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**Interactions between *Escherichia coli* and the New Zealand native
plants *Leptospermum scoparium* and *Kunzea robusta***

A Dissertation
submitted in partial fulfilment
of the requirements for the Degree of
Doctor of Philosophy
at
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by
Minakshi Mishra

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Abstract of a Dissertation submitted in partial fulfilment of the requirements for the Degree of Doctor of Philosophy.

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Leptospermum scoparium and *Kunzea robusta*

by

Minakshi Mishra

Elevated concentrations of human pathogens in waterways is a global environmental issue and can result in widespread human disease and suffering. The provenance of these pathogens is usually from biological wastes, specifically faecal material from humans and livestock. In New Zealand, *Escherichia coli* is used as a reference/indicator organism to indicate pathogen contamination in waterways, with the drinking water standard set at < 1 colony forming unit (cfu)/100 ml and < 260 cfu/100 ml for swimming, following weekly testing. In 2017, the media reported that over half of NZ's rivers are unfit for swimming and in 2016, livestock contaminated drinking water with *Campylobacter jejuni* resulted in some 5500 people becoming ill in Havelock North, the largest recorded *Campylobacter* outbreak in world history.

In 2016, a study showed that the NZ native plants *Leptospermum scoparium* (J.R. G.Forst.) and *Kunzea robusta* (A. Rich.) increased the decimal reduction time of *E. coli* in biosolids amended soil by 90% within five to eight days of bacterial inoculation. Other studies have shown that the antibacterial properties of these plants (with their essential oils and honey) extend to *Candida albicans* and *Salmonella typhimurium*. Potentially, *L. scoparium* and *K. robusta* could be planted in areas receiving human or animal wastes to intercept human pathogens before they enter waterways. However, the mode of action by which these plants increase microbial die-off is unclear. Nor is it understood how these plants affect bacterial fluxes in field conditions, where soils may receive high rates of rainfall or irrigation. This thesis aimed to determine the antimicrobial properties of the roots and leaves of *L. scoparium* and *K. robusta* and to determine whether the provenance of the plants can affect antimicrobial activity. Furthermore, the thesis sought to determine the fluxes of bacteria in soils planted with *L. scoparium* and *K. robusta*. Throughout the thesis, *E. coli* was used as an indicator organism for pathogens. *Lolium perenne* (L.) was used as a comparison plant, as this species is present in 70% of NZ pasturelands.

Laboratory experiments, using nutrient broth and optical density measurements, revealed that after 24 hours, water extracts (containing 50 – 400 mg/l total organic carbon) from *L. scoparium* and *K. robusta* reduced *E. coli* ATCC13706 growth to just 13% - 25% of the control (no extract). In contrast, *L. perenne* leaves extracts significantly increased the growth of bacteria by 63% compared to the control. Root extracts of *L. scoparium* and *K. robusta* inhibited growth more strongly than the leaf extracts. There were significant differences in the inhibitory activities of plants from different provenances, indicating that either genetic or environmental conditions can affect the antimicrobial activities of these species.

Glasshouse experiments using repacked 10 l lysimeters showed that under high flow conditions (14 mm/day), both *L. scoparium* and *K. robusta* exacerbated leaching of *E. coli* compared to *L. perenne*. This was attributed to increased preferential flow caused by the taproot systems of these species. Under low-flow conditions (< 8 mm/day), there was negligible bacterial leaching under all species tested. The examination of soil pore water under *L. scoparium* and *L. perenne* revealed that seven days after inoculation, *E. coli* numbers under *L. scoparium* were significantly lower (1.3×10^3 cfu/ml) than under *L. perenne* (5.9×10^3 cfu/ml). Similar results were obtained for two soil types used: A pallic brown soil (Pawson Silt Loam) and a recent soil (Lismore stony silt loam), but with higher overall leaching from the stony silt loam.

A field experiment, near Duvauchelle on the Pawson silt loam also showed decreased *E. coli* numbers under *L. scoparium* and *K. robusta*, nine days after the inoculation. In contrast, there was little die-off under *L. perenne*. However, this experiment did not reveal whether the disappearance of *E. coli* under *L. scoparium* and *K. robusta* was due to increased die-off or increased preferential flow that removed the *E. coli* from the top soil.

This thesis shows that in some cases, strategic plantings of *L. scoparium* and *K. robusta* may improve the quality of NZ's freshwater resources. The efficacy of planting these species is dependent on the provenance of the plants and the climatic conditions. Although *L. scoparium* and *K. robusta* increased leaching of *E. coli* under high-flow conditions, this may be beneficial in some cases, such as the protection of surface waterways where the trees may result in increased infiltration and decreased surface run off of bacteria. Future work should focus on determining the effect of plants age and rhizosphere chemistry on microbial properties and elucidating whether the regional differences between *L. scoparium* and *K. robusta* are due to the genetic or environmental differences.

Keywords: *Leptospermum scoparium*, *Kunzea robusta*, dairy shed effluent, soil amendments, leaching, lysimeter, preferential flow, pathogens, rhizosphere, antimicrobial properties.

List of Abbreviations

ATCC = American Type Culture Collection

BA = Benzoic acid

CFU = Colony forming units

DOC = Department of Conservation

DSE = Dairy shed effluent

DST = Defined Substrate Technology

ESR = Institute of Environmental Science and Research Ltd.

GH = Glasshouse

JA = Jasmonic acid

LU = Lincoln University

MGO = Dicarbonyl methylglyoxal

MPN = Most probable number

MS Media = Murashige and Skoog's media

MUG = Methylumbelliferyl-D-glucuronide

NA = Nutrient Agar

NB = Nutrient Broth

NI = North Island

NZ = New Zealand

OD = Optical density

OM = Organic matter

ONPG = o-nitrophenyl galactopyranoside

SA = Salicylic acid

SI = South Island

TOC = Total Organic Carbon

UMF = Unique Mānuka Factor

VOC = Volatile organic compound

WHO = World Health Organization

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Chapter 1

Introduction

Increasing incidences of water and food contamination and their direct or indirect association with agricultural wastes has drawn attention towards current waste management practices (Gagliardi & Karns, 2000; Hutchison et al., 2004; Jiang, 2008; Lowman et al., 2013). Dairy farming contributes to 7% of the world's agricultural production (Fyfe et al., 2016). Since 2000, dairy farming activities have become an increasing area of interest in New Zealand (NZ) and is the second largest sector of NZ's international trades bringing economic benefits to the country (NZ \$ 12.8 billion in 2015-16) (Hahner et al., 2014; Statistics New Zealand, 2017).

In NZ, water is a vital resource as a wide range of production is based on agriculture and hence any added contamination puts this at risk and needs immediate attention (Prosser, 2011). *Escherichia coli* > 1 colony forming units (cfu)/100 ml, indicates the presence of other potential pathogens in water samples and is a definite indication of recent faecal contamination (Ministry of Health, 2000, 2016). *Escherichia coli* 0157, at concentrations of just 50 - 100 cfu/ml, can result in human infections (Chart et al., 2000). A media report in 2012 stated that > 52% of the NZ's rivers are absolutely unsuitable for swimming due to faecal contaminations and only 20% rivers were graded as good quality (NZ Herald, 2012). The gastroenteritis outbreak in 2016 in Havelock North, NZ was the result of drinking water contamination with *Campylobacter* spp. sourced from sheep faeces (Department of Internal Affairs, 2017).

Grazing animals harbour infectious organisms in their intestines and their faeces contain pathogens such as bacteria (e.g. *Salmonella* spp., *Campylobacter* spp.), protozoans, helminths and viruses (e.g. Noravirus and Rotavirus) (Ding et al., 2017; Jiang et al., 2010; Sterritt & Lester, 1980). Depending on the available facilities and size of the farm, it is a common practice to herd 600 to 1000 cows and approximately 35 kg of excreta is produced daily by each cow, depending on their health and feed quality and quantity which also affects effluent composition (Jones, 1999; McGarvey et al., 2004). Animals excrete high volumes of nutrients and pathogens in their excrement (Kumar et al., 2005). This study focused on Dairy Shed Effluent (DSE), which is the wash down water from milking shed and a mixture of animal faeces, organic matter, effluent, spoiled milk, pharmaceuticals, sediments, toxins and a large concentration of microorganisms including pathogens (Cools et al., 2001; Dipu et al., 2011; Houlbrooke et al., 2010b; McLeod et al., 2008). The Canterbury region in the South Island is becoming intensively farmed with dairy cattle. It contains 60% of the irrigated land area in NZ (Cichota et al., 2016; Jiang, 2008). Most of the DSE application is practiced in grasslands or pastoral lands with a little

contribution to forestry and horticulture (Sterritt & Lester, 1980). Presence of stony soil in this region increases the potential of bacterial leaching under irrigated land and hence the risk of groundwater contamination (Cichota et al., 2016). Annual DSE production in NZ varies between 50 -70 million m³ and this large amount of faecal production also demands proper storage and management facilities (Monaghan & Smith, 2004). This study focused on the effluent only, commonly used for the land application with low dry matter content and sediments.

Following the (Resource Management Act, 1991), DSE discharge into water streams has been discontinued (Houlbrooke et al., 2010b). Since 1991 under the RMA, land application of DSE is a permitted activity in NZ and uses land in a manner that does not significantly negatively affect aquatic life (Resource Management Act, 1991). Current management practices regulate N application rate to 150 - 200 kg N/ha/year (DairyNZ Auckland, 2012; Waikato Regional Council, 2015), although they do not consider bacterial numbers (Houlbrooke et al., 2010a; Jiang et al., 2002). The land application of DSE represents beneficial reuse of a biological waste, which is also a substitute for conventional chemical fertilizers and irrigation water (Gagliardi & Karns, 2002). In general, DSE that is applied to land is untreated due to the expense of treatment methods, such as of oxidation ponds (Butler et al., 2017; McGarvey et al., 2004). Treatment methods such as DSE storage or oxidation ponds generally do not reduce pathogens due to high nutrient and sediment concentration of the DSE working as the surviving agents for pathogens and as a main factor to increase their numbers (Cameron & Trenouth, 1999). Contamination of fresh water resources can occur from surface run off and/or leaching of land applied pathogens through macro (> 30 µm) or micropores (< 30 µm) in soil (Feeney et al., 2006; Hutchison et al., 2004; McLeod et al., 2003; Viau & Peccia, 2009).

If managed properly, groundwater contamination by the introduced DSE microbial contaminants can be minimised (Ibekwe et al., 2003). Much research has focussed on demonstrating the pros and cons of DSE application to land (Fyfe et al., 2016; Galvin et al., 2010) however, there is only limited research that has focused on the role of vegetation in the receiving environment that may reduce pathogenic loads (Dipu et al., 2011; Prosser et al., 2016). Inappropriate DSE management can cause human diseases following leaching or surface run off of microbial contaminants (Kacprzak et al., 2014a; Kacprzak et al., 2014b; Sterritt & Lester, 1980).

Several studies have investigated bacterial leaching associated with the DSE land application and the potential risk of groundwater contamination with this practice (Aislabie et al., 2011; Brennan et al., 2010; Hutchison et al., 2004; McLeod et al., 2008; McLeod et al., 2003; Mubiru et al., 2000). However, there is a lacuna of information on the role of plants in bacterial leaching. Vegetation can be a principal and cost-efficient way of treating pathogens by using plants antimicrobial activities (Bora et al., 2016; Calo et al., 2015; Dipu et al., 2011; Dorman & Deans, 2000). It has gained popularity with its efficiency

against antibiotic resistant microbial strains and pollution control benefits (Garcha et al., 2016; Neralla & Weaver, 2000; Zhang et al., 2016). Plants with antimicrobial activity release a variety of root exudates in soil against unwanted microbes, which can possibly be utilised for pathogen attenuation without creating any further risk for humans or animals health or for the environment (Abhilash et al., 2012; Mendes et al., 2013).

Native NZ plant species such as *Leptospermum scoparium* J.R.G. Forst. (Allan, 1961) and *Kunzea robusta* A. Rich. (Connor, 1985) have recently been shown to reduce bacterial numbers, including *Salmonella* sp., *Typhimurium* sp., *Campylobacter* sp. (Prosser, 2011; Prosser et al., 2016). Both species belong to Myrtaceae family which is one of the most important plant families in tropical forests and plays important role in soil management with their intensive root systems (Watson & Mardern, 2004; Watson & O'Loughlin, 1985). These plants are hardy in nature, tolerate a wide range of environmental conditions and are therefore, widely used in land rehabilitation projects (Hahner et al., 2014; Lu et al., 2013; Tomblin et al., 2014). Both *L. scoparium* and *K. robusta* are known for their antimicrobial properties and recognised for production of biologically active compounds (reviewed in Chapter 2) (Killeen et al., 2015b; Whitehead et al., 2004). Leaves, bark, seed puree and sap of these plants have been widely used traditionally to treat minor injuries and ailments (Chen & Nelson, 2012; Lu et al., 2014). Essential oils and honey from these plants also have recognised *in vitro* activities against various pathogens including *Staphylococcus aureus*, *Helicobacter pylori* and *Candida albicans* (Douglas et al., 2004; Patton et al., 2006; Perry et al., 1997a; Porter et al., 1998; Tomblin et al., 2014). Various studies have reported increasing use of high value *L. scoparium* and *K. robusta* products such as honey and essential oil, in pharmaceuticals and cosmetics (Song et al., 2013; Zomorodian et al., 2015) due to their antimicrobial properties (Adams et al., 2008; Wyatt et al., 2005). *Leptospermum scoparium* plantations are an effective means to increase economic returns from low fertility soil (Fitzgerald, 2012; Lis-Balchin et al., 2000; Watson et al., 1997). The honey is well known for its medicinal importance and antibacterial properties (Lu et al., 2014) and contributes \$ 281 million each year to the honey industry of NZ (NZ Herald, 2016). Hence, these trees are biologically, medically, and also economically beneficial for NZ society (Robinson et al., 2003; Whitehead et al., 2004). Numerous studies have demonstrated the antimicrobial nature of the honey of *L. scoparium* and *K. robusta* (Chan et al., 2013; Patton et al., 2006; Tomblin et al., 2014) as well as the essential oil (Park et al., 2017; Perry et al., 1997a; Perry et al., 1997b; Song et al., 2013; Van Vuuren et al., 2014). This led to a single research programme (Prosser et al., 2016) that demonstrated increased die-off of *E. coli* in biosolids amended soil under *L. scoparium* and *K. robusta*.

This thesis investigated the antimicrobial potential of *L. scoparium* and *K. robusta* against the bacterial indicator species *E. coli*, determining leaching potential of the model bacterium *E. coli* ATCC13706 and DSE applied *E. coli*, under different soil and irrigation systems using a combination of laboratory,

glasshouse and field studies. The overall aim of this study was to investigate relative effectiveness of *L. scoparium* and *K. robusta* to attenuate bacterial pathogens applied to the soil. Based on the previous study by Prosser et al. (2016) it was hypothesised that the antimicrobial components from the root and shoot system of *L. scoparium* and *K. robusta*, might end up in soil reducing *E. coli* survival and leaching to the groundwater.

The objectives of this study were to determine:

- the relative antibacterial activity of roots and leaves extracts of *L. scoparium* and *K. robusta* compared to *Lolium perenne* (L.), a grass species present in 70% of NZ pasturelands
- the antimicrobial properties of *L. scoparium* and *K. robusta* sourced from different locations in the South Island
- the fluxes of *E. coli* through soil planted with *L. scoparium*, *K. robusta* and *L. perenne* in contrasting soil types and contrasting rates of irrigations
- the persistence of *E. coli* under *L. scoparium*, *K. robusta* and *L. perenne* under field conditions
- the situations in which *L. scoparium* and *K. robusta* may be effectively employed to improve the quality of NZ's freshwater resources

1.1 Chapter outlines

Chapter 2 provides a detailed literature review on the existing knowledge of dairy farming in NZ, DSE production and storage and the related pros and cons concerning its application to land. The chapter presents details of current research on microbial transport and survival in soil, focussing on *E. coli*. Furthermore, the antimicrobial potential of plant species against pathogens is also described focussing on *L. scoparium* and *K. robusta* and the combination of DSE and antimicrobial plants together as a solution towards land rehabilitation projects.

Chapter 3 presents the results of the laboratory studies performed to determine the potential antimicrobial activity of *L. scoparium* and *K. robusta* against a selected *E. coli* laboratory strain, using plants extracts and root exudates.

Chapters 4, 5 and 6 extend the results of the *in vitro* study outlined in Chapter 3 under *in vivo* conditions. These chapters detail three glasshouse experiments determining *E. coli* leaching under *L. scoparium* and *K. robusta* in two different soil systems using different irrigation rates.

Chapter 7 presents the results of a field study investigating the persistence of *E. coli* in soil under *L. scoparium* and *K. robusta*.

Chapter 8 summarises the main results of the previous chapters and presents the overall conclusions of this study. Management practices are suggested for future studies.

Chapter 2

Literature Review

2.1 Background

Dairy farming is a globally recognised industry contributing 7% of global agricultural production (Avery et al., 2004; Fyfe et al., 2016). It is an important industry in NZ (contributing NZ \$ 2.8 billion in 2015-16) as well as in Australia, India, Canada, UK, Europe and the USA (Groot & Van't Hooft, 2016; Jones, 1999; Singh et al., 2016; Statistics New Zealand, 2017). This intensively growing market is also resulting in effluent management issues, which has been recognised as a serious risk for fresh and ground water resources (Banning et al., 2002; Unc & Goss, 2003). With increasing incidences of disease outbreaks and their association with agricultural practices, people now are more aware about proper waste disposal and management, and this brings challenges to government and management authorities (Hutchison et al., 2004).

2.2 Dairy farming: A global view

Dairy farming is practised at small (one to two cows) or large scale (1000 or more cows per herd) worldwide (Garcha et al., 2016). The type of dairy animals, their health, feed provided, weather, government policies and farm management systems differ in each country and so too does their effluent management system (Fyfe et al., 2007). The type of waste treatment depends on the effluent volume, quality and other farm management practices (Longhurst et al., 2000). In Australia, dairy farming is an important industry in rural areas and is largely pasture based with the average herd size of 268 cows (Fyfe et al., 2016). In 2013-14 Australian milk production was 9.2 billion litres, adding A\$ 3.2 billion (1%) to Australia's economy (Dairy Australia, 2014). However, in developing countries such as India, dairy is an unorganised sector where small stakeholders with 1-2 cows run 80% of the total dairy business and only 20% is owned by organised government authorities (Singh et al., 2016). Because a large part of the industry is unregulated, cow faeces are generally dumped onto nearby ground and liquid or effluent is directly washed into the sewage system following washing of milking yards (Garg et al., 2015). Organised sectors focus on using animals waste in biogas production as 1 kg of animal faeces can generate approximately 37 l of biogas at 20 to 30°C (Singh et al., 2016). Land application of effluent is not an option in India due to high temperature, humid weather conditions and any water pooling incidences in the unregulated dairy sectors increasing risk of mosquitoes (Sharma & Bhargav, 2012). Soil injection i.e. injecting animals wastes below the topsoil surface is commonly used in European countries such as the Netherlands, but is not practiced in NZ (Houlbrooke et al., 2010b). In Ethiopia and India, dried animal dung, generally from cows and buffaloes, is also used

as a source of fuel for cooking in rural regions (Groot & Van't Hooft, 2016). In California, USA, farms carry nearly 1000 cows (McGarvey et al., 2004) and effluent management follows national wastewater discharge standards permitted by the States and Environment Protection Agency (United States Environmental Protection Agency, n.d.).

2.2.1 Dairy farming in NZ: Economic opportunities and environmental risks

In 2017, dairy farming was the second largest export earner and the largest agricultural industry in the country (Jiang, 2008). In 2015-2016, dairy exports contributed to NZ \$ 12.8 billion, which was 26% more than all other export sectors in NZ and valued at NZ \$ 2500 per milking cow (Statistics New Zealand, 2017). Dairy farming expanded dramatically in NZ between 1993 to 2003 and during those 10 years a 44% increase in dairy activities and production took place increasing the national cow count to 4 million (Houlbrooke et al., 2010b). With expanding dairy farming, stocking rates have also increased to 2.5 cows/hectare (Houlbrooke et al., 2010a). There has been a dramatic increase in dairy farming in the Canterbury region of the South Island. Canterbury has > 60% of the total irrigated land and the total cow count has increased to 1,386,993 cattle in 2015, increasing the national cattle count to 6.6 million between 2015 and 2016 (Statistics New Zealand, 2017). It is now a common practice to carry 3-17 cows per hectare and nearly 500 to 1000 cows per farm depending on the size of the farm (Jiang, 2008). Intensification is not limited to increasing number of cattle per farm but also increasing number of farms around the country (Jiang, 2008). New dairy farms are now being established in lands that were under flood irrigation initially or were under forestry. These lands are less suitable for dairy production due to the elevated probability of macropore formation, which increases the groundwater contamination risk (Artz et al., 2005). In 2016, the area of exotic forest harvested for dairy farm establishment was 47, 000 hectares (Statistics New Zealand, 2016). The land that is set aside for applications of dairy effluent in farms should be at least 3 ha per 100 cows and no less than 10% of the total farm area (Houlbrooke et al., 2003).

2.3 Dairy shed effluent (DSE) and common management practices

High animal stocking rates result in high volume of nutrient rich dairy shed effluent (DSE) (Banning et al., 2002). New Zealand dairy farms generate 40 to 70 million m³ of DSE annually and appropriate storage and management of such high volumes is a challenge (McLeod et al., 2008). The storage and treatment processes of DSE vary slightly depending on farms size and available facilities (Monaghan & Smith, 2004). Pond treatment is common to manage small quantities of effluent in farms and also helps in reducing the sediment concentration and increases breakdown of organic matter by the residing microbial population before effluent irrigation onto farmland (Fyfe et al., 2016; Ibekwe et al., 2003). However, DSE storage in unlined ponds presents a great risk for groundwater quality through bacterial leaching (Craggs et al., 2003). Large farms also use lagoon systems to store and manage high DSE

volumes and these lagoons can store 10 to 60 million l of effluent (McGarvey et al., 2004). Himathongkham et al. (1999) suggested animal waste storage at 37°C for 45 days to achieve a 5 log reduction in *E. coli* population.

Traditionally in the 1950's, straw beddings were used to treat and compost animal excreta, before applying it to fields (Fyfe, 2013). The method was efficient in decreasing the initially high microbial concentration in DSE due to increased temperature during composting, reducing the risk of microbial contamination. Tallon et al. (2007) suggested composting of animal manure to reduce pathogenic load, however this is not a practical option for effluent containing low dry matter content (Aislabie et al., 2006). Another common traditional DSE management system in NZ includes two ponds system. This system has two separate ponds in which the first pond is anaerobic where organic matter is digested using anaerobic fermentation and the second pond is a facultative pond with larger surface area. In the facultative pond, the bottom layer is anaerobic and the top layer is aerobic, together decreasing the pathogenic microbial population (Sukias et al., 2001). The treated effluent was then directly released into the waterways (Craggs et al., 2003). With the introduction of the Resource Management Act (1991) changes have been made and effluent discharge into water bodies has been discontinued in NZ to avoid its adverse effect on aquatic life and on water resources. Effluent discharge into water bodies is now an illegal or discretionary activity in NZ unless a prior written consent has been obtained (Resource Management Act, 1991). Māori (indigenous people of NZ), advocate that wastes to be recycled on land and not to be discharged into water resources (Cameron & Trenouth, 1999). This is also recommended by the RMA to NZ regional councils to recognise Māori cultural and spiritual values (Houlbrooke et al., 2010a).

Although management and regulations vary between NZ regional councils, all councils agree on guidelines with regards to land application of DSE (Jiang, 2008). Dairy shed effluent can be used as a soil conditioner and as a fertilizer for crops and plants and in this way can reduce 12% of farm fertilizers expenses (Longhurst et al., 2000). Although with variable DSE composition some additional P amendments are required (Fyfe et al., 2016; Ibekwe et al., 2003). Land application of DSE is a good option to recycle a non-desirable waste fulfilling nutrients and water irrigation requirements for cropping or plant growth (Gagliardi & Karns, 2002). Currently, DSE application is limited to pastoral lands and is now a permitted activity (follows guidelines and requires consents) in NZ (Resource Management Act, 1991). Ideally, the land application of DSE should occur in summer with effluent stored for the rest of the year; however, this is seldom achieved in practice (Cools et al., 2001; Hutchison et al., 2004). Land application of DSE has its own drawbacks as it results in the addition of microbial contaminants to the soil, some of which can be potentially pathogenic to humans and animals (Monaghan & Smith, 2004). Pathogenic concentrations and their variability (species) in DSE

depend on various factors such as animal species, health, age of animals, quality of feed and water provided, farm management practices and use of antibiotics (Hashsham et al., 2004). As bacteria need basic nutrients such as C, N, O (depending on facultative, aerobic or anaerobic bacteria), some trace elements, a pH of 4 – 5 and a temperature < 40° C to survive (Berg, 2004), high concentrations of organic matter and nutrients in DSE provides a conducive environment for microbial growth and activity (Garcha et al., 2016).

2.4 DSE source, composition and contaminants

On average, dairy cows excrete 35 kg of effluent per day (Cameron & Trenouth, 1999; McGarvey et al., 2004). While milking, cows spend nearly 2 hours in the milking yards or effluent collection areas which allows only 10% of excreta to be collected and the rest is directly excreted in the field while grazing (Longhurst et al., 2000). Cleaning milking yards requires the use of 50 l water per cow with detergents or washing chemicals (Houlbrooke et al., 2010a). Hence, final effluent collected in collection yards comprises 86% of wash down water with the remaining being 8% excreta, 4% teat washing and 2% of sediments (Sharma & Bhargav, 2012; Tiwari et al., 2006). Dairy shed effluent is a mixture of animal faeces, effluent, spoiled milk, wash down water, detergents along with organic contaminants including nutrients, organic matter, traces of antibiotics and pharmaceuticals, sediments, toxins and high concentration of microorganisms including pathogens (10^6 to 10^9 cfu/ml of faecal bacteria) (Cools et al., 2001; Dipu et al., 2011; McLeod et al., 2003). Most of the total solids in DSE are present as either in dispersed or dissolved form in liquid portion (Fyfe et al., 2016). In DSE, increased biological oxygen demand (BOD) and high organic matter (OM) content providing nutrients for pathogenic survival is a risk for environment, humans and animals (Garcha et al., 2016). The pH of DSE varies from 6.5 to 8 and its application in fields helps in increasing the pH of soil-bacteria-effluent suspension, increasing bacterial survival (Negassa et al., 2015). The microbial composition of DSE is quite complex and varies with animal type, age and health (Unc et al., 2006). *Escherichia coli* 0157, *Salmonella* spp., *Bacillus subtilis*, *Bacillus cereus*, *Brevibacillus* spp., *Bacillus thuringiensis*, *Campylobacter* spp., *Listeria* spp. and viruses including Enterovirus, Norovirus and Rotavirus, are some of the common DSE pathogens (Barak & Schroeder, 2012; Chandler et al., 1981). The total *E. coli* number in DSE can vary between 10^4 cfu/ml to 10^9 cfu/ml (Chart et al., 2000).

An infected cattle beast can shed 10^2 to 10^5 cfu of *E. coli* per g of faeces for 28 to 120 days after the start of infection (Jones, 1999; Omisakin et al., 2003). In fresh animal faeces, *E. coli* can remain in a culturable conditions (on medium) for 30 days (Kudva et al., 1998). Although DSE pathogens are intestinal residents and most of them are not adapted to survive in soil environment, species such as *E. coli*, which resides in the intestines of humans and animals, can survive and grow in soil for a few months by adapting to local environmental conditions (Cools et al., 2001; Horswell et al., 2010). Other

than the pathogenic *E. coli* strain 0157, *Campylobacter* spp. and *Salmonella* spp. are also the faecal pathogens of concern associated with humans and animal infections.

The land applied pathogenic microbial population with DSE also includes antibiotic resistant strains (Sarmah et al., 2006). Continuous use of antibiotics in farms for therapeutic purposes or for increasing animal growth and production in high doses has resulted in microbial strains becoming resistant to these antibiotics (Jechalke et al., 2014; Johnson, 1997). In developing countries, routine vaccination is a common practice to reduce infections in cattle (Singh et al., 2016). Antibiotics are provided to animals generally with feed and 30% - 80% of administered antibiotics are excreted in an unchanged form due to poor absorption by the animal's gut. Excreted antibiotics can still be active in faeces and can impose short to long-term effect on microbial populations. From animal faeces, antibiotics can make their way to groundwater and can be toxic to aquatic life (Srinivasan et al., 2010). In NZ, use of veterinary antibiotics is limited to the pig and poultry industries (Luo et al., 2011) although intra-mammary injectable antibiotics are common in dairy cattle (Srinivasan et al., 2014). The contribution of livestock towards microbial resistance against antibiotics can be 40 times higher than the human contribution (Bui & Choi, 2010). Antibiotics have been reported to bioaccumulate in crops such as beans and radish when present in high concentrations of 160 mg/l, and this requires further investigations using realistic environmental concentrations (Sarmah et al., 2006). Although *E. coli* is common in animal's gut, resistance genes passed on to *E. coli* from various pathogenic bacterial species have developed antibiotic resistance in *E. coli* and it is emerging as a great antibiotic resistant bacterial species (Avery et al., 2004).

2.4.1 *Escherichia coli*: A review

Escherichia coli is a rod shaped, facultative anaerobe, gram-negative bacterium (Brown et al., 2011). It was first recognised by a German scientist, Theodor Escherich, in 1885 in infant stool samples (Chart et al., 2000). It is notable that not all *E. coli* strains are benign (Bell, 1998; Berg, 2004; Brown et al., 2011). After its discovery, some pathogenic *E. coli* strains such as *E. coli* 0157:H7, have always been associated with gastrointestinal illness (LeJeune et al., 2001; Lim et al., 2010). Humans carry > 220 commensal and pathogenic bacterial species along with viruses and fungus both in and outside of their body (Lagier et al., 2017). *Escherichia coli* harmlessly colonises mammals' intestines during birth and after which starts to reside in their gut for lifetime (Jones, 1999).

2.4.1.1. *E. coli* as an indicator of faecal contamination

Some countries routinely test water sources for *E. coli* as a method to reduce the risk of water infection (Brown et al., 2011) as it is not practical to test all microbial strains present in DSE or in other sources of water contamination (Galvin et al., 2010). The World Health Organization (WHO)

specifies a limit of < 1 cfu *E. coli* per 100 ml as the standard for drinking water quality (Boubetra et al., 2011) as presence of *E. coli* assures a recent contamination with faecal pathogens (Barak & Schroeder, 2012; Reddy et al., 1981). *Escherichia coli* has the ability to utilise fluorescence indicator compound MUG (4-methylumbelliferyl β -D-glucuronide) which is widely utilised with *E. coli* as a preferred indicator organism of faecal contamination (Sutton, 2010). *Escherichia coli* is easy to culture and cost-efficient enumeration methods are also available (Aislabie et al., 2011). Optimal *E. coli* growth can be achieved at a temperature of 35°C- 37°C, the temperature of warm-blooded animals, with basic nutrients or medium available for growth (Cools et al., 2001). However, *E. coli* can also grow up to a temperature of 44°C (Jones, 1999). The ability of *E. coli* as an indicator is sometimes challenged by the resistant oocysts released by *Cryptosporidium* spp., although *E. coli* is not present in water without the presence of faecal contamination (Medema et al., 1997; Wilson et al., 1983). An *E. coli* strain that is harmless in one host species can be fatal in others (Mubiru et al., 2000). Pathogenic *E. coli* can reside asymptotically in cattle although the same strain can lead to severe infections or fatality in humans hence, continuous monitoring of water resources using an indicator *E. coli* is important (Cameron & Trenouth, 1999).

2.4.1.2 The pathogenic *E. coli* strains

Pathogenic *E. coli* strains, such as *E. coli* 0157:H7 and *E. coli* 0157:NM, have evolved by acquiring virulence factors from plasmids, bacteriophages and transposons and can be categorised based on their serotype, mechanism of establishing infection and specific virulence factors (Chart et al., 2000). Pathogenic *E. coli* strains produce various types of harmful toxins and can infect the gastrointestinal system of humans and animals with multiplication and release of the pathogenic cells in faeces (Higgins et al., 2011; Lowman et al., 2013) which can be detected using the non-pathogenic *E. coli* as an indicator (Hashsham et al., 2004). Verotoxin (VT1 and VT2) producing *E. coli* strains such as *E. coli* 0157, are considered of the highest health risks and are associated with various food and water outbreaks around the world (Artz et al., 2005; Zimmer, 2008). The sporadic or isolated nature of *E. coli* 0157 makes it difficult to study under field conditions (Vinten et al., 2002). The virulence factors of pathogenic *E. coli* strains are 1) Shiga like toxins (toxins similar to those produced by *Shigella dysenteriae*) 2) product of locus of enterocyte 3) product of the F-like plasmid or p0157, responsible for the transfer of genes and proteins (Berg, 2004; Lim et al., 2010). *Escherichia coli* 0157:H7 is the result of contribution of genes and virulence factors from over 50 different microbial species (Bell, 1998; Omisakin et al., 2003). Somatic coliphages can attach on cell wall of *E. coli* and can present a further risk for human health (Chart et al., 2000). *Escherichia coli* outbreaks result in > 73,000 human infections per year in the USA alone and costs over US \$ 405 million (Hashsham et al., 2004). Jones (1999) stated that the total expense resulting from an *E. coli* outbreak could be as high as US \$ 900 million in the USA alone, including treatment, management costs and economic losses. Increasing

outbreak incidences and resulting economic burden demands an immediate attention to the management of animal waste (Lim et al., 2010).

Increased awareness about infections has not prevented disease associated with pathogen contamination of water and therefore regulations need to ensure that DSE application methods do not result in contamination of ground and surface water (Fyfe et al., 2016; Hahner et al., 2014; Ibekwe et al., 2003). The effect of chemical pollutants from DSE is rapid and obvious, such as eutrophication, and hence it dominates the risk caused by residing bacterial pathogens (Garcha et al., 2016; Hutchison et al., 2004). Current DSE land application policies focus on N application rates, regulating it to 150 - 200 kg N/ha/year (Jiang, 2008; Waikato Regional Council, 2015). None of the NZ council policies currently considers bacterial infiltration associated with DSE application (DairyNZ Auckland, 2008; Jiang et al., 2010).

2.5 DSE land application and associated risks with pathogens

Pathogens associated with faecal contamination of waterways are frequently in the news when organisms such as *Campylobacter* spp., *Salmonella* spp., *Mycobacterium paratuberculosis* and *Listeria* spp. cause disease in humans (Department of Internal Affairs, 2017; Gagliardi & Karns, 2000). These bacteria always occur along with *E. coli* contamination and hence *E. coli* is used as an indicator organism. Drinking water safety is a worldwide issue and land application of DSE can often result in faecal bacteria reaching water resources following surface run off or infiltration in soil (Boubetra et al., 2011; Environment, 2017; Ministry of Health, 2016). Ding et al. (2017) reviewed drinking water safety in China following animal effluent irrigation of fields and found that applied effluent can easily make its way to nearby wells, following infiltration or surface run off. They found faecal pathogens were the main source contaminating well water especially in rural areas due to lack of hygienic water supply. Although, high numbers of animals, point sources, rainfall conditions and distance of the potential water source from the farm also influences water contamination risks (Hashsham et al., 2004; Stoddard et al., 1998). Pathogenic *E. coli* and Norovirus were the dominating pathogenic population causing water borne outbreaks between 1984 and 2014 in China and pathogenic *E. coli* incidences dominated those related to virus (Ding et al., 2017).

Bacterial species present in DSE impose the highest health risks and approximately 95% of non-bacterial human infections are related to viruses especially the human calicivirus (Hashsham et al., 2004). Pathogens distributed in effluent water can also be taken up by plants (Hashsham et al., 2004). Cools et al. (2001) in their preliminary trials found *E. coli* survival in soil after 250 days and stated it as sufficient time for bacteria to enter the edible plant parts such as leaves. In the UK, *E. coli* contamination was found in > 15% of the total animals counted (Vinten et al., 2002). Between 1982-

1998, food poisoning incidences in UK increased by 6 times with more than 100,000 cases reported in 1997. In this time period the mortality rates of those infected were close to 60% (Jones, 1999). According to a report by ESR, in NZ waterborne infection count of humans varies from 18,000 to 34,000 each year (Ball, 2006). Inappropriate management practices are a risk for maintaining water quality including ground and stream water resources (Jiang et al., 2010; McGarvey et al., 2004). Government laws are important to control the amount of contaminants reaching soil and water and analysis of indicator organisms, such as *E. coli*, is important to identify associated risk (Forster-Carneiro et al., 2010). Drinking water standard for *E. coli* in NZ is <1 cfu/100 ml (Houlbrooke et al., 2010b; Ministry for the Environment, 2017).

2.6 Possible ways of pathogenic exposure

Dairy shed effluent directly coming from a reservoir is generally untreated and raw with active bacterial populations residing in it (Aislabie et al., 2006; Bonjoch & Blanch, 2009; Jiang et al., 2005). *Escherichia coli* gets deposited on land while animals are grazing on fields, making their direct way onto the soil and water (Guber et al., 2007). Pathogenic *E. coli* outbreaks are generally associated with direct or indirect contact with animal waste containing the faecal pathogens such as working on farm, handling waste, drinking contaminated water, juice or milk, eating contaminated and undercooked meat, vegetables and fruits, contamination during slaughter management and sometimes even with the ingestion of contaminated soil particles (Hutchison et al., 2004; Jones, 1999). Low concentrations of pathogenic *E. coli* (10-100 cfu/ml) are sufficient to start an infection in either humans or cattle (Chart et al., 2000). Symptoms can include fever, weight loss, bloody or non-bloody diarrhoea, vomiting, haemolytic uremic syndrome, thrombocytopenic purpura (TTP) and infections can also be fatal (Omisakin et al., 2003). Even low DSE volumes reaching to water sources can contain high bacterial concentrations (Monaghan & Smith, 2004). Although, there are various factors that affect the survival and leaching potential of DSE applied bacteria (Monaghan & Smith, 2004).

2.7 Factors affecting survival of bacteria introduced to the soil

Bacterial survival in soil is poorly understood and many studies have found long term *E. coli* survival varying from months to years (Aislabie et al., 2011; Banning et al., 2002; Horswell et al., 2010; McLeod et al., 2008; Wang et al., 1998). Bacterial survival in soil where DSE has been applied depends on pH, temperature, exposure to UV, aeration, ionic strength of soil, native microbial population in soil, application method, adhesion, predation in soil, route of flow, vegetation type, source type (manure or slurry) and root exudates released by the vegetation cover (Medema et al., 1997; Neralla & Weaver, 2000; Zimmer, 2008) and also on bacterial species and variety (Galvin et al., 2010). If the applied pathogens move down the soil profile then UV has limited role to play in bacterial die-off (Jiang, 2008). In general, bacteria are moisture dependent and drought stress can increase their die-off (Craggs et

al., 2003) although, spore forming bacteria can survive in drought conditions (Briancesco et al., 2008) and available moisture in soil pores with applied DSE, is sufficient for increased bacterial survival (Gagliardi & Karns, 2000). Increasing moisture and decreasing temperature also increases bacterial endurance in soil (Avery et al., 2004).

