Captain Cook was reported to have brewed tea and beer from manuka leaves over 200 years ago (Brooker *et al.*, 1981). It is an extremely hardy species, tolerant of varying conditions including infertile and dry soil, or swamp-like habitats (Perry *et al.*, 1997; Stephens *et al.*, 2005). It has been shown to be tolerant to heavy metals (Craw *et al.*, 2007), to grow readily on degraded and erosion prone sites (Watson and O'Loughlin, 1985; Bergin *et al.*, 1995) and to encourage re-vegetation in zones of impaired soil quality (Watson and O'Loughlin, 1985; Craw *et al.*, 2007).

Manuka oil and honey have long been known for their potential medicinal, antiseptic and antimicrobial properties (Porter and Wilkins, 1998; Lusby *et al.*, 2005). The bark, seeds, leaves and sap from manuka trees have been used for centuries in traditional Maori medicinal treatments, including teas and baths (Perry, 1997; Perry *et al.*, 1997; Porter, 1998; Porter and Wilkins, 1998). Concoctions of manuka components were used to treat a myriad of ailments, including urinary complaints, diarrhoea, burns, coughs, fever, skin diseases, inflammation, mouth, throat and eye conditions and as a blood and breath purifier or inhalant for colds (Brooker *et al.*, 1981). Similarly, other species of Myrtaceae were used in Hawaii, Malaysia and in the Moluccas for such things as treating diarrhoea, fever, relieving colic or symptoms of bronchitis (Lis-Balchin *et al.*, 1996). The potential commercial value of manuka oil in New Zealand was investigated as far back at 1924 (Gardner, 1924). More recently manuka products have been investigated for their potential to control pathogenic microorganisms and infection (Allen *et al.*, 1991; Somal *et al.*, 1994; Allen and Molan, 1997; Porter, 1998; Porter and Wilkins, 1998; Christoph *et al.*, 2000; Lis-Balchin *et al.*, 2000; Visavadia *et al.*, 2008; Jeong *et al.*, 2009).

2.3.1. Rehabilitating degraded land

Forest vegetation is known to reduce potential for erosion on erosion prone sites, as well as to reduce runoff and keep the soil profile dry relative to pasture, with

indigenous forest providing the best protection from landslide (Bergin *et al.*, 1995). Much of New Zealand hill country is prone to serious erosion, particularly along the east coast of the North Island (Bergin *et al.*, 1995). Manuka, in particular, has been shown to provide good protection against shallow landslide and erosion due to its shallow and intricate root system (Watson and O'Loughlin, 1985). Manuka roots are strong, occupying 5% of soil volume up to 50cm depths (Bergin *et al.*, 1995) giving added slope stability by providing a reinforced layer of soil at the surface, with a central tap root giving strength and holding the underlying mantle rock in place (Watson and O'Loughlin, 1985). The strength and concentration of manuka roots in the top 50 cm of soil also potentially reduces water movement. The shallow, intricate root system can help dewater the surface layers of soil, preventing leaching of nutrients (Watson and O'Loughlin, 1985) or contaminants. This is important in situations where nutrient containing amendments are being applied to soil or sites are located near waterways. In addition, sandy soils that are prone to erosion are also likely to be prone to leaching of nutrients. Planting species such as manuka on sandy soils may aid in keeping water in the surface layers of soil by providing added organic matter, binding soil in the intricate root system and improving water holding capacity.

Colonisation of exposed soil by Manuka is relatively rapid (Bergin *et al.*, 1995). The plant is a known pioneer/colonising species, and is useful in initial stages of re-vegetation, playing an important part in the natural succession of exposed land to native forest in New Zealand (Mohan *et al.*, 1984). It is often found to be the dominant species on disturbed sites that are prone to salt and wind, or on soil that is too wet, dry or infertile for other forest species (Perry *et al.*, 1997; Scott *et al.*, 2000; Stephens *et al.*, 2005). Manuka's hardy nature makes it a very good species for planting on sites planned for revegetation and rehabilitation, and being a pioneer species it encourages the subsequent colonisation of land with other indigenous plants. Manuka is also tolerant to relatively high soil concentrations of heavy metals, making it useful for indigenous forest regeneration on metal contaminated soil (e.g. old mining sites) (Craw *et al.*, 2007). Manuka has been shown to establish effectively on sites contaminated with high levels of arsenic (As), and shows limited translocation of this metal from roots to shoots and leaves (Craw *et al.*, 2007).

2.3.2. Antiseptic Properties

More than 80% of the world's population relies on traditional medicines, such as herbal remedies, for healthcare (Eloff., 1998; Hassan *et al.*, 2009). The use of plants as medicine is a well documented interaction between humans and their environment (Hassan *et al.*, 2009). Records show that manuka components such as bark, leaves, seeds and sap have been used in New Zealand in beverages and medicinal preparations since before European settlement, particularly as an insecticide, a remedy for fever and a cure for intestinal worms and diarrhoea (Brooker *et al.*, 1981; Porter, 1998; Maddocks-Jennings *et al.*, 2005; Stephens *et al.*, 2005). In modern times manuka oil and manuka products (particularly honey) have been sold widely throughout New Zealand as remedies for minor infections and ailments (Lusby *et al.*, 2005; Visavadia *et al.*, 2008).

Honey has been used as a topical and gastrointestinal remedy for thousands of years (Mundo *et al.*, 2004). In a study into the inhibitory activity of honeys against a range of pathogenic bacteria, it was found that all the tested honeys had some inhibitory effect on the bacteria tested, with manuka honey exhibiting the most pronounced effect compared to other honeys (Lusby *et al.*, 2005). This was particularly obvious with the bacterium *Alcaligenes faecalis*, *Enterobacter aerogenes* and *Staphylococcus aureus*, which were not inhibited by other honeys but were sensitive to manuka honey (Lusby *et al.*, 2005). Many honeys are known to have natural antibacterial properties, primarily due to their mild acidity, ability to maintain moisture, osmolarity, hydrogen peroxide release, and viscosity (which provides a protective barrier), all of which aid in wound healing (Taormina *et al.*, 2001; Cooper *et al.*, 2002; Mundo *et al.*, 2004; Lusby *et al.*, 2005; Visavadia *et al.*, 2008). Hydrogen peroxide is produced during the oxidation of glucose by glucose oxidase from honey bees' hypopharyngeal glands (Taormina *et al.*, 2001) and is therefore found in most honey. However, it has also been noted that the antibacterial activity of some honeys can be dependent on their floral source (Allen *et al.*, 1991). By adding catalase to honey samples to break down hydrogen peroxide, non-peroxide antibacterial activity can be assessed (Taormina *et al.*, 2001). Using this method, Allen *et al. (*1991) found that there was a difference in antibacterial activity

between honeys from different plant sources, with honeys from the New Zealand plants manuka, kanuka, and kamahi all exhibiting non-peroxide antibacterial activity. In this same study it was determined that antibacterial activity of the manuka honey tested was almost entirely due to its non-peroxide components (Allen *et al.*, 1991). It seems that manuka honey has added antimicrobial properties that other honeys do not have (Molan, 2009), and that manuka honey has phytochemical compounds that make a major contribution to this beneficial effect (Cooper *et al.*, 2002). Until recently these phytochemical compounds had not been identified. The principal component responsible has now been suggested to be methyl glyoxal (Adams *et al.*, 2009; Stephens *et al.*, 2010). The source of methyl glyoxal in manuka honey is still not well understood, but thought to be due to non-enzymatic conversion of dihydroxyacetone from manuka flower nectar (Adams *et al.*, 2009). In a recent study by Visavadia *et al*. (2008), manuka honey impregnated dressings applied to wounds were found to effectively combat infections of methicillin-resistant *Staphylococcus aureus* (MRSA). The honey also exhibited anti-inflammatory activity and stimulated an immune response, promoting healing. Alandejani *et al. (*2009) fouund a similar result, with manuka honey effectively killing strains of *Pseudomonas aeruginosa*, and *Staphylococcus aureus* (both methicillin-susceptable and -resistent strains). Manuka honey has been used to treat serious skin ulcers, *Pseudomonas* infections, unhealed skin grafts, abscesses, boils, indigestion, gastritis, rhinosinusitis and many other infections unresponsive to standard antibiotic therapy (Allen and Molan, 1997; Alandejani *et al.*, 2009; Molan, 2009). In a study into the susceptibility of *Helicobacter pylori* to the antibacterial activity of manuka honey, it was found that manuka honey inhibited infection (Somal *et al.*, 1994). *Helicobacter pylori* are gram-negative bacteria known to produce stomach inflammation, gastritis and duodenal ulceration. Manuka honey has also been used in veterinary applications. An example is the inhibition of mastitis causing bacteria in milking cow udders (Allen and Molan, 1997). Manuka honey among others has also been investigated as an alternative food preservative (Mundo *et al.*, 2004), useful in todays climate with people more interested in “green” treatment options for food preservation (Burt, 2004).

In New Zealand, manuka honey is rated according to a scale used to define antimicrobial activity called the unique manuka factor (UMF). The UMF defines a

honey's non-peroxide antibacterial activity, and is calculated according to its antibacterial activity against *Staphyloccus aureus* compared to a phenol reference standard; the UMF rating means the concentration of phenol with the same antibacterial activity as the honey i.e. 15 UMF = 15 % phenol solution (Molan, 2009). Although there is great potential for manuka honeys medicinal use, and much research has been done in this area, it is still not common practice to use honey in western medicine (Molan, 2009). However, this is something that we may see change in the future especially as honey is natural, non-toxic and inexpensive (Alandejani *et al.*, 2009).

Manuka oils have also been shown to have antimicrobial activity against a variety of bacteria, particularly gram-positive strains (Lis-Balchin *et al.*, 1996; Porter and Wilkins, 1998; Christoph *et al.*, 2000; Douglas *et al.*, 2004) and dermatophytes (fungi that can cause infections of the skin, hair, and nails such as ringworm) (Christoph *et al.*, 2000; Maddocks-Jennings *et al.*, 2005). It is thought that the components responsible for this in extracted oil are not the same as those promoting antibacterial ability in honey (Weston, 2000; Stephens *et al.*, 2005). Australian tea tree oil is widely accepted as an antiseptic (Christoph *et al.*, 2000), and whilst its activity is generally greater against some bacteria (Lis-Balchin *et al.*, 1996; Maddocks-Jennings *et al.*, 2005), manuka oil has been shown to have greater effectiveness against many gram-positive strains (Harkenthal *et al.*, 1999). An explanation of gram-positive versus gram-negative bacteria is presented in Figure 2.3. The reason for more inhibitory effect against gram-positive strains is not completely understood, but has been suggested to be due to the lipophilicity of manuka oil and the impact this has on the peptidoglycan of gram-positive bacteria cell walls (Christoph *et al.*, 2000). In addition gram-negative bacteria possess an outer membrane surrounding the cell wall which restricts the diffusion of lipophilic/hydrophobic compounds (Burt, 2004).

Manuka oil is usually extracted from plant material by steam distillation (2000; Douglas *et al.*, 2001). Most commercial harvest of manuka plant material is from wild stands, although in some parts of New Zealand there are plans to develop plantations for harvesting (Douglas *et al.*, 2001; Maddocks-Jennings *et al.*, 2005). Re-growth of plants is rapid (3-5 years) after harvesting and damage is minimal if harvested appropriately

(Maddocks-Jennings *et al.*, 2005). Plantation harvesting will allow for production of higher quality oils through selection of high quality plants, identification of improved lines and exploitation of the more active chemotypes (discussed later) (Douglas *et al.*, 2001; Douglas *et al.*, 2004). Most manuka oil comes from the leaves of the plant, which contain oils sacs called schizogenous secretary cavities (Douglas *et al.*, 2001). However, oil composition is similar between stems and leaves (Perry *et al.*, 1997). Manuka essential oil has many uses other than as an antimicrobial agent, including as a fragrance and in aromatherapy (Lis-Balchin *et al.*, 1996). There have been reports that the oil composition of manuka varies geographically and accordingly, so does its antibacterial activity (Perry *et al.*, 1997; Douglas *et al.*, 2004). Even within geographic locations the oil composition has been shown to vary: this variation is present between individual plants and is persistent throughout maturity (Porter, 1998). Some studies have noted continuing change within and between seasons, with plant chemistry reflecting seasonal, climatic and plant effects (Porter, 1998), whilst others have noted no significant seasonal change in chemical composition (Porter *et al.*, 2000). Taking into account that the chemical composition of manuka oil depends on the ecotype harvested

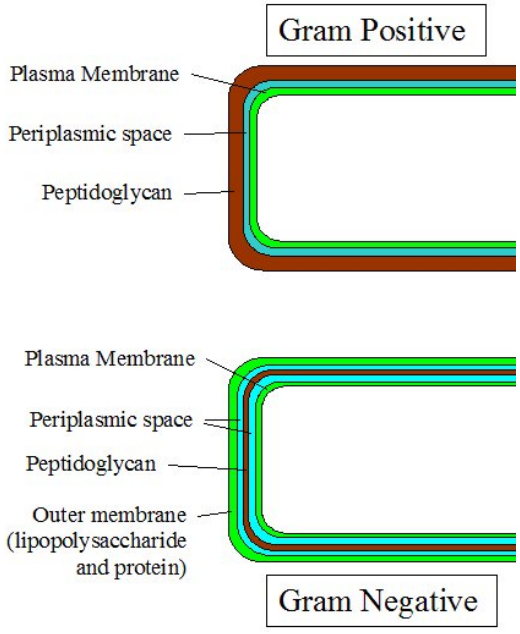


Figure 2.3 *The difference in cell structure between gram-positive and gram-negative bacteria. Gram-positive bacteria stain purple whilst gram-negative stain pink using the gram staining procedure due to differences in cell membranes and peptidoglycan (Search.com, 2009)*

and the locality of harvesting, manuka oil has been classed into three broad chemotypes according to location in New Zealand (Porter *et al.*, 2000) (based on key component chemical markers).

- Far north – high in pinene
- East Cape and Marlborough Sounds – high in triketone
- Remaining locations containing oils high in sesquiterpine

The antimicrobial activity of manuka oil has been associated with the triketone content of the oil, which is identified within the polar fraction (Perry *et al.*, 1997; Porter, 1998; Porter and Wilkins, 1998; Porter *et al*., 2000), primarily, flavesone, leptospermone and iso-leptospermone (Porter, 1998). Non-polar fractions of the oil consist primarily of sesquiterpenes and do not exhibit antibacterial activity (Porter and Wilkins, 1998). Triketones are present in all manuka oils, but it is the ratio of triketone to other chemicals such as pinene and sesquiterpine which distinguishes those with higher antibacterial activity from those with lower. On average manuka oil is made up of 1-7% pinene, 80% sesquiterpine and 15% leptospermone (triketone) (Lis-Balchin *et al.*, 1996), however, it has been noted that manuka plants in the North Island East Cape can have up to 30% more triketone than those in the South Island (Maddocks-Jennings *et al.*, 2005). Leptospermone is the triketone that has been associated with most anti-microbial activity in manuka oil (Weston, 2000; Jeong *et al.*, 2009). It is insoluble in water and present in the leaves, twigs and seeds of manuka (roots have not been tested) (Weston, 2000; Jeong *et al.*, 2009). Leptospermol, as it was originally named, was identified in oils of Australian Myrtaceae species as far back as 1921 (Penfold, 1921) and subsequently in New Zealand manuka by Gardner, in 1924 (Gardner, 1924). Penfold (1921) identified leptospermol as a phenolic compound, but this was disputed by Gardner, who some time later identified it to be a monobasic acid (Gardner, 1925), this was further disputed by Short who found leptospermol to be an acidic phenol (Short, 1926). Today leptospermone is accepted to be a β-triketone, the chemical structure of which can be seen in Fig. 2.4. As well as antimicrobial activity, leptospermone has been shown to have anti-helminthic and insecticidal properties (Lis-Balchin *et al.*, 1996; Lis-Balchin *et al.*, 2000; Douglas *et al.*, 2001). Helminths are parasitic worms that live on or in humans or animals causing disease or illness.

Evidence suggests that leptospermone from bottle brush (*Callistemon citrinus*, also a member of the Myrtaceae family) is an effective herbicide (Cornes, 2005). Cornes (2005) found that leptospermone produced by the roots of the bottle brush suppressed weeds around the plants base. Roots of manuka have not been investigated for leptospermone content, but it may be similar to that found in the bottle brush. Manuka oil also shows a spasmolytic action (suppresses muscle spasms) on smooth muscle and has some antifungal effects (Lis-Balchin *et al.*, 1996; Lis-Balchin *et al.*, 2000).

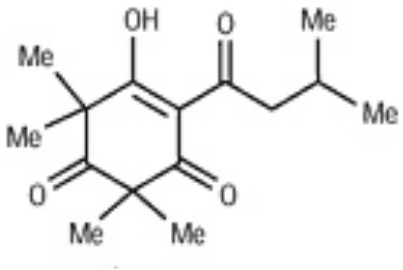


Figure 2.4. *Chemical structure of leptospermone*

Manuka oil has been shown to have activity against strains of *Staphylococcus*, *Listeria, Streptococcus*, *Enterococcus*, *Clostridium* and *Bifidobacterium* (Harkenthal *et al.*, 1999; 2000; Douglas *et al.*, 2001), all of which are gram-positive bacteria. Using the agar disk diffusion method, Jeong *et al*. (2009) found that purchased manuka seed oil strongly inhibited *Clostridium dificile* and *Clostridium perfringens* growth (gastroenteritis causing bacteria), but did not inhibit the growth of *Bifidobacterium breve*, *Bifidobacterium longum*, and *Lactobacillus casei* (widely accepted as being beneficial gut bacterium) or *Escherichia coli* ATCC 11775 (non-pathogenic strain of *E.coli*). Leptospermone alone was also shown to exert a strong growth inhibition effect against *Clostridium perfringins* without adversely effecting beneficial bacteria in the gut (Jeong *et al.*, 2009). These authors concluded that leptospermone isolated from manuka seeds could be used to effectively inhibit harmful intestinal bacterial without adversely affecting beneficial bacteria in the gut, and added that there may be potential to further isolate compounds to improve their antimicrobial potency and stability. Lis-Balchin *et al. (*1996) also found that manuka oil had activity against most of the gram-positive

strains of bacteria tested. However, contrary to Jeong *et al*. (2009), *Escherichia coli*, a gram-negative strain, was also significantly effected. This may be due to a difference in the strain used, or in the quality of the oil. Harkenthal *et al*. (1999) found *Listeria monocytogenes* to be the most susceptible microorganism to manuka oil and *Pseudomonas aeruginosa* (a widespread environmental bacteria that can cause infection in humans and animals) to be completely unaffected. Whilst they agreed that manuka oil was most effective against gram-positive bacteria some inhibition of the gram-negative strains *Salmonella choleraesuis*, *Shigella flexneri*, *Proteus mirabilis*, *Enterobacter aerogenes* and *Citrobacter freundii* was also observed. In the same study it was noted that all strains of *Staphylococcus* tested, including antibiotic resistant strains, were very sensitive to low concentrations of manuka oil (Harkenthal *et al.*, 1999). *Staphylococci* are environmentally prevalent bacteria, most are harmless but many are known for causing mild or acute infections and food poisoning. Methicillin-resistent *Staphylococcus aureus* (MRSA) is one particular strain that has become a worldwide concern due to its resistance to antibiotics and ease of contraction by relatively healthy patients in hospitals (Harkenthal *et al.*, 1999). The apparent susceptibility of this bacterium to manuka oil indicates that future medicinal practices may make greater use of natural manuka products. This is already being investigated in some hospitals around the world (Maddocks-Jennings *et al*., 2009).

2.3.3. Economic uses

Triketone-enriched oils are commercially valuable because of their antibiotic activity (Douglas *et al.*, 2001), and thus the economic value of sources of such oils is clear. However, in addition to the medicinal value of manuka products (oil and honey) there has been interest in the potential for managing manuka plantations for wood production (Bergin *et al.*, 1995). Manuka wood is a very good firewood and its wood chips are used in smoking food (Brooker *et al.*, 1981). Carbon accumulation by manuka is rapid and similar to that of plantation forestry (Scott *et al.*, 2000; Stephens *et al.*, 2005), making its potential as a plantation species more viable. Regeneration of forests plays a major role in global carbon (C) cycling (Scott *et al.*, 2000). It has been suggested that native scrubland comprising of manuka and kanuka accumulates as much

C in biomass as do exotic forestry plantations across New Zealand, which the country uses to offset a portion of its fossil fuel CO_2 emissions (Scott *et al.*, 2000). With their ease of growth and maintenance, reversion of land to manuka scrubland may be a useful C sink for New Zealand, and an economic offset for fossil-fuel emissions through C-sequestration (Scott *et al.*, 2000).

2.4. Study Aims

The aim of the current research is to investigate the potential of manuka as a remediation species, with particular reference to its effect on HM and pathogenic bacteria that may transfer to soil from biosolids recycling to land. It is hypothesised that the antimicrobial ability observed in commercial manuka products might be present in growing manuka and thus aid in the die-off of pathogens in soil where biosolids has been applied. In addition, the intricate root system of manuka may aid in HM stabilisation, whilst its tolerance to HM in soil may reflect an ability to alter HM bioavailability.

2.5. Overview of Study

The following research is an in-depth look into the phytoremediation potential of manuka (*Leptospermum scoparium*), with particular reference to land contaminated through the application of biosolids. The following chapters firstly focus on the potential use of manuka's antimicrobial activity to inhibit survival and growth of pathogenic bacteria from biosolids. Initial experiments consisted of a range of preliminary tests investigating manuka components, and their ability to inhibit the growth of five pathogenic bacterial strains potentially found in biosolids (Chapter Three). Based on these results, further experiments were conducted to ascertain whether these inhibitory components are also present in soil from underneath growing manuka (Chapter Four). The results from these experiments are presented and discussed in Chapters Three and Four of this thesis.

The final experimental chapter in this study (Chapter Five) focuses on manuka's remediation potential in reference to HM contaminated soils. The primary aim of this chapter was to conduct an experiment that would highlight whether manuka has any ability to extract or stabilise metals in soil. The experiment in Chapter Five takes an in-depth look into whether manuka growth has an impact on the bioavailability of spiked HMs in soil. In summary, Chapter Six of this thesis ties together both components of remediation outlined by discussing the overall potential of manuka as a species suitable for planting on biosolids amended land, with reference to the results in Chapters Three, Four and Five.

3. Preliminary Antimicrobial Investigation

3.1. Introduction

Leptospermum scoparium (manuka) components such as bark, leaves and sap have been used in medicinal preparations in New Zealand since before European settlement (Porter, 1998). Many studies have confirmed the antibacterial activity of manuka honey and oil (Porter and Wilkins 1998; Lusby, Coombes et al. 2005) and today manuka products are sold widely throughout New Zealand as natural remedies for minor infections and aliments (Lusby *et al.*, 2005; Visavadia *et al.*, 2008). These factors are discussed in depth in section 2.3 of this thesis.

As mentioned in Chapter Two, biowastes such as biosolids, are routinely disposed of to land in many countries (Warman, 2005). This raises issues around the transfer of pathogenic microorganisms to the environment, and their survival within soil and biosolids. Research has shown that a number of bacterial pathogens are present in sewage sludge and can also be present in biosolids (Garrec *et al.*, 2003; Lemunier *et al.*, 2005; Smith *et al.*, 2005; Horswell and Aislabie, 2006). It is also known that some pathogenic organisms can survive in soil for considerable lengths of time (Horswell and Aislabie, 2006). The proposition is that there may be potential to utilise the antiseptic properties of manuka to reduce risks associated with biosolids application to land. We have carried out preliminary work to investigate whether manuka components, in place of available commercial products, are effective in controlling the pathogenic organisms likely to be found in sewage sludge and other biowastes. Previous research has investigated the antimicrobial affect of manuka honeys and commercially produced oils, however it is manuka in the more natural form which is of interest in this study.

3.2. Aim

The aim of the experiments outlined in this chapter was to prepare and assess the effect of manuka extracts on a range of bacterial pathogens potentially found in sewage sludge, and hence to justify the continuation of investigations into the potential use of manuka as a remediation species for biosolids-amended land.

3.3. Methods

3.3.1. Preparation of plant extracts

Plant extracts were produced by blending plant material at a 1:4 ratio with water. Water was chosen as an extractant to more closely mimic conditions present in soil when herbage decomposes. Previous studies have used an array of solvents when extracting plant products to isolate antimicrobial components (Eloff., 1998), and although solvent extracts have been shown to enhance antibacterial activity, water extracts have also been shown to be effective against some bacteria (Eloff., 1998; Hassan *et al.*, 2009). For the purpose of this research water was determined to be sufficiently effective, more relevant, and its use would avoid any potential inherent toxicity of the solvents themselves.

3.3.1.1. Manuka leaf extracts

Herbage was removed, from the base of the stem upwards, from three manuka seedlings (approx 30 cm in height) obtained from a local nursery, Matatoa Trees and Shrubs, Shannon. The herbage was cut into 1 cm lengths and homogenised. A 5 g sub sample was mixed with 20 ml of deionised water in a Breville mini wizz (blender) and subsequently ground using a mortar and pestle. The ground paste was pressed to obtain liquid which was subsequently filtered using Whatman #1 filter paper and sterilised by passing through Millipore syringe filters (0.2 μm).

3.3.1.2. Manuka roots (Leptospermum scoparium)

All plant matter from the base of the stem down was considered to be roots. As with the manuka leaves, the roots were removed from three manuka seedlings, washed in DI water to remove soil and cut into 1 cm lengths for homogenisation. A 5 g sub sample was mixed with 20 ml of deionised water in a Breville mini wizz (blender) and subsequently ground, pressed and filtered as with the leaf material (Section 3.3.1.1)

3.3.1.3. Rye grass (Lolium perenne)

Approximately 20 g of herbage was taken from a ryegrass field in Porirua by random grab sampling, and immediately transported to the lab. Any herbage other than ryegrass was removed and the remaining ryegrass cut into 1 cm size pieces and homogenised. As with manuka material (sections 3.3.1.1 and 3.3.1.2), a 5 g sub sample was mixed with 20 ml of deionised water in a Breville mini wizz (blender) and subsequently, ground, pressed and filtered using Whatman #1 filter paper, and sterilised by syringe filter (Millipore 0.2 μm)

3.3.2. Agar plate enumeration techniques

Preliminary work was carried out to test the antibacterial efficacy of various manuka products; manuka leaf extract, manuka root extract, manuka honey and commercially produced pure manuka oil. Honey was obtained from a specialty honey store (5+ active), and diluted at a ratio of 2:5 with DI water for analysis. Manuka oil (certified 100%) was used neat. Deionised water was used as a control

3.3.2.1. Sewage sludge colony counts

A serial dilution of sewage sludge was produced by mixing 1 g of anaerobically digested sludge with 9 ml of Ringers solution[1] (Oxoid BR0052, Appendix A2.1), and subsequently transferring 1 ml to 9 ml Ringers solution until a six times serial dilution was achieved (10^{-6}) . Only 10^{-5} and 10^{-6} dilutions were used for the experiment; this was based on previously determined sludge dilutions that gave countable numbers when

[1]An aqueous solution of the chlorides of sodium, potassium, and calcium in the same concentrations as normal body fluids, used to suspend cell cultures.

spread on tryptic soy agar plates (data not shown). These sludge dilutions were spread onto tryptic soy agar plates (a total of 16 plates for each dilution) at the rate of 100 µl per plate and left to dry. Plates were subsequently spread with 100 µl of one of five treatments (water, honey, manuka leaf, manuka root, and oil) in triplicate and incubated at 35°C for 24 hours after which total colony numbers were counted.

3.3.2.2. Pure culture colony counts

Pure cultures of Salmonella *typhimurium-lux* and *Escherichia coli* 0157 were grown from freeze dried stock (Appendix A1.1), in the appropriate growth media, and enumerated according to bacterial enumerating techniques described in Appendix A1.2. A dilution of each broth was prepared such that the broth contained 10^7 colony forming units (cfu) per ml. Broth was then spread onto tryptic soy agar at the rate of 100 µl per plate and left to dry. Plates were subsequently spread with 100 µl of one of four extracts (water, manuka leaf, manuka root, and oil) in triplicate and incubated at 35°C for 24 hours, after which total colony numbers were counted.

3.3.3. Bacterial bioluminescence-based bioassay

The bacterial bioluminescence-based bioassay used in this study was based on that used by Horswell *et al*. (2006) in their investigations of soil heavy metal contamination. This bacterial biosensor, supplied by the University of Aberdeen (UK), was used to investigate the microbial toxicity of manuka-water extracts. The biosensor works by producing luminescence when immersed in favourable conditions, and exhibiting reduced luminescence when in less favourable conditions. The bacteria biosensor consists of lyophilised cells of *Escherichia coli* HB101 genetically modified with the *lux*CDABE genes, originally isolated from *Vibrio fischeri*, using the multicopy plasmid pUCD607 (Rattray *et al.*, 1990; Sousa *et al.*, 1998; Horswell *et al.*, 2006). Freeze-dried cultures of *E. coli* HB101 pUCD607 were resuscitated in 10 ml 0.1 M KCl for 1 h (Sousa *et al.*, 1998; Horswell *et al.*, 2006) and manuka leaf and root extracts were diluted 1 in 10 with sterile deionised water before use. A suspension of biosensor cells (100 µl) was added to 900 µl of manuka leaf or root extract dilution at 15 s intervals and mixed by pipetting. Bioluminescence was measured in a LumiSkan TL (Labsystems, Finland) after a 15 min exposure to each sample. The assay was carried

out in triplicate and sterile water used as a blank. Data were expressed as average luminescence. A reduction in luminescence represents some inhibition of the bacterial biosensor.