Cools et al. (2001) tested the survival of pig slurry derived *E. coli* populations in soils with contrasting textures and at different incubation temperatures (5°, 15° and 25°C) and moisture content. They found that at 5°C, *E. coli* reached the detection limit i.e. 10³ cfu/g of soil, by 68th day however at 25°C, decline in their numbers was rapid and detection limit was achieved in only 26 days. Jiang et al. (2002) tested the survival of *E. coli* O157:H7 at variable temperature range in animal manure and found highest *E. coli* O157:H7 survival at 5°C for up to 70 days and lowest at 37°C for 42 to 49 days. Heat stress or high temperature can slow down the growth of *E. coli*, generally in anaerobic conditions (Lim et al., 2010). Kudva et al. (1998) studied the survival of *E. coli* O157:H7 under varying temperatures in animal waste and found that *E. coli* survived to 100 days at -20°C. Mubiru et al. (2000) compared the die-off pattern of pathogenic and non-pathogenic *E. coli* strains in two different soil types and reported similar die-off patterns for both pathogenic and non-pathogenic strains with slightly higher mortality rate for the non-pathogenic *E. coli*. Bogosian et al. (1996) and Özkanca and Flint (1997) stated *E. coli* (non-O157) survival varying between 1 to 7 days in nonsterile water at 4°C, however survival of *E. coli* O157 has been reported up to 90 days in river water and stated to be dependent on the physiochemical, biological and environmental conditions of the water (Wang & Doyle, 1998).

2.7.1 DSE application method

The land application of DSE is still a new technique, only being used since 1991 in NZ, and many farmers face problems in properly implementing effective management and application systems (Houlbrooke et al., 2003). Other than the chemical, physical and biological properties of the DSE itself, the method used to apply it to land also influences bacterial movement and survival in soil (McLeod et al., 2008). While spray irrigation may generate aerosols containing pathogens, it is widely used because of being inexpensive and effective (Jiang, 2008). Flood irrigation bypasses soil matrix and bacteria flows down through macropores, a process known as preferential flow, allowing deep vertical transport of bacteria into the soil (Craggs et al., 2003). Irrigation below 10 mm/h is reported to reduce the potential for bacterial leaching and with automated irrigators, a minimum of 6 - 7 mm per hour irrigation is possible (Houlbrooke et al., 2010a; Jiang et al., 2010). Over 10 mm/h and generally up to 20 mm/h of irrigation, is considered heavy irrigation (Monaghan & Smith, 2004). High hydraulic loading in soil increases macropore flow and bacteria can move up to 35 times faster in heavily irrigated soils (Unc et al., 2006). When the amount of DSE application or input to soil exceeds the rate of infiltration, surface run off occurs (Jamieson et al., 2002). Run off bacteria can include both active free cells and those attached

to loose soil and vegetation particles (Guber et al., 2007). Following surface run off, bacteria attached to the free-soil particles can directly reach nearby water resources following sedimentation (Cichota et al., 2016). Unattached microbial population in soil is susceptible to leaching (Saini et al., 2003). Studies have also reported that large amounts of bacteria are attached to the suspended solid particles in effluent and hence, reducing sediment flow to water resources will likely be able to reduce bacterial flow (Ding et al., 2017; McCaulou et al., 1995). A sufficient time gap between the first rainfall and bacterial application with animal wastes reduces the chances of bacterial leaching following macropores or cracks in soil (Gagliardi & Karns, 2002; Smith et al., 1985). Ogden et al. (2001) examined *E. coli* leaching in soil and stated that the die-off rate of pathogenic *E. coli* 0157 was similar to the total *E. coli* population and leaching of bacteria was rainfall dependent. Vinten et al. (2002) in their field study inoculated with cattle slurry *E. coli* 0157, found that following no irrigation after bacterial inoculation, maximum *E. coli* population remained in the top soil and that increased rainfall after bacterial application increases bacterial flow into the drainage system. They also stated that hot weather following no irrigation increases bacterial die-off.

Houlbrooke et al. (2010a) stated DSE irrigation can result in the run off of 108 kg N/year, 18 kg P/year, and significant numbers pathogens into surface water resources. They also reported that 30% of DSE applied onto a farm near Palmerston North with silt loam soil, can undergo surface run off with daily irrigation, and stated deferred irrigation systems i.e. storing DSE and irrigating as required can be helpful in decreasing direct contaminant (bacterial and nutrient) flow to the stream water. Under high soil moisture conditions such as in winter and rainy seasons, DSE storage is the best option to avoid contaminant run off or leaching (Cools et al., 2001) although with increasing intensive farming, appropriate DSE storage is a challenge in cold weather due to high soil moisture (Fyfe et al., 2016). In summer, when evapotranspiration increases and rainfall is low, the risk of leaching primarily occurs through macropores under high rates of irrigation (Jiang et al., 2005; McLeod et al., 2008). Under dry soil conditions and due to low water velocity and flow in soil, bacterial movement in soil is limited, although dry soil conditions can increase macropores formation hence, such soil requires an initially low irrigation rate allowing sufficient time to enable the soil to swell and properly moisten (Jiang et al., 2010). Collins (2002) found that for a Waikato farm with poorly drained soil, there was no seasonal difference in the leaching of faecal bacteria however, no similar data is available for the South Island. There is a lack of knowledge and studies regarding bacterial movement with heavy DSE application to soil and the resulting effect on depleting water quality (Monaghan & Smith, 2004).

2.7.2 Effect of soil type on bacterial survival and movement

Bacterial survival and movement are affected by varying soil properties and soil favouring survival of pathogenic microbes can be of great risk (Mubiru et al., 2000). Current management recommendations

for land application of DSE are general and do not account for soil variability (Houlbrooke et al., 2010b; Jiang, 2008). Microbial flow in soil depends on: 1) the physical and chemical properties including pH, texture, pore and particle size 2) environmental factors such as rainfall and temperature and 3) application methods such as irrigation rate and the method of application (Aislabie et al., 2011; Avery et al., 2004; Horswell & Aislabie, 2006; Jamieson et al., 2002; McLeod et al., 2008; McLeod et al., 2003; Smith et al., 1985). Soil with its natural porous structure and physical, chemical and biological properties helps to filter bacteria protecting groundwater from bacterial contamination (Jiang et al., 2010). With intensifying agricultural practices, soil structure, compaction and organic matter content gets depleted and soil loses its ability to act as a perfect filter or barrier between the applied DSE and the water table, allowing direct and rapid transfer of the applied bacteria to the groundwater (Stoddard et al., 1998). Bacterial transport rate depends on the route of water flow, colloid mobilisation mechanism and bacterial die-off kinetics (Vinten et al., 2002). After DSE application to soil, bacterial survival depends on the physical and chemical properties of both the soil and the effluent (Jiang et al., 2005; Jiang, 2008). The bacterial population inoculated with the DSE competes with the native soil microflora, which varies with soil type (Gagliardi & Karns, 2002; Smith et al., 2008). Soil contaminants also affect native microbial communities present in the soil hence, affecting the effective contribution of soil to plant production (O'Donnell et al., 2007). Jiang et al. (2002) tested bacterial survival in sterile and non-sterile soil and found that *E. coli* number rapidly declined in non-autoclaved soil and stated competition with native microorganisms as the main factor. Water is held at low tension in macropores and hence, macropores significantly increase the risk of bacterial leaching (McLeod et al., 2008). While following macropore flow, there will be less interactions between the inoculated and the native microbes meaning there will be no effect on the viability of DSE bacteria (Noguchi et al., 1999).

Soil total porosity can be defined as the sum of micropores (< 30 μm) and macropores (> 30 μm) (O'Donnell et al., 2007). Various definitions of macropores and micropores are available based on matric potential, size of air pores or pore diameter (Germann & Beven, 1981; McLaren, 1996). Applied bacteria follow physical filtration, straining or preferential flow in soil (Jamieson et al., 2002). Filtration occurs when the applied bacterial cells are too large to cross the soil pores and sit on the pore surface and straining occurs when bacteria are entrapped in soil pores following no movement or adsorption. Preferential flow takes place when the bacteria moves independent of the soil pore structure following wide and continuous cracks present in the soil, generally with applied irrigation (Abu-Ashour et al., 1994; Negassa et al., 2015). Bacteria can also independently move a few mm in soil with the help of flagella (Unc & Goss, 2003). Adsorption is a reversible process in soil and is generally common with viruses while the common process responsible for the bacterial movement in soil is filtration (Artz et al., 2005). Smith et al. (1985) reported the recovery of *E. coli* in the drainage water following irrigation

of *E. coli* inoculated soil columns at the rates of 5 - 40 mm/hour to be variable ranging from 8 – 96% of the added *E. coli*. They further confirmed the transfer of *E. coli* from the top to the bottom of the soil columns following macropores. Camesano et al. (1999) stated that bacterial cells act as colloid particles in soil and can block the soil pores or adsorption sites in soil resulting in increased bacterial flow following the macropores. Once bacteria enter the macropore flow, the physical and chemical properties of soil have a minimal role to play in their movement (Noguchi et al., 1999). McLeod et al. (2008) suggested that the high $K_{\text{saturated}}: K_{\text{unsaturated}}$ ratio of soil can provide an idea of the bacterial transport through macropores and that this ratio should be used along with other physical and chemical soil properties to estimate bacterial transport following macropores. Flow following macropores occurs in a wide range of soil types and is generally dependent on the initial soil moisture content, however, there is an ongoing debate between studies on the effect of initial soil moisture status on the bacterial leaching potential (Jiang, 2008; McLeod et al., 2008). Aislabie et al. (2011) studied *E. coli* movement in soil after DSE application in a lysimeter study and found that the leaching potential of *E. coli* was dependent on soil moisture level. Unc and Goss (2003) studied the movement of faecal bacteria present in animal waste (manure and slurry) in two different soil types and found that bacteria primarily travelled down through macropores in both soils although bacterial movement was not restricted to the soils with the initially high moisture status. They found that the depth of bacterial movement in soil was correlated with the moisture content of the applied animal manure. Therefore, solid animal waste such as manure has a high possibility of increasing pathogenic microbial contamination of groundwater. They also mentioned high macropore flow in clayey soil.

McLeod et al. (2008) investigated microbial leaching in variety of NZ soils in both the South and the North Island using the lysimeter studies and reported that following DSE application, well-developed soils such as gley, ultic and granular soils, can increase the leaching potential of bacteria following cracks and macropores, however, underdeveloped or new soils have comparatively less leaching risk. Aislabie et al. (2011) also reported that dense soils increase macropore formation. Fine structure or sandy soils helps the matrix flow of applied bacteria following bacterial sorption (Cools et al., 2001; Mubiru et al., 2000) and sand can also act as a reservoir of *E. coli* (Hashsham et al., 2004). Guber et al. (2007) found 80%, 18% and 2% of the inoculated *E. coli* population attached to silt, clay and sand fraction of the soil respectively. Low conductivity soil can increase the surface run off (Himathongkham et al., 1999) while high clay content in soil can increase crack formation (Jiang et al., 2010). Variability in the inner soil structure increases the variability in the results (Hutchison et al., 2004). Nearly 10% of NZ soils under pastoral agriculture are well structured which increases the chances of bacterial leaching under these soils with applied DSE (Stoddard et al., 1998). The North Island has nearly 50% of soil with a high risk of preferential flow with ~40,500 km² of flat to rolling lands with an average slope of 15 degrees. However, the South Island has ~52,000 km² of flat land with 45% of it at a high risk of

preferential flow (McLeod et al., 2008). Ogden et al. (2001) have found a 5 log decrease in the inoculated *E. coli* population after 8 weeks in sandy soil and after 25 weeks in loam and clay soil stating that the soil type influences bacterial survival. Repacking soil columns removes macropores as the potential factor for bacterial leaching and allows the study of the effect of the bulk density of soil on microbial leaching (Chetochine et al., 2006; Saini et al., 2003).

Tillage activities reduce macropores by disturbing their structure and channels created by earthworms (Artz et al., 2005). Stoddard et al. (1998) observed the effect on the movement of faecal bacterial populations in soil with tillage activity and found no effect on bacterial leaching with different tillage practices. They reported that rainfall sufficient to cause bacterial flow started the leaching process. In fields, a few points with high density of macropores are associated with high bacterial leaching along with installed artificial drainage systems such as mole pipes can increase the risk of contamination of shallow water tables (Aislabie et al., 2011; Monaghan & Smith, 2004). Farms where there is continuous excretion from infected animals can concentrate *E. coli* numbers, supporting long term survival of the pathogens in the soil (McGarvey et al., 2004). LeJeune et al. (2001) performed a long-term study on a dairy farm investigating *E. coli* survival and reported that the farm environment itself acts as a reservoir of *E. coli* rather than the cattle themselves and tracers can sometimes be useful in tracing bacterial pathways in soil. Jiang et al. (2010) studied bacterial movement using bromide as a tracer in the lysimeters study and found that bacteria followed macropores in soil and exchanged less mass between mobile and immobile water however, bromide slowly followed the soil matrix to reach the bottom. Using tracer dyes can be helpful to monitor bacterial flow in soil and can concisely trace the flow pathway (Flury & Flühler, 1995; Jiang, 2008). Various models have been developed to monitor bacterial flow in soil although they require a variety of data inputs which cannot be precisely obtained for different geographical conditions (McLeod et al., 2008).

2.8 Current DSE application practices and their effect on contamination of water resources

Irrespective of soil type and DSE application methods, *E. coli* populations usually decrease to below detectable levels in 2 to 3 months (Cools et al., 2001; Jiang et al., 2002) although the naturalisation of *E. coli* in soil environment and regrowth with available nutrients has also been reported (Avery et al., 2004; Boubetra et al., 2011). *Escherichia coli* population that has reached the detection limits in soil can be challenged by enrichment methods, which shows that the lack of detection of *E. coli* in soil does not guarantee its complete die-off as bacterial population can reside in a Viable But Non-Culturable (VBNC) state (Ogden et al., 2002). Avery et al. (2004) reported that irrespective of the waste application method used, following high organic matter application, *E. coli* could survive for two months in soil. Drainage water from the paddock may contain as little as 2% of the *E. coli* applied with irrigated DSE,

but this can still be sufficient to start an infection in humans or animals (Ross & Donnison, 2003). Gagliardi and Karns (2002) stated that management practices can reduce but cannot eliminate the risk of bacterial leaching to groundwater resulting in infections to humans and animals. Dairy stakeholders demand cost effective, efficient, and long-term DSE treatment and management solutions (Cameron & Trenouth, 1999; Horswell & Aislabie, 2006; Sharma & Bhargav, 2012). There is a shortage of research investigating reducing the bacterial load from DSE, which is important to maintain drinking water quality (Fyfe et al., 2016).

Constructed wetlands are one of the new strategies using antimicrobial plant species and can be useful in the mitigation of the microbial contaminants present in DSE (Ibekwe et al., 2003; Zhang et al., 2016). This system depends on metabolic properties of the plants, released root exudates and the natural microbial population along with vegetation type and hydrology, to treat the effluent (Dipu et al., 2011). Although constructed wetlands are efficient, they can be expensive (McGarvey et al., 2004; Mills et al., 2006).

2.8.1 The need for plant-based treatment strategies

With dairy farming practices more than 50% of the forest land has been converted to grass lands leading to the loss of native vegetation and birdlife (Wall et al., 2010). The Ministry of Environment NZ under the Emission trading scheme (ETS) has recommended a need to increase native vegetation and manage animals wastes (Ministry for the Environment, 2017). Planting native plants and shrubs, in both urban and rural areas and alongside waterbodies, has gained an increasing attention although the potential of these native plants to improve water quality has not been fully explored (Hahner et al., 2014; Robinson et al., 2003). Hahner et al. (2014) estimated the remediation potential of native NZ plants species and found that the rhizosphere system of these plants can reduce the environmental risk associated with applied DSE contaminants. They demonstrated that these native plants modify soil physiochemical properties with selective uptake of deficient trace elements and stated that NZ native plants can add value to the dairy farming activities. They also reported decreasing soil pH down to 3.5 under few natives such as *L. scoparium*, which is a highly acidic environment for bacterial pathogens to survive. However, decreasing soil pH can limit the root growth of some plants (Foy, 1992).

2.9 Native antimicrobial plants as buffers between DSE application areas and water sources

Plant based DSE treatments utilising their rhizosphere microbial activities together to reduce the contaminants, is a slowly growing field receiving attention due to its low cost and high treatment efficiency (Garcha et al., 2016; Rovira, 1965; Saxena & Stotzky, 2001). Plants release a range of low molecular weight C containing substances from their roots for various purpose including increasing

nutrient availability and microbial contaminant degradation (Chen & Nelson, 2012). Root exudates can contribute up to 40% of the photosynthetic C production (Fan et al., 2001). The type of available nutrients to plants and their direct or indirect contact with organic or inorganic components in soil, affects exudates release and their bioavailability (Neralla & Weaver, 2000). Exudates released by plants help in selective enrichment of beneficial microbial communities helping in mineralisation, nutrient transformation, organic matter and contaminant decomposition (Bais et al., 2004; Kim & Owens, 2010). Introduced microbial contaminants can be harmful either to the plants or to the associated microbial population, which can lead the degradation/repel process of unwanted microbes. Both plants and their associated microbes can work together in removal or die-off of the exposed microbial contaminants including unwanted pathogenic or competitive microbes (Mills et al., 2006; Zhang et al., 2016). Root associated bacteria already have a high amino acid demand and any additional microbial population utilising the same nutrient source increases the competition between the native and the inoculated microbial species leading to the microbial contaminants die-off utilising various compounds released by plants or their interaction with associated microflora (Krafczyk et al., 1984). Most compounds released into the rhizosphere are water-soluble and some are volatile (Heil & Bueno, 2007; Kai et al., 2016; Wenke et al., 2010). Some of these compounds exhibit antimicrobial activity against introduced microbial population (Mendes et al., 2013; Wyatt et al., 2005). Plants can maintain selective mechanism for volatile release for their beneficial components such as essential oils and for introduced contaminants such as pathogens (Blom et al., 2011). Chang et al. (2006) demonstrated beneficial removal of nutrient contaminants following plant-microbe interactions and stated use of plants activities as a promising method for contaminant removal such as excessive N. Plants antimicrobial compounds have been reported to present effective antimicrobial activities against the antibiotic resistant phyto-pathogens (González-Lamothe et al., 2009) and in wetland constructions against MS-2 coliphage (Neralla & Weaver, 2000). Zhang et al. (2016) investigated the degradation of pharmaceutical contaminants by plants and found that both plants and their associated root microorganisms can work together or independently to degrade these contaminants. Microbes and the plants roots modify soil properties through their metabolic activities and interactions releasing various polysaccharides and polypeptides, which can increase microbial die-off (Feeney et al., 2006; Hawes et al., 2016). The choice of plant species being used to reduce microbial contamination is important to ensure the proper functioning of the system and should take into consideration their use of water, growth rates, efficiency against contaminant, resistance against insects and grazing animals and tolerance to fluctuating pH and soil moisture content (Neralla & Weaver, 2000). There is limited knowledge regarding the exact mechanism followed by the root exudates affecting different pathogens (Kai et al., 2016; Oburger et al., 2012).

2.9.1 The antimicrobial properties of *Leptospermum scoparium* and *Kunzea robusta*

Leptospermum scoparium J.R.G. Forst. (Allan, 1961) or mānuka and *Kunzea robusta* A. Rich. (Connor, 1985) or kānuka are two native NZ plant species, both members of the Myrtaceae family. These are native pioneer species but are not endemic to NZ as they are also found in south-west Australia and grow widely in Tasmania (Stephens et al., 2010). These plants can grow in varying climatic conditions and can rapidly colonise disturbed or degraded soil (Watson et al., 1997). They are known for their hardy nature and can easily establish themselves in high salt, low pH, low moisture and poor nutrient conditions (Watson & Mardern, 2004). They are often found as the dominant species in high wind regions (Marden et al., 2005). *Leptospermum scoparium* and *K. robusta* are widely distributed woody flora in NZ and can regrow in 3 - 5 years after harvesting (Maddocks-Jennings et al., 2005; Whitehead et al., 2004). Both these plants show abundant flowering and have small, white, bowl shaped flowers pollinated by variety of insects including beetles and flies (Song et al., 2013). Copious production of light weight seeds, which are easily dispersed by wind helps their wide distribution (Lis-Balchin et al., 2000). *Leptospermum scoparium* is often confused with *K. robusta*, although *K. robusta* have comparatively small leaves and flowers and can grow taller than *L. scoparium* (Porter et al., 1998). *Leptospermum scoparium* generally grows from 2 to 5 m although *K. robusta* can grow up to 8 m (Porter & Wilkins, 1999).

2.8.2.1. Benefits of *Leptospermum scoparium* and *Kunzea robusta*

Leptospermum scoparium and *K. robusta* are widely used in rehabilitation of degraded lands with high mining activities (Watson & O'Loughlin, 1985). Both plants promote the soil ecosystem increasing invertebrate activities under their roots (Williams et al., 2014). These plants are often the dominant species in poorly drained and infertile soils (Stephens et al., 2010) and can also reduce soil erosion by stabilising soil with their roots system (Mendes et al., 2013). The roots of these plants can contain ~ 5% of the total soil volume and can reach approximately 50 cm deep providing strength against landslides (Watson & O'Loughlin, 1985). The deep root systems of trees can increase preferential flow of the surface applied microbes (Abhilash et al., 2012; Bargués Tobella et al., 2014; Johnson & Lehmann, 2006) although it has not been yet tested for these two NZ natives. Both plants increase C sequestration in soil and use of these natives helps in restoring native forests (Esperschuetz et al., 2017; Whitehead et al., 2004). *Leptospermum scoparium* is tolerant to arsenic (Craw et al., 2007) however, *K. robusta* can inhibit denitrification, a common problem with land applied DSE (Fitzgerald, 2012) releasing greenhouse gases including N₂O (Lowman et al., 2013). Hahner et al. (2014) stated that *L. scoparium* and *K. robusta* could efficiently manage acquisition of K, P, Zn, Mn, Cu in soil.

Medical benefits: These plants hold a strong social, economic and medical value for NZ society and are also known as tea tree with unpalatable leaves for grazing animals (Chen et al., 2016). Both these plants

produce beneficial biologically active compounds and have been traditionally used in Māori medicines (Chan et al., 2013). Bark, leaves, seeds, honey and essential oil from *L. scoparium* and *K. robusta* are used in medicines and cosmetics. White leaf gums of *K. robusta* are used as medicines against fever, cough and depression (Lu et al., 2014). Seed puree or paste of *L. scoparium* can reduce inflammation, mild pain and diarrhoea and is useful for healing minor injuries Chen and Nelson (2012). Leaf extract from these plants have been proven to reduce eye problems, back stiffness and breast pain and can also be used as mouthwash (Tomblin et al., 2014).

Economic benefits: Of all NZ native trees, *L. scoparium* can provide the highest economic and medical benefits followed by *K. robusta* (Häberlein & Tschiersch, 1998). Honey and essential oils are the main products of these plants and are known for their specific antimicrobial properties (Adams et al., 2008). Both honey and essential oil, especially from *L. scoparium*, are sold widely in NZ and around the world (Chen et al., 2016; Lis-Balchin et al., 2000; Lu et al., 2013). *Leptospermum scoparium* honey in 2014/15 contributed NZ \$ 281 million to countries trade (NZ Herald, 2016).

2.8.2.1.1. Honey

Honey is a traditionally used natural remedy for minor ailments including minor gastrointestinal infections (Adams et al., 2008). The antimicrobial activity attributed to any honey is related to its mild acidity, viscosity, osmolality, ability to maintain moisture and hydrogen peroxide (Lusby et al., 2005). Hydrogen peroxide is produced during glucose oxidation utilising glucose oxidase from the hypopharyngeal gland of honeybees (Chan et al., 2013; Whitehead et al., 2004). *Leptospermum scoparium* honey has been found to exhibit non-peroxidal activities (Jenkins et al., 2011). Caffeic acid, phenethyl ester, polyphenols are important factors for anti-inflammatory effect of *L. scoparium* and *K. robusta* honey (Tomblin et al., 2014). Reactive dicarbonyl methylglyoxal (MGO) in *L. scoparium* honey is known to contribute to its antimicrobial activity (Lu et al., 2014). The MGO is reported to be inhibitory to a number of bacterial species and is derived from the nectar derived dihydroxyacetone (DHA) (Whitehead et al., 2004). The MGO in *L. scoparium* honey have a high concentration of coquinoxaline and o-phenylenediamine (OPD) responsible for non-peroxide antibacterial activity (Stephens et al., 2010). Hydrogen peroxide contributes to the antimicrobial property of *L. scoparium* honey but is not responsible for complete inhibition of bacterial growth (Patton et al., 2006; Schneider et al., 2013). The specific antimicrobial activity of *L. scoparium* honey is measured in the Unique Mānuka Factor (UMF), scale defining the non-peroxidal activity of honey and is calculated based on its activity against *Staphylococcus aureus* (Lusby et al., 2005; Tomblin et al., 2014). *Leptospermum scoparium* honey is effective against a wide range of bacterial and fungal pathogens including antibiotic resistant strains (Lu et al., 2013). It is effective against bacterial bio-films and can stimulate the immune system to increase bacterial resistance (Weston et al., 2000).

2.8.2.1.2. Essential oil

Due to the accumulation of bio-functional and medicinal properties, *L. scoparium* and *K. robusta* oil has increasingly become popular for commercial purposes (Herman, 2014; Maddocks-Jennings et al., 2005). Essential oil is generally obtained following steam distillation of leaves and its composition from these plants varies based on geographical locations, age of plants and climatic conditions (Chen et al., 2016; Demuner et al., 2011; Douglas et al., 2004; Porter et al., 1998; Wyatt et al., 2005). The essential oils contain triketones and monoterpenes, which are known to exhibit high antimicrobial activities (Perry et al., 1997a; Perry et al., 1997b). North Island *L. scoparium* has been found with 30% more triketones than South Island *L. scoparium* (Lis-Balchin et al., 2000). Three broad chemotypes are present in NZ a) far North chemotypes, high in pinene concentration, b) Marlborough sound or East cape chemotypes, high in triketones and c) all other locations high in sesquiterpenes (Porter & Wilkins, 1999). Triketone rich essential oil has high commercial and economic value (Douglas et al., 2004). Approximately 20% of the total *L. scoparium* essential oil composition is of triketones and the remaining 80% can vary between mono- and sesquiterpenes (Lis-Balchin et al., 2000). β -triketones are commonly found in *L. scoparium* essential oil including isoleptospermone, falvescene, leptospermone and grandiflorone (Christoph et al., 2001). Leptospermone is an antibacterial β -triketone commonly found in leaves, twigs and seeds of *L. scoparium* and is responsible for the exhibited antibacterial activity of the essential oils (Weston et al., 2000). It was first identified by Penfold (1921) in Australian Myrtaceae plants and by (Gardner, 1924) in NZ and was initially known as leptospermol. It is now an established β -triketone after being previously recognised as a phenolic, monobasic acid and acidic phenol (Douglas et al., 2004). *Leptospermum scoparium* roots have not been tested for their leptospermone content, although another Myrtaceae plant *Callistemon citrinus* or bottle brush has been found to release leptospermone from their roots (Cornes, 2005). Other than triketones, monoterpenes and sesquiterpenes including α -pinene, β -pinene and myrcene are common components in essential oils (Demuner et al., 2011; Perry et al., 1997a). The concentration of monoterpenes in essential oils can vary from 1% in young plants to 17 - 35% in three-year-old plants (Van Vuuren et al., 2015). *Kunzea robusta* essential oil generally contains high α -pinene concentration (55.5%) compared to *L. scoparium* essential oil (~1-3%) (Porter et al., 1998; Schneider et al., 2013). The antimicrobial activities of *L. scoparium* and *K. robusta* (essential oil and honey) have already been established against many bacterial species including *Brevibacteria* spp., *Staphylococcus* spp., *Listeria* spp., *Clostridium* spp., *Streptococcus* spp. and have been found to be mostly active against gram positive bacterial species (Christoph et al., 2001; Demuner et al., 2011; Van Vuuren et al., 2014). Some studies have also reported the activity of the *L. scoparium* seeds essential oils against intestinal bacterial species such as *E. coli* (Jeong et al., 2009; Lu et al., 2013). *Kunzea robusta* have been reported to produce antiviral compounds (Bloor, 1992). *Leptospermum scoparium* essential oil is utilised in the

treatment of dysentery and in making soaps, toothpastes, cosmetics and pharmaceuticals, generating economic benefits (Porter et al., 1998).

Prosser (2011) found reduced growth of five bacterial species (*Salmonella typhimurium*, *E. coli*, *Listeria monocytis*, *Campylobacter jejuni* and *Clostridium perfringens*) exposed to water extracts of *L. scoparium* and *K. robusta* leaves and roots demonstrating their antimicrobial activities. They also found that soil collected from beneath mature *L. scoparium* plants had no inhibitory effect against faecal pathogens and concluded that active antimicrobial components of these plants, which might be continuously released by roots, are not stable in nature and can rapidly degrade. Fitzgerald (2012) and Prosser et al. (2016) reported reduced *E. coli* growth in biosolids amended soil under *L. scoparium* and *K. robusta* five to eight days after application. The mechanism of the antimicrobial activity is unknown. Hahner et al. (2014) suggested that strategic planting of *L. scoparium* and *K. robusta* in dairy farms could reduce the contamination risks to water resources.

2.10 Research scope

Antimicrobial potential demonstrated by these native plants i.e. *L. scoparium* and *K. robusta*, and their potential against *E. coli*, as demonstrated in a few previous studies (Fitzgerald, 2012; Hahner et al., 2014; Prosser, 2011) indicates that these plants could reduce pathogen risks following DSE application to soil. Potentially active antimicrobial components from *L. scoparium* and *K. robusta* (known from their essential oils and honey chemistry) might decrease microbial leaching from their root zones, thereby reducing contamination risk to groundwater and associated health risks to humans and animals. This study will elucidate leaching potential of the bacterial indicator *E. coli* (a non-pathogenic lab strain ATCC13706 and those growing in DSE), under *L. scoparium* and *K. robusta* and will focus on identifying the potential mechanism responsible for their antimicrobial properties. Use of the indicator bacteria *E. coli* will be helpful to understand how *L. scoparium* and *K. robusta* affect the survival and flux of bacterial contaminants.

Chapter 3

An in vitro assessment of the antimicrobial potential of *Leptospermum scoparium* and *Kunzea robusta*

3.1 Introduction

The land application of Dairy Shed Effluent (DSE) is emerging as a major concern for the health and safety of humans and animals especially with regards to faecal pathogens associated with food, crop and water contamination (Del Serrone et al., 2015). Pathogens exhibiting multidrug resistance, such as β -lactamase-producing *Escherichia coli*, *Pseudomonas* spp. and *Staphylococcus* spp., have been declared by the Infectious Disease Society of America to be especially of concern requiring immediate attention (Marasini et al., 2015). Increasing microbial resistance to drugs and antibiotics has reduced the effectiveness of current medical therapies, which requires the discovery of alternatives (Wyatt et al., 2005).

Plants can be the natural sources of biomedicines, and their extracts have been used in local societies with their recognised efficacy in Ayurveda, Unani and Chinese medicines (Salie et al., 1996). There has been some debate regarding safety issues with the use of plant extracts, generally with their ingestion by humans, but being natural products, biomedicines or plant-products are gaining increasing interest and trust and have been recommended by the World Health Organisation (WHO) (Atsafack et al., 2015; Jain et al., 2015; Marasini et al., 2015). As stated in Chapter 2, *L. scoparium* and *K. robusta* are known for their specific antimicrobial properties (Chen et al., 2016). Bark, leaves and sap of these plants have been used to treat minor ailments and injuries (Adams et al., 2008; Lis-Balchin et al., 2000; Lu et al., 2014; Tomblin et al., 2014). Honey and essential oils of *L. scoparium* and *K. robusta*, have also been shown to have inhibitory properties against microbial pathogenic species including *Staphylococcus aureus* and *Helicobacter pylori* (Häberlein & Tschiersch, 1998; Killeen et al., 2015b; Lu et al., 2013; Patton et al., 2006). *Leptospermum scoparium* and *K. robusta* may also inhibit bacterial growth through changing conditions in the rhizosphere as Hahner et al. (2014) showed that soil surrounding the roots of mature *L. scoparium* can be acidified down to pH 3.5. Other rhizosphere changes may include reduced water content, or the stimulation of other organisms, such as associated microbes, which release antimicrobial compounds (Wicaksono et al., 2016).

Plants with antimicrobial properties, including *L. scoparium* and *K. robusta*, have mostly been tested for food safety in relation to their essential oils (Del Serrone et al., 2015) or in experiments comparing their efficacy to inhibit different microorganisms (Jain et al., 2015; Lis-Balchin et al., 2000). The ease of

plants with antimicrobial properties to treat soil systems contaminated with DSE applied pathogens is gaining attention (Dipu et al., 2011; Ibekwe et al., 2003). There is limited knowledge on the efficacy of these plants and their inhibitory properties against microbial contaminants (Atsafack et al., 2015; Hassan et al., 2009) nor is there much information on how the antimicrobial components from these plants will end up in the soil (Prosser, 2011; Prosser et al., 2014).

3.2 Aim

Using *E. coli* strain ATCC13706 as an indicator species, this study aimed to determine the antibacterial effect of aqueous extracts of leaves and roots as well as root exudates of *L. scoparium* and *K. robusta* in plants from various sites in the South Island. The study also sought to determine the role that rhizosphere pH may have on bacterial survival. It was hypothesised that the antimicrobial components present in the roots and leaves of *L. scoparium* and *K. robusta*, will inhibit the growth of *E. coli* and root exudates will provide an indication of how these plants might reduce *E. coli* survival in the contaminated soil systems. Extracts of *Lolium perenne*, which is present in 70% of NZ pasturelands, was used as a negative control and streptomycin was the positive control.

3.3 Methods and materials

Two-year-old *L. scoparium* and *K. robusta* plants were purchased from the Department of Conservation nursery, Motukarara, Christchurch (43°43'44''S 172°34'42''E). Mature plants samples were collected from 10 to 15-year-old plants growing at different locations: Quail Island (43.6278° S, 172.6902° E), Nikau Gully (43°51'30''S 172°57'00''E) and the Paper road/ L2 Lake (43°41'06.5''S 172°27'05.9''E). For mature *L. scoparium* and *K. robusta*, fully expanded leaves were collected from midway down the stems. Leaves also contained small pieces of the stems or parts of the seed shells.

Commercially prepared East Cape Origin and steam distilled essential oils of *L. scoparium* and *K. robusta* were purchased from Lotus oils, NZ (39°58'44''S 176°32'17''E). The *L. perenne* leaves were collected from an unused experimental field (Field H9) in Lincoln University campus by random grab sampling from two inches above the ground. Murashige and Skoog's (MS) media was kindly obtained from Associate Professor David Leung, Canterbury University.

3.3.1 Origin and maintenance of *Escherichia coli* strain

A non-pathogenic *Escherichia coli* strain ATCC13706 was obtained from the Institute of Environmental Science and Research Ltd (ESR), Wellington. The strain was stored in the glycerol stock (Appendix C.1) at -20°C and for each experiment were freshly cultured from these stocks onto the nutrient agar (NA; Oxoid CM0003) at 30°C for 24 hours.

3.3.2 Plants extract preparation

The plant extract preparations used in the study were prepared using a modification of the methods described by (Prosser, 2011). After preliminary trials using various solvents (Appendix D.1), sterile water was chosen as the best extractant for this study, to more closely represent the natural environment of the plants.

Leaves and roots of *L. scoparium*, *K. robusta* and *L. perenne* were harvested and thoroughly washed with deionised (DI) water to remove dust particles. Plant material was frozen at -20°C until used. Five grams (fresh weight) of either the leaves or roots were weighed and cut into small pieces with clean scissors. Chopped leaves or roots were then crushed into a fine powder using liquid N in a pestle and mortar and the obtained powder was mixed with 20 ml sterile water in 50 ml falcon tubes. Falcon tubes containing crude extracts were first shaken at 320 oscillations per minute in the Stuart™ SF1 flask shaker for an hour at 4°C and then centrifuged at 4175 x *g* for 10 minutes at the same temperature. The supernatant obtained was considered as the extract and was filter sterilised using a Millipore membrane (0.22 µm sterile filter units) under sterile conditions in a laminar flow cabinet. Extracts were frozen at -20°C until used. The plant control i.e. *L. perenne* extracts were prepared in similar way.

A sub sample of the extracts was separated for pH and total organic carbon (TOC) measurement (Appendix C.3 and C.4). For TOC measurement 1:10 dilutions of the extracts were prepared with sterile water. To test any likely change in the plants extracts activity with age, leaves harvested from mature plants were also extracted using the above method and tested against *E. coli*. The mature plant extracts were cited as EXT-3 with the TOC range of 251 mg/l – 526 mg/l (Fig. 3.3).

3.3.3 Effect of plants shoot and root extracts on *E. coli* growth

3.3.3.1. Effect of extracts on *E. coli* growth in Nutrient broth (NB)

Escherichia coli were cultured onto NA plates as stated above (Section 3.3.1) and were incubated at 30°C for 24 hours prior to setting up the experiment. Two colonies taken from these cultured plates were suspended in 5 ml sterile Ringers solution (Prosser, 2011) in sterile falcon tubes and vortexed for 2 minutes to obtain a uniform bacterial suspension. This *E. coli* suspension (10 µl) was used to inoculate nutrient broth (NB; Difco-bacto NB) amended with 100 µl of the extracts containing the TOC range of 52 – 696 mg/l, cited in this chapter as EXT-1, in sterile Eppendorf tubes.

Extract + bacteria composition in Eppendorf tubes: 100 µl extract + 890 µl NB + 10 µl *E. coli* = 1 ml

Table detailing plants fresh dry biomass is presented in Appendix C.13. Extracts were replaced by sterile water for the negative controls and by a streptomycin solution (1 µg /µl) for the positive

controls. The essential oils of *L. scoparium* and *K. robusta* were also tested. For all treatments the Eppendorf tubes were vortexed for a few seconds before incubation for 24 hours in a bench top shaker at 320 rpm at 30°C. Four replicates per treatment were set up. After 24 hours, all Eppendorf tubes were centrifuged at 3219 x *g* for 10 minutes. The supernatant was removed and the pellets re-suspended in 1 ml sterile Ringers solution and vortexed for few seconds to obtain a uniform bacterial suspension. The optical density (OD) was measured at 600 nm using Genesys 10S UV-Vis spectrophotometer, with uninoculated NB used as a blank for OD measurements. To enable the OD measurements at 600 nm to be related to *E. coli* cfu measurements, a growth curve was produced and used to determine a regression formula relating OD at 600 nm to cfu/ ml (Appendix C.5).

The experiment was repeated with *E. coli* exposed to the extracts (480 µl) containing the TOC range of 52 – 696 mg/l (Appendix C.6) and cited as EXT-2. In EXT-2 preparation, 480 µl of each extract was added to 500 µl NB and inoculated with 20 µl of *E. coli* suspension.