3.3.4. Microplate assay

The antibacterial activity of manuka leaf extract was further tested using a spectrophotometric bioassay based on one previously used to determine microbial sensitivity to manuka honey (Patton *et al.*, 2006). This assay was chosen based on its time and cost efficiency when compared to previous assays for detection of antimicrobial activity (Smith *et al.*, 2008), and because broth dilution methods have been found to be more easily reproducible in studies of essential oils than the commonly used agar plate diffusion techniques (Christoph *et al.*, 2000).

Plant leaf extracts were diluted in deionised water to give twelve concentrations such that when 50 µl was diluted to 200 µl in one well of a 96 well microtitre plate the final extract concentrations would be as follows: 25 %, 12.5 %, 6.25 %, 3.13 %, 1.56 %, 0.78 %, 0.39 %, 0.20 %, 0.10 %, 0.05 %, 0.02 %, 0.01 %.

Five bacterial strains were chosen for preliminary analysis based on their presence in sewage sludge (Carrington, 2001; Smith *et al.*, 2005) and the ease of their culture.

- *Salmonella typhimurium*
- *Escherichia coli 0157*
- *Clostridium perfringins*
- *Campylobacter jejuni*
- *Listeria monocytogenes*

Strains of *E.coli* and *Clostridium sp.* are often considered representative of faecal indicator organisms (Eamens and Waldron, 2006), whilst *Salmonella typhimurium* and *Campylobacter jejuni* are enteric pathogens responsible for high rates of gastroenteritis in New Zealand (Horswell and Aislabie, 2006). *Listeria monocytogenes*, a pathogenic

bacterium causing listeriosis, is widely present in the environment and in treated sewage sludge (Garrec *et al.*, 2003; Lemunier *et al.*, 2005).

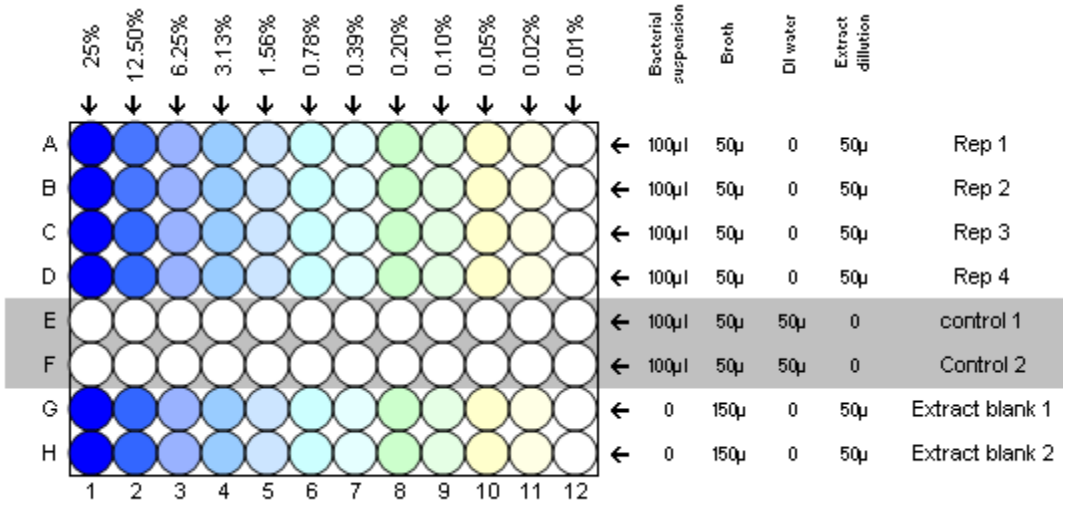


Figure 3.1 *The layout of each 96 well plate. Plant extracts were the last to be added and were diluted in a 1:2 serial dilution with deionised water.*

Each bacterial strain was grown from freeze dried stock (Appendix 1.1) in the appropriate growth media and enumerated according to bacterial enumerating techniques described in Appendix 1.2 to determine the total colony forming units (cfu) present in the stock broth. The optimum dilution of bacterial stock broth to use in the assay was pre-determined by assessing the growth curve of the stock broth at twelve dilutions over 24 hours. The concentration of bacterial stock selected for each bacterium was chosen such that the full growth curve would be visible over the incubation period. Each strain was then grown from the stock broth at optimum conditions in a 96 well plate, in the presence of manuka or ryegrass extracts at twelve concentrations (Fig. 3.1). Photographical representation of an innoculated 96 well plate can be seen in Figure 3.15. Inhibition of growth was determined by measurement of well optical density over time (595 nm). The whole plate was shaken for 10 seconds and read every hour using an automatic absorbance plate reader (Optima) in which the plate remained for the whole incubation period. An increase in optical density was taken to indicate growth of bacteria. Actual conditions for each bacterium are presented in the following sections.

Each strain required different incubation temperatures, lengths of incubation and growth media to obtain the best growth. One plate was used for each extract/bacteria combination, and this equated to 10 plates altogether.

3.3.4.1.Salmonella typhimurium

Salmonella typhimurium was grown in Luria-Bertani (LB) broth (Appendix A2.5) at 30°C for 24 hours in the presence of manuka and ryegrass extracts at 12 concentrations. The optical density (595 nm) of each well was read every hour over 24 hours to give the bacterial growth curve.

3.3.4.2. Listeria monocytogenes

Listeria monocytogenes was grown in tryptic soy broth at 37°C for 24 hours in the presence of manuka and ryegrass extracts at 12 concentrations. The optical density (595 nm) of each well was read every hour over 24 hours to give the bacterial growth curve.

3.3.4.3. Escherichia coli 0157

Escherichia coli 0157 was grown in LB broth at 30°C for 24 hours in the presence of manuka and ryegrass extracts at 12 concentrations. The optical density (595 nm) of each well was read every hour over 24 hours to give the bacterial growth curve.

3.3.4.4. Campylobacter jejuni

Campylobacter jejuni was grown in brucella broth at 37°C for 48 hours in the presence of manuka and ryegrass extracts at 12 concentrations. Plates were placed in aerojars with microaerobic gas producing packs (specified for *Campylobacter*). The optical density (595 nm) of each well was read approximately every 12 hours. This differed from previous bacterial isolates because *C. jejuni* has a slow growth rate and requires microaerobic conditions to grow, requiring the plate to be incubated in the presence of limited oxygen, and the plates had to be removed periodically to be read. Carrying this out every hour would have disrupted the growth of the bacteria.

3.3.4.5. Clostridium perfringins

Clostridium perfringins was grown in cooked meat broth (with the solid meat portions removed) at 37°C for 24 hours in the presence of manuka and ryegrass extracts at 12 concentrations. Plates were placed in aerojars with anaerobic gas producing packs suitable for *clostridium sp.*. The optical density (595 nm) of each well was read every 2 hours up to 10 hours, and then again at 24 hours. This differed from previous bacterial isolates because *C. perfringins* requires anaerobic conditions to grow, requiring the plate to be incubated in the presence of no oxygen. The plate had to be removed from the anaerobic chamber to be read and carrying this out every hour would have disrupted bacterial growth.

3.3.5. Statistical analysis

Data were analysed using Microsoft Excel. Bacterial plate count averages and standard errors were calculated and can be seen in Figs. 3.2 and 3.3. The difference between means were tested for significance using T-tests in Microsoft Excel. For the microplate method data, each bacterial data set was treated in the same manner. The data were first normalised by subtracting the optical density (OD) value at time zero from all subsequent values. Growth curves were then obtained by averaging the replicate wells at each time point and subtracting the respective extract control wells. This was to account for any absorbance caused by the presence of extracts which exhibited some colour. Extinction curves were obtained by plotting the total growth at the endpoint of the assay, calculated as a percent of control (Patton *et al.*, 2006), against the concentration of manuka or ryegrass extract in the well. The graphs were subsequently used to determine environmental effect concentrations (EC_{50} and EC_{20}).

3.4. Results

3.4.1. Agar plate enumeration techniques

3.4.1.1. Sewage sludge colony counts

Analysis of total colony forming units on plates spread with sewage sludge dilutions and five different manuka products showed a significant reduction in colonies when spread with manuka oil and manuka leaf extracts when compared to water or honey dilutions (Fig. 3.2).

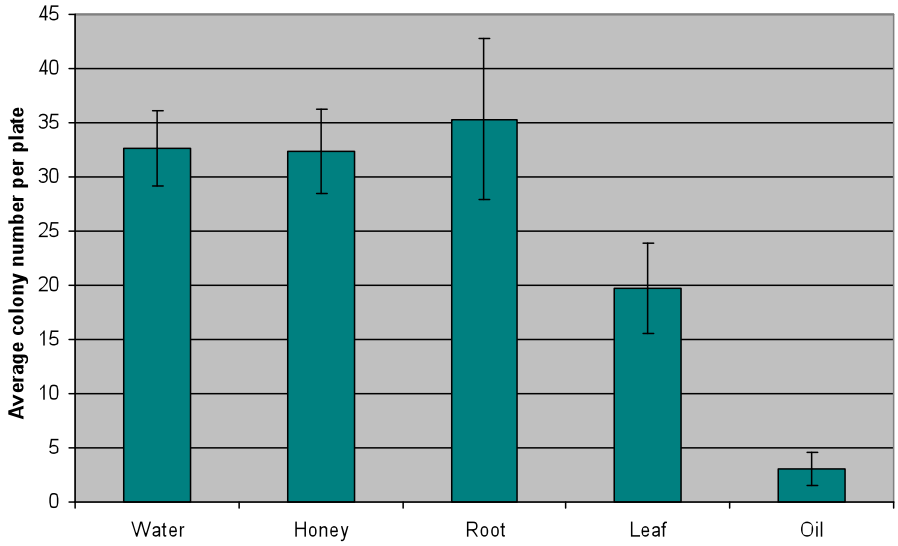


Figure 3.2 *Average total colony numbers counted on tryptic soy agar plates spread with sewage sludge dilutions and five manuka products, incubated for 24 hours at 35° C.*

3.4.1.2. Pure culture colony counts

Total colony numbers were significantly reduced for both salmonellae and *E. coli* in the presence of manuka oil. *S. typhimurium* were also significantly reduced by the presence of manuka leaf extracts and to a lesser extent manuka root extracts (Fig. 3.3). As with salmonellae, *E. coli* were significantly reduced by the presence of both

extracts, however this was not as pronounced for leaf extracts and the leaf and root extracts were not significantly different from each other (Fig. 3.3).

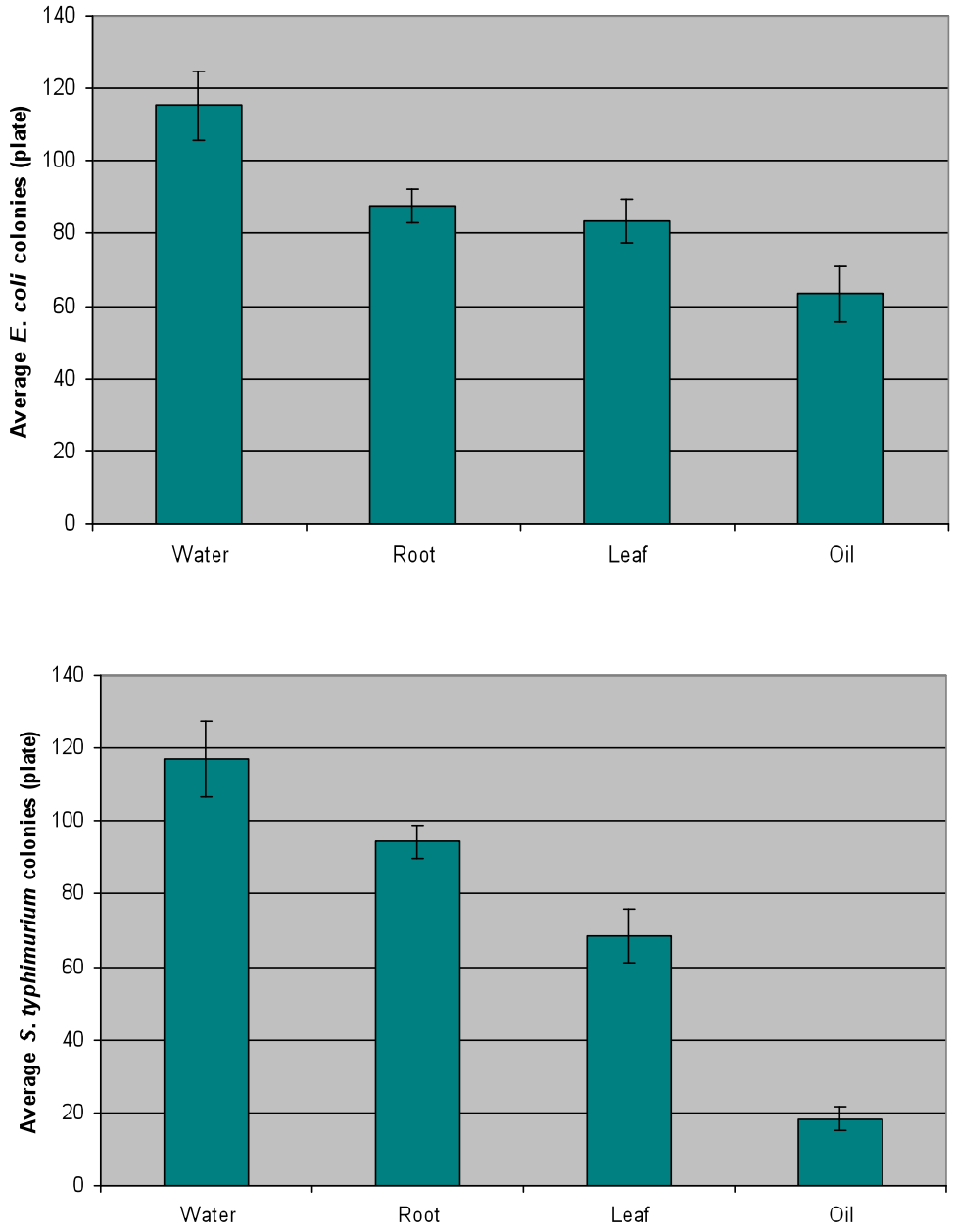


Figure 3.3. *Average total colony numbers counted on tryptic soy agar plates spread with a) E. coli 0157 and b) S. typhimurium in the presence of five manuka products, incubated for 24 hours at 35° C.*

3.4.2. *E. coli* lux biosensor

Results showed a significant reduction of luminescence from the biosensor in the presence of root and leaf extracts when compared to a water control, implying microbial toxicity (Fig. 3.4). The *E.coli* lux biosensor is affected by solution pH and therefore the pH of the extract solutions were measured. Although a difference was observed (Fig. 3.5), this was not considered to be significant enough to cause the observed effect when extracts were diluted at the rate used in the assay (100 µl with 800 µl DI water).

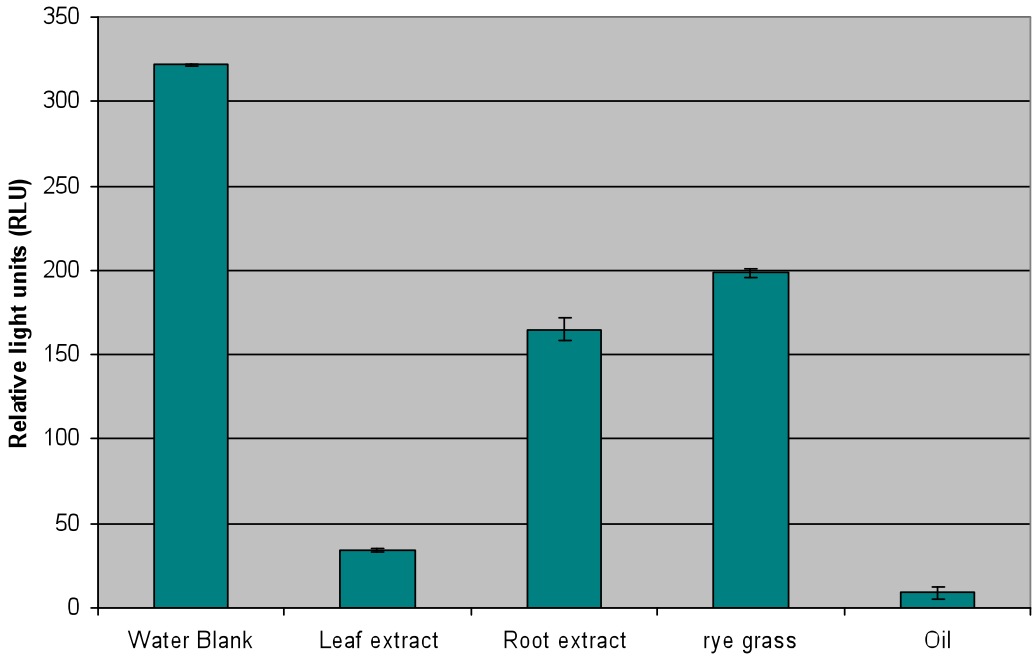


Figure 3.4. *Luminescence of the E. coli lux bacterial biosensor in the presence of manuka root and leaf water extracts.*

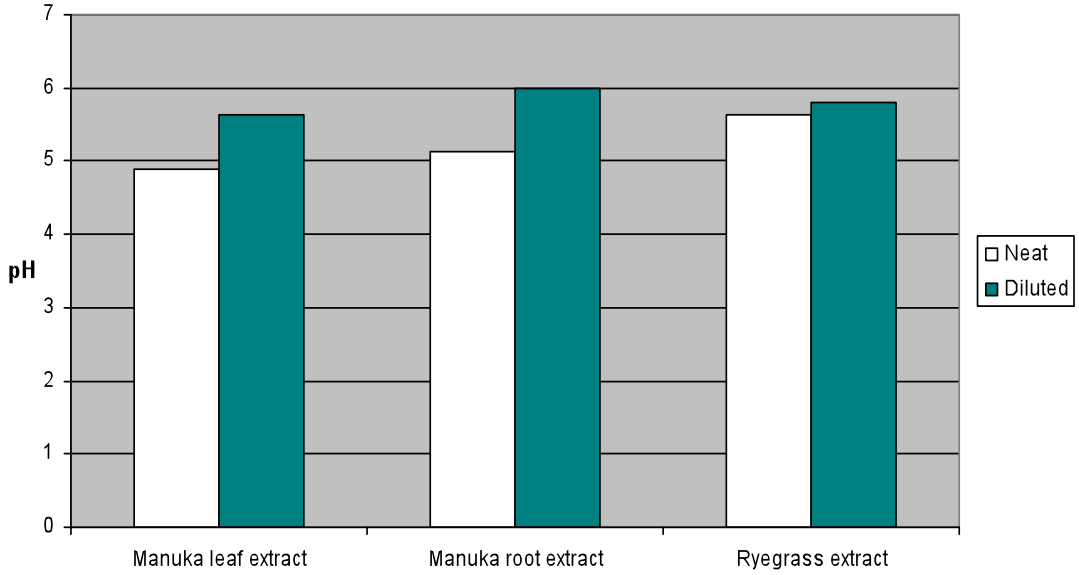


Figure 3.5. *pH of ryegrass, manuka leaf and manuka root extracts before and after dilution to a 1:9 ratio with DI water.*

3.4.3. Microplate assay

3.4.3.1. Salmonella typhimurium

The growth of *S. typhimurium* was significantly impacted by the presence of manuka and ryegrass extracts, the following is a bullet point outline of the key findings:

- A significant reduction in growth of *S. typhimurium* in the presence of manuka extracts was observed (Fig. 3.6).
- 50% and 20% inhibition of growth was observed at manuka extract concentrations of 27.9% and 6.2% respectively (Fig. 3.7).
- In contrast to the manuka extracts, ryegrass extracts gave a small but significant increase in growth (Fig 3.6).
- A significant positive correlation with increasing ryegrass extract concentration can be seen in Figure 3.8.

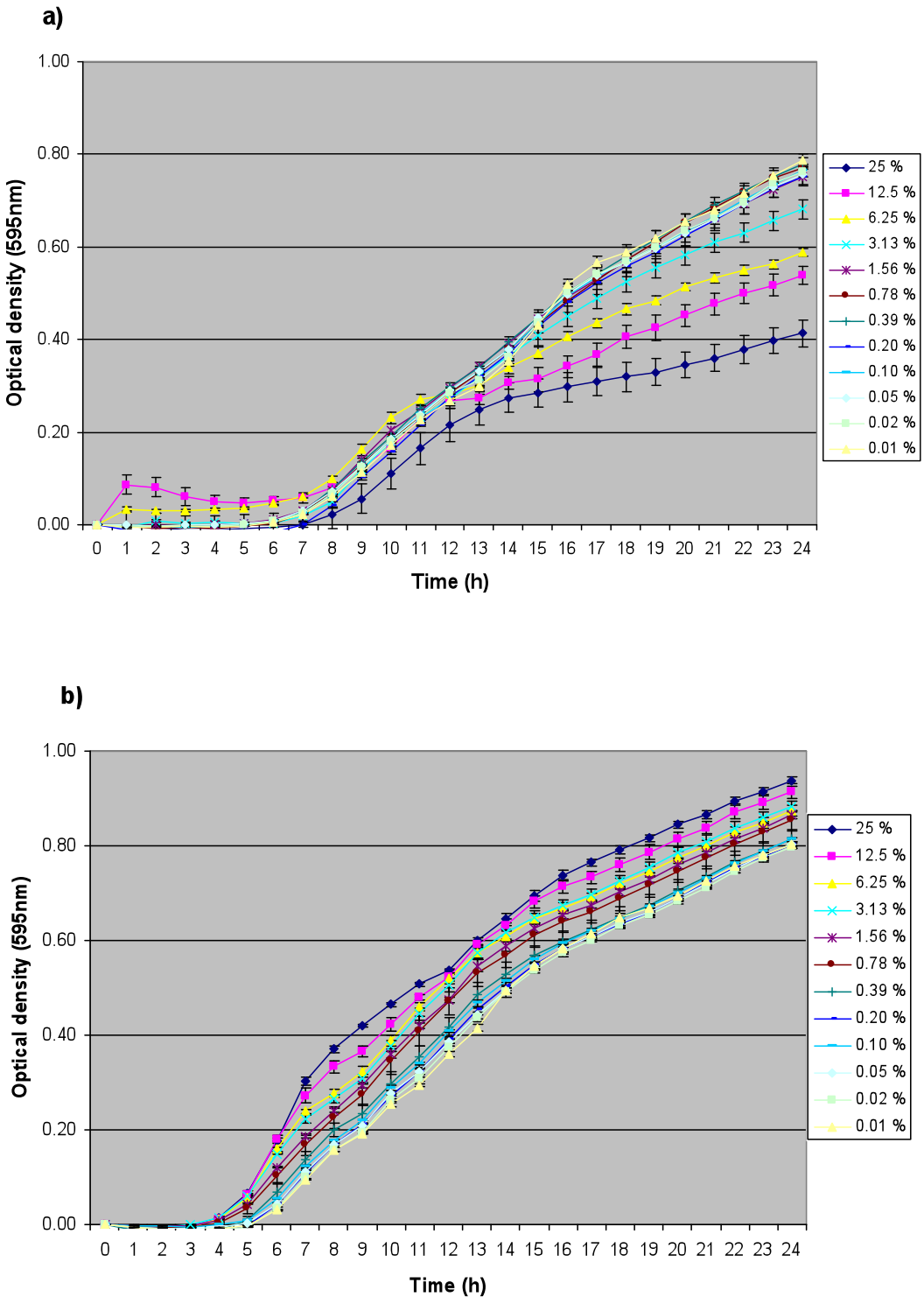


Figure 3.6. *Growth of S. typhimurium over 24 hours in the presence of a) manuka leaf extract and b) ryegrass extract, at twelve concentrations; dark blue lines represent the highest extract concentration and pale yellow the lowest. Error bars represent standard error (SE).*

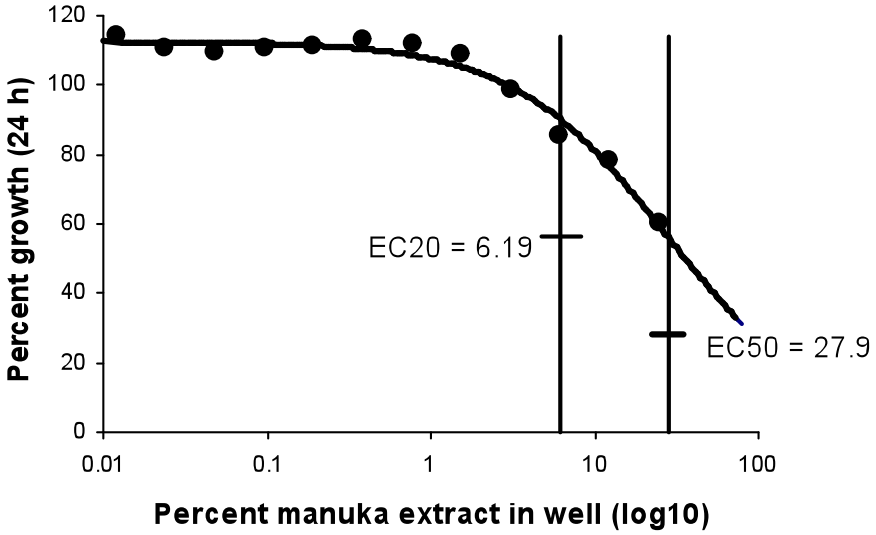


Figure 3.7. *The effect of increasing manuka extract concentration on growth of S.typhimurium after 24 hours of exposure at optimum growing conditions.*

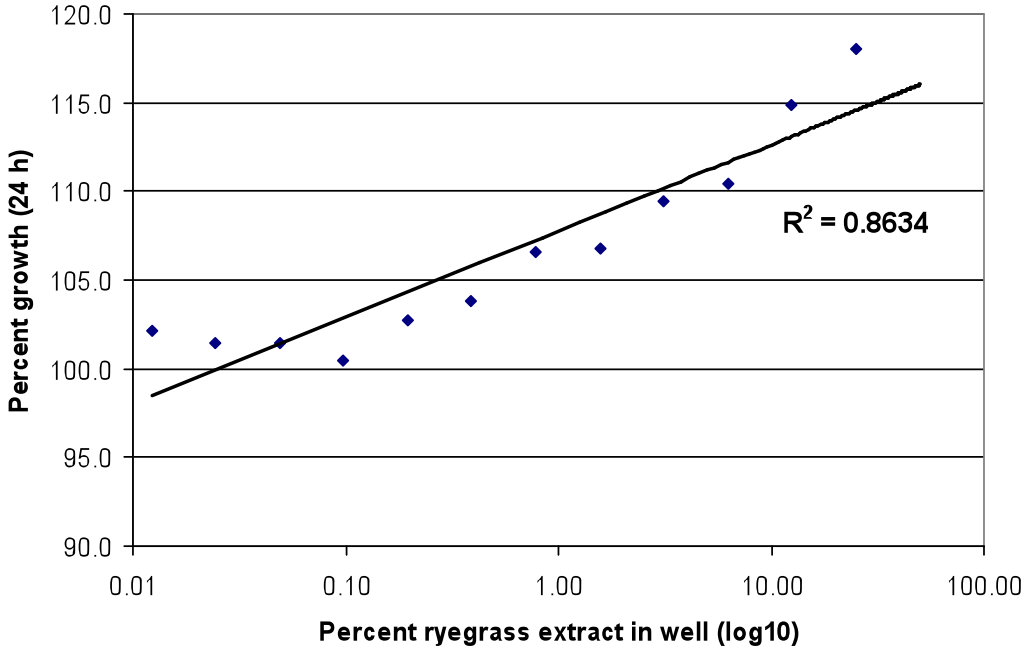


Figure 3.8. *The effect of increasing ryegrass extract concentration on growth of S.typhimurium after 24 hours of exposure at optimum growing conditions.*

3.4.3.2. Listeria monocytogenes

The growth of *L. monocytogenes* was significantly impacted by the presence of manuka and ryegrass extracts, the following is a bullet point outline of the key findings:

- A significant reduction in growth of L. monocytogenes in the presence of manuka extracts was observed, particularly at the highest extract concentration (Fig. 3.9.).
- 50% and 20% inhibition of growth was observed at manuka extract concentrations of 23.3% and 11.9% respectively (Fig. 3.10).
- In contrast to the manuka extracts, ryegrass extracts gave a significant increase in growth (Fig. 3.9).
- A significant positive correlation with increasing ryegrass extract concentration can be seen in Figure 3.11, with higher extract concentration producing as much eight times more growth than those without extract added.