To confirm the viability of the *E. coli* cells after 24 hours in each treatment, a sterile toothpick was dipped into the fourth replicate of the treatment under sterile conditions in a laminar flow, and used to inoculate a NA plate at four equidistant positions, 1 cm from the edge of the plates. All plates were incubated for 24 hours at 30°C and the presence/absence of bacterial growth was assessed.

3.3.3.2. *Escherichia coli* exposure to extracts in NA plates

Two fresh *E. coli* colonies were suspended in 5 ml sterile Ringers solution and vortexed for 2 minutes to give a uniform bacterial suspension. A 100 µl aliquot of this bacterial suspension was spread plated on NA plates. After drying plates for 20 minutes after bacterial addition, a 100 µl aliquot of the extracts were then spread on the same NA plates in triplicates and plates were incubated at 30°C for 24 hours. After 24 hours the number of bacterial colonies on each plate were counted to compare the growth inhibition.

3.3.3.3. Effect of pH on *E. coli* growth

Escherichia coli growth was tested at pH values from 2.5 to 9.5 in both solid and liquid media to determine whether the pH of the extract affects the growth and survival of *E. coli*. The pH of 100 ml NB was adjusted between 2.5 to 9.5 using NaOH or HCl (0.1 M), before autoclaving in tissue culture jars. An *E. coli* cell suspension was prepared by suspending two bacterial colonies in 5 ml sterile Ringers solution and vortexing for 2 minutes in a sterile falcon tube. Ten microliters of this bacterial suspension was then added to the liquid medium in each jar before all jars were incubated in a shaking incubator at 30°C for 24 hours at 320 rpm. Three replicates per treatment were set up. After 24 hours of incubation, bacterial suspension in each jar was mixed vigorously and 1 ml sample was transferred to sterile Eppendorf tubes in triplicates. Eppendorf tubes were centrifuged at 3219 x *g* for 10 minutes. The supernatant was removed using sterile pipette tubes and the pellet was re-suspended in Ringers

solution and vortexed for two minutes to give a uniform bacterial suspension. Optical density of each bacterial suspension was measured at 600 nm using the Genesys 10S UV-Vis spectrophotometer.

For solid medium, the pH of the NA was adjusted between 2.5 to 9.5 using NaOH or HCl (0.1 M) prior to autoclaving as described above. The NA was then poured into Petri plates and a sterile loop was used to inoculate the NA plate with the *E. coli* suspension prepared in Ringers (as described above) at equidistant positions and at 1 cm from the edge of the Petri plate. Three plates were set up for each pH treatment and arranged in a completely randomised design. All plates were incubated at 30°C for 24 hours and the presence/absence of *E. coli* was assessed at the inoculation points after incubation. All plates were photographed to compare the growth rates (Appendix C.7). Both experiments were repeated two times.

3.3.4 Effect of the plants root exudates on *E. coli* growth

3.3.4.1. Surface sterilisation and germination of seeds

Both *L. scoparium* and *K. robusta* seeds were dipped into 96% ethanol for one minute followed by 3 minutes disinfection in 10% commercial sodium hypochlorite solution (Braun & Leung, 1991). Seeds were then washed three times for one minute each in sterile water and allowed to dry for nearly 40 minutes under a sterile airflow in a laminar flow cabinet. Seeds were then transferred into sterile Petri plates, containing sterile tissue paper on the base and the seeds were spread uniformly with the help of sterilised spatula. A 5 ml aliquot of sterile water was added to moisten the tissue paper. Petri plates were sealed and placed in natural light near a window (not facing direct sunlight) at room temperature.

Germinated seeds (seedlings) were carefully transferred onto agar plates using a sterilised spatula, under sterile conditions in a laminar flow. A maximum of 4 to 6 plantlets were plated per agar plate and plates were left at room temperature for three to five days and were observed daily for any contamination. If any contamination appeared, uncontaminated plantlets were transferred into fresh agar plates and further observed for three to five days for sterility. The uncontaminated plantlets were then transferred to sterile tissue culture jars with enough MS agar/broth to cover the base of the jars (~10 ml). All plants were initially maintained at 21°C with 16:8 light: dark regime in small growth chamber for three months. After three months plants with no yellow or dry leaves were considered healthy and were transferred into commercial growth chamber and maintained under similar conditions as the small growth chamber for the next two months and then at room temperature on a bench in the laboratory. After three and six months of growth, plants growth media in which plants root exudates were released was collected using the sterile pipette tips and used to test their antimicrobial activities against *E. coli*.

3.3.4.3. Assay of root exudates against *E. coli*

Plants growing in MS agar were carefully removed with the help of a sterilised spatula in a laminar flow cabinet without disturbing the roots and were transferred onto universal bottles filled with 3 ml sterile Ringers solution. These plants were left in universal bottles for 48 hours (suggested by Associate Professor David Leung, Canterbury University) to enable them to release their root exudates and any associated antimicrobial compounds into the Ringers solution. Sterile filter paper discs of 10 mm diameter were used to test the inhibitory activities of the exudates against *E. coli* using the paper disc method (Carranza et al., 2015; Ericsson et al., 1960). Nutrient agar plates were inoculated with a 100 µl aliquot of *E. coli* suspension using lawn method and two paper discs were placed equidistant and at 1 cm from the edge of the Petri plate (Chan et al., 2013). A 30 µl aliquot of the exudates solution from the universal bottles was pipetted onto the paper discs. For plants growing in MS broth, 30 µl aliquot of the broth containing root exudates was used to inoculate the paper discs. Negative controls consisted of paper discs treated with sterile water. All treatments were set up in three replicates. The plates were incubated for 24 h at 30° C (Appendix D1), after which inhibition of *E. coli* growth by the root exudates was determined by measuring the width of the inhibitory zone around the discs.

3.3.4.4. Freeze drying root exudates

A 1.5 ml aliquot of the Ringers solution or MS broth containing exudates was freeze dried for 24 hours to concentrate any inhibitory compounds present. After freeze drying, the remaining pellet was dissolved in 100 µl sterile water and the inhibitory effect of this solution was tested against *E. coli* using the paper disc method described In Section 3.3.4.3. Sterile water was used as a negative control.

3.3.5 Statistical analysis

Microsoft excel (Version 2013) was used to calculate cfu/ml using the regression formula obtained from the *E. coli* linear regression curve of OD versus cfu (Appendix C.5). When required, data were log-transformed and then analysed using one-way analysis of variance (ANOVA) followed by comparison of individual values of the treatments using Fisher's Unprotected LSD Analysis. Bacterial count below detection limit i.e. 0 cfu/ml, was considered 1 cfu/ml while changing the cfu values to the log₁₀. All data analysis was carried out in GenStat 16th edition (VSN International Ltd.)

3.4 Results

3.4.1 Effect of plant extracts on *E. coli* growth

Exposure of *E. coli* to *L. scoparium* and *K. robusta* extracts i.e. EXT-1, significantly ($p < 0.05$) reduced the cfu/ml compared to the control (Fig. 3.1). Although both root and leaf extracts of *L. scoparium* and *K. robusta* inhibited *E. coli* growth, roots extracts of both species had a significantly greater growth inhibition than the respective leaf extracts. Extract of *L. perenne* leaves on the other hand increased *E.*

E. coli growth significantly compared to the control, with an increase of 63%. Leaf extracts of *L. scoparium* and *K. robusta* resulted in a 15% and 13% reduction in *E. coli* growth, respectively, followed by the root extracts resulting in 25% and 23% reduction in growth, respectively, as compared with the negative control.

Bacterial growth was below the detection limit i.e. 0 cfu/ml, in the media amended with the essential oils of the two native species and streptomycin (positive controls) with there being no significant difference between these treatments. The same results were obtained when the experiment was repeated using EXT-2 and increasing the concentration of the extracts did not increase the level of inhibition (Appendix C.6).

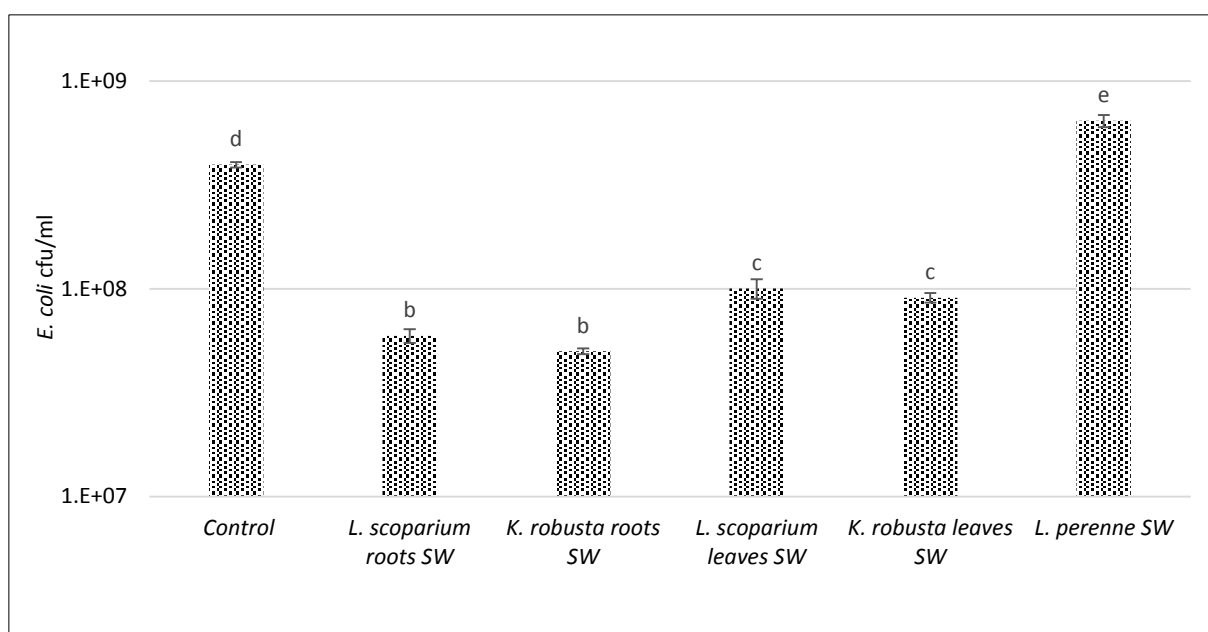


Figure 3-1 Average *Escherichia coli* growth in nutrient broth amended sterile water extracts (EXT-1) of *L. scoparium* and *K. robusta*. *Lolium perenne* was used as a plant control. Control consisted of sterile water replacing the plant extracts (100 μ l) referred as the negative control. Bars with the same letters do not differ significantly ($p < 0.05$) according to Fishers unprotected LSD analysis ($n = 3$). Error bars represent standard error of average value. SW = Sterile water.

The leaf extracts (EXT-3) of all the mature *L. scoparium* and *K. robusta* plants, apart from the leaf extract of *L. scoparium* collected from near Paper road/L2 Lake in Christchurch, significantly inhibited *E. coli* growth compared with the control (Fig. 3.2). The *E. coli* cfu counts differed significantly between extracts produced from *L. scoparium* and *K. robusta* leaves from the different sampling sites, with leaf extract from *L. scoparium* sourced from Quail island significantly reducing *E. coli* cfu counts compared with all other *L. scoparium* and *K. robusta* plant sources. There was no significant difference in the inhibition for leaf extracts from *L. scoparium* from the North Island *L. scoparium*, *K. robusta* from Quail Island and Lincoln University. Extract of *L. perenne* leaves increased *E. coli* cfu counts by 56% compared with the control, while extracts from *L. scoparium* sourced from L2 Lake increased *E. coli* cfu counts by 71% compared with the control and 15% more than *L. perenne* leaf extract. There was no significant

difference in the cfu counts of *E. coli* exposed to leaf extracts from *L. scoparium* sourced from L2 Lake and *L. perenne*. Leaf extract from Quail Island *L. scoparium* trees resulted in an 85% reduction in *E. coli* growth, followed by two-year-old North Island *L. scoparium*, and *K. robusta* leaves collected from Lincoln University and Quail Island, showing 68%, 31% and 34% reduction in *E. coli* growth, respectively compared with the control.

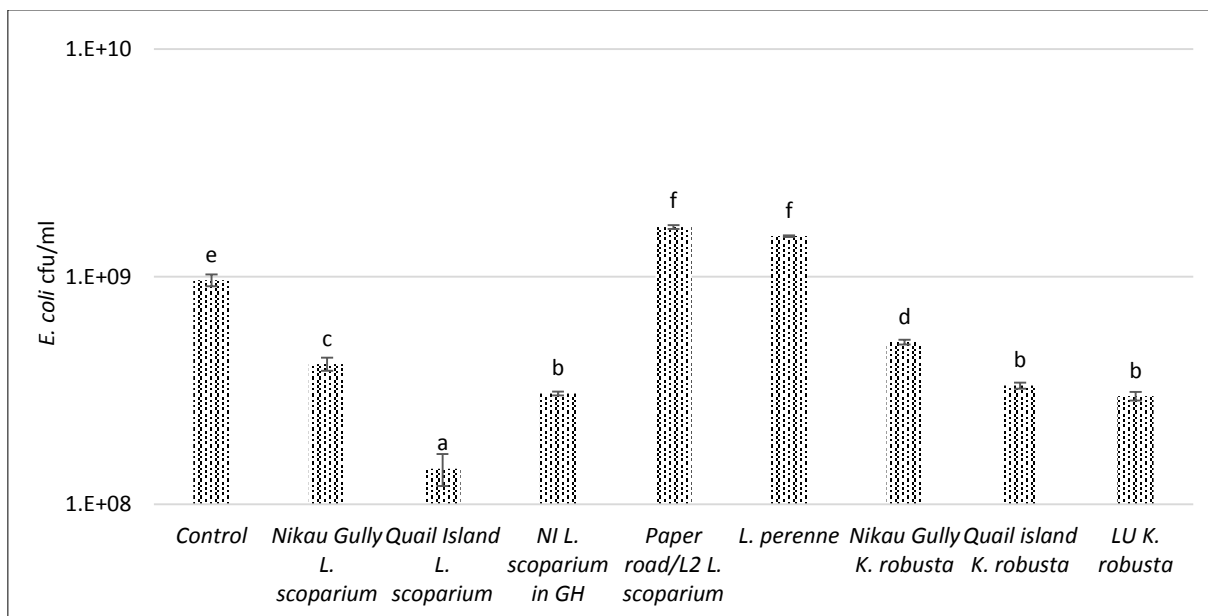


Figure 3-2 Average *E. coli* growth in nutrient broth amended with extracts (EXT-3) from mature *L. scoparium* and *K. robusta* leaves extracts. *Lolium perenne* was used as a plant control. Control consisted of sterile water replacing the plant extracts (100 μ l) and referred to as the negative control. Bars with the same letters do not differ significantly ($p < 0.05$) according to Fishers unprotected LSD analysis ($n = 3$). Error bars represent standard error of the mean. NI = North Island, LU = Lincoln University, GH = glasshouse.

The TOC concentration in the leaf extracts (EXT-3) from the different *L. scoparium* and *K. robusta* plant sources was measured to determine if there was any association with effect on *E. coli* growth. Fig. 3.3 shows EXT-3 TOC exposed to the *E. coli* in the range of 251 mg/l – 526 mg/l. Extracts from *L. scoparium* growing next to L2 Lake had the highest TOC concentration with the lowest TOC concentration found in extracts from *L. scoparium* from Quail Island and the North Island, and *K. robusta* from Quail Island and Lincoln University. Extracts from *L. perenne* had a similar TOC concentration of 423 mg/l to extracts from Nikau Gully *L. scoparium* with 434 mg/l TOC. There was a positive correlation of 81% between the TOC concentration of the extracts (without the sterile water control) and *E. coli* counts in NB i.e. the more was the TOC the more was the growth of *E. coli*.

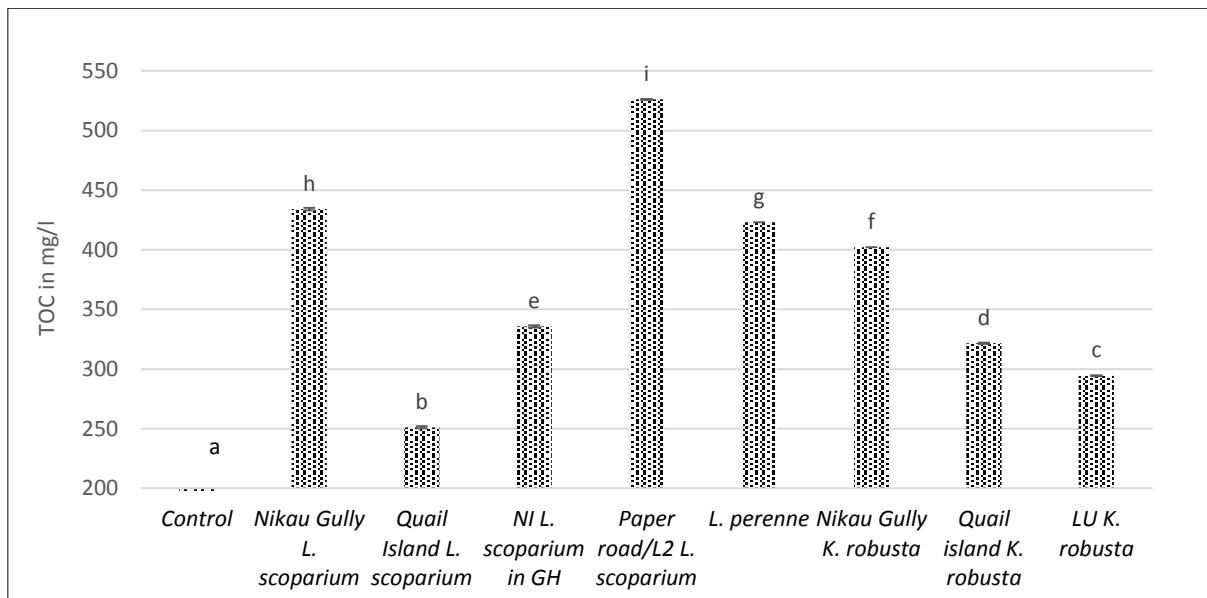


Figure 3-3 Total organic carbon (TOC; mg/l) of EXT-3 leaf extracts from *L. scoparium* and *K. robusta* mature plants from different sampling sites, and *L. perenne*, which were used to amend nutrient broth for *E. coli* culture assays (n = 2). Control consisted of sterile water replacing the extracts. NI = North Island, LU = Lincoln University, GH = glasshouse.

3.4.1.1. Effect of extracts on the viability of *E. coli*

Escherichia coli colonies were seen to grow from all the inoculation points in NA plates from *E. coli* NB cultures exposed to EXT-1 (Fig. 3.4). No colonies developed from the *E. coli* cultures exposed to streptomycin indicating a bactericidal effect on *E. coli*. However, minimal *E. coli* colony growth were seen on the plates inoculated with cultures exposed to *L. scoparium* or *K. robusta* essential oils, which was intermediate to the results seen with streptomycin and sterile water extracts. Negative controls with sterile water generated bacterial growth at inoculation points, similar to the extracts. Similar results were observed for *E. coli* cultures exposed EXT-2 (pictures not shown).

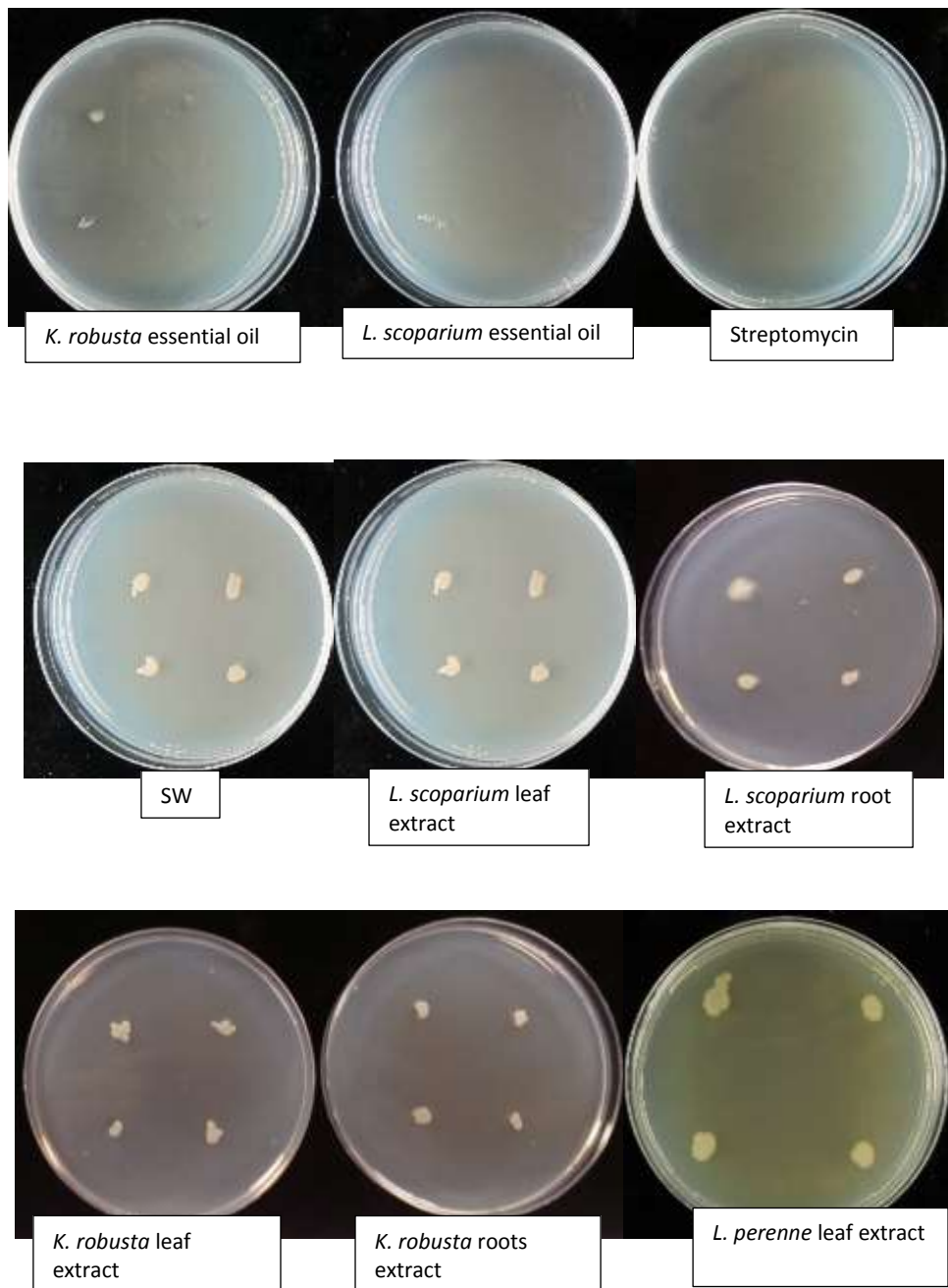


Figure 3-4 *Escherichia coli* colony growth on nutrient agar plates inoculated with *E. coli* nutrient broth cultures exposed to EXT-1 of *L. scoparium* and *K. robusta* and leaf extracts of *L. perenne* compared with cultures exposed to streptomycin or *L. scoparium* and *K. robusta* essential oils. SW = Sterile water used as control.

3.4.1.2. Assay of bacterial treatment with extracts in NA plates

Escherichia coli colonies developed on the NA plates amended with 100 µl of EXT-1 (Fig. 3.5). The number of colonies that formed on the NA plates amended with *L. perenne* and *K. robusta* leaf and root extracts were too high, resulting in a lawn of bacteria that could not be counted (Appendix C.8.). For NA plates amended with *L. scoparium* root and leaf extract the cfu counts were 2.5×10^3 and 2.3×10^3 cfu/ml, respectively, with there being no significant difference between them. Significantly higher cfu counts were seen on the NA plates amended with North Island *L. scoparium* extract, being 4.1×10^3 cfu/ml, compared with all other treatments apart from the sterile water amended control plates.

Essential oils of *L. scoparium* and *K. robusta*, also generated *E. coli* growth of 7×10^2 and 1×10^3 cfu/ml, respectively. No colonies developed on the NA plates amended with streptomycin.

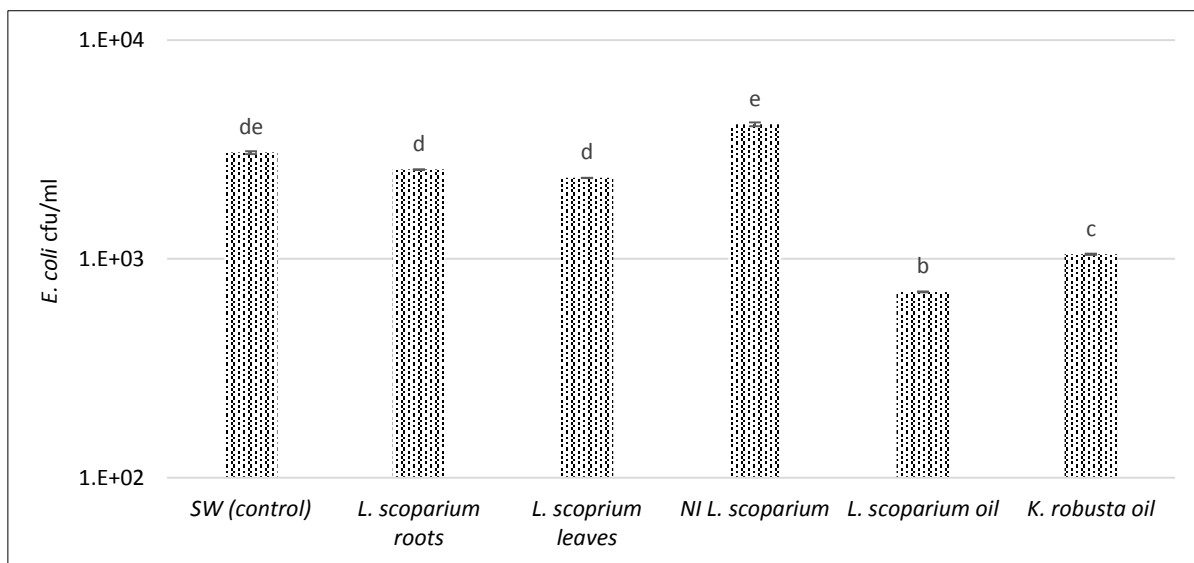


Figure 3-5 Growth of *E. coli* exposed to EXT-1 of *L. scoparium*, *K. robusta* and *L. perenne* on nutrient agar plates after 24 hours. Bars with the same letters do not differ significantly ($p < 0.05$) according to Fishers unprotected LSD analysis ($n = 3$). Error bars represent standard error of average values. NI = North Island, LU = Lincoln University, GH = glasshouse, SW = Sterile water.

3.4.1.3. pH of extracts

The pH value of the extracts differed significantly. There was no significant difference in the pH of the root extracts of *L. scoparium* and *K. robusta*, however the pH of the *L. scoparium* leaf extract was significantly lower compared with that of *K. robusta* leaf extract. The pH of the leaves extract of both species was significantly lower than the pH of the root extracts (Fig. 3.6). The pH of the *L. perenne* leaf extract was above 6.0 while the pH of both *L. scoparium* and *K. robusta* leaf and root extracts was between 4.6 and 5.2.

The pH of the liquid media or NB was 5.98 and the pH of the extracts-broth-bacterial mixture used for OD testing in Section 3.3.3.2, was also measured, which generated a buffering of ~ 0.2 pH in all extracts used.

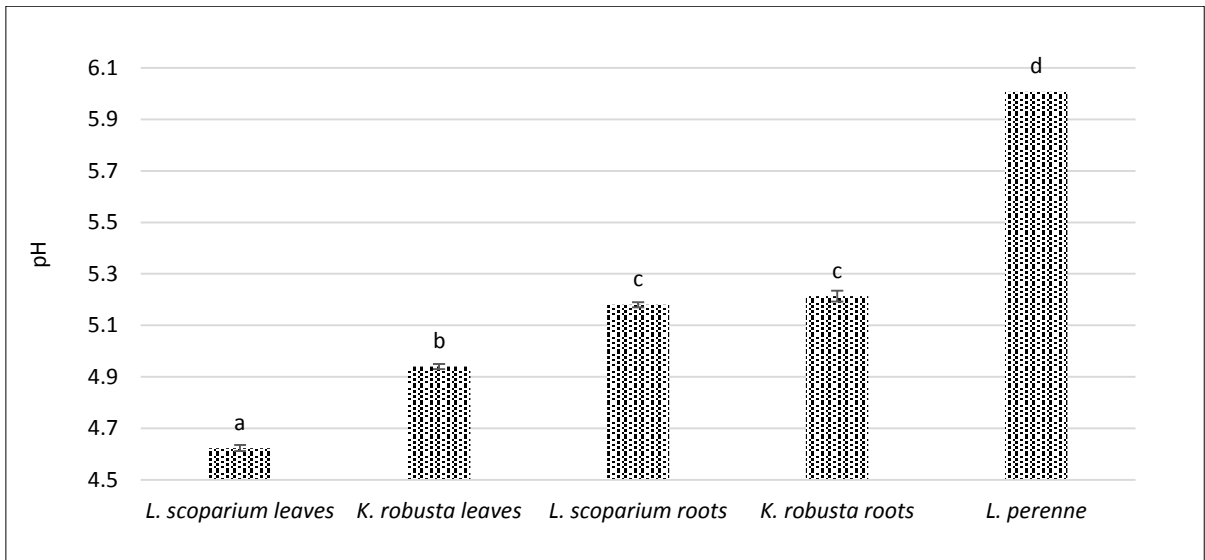


Figure 3-6 The pH of *L. scoparium*, *K. robusta* and *L. perenne* sterile water extracts. Bars with the same letters do not differ significantly ($p < 0.05$) according to Fishers unprotected LSD analysis ($n = 3$). Error bars represent standard error of average values.

3.4.1.4. Effect of pH on *E. coli* growth

Escherichia coli cfu level was below the detection level at pH 4.5 or below (Fig. 3.7). However, at all pH levels tested above 4.5, *E. coli* was seen to grow. *Escherichia coli* counts were between 6.24×10^8 cfu/ml at 8.5 (highest) to 4.76×10^8 cfu/ml at 6.5 (lowest). The results on the pH adjusted NA was also overall similar, although *E. coli* growth was seen at pH 4.5 in solid media (Appendix C.7).

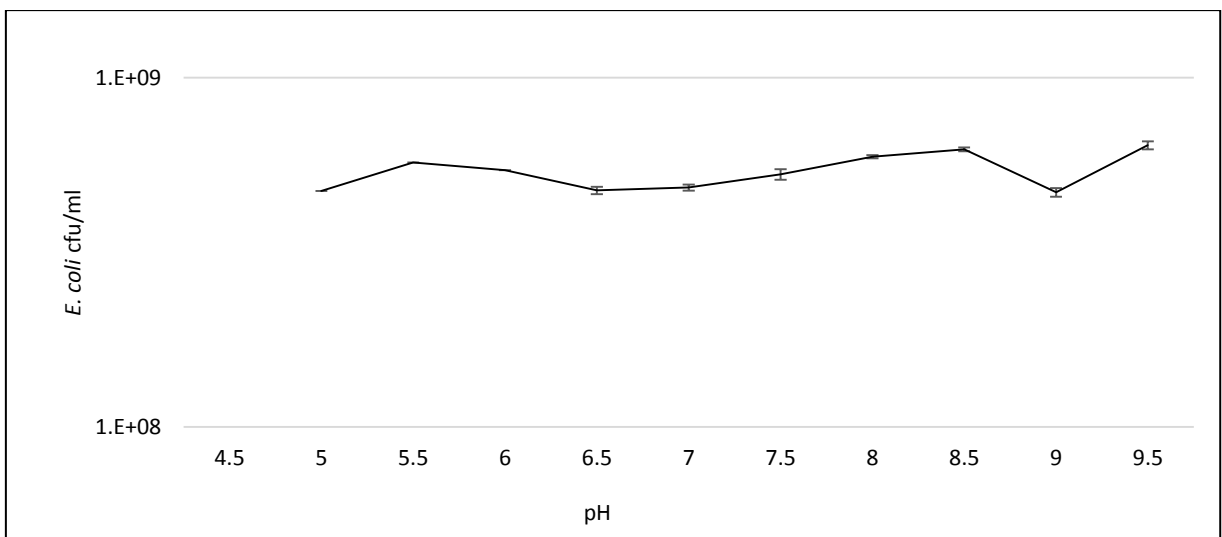


Figure 3-7 *E. coli* growth (cfu/ml) in nutrient broth amended to different pH values. Error bars represent standard error of the average values ($n = 3$).

3.4.2 Testing root exudates activity against *E. coli*

Out of the 66 plantlets grown in MS media, 21 plants survived between three to six months with only eight plants remaining alive until 8 months. Fig. 3.8 shows the different stages of plant culturing in the laboratory.



Figure 3-8 Stages of lab culturing of *L. scoparium* and *K. robusta* plants. A) Plants germination (seedlings) appeared between 12-18 days. B) Seedlings transferred onto agar plates. C) Plants growing into MS media in tissue culture jars.

3.4.2.1. Effect of root exudates on *E. coli* growth

Root exudates of six months old *L. scoparium* and *K. robusta* plants were tested against *E. coli*. None of the plants exudates obtained from the cultured plants amended onto MS agar/broth, inhibited *E. coli* growth on NA plates. Similar results were obtained when exudates obtained after freeze drying

were tested against *E. coli* (Fig. 3.9). As no *E. coli* inhibition was obtained following the methods used, no further chemical analysis of the exudates was performed.

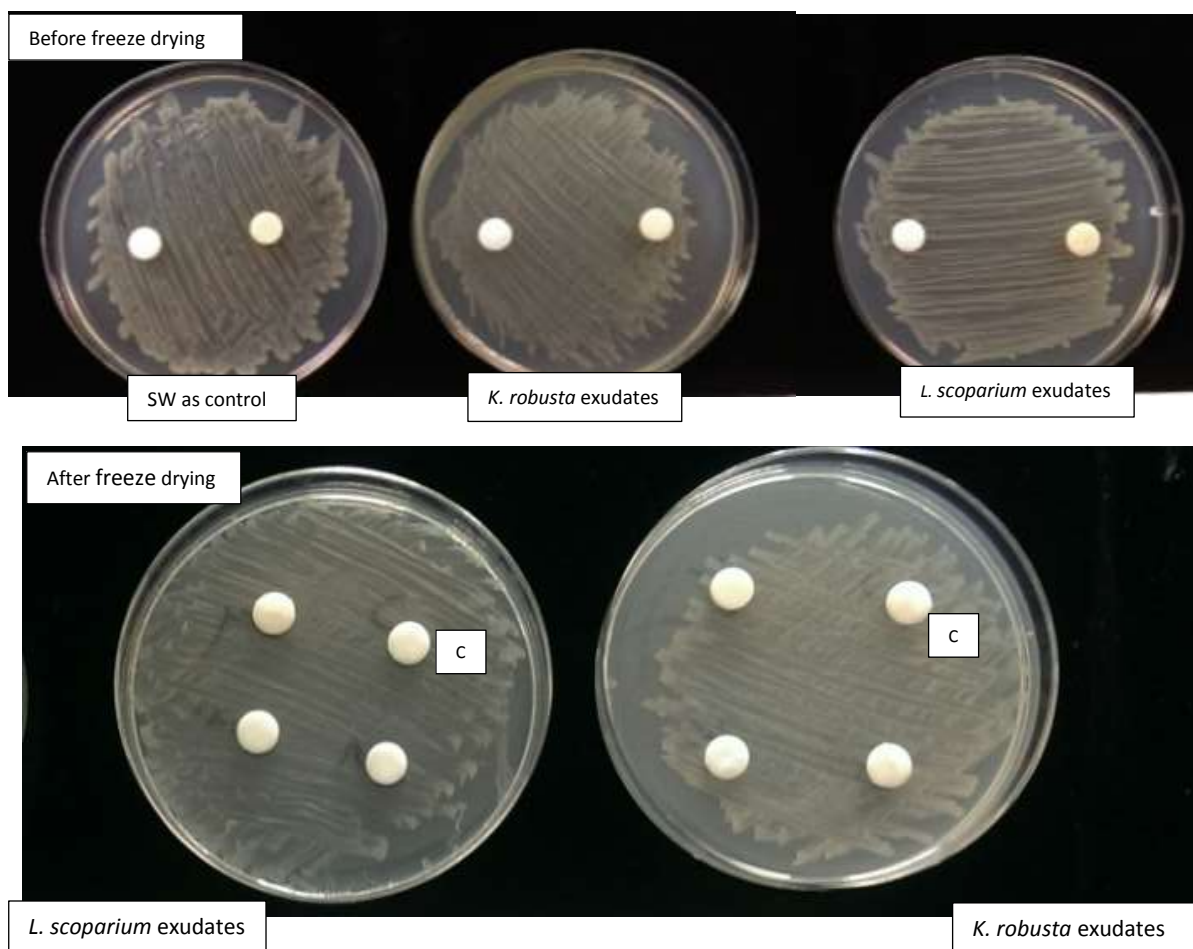


Figure 3-9 No *E. coli* inhibition obtained by the root exudates of 6 months old lab cultured *L. scoparium* and *K. robusta* before or after freeze drying applied onto paper discs and placed onto *E. coli* amended NA plates (n = 3). C = Sterile water used as control.

3.5 Discussion

The results of this study demonstrated a decrease in the growth of *E. coli* exposed to leaf and root extracts of both *L. scoparium* and *K. robusta*. Many studies have shown genotypic and chemotypic differences among North Island and South Island *L. scoparium* and *K. robusta* populations and higher antimicrobial activities of plants from some specific locations such as North Cape in North Island and Marlborough Sounds in South Island (Lis-Balchin et al., 2000; Porter et al., 1998; Porter & Wilkins, 1999). Results from this study are in agreement with Prosser (2011) who demonstrated antibacterial activities of North Island *L. scoparium* and *K. robusta* aqueous extracts against different microbial species including *E. coli*, and also confirm the bacteriostatic nature of the extracts. Leaf extract of *L. perenne* increased the growth of *E. coli* for unknown reasons (Fig. 3.1).

In contrast to the extracts, the root exudates of six months old and laboratory cultured *L. scoparium* and *K. robusta* did not inhibit *E. coli* growth. It is postulated that these laboratory cultured plants were

either too young to release any active antimicrobial components against tested *E. coli* strain or plants might not release the active antimicrobial components without the presence of active rhizosphere environment and the associated microbial populations in soil (Rovira, 1965).

Most studies agree that plant extracts are generally more inhibitory towards gram positive bacterial species than gram negatives with few exceptions (Ghasemi et al., 2015; Marasini et al., 2015; Wyatt et al., 2005). Inhibition of *E. coli*, a gram-negative bacterium, by *L. scoparium* and *K. robusta* aqueous extracts in this study, is thus an interesting result as high efficacy of mainly the essential oils and honey from these native species, has been proven towards gram positive bacterial species with some recent studies showing inhibition of gram negatives (Van Vuuren et al., 2014). Demuner et al. (2011) stated that the high activity of essential oils of Myrtaceae family towards gram positive bacterial strains can be related to the high concentration of Nerolidol, found in some Myrtaceae species such as *Leptospermum flavescens*, although Nerolidol production has not been specifically established for *L. scoparium* or *K. robusta*. The chemistry of the extracts was not determined in this study.