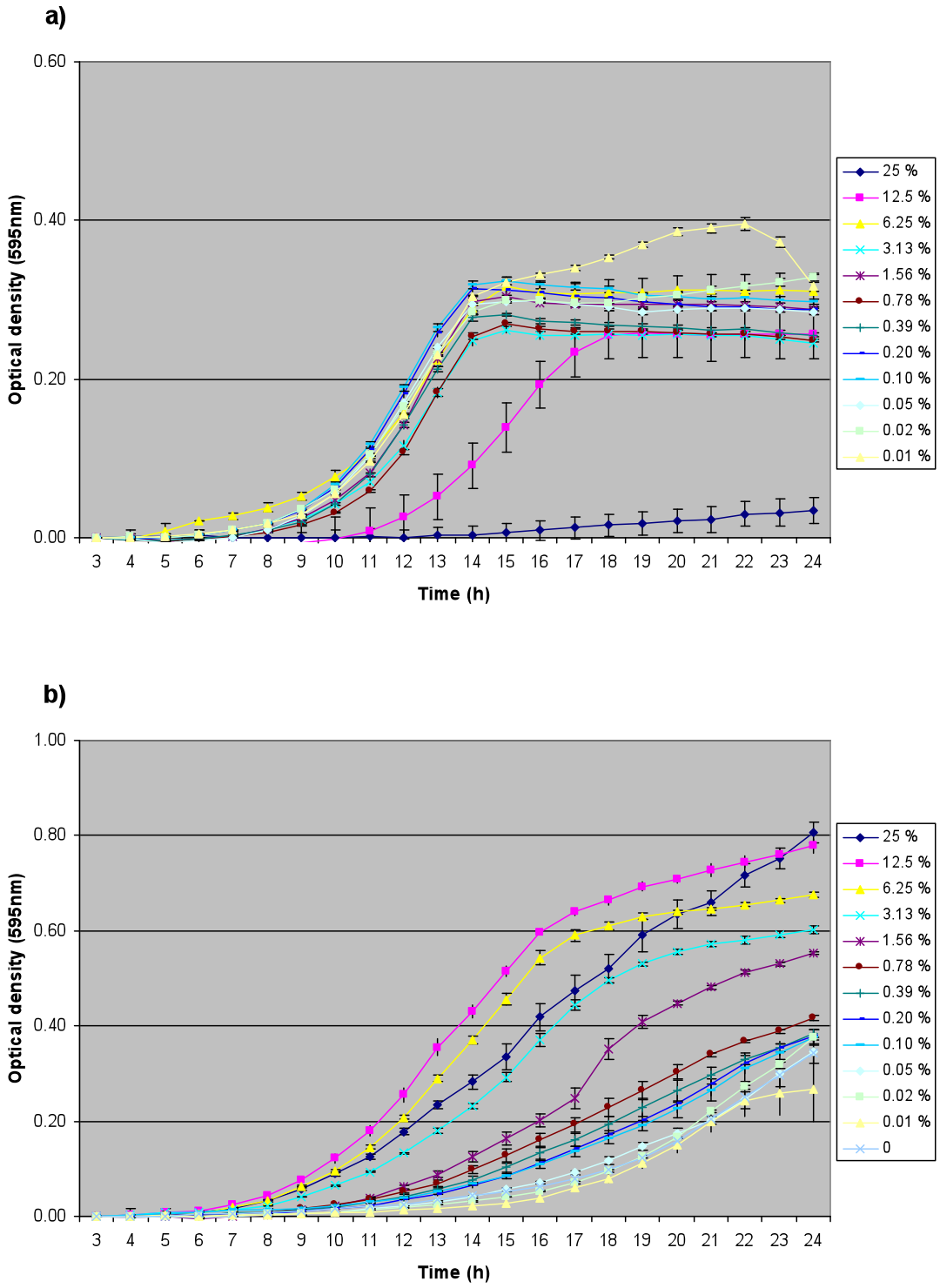


Figure 3.9. *Growth of L. monocytogenes over 24 hours in the presence of a) manuka leaf extract and b) ryegrass extract, at twelve concentrations; dark blue lines represent the highest extract concentration and pale yellow the lowest. Error bars represent standard error (SE).*

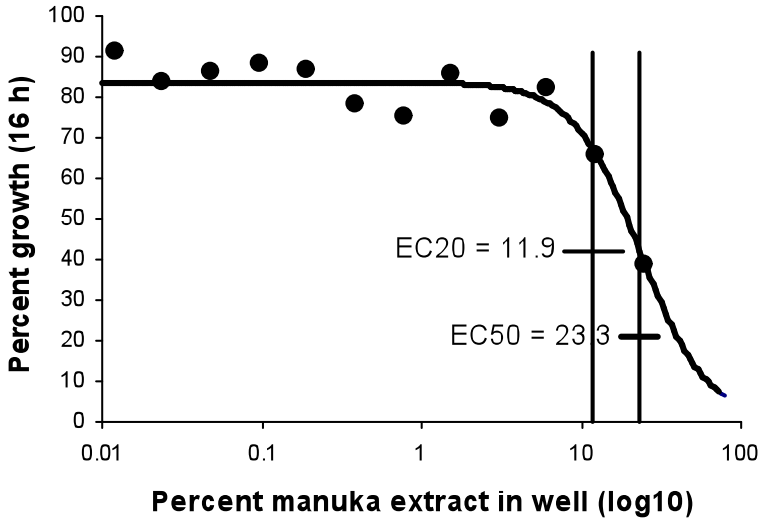


Figure 3.10. *The effect of increasing manuka extract concentration on growth of L. monocytogenes after 16 hours of exposure at optimum growing conditions.*

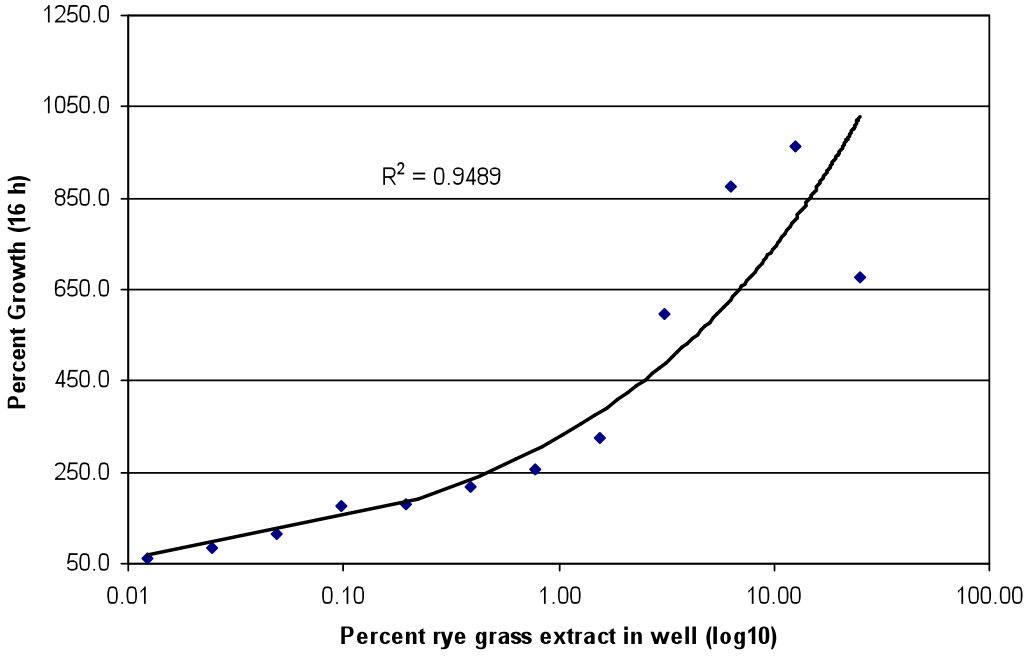


Figure 3.11. *The effect of increasing ryegrass extract concentration on growth of L. monocytogenes after 16 hours of exposure at optimum growing conditions.*

3.4.3.3. Escherichia coli 0157

The growth of *E. coli* was significantly impacted by the presence of manuka and ryegrass extracts, the following is a bullet point outline of the key findings:

- A significant reduction in growth of *E. coli* in the presence of manuka extracts was observed (Fig. 3.12). With 1-25% extract concentrations clustering closely together, similarly with extract concentrations below 1% (Fig. 3.12)
- 50% and 20% inhibition of growth was observed at extract concentrations of 27.8% and 1.06% respectively (Fig 3.13). The graph in Figure 3.13 shows wide error bars, however there is still a clear inhibition of growth.
- In contrast to the manuka extracts, ryegrass extracts gave a significant increase in growth (Fig. 3.12).
- A significant positive correlation with increasing ryegrass extract concentration can be seen in Figure 3.14.

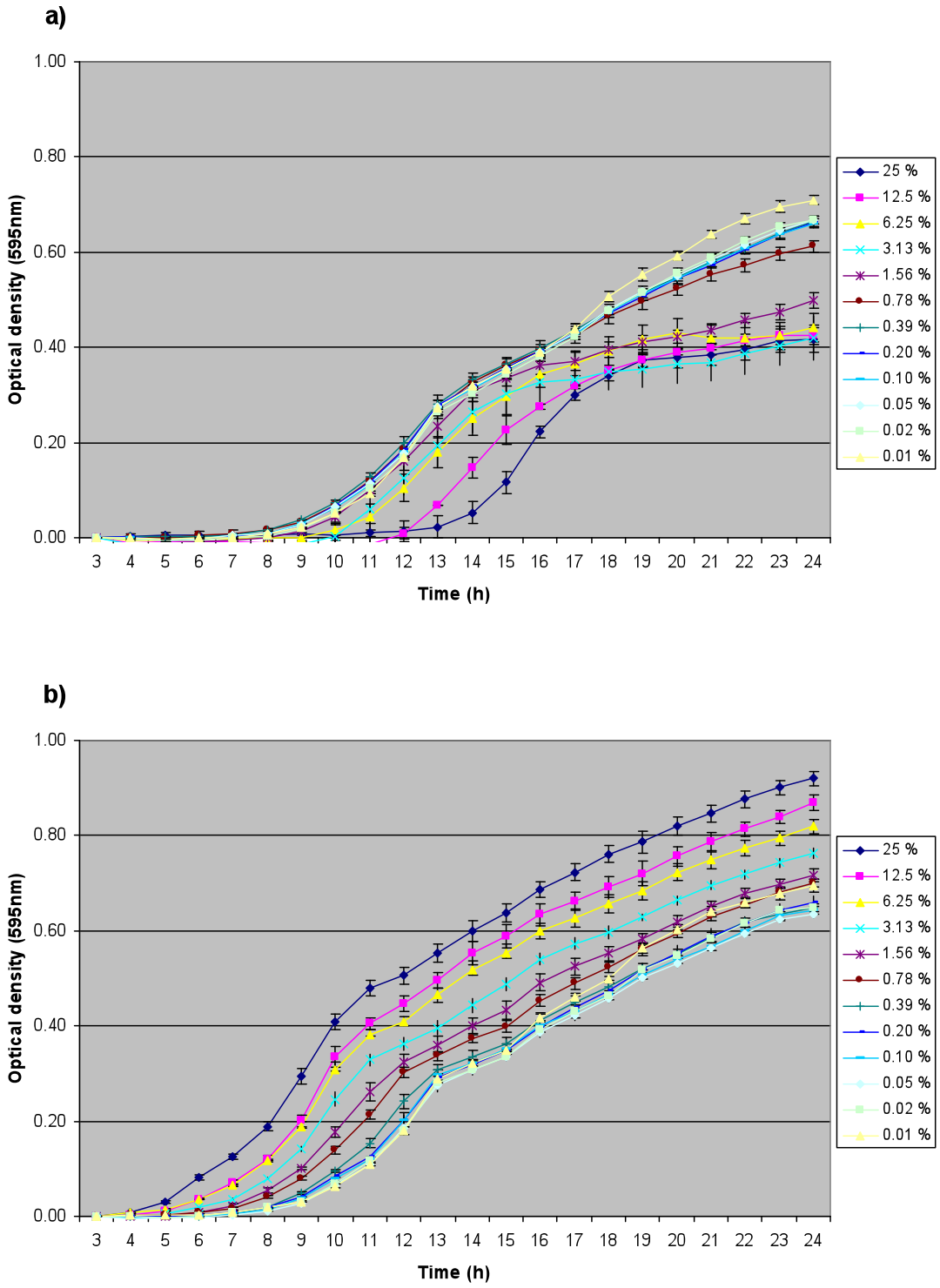


Figure 3.12. *Growth of E. coli 0157 over 24 hours in the presence of a) manuka leaf extract and b) ryegrass extract, at twelve concentrations; dark blue lines represent the highest extract concentration and pale yellow the lowest. Error bars represent standard error (SE).*

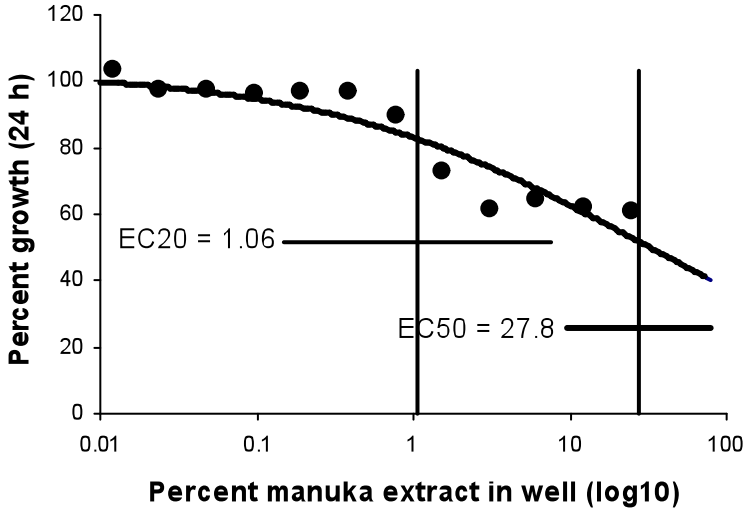


Figure 3.13. *The effect of increasing manuka extract concentration on growth of E. coli 0157 after 24 hours of exposure at optimum growing conditions.*

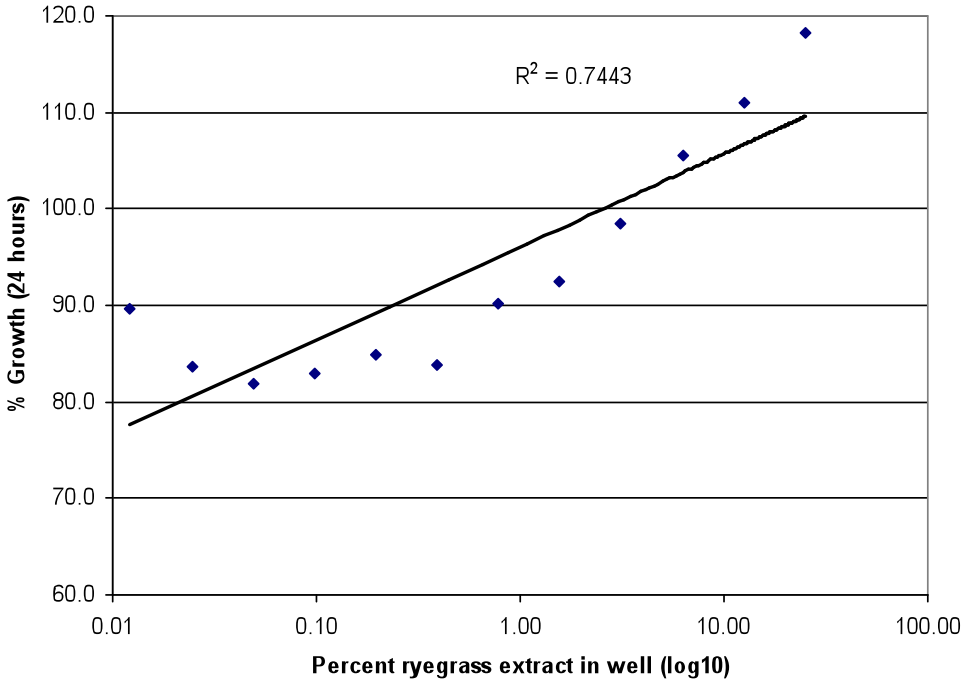


Figure 3.14. *The effect of increasing ryegrass extract concentration on growth of E. coli 0157 after 16 hours of exposure at optimum growing conditions.*

3.4.3.4. Campylobacter jejuni

A photographical representation of *C. jejuni* after growth with manuka extracts can be seen in Figure 3.15. The growth of *C. jejuni* was significantly impacted by the presence of both manuka and ryegrass extracts, the following is a bullet point outline of the key findings:

- A significant reduction in growth of *C. jejuni* in the presence of manuka extracts was observed. This inhibition was observed to be 100% from well concentrations of 0.78% and above.
- 50% and 20% inhibition of growth was observed at well extract concentrations of 0.597% and 0.541% respectively (Fig. 3.16). Judging by the size of the error bars in Figure 3.16, the statistical model used here does not appear to be appropriate for this data set, however, the curve does highlight the clear cut off in growth between extract concentrations of 0.39% to 0.78%.
- In contrast to the manuka extracts, ryegrass extracts gave a small but significant increase in growth (Fig. 3.17).

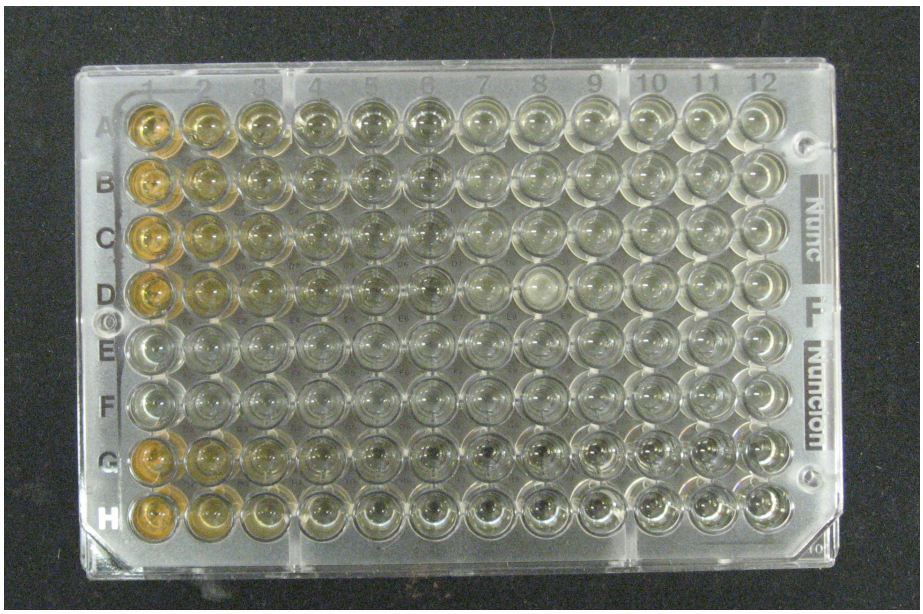


Figure 3.15. *Campylobacter jejuni after 48 hours in a 96 well plate with manuka leaf extracts at 12 concentrations. A reduction in turbidity can be seen rows one to six indicating a reduction in growth.*

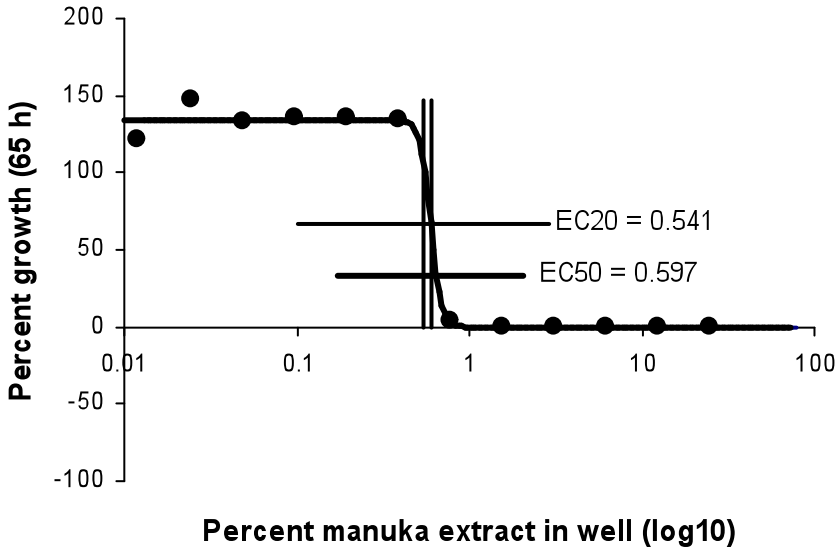


Figure 3.16. *The effect of increasing manuka extract concentration on growth of C. jejuni after 48 hours of exposure at optimum growing conditions.*

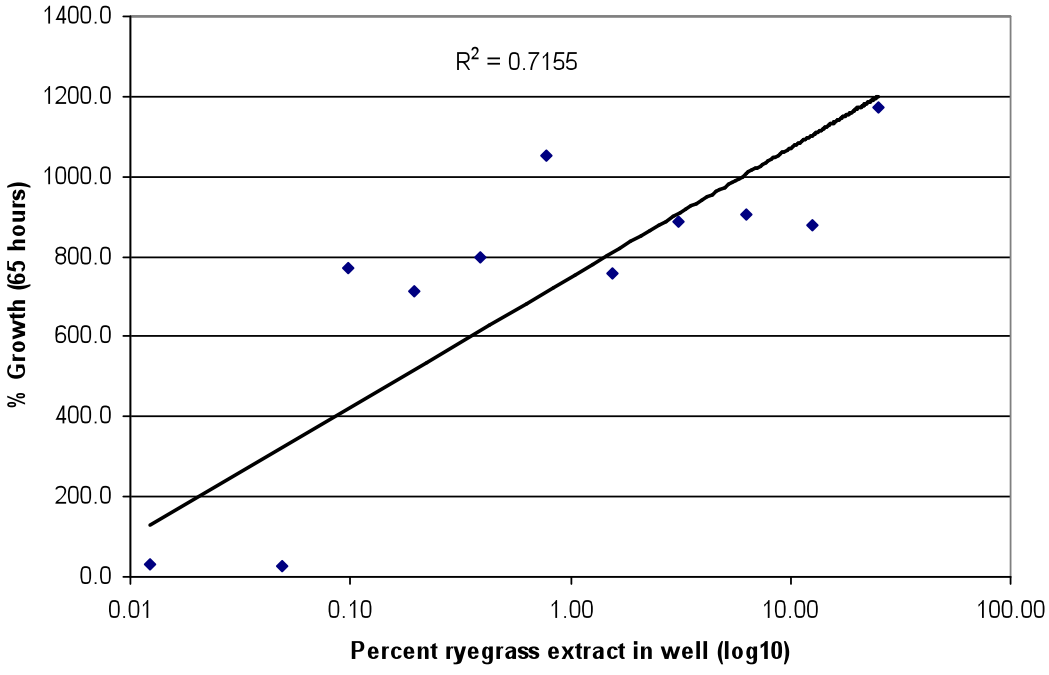


Figure 3.17. *The effect of increasing ryegrass extract concentration on growth of C. jejuni after 48 hours of exposure at optimum growing conditions.*

3.4.3.5. Clostridium perfringins

The growth of *C. perfringins* was significantly inhibited by the presence of both manuka and ryegrass extracts, the following is a bullet point outline of the key findings:

- A significant reduction in growth of *C. perfringins* in the presence of manuka extracts was observed.
- 50% and 20% inhibition of growth was observed at well extract concentrations of 0.070% and 0.019% respectively (Fig. 3.18). The curve in Fig. 3.18 illustrates the rapid decline in growth of *C. perfringins* in the presence of the manuka extract.
- Surprisingly, ryegrass extracts also gave a small but significant decrease in growth of *C. perfringins*, with an observed 50% and 20% inhibition of growth at well extract concentrations of 33.4% and 14.4% respectively (Fig. 3.19).

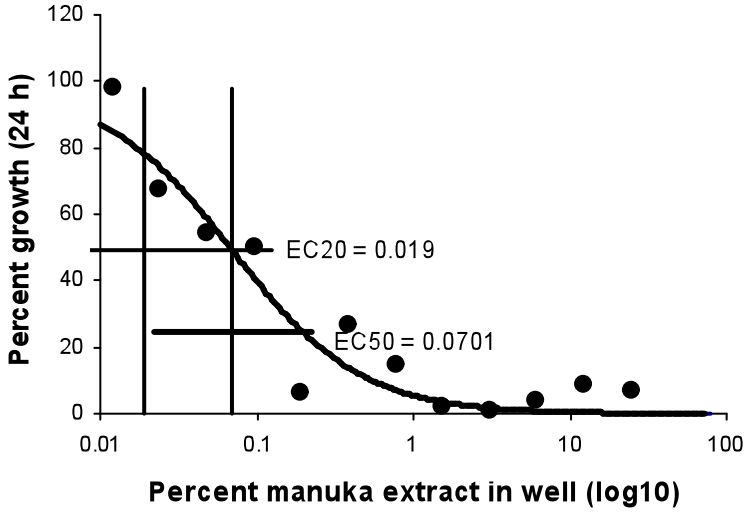


Figure 3.18. *The effect of increasing manuka extract concentration on growth of C. perfringins after 24 hours of exposure at optimum growing conditions.*

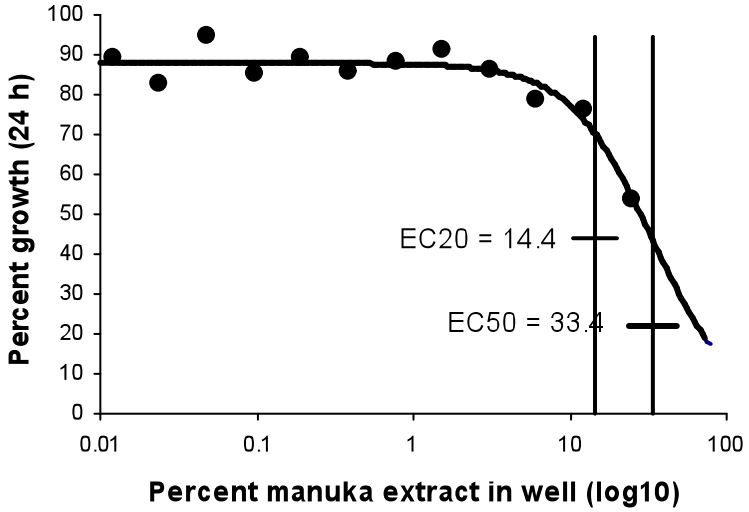


Figure 3.19. *The effect of increasing ryegrass extract concentration on growth of C.perfringins after 24 hours of exposure at optimum growing conditions.*

3.5. Discussion

The presence of manuka extracts on agar plates significantly reduced the numbers of bacterial colonies from sewage sludge dilutions (manuka leaf only) as well as *Salmonella* and *E.coli* pure cultures (leaf and root extracts). The presence of manuka leaf extract also significantly affected the growth rate and reduced overall growth of *S. typhimurium, E. coli* 0157, *C. perfringins, C. jejuni, L. monocytogenes,* with the most pronounced effects observed in *Clostridium* (EC_{50} = 0.070%) and *Campylobacter* (EC_{50}=0.597%). Pure manuka oil has been found to have activity against many pathological bacteria, including *Staphylococcus*, *Listeria* and *Enteroccus* species (2000; Douglas *et al.*, 2001), but predominantly against gram-positive strains (Christoph *et al.*, 2000; Maddocks-Jennings *et al.*, 2005). This explains the inhibition observed for both *Listeria* and *Clostridium* in the presence of our manuka extracts, however, it does not explain the inhibition in the other three pathogens, which are all gram-negative. Although some research has shown effects of manuka oil against gram-negative bacteria

(Christoph *et al.*, 2000) the inhibition observed towards C*ampylobacter*, *E. coli*, and *Salmonella* with our water extracts is extremely surprising. A survey of the literature provides no previous evidence for manuka products having such a strong impact against these bacterial strains. This is a significant finding and would indicate more research is required in this area. One explanation for this finding may be that because the extracts in the current experiment were made with water and were not lipid based, as most essential oils are, the outer membrane of gram-negative bacteria which restricts the diffusion of lipophilic/hydrophobic compounds (Burt, 2004) does not protect it from hydrophilic compounds as efficiently. This would not however explain the impact on gram-positive bacteria, which in previous experiments was thought to be due to the degradation of peptidoglycan in cell walls caused by the lipophilicity of manuka oil (Christoph *et al.*, 2000).

The experiments in this chapter are considered to be preliminary investigations. All five bacterial species assayed have shown significant reduction in growth, and in some cases complete inhibition of growth and significant toxicity, in the presence of water extracts of manuka. This can be attributed to components unique to the manuka extracts, highlighted by the observed increase in growth in the presence of rye grass extracts (in all but one), although it is understood that a wider range of plants would need to be tested to confirm this conclusion. This is a surprising result, as it has previously been suggested that the manuka component responsible for the antimicrobial activity of manuka oils (leptospermone) is not soluble in water (Weston, 2000). It is therefore uncertain what is present in the extracts causing the observed inhibition, and whether the components responsible would persist in soil once herbage decay is underway. However, it does indicate that there may be potential for manuka in rehabilitation of waste amended sites through the mitigation of growth and survival of pathogenic microorganisms. Although it has been noted that a more in-depth investigation needs to take place before this principle can be confirmed, the results do justify further investigation into its potential. Further investigations may include analysis of the manuka-water extracts to determine if the components previously described in the literature to be responsible for manuka antimicrobial activity are present, and larger scale experiments looking into how the presence of manuka affects the survival of bacterial pathogens in whole soil.

4. In-situ Effects of Manuka Growth on Survival of Bacterial Pathogens in Soil.

4.1. Introduction

Many manuka components have known antibacterial activity, and these have been discussed in depth in Chapters Two and Three. The results from the experiments described in Chapter Three demonstrated an inhibitory effect of manuka/water extracts on five pathogenic bacterial strains that are potentially found in biowastes (in particular biosolids). The results of this preliminary work warranted further investigations into whether inhibitory effects can also be observed within soil. As previously discussed, there is a growing need for an environmentally sound means of disposing of organic wastes such as biosolids, and it is becoming common to look to recycling them onto land to make use of their potential nutritional value (Cameron *et al.*, 1997; Warman, 2005; Alvarenga *et al.*, 2009; Maiaomiao *et al.*, 2009). The volume and quality of biosolids allowed to be applied to land in New Zealand is currently controlled by guidelines that also set criteria for pathogens (NZWWA, 2003; Horswell *et al.*, 2009). These guidelines specify that biosolids must be treated so that pathogen numbers are below detection limits (Grade A biosolids). Or alternatively, a time limit between application and public use may be put into place (exclusion period or period of restraint, grade B biosolids) (NZWWA, 2003; Horswell *et al.*, 2009). In the latter case, the inactivation of enteric pathogens in the soil matrix is an essential component of a multi-barrier approach for protecting human health (Cass, 2007). However, inactivation within the soil does not always occur, and studies have shown survival of many human pathogens for considerable lengths of time in soil (Yeager and Ward, 1981; Lemunier *et al.*, 2005; Eamens and Waldron, 2006; Horswell and Aislabie, 2006), and even apparent re-growth (or re-colonisation) of pathogenic bacteria in biosolids (Vasseur *et al.*, 1996; Zaleski *et al.*, 2005; Eamens and Waldron, 2006). Eamens *et al*. (2006) found that levels of spiked *E.coli*, *Salmonella* and *Clostridium* were still above background levels more than six months after addition, which is longer than the recommended restraint periods for some land uses (e.g. forestry) (NZWWA, 2003).

The rate of pathogen die-off during the period of constraint is affected by many factors including season, sunlight, soil temperature, soil moisture, soil type, pH and indigenous microbial population (through microbial competition and predation) (Carrington, 2001). The major disadvantage of determining and applying a period of constraint is that most of these factors cannot be controlled or predicted (Carrington, 2001). If the length of time between application and use could be shortened it may encourage land application of biosolids, thus allowing for the recycling of valuable nutrients. If the presence of manuka on soil amended with biowastes (in particular biosolids) was able to reduce pathogen numbers more rapidly than sites without manuka, it may be a useful tool to speed up the treatment process. In addition, surface application of biosolids can enhance pathogen die-off due to UV inactivation and increased moisture loss (Avery *et al.*, 2004; Eamens and Waldron, 2006), but this practise is not favoured due to the potential exposure of waste to humans. Incorporation is laborious and if the need for this step could be reduced it would remove another barrier preventing biosolids recycling. Previous work has already shown manuka responds well to biosolids amendment, exhibiting increased growth with increased biosolids application.

Manuka antibacterial agents may end up in the soil via a number of pathways. One potential pathway is rhizodeposition, which is the altering of rhizoshpere soil through the release of root exudates, mucilage and sloughed cells (Castaldi *et al.*, 2009a). Through the release of carbon sources/organic compounds (organic acids, amino acids) and protons, plant roots are able to alter soil pH, select for different bacterial populations and enzymatic activity (Cheng *et al.*, 2004; Yang *et al.*, 2007; Castaldi *et al.*, 2009a; Quartacci *et al.*, 2009). Studies have found that many root exudates remain stable in soils for significant lengths of time (Quartacci *et al.*, 2009). Cornes (2005) found that leptospermone (the component thought to be responsible for antimicrobial activity in manuka oil) produced by the roots of bottle brush suppressed weeds around the plant's base. Roots of manuka have not been investigated for leptospermone content, but it may be similar to that found in the bottle brush. If this is the case, leptospermone released by manuka roots may also inhibit bacterial populations in soil, including pathogenic organisms, and we would expect to see greater pathogen

inhibition in soil layers associated with root growth. A second potential pathway is leaf fall, which is very common in manuka all year. The soils underneath long-standing manuka forests have been exposed to leaf fall for considerable lengths of time. These leaves will eventually degrade in the soil, potentially releasing antibacterial agents. It has been shown that water extracts of manka leaves can inhibit growth of pathogenic bacterial species (Chapter Three), and it may be that these same antimicrobial agents are released when litter is decomposed. If this is the case, increased inhibition of pathogens would be expected in surface soil layers where leaf litter collects.

In the present study two pathogenic bacterial species have been chosen from those tested in Chapter Three; *Salmonella typhimurium* and *Campylobacter jejuni.* These were chosen based on the results in Chapter Three and the relevance of the species to New Zealand. *Salmonella typhimurium* is the most common cause of enteric infections in NZ [reported incidence rate was 32.2 cases per 100 000 persons in 2008] (ESR, 2008). New Zealand also has the highest rate of *Campylobacter* infection in the developed world (Horswell and Aislabie, 2006; ESR, 2008) and campylobacteriosis is the most commonly notified disease in New Zealand comprising 46% of all notifications [156.8 per 100 000 population in 2008](ESR, 2008). In addition, data shows that *Campylobacter* and *Salmonella* species are the leading causes of bacterial gastroenteritis worldwide (Donnison, 2003; Sindhu and Toze, 2009).

Soil biological (microbial biomass, soil microbial community structure), biochemical (*3.1.3.2; acid phosphatase*, and *3.1.6.1; Aryl- sulphate sulphohydrolase* activities) and chemical (pH, total carbon (TC) and total nitrogen (TN)) properties were also investigated along with pathogen survival to help ascertain what influence, if any, manuka has on soil biological processes. The enzymes phosphatase and sulphatase were chosen because of their well understood chemistry, abundance in soil, and the recognised importance of their functioning for soil health (Gianfreda and Bollag, 1996; Dick, 1997; Macdonald, 2006). Both enzymes are hydrolases, catalysing the hydrolysis of various bonds (Dixon, 1979; Mathews, 2000) by the addition of the H^+ and OH^- ions of water (Raven, 1999). Phosphatase enzymes hydrolyse *organic* ester-phosphorus (P) compounds in soils and are a key component in the P cycle (Malcolm, 1983; Doelman,

1989). Sulphatase enzymes hydrolase *organic* ester-sulphur (S) compounds in soil, releasing inorganic sulphate (Tabatabai, 1970; Speir and Ross, 1978).

4.2. Aim

The aim of the experimental work described in this chapter was to further investigate the antibacterial properties of manuka, focussing on potential in–situ soil effects. This was achieved by way of bacterial die-off experiments, comparing the rate of death of *Salmonella typhimurium-lux* and *Campylobacter jejuni* in soils collected from underneath a manuka stand with those from a control pasture site. Soil chemical and biochemical parameters will also be assessed to investigate potential impacts of plant species on these properties.

4.3. Methods

4.3.1. Soil collection

Soil samples were taken from two sites in close proximity, located in the eastern hills of Lower Hutt, New Zealand. One site had a known history of manuka presence for at least 50 years, and the other, with no previous manuka growth, was used as a control. Samples were randomly taken as 10 replicate cores (2 cm diameter) from underneath each of three manuka trees at depths of 0-5 cm, 5-10 cm, and 10-20 cm. Soil depths were taken separately to investigate whether antimicrobial activity of manuka is the result of root exudation or leaf-fall and subsequent degradation. Samples were then bulked into separate plastic bags to give a single sample for each depth. The same method was applied for collection of control samples except that cores were not from underneath growing trees but on grass-land. This generated six-bulked samples in total (3 manuka, 3 control). On returning to the laboratory soil samples were sieved to 4 mm, this was to remove large objects and homogenise the sample, without removing all leaf

and root material that may be relevant to the experiment. A sub sample of sieved soil was dried for chemical analysis and the remainder stored in the fridge (4°C) until use.

4.3.2. Chemical and biochemical analysis

Soil pH was measured immediately after collection. Soil samples were mixed with deionised water at a 1:2.5 ratio, stirred with a glass rod, and left to stand overnight before measurment using a glass pH meter probe (Thermo Orion perpHect LogR meter, model 310).

Soil phosphatase (*3.1.3.2, acid phosphatase*) and sulphatase (*3.1.6.1, Aryl- sulphate sulphohydrolase*) activities were measured using the p-nitrophenyl colorimetric method of Speir *et al*. (1984), based on the methods of Tabatabai and Bremner (1969, 1970). Exceptions were that each assay was run with 0.5 g of fresh soil and incubation times were 1 hour for phosphatase and 4 hours for sulphatase. Each soil sample was run in triplicate with one non-substrate control.

Air-dried, ground soil samples from all three depths were sent to Landcare Research, Palmerston North, for analysis of total carbon (TC) and total nitrogen (TN). This was determined by the use of a Leco furnace (Leco (Laboratory Equipment Corporation), St Joseph, Michigan, USA) and subsequent analysis by thermal conductivity for N and infrared detection for C.

4.3.3. Biological analysis

Sub-samples of fresh soil from all three depths were sent to Landcare Research, Hamilton, for analysis of microbial biomass carbon, determined according to the methods of Daly and Wainiqolo (1993) and Sparling (1994) adapted from that of Vance *et al*. (1987).

Analysis of soil microbial community structure can provide evidence to whether manuka has effected the microbial population within the soil profile. If there is evidence of a change in microbial community between the soil samples this may provide evidence that manuka can also effect the survival of introduced bacteria. Variations in

microbial community structure between the six soil samples was investigated by first extracting bulk soil microbial DNA, and subsequently analysing it using multiplex Terminal Restriction Fragment Length Polymorphism (T-RFLP) (Parkinson *et al*., *In Press*, see Appendix A1.6 for method details and further explanation). This work was carried out by Dr Rachel Parkinson at ESR, Porirua.

4.3.4. Pathogen Experiment

4.3.4.1. Experiment set up

Pure cultures of *Salmonella typhimurium-lux* (Horswell *et al.*, 2009) and *Campylobacter jejuni* were grown from freeze-dried stock (Appendix A1.1), in the appropriate growth media, and enumerated according to bacterial enumerating techniques described in Appendix A1.2.

For each of the six soils, 10 g of fresh soil was weighed into 15 x 100 mL sterile glass bottles, (total of 90 bottles), this was repeated for both bacteria strain (total of 180 bottles). Each 10 g sub-sample was subsequently spiked with the equivalent of 10^6 colony forming units (cfu) of *Salmonella typhimurium-lux* or *Campylobacter jejuni,* per g of wet soil. All of the bottles were incubated in the dark at 16º C (average summer soil temperature) with loosened caps to allow for aeration, for the duration of the experiment. During an initial test run, it was observed that very few *Campylobacter* survived past one week from spiking, but a majority of the *Salmonella* did survive. As a result, the decision was made to run the two bacterial strains as separate experiments instead of the initial idea of running them together. Pathogen enumeration was carried out on soil samples on days 1, 7, 14, 28 and 42 for *Salmonella* and on days 1, 2, 4 and 14 for *Campylobacter,* using the methods outlined below. Only four sampling dates were carried out for *Campylobacter* instead of the five initially planned. This was due to there being no culturable colonies remaining at the 14 day time point, and subsequently the remaining bottles of spiked soil were discarded.

4.3.4.2. Soil harvesting

On each sampling date, three bottles of each soil type were removed from the 16º C incubator for enumeration (total of 18 bottles). Each bottle was filled with 90 mls of ringers solution (Appendix A2.1) using a sterile measuring cylinder. Bottles were subsequently placed on a reciprocal shaker at 180 rpm for 15 minutes to disperse soil and suspend bacteria. Samples were diluted decimally by transferring 1 ml to 9 ml PBS solution (Appendix A2.3) until a six times serial dilution was achieved (10^{-6}). Serial dilutions were enumerated for bacteria according to the methods outlined in section 5.3.2.3.

4.3.4.3. Pathogen enumeration

Both methods of pathogen enumeration involve the use of an initial resuscitation step followed by a growth phase using selective media designed to eliminate background contamination (Appendix A1.3 and A1.4). The initial resuscitation phase is to allow for the recovery of cells that might have been sub-lethally damaged by physical changes in the environment due to incubation in soil. All six soils were enumerated for *Campylobacter* and *Salmonella* prior to the experiment and no cells of either were detected, removing the need for control samples throughout the experiment.

4.3.4.3.1. Campylobacter

After surveying the literature, an array of methods for enumerating *Campylobacter* in environmental samples were tested (Karmali *et al.*, 1986; Bolton *et al.*, 1988; Endtz *et al.*, 1991; Höller, 1991; UKWIR, 2000; Donnison, 2003). Of these, one method was deemed to be most suitable and effective for the current experiment. This was due to inefficiencies or associated costs of the other methods investigated. The method chosen was based on the most-probable-number (MPN) method of Donnison (2003). This method was, however, slightly modified in that Exeter broth was substituted with Boltons media (Fort Richard code#3162) and mCCDA selective supplement was substituted with C.A.T. selective supplement (Oxoid SR 174E). Boltons broth was chosen due to its observed effective performance, reduced cost and the ease with which it could be obtained compared to Exeter broth. Full media recipes from the supplier can be seen in appendix A2.2. The only difference between C.A.T

supplement and mCCDA is that C.A.T. contains the extra ingredient teicoplanin. The combination of cefoperazone, amphotericin and teicoplanin in C.A.T. supplement has previously been shown to give the greatest reduction in background and least diminution in *C. jejuni* numbers compared to three other inhibitory agents (UKWIR, 2000). All resuscitation and detection methods for *campylobacter* were carried out microaerophilically using anaerobic jars and Campygen sachets (Oxoid). A three-tube per row most probable number (MPN) format was used in this experiment, this is widely used in NZ and is more cost-effective than a five-tube MPN (Donnison, 2003).

4.3.4.3.2. Salmonella

Surviving *S. typhimurium-lux* in the soil samples were enumerated using a plate count method developed by the UK Water Industry Research (2000). This method is based on a membrane filtration technique using solidified tetrathionate broth (BD Difco™ 210430) as a resuscitation medium followed by Rambach agar as a growth medium. This method has the benefit of only two steps, and performance was found to be at least as effective as previous methods (UKWIR, 2000; Horswell *et al.*, 2009). Specific method details can be seen in Appendix A1.3. In the present study Rambach agar was substituted with CHROMagar™ Salmonella plates (Fort Richard code#1078), which are a newer version of Rambach and are reported by the supplier to be more effective. On testing the plates this was determined to be the case. All plates were conducted in duplicate and plate counts averaged. The lowest dilution that produced plate counts of between 20-200 cfu was used to estimate the total number of colonies in the soil samples.

4.3.5. Statistical analysis

Microsoft excel was used for the calculation of averages, standard deviations (SDs) and standard errors (SEs) for all of the soil chemical and biochemical data. Most probable numbers (MPN) for *Campylobacter* were calculated using prepared MPN tables based on the rate of dilution and the number of tubes used. For pathogen data, log averages and associated SDs were calculated from each set of three replicates for each sample time. Decimal reduction times or D-values (the number of days required to

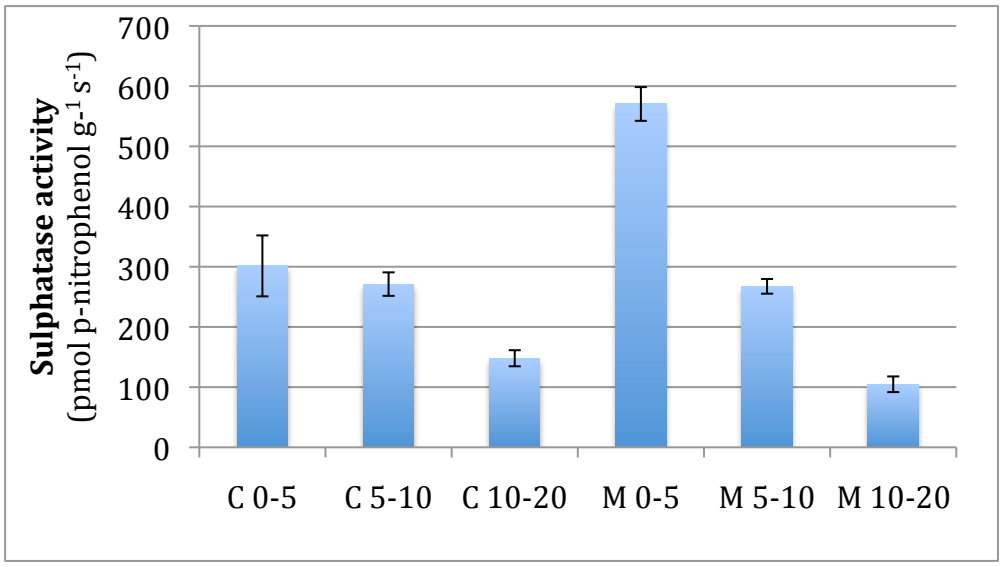
cause one $\log_{10}$ or a 90% reduction from the initial bacterial population) (Hutchison *et al.*, 2004; Horswell *et al.*, 2009) for pathogen die off were calculated from pathogen data over the four (*Campylobacter)* or five (*Salmonella*) time periods. After fitting curves to the data (exponential or power with an R^2 of between 0.87-0.99), D-values were determined by first calculating 90 % reduction of the original colony numbers, and using this number to solve curve equations. D-values, enzyme activities and microbial biomass were compared for statistical significance using two-way analysis of variance (ANOVA) in SPSS and two tailed t-tests in microsoft excel 2008.

4.4. Results

4.4.1. Chemical and biochemical analysis

Results from the analysis of soil moisture content, pH, TN, and TC can be seen in Table 4.1. The pH data shows that soil pH under manuka was generally lower than that found at the control site. Total carbon was most affected by depth, with the highest percentage of C in the surface layers for both manuka and control soils. The 0-5 cm manuka soil sample exhibited the highest percent of C, between 3-6% higher than the other five samples. As with TC, TN appears to be mostly affected by soil depth rather than plant type, with the greatest percent of N in the 0-5 cm depth for both manuka and control soils. Average enzyme activity can be seen in Table 4.2. Phosphatase and sulphatase activities exhibited a similar trend to soil chemistry, with greater activity in the surface layers (i.e. 0-5 cm > 5-10 cm > 10-20 cm) (Fig. 4.1) and a prominent depth effect in the manuka soils. Both enzymes exhibited less of a depth effect in the control soils compared to the manuka soils. Sulphatase activity was significantly greater in the manuka surface soil compared to all other samples ($p < 0.01$), whilst both 10-20 cm depth samples were significantly lower than the remaining four ($P<0.05$) (Table 4.2). There was an overall increase in phosphatase activity in the manuka soils compared to the control soils (Fig. 4.1). This difference was shown to be significant in the top two layers ($p < 0.01$) (Table 5.3).

a)



b)

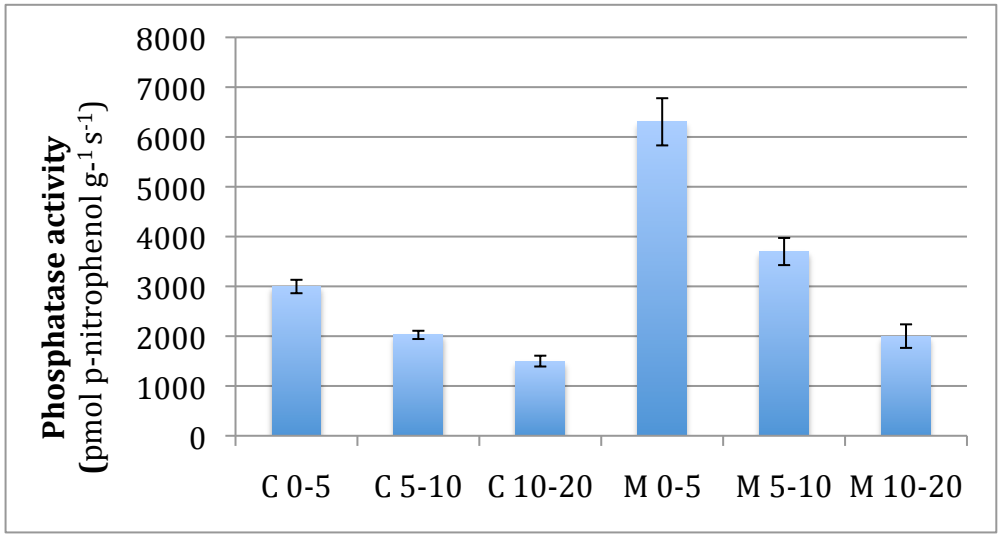


Figure 4.1 *Average soil enzyme activity for a) Sulphatase and b) Phosphatase in six soils. Error bars indicate SE.*

Table 4.1 *Soil chemical and biochemical properties.*

Sample	Moisture factor	pH	TC (%)	TN (%)
Control 0-5	1.46	5.0	4.6	0.4
Control 5-10	1.29	4.8	3.0	0.3
Control 10-20	1.25	4.9	2.4	0.2
Manuka 0-5	1.54	4.4	7.9	0.5
Manuka 5-10	1.32	4.5	4.0	0.3
Manuka 10-20	1.27	4.7	1.6	0.1

Table 4.2 *Soil phosphatase, sulphatase and microbial biomass activities. Numbers represent arithmetic means ± SE. Arithmetic means followed by the same letter in a column are not significantly different.*

Sample	Phosphatase (pmol p-nitrophenol g^{-1} s^{-1})	Sulphatase (pmol p-nitrophenol g^{-1} s^{-1})	Microbial Biomass C (ug C g dry $soil^{-1}$)
Control 0-5	2997 ± 135^{ad}	301 ± 51^{a}	160 ± 22^{a}
Control 5-10	2026 ± 83^{b}	271 ± 20^{a}	140 ± 3^{ab}
Control 10-20	1499 ± 109^{b}	148 ± 13^{b}	125 ± 2^{ac}
Manuka 0-5	6304 ± 474^{c}	570 ± 28^{c}	334 ± 11^{d}
Manuka 5-10	3700 ± 273^{d}	267 ± 12^{a}	200 ± 7^{ae}
Manuka 10-20	2001 ± 237^{ab}	105 ± 13^{b}	100 ± 3^{af}

4.4.2. Biological analysis

4.4.2.1. Microbial biomass

Microbial biomass C data showed a similar trend to that observed with enzyme activity, and TC (Fig. 4.2). Manuka soil samples in the two top soil layers had the highest microbial biomass, with the 0-5 cm sample having significantly higher microbial biomass C than the remaining five ($P < 0.05$, Table 4.2.). In contrast, each of the three control samples were not significantly different from each other ($P < 0.05$) and had considerably lower microbial biomass than the top two manuka soil layers.

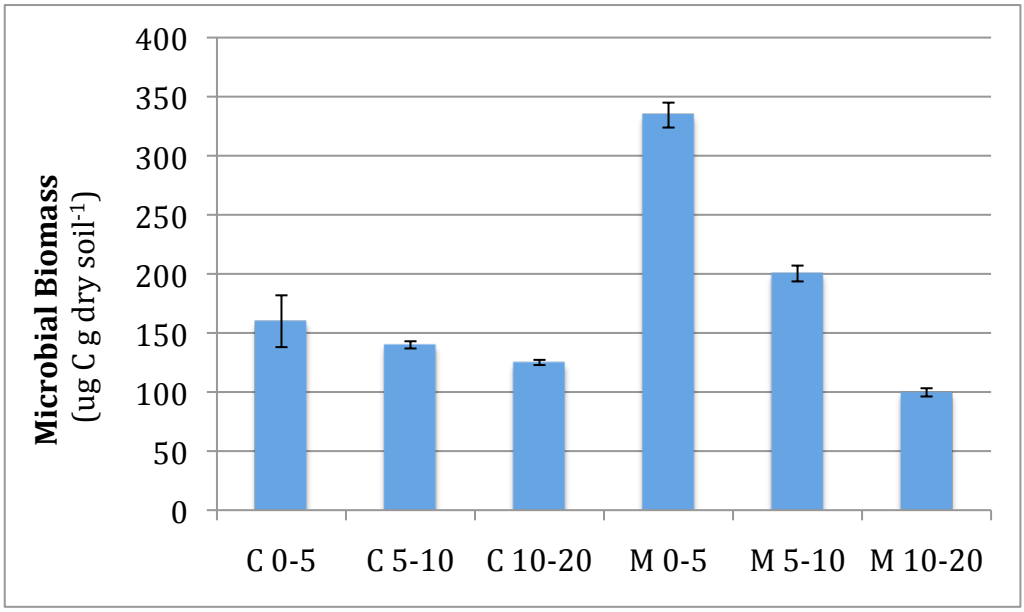


Figure 4.2. *Microbial Biomass carbon by fumigation extraction of six soil samples taken at varying depths from underneath a manuka stand and a nearby control site.*

4.4.2.2. Soil microbial community

Analysis of microbial community structure using multiplex T-RFLP analysis of soil DNA showed that the microbial community was highly different between control and manuka soils, independent of soil depth (Fig. 4.3). This can be seen diagrammatically in Figure 4.3 by the clear grouping of control soils versus manuka soils when all microbial groups were examined. This same trend was most clearly observed within the fungal community when surveyed independently from the other microbial groups (Fig. 4.4). On analysis of the raw data for fungi, a greater number of total peaks were observed in the manuka soil group, suggesting greater fungal diversity in the presence of manuka. A peak in T-RFLP data represents a particular length fragment of DNA, a greater range of fragments in a sample is said to represent a more diverse microbial community (Parkinson, 2004). The bacterial community exhibited a similar effect in the presence of manuka, except that the 10-20 cm depth soil under manuka clustered closer to the control soils whilst the 0-5 and 5-10 cm depths clustered separately.