The type of solvent and process used for the extracts preparations is critical as results can vary depending on the type of solvents, microbial species used, resistance development levels in the microbes, season of the biomass collection and type of biomass (flowers, twigs or stems) (Hassan et al., 2009; Lis-Balchin et al., 2000). The antimicrobial effects of the essential oils on gram negative bacteria depends on the specific compounds, as some antimicrobial components can displace the hydrophobic layer present on the outer cell wall of the gram-negative bacteria resulting in inhibition (Calo et al., 2015; Skala et al., 2016). Bacterial growth inhibition requires the interaction of the plants/extracts antimicrobial components with those of the bacterial cell membrane possibly resulting in the disturbance of the transport proteins or other critical components in bacteria such as Indole (Begley et al., 2005; Brandl et al., 2001; Hentzer et al., 2003). Sterile water used here, presented bacterial inhibition possibly by extracting the hydrophilic antimicrobial components from plants, which might have not been possible if the hydrophobic/lipid solvents had been used. The inhibition of the gram-negative bacteria by sterile water extracts indicates the presence of hydrophilic antimicrobial compounds in the leaves and roots of *L. scoparium* and *K. robusta*, different from those present in essential oils (due to the hydrophobic nature of essential oils) however, the following mechanism was unknown. The use of water and a cold temperature extraction process with liquid N has been chosen for preparing biomass powder as high temperature present at the time of extract preparation, such as with the use of Soxhlet extractor apparatus, can reduce the effectiveness of antibacterial plant compounds through the denaturing of proteins and amino acids (Marasini et al., 2015).

Jain et al. (2015) tested the antimicrobial activity of extract of several plants used in traditional Indian medicines using organic, aqueous and crude extracts and stated that efficacy differed depending on

the solvent type used. They found that aqueous extract of amla (*Phyllanthus emblica*) showed higher antibacterial activity than its organic solvent extracts, however, organic extracts of ginger (*Zingiber officinale*) were most active, all against *Streptococcus mutans*. They suggested that during the extraction of the natural components such as essential oils, hydrophobic active compounds in the essential oils, showing high antimicrobial activity, might not be effectively extracted with water only and obtaining a uniform solution of essential oils or their active hydrophobic compounds is quite difficult in aqueous solutions. Wyatt et al. (2005) found highest efficacy of the leaf lipid extracts of *K. robusta* in extracting bioactive components such as terpene. They stated that some extraction processes such as lipid extraction, might result in the extraction of a high abundance of fatty acids such as lauric acid, which might be responsible for the high antimicrobial effect observed. In contrast extracts using other extraction processes, for example using terpene, might only show a moderate inhibitory effect against microbes due to minor amounts of antimicrobials such as butinilic acid being extracted (Hernández-Pérez et al., 1994; Ouattara et al., 1997). Eloff (1998) suggested acetone as being a better option as a solvent, being less toxic and able to dissolve a variety of hydrophobic, hydrophilic, lyphophilic and volatile components from plant tissues. Other studies have reported similar antimicrobial activities by both water and ethanol extracts of plants (Häberlein & Tschiersch, 1998; Hassan et al., 2009), which does not fit with this study as the ethanol and sterile water used in preliminary trials (Appendix D.1) presented variable results with toxic effect of ethanol extracts against bacteria.

In this study, some regrowth of *E. coli* exposed to the extracts was observed, including for colonies exposed to inhibitory roots extracts of both *L. scoparium* and *K. robusta*. These results indicate that the extracted water-soluble components from both plant species had a bacteriostatic effect against the tested *E. coli* strain, i.e. they were able to prevent bacterial growth but did not kill the bacterial cells. Hence, favourable environmental conditions on NA media resulted in regrowth of extract treated *E. coli*. Hassan et al. (2009) suggested that extracts can show bacteriostatic effect at lower concentrations while a bactericidal effect can be observed at high concentrations. This was not applicable in this study as exposure to either EXT-1 i.e. 100 µl or EXT-2 i.e. 480 µl extracts (Appendix C.6) resulted in a bacteriostatic effect only. This indicates that in the natural environment these plants might inhibit bacterial activity, although bacterial growth might return in the absence of sufficient concentration of plant compounds. Interestingly, essential oils of both *L. scoparium* and *K. robusta*, which inhibited *E. coli* growth in liquid medium to below detection limit, did not inhibit bacterial growth in NA (Fig. 3.5). This may be because incubation of *E. coli* in liquid media containing the essential oils in a shaking incubator might have resulted in increased exposure of *E. coli* to the essential oil components, which did not occur in the solid agar. Nutrient agar also sometimes interferes with the diffusion of components generally due to its sulphur components (Jain et al., 2015).

The inhibitory activity of leaf extracts (EXT-3) obtained from different mature *L. scoparium* and *K. robusta* plant sources (Fig. 3.2) against *E. coli* was demonstrated to vary. Extracts from most of the mature plant samples inhibited *E. coli* growth compared to the control, however extracts from *L. scoparium* plants from a site near the L2 Lake in Christchurch was shown to increase *E. coli* growth to 71% more than the control. Total organic carbon analysis of the mature plant extracts (without the sterile water control), demonstrated a high correlation of 81% between bacterial growth rates and extracts TOC i.e. the higher the TOC concentration, the higher the *E. coli* growth. Although, the TOC concentration of extracts from Nikau Gully sourced *L. scoparium* and *L. perenne* was similar (434 mg/l and 423 mg/l, respectively), bacterial inhibition was higher for the Nikau Gully sourced *L. scoparium* than the *L. perenne*, indicating that the active inhibitory components were present in the native plant extracts along with high TOC concentration. It is likely that the TOC concentrations in the plant extracts provided nutrients to the *E. coli* in the form of sugars (not tested), and in some extracts this high concentration enabled the *E. coli* cells to overcome the antimicrobial activity of any inhibitory components present. Hence, to elucidate further the role of these native plants as a remediation species, chemical analysis to identify the hydrophilic inhibitory components and their variability with plants age, tissue type and location is required. The pH of the extracts had no effect on bacterial growth, indicating the results obtained were due to the interactions between the bacteria, TOC concentration and the concentration of inhibitory compounds in the extracts.

Prosser (2011) stated the possibility that the specific antibacterial compounds of *L. scoparium* and *K. robusta*, might be strongly bound to the inner layers of the cell wall and only be released at high temperatures, such as steam distillation used during essential oils and honey production. In the present experiment, *E. coli* were exposed to crushed leaves and roots thereby making available chemicals that were attached to, or enclosed within, the cell walls. To demonstrate whether these native species will release their inhibitory root components showing activity in extracts, a microcosm study was performed elaborating released root exudates activities of laboratory cultured *L. scoparium* and *K. robusta* against *E. coli*. The results showed no effect of the released root compounds of either *L. scoparium* or *K. robusta* against the tested *E. coli* strain. As the presence of root exudates was not tested using any specific method, it cannot be guaranteed that the root exudates were even released by plants in the growth media. These 6-month-old plants were relatively young and this may have affected release of any inhibitory or antimicrobial components active against *E. coli*, with the method used and within the time tested, resulting in no inhibition of *E. coli*. Studies have suggested changing plant activities and their endophytic communities, responsible for various selective processes including release of root exudates, with age (Chen & Nelson, 2012; Demuner et al., 2011; Douglas et al., 2004; Wicaksono et al., 2016) and has also been demonstrated with our mature plants extracts (EXT-3) activities. Hence, the lack of inhibition of *E. coli* found with root exudates could be because no

antibacterial components were released by the young plants or the components were below the detection limit to present inhibition with paper disc method. It also indicated that the inhibitory components released by crushing the leaves or roots in extracts might have resulted in the exposure of *E. coli* cells to those inhibitory components, which are not generally released by plants under more natural situations. Further, the release of inhibitory root exudates by plants is a result of various complex plant-plant, plant-microbe and microbe-microbe interactions in the rhizosphere (Bais et al., 2004; McNickle & Dybzinski, 2013; Mendes et al., 2013), which was absent in this laboratory study.

Fan et al. (2014) stated that many antibacterial compounds released by plants are readily decomposable and hence to maintain their fitness, plants continuously release these components in natural environment. In this study, the 24-hour duration of concentrating the active antibacterial components with freeze drying might have resulted in the loss of any active compound against *E. coli* (such as volatiles) due to their fast degradation time (Kai et al., 2016). Prosser (2011) also reported fast degradation of the active antimicrobial components from soil samples collected from under *L. scoparium* trees. They stated the possibility of losing active antimicrobial components between field sampling and laboratory tests demonstrating no bacterial inhibition. One of our experiment (Appendix A) tested the VOCs of laboratory cultured *L. scoparium* and *K. robusta* and showed their potential against *E. coli*, which needs further testing.

Although, nutrients from the MS media used in this study might have been another factor affecting the effective release of inhibitory root exudates by plant roots. Future studies using different media with different salt and nutrient concentrations, for plants culturing and growth, might generate different results. Gagliardi and Karns (2000) found that N concentration in the media could affect bacterial survival and growth. Krafczyk et al. (1984) stated that a high N concentration in the growth medium can override the inhibitory effect of exudates released by plants and plants start to utilise the high N concentration to support fast growth. Although, in the present study, rapid root growth and increased plants biomass was only observed after 4 ½ months of plants germination, with no effect observed on plants height. Increased bacterial activity at low K concentrations was also reported by Krafczyk et al. (1984) indicating that nutrients available to plants do not only affect the released roots exudates but also the activity of the associated microbial population. They stated that hydrophobic components released by plants are in general excluded in laboratory studies using seedlings. As in this study, plants were growing in hydroponic solution and freeze-dried pellets were dissolved in Ringers solution (hydrophilic), any active hydrophobic compound released by plants active against *E. coli* might have been excluded. The no visible antibacterial activity from root exudates result that was observed also indicates the possibility of an induced antimicrobial response by these species, whereby the plants release specific antimicrobial components or gases only during an encounter with a non-native microbe (Appendix A), following a specific pathway as their defence mechanism (Kinkel et al., 2011;

Kinkel et al., 2012). Analytical methods such as chromatography, are helpful in detecting the blend of compounds released by plants in the rhizosphere (Fan et al., 2001). However, as no *E. coli* inhibition or active antibacterial effect was observed, this laboratory experiment was not extended to analyse the chemistry of the released root components.

Based on the results of this study indicating a bacteriostatic effect of the extracts on *E. coli*, it is likely that these plants will most probably prevent the growth of *E. coli* contaminants in soil rather than affecting their survival. If the bacterial population is residing under these plants, further study of their leaching potential and the activity of plants root exudates in natural systems, is essential in determining their effect to reduce water contamination risks. These experiments showed that *E. coli* cannot survive in nutrient broths with a pH of < 4.5 (Fig. 3.7), well above the levels reported in the rhizosphere soil of *L. scoparium* (Hahner et al., 2014). Therefore, the antimicrobial properties of these plants in the field may be partly due to rhizosphere acidification. Given that acidification is a relatively slow process (Helyar & Porter, 1989), it might be expected that older stands of *L. scoparium* would be more effective than fresh plantings in inhibiting pathogens.

3.6 Conclusion

This study demonstrated the antibacterial activity of the sterile water extracts of leaves and roots of *L. scoparium* and *K. robusta* against a non-pathogenic *E. coli* strain ATCC13706, unlike *L. perenne*, which significantly increased bacterial growth rate. Plants provenance presented variability in plants activities. Results demonstrated high inhibitory activities of the root extracts of both natives however; all extracts presented a bacteriostatic effect. Roots exudates from six months old *L. scoparium* seedlings showed no bacterial inhibition, indicating that either the plants were too young to release any antibacterial root components or they do not release these exudates without the natural rhizosphere conditions with associated microbes. To establish these natives as a remediation species for DSE contaminated lands, glasshouse and field studies, being more representative of the natural field situation, are required as these may show increased bacterial inhibition rates under *L. scoparium* and *K. robusta*. Future studies should focus on identifying the changing rhizosphere chemistry of the plants such as pH, which might be a potential factor inhibiting bacterial growth.

Chapter 4

The effect of *Leptospermum scoparium* and *Kunzea robusta* on bacterial fluxes in soil

4.1 INTRODUCTION

The land application of dairy shed effluent (DSE) introduces infectious microbes including pathogens such as *Salmonella* spp. and *Pseudomonas* spp. to the soil (Hashsham et al., 2004). Of these pathogens, *Campylobacter jejuni*, is of greatest concern in NZ as NZ had the highest rate of Campylobacteriosis during 2006 (Müllner et al., 2010; Sears et al., 2011). The land application of DSE is currently limited to the pastoral farms with little contribution towards forestry and horticulture (Zaleski et al., 2005) and can result in the bacterial leaching and surface run off to the nearby water resources (Aislabie et al., 2006). Houlbrooke et al. (2010a) mentioned that 30% of the land applied DSE can undergo surface run off following daily irrigations, which is a big concern for maintaining the water quality. In NZ, guidelines require <1 cfu/ml of *E. coli* in the drinking water supplies (Ministry for the Environment, 2017; Ministry of Health, 2000).

With antibiotic resistance developing in pathogens, including pathogenic strains of *E. coli* (Filoche et al., 2005; Jenkins et al., 2011) the use of antimicrobial plants, which contain novel antimicrobial components against microbial contaminants is gaining attention (Atsafack et al., 2015). Antimicrobial plants have been used for millennia for treating minor ailments and injuries (Marasini et al., 2015; Salie et al., 1996) and the inhibitory actions of their extracts against human pathogens have been demonstrated in various laboratory studies (Bora et al., 2016; Prosser, 2011). *Leptospermum scoparium* J.R.G. Forst. (Allan, 1961) and *Kunzea robusta* A. Rich. (Connor, 1985) are gaining interest due to specific antimicrobial properties of their triketone rich essential oils and honey, and also with their commercial importance for the country (Chen et al., 2016; Lu et al., 2014; Perry et al., 1997a; Perry et al., 1997b). Regardless of the known antimicrobial activities of medicinal plants their potential for pathogen attenuation in contaminated soil systems has received scant attention (Khan et al., 2013).

Studies monitoring stem-flow from canopies of trees and shrubs have reported that the precipitation on the leaves of the trees can dissolve compounds that then reach the soil near the tree trunks, depositing these compounds into the rhizosphere (Johnson & Lehmann, 2006; Li et al., 2009) although none of these studies report the deposition of the potential antimicrobial components from the leaves of the medicinal plants with the rain water. Litter deposition under trees might also deposit any potential antimicrobial component along with the organic matter from the leaves of the antimicrobial plants to the ground (Bargués Tobella et al., 2014).

Increasing plant cover reduces contaminant mobility and hence the risk of their off-site movement or surface run off (Bargués Tobella et al., 2014; Bolan et al., 2003) although studies have also mentioned that preferential flow under trees as compared to the shallow rooted crops or pasture (Yunusa et al., 2002) may exacerbate the downward movement of contaminants. Root exudates released by the trees are deposited into the preferential flow pathways created by the roots or into the adjacent soil systems (Bertin et al., 2003). These exudates are broad spectrum in action and may contain natural antibiotics protecting the plants from herbivores and pathogens (Bais et al., 2004; Bakker et al., 2014). Therefore, root induced preferential flow pathways are nutrient rich compared to the other cracks in the soil (Johnson & Lehmann, 2006) and the deposited nutrients and exudates in these pathways might expose the introduced pathogens to antimicrobial environment resulting in the decrease in bacterial numbers in groundwater.

Prosser et al. (2014) reported the antimicrobial potential of the roots and leaves extracts of *L. scoparium* and *K. robusta* against *E. coli*, and also reported rapid *E. coli* die-off under both species in a pot trial (Prosser et al., 2016). Our previous experiment (Chapter 3), has added to this knowledge showing the variation in efficacy of *L. scoparium* and *K. robusta* extracts depending on the provenance and that the young seedlings root exudates were ineffective against *E. coli*. Based on the results of Chapter 3, it was hypothesised that the antimicrobial components of *L. scoparium* and *K. robusta*, which exhibited bacterial inhibition in sterile water extracts (Fig. 3.1) might enter the soil contaminated with *E. coli*, resulting in its growth inhibition. This study aimed to elucidate the effect of antimicrobial components of *L. scoparium* and *K. robusta* on the leaching of a non-pathogenic bacterial strain *E. coli* ATCC13706, in two different soil types and to determine the path followed by the inoculated *E. coli* in the soil.

4.2 Materials and methods

4.2.1 Experimental set up

One-year-old *L. scoparium* and *K. robusta* plants were purchased from the Department of Conservation (DOC) nursery, Motukarara, Christchurch (43°43'46''S, 172°34'42''E). *Lolium perenne* seeds were obtained from Field Research Centre (FRC) Facility of Lincoln University. Soils were collected from Akaroa 43°47'32''S, 172°58'15''E (Pawson silt loam, Pallic Firm Brown soil) and Eyrewell forest 43°27'03''S, 172°05'20''E (Stony silt loam, Lismore soil) in Christchurch, NZ. These soils were selected to represent variable soil environment (common in South Island), where waste DSE can be applied. Sand and gravel (2.5 – 5 cm) were collected from Fulton and Hogan Construction Company, Christchurch, and a non-pathogenic strain of *E. coli* ATCC13706 (used in Chapter 3) was obtained from The Institute of Environmental Science and Research (ESR) Wellington.

Around 700 kg of each soil was collected from 0 - 30 cm top soil after removing any vegetation on the soil surface. Soils were homogenised and passed through a 4 mm sieve to remove large stones and roots. Subsamples of soils were frozen for the physical and chemical analysis following the laboratory manual for the Department of Soil and Physical Sciences (unpublished by Roger Cresswell and Leanne Hassall) (Table 4.1). Eyrewell soil was mixed with 20% sand to maintain porosity (Appendix D.2). Sixty 10 l lysimeters (diameter = 25.3 cm, height = 27.4 cm) were used in the experiment after 20% bleach wash (from 4% commercial bleach). After 30 minutes of bleach wash, lysimeters were further washed with the tap water and dried for 24 hours before filling with soil. Lysimeter bases were layered with 1.5 kg gravel, covering 3 cm of the bottom of the lysimeters, sandwiched between single layers of the winter sheets (knitted cloth by Egmont Commercials, provides a shade level of 45 - 50%) on both sides. The lysimeters were then filled with the soil (8.5 kg). Petroleum jelly was injected down the sides of each lysimeter (Appendix D.2), to eliminate edge flow.

Control lysimeters were established with 2 g of *L. perenne* seeds. All plants were established for controls and treatments in both soils with five replicates each (3 plants species x 2 treatments x 5 replicates x 2 soils = 60 pots) (Fig. 4.1.a). Plants were randomised following randomised complete block design (RCBD).

Table 4-1 Physical and chemical properties of the Eyrewell (43°27'03"S, 172°05'20"E) and the Akaroa (43°47'32"S, 172°58'15"E) soil. Values in brackets represent standard error of the mean value (n = 5). n.d. = not detected.

	Eyrewell Stony silt loam/Lismore soil	Akaroa Pawson silt loam/ Pallic Firm Brown soil
pH	5.2 (0.01)	4.9 (0.01)
CEC [meq/100 g]	13	13
Total N [%]	0.23 (0.01)	0.38 (0.05)
C/N	20 (0.4)	10 (0.1)
NH ₄ ⁺ - N	3.1 (0.1)	6.8 (0.2)
NO ₃ ⁻ - N	25 (1.2)	22 (1.1)
Al (mg/kg)	30477 (389)	27516 (232)
Ca (mg/kg)	2472 (41)	4735 (32)
Cd (%)	0.43 (0.00)	≤ 3 x10 ⁻⁴
Cr (mg/kg)	22 (0.3)	18 (0.2)
Cu (mg/kg)	3.4 (0.3)	8.2 (0.1)
Fe (mg/kg)	22293 (270)	20614 (109)
K (mg/kg)	4468 (37)	4818 (77)
Li (mg/kg)	34 (0.4)	n.d.
Mg (mg/kg)	3768 (33)	4374 (44)
Mn (mg/kg)	288 (3)	592 (5)
Na (mg/kg)	268 (4)	225 (9)
Ni (mg/kg)	7.3 (0.1)	8.7 (0.1)
P (mg/kg)	383 (7)	826 (10)
Pb (mg/kg)	14 (0.1)	13 (0.2)
S (mg/kg)	210 (6)	334 (3)
Zn (mg/kg)	75 (2.6)	71 (0.8)

Lysimeters were irrigated daily to field capacity for four months. During the experimental period, plants were watered to give ca. 300 ml of leachate daily. This required 14 mm of irrigation to the *L. scoparium* and *K. robusta* and 7 mm of irrigation to the *L. perenne* (Appendix D.2). Leachates were collected in 2.5 l plastic containers (Fig. 4.1.b). These containers were disinfected using 20% bleach solution before the start of the experiment and were thoroughly washed with water and dried before

leachate collection. Containers were also washed between leachate collections. Collected leachate were measured using the measuring cylinders and the volumes were recorded. Once a nearly constant volume of drainage started to leach for all the species, these irrigation rates, were maintained until the pots were spiked with *E. coli* ATCC13706.



Figure 4-1 Shows **a)** experimental set up in the glasshouse and **b)** leachate collection.

4.2.2 Preparation of the *E. coli* culture

Before the addition of bacteria to the plants, the background soil *E. coli* numbers leaching with the applied irrigations were enumerated for each lysimeter using Colilert-18 (IDEXX laboratories) at 36°C (Boubetra et al., 2011; Brown et al., 2011).

Escherichia coli from the glycerol stock (Appendix C.1) was cultured into the Nutrient Agar (NA; Oxoid CM0003) plates 24 hours before Nutrient broth (NB; Difco-bacto NB) inoculation. The bacterial culture for inoculation was prepared in sterile NB. One *E. coli* colony was inoculated into 500 ml NB in Schott bottles and the inoculated broth was incubated in a shaking incubator at 180 rpm overnight (18 to 22 hours) at 30°C. The bacterial culture was then transferred into 50 ml sterile falcon tubes and centrifuged at 4175 x *g* for ten minutes. Each bacterial pellet was washed twice with sterile Ringers solution and dilutions were prepared in Ringers to obtain a final OD of 0.4 at 600 nm corresponding to 10⁷ cfu/100 ml according to the previous calibration curve (Appendix C.5). Five sub samples of the *E. coli* culture were plated on NA following serial dilutions and plates were incubated at 30°C for 24 hours to confirm the bacterial count. Bacterial enumerations were performed from the plates generating 30 - 300 cfu/ml.

A solution of 500 mg NaBr/l (400 mg Br/l) was prepared in water and used as a tracer in soil. The concentration of NaBr used was non-toxic to *E. coli* (Appendix C.9).

4.2.3 Bacterial spiking

Each treatment plant received 100 ml of *E. coli* culture containing 2.6 x 10⁵ cfu/ml. The culture was applied using a measuring cylinder at a rate of 25 ml per hour. One hour after spiking with *E. coli*, 100 ml of NaBr solution was added at a similar rate to both treatment and control plants. All irrigations were completed in nine hours and plants were left overnight to allow *E. coli* interaction with the root compounds. After 24 hours, plants were maintained using the previous irrigation rates (14 mm for *L. scoparium* and *K. robusta* and 7 mm for *L. perenne*).

4.2.4 Bacterial enumeration and NaBr determination

Leachate volume was measured daily and a 100 ml subsample was separated for *E. coli* enumeration using Colilert-18 (IDEXX laboratories). Colilert-18 is a Defined Substrate Technology (DST) based method of IDEXX Laboratories that detects and enumerates total coliforms and *E. coli* simultaneously in water. Colilert-18 utilises the ability of coliforms to metabolize o-nitrophenyl galactopyranoside (ONPG) changing the sample colour to yellow and *E. coli*'s ability to metabolize ONPG and methylumbelliferyl-D-glucuronide (MUG), resulting in the colour change of the sample along with fluorescence under UV light (Boubetra et al., 2011). This study did not enumerate total coliforms.

A subsample of each leachate (30 ml) was also collected for the bromide analysis using Ion Chromatography System (Dionex DX-2100). The system has an Anion Self-Regeneration Suppressor (AERS 500, 4 mm) with conductivity detector. Eluent Source was EGC III KOH cartridge with CR-TC and a flow rate of 1.0 ml/min. The auto-sampler was a Dionex AS-AP and the analytical columns were IonPac AS18 (4 × 250 mm) and the guard column was IonPac AS18 (50 × 4 mm) with the column temperature of 30°C. All samples were filtered with 0.45 µm NY membrane filters and 25 µl sample was injected in the system. A standard stock solution of 1 mg/l bromide was used for preparing the calibration curve with deionised (DI) water. The concentration range of the calibration curve was from 0.02 ppm to 100.00 ppm.

Leachate samples for bromide were frozen at – 20°C until analysis, while *E. coli* enumeration was performed within 4 hours of collection.

4.2.5 Dye application and harvesting soil columns

At the end of the experiment, shoots of *L. scoparium* and *K. robusta* were removed by cutting approximately 2.5 cm above the soil surface and *L. perenne* was harvested approximately 5 cm above the soil surface. Roots of all plants were left undisturbed. Immediately after removing the shoots, all soil columns were slowly irrigated with 7 mm of brilliant blue dye (4 g/l solution) to trace the preferential flow path. Soil columns in the lysimeters were left undisturbed for 48 hours after which they were removed from the lysimeters into clean plastic sheets (Appendix C.10) and were incised from the centre into two parts using a saw and hammer. Pictures of the incised soil columns were taken and the soil was mixed uniformly. Soil sub sample was collected to measure the moisture content after drying the soil at 75°C for two days (results not shown).

4.2.6 Statistics

Standard errors were calculated using Microsoft Excel (Version 2013). To identify the significant differences in *E. coli* leaching between species, cfu/ml was log-transformed, and two-way ANOVA was performed on these log numbers using the Fishers unprotected LSD analysis ($p < 0.05$) in Genstat 16th edition (VSN International Ltd). Bacterial count below detection limit i.e. < 1 cfu/100 ml, were considered 1 cfu/ 100 ml prior to \log_{10} transformation.

4.3 Results

Fig. 4.2 – 4.5 show the highest bacterial count leached within two days of the *E. coli* addition, from all plant species in both soils. At the end of the experiment, an average total of 8.5×10^4 cfu/ml and 4.8×10^4 cfu/ml of the applied bacterial population was recovered from the Akaroa and the Eyrewell soil, respectively, from beneath *L. scoparium* and *K. robusta*, which accounted for 1 – 3% of the applied *E.*

coli population, respectively. Bacterial leaching under *L. perenne* was < 1% of the inoculated bacterial population in both soils.

Fig. 4.2.a shows *E. coli* leaching in the Akaroa soil under all species. Significantly higher bacterial leaching was observed under the native plants compared to *L. perenne* throughout the experiment. Until the sixth leachate event, there was no significant difference between *L. scoparium* and *K. robusta* leaching patterns, with *K. robusta* leaching the highest bacterial counts during the experiment with the highest of 2.48×10^4 cfu/ml *E. coli* on the second day followed by *L. scoparium* leaching 9.9×10^3 cfu/ml. *Lolium perenne* leached the highest of 4.8×10^3 cfu/ml during the third leachate collection. From the eleventh irrigation onwards, bacterial numbers in the drainage were below detection limit (< 1 cfu/100 ml) under *L. perenne*. However, a detectable number of *E. coli* continued to leach under *L. scoparium* and *K. robusta* until the fourteenth drainage event. At the end of the experiment, *E. coli* numbers in the leachate reduced to 4.1 cfu/ml, 13.6 cfu/ml and < 1 cfu/ml under *L. scoparium*, *K. robusta* and *L. perenne* respectively.

Contrary to the Akaroa soil, highest bacterial leaching occurred under *L. scoparium*, in the Eyrewell soil (Fig. 4.2.b) leaching 1.68×10^4 cfu/ml on the third leachate collection followed by *K. robusta* leaching 8.9×10^3 cfu/ml on same day. *Lolium perenne* drained highest bacterial concentration on the second leachate event generating 3.7×10^3 cfu/ml of *E. coli*. Up to the eighth leachate event, there were no significant differences between the three species. A detectable number of bacteria leached under all the species throughout the experiment and reached to 12 cfu/ml, 3 cfu/ml and < 1 cfu/ml under *L. scoparium*, *K. robusta* and *L. perenne*, respectively, at the end of the experiment. In the Eyrewell soil, bacterial leaching continued for longer period compared to the Akaroa soil under all species.

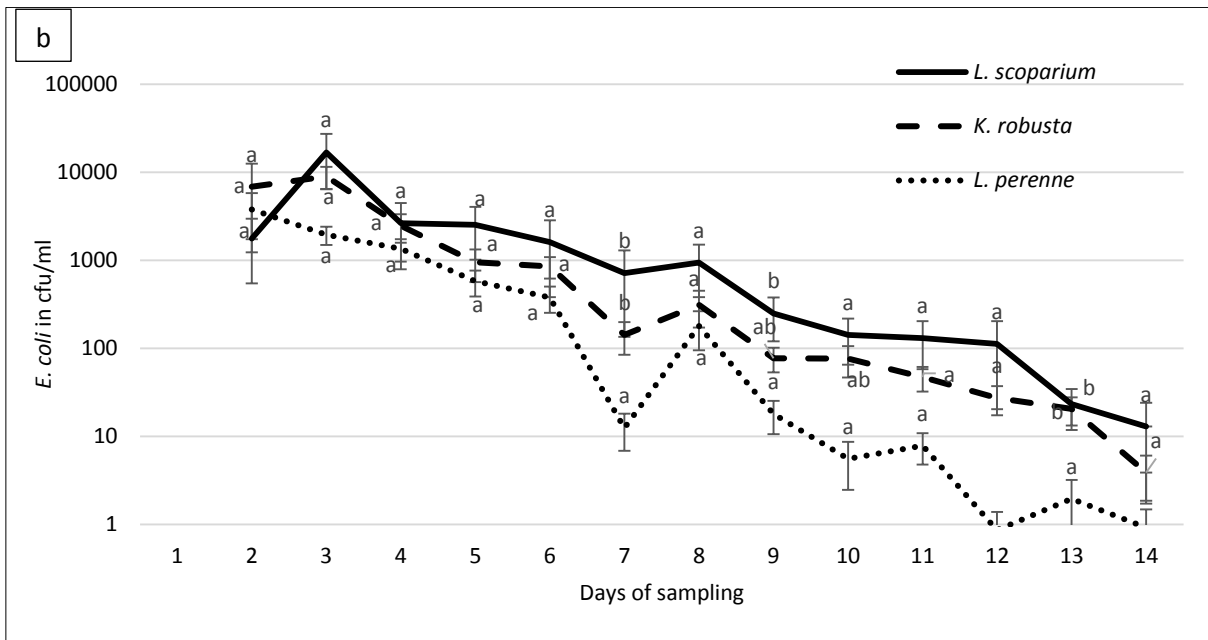
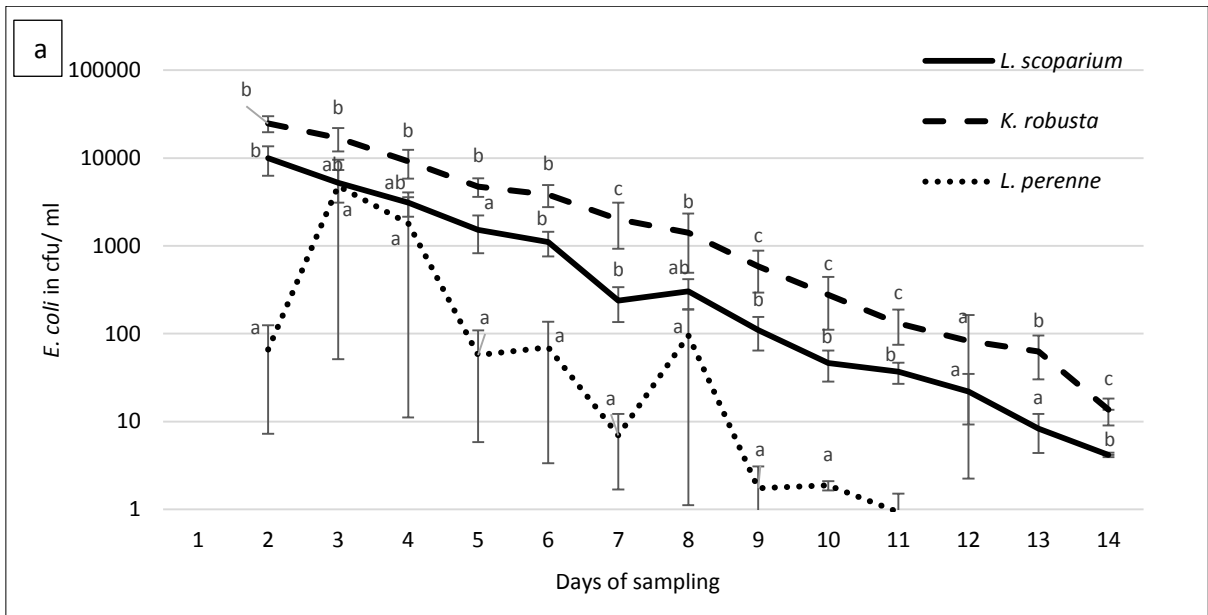


Figure 4-2 Average total *E. coli* leaching under *L. scoparium*, *K. robusta* and *L. perenne* in **a)** the Akaroa soil and **b)** the Eyrewell soil. Error bars represent standard errors of the mean (n = 5). Data points with the same letters each day, are not significantly different based on Fishers unprotected LSD analysis (p < 0.05).

At the end of the fourteen days experiment in the Akaroa soil, both *L. perenne* and *L. scoparium* leached < 1% of the inoculated *E. coli* while *K. robusta* leached nearly 3% of the total added bacterial population (Fig. 4.3.a). However, in the Eyrewell soil (Fig. 4.3.b) *L. perenne* and *K. robusta* leached an average total of < 1% of the inoculated *E. coli* while *L. scoparium* leached the highest *E. coli* with 1.2% of the inoculated bacteria. Highest *E. coli* populations were recovered in the soil columns of the *L. perenne* rhizosphere in both soils (Appendix C.12).

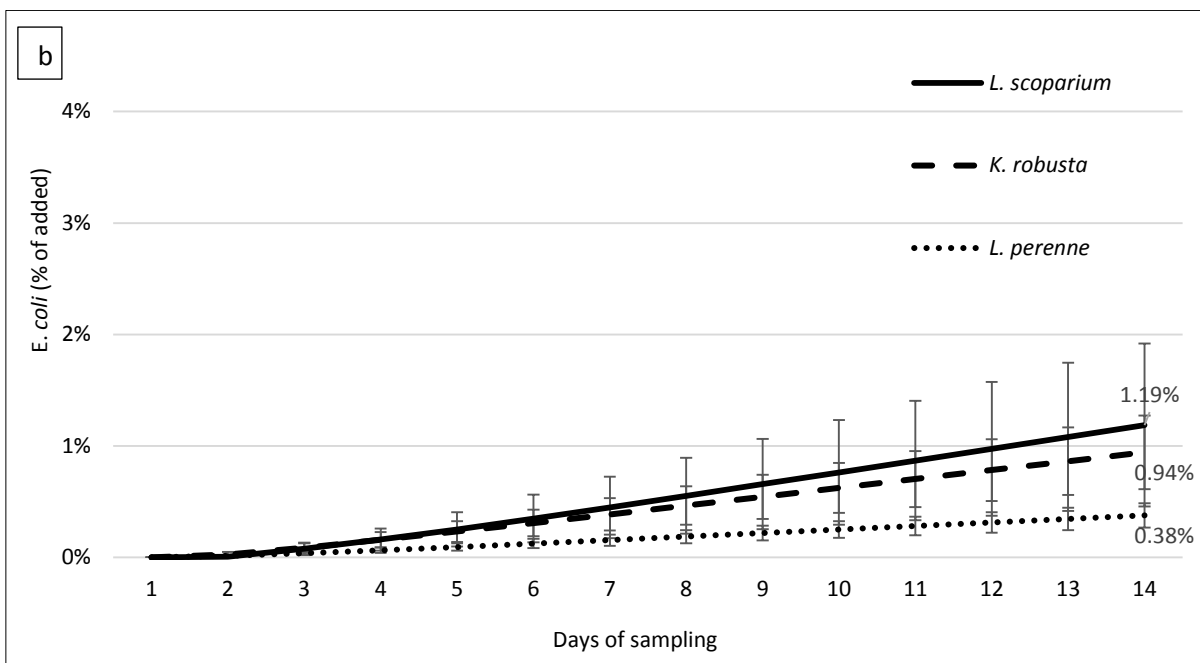
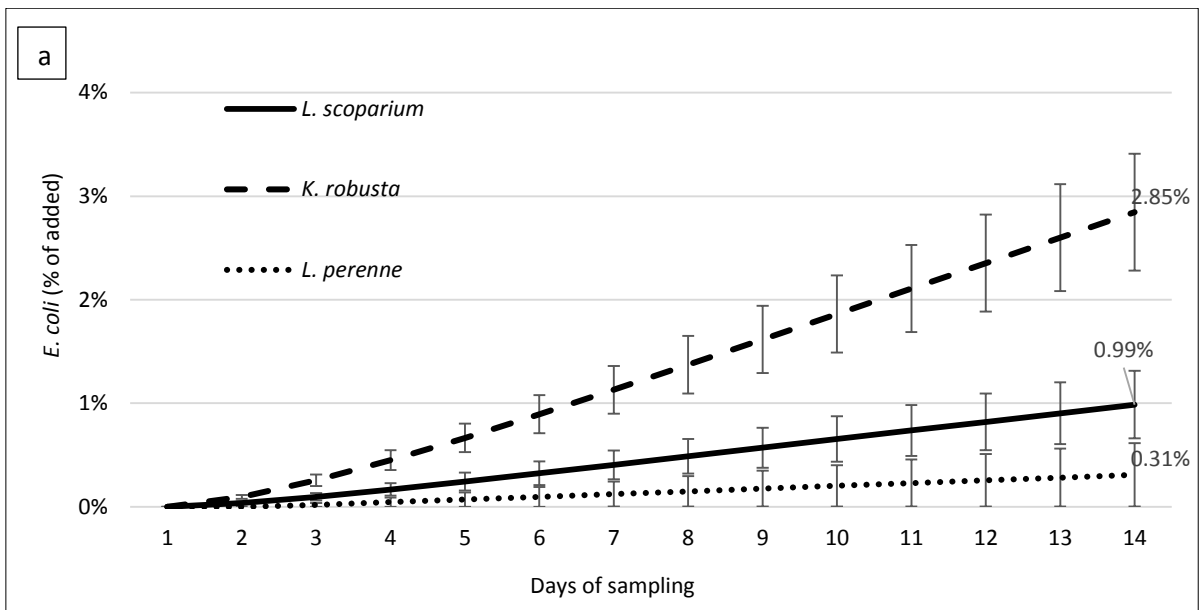


Figure 4-3 Cumulative *E. coli* leaching percentage (of added *E. coli*) in **a**) the Akaroa soil and **b**) the Eyrewell soil, under *L. scoparium*, *K. robusta* and *L. perenne*. Error bars represent standard errors of the mean (n = 5).