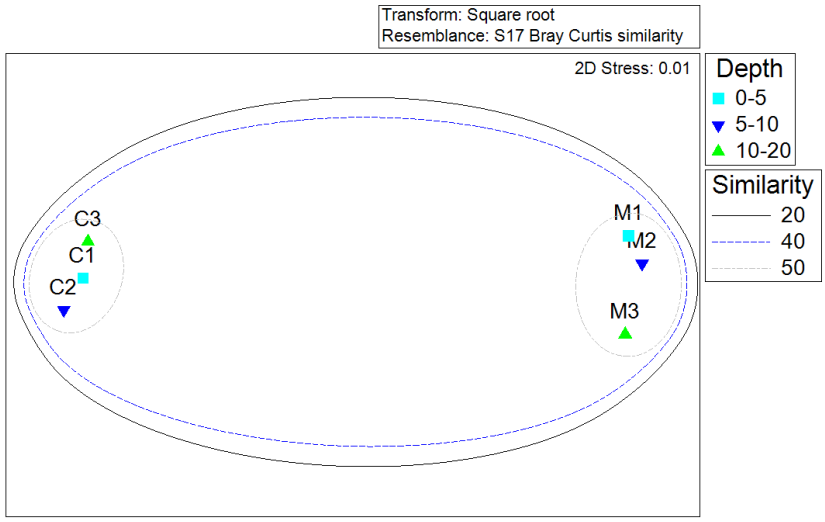


Figure 4.3 *MDS plot of T-RFLP soil profiles showing the entire microbial community (bacteria, fungi, archaea and rhizobia). Samples clustered close together are considered more similar than those further apart.*

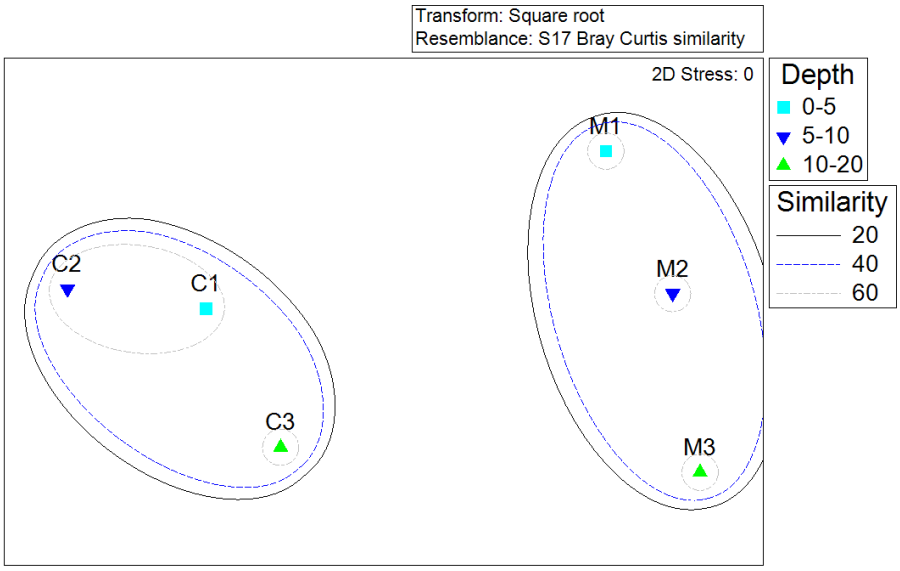


Figure 4.4 *MDS plot of T-RFLP soil profiles for fungi. Samples clustered close together are considered more similar than those further apart.*

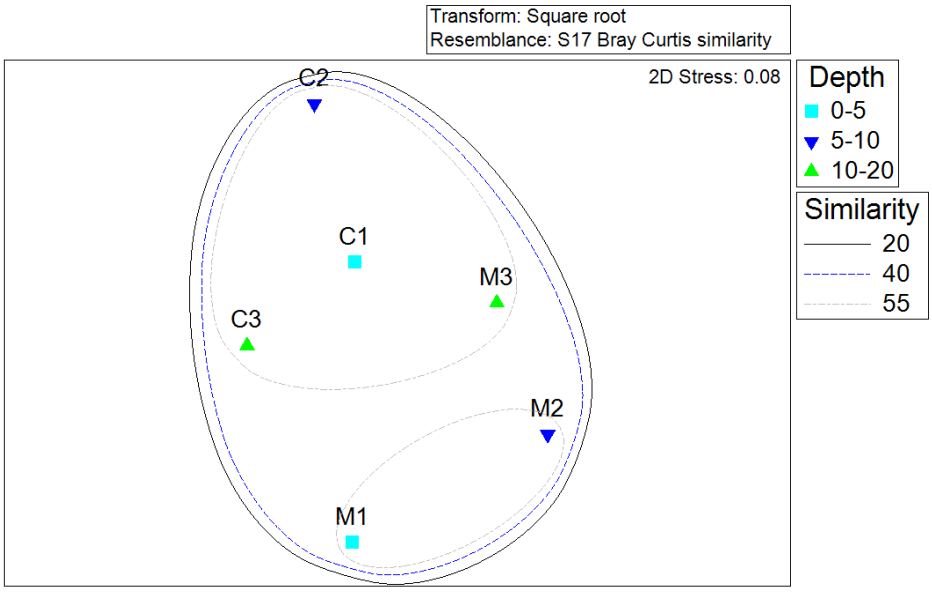


Figure 4.5 *MDS plot of T-RFLP soil profiles for bacteria. Samples clustered close together are considered more similar than those further apart.*

4.4.3. Pathogen enumeration

4.4.3.1. Campylobacter

Overall, survival of *Campylobacter jejuni* in the six soil samples was poor. The total number of viable *Campylobacter* rapidly declined from Day One to Day 14, with no colonies present at Day 14 for all samples. Figure 4.6 indicates that the die-off of *Campylobacter* was fastest in the control 0-5 cm soil sample, whilst survival was the same in the remaining five soil samples. Decimal reduction times (D-values) were calculated from the MPN of *Campylobacter* colonies over four time points. After analysis of R^2 values, power series curves were fit to the decay data and 90 % reduction times calculated from the resulting curve equations. The D-values for *Campylobacter* can be seen in Table 4.3 and Fig. 4.7. A larger D-value indicates survival of bacteria was greater whilst a smaller D-value indicates more rapid die-off. For both control and manuka soils, the 10-20 cm soil samples yielded larger D-values for *Campylobacter* (Fig 4.7), indicating these soils facilitated greater pathogen survival. The control 10-20 cm soil exhibited a significantly greater D-value than Control 0-5 cm ($P < 0.05$), however, there were no significant differences between the remaining D values of the soils for *Campylobacter* (Table 4.3).

Table 4.3 *Length of time (days) required to achieve a 90% reduction (1-log decrease) in inoculated bacterium (D-value) in six soil samples. Numbers represent arithmetic means ± SE. Arithmetic means followed by the same letter in a column are not significantly different.*

	Salmonella (Days for 90% reduction)	***Campylobacter*** (Days for 90% reduction)
C 0-5	2.1 ± 0.86^{a}	1.5 ± 0.09^{a}
C 5-10	4.8 ± 0.87^{b}	1.5 ± 0.14^{ab}
C 10-20	5.9 ± 0.96^{b}	1.8 ± 0.04^{b}
M 0-5	4.6 ± 0.82^{b}	1.4 ± 0.06^{a}
M 5-10	6.9 ± 0.93^{b}	1.6 ± 0.04^{a}
M 10-20	10.7 ± 1.0^{c}	1.7 ± 0.14^{ab}

4.4.3.2. Salmonella

The die-off of *Salmonella typhimurium* was at a steady rate for all soils, with some colonies still present at the end of the experiment (day 42) (Fig. 4.8). In Figure 4.8 it can be seen that the six soil samples behave in a similar manner with regards to *Salmonella* die-off. However, the manuka soils had more total cfu at each time point than did the control soils. D-values were calculated from the decline of *Salmonella* over five time points. After plotting data on XY scatter plots, either power series or exponential curves were fitted to decay data, depending on which had the best R^2 (R^2 values ≥ 0.85, example in Fig. 4.10). Ninety percent reduction times were subsequently calculated from curve equations. D-values for *Salmonella* can be seen in Figure 4.9. Data in table 4.3 shows that soil depth significantly affects D-values for both manuka and control soils. The most prominent effect is observed in the manuka 10-20 cm depth, this soil sample exhibited a significantly greater D-value than all the other soils (Table 4.3), suggesting something in this sample has the affect of enhancing *Salmonella* survival. Additionally, control 0-5 cm has a significantly lower D-value than all other soils ($P < 0.05$). Overall, manuka appears to have a higher D-value than the respective depth in the control sample (manuka vs. grass-land), this difference was significant in the 0-5 cm and 10-20 cm samples, but not the 5-10 cm sample.

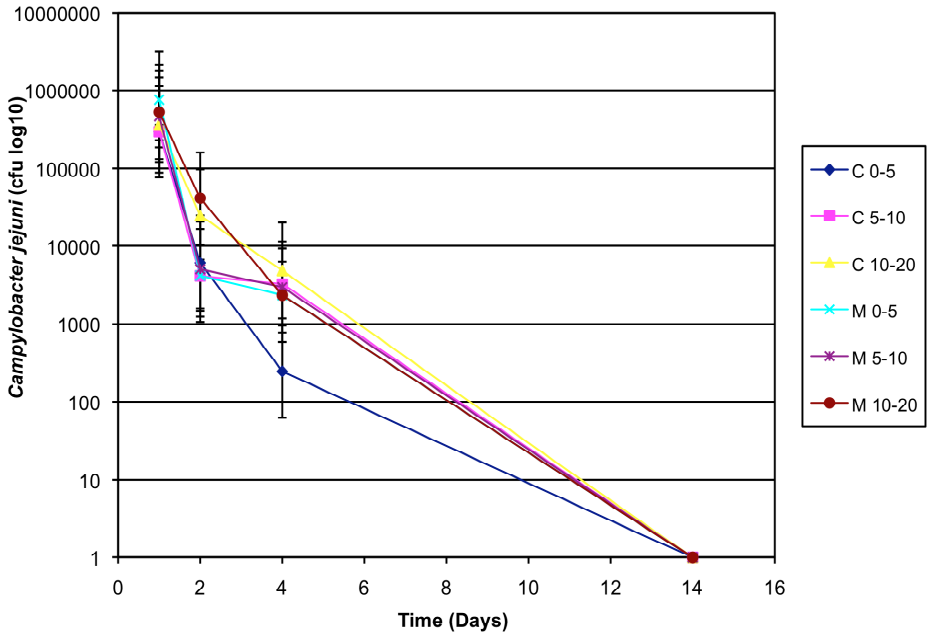


Figure 4.6 *Decline of inoculated Campylobacter jejuni in six soil samples over 14 days. Each data point is the log average of three independent reps. Error bars indicate 95% confidence intervals. A value of 1 cfu g soil^{-1} was given to samples in which no bacteria were detected to enable the plotting of log data.*

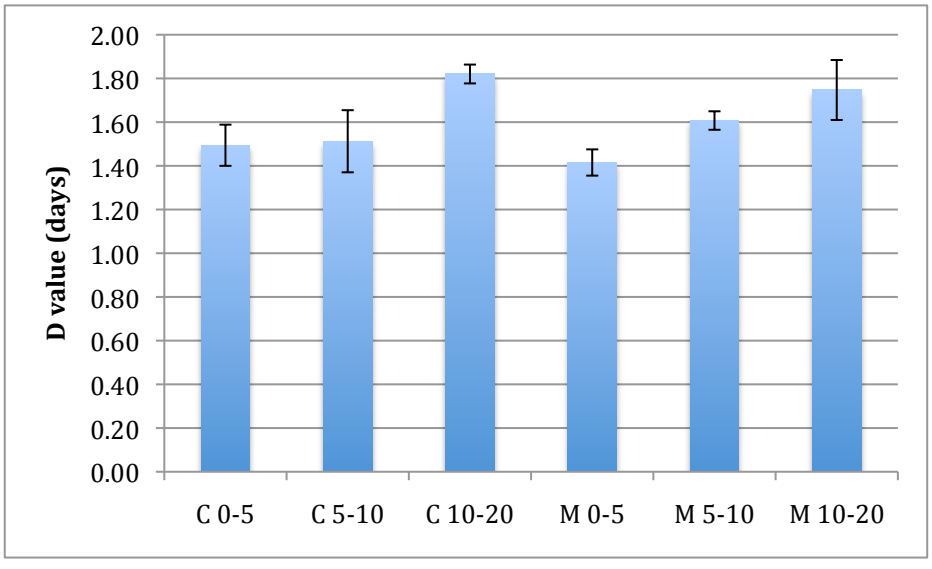


Figure 4.7 *Length of time (days) required to achieve a 90% reduction in Campylobacter (D-value) for the six soils. Error bars represent SE.*

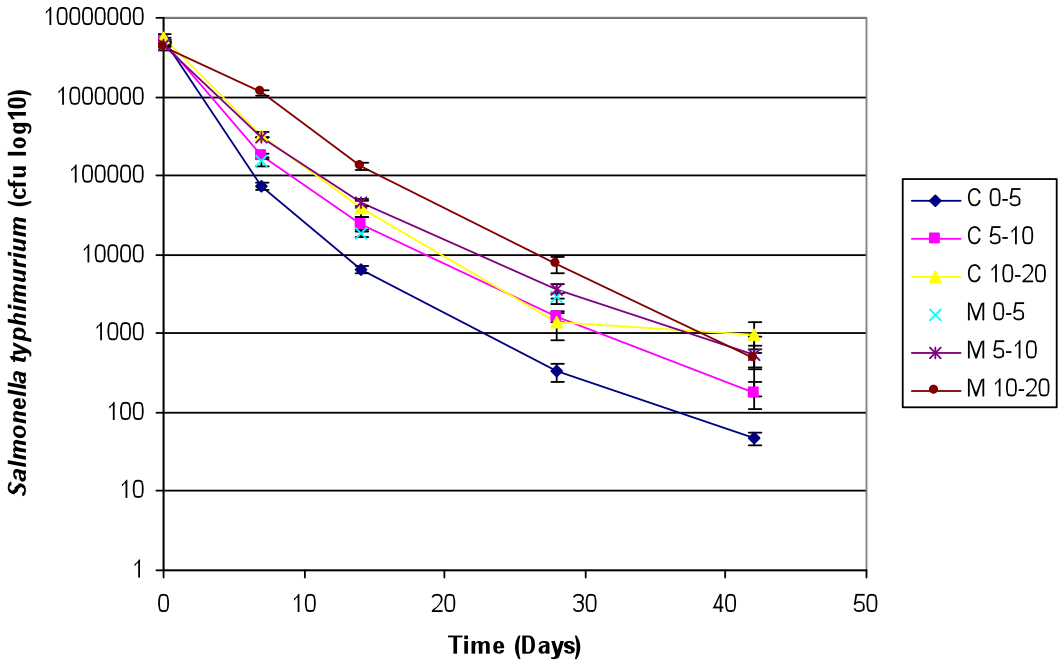


Figure 4.8 *Decline of inoculated Salmonella typhimurium in six soil samples over 42 days. Each data point is the log average of three independent reps. Error bars indicate log SE.*

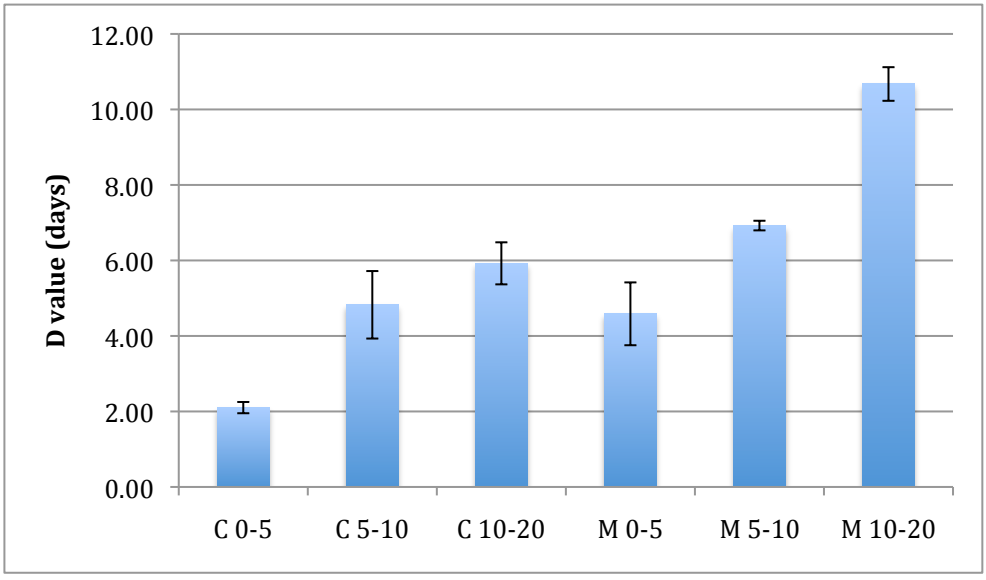


Figure 4.9 *Length of time (days) required to achieve a 90% reduction in Salmonella (D-value) for the six soils. Error bars represent SE.*

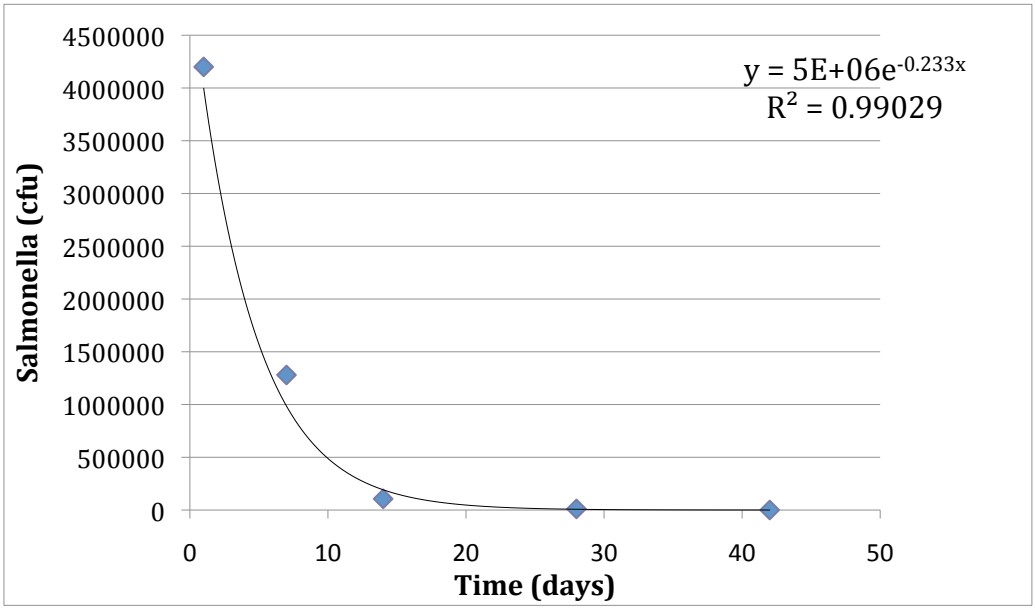


Figure 4.10 *Die off of Salmonella in soil from underneath manuka 10-20 cm depth over 42 days. Line represents one of three replicate curves.*

4.5. Discussion

Results of the current experiments demonstrated no obvious effect of manuka antimicrobial ability on pathogenic bacteria from biosolids in soil taken from underneath a long-standing manuka forest. There were few significant differences between the soils from under manuka and those from the control site in the die-off rate (D-value) or survival time of the pathogenic strains of *Campylobacter* and *Salmonella* tested. The exception was that spiked *Salmonella* had a significantly longer decimal reduction time (D-value) in soil from underneath manuka at the depth of 10-20 cm than it did in all other samples. Overall, when comparing relative soil depths between treatments, manuka appeared to increase the survival-rate of s*almonella* (up to two-fold), and this was significant at the 0-5 and 10-20 cm soil depths (Fig. 4.9). This trend was the reverse from what was expected. It may be that the components that give rise to

antimicrobial activity in manuka are quickly degraded in soil, by other organisms or enzymes, and thus storage in the fridge (four weeks) before the experiment took place has reduced any activity that may have initially been present. Additionally, manuka plants may release antimicrobial agents from roots continuously, which do not remain stable in soil. Future research using experiments with live plants in pots may be useful in investigating this further. It should also be noted that the mechanism of plant extraction used in Chapter Three, as well as those used in commercial production of manuka products, may mechanically or thermally degrade plant cells in such a way that the antimicrobial agents are released, and that this process does not occur through natural degradation (decomposition).

The data show that overall pathogen survival was more related to soil depth than treatment (plant type). This trend was the same for soil pH, moisture content, total nitrogen (TN) and total carbon (TC), as well as sulphatase and phosphatase activities, which showed significant depth effects. Soil C, N, phosphatase and sulphatase activities increased closer to the surface, as did soil moisture content. However, pathogen survival was decreased in these more productive nutrient rich surface soil layers. Research has shown that increased soil moisture enhances pathogen survival (Carrington, 2001), contradicting what was seen here. It may be that increased TC, TN and moisture content in the surface layers has encouraged greater microbial activity (also reflected in the enzyme activities and microbial biomass data). If this is the case, it is likely that inoculated pathogen die-off is enhanced due to the greater native bacterial population in these upper layers, through predation or competition. Studies have found that where there is an increased or more active indigenous microbial population in soil the survival of introduced pathogens is reduced (Pepper, 2006). In addition, it has been noted that increased clay content of soil can increase the survival rate of pathogens, and introduced bacteria (England *et al.*, 1993; Pepper, 2006), due to enhanced protection from predation through increased protective pore space (England *et al.*, 1993). This may further explain why D-values of both *Salmonella* and *Campylobacter* were greater in the 10-20 cm soil layers. On visual inspection the soil samples appeared to have an increased relative abundance of clay with depth down the soil profile.

Soil pH close to neutral has been related to enhanced pathogen survival (Pepper, 2006), however, this was not considered to be a main controlling factor in the current study. The control soil samples exhibited a higher pH (closer to neutral) when compared to the relative manuka soil depth. This was not reflected in the pathogen survival as the more acidic manuka soils exhibited larger D-value (greater survival) for *Salmonella* than the control soils. There was a marked decrease in soil pH in the 0-5 cm and 5-10 cm soil sample under manuka (pH 4.4 and 4.5 respectively). Therefore, unlike the other soil chemical and biochemical factors, soil pH does appear to be influenced by plant type, most likely through rhizodeposition (in 5-10 cm depths) and leaf fall (in surface soil). The observed decrease in soil pH in the manuka soils is in agreement with results in Chapter Three, which demonstrated water extracts of manuka leaves were more acidic than water extracts of ryegrass or manuka roots.

Overall enzyme activity increased in the surface soil layers for both sites, with soil collected under manuka at 0-5 cm and 5-10 cm exhibiting the highest phosphatase and sulphatase activity. This differential activity is highlighted in the microbial biomass C and T-RFLP data, which showed soil under manuka at 0-5 cm and 5-10 cm to be different from the other four samples. Soil phosphatase and sulphatase activities were greater in the manuka soils compared to the same depths for the control soils, which was in agreement with the microbial biomass data. Enzyme activity exhibited a similar trend to soil TN and TC which was to be expected. It has been said that the microbial community of a soil can be reflected in the level of the soil's enzymatic activity, and that changes in enzyme activity can be attributed to modifications in the microbial community structure (Speir and Ross, 1978; Kandeler, 1996). It appears that the total bacterial abundance is related to soil depth (seen in enzymes and biomass C) in the current experiment. However, when taking into account the T-RFLP data, there is a clear difference between control and manuka soil, unrelated to soil depth. In addition, there was a greater total fungal diversity under manuka. This would imply that the observed difference in community structure of fungi and bacteria is somewhat related to plant type and not just soil chemical and biochemical factors. Manuka root exudates may be selecting for specific microbial groups over others influencing the indigenous microbial population.

The current experiments found no effect of antimicrobial agents in soil from underneath a manuka stand on the pathogenic bacteria investigated. Observed die-off of pathogenic bacteria over time was equivalent for both plantings. It may be that due to the increased productivity (and soil biological activity) through increased C, N and moisture in the soil, potential inhibitory effects were masked. In the present study, the decision to leave biosolids out of the experiment was made based on the assumption that its presence could confound the effect of soil on pathogen die-off by masking inhibitory responses due to the added nutrients and microbial biomass. Our results suggest that may have been the case. However, in a study by Horswell *et al*. (2009) it was noted that the presence of biosolids had the effect of enhancing survival of *Salmonella*. In our experiment, *Salmonella* colonies were still viable six weeks after spiking. If survival rates of *Salmonella* are higher in the presence of biosolids or other organic waste, survival may have been enhanced further had it been included. Therefore, introducing biosolids should be a consideration for future research. Lemunier *et al*. (2005) found that *Salmonella serovar enteritidis* survived up to 3 months in composts made from manure that had been applied to land. Hutchinson et al. (2004) calculated decimal reduction times of between 0.76 and 5.2 for *Salmonella* inoculate in livestock waste that was applied to land. These rates are lower to within range of those exhibited in the current experiment, which were between 2.1 and 10.7 days. The infective dose of *Salmonella* for human infection has been reported to be between 10^3 – 10^6 cells, depending on the health and age of the person (Blaser and Newman, 1982; Kothary and Babu, 2001; Klotchko and Wallace, 2009). Many factors interact and contribute to an organism's ability to infect the host, and therefore the dose of infection is not the same for every individual (Johnson, 2003). In the current experiment there was at least 10^3 cfu per gram of soil remaining in all samples after two weeks of incubation, and in all but four after 28 days. At the final sampling time of 42 days there was an average number of 450 cfu per gram of soil remaining, requiring the ingestion of 3 g of soil for infection, and although this is unlikely to occur in humans it poses a threat to grazing animals. Avery *et al*. (2004) found that spiked *E. coli* 0157:H7 (an indicator species for pathogenic bacteria in sewage) applied to soil in organic waste (cattle slurry and ovine stomach contents) was able to survive for 2 months at cell numbers above the infective dose (Avery *et al.*, 2004). The current result is in line with those exhibited here. It should be noted, however, that the spiked levels of pathogenic

organisms used here (10^6 cfu per g soil) is much higher than what is typically expected in treated biosolids, and therefore, if lower levels are present initially then the threat of infection would be markedly reduced.

Campylobacter is considered fragile and sensitive to environmental stresses (Pepper, 2006), this may be why we observed a rapid decline in *Campylobacter jejuni* over the two-week sampling period. Stelzer and Jacob (1991) found that sewage sludge samples only before digestion contained *Campylobacter*, and that *Campylobacter* was never found in sewage sludge that had been digested for more than 90 days. Studies have shown that survival of *Campylobacter* in aerobically treated biosolids is low, but that when treated anaerobically survival rates are much higher (Sindhu and Toze, 2009). Colonies are highly susceptible to aeration and survive longer in the absence of oxygen. One reason why *Campylobacter* numbers declined so rapidly in the current experiment may be due to the soil samples being small and exposed to the air. During land application of biosolids there would be more opportunity for anaerobic spaces within the soil/biosolids matrix (particularly if incorporated as opposed to surface applied), potentially facilitating an increased survival of *Campylobacter*. In a study into the effect of incorporation into soil of infected organic wastes on the reduction of pathogenic bacteria counts, Hutchinson *et al*. (2004) found decimal reduction times (D values) of between 0.38 and 4.03 days for *Campylobacter jejuni*, with most between 1 and 2. This is in agreement with the current study, which had D-values of between 1.4 – 1.8 days for *campylobacter*. The infective dose of *Campylobacter jejuni* has been reported to be as low as 500 cells (Robinson, 1981; Kothary and Babu, 2001). The results in this chapter show that after four days there would be enough colonies remaining to infect humans (when applied at the rate of 10^6 cfu per g soil), but by two weeks this had completely diminished, eliminating potential threats from this organism.

Research has shown that it is possible for pathogenic bacteria to remain in a viable but non-culturable (VBNC) form when submitted to environmental stresses, such as those present in biosolids and in land applied biosolids (England *et al.*, 1993; Wery *et al.*, 2006). This is a potential hazard for human health as it suggests that the culturing techniques routinely used in sewage sludge analysis may underestimate the concentration of pathogens present. In future experiments it may be beneficial to use an

additional method such as real-time quantitative PCR (qPCR) alongside traditional culturing techniques. This method was shown by Wery *et al*. (2006) to pick up viable cells of *Listeria monocytogenes* that the culture techniques alone did not.

4.6. General Conclusion

Overall, it would appear that the soil chemical and biochemical factors measured are indirectly influencing pathogen survival due to their effect on indigenous microbial populations. This could potentially be masking any antimicrobial effects of the presence of manuka. There is a limited amount of research in this area and therefore comparisons cannot be made to previous research. Further studies would help illustrate which soil factors most effect pathogen survival. There is a myriad of future work that could be carried out based on the results of the current research. For example, we would suggest introducing spiked biosolids into the experimental procedure as mentioned previously. Also, amendment of soil samples with varying manuka components, such as leaves, prior to inoculation of pathogens may give better results. Initial stages could look into the addition of manuka water extracts, such as those in Chapter Three, to soil or biosolids samples. Additionally, identification and isolation of the components that cause pathogen die-off in extracts in Chapter Three would give significant additional insight. Further experiments using all five bacterial strains used in chapter two would provide a greater understanding of how these bacteria behave in the presence of manuka, as well as introducing other pathogenic organisms such as viruses and protozoa. In addition, as mentioned previously, the use of live plants grown on pathogen-spiked soil for long periods of time would add further value to this study.

5. Metal Salt Pot Trial

5.1. Introduction

Heavy metal (HM) contamination of land is a growing concern worldwide. HMs can enter a soil system in many ways and, although there are low levels of HM in all soils and living organisms (Robinson *et al.*, 2006), it is generally accepted that contamination of land with high quantities of HM is anthropogenic. Plants have a major influence on soil formation and development and consequently have a major role in land rehabilitation and remediation. For heavy metal (HM) contaminated land, there are two principal approaches to remediation using plants (phytoremediation); phytoextraction and phytostabilisation (Ghosh and Singh, 2005; King *et al.*, 2008; Vamerali *et al.*, 2009). Phytoextraction is primarily carried out by enhanced uptake of metals in plant material and subsequent harvesting, thereby removing a proportion of the contaminants from the site altogether (Ghosh and Singh, 2005; Craw *et al.*, 2007; King *et al.*, 2008). This is usually achieved through the use of known HM hyperaccumulating plants (plants that accumulate large quantities of HM in their biomass (Reeves, 2006)), but also through the addition of chelating agents to the soil to improve metal bioavailability and thus uptake by 'normal' plants. Studies have shown potential for HMs to be extracted from hyperaccumulating plant material and recycled (phytomining) (Anderson *et al.*, 1999), thereby physically removing the HMs from the contaminated site. The main benefit of using hyperaccumulating plants to remove metals is that de-contamination can be achieved without chemical intervention (Reeves, 2006) and at relatively low costs compared with conventional engineering techniques (Vamerali *et al.*, 2009). Phytostabilisation on the other hand is the process of stabilising and limiting the movement, mobility or bioavailability of HM contaminants within the soil profile using plants (Ghosh and Singh, 2005; King *et al.*, 2008; Santibanez *et al.*, 2008). Through the establishment of metal tolerant species, plants can achieve metal stabilisation by; decreasing the amount of water in the profile (preventing leaching or water movement), providing wind shields that prevent erosion and distribution of soil to other areas, and by encouraging sorption, precipitation or complexation of contaminants

within the soil profile (Ghosh and Singh, 2005; Mertens *et al.*, 2007; Brunner *et al.*, 2008; King *et al.*, 2008; Santibanez *et al.*, 2008). Phytostabilisation of metal contaminated sites such as mine tailings is considered a cost-effective remediation strategy that prevents human and animal exposure to HMs, and reduces further environmental contamination (Santibanez *et al.*, 2008). In situ immobilisation of HM can be further enhanced by addition of soil amendments known to reduce mobility (Robinson *et al.*, 2006). Not all plant species are suited to either phytoextraction or phytostabilisation, and it has been noted that whilst some trees can decrease metal movement, others can in fact enhance it (Mertens *et al.*, 2007).

The influence of different tree species on metal mobilization is not totally clear, and only a few species have been investigated so far (Mertens *et al.*, 2007). However, it has been noted that some plants can influence the speciation and solubility of HMs in soil (Cheng *et al.*, 2004). They do this through altering rhizosphere soil properties, such as pH, organic carbon or total nitrogen, or through altering microbial community structure or soil enzyme activities (Cheng *et al.*, 2004; Yang *et al.*, 2007; Castaldi *et al.*, 2009a; Quartacci *et al.*, 2009; Wang *et al.*, 2009). This is primarily by way of rhizodeposition (Castaldi *et al.*, 2009a), the altering of rhizosphere soil through the release of root exudates, mucilage and sloughed cells (Castaldi *et al.*, 2009a). Studies have found many root exudates remain stable in soils for significant lengths of time (Quartacci *et al.*, 2009). Furthermore, through the release of carbon sources, protons and organic compounds (organic acids, amino acids) plant roots are able to select for different bacterial populations and enzymatic activity (Cheng *et al.*, 2004; Yang *et al.*, 2007; Castaldi *et al.*, 2009a). If plant roots are able to influence soil properties, then it seems likely that different plant species may in turn have different effects on the availability of HM in soil. The rhizosphere is the interface between soil and roots, where all soil:plant:organism interactions occur, therefore solubility of HM here is the most important determinant for evaluation of HM bioavailability (Wang *et al.*, 2009). Manuka is a plant species that is used in re-vegetation operations in New Zealand as it has a high tolerance to varying climatic and soil conditions including elevated levels of HM (Craw *et al.*, 2007). However, the potential use of manuka for the phytoextraction or phytostabilisation of HMs has not yet been quantified.

Heavy metals can exist in soil in many forms which are generally defined as; exchangeable, carbon bound, bound to iron and manganese oxides, bound to organic matter or in residual minerals (Tessier *et al.*, 1979). Many studies focus on the total quantities of a particular HM to determine its toxicity, but it is well known that it is the form or type of association of metal with soil particles (speciation) that determines its availability to organisms and the environment (Tessier *et al.*, 1979; Speir *et al.*, 1999a; Maiaomiao *et al.*, 2009). The most loosely bound forms, such as exchangeable are more available (bioavailable) to plants and organisms than the more tightly bound forms in stable organic or mineral complexes (Fujii and Kaneko, 2009; Smith, 2009). High levels of loosely bound, and thus highly available metals can have adverse environmental implications, such as movement into the food chain, or even plant death in extreme situations. The speciation of HMs is influenced by many factors, including soil pH, soil organic carbon content, cation exchange capacity (CEC), microbial population and clay content (Speir *et al.*, 1999a; Santibanez *et al.*, 2008; Castaldi *et al.*, 2009a; Smith, 2009; Yadav *et al.*, 2009). As mentioned, many of these factors are influenced by plant species, and if a particular plant species can alter the availability of HMs in soil from a more or less available form it may be useful in land remediation operations.

A number of sequential extraction procedures exist for determining the concentration and proportions of the different metal forms in soil. Many of these methods have been criticised due to method complexity and difficulty in interpretation (McLaren and Clucas, 2001; Jeyakumar *et al.*, 2008). However, despite criticism, sequential fractionation (one method for analysis of HM fractions in soil) is still the best means currently available for analysis of HM speciation, and provided the limitations are understood, the method can add great insight into bioavailability of HMs in soil (McLaren and Clucas, 2001).

The focus of this chapter is on the potential use of manuka in the remediation of HM contaminated land (with particular reference to biosolids application), and to investigate the impact of manuka on HM bioavailability in soil in relation to the restoration of HM contaminated sites. Ryegrass was chosen as a control plant species due to the prevalence of biosolids application to pastural/agricultural soil in many countries. Ryegrass is the primary grass species used in pastoral fields in New Zealand, and uptake of HM by

pastoral species is of significance due to the potential for ingestion by animals and contamination of the food chain. The second control species chosen for this experiment was the woody shrub/small tree *Coprosma robusta* (karamu). *Coprosma robusta* is a hardy native found throughout New Zealand, known to be tolerant to varying conditions (Landcare, 2009) and used in land re-vegetation operations in much the same way as manuka.

The direct spiking of soil or biosolids with HM salts in order to investigate the potential impacts of HM contaminated biosolids application to land is a technique that is widely used (McLaren and Clucas, 2001; Jeyakumar *et al.*, 2008). However, the use of metal salts as a substitute for biosolids naturally contaminated with metals has been widely criticised. This is primarily due to the metals in biosolids being strongly complexed with mineral and organic components, making them less biologically available than those in metal salt form (Speir *et al.*, 2007; Speir, 2008). This is partly due to sewage treatment procedures as well as the source of HM contamination (Jeyakumar *et al.*, 2008). However, justification for the use of metal salts are numerous. Use of metal salts allows the separation of negative effects caused by the HM of concern from other HMs or contaminants that may be present in the biosolids (McLaren and Clucas, 2001). It is also impossible to achieve concentrations of HM in soil to levels near guideline rates using biosolids alone, without repeat applications over many years, or at an excessive application rate that is unrelated to actual practices (Speir *et al.*, 2007). In addition, it has been shown that metals added through biosolids can mask the adverse effects of HMs due to enhanced microbial and biological activity that is promoted by nutrients in biosolids, making results hard to interpret (Speir *et al.*, 2007). The use of HM salts on their own as an alternative to spiked biosolids gives the benefit of accurate dosing, and an immediate impact that can be assessed from a single application (Speir *et al.*, 2007). In order to accurately observe the interaction between the chosen plants and HMs without added complexity, the decision was made to remove biosolids from the current experiment. This would have the benefit of making the results relevant to other HM contamination situations.

5.2. Aim

The aim of the experiment outlined in this chapter is to assess whether manuka plants grown on copper (Cu) and zinc (Zn) salt-spiked soil will have an impact on soil chemistry and the availability of Cu or Zn over time, and what impact the HM will have on plants. This is to be achieved by 1) investigating the impact of manuka, and other plant species, on Cu and Zn bioavailability and 2) analysing the plants to assess their uptake and accumulation of Cu and Zn.

5.3. Methods

5.3.1. Soil collection

Soil (150 kg) was collected from a pasture site in Palmerston North (Manawatu silt loam). On returning to the laboratory, soil samples were sieved to less than 8 mm, and a subsample weighed out for pH analysis, water holding capacity and moisture content. Based on the moisture content values, soil was dried slightly in large plastic trays (612 mm x 885 mm) to approx 40 % water holding capacity. Soil was subsequently mixed and divided into three equal volumes in separate trays for amendment with metal salts.

5.3.2. Experiment set up

5.3.2.1. Metal salt amendment of soil

Soil was amended with copper sulphate ($CuSO_4$) and zinc sulphate ($ZnSO_4$) at three rates, based on current regulations for biosolids application to land in New Zealand (zero, guideline and double guideline) (NZWWA, 2003). These quantities amounted to the following concentrations: Zn = 0, 300 and 600 mg kg^{-1} dry soil, Cu = 0, 100 and 200 mg kg^{-1} dry soil, (the actual amounts added to bulk soil can be seen in Table 4.1).

Table 5.1. *Total copper ($CuSO_45(H_2O)$) and zinc ($ZnSO_47(H_20)$) sulphate added to bulk soil samples in 1L H_2O*

Treatment	Field moist soil (Kg)	Equivalent dry soil (Kg)	Total $CuSO_4$(g)	Total $ZnSO_4$(g)
Zero	45	36.59	0	0
HM1	45	36.59	14.38	48.28
HM2	45	36.59	28.68	96.54

For each treatment, metal salts were first dissolved in 1 L deionised water and applied using a 1 L spray bottle, with thorough mixing in the tray throughout application. Soil was then left for one month covered in black plastic sheets (to keep dark), at room temperature to allow for equilibration. Moisture was checked regularly and the soil was mixed weekly by turning. After one month a subsample of soil was collected from each treatment for the analysis of total Cu and Zn to determine whether the correct amount of metal salts had been applied. Soil Cu and Zn fractionation analysis was also conducted to check metal bioavailability, and to ascertain whether metals were sufficiently bound into soil to prevent immediate toxicity to plants.

5.3.2.2.Pot experiment preparation

After one month equilibration, the amended soil was used to fill pots for planting. Two litre (72 in total) straight sided polypails (polypropolene buckets) were purchased from Stowers plastics in Petone (Lower Hutt, New Zealand), these can be seen in Figure 5.1. Eight holes were drilled into the bottom of each and the pots filled to approximately 2 cm (300 g) with small pebbles for drainage. The pots were subsequently packed with 1600 g of soil according to treatment; Zero, HM1 (guideline Cu and Zn addition) or HM2 (double guideline Cu and Zn addition), and compacted lightly to maintain some structure. Seedlings of *Leptospermum scoparium* (manuka) and *Coprosma robusta* (karamu) were obtained from a local nursery, Matatoa Trees and Shrubs, Shannon (approx 30 cm in height) and seeds of ryegrass (*Lolium perenne)* from stock stored in the ESR laboratory (Kenepuru Science Center, Porirua). Seedlings (one per pot) and seeds (0.20 g per pot) were subsequently planted in the pots, a total of six

for each soil treatment. Plants were grown for 12 months outside, and watered twice weekly when no rain occurred.

Figure 5.1. *Polypails containing ryegrass, manuka and coprosma after six months of growth*

5.3.3. Soil and plant harvesting

Plants and soil were removed from the pots at six months and 12 months after set up. Removal was by destructive sampling of three replicates of each treatment (zero, HM1 and HM2) at each time point (9 x manuka, 9 x coprosma, 9 x ryegrass and 9 x no plant control), giving a total of 36 samples for analysis at each time point. For the purpose of this experiment rhizosphere soil was determined to be any soil that was attached to the roots and removed by gentle shaking by hand. All root adhering soil was collected from each pot and sieved to <2 mm. A sub sample of fresh soil was stored in the fridge at 4°C and the remaining soil was air-dried overnight and mixed by quartering. One quarter of this was subsequently ground using a 250 μm sieve and mortar and pestle for chemical analysis.

Plant material was harvested from the pots at the same time as soil. All plant material was washed thoroughly with running tap water and subsequently rinsed in deionised water to remove soil. Roots were separated from stem and leaf herbage by cutting; roots were determined to be any plant material from ground level downwards

and similarly leaf and stem was considered to be all aboveground biomass (Wang *et al.*, 2009). All plant material was weighed, cut into approximately 1 cm lengths and oven dried at 70°C for 24 hours to determine dry weights. Dry samples were then ground using a Yellowline grinder for later digestion.

5.3.4. Soil and Plant analysis

5.3.4.1. Soil total Cu and Zn

Prior to analysis, a sub sample of each ground air dry soil sample was oven dried at 105°C overnight for use. The total Cu and Zn concentrations of the soil samples was determined by the method of Kovacs *et al*. (2000). For quality control, one blank tube and one known standard reference sample was analysed with each analytical batch (SRM 2710). A sub sample of dried and ground soil (1 g) was weighed into one 50 ml digest tube for each sample. To all tubes, 5 ml of 65% HNO_3 was added and pre-digested for 30 minutes at 60°C using a dry heat block. Subsequently, 5 ml H_2O_2 was added and the temperature increased to 120°C for 270 minutes with occasional agitation. Samples were subsequently diluted to 50 ml with DI water, and filtered before analysis. Analysis was conducted using flame atomic absorption spectrometry (FAAS) (GBC Avanta) for both Zn and Cu.

5.3.4.2. Plant total Cu and Zn

Total concentrations of Cu and Zn in the plant material were determined by the method of Gray *et al*. (1999). A sub sample of dried and ground plant material (0.5 g) was weighed into one 15 ml digest tube for each sample, with the exact weight recorded for later reference. Concentrated HNO_3 (10 ml) was added to each tube and the mixture digested on a dry heat block in four stages:

Step 1 40° C 30 mins
Step 2 80° C 120 mins
Step 3 120° C 120 mins
Step 4 140° C 120 mins

The digest solutions were subsequently transferred into 25 ml volumetric flasks, taking care to rinse out the tube, and made up to 25 ml with deionised water. Samples were filtered and stored at 4°C until analysis. Analysis was by flame atomic absorption spectrometry (FAAS) (GBC Avanta) for both Zn and Cu.

5.3.4.3. Cu and Zn fractionation of soil (sequential extraction)

Preparation of standard reference sample

The reference/QC sample used for this experiment was a 1:1 combination of two soils that had been spiked two years prior with Cu and Zn at ESR Kenepuru Science Centre. The soils had been spiked with 1000 mg Zn kg soil^{-1} ($ZnSO_4$) and 750 mg Cu kg soil^{-1} ($CuSO_4$) respectively. A sub-sample of this soil was air-dried, and ground using a mortar and pestle and 250μm sieve. Ten replicate samples of this reference sample were run through the fractionation procedure outlined below to use as a comparison to the samples in the current study.

Method protocol

Sequential extraction of soil samples was carried out using the method of Tessier (1979), with alterations by McLaren and Clucas (2001). This method is based on the sequential extraction of different metal fractions with different binding forces to varying components of the soil matrix (Fujii and Kaneko, 2009). One gram of air dried, finely-ground soil was placed into a 50 ml polypropylene centrifuge tube for each sample. Each run consisted of ten samples, one known control soil sample and one blank. Samples were sequentially extracted using a series of five extraction solutions related to the fraction of interest. A brief overview of the extractants used can be seen in Table 5.2, for full method details see Appendix A1.5. Solutions generated by each step of the fractionation procedure were filtered (Whatman #41) and made up to the appropriate dilution (specified in the methods) with deionised water in volumetric flasks. The Zn and Cu concentrations of extracted solutions were subsequently determined using atomic absorption spectrometry (FAAS) (GBC Avanta).

Table 5.2. *A brief overview of the sequential extraction procedure (after Tessier et al., 1979)*

Step	Metal Fraction	Extractant Used	Extraction Conditions
1	Soluble and exchangeable	1 M $Mg(NO_3)_2$, pH 7.0	1 g soil, 8 ml extractant; 1 h shaking
2	Bound to carbonates	1 M sodium acetate, adjusted to pH 5.0 with acetic acid	Residue from step 1, 8 ml extractant; 5 h shaking
3	Bound to oxides	0.4 M $NH_2OH.HCl$ in 25% v/v acetic acid	Residue from step 2, 20 ml extractant; heated for 6 h at 96C
4	Bound to organics	H_2O_2 adjusted to pH 2 with HNO_3	Residue from step 3, 8 ml extractabt; heated for 2 h at 85C then 3 ml extractant added and heated a further 3 h at 85C
5	Residual	H_2O_2 and HNO_3 digestion	Residue from step 4 dried and 0.5 g subsmple digested according to Kovacs et al., (2000)

5.3.5. Soil chemical analysis

Air-dried, ground soil samples from the one year sampling only were sent to Landcare Research, Palmerston North, for analyses of total carbon (TC) and total nitrogen (TN). This was determined by the use of a Leco furnace (Leco (Laboratory Equipment Corporation), St Joseph, Michigan, USA) and subsequent analysis by thermal conductivity for N and infrared detection for C.

Soil pH was determined immediately upon transfer of field moist soil to the laboratory and then again when required. Soil samples were mixed with deionised water at a 1:2.5 ratio, stirred with a glass rod, and left to stand overnight before measurment using a glass pH meter probe (Thermo Orion perpHect LogR meter, model 310).

5.3.6. Statistical analysis

Microsoft excel was used for calculation of averages, standard deviations and standard errors, for both total soil and total herbage Cu and Zn. Percent distributions of Cu fractions and Zn fractions were also calculated using Microsoft excel. Comparisons between variables were analysed by ANOVA using SPSS 16.0, statistical significance was interpreted using Tukey post-hoc tests.

5.4. Results

5.4.1. Soil chemical analysis

The pH of the bulk soil after the one month equilibration period, but before planting, can be seen in Table 5.3. The pH of the fresh soil at time one (six months after planting) and time two (12 months after planting) can be seen in Table 5.4. These data show that the decrease in soil pH caused by amendment of bulk soils with metal sulphates at time zero has subsequently changed over time to be within the range of the

unspiked soil, possibly due to soil buffering abilities. Very few significant effects of plant type on soil pH were observed (Table 5.4).

Results for the analysis of soil TC and TN can be seen in Table 5.5. There were no significant differences in percent of soil C or N between plant types (Table 5.5). However, unspiked soil was shown to be significantly higher in C than spiked soils ($p < 0.01$)

5.4.2. Soil total Cu and Zn

Total Zn and Cu in the bulk soil samples at time zero, before planting, can be seen in Table 5.3. The data shows that the target soil concentrations were achieved through spiking. The averages and standard error concentrations for total Cu and Zn in the soil samples after six months (time one) are presented in Table 5.6. The data in Table 5.6 shows that all plant species at time one had little or no impact on the total concentrations of either Cu or Zn in the soil. This is illustrated by the consistency across plant species and indicates all the soils were behaving in a similar manner. Similarly, Table 5.7 shows that the average total Cu and Zn concentrations in soil after one year (time two), showed little difference between the plant species. Analysis of variance (ANOVA) between species for each treatment showed only three significant differences between plants over both time periods and HM treatments (Tables 5.6 and 5.7). These were as follows;

1) At six months, untreated soil under coprosma had significantly less Zn than control, ryegrass and manuka ($P<0.01$)
2) HM1 treated soil, at one year, exhibited significantly less Zn under coprosma compared to ryegrass, but not the control soil ($P<0.01$).
3) HM2 treatment showed a significantly lower Cu concentration under manuka when compared to the control soil, but not when compared to ryegrass or coprosma ($P<0.01$).

Some loss of both Zn and Cu over time has been observed in all treatments, this may be due to leaching and/or plant uptake. Analyses of standard reference samples resulted in concentrations of Zn and Cu within 3.5% and 2.5 % respectively of the certified values (2950 $\mu g\ g\ soil^{-1}$ for Cu and 6952 $\mu g\ g\ soil^{-1}$ for Zn).

Table 5.3. *Total copper and zinc concentrations in bulk soil samples after spiking (time zero)*

Sample	Spiked Cu mg/kg	Spiked Zn mg/kg	Total Cu mg/kg	Total Zn mg/kg	pH
ZERO	0	0	17	101	5.75
HM 1	100	300	119	447	5.26
HM 2	200	600	222	747	5.10

Table 5.4. *Soil pH of fresh soil samples after six months (time one) and one year (time two)*

**Means followed by the same letter in a row were not significantly different (P>0.05).*

Treatment	pH time one (6mths)				pH time two (1yr)			
	Control	Ryegrass	Coprosma	Manuka	Control	Ryegrass	Coprosma	Manuka
ZERO	5.74 ± 0.21[a]	5.91 ± 0.03[a]	5.87 ± 0.11[a]	5.94 ± 0.13[a]	5.51 ± 0.14[a]	5.64 ± 0.04[b]	5.43 ± 0.10[a]	5.65 ± 0.03[b]
HM 1	5.75 ± 0.03[a]	5.74 ± 0.06[a]	5.75 ± 0.05[a]	5.83 ± 0.02[b]	5.52 ± 0.05[a]	5.74 ± 0.05[b]	5.53 ± 0.10[a]	5.59 ±
HM 2	5.66 ± 0.01[a]	5.70 ± 0.03[a]	5.74 ± 0.08[a]	5.68 ± 0.06[a]	5.53 ± 0.03[a]	5.61 ± 0.05[a]	5.47 ± 0.04[a]	5.52 ± 0.02[a]

Table 5.5. *Percent carbon and nitrogen (mean ± s.e., n=3) in air-dried soil samples after one year (time two)*

Treatment	Total Carbon (%)				Total Nitrogen (%)			
	Control	Ryegrass	Coprosma	Manuka	Control	Ryegrass	Coprosma	Manuka
ZERO	4.32 ± 0.09	4.36 ± 0.21	4.43 ± 0.04	4.26 ± 0.10	0.45 ± 0.01	0.46 ± 0.01	0.46 ± 0.01	0.45 ± 0.01
HM 1	4.14 ± 0.07	4.20 ± 0.08	4.21 ± 0.10	4.19 ±	0.43 ± 0.01	0.44 ± 0.01	0.45 ± 0.002	0.46 ±
HM 2	4.20 ± 0.10	4.19 ± 0.05	4.24 ± 0.08	4.19 ± 0.05	0.47 ± 0.01	0.46 ± 0.01	0.47 ± 0.01	0.46 ± 0.01

Table 5.6. *Total copper and zinc concentrations (mean ± s.e., n=3) in oven dried soil samples after six months (time one)*

**Means followed by the same letter in a row were not significantly different (P>0.05).*

Treatment	Total Cu (mg kg^{-1} dry soil)				Total Zn (mg kg^{-1} dry soil)			
	Control	Ryegrass	Coprosma	Manuka	Control	Ryegrass	Coprosma	Manuka
Zero	11.3 ± 0.2[a]	12.2 ± 0.7[a]	11.0 ± 0.0[a]	12.2 ± 0.2[a]	95.8 ± 0.2[a]	95.7 ± 0.6[a]	80.0 ± 5.2[b]	95.7 ± 1.0[a]
HM1	101 ± 0.6[a]	106.8 ± 2.4[a]	109.0 ± 2.5[a]	104.5 ± 1.5[a]	363.9 ± 6.1[a]	353.8 ± 0.7[a]	364.9 ± 5.2[a]	352.4 ± 6.6[a]
HM2	198.7 ± 1.5[a]	195.2 ± 1.9[ab]	195.8 ± 1.8[ab]	189.5 ± 0.3[b]	597.7 ± 2.0[a]	588.0 ± 5.3[a]	570.0 ± 9.3[a]	565.2 ± 19.1[a]

Table 5.7. *Total copper and zinc concentrations (mean ± s.e., n=3) in oven dried soil samples after one year (time two)*

**Means followed by the same letter in a row were not significantly different (P>0.05). Lack of s.e. is due to plant death.*

Treatment	Total Cu (mg kg^{-1} dry soil)				Total Zn (mg kg^{-1} dry soil)			
	Control	Ryegrass	Coprosma	Manuka	Control	Ryegrass	Coprosma	Manuka
Zero	8.2 ± 0.5[a]	7.5 ± 0.1[a]	7.3 ± 0.04[a]	7.5 ± 0.3[a]	73.0 ± 0.8[a]	77.9 ± 1.5[a]	75.1 ± 2.0[a]	73.1 ± 1.3[a]
HM1	66.6 ± 0.8[a]	70.8 ± 1.4[a]	67.3 ± 1.8[a]	71.2 ±	317.1 ± 0.6[ab]	327.3 ± 4.1[a]	307.0 ± 2.8[b]	316 ±
HM2	127.7 ± 1.7[a]	137.3 ± 0.4[a]	134.1 ± 4.7[a]	134.5 ± 1.7[a]	540.4 ± 19.7[a]	579.6 ± 5.7[a]	548.3 ± 8.5[a]	542.4 ± 8.7[a]

5.4.3. Herbage total Cu and Zn

At both the six month and one year sampling times all surviving plants appeared healthy and no apparent negative effects or toxicity due to metal salt addition were observed. Average and standard error concentrations for total Cu and Zn in the root and shoot samples for time one (six months) are presented in Tables 5.8 and 5.9, and for time two (one year) in Tables 5.10 and 5.11. Due to plant death the manuka HM1 treatment was reduced to only one plant replicate at time two, this is represented in Tables 5.10 and 5.11 by the lack of standard error.

Based on the reported data, it appears that manuka takes up a significantly higher concentration of Cu into shoots than coprosma or ryegrass at both time one ($P<0.05$ and $P<0.01$ respectively) and time two ($P<0.01$ for both). The Cu concentration in the leaves and shoots of coprosma and ryegrass was not significantly different at either time point ($P=0.24$, $P=0.29$). At time one, it was observed that uptake of Zn by manuka was significantly less than that of both ryegrass or coprosma ($P<0.01$), while there was no difference between Zn in coprosma and ryegrass ($P=0.07$). However, by time two, all three plants exhibited significant differences in their uptake of Zn ($P<0.01$) in the order of manuka<ryegrass<coprosma.

After six months it was shown that manuka and coprosma both accumulated significantly more Cu in their roots than ryegrass ($p<0.01$), however by the end of one year, coprosma had significantly more Cu in roots than ryegrass ($P<0.01$) and manuka ($p<0.01$), but the difference between manuka and ryegrass was not significant. It is clear from the tables that at time one coprosma accumulated significantly more Zn in the roots than either manuka or ryegrass ($P<0.01$). Each species behaves differently in the accumulation of root Zn ($P<0.01$) in the order of ryegrass<manuka<coprosma. The same trend was observed at time two ($P<0.01$), however the accumulation by manuka had increased markedly from six months to one year.

Table 5.8. *Total copper and zinc concentrations (mean ± s.e., n=3) in oven dried shoot and leaf samples after six months (time one)*

Treatment	Total Cu (mg kg $^{-1}$ dry leaves)			Total Zn (mg kg $^{-1}$ dry leaves)		
	Ryegrass	Coprosma	Manuka	Ryegrass	Coprosma	Manuka
Zero	4.5 ± 0.51	21.5 ± 9.87	15.5 ± 2.30	65.1 ± 4.86	84.3 ± 1.17	37.2 ± 3.87
HM1	12.9 ± 0.72	25.0 ± 13.06	29.6 ± 6.34	211.4 ± 34.25	330.5 ± 17.0	78.4 ± 11.28
HM2	15.1 ± 0.87	10.7 ± 0.99	57.5 ± 4.48	631.6 ± 26.58	764.4 ± 39.9	139.6 ± 11.28

Table 5.9. *Total copper and zinc concentrations (mean ± s.e., n=3) in oven dried root samples after six months (time one)*

Treatment	Total Cu (mg kg $^{-1}$ dry roots)			Total Zn (mg kg $^{-1}$ dry roots)		
	Ryegrass	Coprosma	Manuka	Ryegrass	Coprosma	Manuka
Zero	9.5 ± 1.23	13.3 ± 1.36	9.4 ± 0.43	119 ± 5	163 ± 31	147 ± 24
HM1	59.3 ± 5.12	99.0 ± 7.79	72.3 ± 5.49	670 ± 24	2468 ± 58	1501 ± 101
HM2	121.0 ± 8.95	245.0 ± 27.4	221.0 ± 8.28	1473 ± 82	3470 ± 260	1926 ± 240

Table 5.10. *Total copper and zinc concentrations (mean ± s.e., n=3) in oven dried shoot and leaf samples after one year (time two). * indicates insufficient replication for s.e*

Treatment	Total Cu (mg kg $^{-1}$ dry leaves)			Total Zn (mg kg $^{-1}$ dry leaves)		
	Ryegrass	Coprosma	Manuka	Ryegrass	Coprosma	Manuka
Zero	2.3 ± 0.32	4.51 ± 0.85	17.00 ± 3.85	24.1 ± 3.41	64.53 ± 8.87	37.3 ± 0.70
HM1	4.6 ± 0.96	11.54 ± 3.74	10.49 ± *	332.8 ± 42.3	475.49 ± 50.9	80.25 ± *
HM2	5.6 ± 0.57	5.33 ± 0.06	21.17 ± 2.18	528.2 ± 21.8	789.8 ± 73.5	189.31± 35.1

Table 5.11. *Total copper and zinc concentrations (mean ± s.e., n=3) in oven dried root samples after one year (time two). * indicates insufficient replication for s.e.*

Treatment	Total Cu (mg kg $^{-1}$ dry roots)			Total Zn (mg kg $^{-1}$ dry roots)		
	Ryegrass	Coprosma	Manuka	Ryegrass	Coprosma	Manuka
Zero	7.5 ± 1.11	11.23 ± 0.66	9.0 ± 1.34	40.7 ± 2.07	86.1 ± 14.2	84.9 ± 2.07
HM1	35.6 ± 2.71	87.8 ± 4.32	57.27 ±*	505.9 ± 6.98	1921 ± 318	1007 ± *
HM2	81.33 ± 1.52	192.1 ± 4.54	118.82 ± 20.3	1158 ± 57.7	3241 ± 85.8	2729 ± 62.4

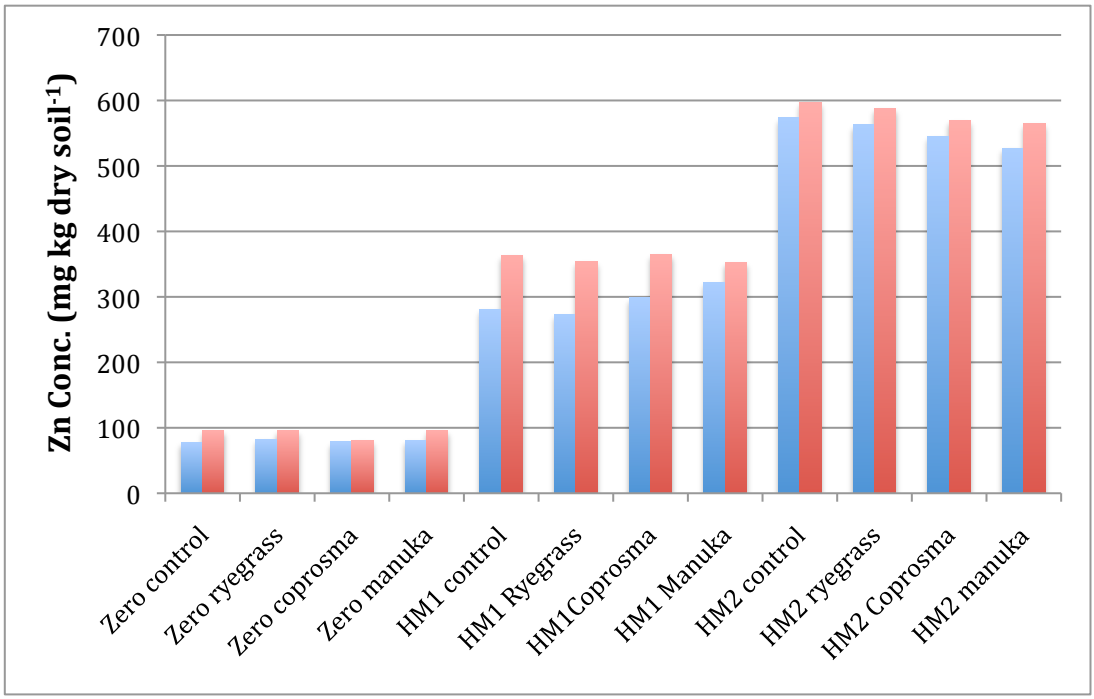
5.4.4. Copper and zinc fractionation of soil.