Fig. 4.4.a and b, shows the breakthrough curve of tracer bromide. Bromide in the Akaroa soil generated similar leaching pattern as *E. coli*, demonstrating significantly higher leaching under *K. robusta* although the peaks were more stable than for the *E. coli*, especially between the second to seventh leachate events under all species (Fig. 4.4.a). *Kunzea robusta* leached the highest of 28 mg bromide mass on the second day and continued to leach nearly similar amount until day seven. The second highest bromide leaching was observed under *L. scoparium*, leaching highest of 21.3 mg bromide on day four. *Lolium perenne* initially generated a highest bromide peak of 6.3 mg on the first day followed by the other highest peak of 7.3 mg bromide mass leached on day twelve. Following day seven onwards, bromide leaching under all species generated lowering peaks, except *L. perenne* on day

twelve. Until the end of the experiment the bromide concentration did not fall below the detection limit under any species. *Leptospermum scoparium* represented an intermediary behaviour between *K. robusta* and *L. perenne* in this soil with no significant difference from either of the other species.

The bromide in the Eyrewell soil (Fig. 4.4.b) exhibited an initial rapid movement of the tracer confirming the initial flow dominated by the preferential flow pathways, especially under *K. robusta* and *L. perenne*, under this stony silt loam, with highest peaks obtained on the first leachate event under all the species. *Leptospermum scoparium* generated a significant difference from *L. perenne* and *K. robusta* on the first leachate event and between the fifth to eighth leachate events. There was no significant difference between *L. perenne* and *K. robusta* throughout the experiment. From the second day onwards, *L. scoparium* generated a slowly increasing bromide peak leaching highest of 23.32 mg bromide on the sixth drainage event after which the peak came down to 1.6 mg bromide on day fourteen, leaching a cumulative total of 132 mg out of the 400 mg added, by the end of the experiment. However, *L. perenne* and *K. robusta*, did not leach more bromide than the first day and leached the lowest concentration of 0.9 mg and 1.7 mg respectively, at the end of the experiment.

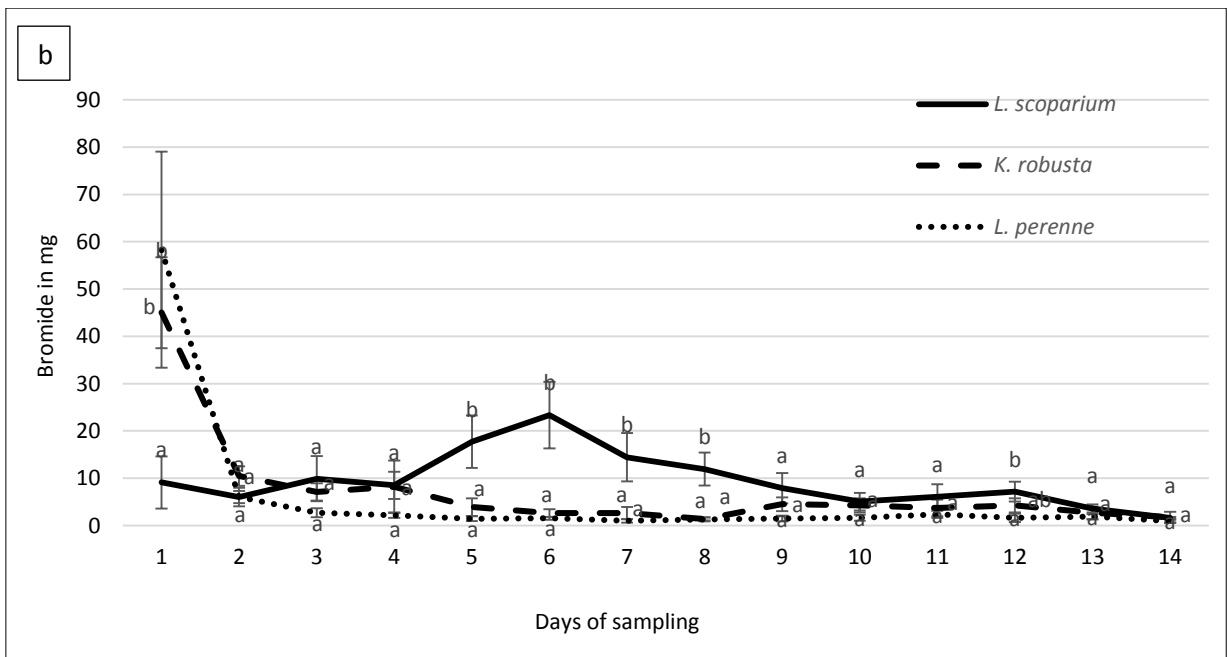
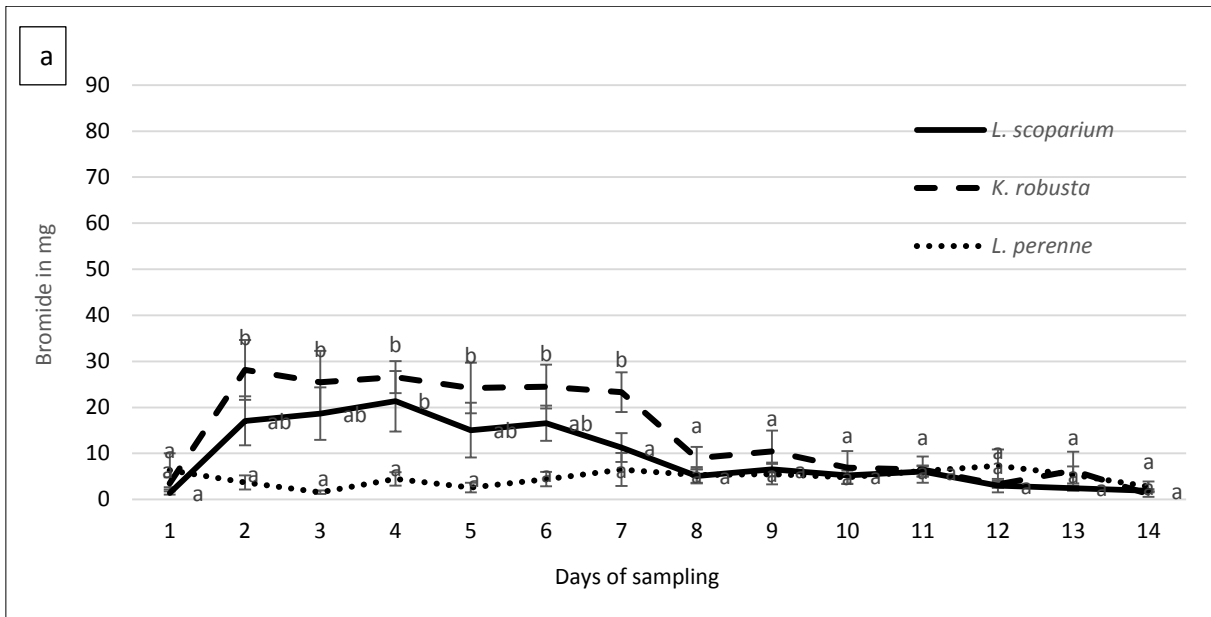


Figure 4-4 Average bromide mass leaching (in mg), under *L. scoparium*, *K. robusta* and *L. perenne* in **a)** the Akaroa soil and **b)** the Eyrewell soil. Error bars represent standard error of the mean (n = 5). Data points with the same letters each day, are not significantly different based on Fishers unprotected LSD analysis (p < 0.05).

The cumulative bromide leaching percentages under both the Akaroa and the Eyrewell soil are presented in Fig. 4.5.a and b, respectively. At the end of the experiment, *K. robusta*, *L. scoparium* and *L. perenne* leached 49%, 32% and 16% of the mass of the bromide added respectively in the leachate obtained from the Akaroa soil (Fig. 4.5.a) being significantly different. However, no significant difference in bromide leaching was observed under *K. robusta* and *L. perenne* in the Eyrewell soil, throughout the experiment (Fig. 4.5.b). In this soil, *L. scoparium* leached the highest of 33% of the

added bromide mass at the end of the experiment followed by *K. robusta* and *L. perenne* leaching 25% and 21% of the added bromide mass, respectively.

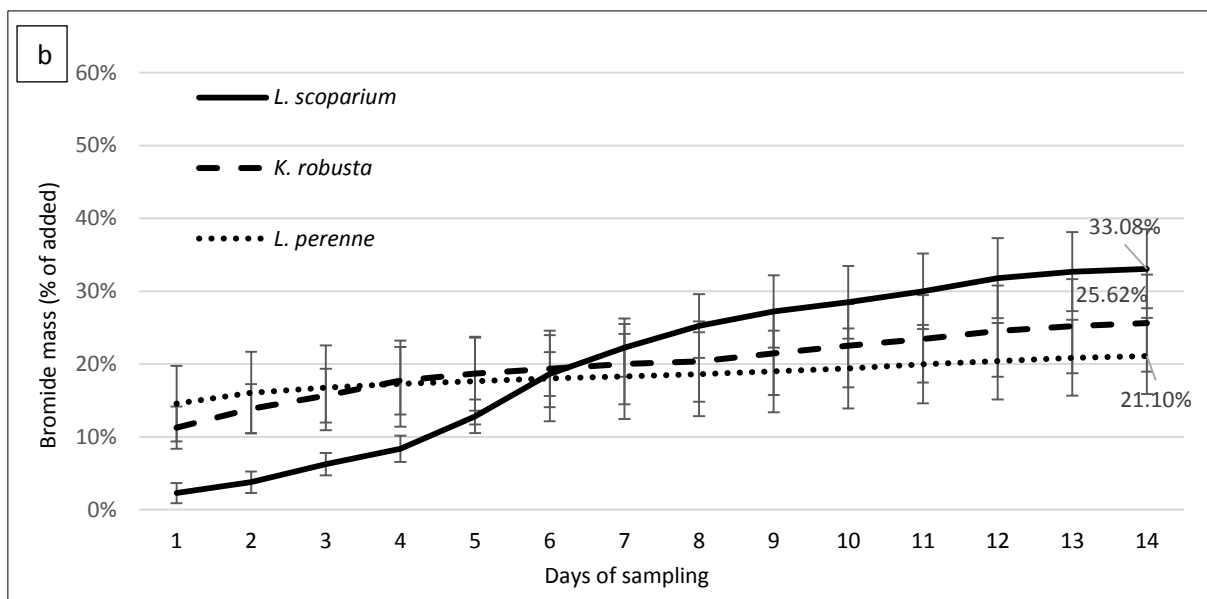
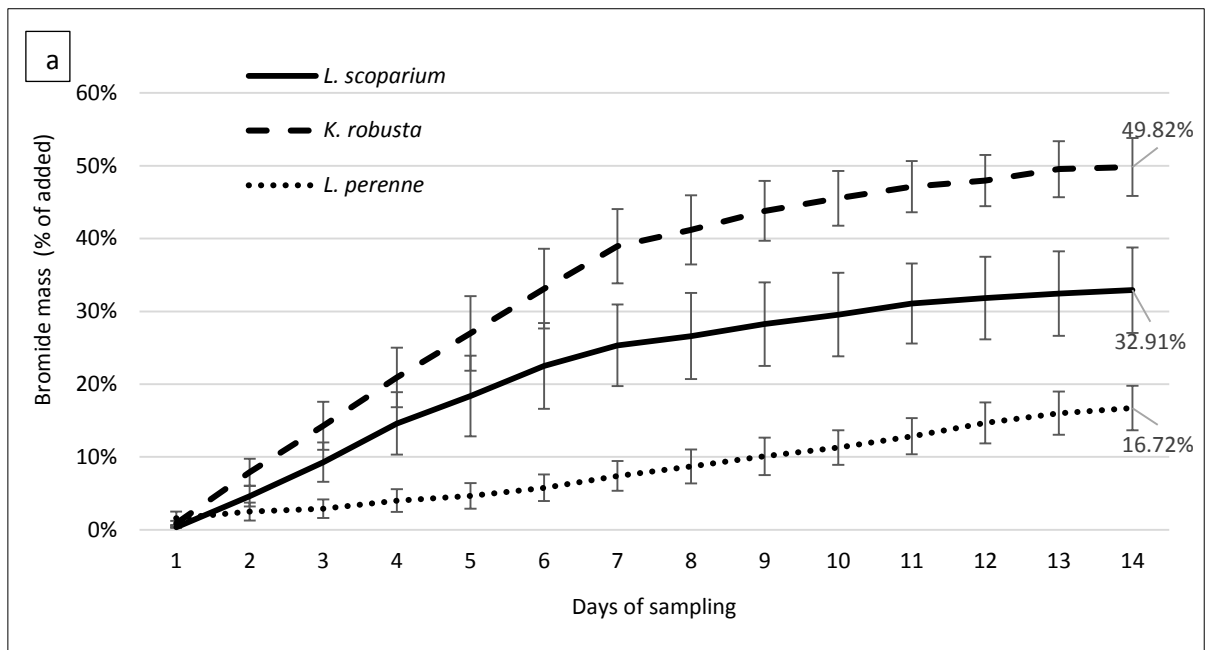


Figure 4-5 Cumulative bromide leaching percentage under *L. scoparium*, *K. robusta* and *L. perenne* in **a)** the Akaroa soil and **b)** the Eyrewell soil. Error bars represent standard errors of the mean (n = 5).

Fig. 4.6 shows brilliant blue dye traced in the harvested soil columns of *L. scoparium*, *K. robusta* and *L. perenne* from both soils. The evenly distributed dye in the top soil of both native trees, followed the taproot system of these plants in both soils right to the bottom, showing the potential route followed by the inoculated *E. coli* under these trees. Interestingly, *L. perenne* retained the whole volume of the added dye solution in the top soil column only. With 7 mm of the dye solution irrigated for over 6 hours (with no irrigation following), none of the plants generated any dyed leachate at the bottom.

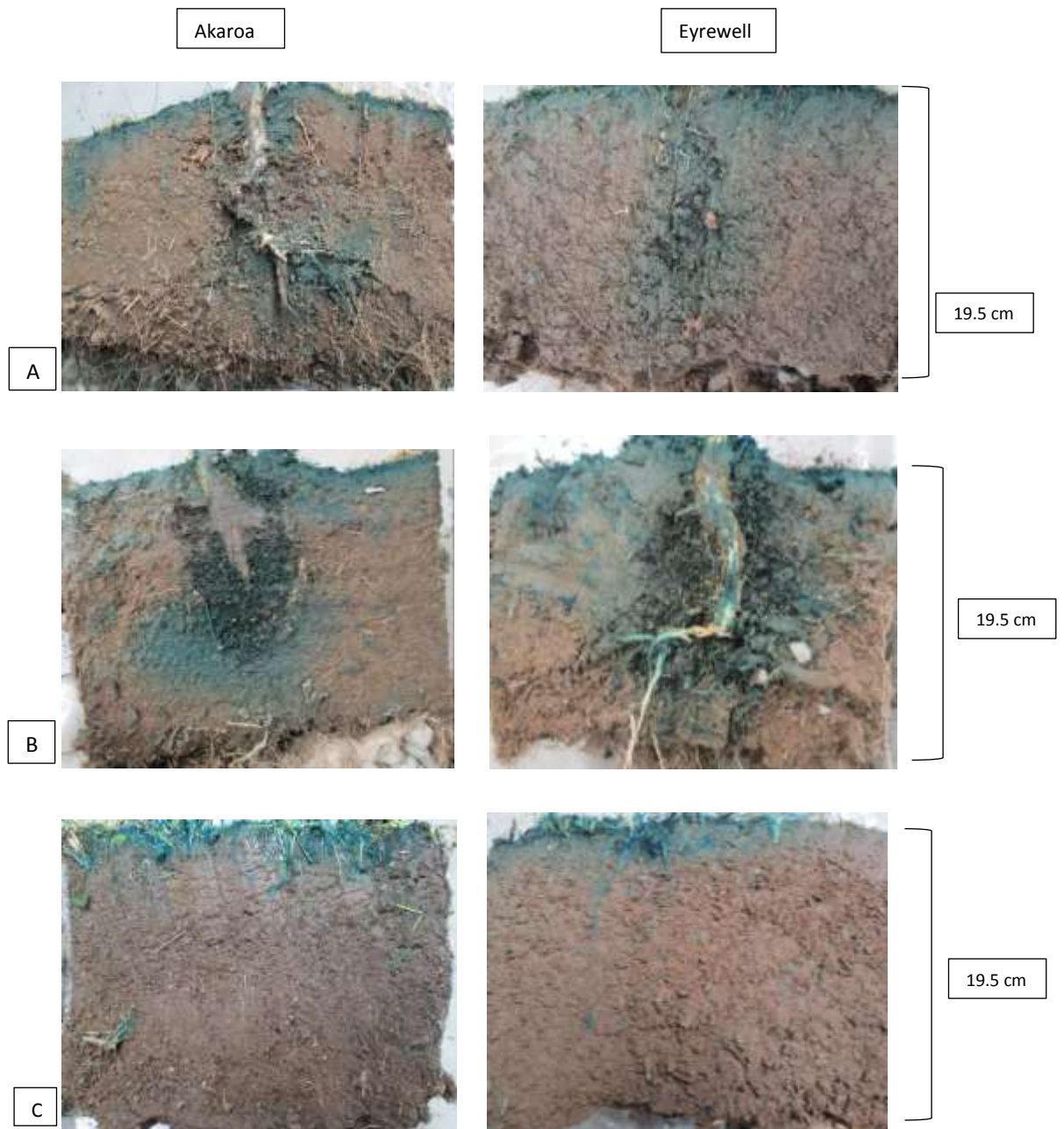


Figure 4-6 Brilliant blue dye stained and dissected soil columns of **A** = *K. robusta*, **B** = *L. scoparium* and **C** = *L. perenne*.

4.4 Discussion

Increased preferential flow under the native shrubs was expected following their growing root-induced preferential flow pathways (Bargués Tobella et al., 2014; Yunusa et al., 2002) and consistent bacterial leaching up to 3% of the inoculated bacteria i.e. 2.6×10^7 cfu/100 ml, was observed under *L. scoparium* and *K. robusta* in both soils following heavy irrigations. As highest bacterial numbers leached within the first two to three days in both soils under all species, it was concluded that the initial bacterial leaching was dominated by the preferential flow pathways present in the soil (Banning et al., 2002). Although the bacterial inhibition results varied between the two soils, it was assumed that the unrecovered *E. coli* (> 97%) from beneath *L. scoparium* and *K. robusta*, was attenuated following the

antibacterial properties of the root exudates (Johnson & Lehmann, 2006) of these species (along with the natural die-off when introduced to the soil). During the preliminary runs (Appendix C.12), bacterial recovery from soil under the natives was below detection limit i.e. < 1 cfu/100 ml. This was in contrast to *L. perenne*, where *E. coli* was detected until two months after the twelve days leaching trial, which confirms that the *E. coli* not recovered into the leachate under *L. scoparium* and *K. robusta* are susceptible to the increased die-off under these native species. Although the exact mechanism responsible for the bacterial die-off under *L. scoparium* and *K. robusta*, could not be established, it was hypothesised/assumed that the roots exudates and roots associated microorganisms had a major role to play here (Perry et al., 2007).

Other than the vegetation, soil type and its quality also influence bacterial movement into the soil (Brennan et al., 2010) and rate of bacterial die-off further depends on the soil porosity and structure, irrigation rates and intensity, moisture content, temperature, pH and exposure to UV (Aislabie et al., 2011; Horswell & Aislabie, 2006; Mubiru et al., 2000). Plants behaviours in this study were also influenced by the soil type with the highest bacterial leaching in the Akaroa soil with *K. robusta* while the highest bacterial leaching in the Eyrewell soil was for *L. scoparium* (with no significant difference). Depending on the environment, soil type and the microbial competition, plants can alter the composition of the released exudates (Perry et al., 2007) and hence the rhizosphere activities, resulting in variable results presented by the same species in different environment (Bakker et al., 2014; Kinkel et al., 2011). The two soils used in this study were, *first*, a Pawson silt loam or Pallic firm brown soil with high clay content and defined soil structure, and *second*, a stony silt loam or Lismore soil from Eyrewell pine forest with 20% increased sand content than naturally present. The Eyrewell soil generated slow but long-term leaching of both bromide and *E. coli* as compared to the Akaroa soil. The highest *E. coli* recovery percentage from beneath the natives, in the Akaroa soil was less than 3% of the inoculated bacteria and in the Eyrewell soil was less than 1.5% of the inoculated bacteria at the end of the experiment.

As DSE contains between 10^5 - 10^7 cfu/ml of faecal bacteria, higher than that used in this study (Cools et al., 2001; Dipu et al., 2011) comparing these leaching rates in a field environment (per hectare), bacterial leaching could be as high as 10^{10} cfu/ml per hectare to the groundwater resources. This leaching rate of microbial contaminants, which may include some potentially pathogenic bacteria is sufficient to establish an infection in animals and humans as pathogenic strains of *E. coli* requires just 10 - 100 cfu/ml for infection (Avery et al., 2004) and those of *Campylobacter* spp. requires $< 8 \times 10^2$ cells to start infection in humans and animals (Black et al., 1988). Ross & Donnison (2003) stated that leachate from paddocks may contain a minimum of 2% of the applied *E. coli* with irrigated DSE, but that can still be sufficient to start the infection in humans and animals.

The high irrigation rate of 14 mm/day in this study was required to obtain equal leaching from all species. However, this is an uncommon condition in NZ. Irrigation rates below 10 mm have been suggested to reduce the preferential flow (Jiang, 2008; Monaghan & Smith, 2004). With high irrigation rates, small soil pores fill immediately and water follows macropores or cracks present in the soil. Water passing through these cracks, flows with high velocity, allowing minimum time of interaction between the soil particles and *E. coli*, increasing their numbers in the leachate (Aislabie et al., 2011; Brennan et al., 2010; Johnson & Lehmann, 2006). Our preliminary trials (Appendix D.2), followed daily irrigation rates of around 7 mm/day and *L. scoparium* and *K. robusta* did not generate any leachate at this irrigation rate, which suggests that irrigation strategies following low irrigations might result in an absolute no bacterial leaching under these species, which would also increase the time that bacteria spend in the vicinity of the roots. Soil type and quality should also be considered as the variables affecting the bacterial leaching results.

Non-adsorbent tracer compounds such as bromide are also susceptible to follow the preferential flow routes under heavy irrigations (Jiang et al., 2010). High and rapid leaching of bromide (30% – 49%) in this study from under the natives supports this. Compared to the bacteria, bromide leached at a nearly constant concentration for few days under both the natives in the Akaroa soil, and under *L. scoparium* only, in the Eyrewell soil. Low bacterial recovery as compared to the bromide at same time points from beneath the natives, suggests antimicrobial interactions under the *L. scoparium* and *K. robusta* soil. Although, similar bromide and bacterial leaching trend was also found under *L. perenne*, which could be because of the fibrous root systems of *L. perenne* holding the bromide in soil (not tested) similar to dye (Fig. 4.6). This also suggests increased probability of surface run off of the contaminants (nutrients) other than bacteria applied with DSE under *L. perenne*. The larger size of the bacterial cells (0.5 – 2 µm) (Bell, 1998) compared to the bromide ion (182 pm) could also be a possible reason for the high bromide leaching from under all species.

The preferential flow routes under both natives have also been confirmed by the dye tracer (Flury & Flühler, 1995). Soil columns harvested after the dye application confirmed that the dye flow was restricted to the primary taproot section of *L. scoparium* and *K. robusta* in both soils. However, *L. perenne* retained the entire dye volume on the top soil surface. Low bacterial recovery in the leachate from *L. perenne* and the dye retention in its top soil column demonstrates low preferential flow under this species and its ability to hold the bacteria in its fibrous roots system. Bromide retention in the soil was not tested for any species. The reduced preferential flow under *L. perenne* may exacerbate surface runoff of the contaminants sitting on the top soil surface of *L. perenne*, during heavy irrigation or rainfall and therefore increase the risk of freshwater contamination. Bacterial retention by pasture in the top soil has also been explained in previous studies (Chandler et al., 1981; Vinten et al., 2002; Zhai et al., 1995).

The preferential flow of the bacteria under *L. scoparium* and *K. robusta* may increase bacterial movement towards the roots of these antimicrobial plants, decreasing the risk of the surface run off of the bacteria compared to *L. perenne*. This may increase bacterial interactions with the antimicrobial root components. Bargués Tobella et al. (2014) reported less surface run off under trees as compared to bare soil and stated that the trees can be helpful in maintaining the dry soil conditions beneficial for reducing bacterial leaching, and decreasing the surface run off of the applied contaminants. Johnson and Lehmann (2006) reviewed stem-flow and roots induced preferential flow under trees and reported that trees concentrate nutrients in the rhizosphere by dissolving the precipitations concentrated on the leaves and transporting them to the root channels.

This study confirms bacterial leaching following the root induced preferential flow pathways under *L. scoparium* and *K. robusta* and that the leaching behaviour of these natives can be affected by the soil type. Comparing the high bromide leaching (30% - 49%) and low bacterial leaching (<1% - 3%) following high irrigation rates in both soils, antimicrobial effect of *L. scoparium* and *K. robusta* can be established against *E. coli* and is expected to be more prevalent under low irrigation rates. Similar bromide and bacterial leaching trend under *L. perenne* are expected to be the result of its fine root system, similar to dye. As this study was aimed to produce the leachates and followed irrigation rates higher than those regulated in NZ, further studies with low irrigation rates will be required to answer the effect of increased time of interactions between effective antimicrobial root components of *L. scoparium* and *K. robusta* and the surface applied *E. coli*. As this study used a lab strain *E. coli* suspension prepared in Ringers solution to inoculate the plants, testing the DSE *E. coli*, which is exposed to the high organic matter contents, under these plants, might present a different aspect of the plants and bacterial behaviour.

4.5 Conclusion

This study established the antimicrobial effect of *L. scoparium* and *K. robusta* on the leaching of a non-pathogenic bacterial strain *E. coli* ATCC13706 in two different soil types. The dye tracer confirms that bacterial flux followed the root induced preferential flow pathways under the natives. The preferential flow under the native species may decrease the risk of surface run off of the applied *E. coli*, which may otherwise occur with *L. perenne* following irrigation/rainfall. Increased infiltration would increase the interactions of the bacteria with the antimicrobial roots. However, these woody species may exacerbate the leaching of contaminants, including microbes, into shallow groundwater. Future studies following low irrigation rates might result in no bacterial leaching from under these natives. Field studies, which have natural soil cracks along with root induced preferential flow pathways, are also important to evaluate plants antimicrobial potential

Chapter 5

Bacterial fluxes under *Leptospermum scoparium* and *Kunzea robusta* in soil under low-flow conditions

5.1 Introduction

Macropores (> 30 μm pores) present in the soil reduces its capacity to remove suspended or dissolved matter from soil solution (Brennan et al., 2010). Macropore flow or preferential flow occurs under high hydraulic loading following flood irrigation or rainfall > 10 mm/hour (Gagliardi & Karns, 2000; Jiang, 2008). Preferential flow can exacerbate the leaching of bacteria through soil (Abu-Ashour et al., 1994; Aislabie et al., 2011). As water is held at low tension in macropores and moves with high velocity, it allows less time for interactions between the applied bacteria and the soil particles, hence, increasing bacterial transport (Aislabie et al., 2011; Aislabie et al., 2006; Brennan et al., 2010; McLeod et al., 2008). Neralla and Weaver (2000) stated that high bacterial numbers in the leachate indicates lower sorption and less interaction of bacteria in soil. Although macropores comprise just a small fraction of a soil's porosity, they have a disproportionately large contribution in microbial transport to groundwater (Jiang, 2008).

Stoddard et al. (1998) stated that organic applications, such as animal manure, in soils might exacerbate the preferential flow of bacteria depending on the rainfall intensity and soil structure. Hence, following animal waste applications, microbial contaminants can effectively move into the soil with low irrigation rates. Although, physiochemical properties of both the soil and the DSE, along with the DSE application methods, affect bacterial movement and survival in soil (Jiang et al., 2005; McLeod et al., 2008; Mubiru et al., 2000; Neralla & Weaver, 2000).

Other than the natural soil aging and agricultural activities, trees also contribute to the preferential flow in soil, induced by stem-flow (Johnson & Lehmann, 2006; Li et al., 2009) and by their extensive root systems (Bargués Tobella et al., 2014; Noguchi et al., 1999; Yunusa et al., 2002). As roots grow, they apply forces to extend deep into the soil resulting in the discontinuity and preferential flow pathways (Bargués Tobella et al., 2014). When roots die and rot away, they leave hollow spaces in soil, which also works as preferential flow pathway for microbes (Benegas et al., 2014). If roots cross the complete soil mantle, they can create a direct hydrological link between inoculated bacterial contaminants and the water table (Noguchi et al., 1999). *Leptospermum scoparium* has a shallow lateral root structure and can grow to a depth of nearly 3 m into the soil proliferating a mass of fine structured roots throughout the soil (Watson & O'Loughlin, 1985). Roots of *L. scoparium* have been

found to provide protection against shallow landslides and to maintain a reinforced soil structure (Bergin et al., 1995). Although the extent of the improvement in soil reinforcement depends on the root to soil ratio and on specific root characteristics (Easson & Yarbrough, 2002) such as root morphology, which influences the degree of mechanical reinforcement provided to the soil by the trees (Watson & Mardern, 2004). The main roots of *L. scoparium* can occupy nearly 5% of the soil volume in the top 50 cm of the soil layer (Watson & O'Loughlin, 1985). Stands of *K. robusta* (pure and mixed) are commonly grown around river banks in NZ, to provide stability to the bank soil (Watson et al., 1997; Watson & O'Loughlin, 1985).

Chapter 3 showed that the sterile water extracts of *L. scoparium* and *K. robusta* inhibit the growth of *E. coli* (Fig. 3.1) but Chapter 4 showed that root induced preferential flow pathways increase bacterial leaching in soil following high irrigation rates. Based on the results of the previous two chapters, it was hypothesised that under low-flow conditions, where preferential flow should be less prevalent, the antimicrobial rhizosphere components of *L. scoparium* and *K. robusta* should reduce bacterial leaching under these plants. This study aimed to determine the effect of low irrigation rates and increased time of interaction between DSE-applied *E. coli* and the roots of *L. scoparium* and *K. robusta*.

5.2 Materials and methods

5.2.1 Experimental set up

Chapter 4 details of the plants and soils were used in this experiment (Section 4.2.1). Swamp *L. scoparium* were purchased from Forest Flora nursery, Ngaruawahia, North Island (37°39'44''S 175°08'13''E) and the DSE was collected from the Lincoln University Research Dairy Farm (48°38'22''S, 172°27'24''E).

Around 700 kg of each soil was collected from 0 - 30 cm top soil after removing any vegetation on the soil surface. Both soils were homogenised and passed through a 30 mm sieve to remove large stones while maintaining soil aggregate structure. The Eyrewell soil was mixed with 20% sand to maintain the porosity (Appendix D.2). One hundred 10 l lysimeters (diameter = 25.3 cm, height = 27.4 cm) were used in the experiment and the lysimeters were filled with soil as described in Chapter 4 (Section 4.2.1). After filling, and before planting, the lysimeters were maintained at field capacity for one month to settle the soil and then were dried for five days or until the soil separated from the sides of the lysimeters. This gap was filled with petroleum jelly. The lysimeters were maintained at field capacity for a further week. Plants were established in both soils and were maintained under field capacity for the next three months. Control species were established with 2 g of *L. perenne* seeds and unplanted controls were included. The lysimeters were established for controls and treatments in five replicates each, for all species ((5 + 5 = treatment + control) x 5 species x 2 soils = 100 lysimeters). Weeding was

performed twice per week. Plants were randomised following a randomised complete block design (RCBD).

5.2.2 Bacterial spiking

Before spiking, plants were irrigated at field capacity to wet the soil uniformly and were left for 24 hours for excess water to drain. After 24 hours, rubber stoppers were installed in the hole at the bottom of the lysimeters to prevent drainage with the aim of increasing the time of interaction between roots and the applied bacteria. Fresh DSE was collected 48 hours before the experiment and was stored at 4°C. The *E. coli* count of DSE was obtained using Colilert-18 (IDEXX laboratories) (details in Section 4.2.4) and 8 mm (400 ml) DSE per treatment pot was applied to inoculate *E. coli*. With 8 mm DSE, 2.1×10^7 cfu of total *E. coli* was applied in each treatment pot. After *E. coli* inoculation, 400 mg NaBr (1 mg/ml) was applied per treatment plant only. Control plants were irrigated with same volume of tap water only. All irrigations were performed using a pressurised spray bottle at the rate of 2 mm/hour and all irrigations were completed in nine hours. Plants (both controls and treatments) were further irrigated with tap water on day 4, 6, and 9 after DSE application, with 4 mm to 6 mm irrigation rates, respectively, to avoid anaerobic conditions in the lysimeters while maintaining the soil moisture and to ensure leachate at the end of the experiment i.e. on the tenth day.

5.2.3 Leachate collection and analysis

Four hours after the irrigation on the 9th day, the rubber stoppers at the base of the lysimeters were removed and lysimeters were connected with clean (20% bleach washed and dried) containers to collect the leachates. Twenty-four hours after removing the rubber stoppers from the lysimeters, no leachate was obtained from under any plants and plants were further irrigated with tap water (7 - 8 mm per day) to generate leachate. Released leachates were then used for *E. coli* enumeration using Colilert-18. Irrigations and daily leachate collections were performed for five days after removing the rubber stoppers and the study finished on the sixteenth day with leachate collection. Leachate volumes were measured using clean measuring cylinders, and volumes were recorded. Sub samples of leachate for bromide and *E. coli* analysis were collected daily.

Details of the bacterial and NaBr enumeration using Colilert-18 (IDEXX laboratories) (Boubetra et al., 2011) and Ion Chromatography System (Dionex DX-2100), respectively, have been provided in Section 4.2.4 of Chapter 4.

5.2.4 Statistics

Standard errors were calculated using Microsoft Excel (Version 2013). To identify significant difference in *E. coli* leaching between species, cfu/ml was log-transformed and two-way ANOVA was performed

on these log numbers using Fishers unprotected LSD analysis ($p < 0.05$) in Genstat 16th edition (VSN International Ltd). Bacterial count below detection limit i.e. < 1 cfu/100 ml, was considered 1 cfu/ml prior to the \log_{10} transformation.

5.3 Results

Fig. 5.1 (a and b) shows the average *E. coli* leaching under all treatments after ten days of interaction with the roots of the plant treatments, as well as the unplanted controls (bare soil) in the Akaroa and the Eyrewell soil, respectively. Bacterial leaching under all species in both soils was negligible. In the Akaroa soil the highest *E. coli* leaching was obtained under *K. robusta* with 1.5×10^3 cfu/ml leached during the fourth leachate collection followed by North Island swamp *L. scoparium* leaching the highest count of 9.1×10^2 cfu/ml on the third leachate collection (Fig. 5.1.a). Unplanted controls (unplanted lysimeters) leached the third highest bacterial count throughout the experiment. *Leptospermum scoparium* was similar to *L. perenne*, leaching the lowest *E. coli* population in the leachate with no significant difference between the two species. Except for the second leachate event, there were no significant differences between *K. robusta* and *L. perenne*. A significant difference existed between *L. scoparium* and the unplanted controls in bacterial leaching. At the end of the experiment, *E. coli* count decreased to 1 cfu/ml under *L. scoparium* however, the unplanted controls were still leaching 50 cfu/ml of *E. coli* on fifth leachate event.

Unlike the Akaroa soil, in the Eyrewell soil *L. scoparium* leached the highest bacterial numbers at the second leachate event with 1.7×10^3 cfu/ml of *E. coli* with this slowly decreasing to < 10 cfu/ml by the end of the experiment (Fig. 5.1.b). *Kunzea robusta* and swamp *L. scoparium* showed the second and third highest bacterial leaching, respectively, with the peaks decreasing to 260 cfu/ml and < 2 cfu/ml, respectively, under both species by the fifth leachate collection. *Leptospermum scoparium* and *K. robusta* represented no significant difference in their *E. coli* leaching patterns. The unplanted controls showed a slowly increasing peak leaching 2×10^2 cfu/ml *E. coli* on the fifth leachate, and generating a significant difference between *L. scoparium* and unplanted controls during the fifth leachate event. Similar to the results for the Akaroa soil, *Lolium perenne* had the least *E. coli* leaching compared to the other species with the highest peak of 75 cfu/ml on the fourth leachate event.

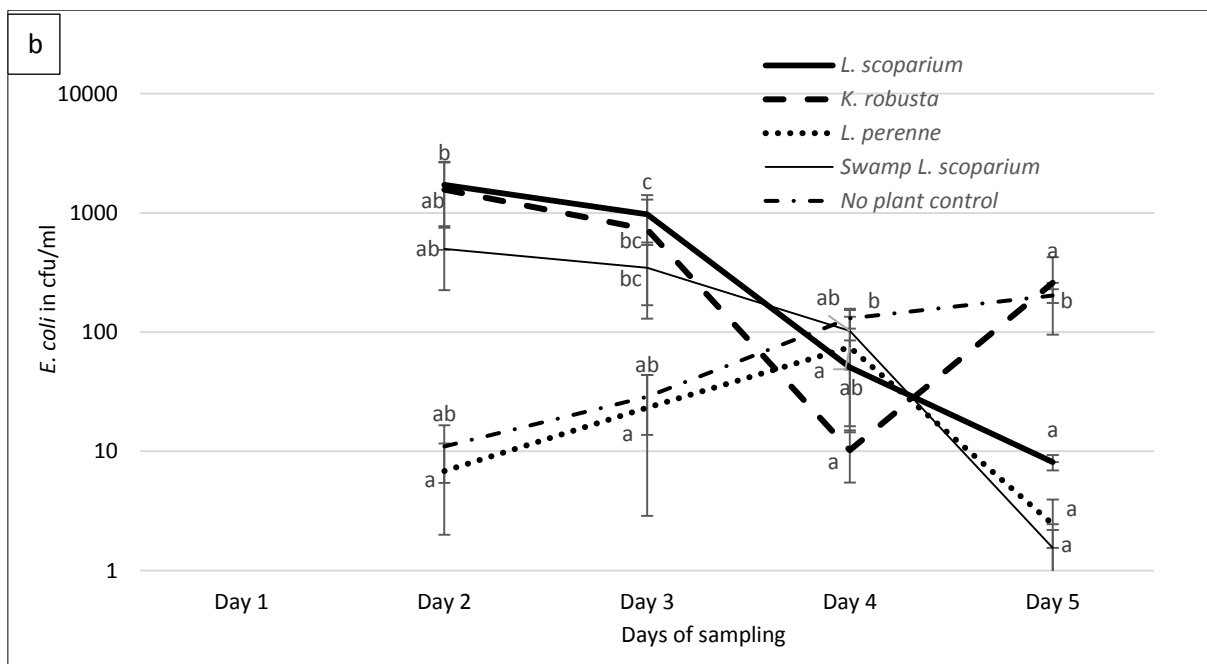
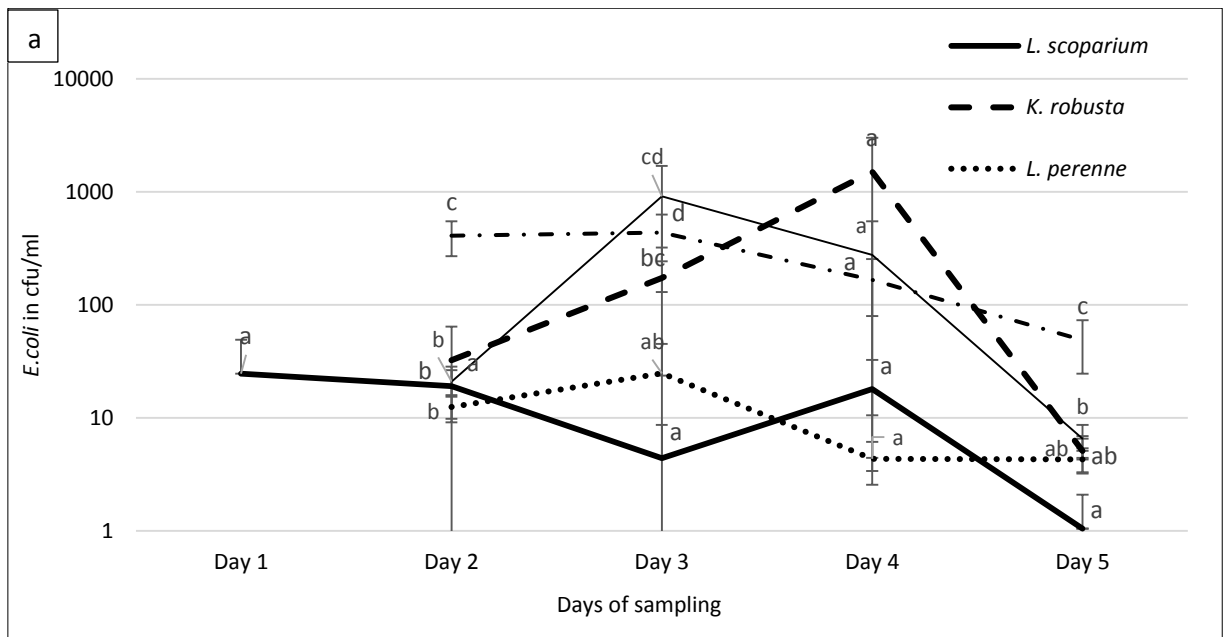


Figure 5-1 Average total *E. coli* leaching under all plants species in **a)** the Akaroa soil and **b)** the Eyrewell soil. Error bars represent standard error of the mean value (n = 5). At each sample time, data points with the same letters are not significantly different based on Fishers Unprotected LSD analysis (p < 0.05).