The reliability of the fractionation method to account for all metal in the soil was checked by comparing the sum of the metals in individual fractions with the concentration of metals found through a single total metal digest. Figures 5.2, 5.3 and 5.4 present the relationship between the sum of the metal concentration recovered by fractionation and the total metal concentration of the single digest. In all cases, the sum of fractions produced a lower value than the total digest (Fig. 5.2), considered to be due to the small cumulative losses of material during fractionation (McLaren and Clucas, 2001). However, the agreement was good, indicating the validity of the fractionation procedure to account for soil metal.

The percentage distribution of Cu and Zn between solid phase fractions is presented in Figures 5.5 and 5.6. The results in these graphs indicate that there are no obvious differences in the proportional distribution of Cu or Zn between plant species after six months or one year of growth. The one exception is that under coprosma there appears to be a lower proportion of exchangeable- and residual-, and greater proportion of organic bound-copper, compared to soil under the other plant species at both time one and time two.

From Figures 5.5 and 5.6 it can be seen that the highest proportion of Zn in spiked soils remained in the soluble/exchangeable fraction even one year after spiking. This is not observed with Cu, which by six months has a larger proportion bound onto the organic and oxide fractions for all soil samples. In all unspiked (zero) soils the highest proportion of both Zn and Cu was in the residual portion, however for both metals this was slightly reduced at time two. As was expected, the proportion of HM in the residual fraction of spiked soils was markedly reduced, though the actual concentrations of these fractions remained the same or increased with metal salt addition. It should be noted that the total concentration of Cu in the residual portion increased markedly with metal salt addition, and the subsequent fraction in Zn was unchanged with metal salt addition (seen in the values at the top of Figs 5.5 and 5.6), suggesting dissolution of the residual portion does not explain the high levels of Zn remaining in the soluble fraction.

a)



b)

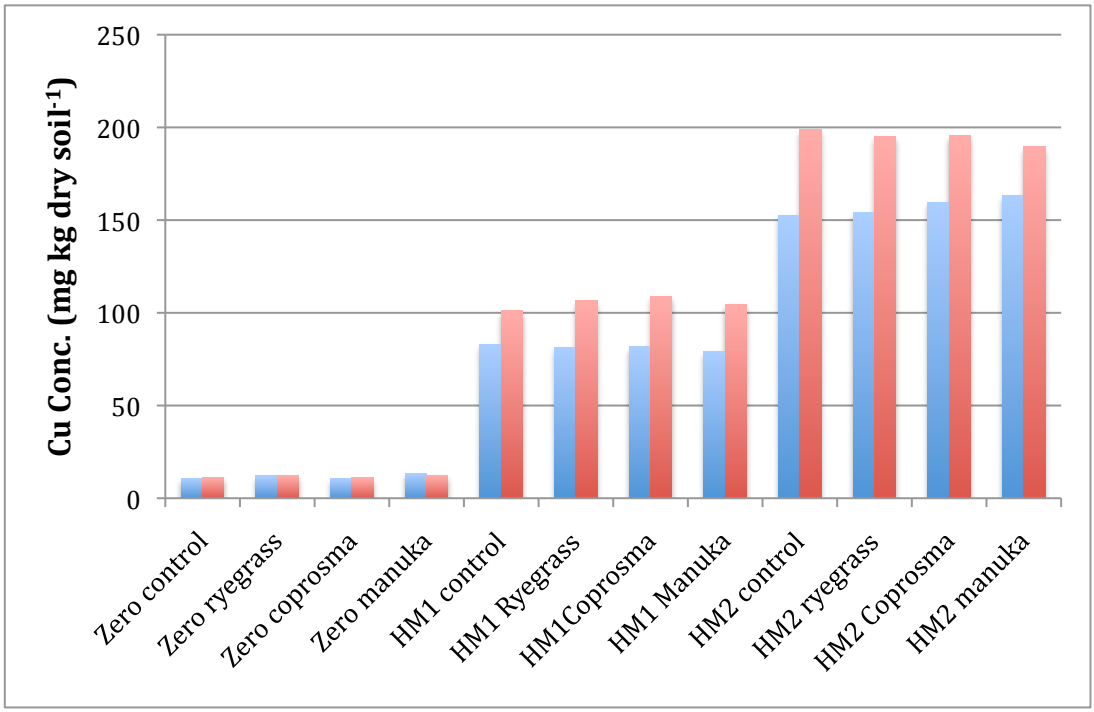
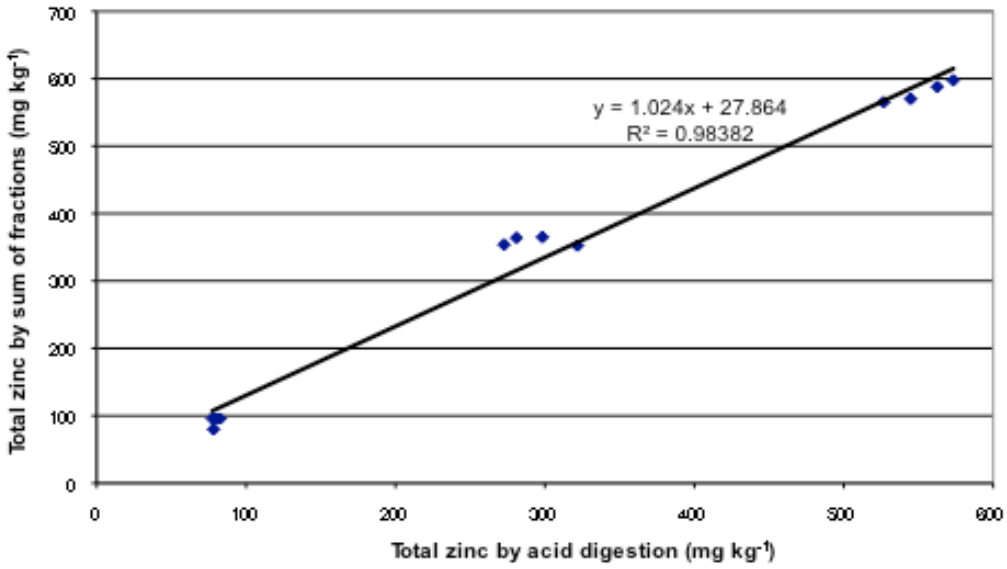


Figure 5.2 *The comparison of the metal concentrations calculated, between the sum of all fractions (blue) and total digest (red) for a) Zn and b) Cu*

a)



b)

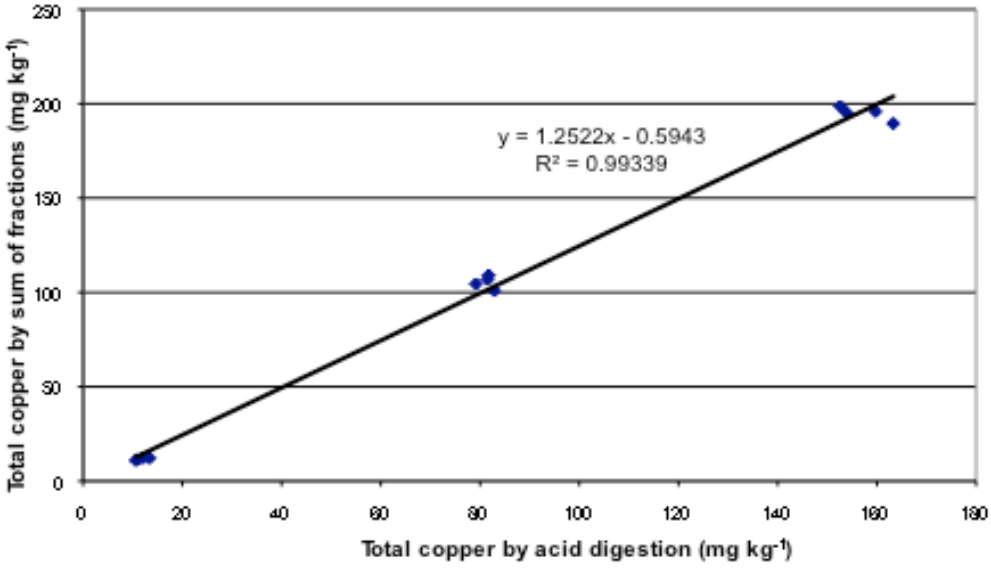
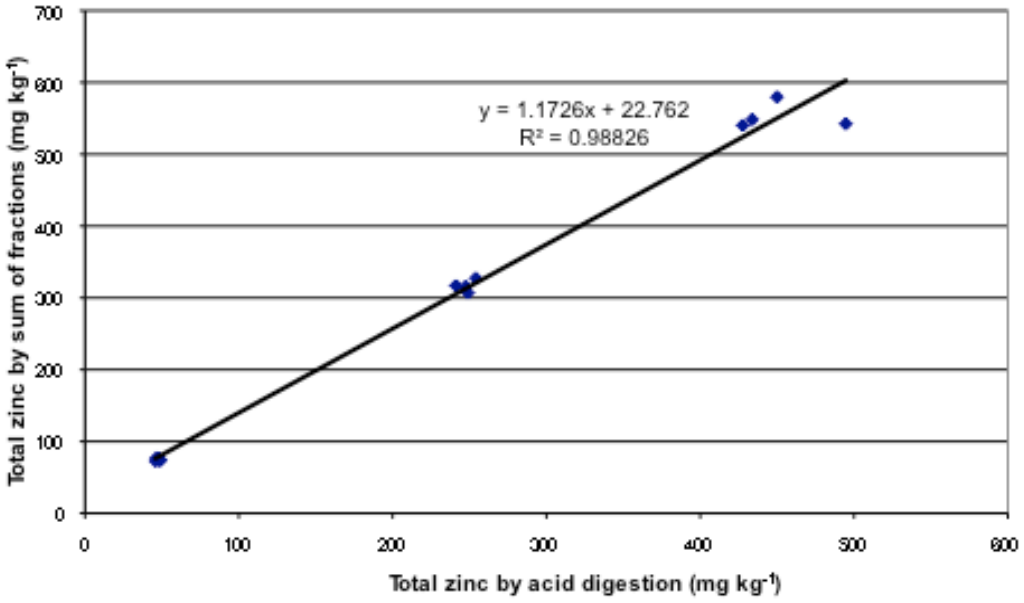


Figure 5.3. *Relationship between the sum of all metal fractions and the total metal concentration determined by acid digestion for a) zinc and b) copper, after six months (time one).*

a)



b)

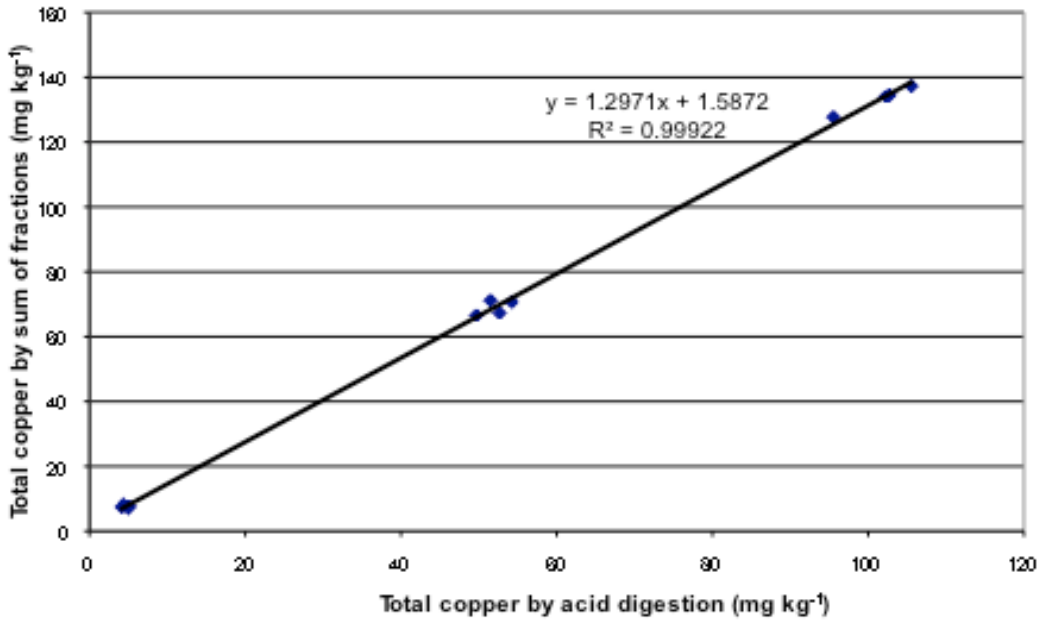
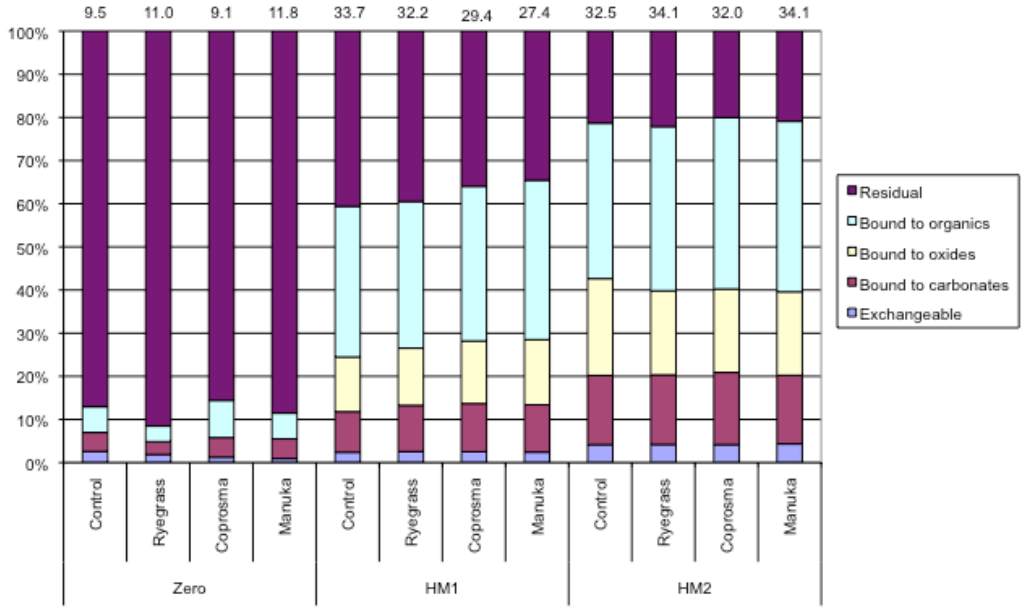


Figure 5.4. *Relationship between the sum of all metal fractions and the total metal concentration determined by acid digestion for a) zinc and b) copper, after one year (time two).*

a)



b)

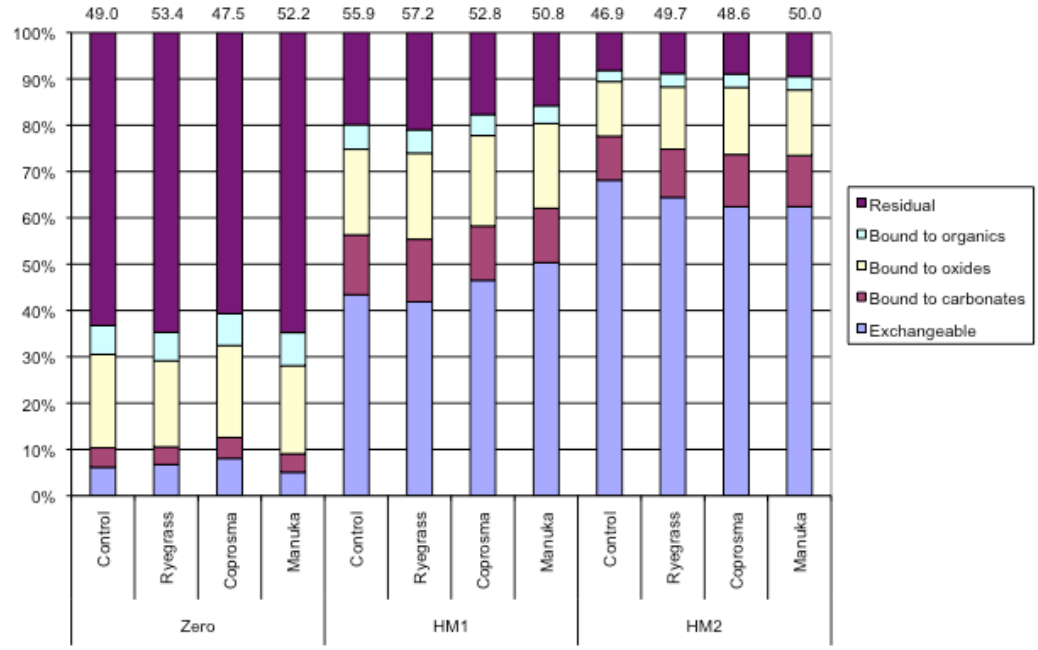
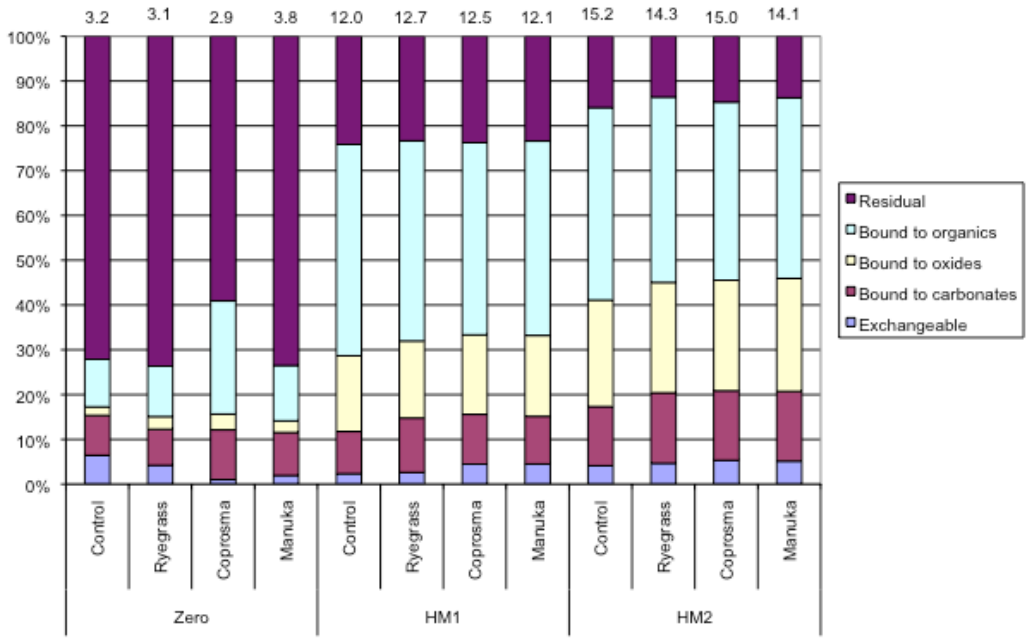


Figure 5.5. *Percentage distribution of Cu fractions (a) and Zn fractions (b) in soil samples after six months (time one). Values at the top represent the average concentration of the residual fraction (mg kg^{-1}).*

a)



b)

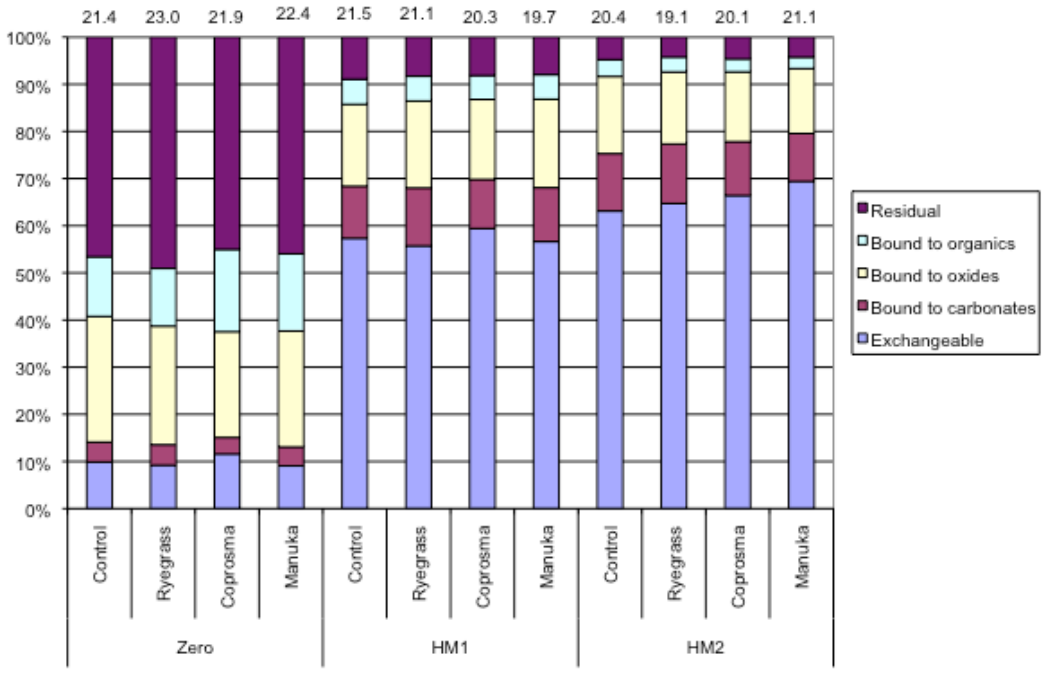


Figure 5.6. *Percentage distribution of Cu fractions (a) and Zn fractions (b) in soil samples after one year (time two). Values at the top represent the average concentration of the residual fraction (mg kg^{-1}).*

5.5. Discussion

The results gained from this experiment gave some valuable insight into the behaviour of three different plant species growing in Zn and Cu spiked soils. The results clearly showed that the three plant species investigated differ in their ability to uptake and accumulate both metals, but have no apparent effect on HM fractionation over the relatively short time period of one year. Some limiting factors have been noted and these will be discussed in more depth.

In a study conducted by Santibanez *et al*. (2008) on the stabilisation of metal mine tailings using biosolids and ryegrass (*Lolium perenne*), investigations into the uptake of HM by ryegrass found more Cu accumulation in the roots compared to the shoots. Similarly in our study, the concentration of Cu in the roots was up to eight times greater than that in the shoots. This was the same for all three plants in our experiment in that whilst some Cu was taken up into shoots, the majority was confined to the roots. Santibanez *et al*. (2008) also found accumulation of Zn in the roots of ryegrass. In our study, accumulation of Zn in the roots was also observed, with the Zn concentration in the roots being between 1.8 and 3 times greater than in the shoots. However, much more Zn than Cu was taken up into the shoots by all three plants, possibly due to the high proportion of Zn in the exchangeable form. Castaldi *et al*. 2009 found that two plant species (pea and wheat) investigated for their effect on metal mobility and soil chemical and biological parameters, accumulated more Pb, Cd and Zn in roots than in leaves. They found translocation of metals to be crop dependent, with pea plants translocating higher levels of Zn and Cd whilst wheat translocated higher levels of Pb (Castaldi *et al.*, 2009a). Accumulation of HM into stems and shoots of plants is controlled by the rate of uptake by roots and the rate of transport to roots, this is in turn controlled by plant water uptake (Robinson *et al.*, 2006). The different need for water, evapotranspiration rate and rate of uptake between species may be one reason for differences in HM translocation.

All three plants had some accumulation of Zn in their roots, however, manuka appeared to be the most effective plant at restricting movement of Zn from roots to shoots, with up to 19 times more Zn in the roots compared with the shoots. In addition,

significantly less Zn was found in the roots and shoots of manuka than the other two plants, indicating it may have a mechanism of limiting Zn uptake. Manuka took up significantly more Cu into above ground biomass than did ryegrass or coprosma for all treatments including control, but this same trend was not observed in the roots. Research shows that the degree of HM uptake varies widely from species to species, and also depends on the HM of concern (Yadav *et al.*, 2009). Zinc is relatively labile and therefore easily taken up into plant tissue whilst Cu is strongly sorbed to soil components, and therefore plant uptake is more limited (Smith, 2009). This makes the increased uptake by manuka compared to the other plant species of some degree of interest.

The criteria for hyperaccumulating plants have been set by a number of researchers. A review by Reeves (2006) suggests the limit for hyperaccumulation status for Cu to be >1000 mg kg^{-1} and for Zn >3000 mg kg^{-1} (or 10 000 mg kg^{-1} depending on opinion) in any above ground biomass, so long as the metal uptake was not considered "forced" and was under natural conditions. In the present study, although the soil conditions were not natural due to the addition of metal salts, the soil was left to equilibrate before seedlings were planted and was not considered conducive to "forced" uptake. In any case, the plants in the current study were not deemed to be hyperaccumulators. Although some elevated uptake of Zn and Cu was observed these levels did not reach greater than 789 mg kg^{-1} for Zn or 57 mg kg^{-1} for Cu. Even though it was not considered a hyperaccumulator, manuka did exhibit Cu uptake that was outside the normal range of 5-25 mg kg^{-1} (Reeves, 2006). It cannot be determined if the plants had been left to grow longer whether the concentrations of metals in herbage would increase significantly, or if accumulation would plateau or even kill the plants. Further investigation by means of a longer study with greater replication (to allow for some plant die-off) may be worthwhile to assess this. Based on what was found, the phytoremediation of a contaminated site by the means of phytoextraction would not be recommended for any of the plants investigated. All species did appear to have a good level of tolerance to Cu and Zn at the levels tested. Further to this, manuka significantly limited the uptake of Zn compared to the other two plants, potentially making it a good candidate for phytostabilisation of Zn contaminated sites. In addition, other studies have shown manuka to establish effectively on sites contaminated with high levels of arsenic

(As), and limit translocation of this metal from roots to shoots and leaves (Craw *et al.*, 2007). Phytostabilisation has been accepted as a possible remediation strategy for HM polluted soils (Brunner *et al.*, 2008), and successful phytostabilisation involves holding contaminants in place to prevent movement (Brunner *et al.*, 2008). It has been suggested that the extended root system of trees is more suitable than that of herbaceous species for remediating sites with deeper pollution (Vamerali *et al.*, 2009). The two woody species in the current trial accumulated significantly more Cu and Zn in their roots than did the grass, highlighting potential for their use in stabilising these metals in the soil profile. In addition, manuka is known to be good for prevention of erosion and it has a strong, intricate and shallow root system (Bergin *et al.*, 1995) that would aid in containment of contaminants. Wang *et al*., (2009) noted that rhizosphere soils accumulated more heavy metals with the re-vegetation time than bulk soils from outside the root zone. They put this down to the movement of HM ions towards plant roots through mass flow of soil water, and diffusion when roots absorb water or nutrients (Wang *et al*. 2009). Wang *et al*., (2009) suggest that this highlights the fixing and stabilizing effects of re-vegetation on soil HM caused by plant roots and exudates. The current study did not compare rhizoshpere and bulk soils due to time and financial constraints, however, this would be an interesting aspect to look at in future work.

In a similar study to this, King *et al*. (2008) looked into the effect of four *Eucalyptus* species on As availability in an arsenical, sulphidic gold mine tailings. Plants were grown on soil for five years before sampling took place, and King *et al*. (2008) found higher concentrations of As in more mature leaves compared to younger shoots. Brunner *et al*. (2008) found that accumulation of Al, Cr and Ni in roots of spruce and poplar reached a maximum concentration after three or four years from planting. The same result was not observed for Zn and Cu in their study (Brunner *et al.*, 2008). In the present study, plant material was sampled after only six months and one year, suggesting again that a longer length of growth may have shown more significant results. However, running a longer experiment was not within the scope of this research. It has also been noted in studies of metal uptake by plants, that when leaves and stems have been analysed separately, the levels of metals in the stems were much lower than what was found in the leaves. In some instances, the concentrations in leaves were 5 to 10 times higher than that found in woody stems (Brunner *et al.*, 2008; King *et al.*,

2008). Mertens *et al*. (2007) found Zn concentrations in wood of mature poplar trees were much less than that in the litter fall after a 33 year growing period. In the present study, leaves and stems were analysed together and perhaps had they been separated, a higher concentration of metals may have been observed in the leaves.

It has been suggested that the distribution of HM in the rhizosphere soil is more important for the evaluation of the bioavailability of HM than in bulk soil (Wang *et al.*, 2009). As the focus of this work is on the impact of different plants on the bioavailability of Cu and Zn in soil, only soil from near the plant roots was sampled. Sequential extraction (or fractionation) procedures have been developed for determining the proportions of HM present in a soil sample in different forms (McLaren and Clucas, 2001). There are known limitations to these methods, nevertheless they provide useful information of HM distribution in soil and hence HM availability (McLaren and Clucas, 2001). A good check on the reliability of sequential fractionation data is whether, within acceptable error, the sum of the metal in the individual fractions is equal to a single total metal determination. In the present study the metal concentrations recovered during fractionation were considered acceptable to give a good picture of HM availability, although, they were generally lower (in some cases up to 25% in spiked soils) than the single total metal determinations. This was considered mainly due to small cumulative losses of material during fractionation (McLaren and Clucas, 2001).

No readily apparent and significant effect of plant on Cu and Zn availability, TC, TN or soil pH was detected. This result is in agreement with that of King *et al*. (2008), who found no effect of four *Eucalyptas* species on As availability or soil pH after five years. Although the presence of plant species in our study did not reduce the bioavailability of Cu or Zn, it should be noted that nor did they increase bioavailability, and this is a favourable trait if phytostabilisation is the main goal of remediation. Many studies, however, have reported an effect of plant species on pH, soil chemistry and metal mobility in soil. Castaldi *et al*. (2009) found that the pH of soils decreases in the presence of plant growth compared to unplanted soil. Furthermore plant growth caused a significant increase of total organic carbon and total nitrogen compared to unplanted soil. A significant decrease in total HM's was also observed after plant growth, Cd solubility was increased under both plant species and pea growth increased soluble and

exchangeable Zn, whereas wheat had no effect (Castaldi *et al.*, 2009a). In another study, proportions of carbonate, Fe-Mn and organically bound fractions (of Cu, Pb, Cd and Zn) in the rhizosphere soils were higher than in the bulk soil and the proportions of exchangeable and residual were less (Wang *et al.*, 2009). Wang *et al*., (2009) also found that pH, OM, TN and TP were all significantly higher in the rhizosphere soil compared to bulk soil, which they attributed to the release of root exudates and metabolites. Mertens *et al*. (2007) found that soil pH and organic carbon were significantly different under different tree species after 33 years growth on a contaminated sediment disposal site in The Netherlands. Heavy metals in soil are in an equilibrium and, as some are removed from soil solution by plant uptake or leaching, more will be released into soil solution (Robinson *et al.*, 2006). This may explain why although different amounts of uptake between plants was observed in this study, this was not reflected in the measured soluble fractions of metal in the soil samples.

Independent of plant species, some differences in the behaviour of Cu and Zn were observed. In the current study it was found that the proportion of Cu that remained in solution was much lower than that of Zn, in agreement with the results of Jeyakumar et al. (2008) and McLaren and Clucas (2001) in their studies involving metal salt spiking of biosolids samples. Negatively charged binding sites in the soil, located predominantly on organic matter, clays and oxides of Fe, Mn and Al, bind positive metal ions (Robinson *et al.*, 2006). As the total concentration of trace elements in soil increases, there is a decrease in the soil's ability to absorb further additions due to saturation of chemical binding sites (Robinson *et al.*, 2006), causing more metal to be present in soluble forms. This may explain why the higher proportions of Cu in the present study are located in the organic and oxide bound forms, where negative binding sites are located.

The use of metal salts in investigations of metal contamination of soil as a representation of organic waste contamination has been criticised in the past for a number of reasons. Firstly, metals applied in salt form are known to significantly acidify the soil (Speir *et al.*, 1999b). This was observed in the current experiment at time zero, however, it was noted that the pH values had returned to near control soil values by the one-year sampling time. Secondly, metals in biosolids are strongly complexed with

mineral and organic components, making them less biologically available than those in metal salt form (Speir, van Shaik et al. 2007; Speir 2008). It is suggested that when a soil ecosystem is spiked with metal salts they are subjected to a sudden increase in metals unlikely to occur through biosolids application (Speir *et al.*, 2007). However, for the purpose of this experiment it was considered appropriate to remove the variability that would have been caused by biosolids addition. This was so that the interaction between plants and HM could be easily observed without complication. It is also impossible to achieve HM concentrations in soil at the levels needed without repeat application of contaminated biosolids over long time periods, or applying a large dose that is not conducive to real life practices. Future research may include metal spiked biosolids (or other organic wastes).

6. General Discussion and Conclusion

The experiments in this research were able to closely evaluate the potential of manuka as a remediation species for biosolids-amended land. Results found that components of manuka extracted into water cause an inhibitory response of the *E. coli* lux biosensor and inhibit growth of pathogenic bacteria potentially found in biosolids. This was most evident for aqueous manuka leaf extracts, which significantly inhibited the growth of *S. typhimurium, E. coli 0157, C. perfringins, C. jejuni* and *L. monocytogenes*. In the case of *C. Jejuni,* inhibition of growth was 100% (complete toxicity) when in the presence of only 0.78% manuka leaf extract (dilution of a 5 g into 20 ml extract). These results are both surprising and noteworthy, particularly as a survey of the literature did not provide evidence of such strong inhibition of these bacteria occurring in the presence of manuka products previously. However, this inhibitory response was not replicated in the soil experiment carried out. The nutritional value of the manuka soils (exhibited in soil chemistry and biochemistry) may have masked any antimicrobial activity that many have initially been present. Furthermore, soil samples had been left for some length of time before the experiment was carried out, and antimicrobial agents from manuka may have been degraded over time by soil organisms or enzymes. I believe it is also important to note that in the production of commercial manuka products, as well as the manuka-water extracts used in this research, manuka biomass is mechanically and/or thermally degraded in order to extract the antimicrobial agents from plant cells. It may be that these agents are not readily released through natural biodegradation. Although pathogen die-off appeared unaffected, analysis of microbial community structure (T-RFLP) revealed a difference in both bacterial and fungal community structure (and diversity for fungi) for soils samples that had been exposed to manuka growth for a number of years, relative to a control area under grass. This indicated that plant type has some influence on the microorganisms present in the soil. Whether this is through root exudation, selection for specific microbial groups or species-specific symbiotic relationships, or other factors the observation merits further investigation.

The current results do indicate that further research into the potential use of manuka to manage levels of pathogenic soil microorganisms is warranted. Future research could include the use of various manuka biomass components or water extracts, such as those in Chapter Three, mixed into pathogen-spiked soil samples. Experiments should eventually lead to the inclusion of pathogen-spiked biosolids. This would give a greater understanding of how the nutrients present in biosolids influence pathogen survival in the presence of antimicrobial agents. In addition, studies using live plants grown on spiked soil/biosolids would help to further distinguish the source of antimicrobial activity (for example root exudation vs. leaf fall). However, the current research tentatively suggests that it is the mechanical destruction of plant cells, particularly the leaves, that releases the antimicrobial agents. Alternatively, if roots do release antimicrobial chemicals through root exudation, these may be rapidly degraded in the soil environment. Identification and isolation of the components that caused the observed pathogen die-off in the extracts of Chapter Three would give significant additional insight also.

From the perspective of heavy metal (HM) phytoremediation, experiments in Chapter Five showed that manuka has the ability to grow on HM contaminated soils, a desirable trait for planting on biosolids-amended sites. Although results show that manuka does not reduce the bioavailability of HM in soil, manuka does not increase bioavailability either, which is a desirable trait if stabilisation of metals is the aim of remediation. Manuka also exhibited an increased uptake of Cu, whilst it restricted the uptake of Zn (accumulating it in roots), when compared to two other plant species. Plants in the current experiment were grown for only one year whilst many studies have shown that metal accumulation in plants can occur over longer periods than this, and that eventually accumulation may reach a plateau, or cause plant death. In light of this, future research should include a longer study with greater replication to assess whether concentrations of metals in herbage would continue to increase or if levels would plateau at a maximum level specific to each plant species. It would be interesting to know the maximum levels at which manuka takes up Cu and Zn. Furthermore, conducting this study using a range of HMs to give a complete picture of how manuka reacts differently in the presence of different metals would add further insight. Experiments including HM spiked biosolids also constitute essential future work, as

biosolids alone has an impact on HM availability in soil. The results presented here indicate that manuka can stabilise some metals, highlighted by the fact that it accumulated Cu and Zn in roots. The intricate, shallow, root system of manuka would aid in the containment of these contaminants. These factors, combined with manuka's ability to stabilise erosion prone soils and its use as a regeneration species for native vegetation, makes it an ideal species for land restoration projects including the remediation of biosolids-amended land.

Manuka components have economic value through commercial production of honey and essential oil-related products. This makes establishment of manuka plantations a viable option, and if this can be achieved on otherwise unusable land (due to contamination or degradation), where biosolids can be recycled then this system has added potential to generate an economic return. In a scenario such as this, where manuka is planted for regeneration purposes, or as part of commercial production, the beneficial re-use of biosolids could become a sustainable option. This may occur on a rotational basis to manuka stands, so that biosolids is added to the same site only once in a number of years. This could act both as an aid to enhance growth of manuka, mitigating the need for commercial fertilisers, as well as a means of 'treating' both bacterial and inorganic contaminants in the biosolids. Metals may be stabilised by manuka roots whilst at the same time, continual degradation of leaf fall, or rotational cropping and mulching of manuka biomass, would aid in the attenuation of introduced bacteria, including pathogenic strains. In the current climate where society is ever increasingly conscious of waste reduction and contaminant control, this remediation system may represent an economically, socially and environmentally acceptable solution to the ever-present question of what to do with biosolids

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Appendix 1: Methodology

A1.1 Resuscitation of freeze dried cultures

1) Remove freeze dried stock (in glycerol) from freezer and inoculate a 1ul loop full into three tubes of the relevant resuscitation broth (Table A.1).
2) Grow tubes in conditions according to Table A.1
3) Mix all three tubes together and centrifuge cultures at 2500 rpm for 1 hour
4) Remove supernatant using pipette and re-suspend culture in 3 ml Ringers solution to wash
5) Centrifuge at 14000 rpm for 1 minute.
6) Remove supernatant carefully using pipette and again re-suspend culture in 3 ml ringers solution for storage at 4° C

A1.2 Quantification of Ringers Bacterial Stocks

1) Prepare 10 x 1.5 ml eppendorf microcentrifuge tubes with 900 μl of ringers solution and label from 10^{-1} to 10^{-10}.
2) Add 100 μl of bacterial stock from the fridge to the first eppendorf tube.
3) Dilute samples decimally by transferring 100 ul to 900 ul ringers solution until a ten times serial dilution is achieved (10^{-10})
4) Using a 10 ul pipette, pipette three 10 ul dots of each dilution onto the relevant quantification media (Table A.1.)
5) Incubate plates at 37°C overnight and count colonies.
6) Total bacteria in the stock can be calculated from the lowest dilution that produced countable colonies, and averaging the colonies over the three dots

Bacterial Strain	Resuscitation broth	Growth conditions	Quantification media
Salmonella typhimurium-lux	Luria-Bertani (LB) broth supplemented with 50 µg ml^{-1} of kanamycin (Sigma K1377) and 12.5 µg ml^{-1} of tetracyclin (Sigma T7660)	30° C for 24 hours shaking at 180 rpm	Luria-Bertani (LB) agar supplemented with 50 µg ml^{-1} of kanamycin (Sigma K1377) and 12.5 µg ml^{-1} of tetracyclin (Sigma T7660)
Escherichia coli 0157	Luria-Bertani (LB) broth supplemented with 50 µg ml^{-1} of ampicillin (Sigma)	30° C for 24 hours shaking at 180 rpm	Luria-Bertani (LB) broth (supplemented with 50 µg ml^{-1} of ampicillin (Sigma)
Listeria monocytogenes	Tryptic soy broth (TSB)	37° C for 24 hours shaking at 180 rpm	Tryptic soy agar (TSA)
Campylobacter jejuni	Brucella broth	37° C for 48 hours static. Place in aerojars with microaerobic gas producing packs	5 % sheep blood agar
Clostridium perfringins	Cooked meat broth (with the solid meat portions removed)	37° C for 24 hours static. Place in aerojars with anaerobic gas producing packs	Anaerobic blood agar

Table A1.1. *A break down of resuscitation media and growth conditions required for the resuscitation of frozen glycerol stocks of five pathogenic bacteria*

A1.3 Enumeration of *Salmonella typhimurium* in soil

Adapted from UKWIR, (2000).

Media

Resuscitation media - solidified Tetrathionate Broth.

Add 46 g of Tetrathionate Broth base (Oxoid) and 15 g bacteriological agar to 1 litre of water and bring to the boil whilst stirring. Allow the medium to cool to 50°C and add 50 µg ml^{-1} of kanamycin (Sigma K1377) and 12.5 µg ml^{-1} of tetracyclin (Sigma T7660). Add 10 ml of iodine-iodide solution (6 g iodine, 5 g potassium iodide dissolved in 20 ml sterile DI H_2O) to each litre of agar. Mix and pour into 55 mm plastic petri dishes.

Growth media – Chromagar

Chromagar *salmonella* is made up to the manufacturers instructions and poured into 55 mm plastic petri dishes.

Membrane filtration technique

1) Take three bottles of 10g soil for each treatment from the 16°C incubator. Using a sterile measuring cylinder pour 90 ml of phosphate buffered solutions (PBS) into each bottle and replace cap.
2) Shake bottles on a rotary shaker at 180rpm for 15 minutes.
3) Dilute samples decimally by transferring 1 ml to 9 ml PBS solution until a seven times serial dilution is achieved (10^{-7}).
4) Seperately filter diluted samples through disposable 0.45 µm cellulose nitrate filter units (Nalgene) beginning from the highest dilution and working backwards. Filter each dilution in duplicate: Aseptically place filter papers onto filter base of the manifold. Transfer 1 ml of sample onto the membrane filter and add 10 ml of sterile rinse water to disperse. Apply vacuum until liquid has passed over filter. Add a further 10 ml of rinse water to the manifold to flush away remaining bacteria and apply vacuum.

5) Using sterile tweezers remove filter from housing and place onto the surface of solidified Tetrathionate Agar plates, ensure no bubbles are trapped beneath the filter paper.

Incubation

1) Incubate plates in aerobic condition for 24 hours at 37°C
2) Using sterile tweezers, aseptically transfer membrane filters to Chromagar salmonella plates, ensure no bubbles are trapped beneath the filter paper
3) Incubate plates in aerobic conditions for 24 hours at 37°C

***Salmonella* Enumeration**

1) The total number of colonies in the soil samples were estimated by counting pink colonies on the lowest dilution that produced plate counts of between 20-200 cfu. Averages of the duplicate plates were used to extrapolate total cfu g soil^{-1}

A1.4 Enumeration of *Campylobacter jejuni* in soil

Adapted from Donnison, (2003).

Media

Primary enrichment media – Boltens broth (Fort Richard code#3162)**.**

Boltons broth is purchased premade in 5 ml tubes.

Secondary enrichment media – Campylobacter blood free medium (mCCDA)

The mCCDA base can be purchased from Oxoid (CM739) and made up to the manufacturers instructions. Ingredients and instructions are as follows:

Ingredients	g L^{-1}
Lab-Lemco powder	10.0
Peptone	10.0
Sodium chloride	5.0
Bacteriological charcoal	4.0
Caesin hydrolysate	3.0
Sodium desoxycholate	1.0
Ferrous sulphate	0.25
Sodium pyruvate	0.25
Agar	12.0

Distilled water

1000 ml

pH 7.4 ± 0.2

Suspend 22.75 g of mCCDA agar base in 500 ml of distilled water, mix well and boil to dissolve agar. Sterilise by autoclave (121°C for 15 min). Reconstitute one vial of C.A.T supplement in 2 ml of sterile distilled water, using a pipette to mix. Cool base media to 50°C and add 1 vial of reconstituted C.A.T. selective supplement (Oxoid SR 174E) per 500 ml of mCCDA. Mix and pour into 55 mm plastic petri dishes

Procedure

1) Take three bottles of 10g soil for each treatment from the 16°C incubator. Using a sterile measuring cylinder pour 90 ml of phosphate buffered solutions (PBS) into each bottle and replace cap.
2) Shake bottles on a rotary shaker at 180rpm for 15 minutes.
3) Dilute samples decimally by transferring 1 ml to 9 ml PBS solution until a seven times serial dilution is achieved (10^{-7}), capping ans shaking inbetween.
4) Pre-label the required number of Boltons broth tubes.
5) Inoculate 3 x labelled Boltons broth tubes for each dilution with 1 ml of diluted sample. At lease three tubes per dilution must be inoculated because results for at least three consecutive rows are required to obtain an MPN score.
6) Loosen caps and place Boltons tubes into an airtight container with an adequate number (based on packet instructions) of Campygen sachets (Oxoid) to create a microaerobic environment.
7) Place containers into a 37°C incubator for 48 hours
8) Pre-label the required number of mCCDA agar plates.
9) Remove tubes from incubator and streak each tube onto half a plate of mCCDA agar using a 1 μl inoculating loop.
10) Re-seal plates inside the airtight container with required number of Campygen sachets.
11) Incubate container at 37°C for 48 hours.

A1.4.4 *Campylobacter* enumeration

1) The total number of colonies in the soil samples are estimated by presence/absence scores of campylobacter colonies at each dilution, and subsequently using an MPN table to extrapolate total cfu g $soil^{-1}$.
2) *Campylobacter* colonies appear as flat, moist, grey-white coloured colonies with irregular margins on mCCDA agar. An additional

confirmation step may be required when looking for indigenous bacteria in samples that have not been inoculated with known *Campylobacter Sp*. For additional confirmation tests see Donnison, (2003)

A1.5 Sequential extraction procedure

Adapted fom Tessier et al. (1979) and Mclaren and Clucas (2001)

Soil samples for analysis are air-dried overnight and thoroughly mixed by quartering, before being ground using a 250 um sieve and mortar and pestle. Soil samples are then subjected to five extraction steps as follows.

1) Soluble and Exchangeable:

- Weigh 1 g of air-dried ground soil into a 50 ml polypropylene centrifuge tube.
- Add 8 ml of magnesium nitrate (1 M $MgNO_3$, pH 7.0) to the tubes and shake on a rotary shaker for 1 hour.
- Centrifuge tubes at 4500 rpm for 30 minutes. Filter supernatant into 25 ml volumetric flasks.
- Add 8 ml deionised water to the soil residue from 1b and shake well.
- Centrifuge again at 4500 rpm for 30 minutes and filter again into the same 25 ml volumetric flask
- Rinse filter papers with a little deionised water and make flasks up to 25 ml
- Determine Zn and Cu concentration in the resulting solution by flame atomic absorption spectrometry (FAAS)

2) Specifically sorbed (bound to carbonates):

- To the residue from step 1, add 8 ml of 1M sodium acetate (adjusted to pH 5.0 with acetic acid) and shake for 5 hours.
- Centrifuge tubes at 4500 rpm for 30 minutes and filter supernatant into 25 ml volumetric flasks
- Add 8 ml deionised water to the soil residue from 2.b and shake well.

- Centrifuge again at 4500 rpm for 30 minutes and filter again into the same 25 ml volumetric flask
- Rinse filter papers with a little deionised water and make flasks up to 25 ml
- Determine Zn and Cu concentration in the resulting solution by flame atomic absorption spectrometry (FAAS)

3) Oxide bound (bound to Fe and Mn oxides):

- To the residue from step 2, add 20 ml of 0.04M hydroxylamine hydrochloride ($NH_2OH.HCl$, in 25% v/v acetic acid) and mix gently.
- Place on digest block at 96°C, and leave for 6 hours with occasional agitation.
- Centrifuge tubes at 4500 rpm for 30 minutes and filter supernatant into 50 ml volumetric flasks
- Add 8 ml deionised water to the soil residue from 2.b and shake well.
- Centrifuge again at 4500 rpm for 30 minutes and filter again into the same50 ml volumetric flask
- Rinse filter papers with a little deionised water and make flasks up to 50 ml
- Determine Zn and Cu concentration in the resulting solution by flame atomic absorption spectrometry (FAAS)

4) Organic bound

- To the residue from step 3, add 8 ml of 30% hydrogen peroxide (H_2O_2 adjusted to pH 2.0 with HNO_3), add 2 mls at a time to prevent overflow and mix gently.
- Place on digest block at 85°C, and leave for 2 hours with occasional agitation.
- Add a further 3 ml H_2O_2 (pH 2.0) and digest at 85°C for 3 hours
- Cool sample and add 5 ml 3.2 M ammonium acetate (NH_4OA_c) in 20% v/v in HNO_3.
- Make up to 20 ml with deionised water and shake on rotary shaker for 30 minutes.

- Centrifuge tubes at 4500 rpm for 30 minutes and filter supernatant into 50 ml volumetric flasks
- Add 8 ml deionised water to the soil residue from 2.b and shake well.
- Centrifuge again at 4500 rpm for 30 minutes and filter again into the same 50 ml volumetric flask
- Rinse filter papers with a little deionised water and make flasks up to 50 ml
- Determine Zn and Cu concentration in the resulting solution by flame atomic absorption spectrometry (FAAS)

5) Residual: Based on the method of Kovacs et al. (2000).

- Dry the tube, including the residue from step 4 in an oven at 105°C
- Finely grind sediment and weigh 0.5 g into clean 50 ml polypropylene centrifuge tubes. Include one blank and one known standard sample with each run.
- Add 5 ml of 65% HNO_3 to the tube and digest for 30 minutes at 60°C using a dry heat block.
- Add 5 ml H_2O_2 and increase the temperature to 120°C
- Digest for a further 270 minutes with occasional agitation.
- Dilute samples to 50ml with deionised water in glass volumetrics and filtered before analysis by flame atomic absorption spectrometry (FAAS)

A1.6 Multiplex T-RFLP method for assessing microbial community structure.

Method details courtesy of Dr Rachel Parkinson (Parkinson et al., 2010; In Press).

Sub-samples of 0.3 g of each soil sample were weighed into DNA extraction tubes and stored at -80°C. Tubes containing the soil sub-samples were removed from storage and allowed to defrost on ice before processing. Bulk DNA was extracted using the Bio101 FastDNA Spin Kit for Soil (QBIOgene) as per the manufacturer's instructions. Total DNA was quantitated using the PicoGreen dsDNA Quantitation Kit (Molecular Probes Inc.).

Primer Pairs	Target	Sequence (5' to 3')	Reference
HEX-F63	Bacterial 16S	AGGCCTAACACATGCAAGTC	(Marchesi et al., 1998)
R1087		GTTGCGGGACTTACCCC	(Hauben et al., 1997)
FAM- ITS1	Fungal ITS region	CTTGGTCATTTAGAGGAAGTAA	(Gardes and Bruns., 1993)
ITS4		TCCTCCGCTTATTGATATGC	(White et al., 1990)
NED-Ar3F	Archaea	TTCCGGTTGATCCTGCCGGA	
Ar927R		CCCGCCAATTCCTTTAAGTTTC	(Giovannoni et al., 1988) (Jurgens et al., 1997)
PET-Rhiz1244R	Rhizobia	CTCGCTGCCCACTGTCAC	
F63		AGGCCTAACACATGCAAGTC	(Tom-Petersen et al., 2003) (Marchesi et al., 1998)

Table A1.2 *Primers used for T-RFLP analysis.*

Terminal Restriction Fragment Length Polymorphism (T-RFLP) analysis was performed separately using the extracted DNA. T-RFLP is commonly used to

profile microbial communities. Because T-RFLP allows the examination of non-culturable organisms (Thies, 2007), it enables investigation of the effects of environmental factors on whole microbial community structure. Two multiplex PCR reactions were performed for each sample, each using two primer pairs (Table 1). The first amplified target DNA from the bacterial 16S gene and the fungal ITS region. The second amplified regions from archaea and rhizobia. Each reaction contained 25 µl 2x Multiplex PCR Mastermix (Qiagen); 200 nM each primer; 1 µl Q solution (Qiagen); 20 ng template DNA; and DNase-free H_2O to a total volume of 50 µl.

Reactions were heated to 95°C for 15 minutes, followed by 30 cycles of 94°C for 30 seconds, 56°C for 45 seconds, 72°C for 90 seconds and a final extension of 72°C for 10 minutes using a Palmcycler (Corbett Life Science) thermocycler.

The products from the two multiplex PCR reactions were combined and purified using a QIAquick PCR Purification Kit (Qiagen). Restriction enzyme digestion was performed using *Msp*I (Roche Molecular Biochemicals) as per the manufacturer's instructions with a 3 hour incubation at 37°C followed by a 20 minute deactivation at 60°C. The MinElute Enzymatic Reaction Cleanup Kit (Qiagen) was used as per the manufacturer's instructions to remove components of the enzymatic reaction prior to fragment analysis.

The resulting T-RFs were analysed by the Allan Wilson Centre for Biodiversity (Palmerston North) using an ABI3700 Genetic Analyser (Applied Biosystems). The raw T-RFLP profile data generated were collated and analysed using Genemapper v.4 software (Applied Biosystems). Only T-RFs that fell within the accurate sizing range (50-500 bp) were included in analysis. Any T-RFs with fluorescence units of less than 100 were also discarded to minimise the effect of artefacts, as were any T-RFs that contributed less than 1% of total fluorescence after exclusion of those under 100 fluorescence units.

Multi Dimensional Scaling (MDS) analysis was performed using PRIMER 6 (Plymouth Marine Laboratory) software (Clarke, 1993). Data were square root transformed. A Bray-Curtis Similarity matrix was constructed for each dataset and the MDS plot generated from this, using 50 restarts to ensure the best solution, was presented. Statistical significance of groupings was performed using the ANOSIM function in PRIMER. Multi Dimensional Scaling was used to examine the similarity between DNA profiles. This method allows profiles to be plotted spatially, depending on how similar they are to each other and is based on a similarity matrix of all the profiles.

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Appendix 2: Media recipes

A2.1 Ringers Solution

Ingredients	**g L^{-1}**
Sodium chloride	2.15
Potassium chloride	0.075
Calcium chloride $6H_2O$	0.12
Sodium thiosulphate $5H_2O$	0.5

Dissolve ingredients in 1000 ml of deionised water and sterilise by 121°C for 15 minutes

A2.2 Bolton Broth

Bolton Broth Base (Oxoid CM0983)

Ingredients	**g L^{-1}**
Meat peptone	10.0
Lactalbumin hydrolysate	5.0
Yeast Extract	5.0
Sodium chloride	5.0
Alpha-ketoglutaric acid	1.0
Sodium pyruvate	0.5
Sodium metabisulphite	0.5
Sodium carbonate	0.6

Haemin	0.01
pH 7.4 ± 0.2 @ 25°C	

Bolton Broth Selective Supplement (Oxoid SR0183)

Ingredients	**g L^{-1}**
Cefoperazone	20.0mg
Vancomycin	20.0mg
Trimethoprim.	20.0mg
Cycloheximide	50.0mg

Directions: Add 13.8g of Bolton Broth to 500ml of distilled water. Sterilise by autoclaving at 121°C for 15 minutes. Cool to 50°C. Aseptically add 25ml Laked Horse Blood and 1 vial of Bolton Broth Selective Supplement, reconstituted in 2 ml sterile deionised water. Mix well and distribute into sterile screw top tubes.

A2.3 Phosphate Buffered Saline (PBS)

Ingredients	**g L^{-1}**
Sodium chloride	8.0
Potassium chloride	0.2
Disodium hydrogen phosphate	1.15
Potassium dihydrogen phosphate	0.2
pH 7.3	

Directions: Dissolve in 1 l of distilled water and autoclave for 10 minutes at 115°C.

A2.4 Rinse water

Ingredients	**g L^{-1}**
Sodium chloride	8.5
Potassium dihydrogen phosphate	0.3
Sodium hydrogen phosphate	0.6

Directions: Adjust to pH 7.0 and sterilise by autoclave. Once cooled add 5 ml of sterile 4% MgCl2

A2.4 Tryptic Soy Broth (TSB) (Oxoid CM0876)

Ingredients	**g L^{-1}**
Pancreatic digest of casein	17.0
Papaic digest of soybean meal	3.0
Sodium chloride	5.0
Dibasic potassium phosphate	2.5
Glucose	2.5
pH 7.3 ± 0.2	

Directions: Add 30 g to 1 litre of distilled water, mix well and distribute into tubes. Sterilise by autoclaving at 121°C for 15 minutes.

A2.5 Luria-Bertani (LB) broth

Tryptone	10
Yeast extract	5
Sodium chloride	10

Directions: Dissolve in 1000 ml deionised water and sterilise by autoclaving at 121°C for 15 minutes.