Cumulative percentage of *E. coli* leaching in both soils is presented in Fig. 5.2.a and b. In the Akaroa soil (Fig. 5.2.a) leaching under all species were negligible and followed *L. perenne* < *L. scoparium* < unplanted controls < swamp *L. scoparium* < *K. robusta*. *Lolium perenne* used as the control leached a minimum of 2.1×10^{-4} % of the inoculated bacterial population followed by *L. scoparium* leaching 3.1×10^{-4} % while *K. robusta* leached highest and an average total of 7.9×10^{-3} % of the inoculated bacterial population.

Bacterial leaching percentage was also negligible in the Eyrewell soil, where *L. scoparium* leached an average total of $5.2 \times 10^{-3}\%$ of the inoculated *E. coli* at the end of the experiment, while unplanted controls leached $2.9 \times 10^{-3}\%$ only. *Escherichia coli* leaching pattern followed *L. scoparium* > *K. robusta* > swamp *L. scoparium* > unplanted controls > *L. perenne* in the Eyrewell soil (Fig. 5.2.b).

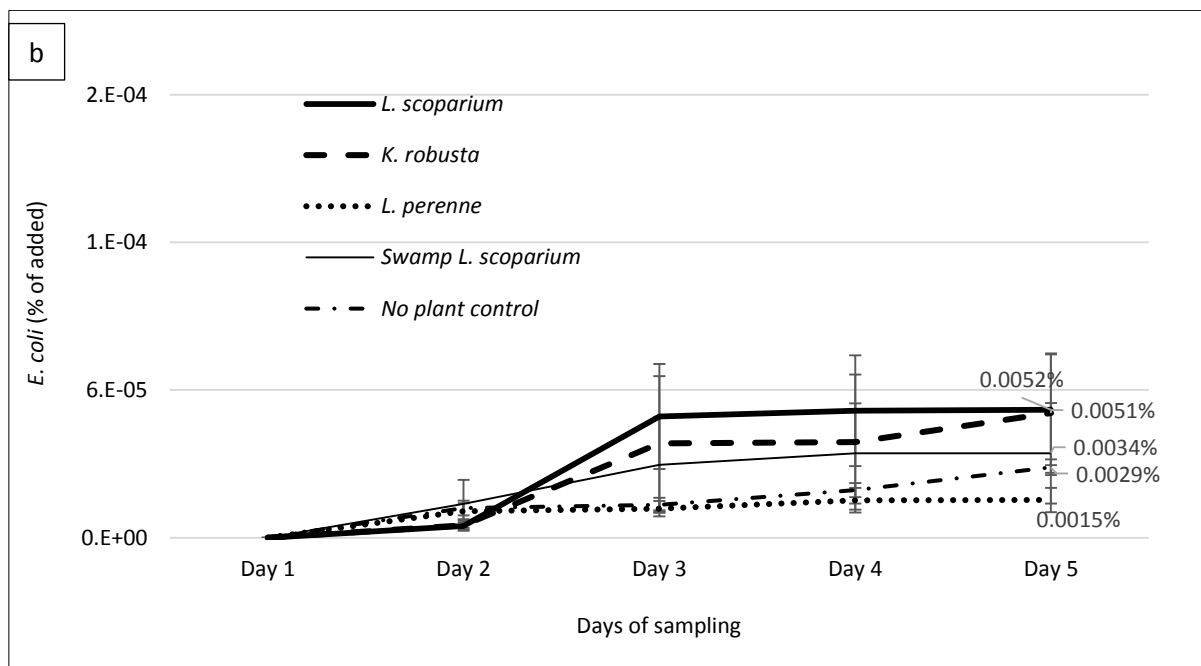
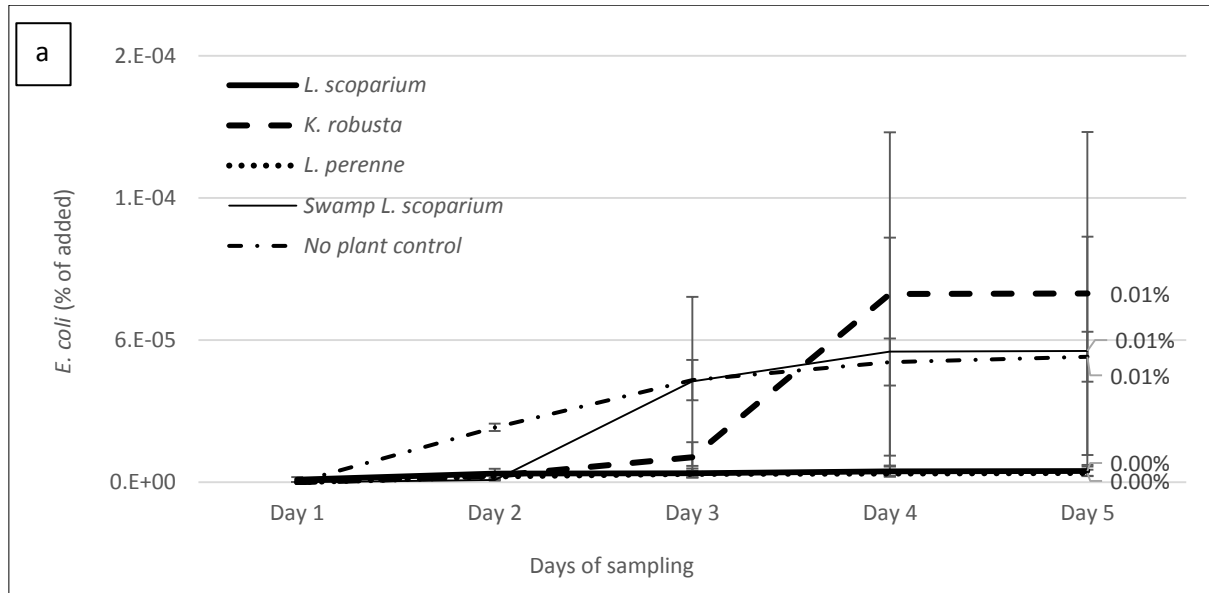


Figure 5-2 Cumulative percentage of *E. coli* leaching in **a)** the Akaroa soil and **b)** the Eyrewell soil. Error bars represent standard error of the mean value (n = 5).

Fig. 5.3 shows the breakthrough curve of the bromide tracer in both soils. In the Akaroa soil (Fig. 5.3.a), the bromide curve suggests bromide following the preferential flow routes under all species while the unplanted control, on day 2 only, generated a significantly higher bromide leaching compared to all the other plant species. At day 3, the significant difference existed between the unplanted control, *L.*

perenne, and swamp *L. scoparium*. *Leptospermum scoparium* started early bromide leaching, from the second leaching event and showed the highest peak of 11.8 mg bromide mass leaching on day four. *Kunzea robusta* on the other hand, started bromide leaching from the third leachate event similar to swamp *L. scoparium* and *L. perenne* and showed the peak of 31.9 mg bromide mass on the fifth leachate collection.

The unplanted control in the Eyrewell soil (Fig. 5.3.b) presented highest bromide leaching from the second day onwards and reached the peak concentration of 50 mg bromide mass on the fourth day. All other species in this soil presented a slowly increasing peak reaching the highest concentration of < 10 mg bromide mass leaching under *K. robusta* and *L. perenne* by the end of the experiment. *Leptospermum scoparium* leached the minimum with an average total of 3.6 mg bromide by the end of the experiment.

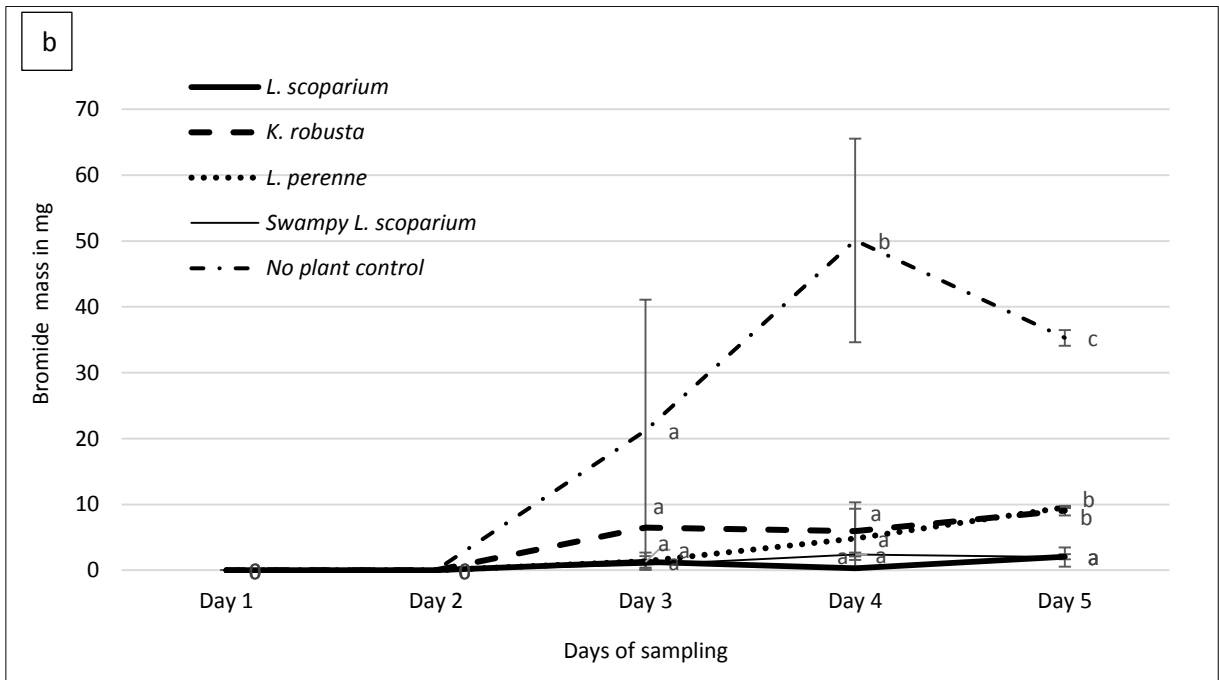
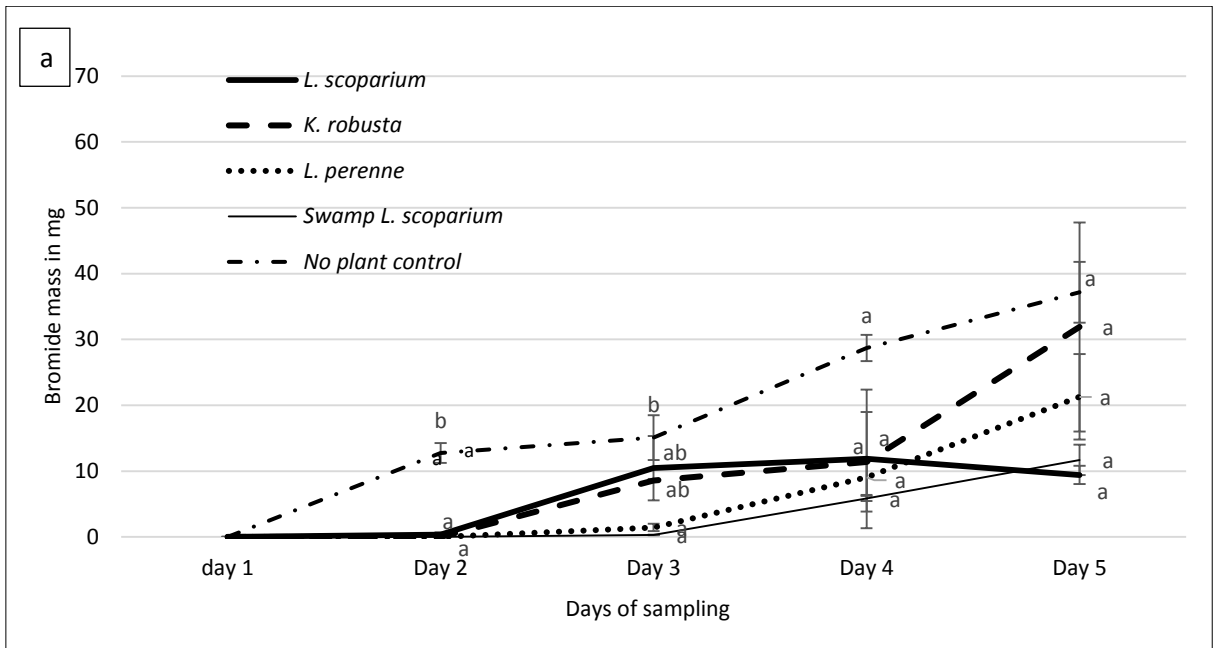


Figure 5-3 Bromide leaching in **a)** the Akaroa soil and **b)** the Eyrewell soil under all species. Error bars represent standard error of the mean value (n = 5). At each sample time, data points with the same letters are not significantly different based on Fishers Unprotected LSD analysis (p < 0.05).

Fig. 5.4 shows the cumulative percentage of bromide leaching in both soils. At the end of the experiment, in the Akaroa soil (Fig. 5.4.a) the unplanted control leached the highest of 23.4% of the added bromide mass while a minimum of 4.5% of the total bromide leaching occurred under swamp *L. scoparium*. Bromide leaching followed unplanted control > *K. robusta* > *L. scoparium* > *L. perenne* > Swamp *L. scoparium* in the Akaroa soil.

In the Eyrewell soil (Fig. 5.4.b), unplanted control leached > 26% of the added bromide mass by the end of the experiment while *L. scoparium* leached < 1% of the inoculated bromide mass only.

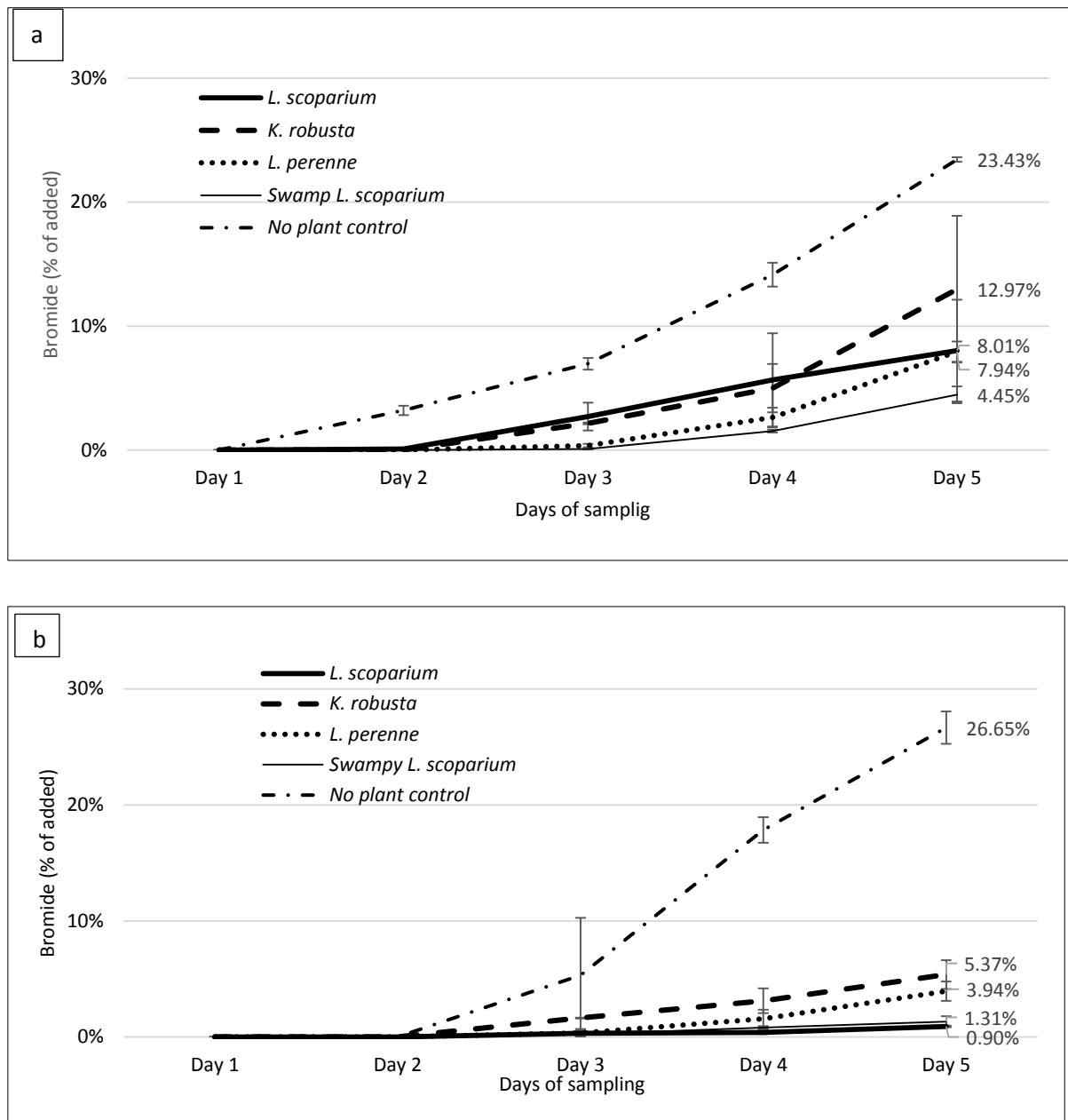


Figure 5-4 Cumulative percentage of bromide leaching in **a)** the Akaroa soil and **b)** the Eyrewell soil. Error bars represent standard error of the mean value (n = 5).

5.4 Discussion

Compared to Chapter 4, this study allowed increased time of interaction (11 - 15 days) between DSE applied *E. coli* and plants roots or unplanted soils. As expected, bacterial leaching decreased with increased interaction time and slow irrigation rates although, the soil type affected bacterial leaching or plants activities against *E. coli*.

With increased time of interaction in the Akaroa soil, maximum bacterial leaching occurred was 0.01% of the inoculated *E. coli* population under *K. robusta*, swamp *L. scoparium* and unplanted control while *L. perenne* and *L. scoparium* leached negligible bacterial numbers with $3 - 4 \times 10^{-4}$ % of the inoculated *E. coli*, respectively. A significant difference in bacterial leaching existed occasionally between all the species throughout the experiment and any effect of preferential flow dominating the *E. coli* leaching in this soil could not be established. Contrary to the Akaroa soil, in the Eyrewell soil, *L. scoparium* represented the effect of preferential flow for *E. coli* leaching the highest of 1.7×10^3 cfu/ml on the second day of leachate collection and accounting for 5.0×10^{-4} % of the inoculated bacterial population. It was followed by *K. robusta* and swamp *L. scoparium* leaching 1.5×10^3 cfu/ml and 5×10^2 cfu/ml, respectively, on the same day. Variability in the bacterial leaching intensity under *L. scoparium* in the two soil types used was observed, however in both soils occasional significant differences existed between *L. perenne* and *L. scoparium*. These results explain that the increased DSE residing time in soil, following deficit irrigation, results in limited bacterial leaching under plants, which varies with the soil type. The results also present variable effect of *L. scoparium* roots components against *E. coli*, in different soil types. All other plant species followed similar leaching pattern in both soils i.e. *K. robusta* > swamp *L. scoparium* > unplanted control > *L. perenne* with no significant difference.

Comparing the minimum *L. scoparium* bacterial leaching in the Akaroa soil i.e. 4.1×10^{-4} %, to a field environment, can present a different aspect. An average stocking rate for dairy farms in the South Island is four cows per hectare (Farmlands, 2016) and each dairy cow produces nearly 57 l of effluent per day (Jones, 1999; McGarvey et al., 2004) containing an average of 10^5 cfu/ml of *E. coli* (LeJeune et al., 2001; Omisakin et al., 2003). For example, if there were $10,000 \text{ m}^3$ of the groundwater per hectare receiving 4.1×10^{-3} % the effluent from 20 cows (following rotational grazing), the microbial contaminant concentration would be around 4.5×10^6 cfu/ml in the groundwater. This microbial numbers is more than sufficient to infect humans or animals (Avery et al., 2004; McLeod et al., 2008; Sears et al., 2011; Trimble et al., 2013). However, minimum/deficit irrigation used in this study to maintain the soil moisture resulted in no leachate (Section 5.2.3) and continuous irrigations were performed to produce the leachate. Hence, following a gap of twenty or more days between effluent irrigation might provide more chances of bacterial inhibition under these antimicrobial trees planted in dairy farms vicinity. Vinten et al. (2002) in their field study with *E. coli* inoculated with animal slurry found that following no irrigation after bacterial inoculation, maximum *E. coli* population remained in the top soil and stated that increased rainfall after bacterial application into the soil can exacerbate its flow to drainage water. Ogden et al. (2001) and Cools et al. (2001) stated that hot weather and no irrigation following bacterial application in soil decreases the chance of bacterial leaching and increases the die-off rates. Hence, bacterial inoculation with DSE application in a dry summer season under *L. scoparium* might further reduce the risk of bacterial leaching increasing their die-off rate. Wicaksono

et al. (2016) found changing microbial endophyte communities in *L. scoparium* with plant maturity and suggested that these changing microbial communities might be the responsible factor for the variable chemotypic/antibiotic composition of these plants with age. This suggests that mature *L. scoparium* trees might have a different and more stable composition of antimicrobial components and future studies with mature and established *L. scoparium* trees might provide bacterial attenuation independent of the soil type. Glasshouse studies using repacked soil columns ignore the potential natural cracks present in soil and hence, field studies are important to further evaluate plants antimicrobial activities (Kung et al., 2000; Smith et al., 1985). Survival of *E. coli* residing in the soil was not tested in this study, as that was not the aim. Studies suggest naturalisation or regrowth of *E. coli* in soil with time (Avery et al., 2004) however, during preliminary trials (Appendix C.12) bacterial enumeration in soil was performed for all species and suggested no bacterial regrowth under the natives within tested time period.

Zaleski et al. (2005) stated that the bacterial attenuation results differ based on the soil type and similar results were observed in this study. The previous study by Prosser et al. (2016) demonstrated rapid bacterial decline under the soil systems of *L. scoparium* and *K. robusta*, although they used a Templeton silt loam soil in their study. Variable *E. coli* inhibitory action of *L. scoparium* in the two soil types used in this study, might have been driven by the specific factors present in each soil such as native microbial population including antagonistic soil microbes (fungi or bacteriophages), competition present between the inoculated and the native microbial population, released root exudates, soil structure and its water holding potential, presence of nutrients and the clay concentrations (Johnson & Lehmann, 2006; McLeod et al., 2008; Mendes et al., 2013). Plant processes are always linked with the soil microflora (Bakker et al., 2014; Robinson et al., 2003) and the rhizosphere is a competitive environment for the non-natives to survive (Neralla & Weaver, 2000). Depending on the soil type and the extent of competition, native soil microbes or the roots associated microbes of plants can implicate and inactivate the inoculated bacteria to reduce the competition (Jiang et al., 2002; Unc & Goss, 2003). Jiang et al. (2002) compared sterilised and unsterilized soil with manure amendments containing *E. coli* 0157:H7 and reported that *E. coli* 0157:H7 reduced faster in the unsterilized soil because of the competition and the interaction with the native soil microbes. Mubiru et al. (2000) stated that bacterial mortality rates vary based on the soil type used. Along with the chemotypic effects, roots biomass, spread, depth and distributions are also controlled by the locations and soil types and hence the same plant species growing in one region can have a different roots morphology in another region with another soil type, which might affect roots/plants activities (Watson & Mardern, 2004).

In this study high nutrients concentration were provided to the plants with DSE application, which might have affected the activities of the plants rhizosphere communities. Zhang et al. (2016) stated that depending on the preferences and availability of nutrients, either plants or the root-associated

microorganisms can lead the contaminant removal process. They found that root associated microorganisms lead the contaminant removal from the soil showing correlation with the root organic C content. Prosser et al. (2016) stated that the antimicrobial effect of the plants could differ based on the soil type due to differences in the phytochemical sorption released by the plants, which can affect their bioavailability to the beneficial microbes or to the pathogens. Bakker et al. (2014) studied the soil microbiome associated with *Schizachyrium scoparium* and several other plants species in different soil types. They reported that specific rhizosphere taxa associated with plants generates antagonistic activities against pathogens and that the outcome of the species (plant-rhizosphere microbe-pathogen) interaction is dependent on the environment including the soil type and the richness of the plant species in a community. As the activities of the rhizosphere microbes was not tested in this study, future studies should focus on identifying how the associated rhizosphere microbes of *L. scoparium* and *K. robusta* interact with the native soil microbes and affect the survival of introduced microbial contaminants.

A complex network of interactions between plant-microbes and microbe-microbe (native and foreign) influences the release of root exudates by plants. Stable plant microbial communities including endophytes (Wicaksono et al., 2016) and roots associated microbes are specifically associated with a particular plant species and plants can alter their dynamics to obtain the best environment for their fitness (Bakker et al., 2014). This procedure of achieving fitness, might result in a co-evolutionary competitive environment or different root exudates released by the same species in different soil environments (Johnson & Lehmann, 2006). As plants secretions might vary based on the plants need in a particular soil type, these compounds, can be either beneficial, inhibitory or antimicrobial for the inoculated microbes including pathogens (Segura et al., 2009). Association networks within the plant microbial communities can shift the competition for nutrients by influencing the production of antibacterial metabolites by plants (Bakker et al., 2014; Kinkel et al., 2011). However, individual plant species may have contrasting interactions with soil contaminants (Fan et al., 2001) in the same soil or the same contaminant in different soil types (Johnson & Lehmann, 2006; Zhang et al., 2015). It is well known that roots release antimicrobial components, although the selection procedure of the pathogens by the root exudates is not well understood (Bakker et al., 2014). The analysis of the released root exudates was not performed in this study and neither any information exists on the rhizosphere chemistry of *L. scoparium* and *K. robusta* to relate the variability observed in the results of this study with a particular mechanism. In future studies analysis of the rhizosphere chemistry of these plants will expand the understanding of plants behaviour against microbial contaminants.

This study infers that the lowest *E. coli* leaching in the Akaroa soil under *L. scoparium* was the result of the antibacterial interactions in its rhizosphere in this soil. However, its highest bacterial leaching in the Eyrewell soil might have been the result of soil properties varying the effective antimicrobial

interactions in its rhizosphere, resulting in the preferential flow routes dominating the *E. coli* movement down to the leachate. Lysimeters were not harvested to confirm the preferential flow routes in this study and only the bromide leaching was used as an indicator for the preferential flow.

Nutrient rich applications such as DSE can increase the survival time of the inoculated bacterial population by slowly releasing nutrients, formation of microhabitats, and affecting aggregate formation by the soil and its water retention capacity by blocking the soil pores with high organic matter content (Howell et al., 1995; Mubiru et al., 2000). It was concluded that most of the DSE applied *E. coli* in the Eyrewell soil leached down to the bottom of the lysimeters, gaining protection from the active and competitive rhizosphere microbes or any released antibacterial root components by *L. scoparium* resulting in the highest bacterial leaching in this soil. The DSE compounds might also have provided nutrients to support bacterial survival or even for their regrowth during the experimental period in this soil. Although, low bacterial numbers under *L. scoparium* in the Akaroa soil suggests that the specific antibacterial rhizosphere interactions in this soil overrode the nutrients in the DSE that could increase the survival of *E. coli*. Sandy soils have previously been stated to increase the survival time of bacteria as sand can act as the reservoir of the applied bacteria (Aislabie et al., 2011; Cools et al., 2001; Hashsham et al., 2004; McLeod et al., 2008; Mubiru et al., 2000).

Lolium perenne generated the lowest total bacterial leaching of 47 cfu/ml and 107 cfu/ml in the Akaroa and the Eyrewell soil, respectively by the end of the experiment with no significant difference with *L. scoparium*, especially in the Akaroa soil. Our previous experiment (Chapter 4) also indicated that *L. perenne* holds the inoculated *E. coli* populations in its fibrous root system decreasing their numbers in the leachate and increasing the risk of their surface run off. Similar to the low *E. coli* leaching results of this study under *L. perenne*, Di et al. (1998) observed low nitrate concentrations in the leachate from pastoral fields under flood irrigation. Bacterial leaching risk is high under saturated soil conditions as compared to non-saturated soils due to the air-water interface formation, which creates strong capillary forces reducing the bacterial movement (Bergin et al., 1995; Jiang, 2008) and forests can maintain dry soil conditions better than the pasture (Prosser, 2011). Bargués Tobella et al. (2014) also reported lower surface run off potential of trees.

In the Akaroa soil, where *L. scoparium* had lower bacterial leaching, the unplanted control leached significantly high *E. coli* populations in the leachate, however in the Eyrewell soil, all Myrtaceae species increased the risk of bacterial leaching following preferential flow, as compared to the unplanted control, showing slowly increasing bacterial peak with a significant difference. Higher bromide leaching percentage in both soils compared with *E. coli* suggests preferential flow along with varying levels of bacterial inhibition by *L. scoparium* in both soils. Hence, the first rainfall sufficient to create a flow after long term interaction, resulted in bacterial leaching. Watson and O'Loughlin (1985) suggested that the

root system of *L. scoparium* could prevent nutrient leaching, which only fits with the bacterial leaching results in the Akaroa soil.

The results of this study confirmed that increased time of interaction of *E. coli* with the root components of *L. scoparium* following minimum/deficit irrigation, decreases their numbers in the leachate to a varying degree and that the bacterial inhibition intensity of *L. scoparium* is dependent on the soil type. As all other plant species followed the same bacterial leaching pattern in both soils, any antibacterial potential of these plants could not be established, although they represented their potential of increasing the preferential flow of the surface applied bacteria independent of the irrigation scheme followed (compared to Chapter 4). These results also confirm that the fine textured soil increases the risk of bacterial leaching, under these plants, and hence, further studies will be required to identify *L. scoparium* potential against *E. coli* in a range of soil types.

5.5 Conclusion

The increased interaction time of DSE-applied *E. coli* with *L. scoparium* roots, following deficit irrigation presented varying level of bacterial leaching ranging from $4.7 \times 10^{-4}\%$ in the Akaroa soil to $5.2 \times 10^{-3}\%$ in the Eyrewell soil presenting differing *L. scoparium* activities in different soil types. All other plant species presented similar leaching patterns in both soils following *K. robusta* > swamp *L. scoparium* > unplanted control > *L. perenne* with no significant difference from each other and no effect on bacterial leaching. Increased risk of surface runoff of surface applied bacteria under *L. perenne* is predicted. For future work, it will be interesting to compare the antibacterial activities of mature *L. scoparium* trees in different soil types to evaluate their potential against bacterial leaching.

Chapter 6

Survival of *Escherichia coli* in soil pore water under *Leptospermum scoparium* following dairy effluent application

6.1 Introduction

Soil micropores (< 30 µm) have the highest microbial activity (Negassa et al., 2015) because they contain thin films of surface-bound solution where bacteria may survive and grow (O'Donnell et al., 2007). In contrast, macropores (> 30 µm), allow oxygen exchange and are responsible for most of the preferential flow of surface-applied pathogens. Soil pore structure and water content affects bacterial infiltration along with irrigation intensity (Feeney et al., 2006; Gagliardi & Karns, 2000; Karube & Kawai, 2001).

Root-induced macropores in soils contribute to the vertical transport of the surface applied contaminants (Bais et al., 2004; Bargués Tobella et al., 2014; Johnson & Lehmann, 2006). Roots interact with nutrients, air, and water and exude compounds, including volatiles and antibiotics (Abhilash et al., 2012; Kai et al., 2016; Mendes et al., 2013). Surface applied bacterial populations, not recovered in the leachate, can reside in the porous and nutrient rich environment in soil near plants roots and may persist, multiply or die-off (Bertin et al., 2003; Blom et al., 2011). Ogden et al. (2001) stated that some bacterial populations are not susceptible to leaching, even after continuous heavy rainfalls.

The results of our previous experiments (Chapter 4 and 5) showed that the root-induced preferential flow pathways under *L. scoparium* and *K. robusta* increase the leaching risk of surface applied *E. coli*, irrespective of the irrigation regime followed however, soil type was a crucial factor affecting leaching behaviour. Chapter 5 showed that even after ten days of interaction between the roots and the bacteria, < 1% of *E. coli* were still recoverable in the leachate with continuous irrigations. As Chapters 4 and 5 focussed on the bacterial leaching potential under *L. scoparium* and did not assess the bacterial population residing in the soil pore space under *L. scoparium*, this study aimed to identify the survival of DSE-applied *E. coli* in the soil pore water under *L. scoparium* with time. As no antibacterial activity of *K. robusta* was observed in Chapter 4 and 5, only *L. scoparium* was used in this study because of its demonstrated antimicrobial activities (Douglas et al., 2004; Lu et al., 2014; Porter et al., 1998; Porter & Wilkins, 1999). It was hypothesised that a lower water flux will reduce bacterial preferential flow allowing uniform distribution of the applied DSE within the soil profile and the results will provide a pattern of bacterial die-off as the interaction time increases (which could not be recovered in Chapter 5).

6.2 Methods and materials

6.2.1 Experimental set up

Details of the materials i.e. plants, soils and DSE collected are presented in Chapters 4 and 5. The lysimeter set up followed that described in Chapter 5 and used the Eyrewell soil with *L. scoparium* or *L. perenne*. There were nine lysimeters for each species, divided into three blocks (Fig. 6.1).

6.2.2 Bacterial inoculation with DSE application

Fresh DSE load was collected 48 hours before the experiment and was stored at 4°C. A 100 ml DSE subsample was collected for *E. coli* enumeration using Colilert-18 (IDEXX laboratories) (details in section 4.2.4). Plants were irrigated with the tap water at field capacity, 24 hours prior to the experiment, using a sprinkler to moisten the soil uniformly and were left undisturbed. After 24 hours, rubber stoppers were installed in the hole at the bottom of each lysimeter before DSE application.

Eight mm (400 ml) DSE was slowly irrigated within 5 hours using pressurised spray bottle. The irrigation rate of 2 mm/hour was chosen to avoid anaerobic conditions in the lysimeters. With 8 mm DSE irrigation to each lysimeter, 1.3×10^5 cfu of total *E. coli* was added. After DSE application, all lysimeters were weighed (with growing plants and added DSE) and plants were left undisturbed. Throughout this experiment, the moisture content of each lysimeter was maintained constant (per lysimeter and not between lysimeters) (Fig. 6.4) by daily weighing the lysimeters and adding/spraying tap water to recover the lost moisture. The irrigation rate was chosen based on the preliminary experiments (Appendix D.2) up to the field capacity.

6.2.3 Lysimeter harvest

One block at a time was randomly chosen to be harvested on day 2, 5, and 7 after DSE application. Shoots of *L. scoparium* were removed by cutting nearly 2.5 cm above the soil surface and *L. perenne* leaves were harvested nearly 5 cm above the soil surface. Each soil column was separately harvested in a clean plastic tray to avoid cross contamination. Any lost soil pore water during the harvest was calculated by collecting the maximum water from the tray (avoiding soil) in a measuring cylinder. The collected volume was recorded for the calculation of the total soil pore water. Soil from each lysimeter was homogenised, and a subsample was collected. Bacterial enumeration started within 2 hours of the soil collection.

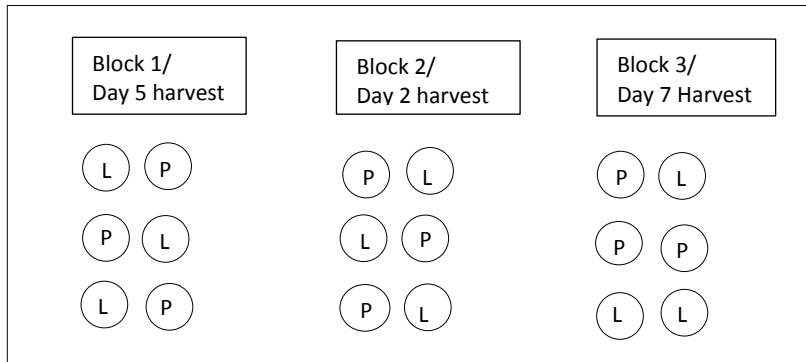


Figure 6-1 Layout of the experiment set up and the lysimeter harvesting plan. L = *Leptospermum scoparium*, P = *Lolium perenne*.

6.2.4 Bacterial enumeration in soil pore water

A subsample of each soil (180 g) was collected for extracting soil pore water using Jumbosep™ centrifugal devices (PALL laboratories), which were chosen for this study after personal communication with Jacqui Horswell (ESR, Wellington). Jumbosep™ were layered with a 0.4 µm filter paper and were filled with 50 g of fresh soil in three replicates each. Each Jumbosep™ with the weighed soil was sealed using Parafilm® and were left overnight in the fridge. After overnight storage, each Jumbosep™ was centrifuged at 258 x g for 1 hour to release the pore water. If no soil pore water was collected in the collection container after the first hour of centrifugation, Jumbosep™ for that particular sample (all *L. scoparium*) was centrifuged again for another hour. Collected pore water was used within 4 hours for *E. coli* enumeration using Colilert-18 (IDEXX laboratories) after required dilutions. Subsamples of pore water were also used for pH measurement (Fig. 6.3). After centrifugation, the collected volume of the pore water was used to calculate the total pore water in each lysimeter. The measured *E. coli* concentration (in cfu/ml) from Colilert-18 (IDEXX laboratories) was multiplied with the total pore water calculated, to estimate total bacterial population in each lysimeter.

6.2.5 Statistics

Standard errors were calculated using Microsoft Excel (Version 2017). To identify significant difference in *E. coli* leaching between species, cfu/ml was log transformed and two-way ANOVA was performed on these log numbers. Difference in LSD (5%) is presented as a measure of variability using Genstat 16th edition (VSN International Ltd). Bacterial count below detection limit i.e. < 1 cfu/100 ml, were considered as 1 cfu/ml for log₁₀ transformation.

6.3 Results

Fig. 6.2 shows total *E. coli* residing in the soil pore water under *L. scoparium* and *L. perenne*. The plants maintained a significant difference in *E. coli* leaching other than on day five. *Lolium perenne* had highest bacterial count throughout the experiment between 5.4×10^3 cfu/ml and 2.7×10^3 cfu/ml.

However, bacterial count under *L. scoparium* decreased from day two to day seven with highest *E. coli* count of 1.3×10^3 cfu/ml on day five and lowest of 1.3×10^2 cfu/ml *E. coli* by day seven.

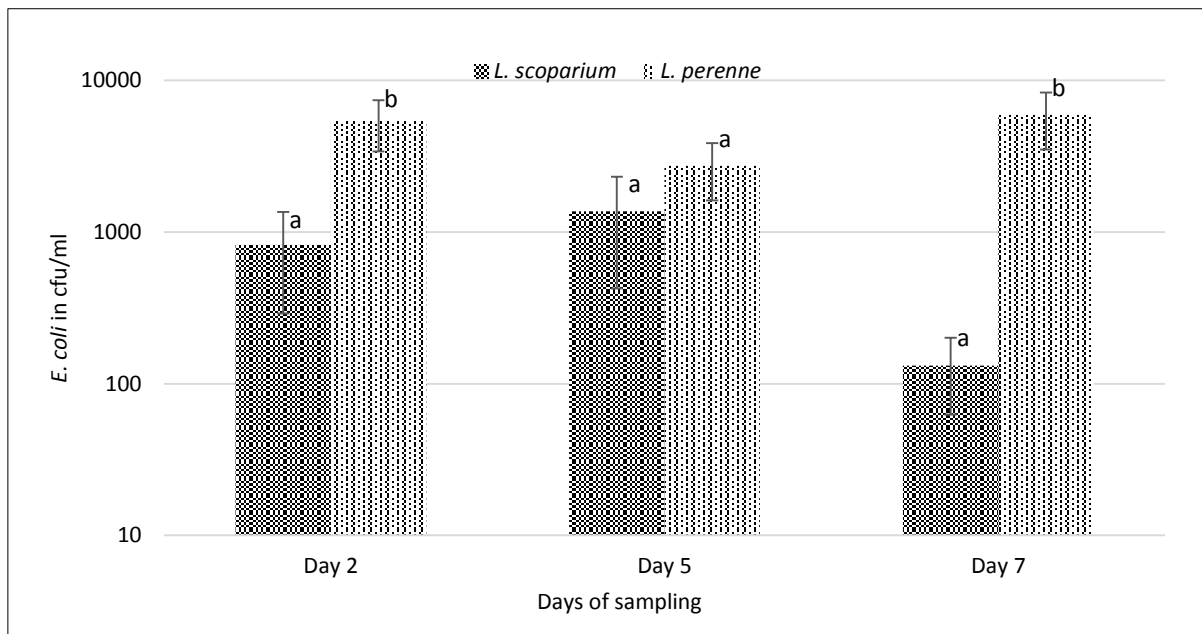


Figure 6-2 Average total *E. coli* population residing in the soil pore water under *L. scoparium* and *L. perenne*. Error bars represent standard errors of the mean value (n = 3). At each sample time, data points with the same letters are not significantly different based on Fishers Unprotected LSD analysis ($p < 0.05$).

Fig. 6.3.a shows the pH of the extracted pore water from the soil under both species. A significant difference existed between the pH of *L. scoparium* and *L. perenne* with pH values of 5.8 and 6.1, respectively. These pH values were within the tolerable range for *E. coli*. Fig. 6.3.b shows the average soil moisture content (%) of *L. scoparium* and *L. perenne* after DSE application. *Leptospermum scoparium* had a significant low soil moisture content of 47.40% than *L. perenne* with 66.98% of soil moisture. *Lolium perenne*, with highest soil moisture also had high bacterial survival.

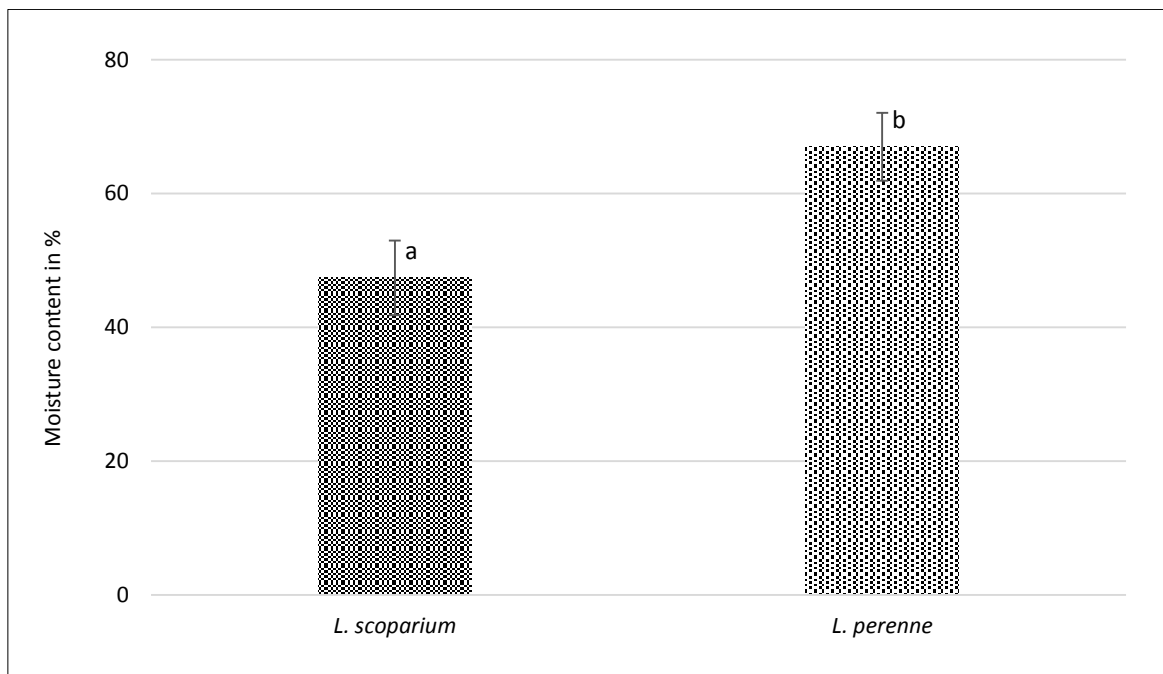
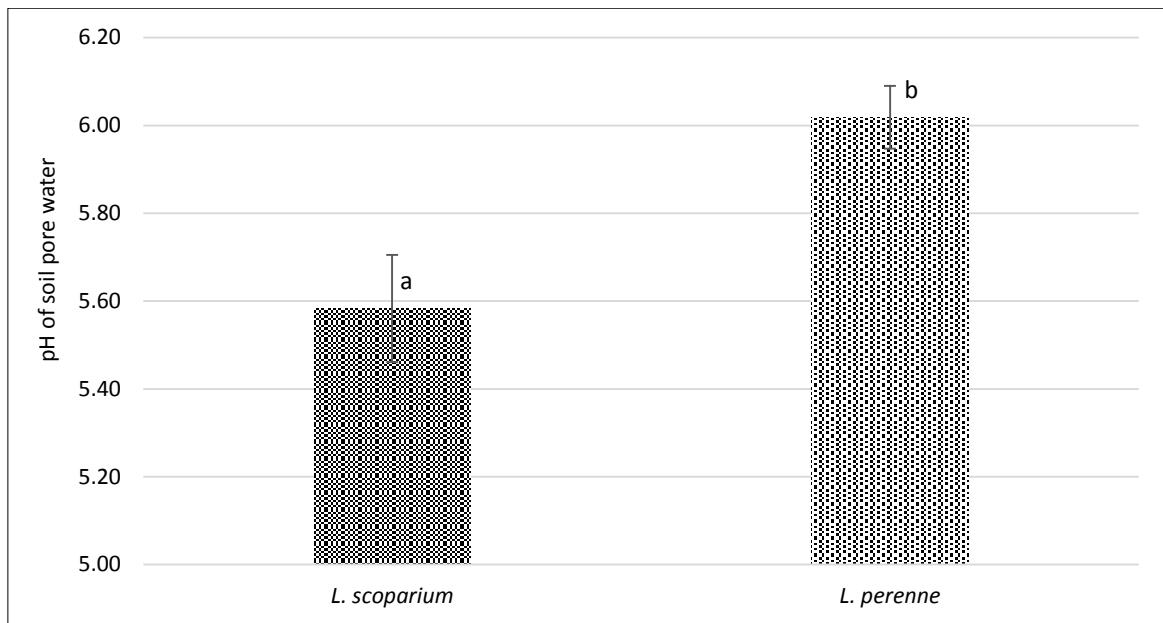


Figure 6-3 Shows **a)** the pH of the soil pore water under *L. scoparium* and *L. perenne* and **b)** the average soil moisture content under *L. scoparium* and *L. perenne* in percentage. Error bars represent standard errors of the mean value (n = 3). Data points with the same letters are not significantly different based on Fishers Unprotected LSD analysis ($p < 0.05$).

6.4 Discussion

After the seven-day experiment, a significant difference in bacterial leaching was observed between *L. scoparium* and *L. perenne* with the bacterial counts of 1.3×10^2 cfu/ml and 5.9×10^3 cfu/ml *E. coli*. Similar to the results of previous chapters, *L. perenne* showed highest bacterial survival throughout the

experiment, which could be a result of its high soil moisture content however; lowering bacterial numbers under *L. scoparium* confirm the antibacterial effect of its roots/rhizosphere components.

Although the exact mechanism of reducing the *E. coli* population by *L. scoparium* is unknown, the results indicate that along with the natural die-off, soil processes in the root-zone of *L. scoparium*, may attenuate bacteria (Kinkel et al., 2011; Mendes et al., 2013). Long-term studies might present no bacterial recovery from under *L. scoparium* further confirming its antimicrobial potential. Similar to this study, Prosser et al. (2016) who used *E. coli* spiked biosolids instead of DSE, also demonstrated decreased *E. coli* population under the soil system of *L. scoparium* and *K. robusta* within seven days of bacterial inoculation, however, the responsible mechanism for bacterial reduction was unknown. While various studies have mentioned roots exudates as the potential antimicrobial agents released by the plants in soil (Johnson & Lehmann, 2006; Kinkel et al., 2012; Lau & Lennon, 2011) their mechanism of choosing a particular pathogen still lacks knowledge (Mendes et al., 2013). Prosser (2011) suggested that plants could deposit antimicrobial root components into the soil via rhizodeposition, which can affect the rhizosphere soil chemistry (Castaldi et al., 2009; Hawes et al., 2016). Analysing the rhizosphere chemistry of the plants was beyond the scope of this study. As the rhizosphere chemistry of the NZ native species including *L. scoparium*, has not been explored, it is difficult to derive the main factors (exudates, volatiles or the associated microorganisms) responsible for the bacterial growth inhibition, and further studies will be required to investigate the same. Such studies may follow (Abhilash et al., 2012; Bais et al., 2004; Hawes et al., 2016; Mendes et al., 2013) who reviewed root-microbe interactions, rhizosphere microorganisms and the associated techniques to elucidate how the complex chemical exchanges and root exudates including volatiles increase plant-plant, plant-microbe and microbe-microbe interactions in the rhizosphere.

As stated in Chapter 2, organic applications increase bacterial survival by slowly releasing the nutrients (Avery et al., 2004; Horswell et al., 2007; Jiang, 2008; Kumar et al., 2005; McLeod et al., 2008; Solomon et al., 2002; Stoddard et al., 1998). However, bacterial decline under *L. scoparium* suggests that the interactive processes in the rhizosphere were unaffected by the DSE nutrient supply providing any support for bacterial survival. It also suggests that in natural conditions, absence of the inhibitory components in soil such as those from *L. scoparium*, microbial contaminants can grow utilising the DSE nutrients (Banning et al., 2002) and water flowing with high velocity can drain these bacteria down to the leachate (Gagliardi & Karns, 2000).

As expected, the soil pore water under *L. perenne* contained highest bacterial population and soil moisture throughout the experiment. Chapters 4 and 5 have also demonstrated high bacterial recovery and survival in the top soil system under *L. perenne* and confirmed the same with the dyed soil columns (Fig. 4.6). Chandler et al. (1981) stated that ryegrass roots can support and increase *E. coli* survival for

long time period in soil and that the high soil moisture can increase the *E. coli* concentration/numbers. As *L. perenne* can effectively cover the top soil layer as compared to the natives, and had the highest moisture content during harvest, temperature difference of the soil, between species, can also be a potential factor demonstrating high bacterial survival in its soil pore water (Joergensen et al., 1990; Reddy et al., 1981). Soil temperatures were not evaluated in any of the experiment of this thesis and this should be included in future studies. The average glasshouse temperature during the trial was 14.95°C – 19.2°C. These results indicate that the low infiltration under *L. perenne* can result in high surface run off potential of the bacteria residing in its roots, following high rainfall/irrigation (Chandler et al., 1981; Jamieson et al., 2002; Zhai et al., 1995).

Ruamps et al. (2011) reported that the size of the microbial colony in the soil depends on its pore size and hence, on the available pore water. Microbial abundance normally correlates with the soil pore water volume and increased pore structure in the soil can increase microbial interactions with antimicrobial components released by the plants (Bais et al., 2004; Nielsen et al., 2008). Trees actively maintain a porous rhizosphere region and dry soil conditions beneficial for reducing bacterial leaching risks as compared to *L. perenne* (Bargués Tobella et al., 2014; Smith et al., 1985; Zaleski et al., 2005). As *L. scoparium* had low soil moisture content during the harvest and represents tightly bound soil pore water than *L. perenne*, low bacterial numbers under *L. scoparium* can also be a result of low soil moisture or high water use by *L. scoparium*. Dry soil conditions under trees can result in high exudates concentration in the soil, as increasing moisture can dilute the components concentrations increasing bacterial growth (Blom et al., 2011; Cools et al., 2001; Dicke et al., 2009; Dorman & Deans, 2000; Zaleski et al., 2005). It was concluded that the significant high soil moisture content (and the associated DSE nutrients) under *L. perenne* compared to the *L. scoparium* increased bacterial survival in their soil pore water and hence, bacterial numbers were higher under *L. perenne* throughout the experiment. Jamieson et al. (2002) reported that excessive soil moisture can lower down *E. coli*'s ability to utilise available organic components and hence, a reduction in their numbers can be evident. However, this does not fit with the results of *L. perenne* in this study.

Low irrigation rates following spray irrigation is beneficial in reducing the preferential flow (Jiang, 2008). As the study followed low water flux conditions, it excluded bacterial leaching (Appendix D.2) following preferential flow and allowed more time of interaction between applied bacteria and any inhibitory rhizosphere components. As stated in previous chapters, *E. coli* leaching can be avoided following no rainfall and dry weather conditions after bacterial application (Ogden et al., 2001; Saini et al., 2003). The total irrigation applied in this study was 8 mm (400 ml) of DSE, which resulted in no leachate during the harvest and lowest bacterial recovery from the soil pore water under *L. scoparium*. This shows that following no irrigation after slowly irrigated DSE (2 mm/hour) under *L. scoparium*, increased bacterial die-off can be expected (Smith et al., 1985; Vinten et al., 2002; Yunusa et al., 2002)

in soil, but will need further investigations using varying soil types. Unc and Goss (2003) stated that applying low bacterial loads at a time and increased time for containment can be beneficial to increase bacterial die-off increasing the effect of present antimicrobial roots exudates (Bais et al., 2004).

Fine structured soils have been shown to increase bacterial survival (Bakker et al., 2014; Linn & Doran, 1984; Mubiru et al., 2000; Strange & Scott, 2005; Wolf et al., 2013) especially by sorption with the DSE colloids (McLeod et al., 2008). Cools et al. (2001) tested survival of *E. coli* after pig slurry application in soils of different textures and found that sandy soils are the best for *E. coli* survival. Although in this study bacterial die-off was observed in the Eyrewell soil (Stony silt loam, Lismore soil) with 20% increased sand content. This indicates that in future studies use of variable soil types will be helpful to target effective *L. scoparium* activities against *E. coli*. As the DSE was irrigated to avoid the anaerobic conditions in the lysimeters, and further, *E. coli* is a facultative anaerobe, anaerobic conditions in the lysimeters had no contribution towards bacterial die-off.

Roots of *L. scoparium* stabilise soil (Watson et al., 1997; Watson & Mardern, 2004; Watson & O'Loughlin, 1985) and can decrease soil pH to as low as 3.5 under mature trees (Hahner et al., 2014), which is highly acidic for bacterial pathogens (Bååth, 1996). In this study, pH of the soil pore water under both species was not in the range to affect *E. coli* survival (Fig. 3.7), although high pH of *L. perenne* might have affected bacterial retention in the top soil (Fig. 6.3), which needs further investigations.

The lowest *E. coli* population recovered from the soil pore water under *L. scoparium* confirms the potential of its antimicrobial rhizosphere components/activities against *E. coli*. However, further studies are required to identify the particular mechanism or component responsible for this bacterial decline under *L. scoparium*. The low irrigation rates up to the field capacity allowed maximum interaction time between the rhizosphere components and the applied bacteria, avoiding preferential flow pathways. Previous chapters (Chapter 4 and 5) have confirmed bacterial leaching following the root induced preferential flow pathways under *L. scoparium* and *K. robusta* and that the leaching was dependent on irrigation rates and the soil type. As the *L. scoparium* and *K. robusta* are of great interest as riparian plants (Watson & O'Loughlin, 1985) it will be interesting to study if the antimicrobial root components of *L. scoparium* present inhibitory actions against DSE bacteria in a riparian region and how the presence of a water source affects bacterial survival under *L. scoparium*.

6.5 Conclusion

A reduction in the DSE applied *E. coli* numbers surviving in the soil pore water under *L. scoparium* was observed with time, with a significant difference from *L. perenne* showing the highest bacterial survival throughout the experiment, other than on second day. These results demonstrated the antimicrobial

effect of *L. scoparium* in soil pore water, although the mechanisms are still unclear. Potentially, *L. scoparium* may have inhibited *E. coli* growth through root exudates, rhizosphere acidification, or removal of soil pore water with high water use. This drying action of *L. scoparium* should increase the soils ability to absorb moisture and hence reduce surface run off. Future studies should focus on examining bacterial survival under mature *L. scoparium* trees following varying soil moisture content to understand how soil moisture content can affect plants inhibitory activities against the bacteria.

Chapter 7

The effect of *Leptospermum scoparium* and *Kunzea robusta* on *Escherichia coli* survival in the field environment

7.1 Introduction

Chapters 3 - 6 of this research demonstrated that *L. scoparium* and *K. robusta* can attenuate introduced microbial contaminants in soil but may exacerbate preferential flow leading to the increased microbial leaching under high flow conditions. The taproot system (Watson & O'Loughlin, 1985) of these plants can create preferential flow pathways, which under irrigation rates in some soil types can significantly increase bacterial leaching. While glasshouse or laboratory conditions permit the control of many environmental conditions, they may not accurately represent the field situations (Limpens et al., 2012).

Kung et al. (2000) stated the importance of the field studies as repacking of the soils in the laboratory or glasshouse experiments can exclude the natural cracks or macropores present in natural soil conditions, leading to lower preferential flow than would occur in the field. Lower contaminant leaching in glasshouse trials may result in the regulatory approval for contaminants to be applied to soil at rates that would cause unacceptable leaching in the field situation. Smith et al. (1985) stated that repacked soils generate minimum preferential flow showing no relationship between the soil properties and the bacterial transport. Hence, in the field, there may be more preferential flow under *L. perenne* than occurred in the glasshouse studies (Chapters 4 - 6). Field trials are a key part of determining the effect of plants on the fate of pathogens (Jiang, 2008; Jiang et al., 2010; Matteson et al., 2014; Zaleski et al., 2005) as field studies provide the opportunity to integrate various practical and theoretical concepts together (Kent et al., 1997).

Wang et al. (1998) reported that in natural environment, effective soil connection is lost with growing roots, and cracks in the soil can dominate the vertical bacterial flow contributing to the groundwater contamination (Artz et al., 2005; McLeod et al., 2003; Smith et al., 1985). Roots of *L. scoparium* and *K. robusta* have previously been recognised for their soil stabilisation ability (Watson & O'Loughlin, 1985) and to modify soil physiochemical properties (Hahner et al., 2014). However, their potential for increasing preferential flow in soil was shown in Chapters 4 - 6.

Following the results of Chapter 6, deficit irrigation under *L. scoparium* and *K. robusta*, can avoid bacterial leaching. However, higher bacterial leaching may occur under these natives and *L. perenne* due to macropores that were not present in the lysimeter experiments, which used repacked soil (Jiang, 2008; Kung et al., 2000; McLeod et al., 2003). Therefore, there may be less difference in leaching

between the *L. perenne* and the tree species because preferential flow, because of the native soil structure, may also occur under *L. perenne*. As trees can have higher evapotranspiration than pasture (Chandler et al., 1981; Smith et al., 1985) because much of the rainfall/irrigation is evaporated from the canopy (Bais et al., 2004; Bargaés Tobella et al., 2014) this may result in lower bacterial leaching under *L. scoparium* and *K. robusta*. Hence, it was hypothesised that natural cracks present in soil will result in less difference in bacterial preferential flow under all species (trees and *L. perenne*) and high evapotranspiration from trees will result in less bacterial movement downwards, hence more bacterial persistence and chances of die-off under trees. This study aimed to evaluate the persistence of an indicator *E. coli* ATCC13706 under two-year-old *L. scoparium* and *K. robusta* growing in the field under natural conditions to determine how field plantings may affect the pathogen numbers in the topsoil.

7.2 Methods and materials

One-year-old *L. scoparium* and *K. robusta* plants were grown in a field located in Duvauchelle region of Canterbury (44°44'59''S, 172°56'03''E) (Fig. 7.1) along with nine other native species growing in total of 27 blocks in a ca. 1000 m² of land. The soil type of the field plot was Pawson silt loam, which was used in glasshouse trials. As out of 27 plots, 12 were under the Treated Municipal Wastewater (TMW) irrigation, plants for this field trial were chosen from the control plots i.e. those not receiving any TMW. After one year of plantation from June 2016, and growth under natural rainfall, this field experiment was conducted at the end of summer. Plants with no yellow and deteriorated leaves and stems and with no visual fungal infection (mānuka blight) were considered healthy and five of these healthy plants, per species, were selected randomly in the field for trial. Any grass growing in a 60 cm radius around the main stem was cleared using a spade without disturbing the roots of either the plants or of the nearby growing grass. Steel rings (50 cm diameter) were then pushed around the selected plants in ground to a depth of nearly 2.5 cm to guide the bacterial solution into the soil. Five areas without *L. scoparium* and *K. robusta* growth, containing naturally established *L. perenne* (mix of naturally growing grasses) were selected as plant control and were treated in similar manner.



b

1C	3E	3C	1E	2C	2E	1C	3E	C
3C	2E	2C	2E	1C	3E	3C	2E	C
2C	1E	1C	3E	3C	1E	2C	1E	C

Figure 7-1 a) Experimental field site at Duvauchelle, Canterbury (44°44'59''S, 172°56'03''E) and **b)** the layout of the field site planting plan. Bold letters (1C) indicate the blocks growing with *L. scoparium* and *K. robusta* plants. C = Control plots with no treated municipal wastewater (TMW) irrigation. All other plots indicate other native vegetation, not used in this field trial.

7.2.1 Bacterial culturing and inoculation

Bacterial culture was prepared following the methods used in Section 4.2.2 in sterile Nutrient broth (NB). Inoculated broth was incubated in a shaking incubator at 180 rpm overnight (18 to 22 hours) at 30°C. This culture was transferred into 50 ml sterile falcon tubes and centrifuged at 4175 x g for ten minutes. Each bacterial pellet was washed twice with sterile Ringers solution and dilutions were prepared in Ringers to obtain a final OD of 0.4 at 600 nm corresponding to 10⁶ cfu/ml according to previous calibration curve (Appendix C.5). Five sub samples of this *E. coli* culture were plated in Nutrient agar (NA) following serial dilutions and plates were incubated at 30°C for 24 hours to confirm bacterial count. Bacterial stock was enumerated from plates with 30 - 300 colony forming units.

Before the application of *E. coli* ATCC13706, soil samples were collected from under each plant species for background *E. coli* reading using three tube most probable number (MPN) method (Sutton, 2010)

and for moisture analysis (result not shown). One hundred ml of the prepared bacterial stock with total *E. coli* count of 5.3×10^8 cfu/100 ml was added near the roots of *L. scoparium*, *K. robusta*, and *L. perenne* within the steel rings. Two hours after the bacterial stock addition, 100 ml tap water was added to the same plants (50 ml/hour) to guide the bacteria below the top soil. The irrigation rate followed for both bacterial and the tap water addition was 1 mm/4 hours or 0.25 mm/hour. After two hours of the tap water addition, steel rings were removed and plants were left undisturbed.

7.2.2 Sample collection and bacterial enumeration

Soil samples were collected from the area of the bacterial application under *L. scoparium*, *K. robusta*, and *L. perenne* from 0 - 10 cm topsoil layer using soil core samplers on day 2, 6, and 9 after bacterial application. Samples were collected in three replicates from under each plant. All three replicates were mixed together and soil samples were left overnight in the fridge at 4°C and bacterial enumeration was performed the next day (after 16 hours) using three-tube MPN method (Sutton, 2010). This method is based on the two specific bacterial enzymes i.e. β -D-galactosidase and 4-methylumbelliferone responsible for the colour change of the sample to yellow and gas and fluorescence production respectively from *E. coli* positive samples.

Fresh soil (1 g) was weighed and dissolved into 9 ml Ringers solution. This mixture was vortexed for three minutes to give a uniform soil slurry. Serial dilutions were prepared for this soil slurry up to 10^{-8} dilutions and each dilution was vortexed for three minutes. One ml of soil slurry from each dilution was added onto three Lauryl Broth (LB) tubes and tubes were sealed tightly. Any air bubbles present inside these LB tubes were removed and all tubes were incubated at 36°C for 24 hours. After 24 hours, LB tubes with air bubbles were considered positive and numbers were recorded. Negative LB tubes (with no air bubbles) were re-incubated for 24 hours at 36°C and if the air bubbles appeared after next 24 hours, these tubes were considered positive. One ml sample from each positive LB tube was immediately transferred onto the *Escherichia coli* (EC) media tubes and after removing air bubbles, all EC tubes were incubated at 44°C for 24 hours. After 24 hours, EC tubes with air bubbles were considered faecal coliforms positive and were observed under UV light (590 nm) for fluorescence (Fig. 7.2). Fluorescing EC tubes were considered *E. coli* positive and their numbers were recorded. These numbers were converted into cfu/ml by using the MPN table and making required calculations.

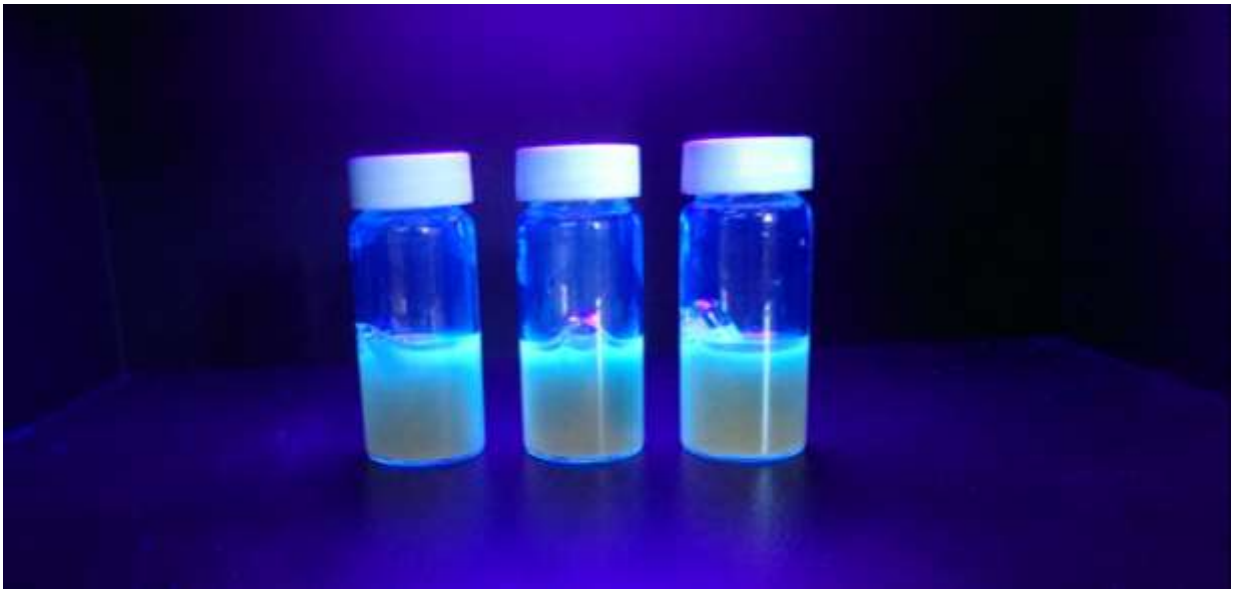


Figure 7-2 The *Escherichia coli* (EC) media tubes fluorescing under UV light (590 nm) showing EC + 4-methylumbelliferyl β -D-glucuronide (MUG) positive tubes. The tubes indicate the presence of *E. coli* as *E. coli* utilises fluorescence indicator compound MUG to give a bright blue colour under UV light.

Soil samples were also tested for pH and moisture content before and after bacterial inoculation. The climate data was obtained from National Institute of Water and Atmospheric Research (NIWA).

7.2.3 Statistical Analysis

Total *E. coli* numbers obtained in cfu/ml in one gram of fresh soil were log transformed and one way ANOVA was performed on these log numbers using Genstat 16th edition statistical software (VSN International Ltd). The significant differences were predicted based on the Fishers Unprotected LSD analysis ($p < 0.05$).

7.3 Results

Fig. 7.3 shows *E. coli* survival under *L. scoparium*, *K. robusta*, and *L. perenne* for the nine-day trial at the field site in Duvauchelle. The climate data (Fig. 7.4) shows a total rainfall rate of 52 mm in the region during the experimental period. *Leptospermum scoparium* and *K. robusta* represented similar pattern of bacterial reduction throughout the experiment with no significant difference between the two species. However, *E. coli* numbers beneath *L. perenne* were significantly higher than both native species throughout the experiment. After only 24 hours of *E. coli* application, on day 2, bacterial numbers reduced to 5.4×10^6 cfu/g of fresh soil under *L. scoparium* followed by *K. robusta* with 6.8×10^6 cfu/g of fresh soil. However, *L. perenne* presented the highest bacterial count of 7.5×10^7 cfu/g of fresh soil until sixth day. By the end of the experiment, bacterial population under *L. scoparium* and *K. robusta* reduced to 1.3×10^5 cfu/g and 1.1×10^5 cfu/g of fresh soil, respectively, while bacterial population under *L. perenne* was 6.1×10^6 cfu/g of fresh soil.

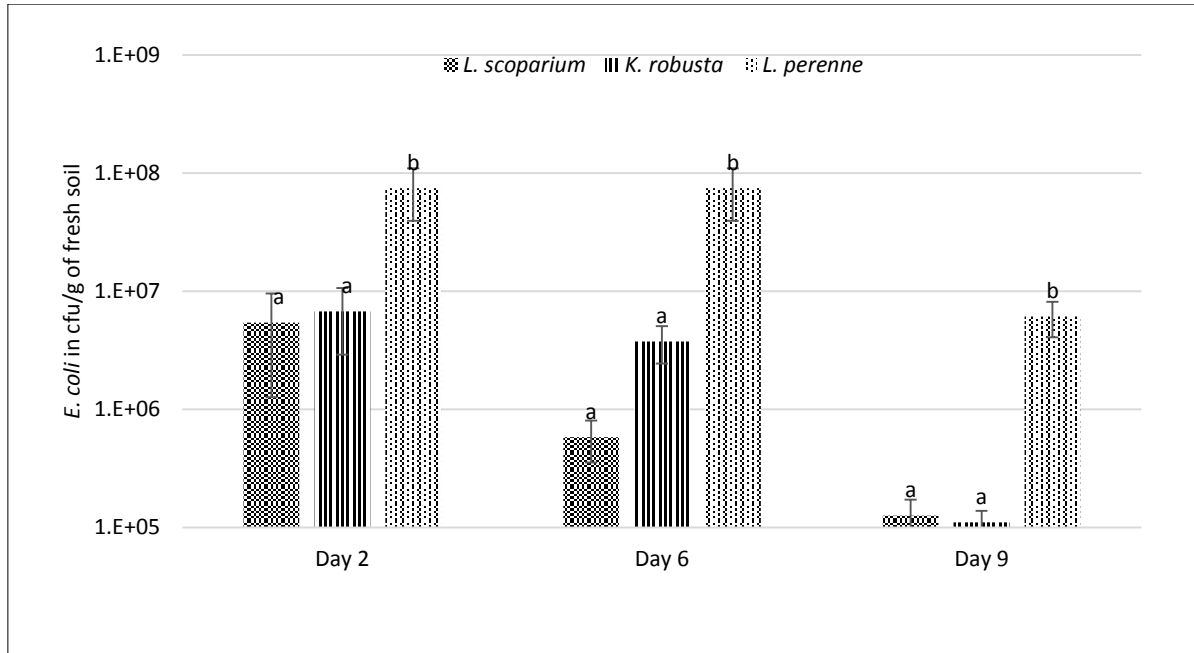


Figure 7-3 *Escherichia coli* survival in soil under *L. scoparium*, *K. robusta* and *L. perenne* in Duvauchelle field trial. Error bars represent standard error of the mean value (n = 5). Data points with the same letters each day are not significantly different based on Fishers unprotected LSD analysis (p < 0.05).

Fig. 7.4 shows the rainfall and the evapotranspiration data of the Duvauchelle field site for the experimental period. A heavy rainfall of 10 mm was observed before second soil sampling on day six and highest rainfall was observed on day eighth i.e. before the last soil sampling on day ninth, with nearly 20 mm of rainfall over 24 hours. The total cumulative rainfall and evapotranspiration for the experimental time was 52 mm and 7.5 mm, respectively. This heavy rainfall might have supported vertical bacterial movement.

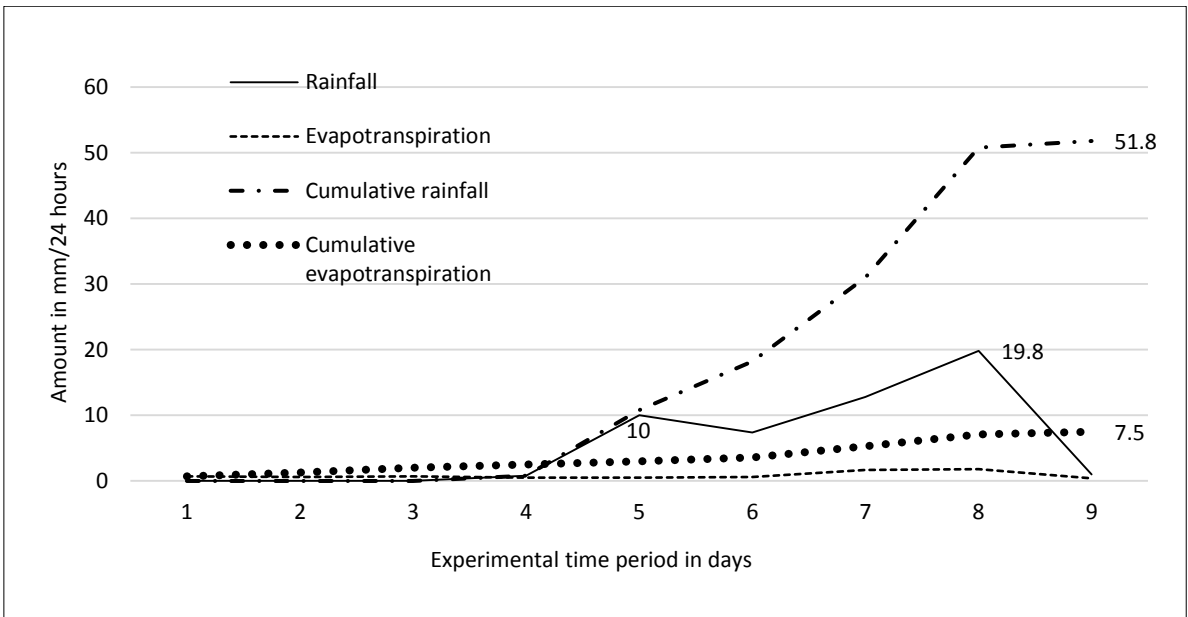


Figure 7-4 The rainfall and evapotranspiration data (in mm) of the Duvauchelle field site. The data was obtained from NIWA.

Fig. 7.5 shows the average soil pH beneath each plant species in Duvauchelle field. The pH of both *L. scoparium* and *K. robusta* showed a slight increase after bacterial addition reaching an average of 5.9 and 5.7 respectively, by the end of the experiment. *Lolium perenne* maintained a stable pH of 6.3 throughout the experiment.

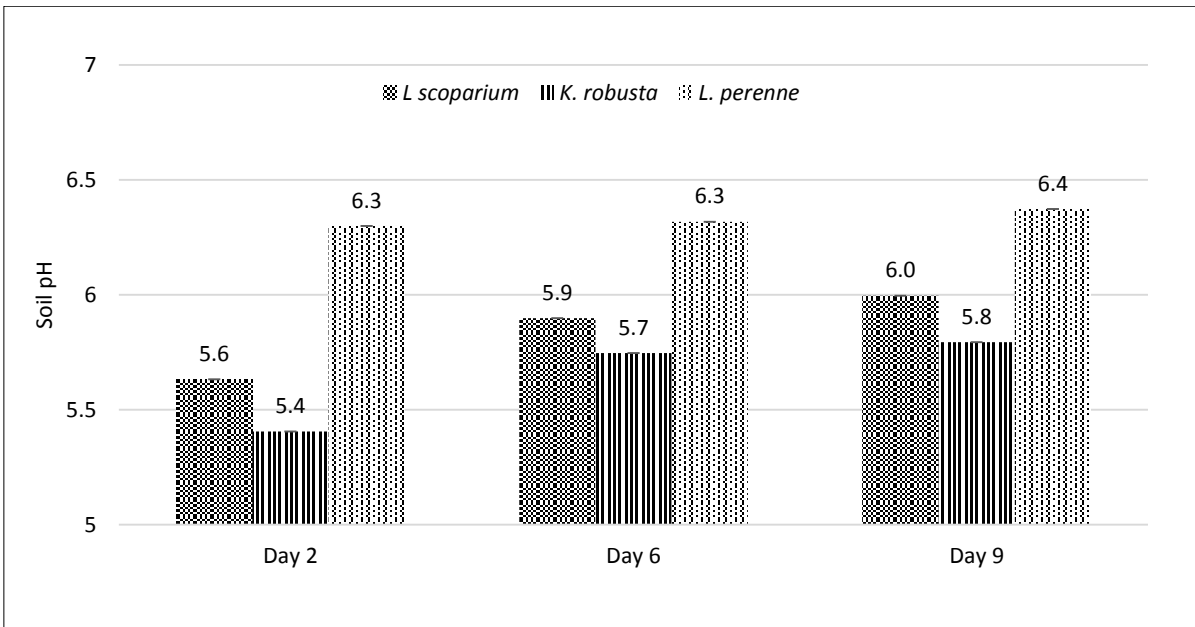


Figure 7-5 Shows changes in soil pH from beneath *L. scoparium*, *K. robusta* and *L. perenne* growing in Duvauchelle field. Error bars represent standard error of the mean value (n = 5).

7.4 Discussion

This study was conducted to identify the potential of preferential flow affecting bacterial persistence in the soil under *L. perenne*, *L. scoparium*, and *K. robusta* in the field environment. As expected, the results showed a significant decrease in bacterial numbers under both natives within nine days of application. However, *L. perenne* sustained significantly higher bacterial numbers compared to both natives throughout the experiment. The factors affecting the declining bacterial numbers are unknown as this preliminary field trial was not exceeded to analyse the rate of preferential flow, bacterial leaching or surface run off. However, the degree of preferential flow was not estimated in this study, the total heavy rainfall of about 58 mm (Fig. 7.4) during the experimental time period was postulated to be a possible reason for increased preferential flow resulting in the declining bacterial numbers under the natives (concluding from the glasshouse results). If this rainfall rate was able to assist bacterial leaching below roots perimeter or below the sampling layer i.e. 10 cm from topsoil, needs further investigations by monitoring belowground bacterial movement.

The small error bars of the *L. perenne* (Fig. 7.3) and soil pH (Fig. 7.5), are generally not anticipated in a field study due to the heterogeneity of the land (Limpens et al., 2012). This suggests that possibly all the replicates were affected in similar manner, weather it was due to preferential flow, die-off, or surface run off. Minimum variation in soil pH also indicates the possibility of less soil heterogeneity at the filed site, although it cannot be established as none of the soil physiochemical properties were analysed. The small error bars of the *E. coli* data could be due to: 1) the preferential flow or surface run off (if occurred) washing all the applied bacteria under the plants (Heeren et al., 2010; Kung et al., 2000) or 2) the limitation of the enumeration method used i.e. MPN, which relies on fluorescence development by *E. coli*. Nevertheless, this method can provide a 95% confidence interval (Fredslund et al., 2001). In future agar plating method along with the MPN, might be more useful in enumerating the exact bacterial counts from under each species (Chen et al., 2003; Ray & Speck, 1978).

A total evapotranspiration of 7.5 mm was measured during the experimental period. As stated before, high evapotranspiration resulting in low preferential flow can be expected under trees due to the canopy effect (Li et al., 2009). However, if this 7.5 mm evapotranspiration was sufficient to decrease downward bacterial movement under *L. scoparium* and *K. robusta*, needs further investigations. The stem-flow and through-fall examining studies also suggest that the canopy effect under trees protects the underground soil from raindrop effect (Levia Jr & Herwitz, 2000) and deposit nutrients from leaves and stems in rhizosphere (Johnson & Lehmann, 2006). As the plants used for this field study were only two-year-old, there is less probability of the stem-flow or through-fall to affect bacterial persistence under these plants. The collected soil samples from under any species were not tested for rhizosphere chemistry.

Similar to the glasshouse experiments (Chapter 4 - 6), high bacterial numbers under *L. perenne* indicated no bacterial die-off. Smith et al. (1985) suggested that the high probability of the preferential flow pathways in field studies could increase the preferential flow of the introduced contaminant, which cannot be acknowledged under *L. perenne* in this study.

As this field trial followed a deficit irrigation rate of 0.25 mm/hour during bacterial application, and 0 mm/24 hours rainfall occurred during the first three days, there might have been minimal preferential flow during first three days increasing the chances of bacterial interactions with the roots and its components, however this study had insufficient evidence to establish this. Aislabie et al. (2011) stated that while passing through the soil cracks following low irrigations, microbes have sufficient time to interact with the soil and its components, however high water-flux could decrease the efficient interaction. Kung et al. (2000) agreed to the same and stated that during continuous irrigation events, contaminants flow get shifted towards larger pores or cracks and hence, maintaining gaps between irrigations helps to avoid bacterial leaching and increasing interactions with the soil components. Consequently, for future studies testing the microbial persistence under *L. scoparium*, *K. robusta* and *L. perenne* during summer season, where unwanted rainfall/irrigations can be avoided will be helpful to develop the data establishing microbial persistence under *L. scoparium* and *K. robusta* (Cools et al., 2001; Ogden et al., 2001). Similar to this study, Prosser et al. (2016) in a glasshouse experiment demonstrated rapid *E. coli* die-off under *L. scoparium* and *K. robusta* compared to *L. perenne* within seven days of the bacterial application. Although, glasshouse studies allow repacking of the soil where factors such as macropores are avoided presenting effective results (Limpens et al., 2012).

None of the soil pH sampled from beneath plants used in this study was in the range to affect bacterial survival (Fig. 3.7). Tawfik et al. (2004) reported that pH range of 6.5 to 8.4 does not affect *E. coli* removal/die-off from the soil and natural bacterial die-off has minimum role in decreasing their population as compared to the other soil properties. However, the soil pH under *L. scoparium* and *K. robusta* in this study may have not affected bacterial persistence, Hahner et al. (2014) found the pH of 3.5 under mature *L. scoparium* trees, which is an acidic environment for bacteria to survive. They confirmed that the rhizosphere activities of these trees modify soil physio-chemical properties such as increasing the mobility of the trace elements. McCaulou et al. (1995) reported that bacterial attachment with soil particles increases with decreasing pH, which shows that future studies using mature *L. scoparium* trees might represent bacterial die-off in field conditions if unwanted bacterial flow (following preferential flow or surface run off) can be avoided (Hahner et al., 2014; Perry & Brennan, 1997; Porter et al., 1998; Wicaksono, 2016).

The results of this preliminary field trial showed similar results as the glasshouse experiments with a significant high bacterial persistence under *L. perenne*, which was expected to be the result of its

fibrous roots holding the bacteria and increasing the risk of bacterial surface run off following rainfall. The decreasing bacterial numbers under *L. scoparium* and *K. robusta* were expected to be the result of preferential flow or die-off, although this study had insufficient evidence to establish this and future studies are required to investigate this further. As the heavy rainfall occurred during the experimental period and rate of preferential flow was not monitored, any antimicrobial behaviour of these plants could not be established in this study. As belowground bacterial movement was not traced, future studies should investigate the same using dye tracers, fluorescence antibodies and cellular markers in summer season when unwanted rainfall/irrigations can be avoided tracing effective bacterial movement below topsoil (Artz et al., 2005; Banning et al., 2002; Vinten et al., 2002).

7.5 Conclusion

This preliminary field trial tested the persistence of *E. coli* ATCC13706 under the topsoil systems of *L. scoparium*, *K. robusta*, and *L. perenne* in field environment and showed lower bacterial numbers under *L. scoparium* and *K. robusta*, with a significant difference from *L. perenne* presenting highest bacterial numbers. Factors affecting bacterial persistence under the species i.e. preferential flow, die-off or surface run off, cannot be established, as the study was not expanded to analyse these. High run off risk under *L. perenne* was estimated. More evidences are required to confirm the results of this study in future.

Chapter 8

General discussion and conclusions

This study aimed to quantify the antimicrobial potential of the two New Zealand (NZ) native plant species, *Leptospermum scoparium*, and *Kunzea robusta* using *Escherichia coli* as an indicator species. In particular, the study sought to determine the survival and leaching patterns of *E. coli* under *L. scoparium* and *K. robusta* in contrasting soil and irrigation systems. Based on the study by (Prosser et al., 2016) it was hypothesised that the antimicrobial components released by the roots of these native species will reduce bacterial survival and therefore their movement in the soil reducing the risk of groundwater contamination.

The laboratory results using the bacterial suspensions in nutrient broth (NB) demonstrated inhibitory potential of the sterile water extracts (root and leaf) of both native species against the chosen *E. coli* strain (Chapter 3) with root extracts showing the greatest inhibition of *E. coli*, unlike the *L. perenne* extracts, which increased the bacterial growth. The extracts did not kill the *E. coli* but arrested their growth – a bacteriostatic effect. Although, extraction of the inhibitory components using sterile water suggests that plants contain antimicrobial compounds that are hydrophilic in nature, unlikely to those in essential oils i.e. leptospermone, triketones and polygodial (Killeen et al., 2015a; Park et al., 2017). Chapter 3 also showed that these inhibitory plants activities differ with plants age and geographical locations. However, the South Island plants, which were stated to be less effective than those from North Island (Douglas et al., 2004; Maddocks-Jennings et al., 2005; Porter & Wilkins, 1999) were still effective against bacterial growth inhibition, compared to *L. perenne*. The bacteriostatic nature of the extracts suggests that in contaminated soil systems, these native plants species might slow down the bacterial growth rate, although following favourable growth conditions such as the presence of nutrients, bacterial regrowth could be observed. It was postulated that the presence of sugar (not tested) in extracts TOC composition that can promote bacterial growth, may have offset the antimicrobial components present in some of the extracts, as a comparison between the TOC concentrations of *L. perenne* and the Nikau Gully sourced *L. scoparium*, indicated the presence of antimicrobial components in the plant extracts. As determining the extracts chemistry was beyond the scope of this study, future studies should consider identifying the chemistry of the plant extracts and to demonstrate how the varying concentration of different components including TOC, affects microbial growth inhibition.

The following glasshouse study (Chapter 4) showed that under high-flow conditions (14 mm/day), preferential flow was the overriding process and leaching of *E. coli* under *L. scoparium* and *K. robusta* was greater than under *L. perenne*. The results indicated that the taproot system of *L. scoparium* and

K. robusta (Watson & O'Loughlin, 1985) increases preferential flow of the surface applied bacteria and that the bacterial leaching rates differ with the soil type and properties (Bakker et al., 2014; Mendes et al., 2013). Testing the antimicrobial activity of *K. robusta* in a different soil type might present its alternative inhibitory potential as *K. robusta* has been shown to have antiviral activities (Bloor, 1992). Chapter 5 showed that under low-flow conditions (8 mm/day), bacterial leaching could be eliminated.

In Chapter 6, following deficit irrigation, a reduction in the bacterial numbers residing in the soil pore water was observed under *L. scoparium* indicating its antimicrobial effect against the DSE-*E. coli*. However, the responsible mechanism was unknown. Hence, future studies should focus on testing the rhizosphere components, root exudates, root released volatile organic compounds (VOCs) and native or associated plant microbes to identify the mechanism(s) responsible for the plant's behaviour against *E. coli*. Similar to other studies (Aislabie et al., 2011; McLeod et al., 2008) this research also determined sandy soil (Stony silt loam, Lismore soil) as of high risk for bacterial leaching and confirmed that plants activities differ with the soil type as soil type defines their macropore building potential. McLeod et al. (2008) and Tallon et al. (2007) reported that clayey soil can increase the cracks/macropores formation, especially when exposed to continuous drying and wetting series. This indicates that the increased root induced preferential flow under *L. scoparium* and *K. robusta* can further increase microbial contaminants leaching risk in clayey soil. Previous study by Prosser et al. (2016) used a Templeton silt loam in their glasshouse experiment and demonstrated rapid bacterial decline under the soil systems of *L. scoparium* and *K. robusta*. Future studies should also focus on investigating how the antimicrobial mechanisms of plants varies with the soil type, which will be a great addition to the existing knowledge of *L. scoparium* and *K. robusta* antimicrobial activities against indicator bacterium *E. coli*.

The preliminary field study (Chapter 7), similar to the glasshouse studies, demonstrated decreasing bacterial numbers under *L. scoparium* and *K. robusta*, with a significant difference from *L. perenne*, however the responsible factor i.e. preferential flow or die-off with the antimicrobial effect from the natives rhizosphere components, could not be established in this case. Following the climate data and concluding from the results of the previous experiments of this research, preferential flow was anticipated as the responsible factor for decreasing bacterial numbers under the natives. For futures studies, use of dye tracers, fluorescence antibodies, and cellular markers in lysimeter or field trials is recommended to follow the deep surface movement of the inoculated *E. coli* (Artz et al., 2005; Banning et al., 2002; Vinten et al., 2002).

The overall results of this research indicate that both *L. scoparium* and *K. robusta* have antimicrobial action, however the taproot system of these natives can exacerbate preferential flow of the surface applied bacteria following high irrigation rates of 14 mm/day (the highest irrigation rate used in this study).

Under high irrigation or precipitation rates, where preferential flow processes will dominate under *L. scoparium* and *K. robusta*, these natives may increase infiltration and therefore reduce run off of bacteria into surface waters. (Fig. 4.6.c). Similar to Hahner et al. (2014) this study also favours strategic planting of *L. scoparium* and *K. robusta* around dairy farms. Some possible scenarios outlining the strategic planting are presented in Fig. 8.1. a and b, and Fig. 8.2. The South Island has ~52,000 km² of flat land with 45% of it at a high risk of preferential flow as compared to the North Island with nearly 50% of soil with a high risk of preferential flow with ~40,500 km² of flat to rolling lands, with an average slope of 15 degrees (McLeod et al., 2008).

Fig. 8.1.a and b show a scenario with and without the antimicrobial trees planted in riparian regions. With natives growing as buffer zones in between the dairy farms and the fresh water resources, time for the applied microbial contaminants to reach water sources increases due to increased preferential flow under the natives, unlikely with *L. perenne* (Fig. 8.1.b) where time for microbial contaminants released from dairy farms to reach the water resources is minimum. Increased preferential flow under the natives also provides increased chances of microbial die-off due to increased interactions with the plants rhizosphere components. Preferential flow can still be a risk with shallow water aquifers as microbes that reach the bottom of the roots may still be mobile. However, as the roots of *L. scoparium* and *K. robusta* generally grow between 0.5 – 3 m deep (Watson & O'Loughlin, 1985), and the average depth of groundwater in Canterbury is over 6 m (Canterbury Open Data, 2017), microbes have to pass through a lot of soil to reach groundwater. This further increases the chances of their filtration in soil before reaching the water table (Abu-Ashour et al., 1994; Jamieson et al., 2002; Li et al., 2009; Negassa et al., 2015). If aquifer contamination is not a concern (e.g. in a coastal environment, or if the aquifer is deep) then *L. scoparium* and *K. robusta* may permit increased irrigation rates without run off.

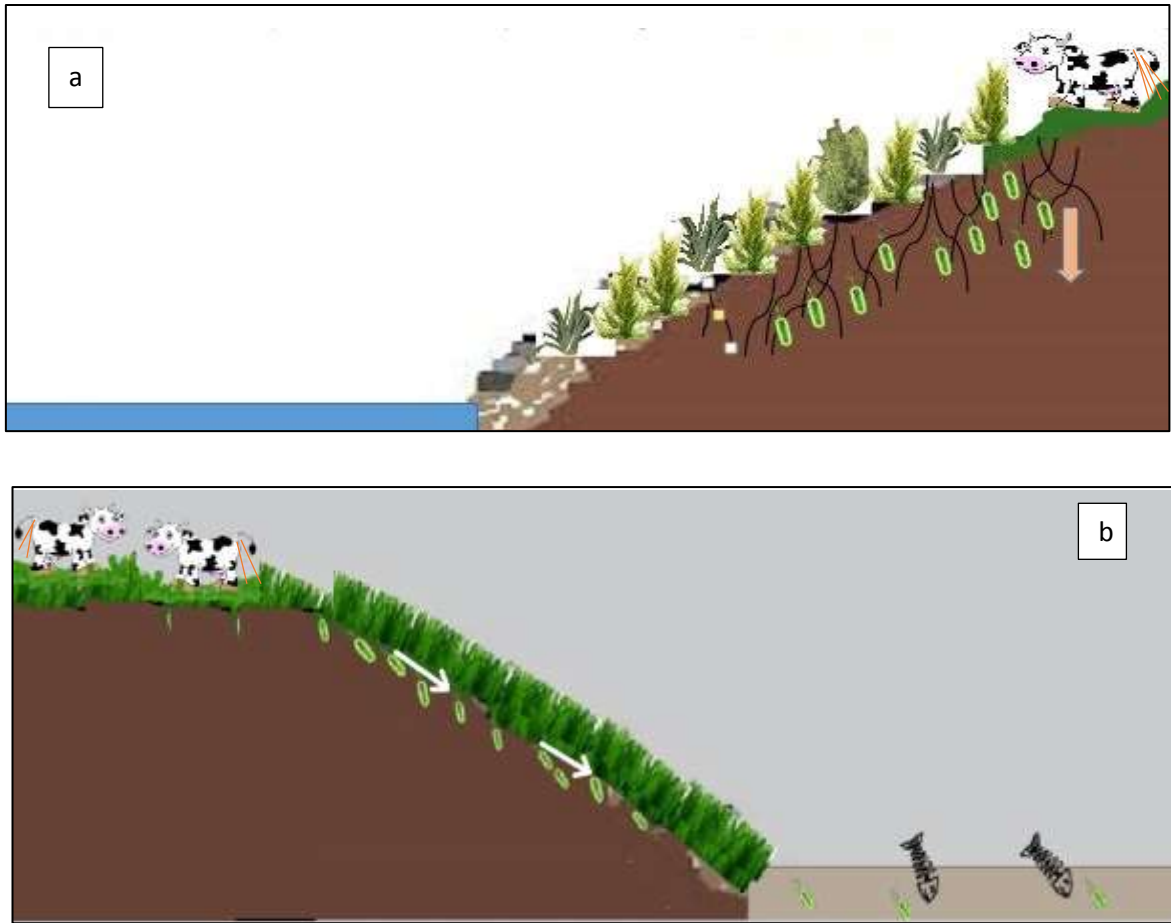


Figure 8-1 Demonstrates the movement of the microbial contaminants released from the dairy farms near riparian region **a)** with *L. scoparium* and *K. robusta* growing as buffer zones between dairy farm and water and **b)** with *L. perenne* where microbial contaminants can follow surface run off with minimum inhibition.

Another scenario is described in Fig. 8.2 where *L. scoparium* and *K. robusta* are presented as the DSE disposal blocks near milking yards in dairy farms. This strategy to use natives as the disposal blocks not only provides an easy effluent disposal option to the farmers but also reutilises the nutrient rich effluent, which is otherwise disposed as waste in farms, creating further problems for water contamination. A recent study by (Gutiérrez-Ginés et al., 2017) who used biosolids amendments to grow NZ natives, suggested that the natives present a positive growth response with the nutrient rich amendments. This suggests that *L. scoparium* and *K. robusta* grown as the DSE disposal blocks around farms also provide diversification to farmers and plantation costs can be offset by the generation of high quality NZ products i.e. essential oils and honey (Allen et al., 1991; Badet & Quero, 2011; Chen et al., 2016).

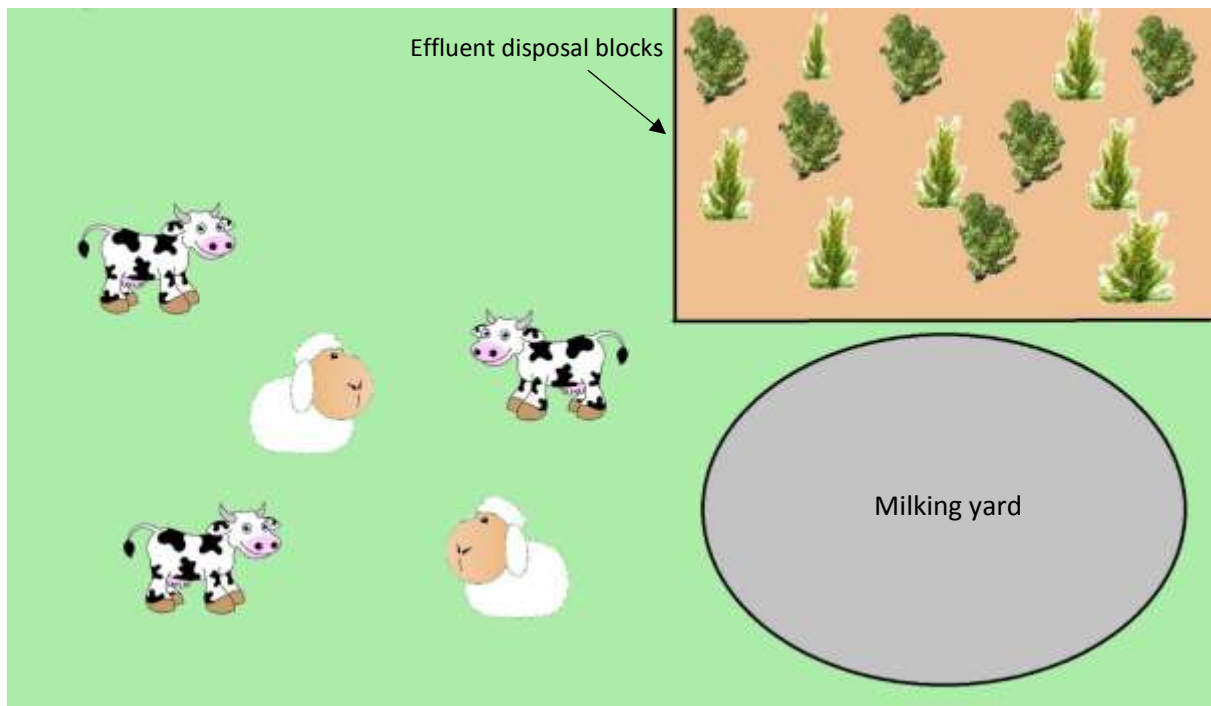


Figure 8-2 A scenario of a strategic plantation of *L. scoparium* and *K. robusta* for an effluent disposal blocks in dairy farms.

As food and water contamination with faecal pathogens is a global environmental issue (Jones, 1999; Marasini et al., 2015) and as antimicrobial plants are found worldwide, there is potential to develop this research overseas. Candidate species with antimicrobial properties include *Azadirachta indica* (Del Serrone et al., 2015), *Phyllanthus emblica* (Jain et al., 2015), *Cinnamomum verum* (Zhang et al., 2016), *Heteromorpha trifoliata* and *Maesa lanceolata* (Adamu et al., 2013). As the interaction between plants and pathogens is dependent on both the plant and pathogen species (Kai et al., 2016; Mendes et al., 2013), one plant species may not be sufficient to act against the variety of pathogens found in biological wastes (Blom et al., 2011). Studies monitoring bacterial leaching in a mixed native species forest (polyculture) where different pathogenic species can be targeted by different plants species (Appendix B) is recommended (Brockerhoff et al., 2008; Kelty, 2006; Lamb, 1998). Plant monocultures can lead unwanted dieback through the advent of new pests such as root herbivores or fungal diseases such as *Puccinia psidii* (myrtle rust) (Carnegie et al., 2016), hence mixed communities should be preferred (Carnegie et al., 2016; Cook-Patton & Bauerle, 2012; Hunter & Aarssen, 1988; MONTAGNINI et al., 1995). The antimicrobial properties of most of the NZ natives were recognised by Māori (indigenous people of New Zealand), for their medicinal and antimicrobial properties (Brooker et al., 1981; Earl et al., 2010; Porter et al., 1998). Hence, testing the antimicrobial potential of these natives against DSE pathogens might present their remediation potential for the DSE contaminated soil systems (Appendix B). Although essential oil production by the roots of either *L. scoparium* or *K. robusta* have not been tested yet, a recent study by Skala et al. (2016) have extracted the essential oil from the hairy roots of *Rhaponticum carthamoides* following hydro-distillation method and found high

oil activity against *Enterococcus faecalis* and *Pseudomonas aeruginosa*. They stated that different plant parts can synthesise essential oils with varying component concentrations, such as of sesquiterpenes, and hence, the pharmacological activities can differ based on the source (leaves or roots) of the oil. For future studies, it will be interesting to identify the essential oil production by *L. scoparium* and *K. robusta* roots, which might present a potential mechanism for the bacterial inhibition in their rhizosphere with the associated volatiles components. As the plants used in this study were only two years old, it is possible that the active inhibitory components synthesis was lacking in these plants and future studies with mature *L. scoparium* and *K. robusta* may show different results (Chen et al., 2016; Wicaksono et al., 2016).

This thesis evidence the antimicrobial potential of *L. scoparium* and *K. robusta* and advocates the strategic establishment of these natives around dairy farms to reduce the surface run off risk of the applied bacterial contaminants, although the antibacterial behaviour of the plants against *E. coli*, needs further investigations. In the progressing society, where people are being more aware and conscious about the waste management strategies and the food and water contaminations, finding a solution with the native plant species might present an effective way of reducing contamination risk with additional benefits of native species regeneration and economic benefits for the country. The results of this study will be helpful to foster the hopeful prospect from the natives.

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Appendix A

Effect of volatile organic compounds released by *Leptospermum scoparium* and *Kunzea robusta* on *Escherichia coli*.

A.1 Aim

The aim of this study was to test the antibacterial activities of the VOCs released by the *L. scoparium* and *K. robusta* plants against a non-pathogenic *E. coli* strain ATCC13706 and to observe any changes in the growth pattern or in the released volatiles composition of either the plant or the bacteria, as the result of their interactions.

A.2 Methods and materials

Seeds of *L. scoparium* and *K. robusta* originally sourced from the Port Hills, NZ (43°36'38''S 172°36'47''E) were obtained from the Department of Conservation nursery, Motukarara, Christchurch (43°43'46''S, 172°34'42''E). Murashige and Skoog's (MS) media was kindly provided by Associate Professor David Leung, Canterbury University.

A.2.1 Surface sterilisation and germination of seeds

Seed sterilisation and germination was performed following the method described in Section 3.2.4.2, in Chapter 3. All seedlings were maintained on agar plates for one month and then transferred to split Petri plates (Petri plates divided into two compartments). Split Petri plates were filled with 1/10 strength MS agar on one side and Luria-Bertani (LB) agar on the other side (Fig. A.1). After assuring sterility i.e. no fungal growth on the germinated seedlings in agar plates, five plantlets per species were transferred on MS agar on split Petri plates and plates were sealed with parafilm®. All plates were maintained at room temperature at an angle of 45°, in natural light near a window (not facing direct sunlight). Plants were grown on split Petri plates for 25 days before bacterial inoculation on LB agar. After 25 days of plants growth in MS media, split Petri dishes with all five plants showing no microbial growth near roots or shoots and no dried leaves or roots, were considered healthy and were chosen for the experiment. Plates were divided into treatments and controls in single replicates (plants with and without bacteria) and the experiment was repeated two times. Split Petri dishes were also maintained for unplanted controls (only *E. coli*). Plates with sterile media only were used as blanks.

A.2.2 Bacterial inoculation

Fresh *E. coli* was cultured on Nutrient Agar (NA), 24 hours prior to inoculation on split Petri plates. One fresh bacterial colony was dissolved into 3 ml Ringers solution and vortexed to make a uniform bacterial slurry. Serial dilutions of up to 10^{-3} were prepared from this bacterial slurry and the last dilution (10^{-3}) was used to inoculate LB agar on split Petri plates. Five separate 10 μ l drops of bacterial slurry were carefully inoculated on LB agar. Plates were covered with lids and were allowed to dry for forty minutes under sterile air-flow in a laminar flow hood. Bacterial controls were inoculated in the same way and all plates were sealed and maintained for three more days at room temperature (25°C) to allow volatile exchange between plants and bacteria using the headspace.

A.2.3 Sample collection and Gas Chromatography (GC) analysis

After three days interactions between plants and bacteria, any observed changes such as contamination or changed growth, in either plants or bacteria were noted and all plates were photographed to record the observations. The respective agar sections from under plants and bacteria were cut using a sterile blade with nearly 2 ml of total agar attached to the plant or bacteria. These agar sections with attached plant or bacteria were sealed into 20 ml amber Solid Phase Micro-extraction (SPME) vials ready for Gas chromatography–mass spectrometry (GC-MS) Volatile organic compound (VOC) analysis. These vials were further maintained at room temperature for 24 hours before GC analysis at the Analytical Services Unit of Lincoln University.

Samples were analysed using methods drawn from the work of Stoppacher et al. (2010) and Nieto-Jacobo et al. (2017) on VOCs from *Trichoderma* fungi. A Shimadzu GC-MS QP2010 Ultra (Shimadzu, Japan) gas chromatograph mass spectrometer fitted with a Restek Rtx-5ms (5% diphenyl - 95% dimethyl polysiloxane) fused silica capillary column (30 m x 0.25 mm i.d. x 0.25 μ m, Bellefonte, PA, USA) was used to analyse the VOCs sampled. A CTC-Combi PAL auto sampler (PAL LHX-xt) fitted with a 23 gauge 65 μ m PDMS/DVB-SPME fibre (Supelco, Sigma-Aldrich) was used to sample the headspace of 20 ml amber vials containing 2 ml of agar which had been incubated at 25°C for 24 hours. Extraction of the VOCs involved exposing the SPME fibre to the headspace of the 20 ml vials for 30 minutes at a temperature of 35°C, an adaption of that described by Tait et al. (2014). The SPME fibre was then desorbed in the GC injection port, operating in splitless mode at a temperature of 250°C (post injection the split vent was opened at a ratio of 30:1). The column oven was initially held at 40°C for 2 minutes, before being heated to 200°C at 10°C min⁻¹ and then further ramped to 260°C at 25°C min⁻¹ where it was held at this temperature for 5 minutes. Helium was used as the carrier gas with a constant linear velocity set at 36.1 cm/sec (1.0 ml/min column flow). The mass spectrometer (MS) was operated in electron impact ionization mode with 70 eV and mass range of 33 to 500 m/z. The temperature of the capillary interface was 260°C, with the source temperature set at 230°C.

Initial identification of detected peaks was made by matching their mass spectra with the spectra of reference compounds found in the databases NIST EPA/NIH Mass Spectral Library database (National Institute of Standards and Technology, NIST11) and Wiley Registry of Mass Spectral Data 10th edition (John Wiley & Sons, Hoboken, New Jersey). An alkane mixture (C8-C20, Sigma-Aldrich) of 20 µl volume was run under the same GC conditions as described above and this was used in conjunction with published retention index information for 5% diphenyl - 95% dimethyl polysiloxane capillary columns to confirm the initial mass spectral identifications.

A.3 Results

Fig. A.1 shows the results obtained before and after the interaction between the VOCs of *E. coli* and plants. Although the colonies in both split Petri plates (A.1.b) were uncountable due to high cell density, difference in the cell density was visible with and without the presence of *L. scoparium* volatiles. *E. coli* cells were dense in the absence of *L. scoparium* VOCs however, an inhibition of *E. coli* growth was observed when *L. scoparium* was present. The plates showing interaction of *K. robusta* VOCs with *E. coli* are presented in Fig. A.1.c. The *E. coli* colonies growing on the LB agar were observed to spread, indicating no inhibition by *K. robusta* VOCs.



Figure: A.1. Shows **A:** split Petri plates set up (blank and plant control). **B:** *E. coli* growth with and without the *L. scoparium* volatiles. **C:** *E. coli* growth with and without *K. robusta* volatiles

The VOC components released by *E. coli*, *L. scoparium* and *K. robusta*, before and after the interactions, is presented in Fig. A.2 to A.4. Fig. A.2 shows the peak area ratios (in percentage) of some *E. coli* VOCs released independently and their interaction results with *K. robusta* and *L. scoparium* volatiles. Indole, a component released in the highest concentration by *E. coli* in the unplanted Petri plates, was

completely inhibited during the presence of *L. scoparium* along with 2, 4-dimethyl-1-heptene. However, *K. robusta* volatiles partially inhibited the bacterial volatiles with no effect on indole and a slight inhibition of 2, 4-dimethyl-1-heptene. During interaction of *E. coli* with *L. scoparium*, new components including β -elemene and benzyl alcohol appeared in the bacterial volatile blend.

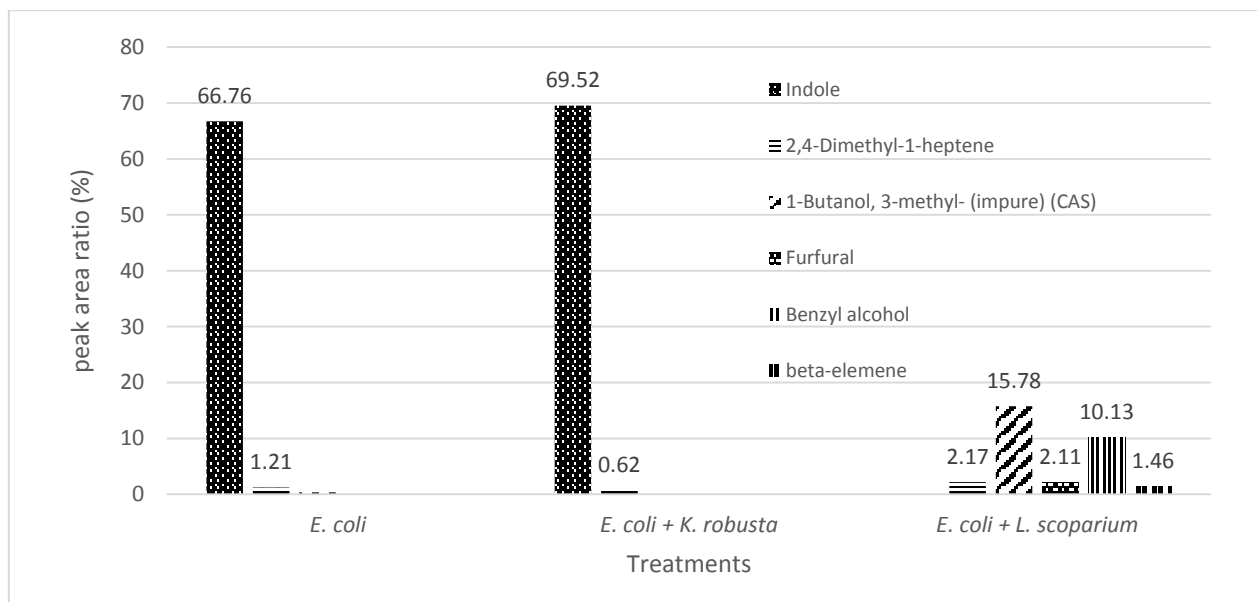


Figure. A.2. Shows *E. coli* VOCs and the effect of their interactions with *K. robusta* and *L. scoparium* volatiles. Each column represents the peak area ratio percentage of each component.

Fig. A.3 shows the VOCs released by *L. scoparium* with and without the presence of *E. coli*. Benzoic acid (BA) which was the highest released *L. scoparium* component, was inhibited to less than a third of its level in the *L. scoparium* control in the presence of *E. coli*. The levels of many other components such as ethyl tiglate, an important insect attractant were also inhibited in the presence of *E. coli*. Other than inhibiting most VOCs in the interaction with *E. coli*, higher levels of other VOCs were released by *L. scoparium* in the presence of *E. coli*, including 1-butanol-3-methyl, an insect repellent and 1-butanol, 3-methyl benzoate, a plant protectant component.

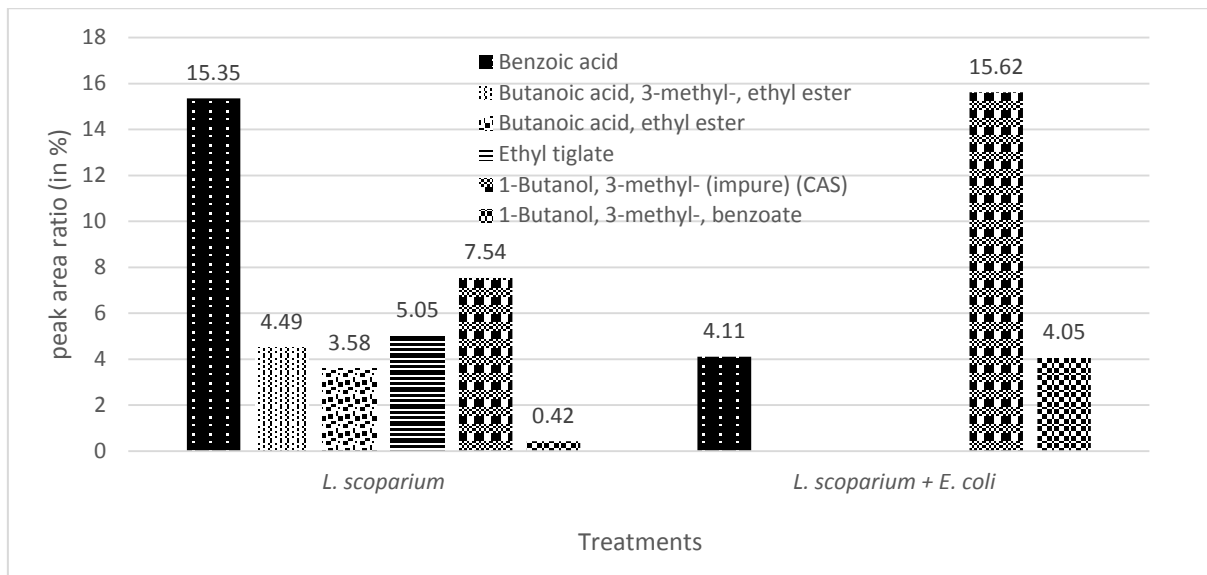


Figure. A.3. Shows *L. scoparium* VOCs and the effect of plant interaction with *E. coli* volatiles. Each column represents the peak area ratio percentage of each component.

Fig. A.4 shows *K. robusta* volatiles released with and without the *E. coli* presence. Similar to *L. scoparium*, BA which was released in highest concentration by *K. robusta* independently, was inhibited nearly eight times in the presence of *E. coli*. Other components i.e. ethyl tiglate (not shown), an insect attractant, and a BA derivative butanoic acid, 3-methyl-, ethyl ester were also inhibited in the presence of *E. coli*. A common essential oil monoterpene i.e. 2-pinene, which works as an insect repellent, was released at nearly six times higher concentrations by *K. robusta* during exposure to *E. coli*, along with 1-butanol, 3-methyl-, benzoate released at three times higher concentration. Indole, a new component, also appeared in the *K. robusta* volatile blend during the presence of *E. coli*.

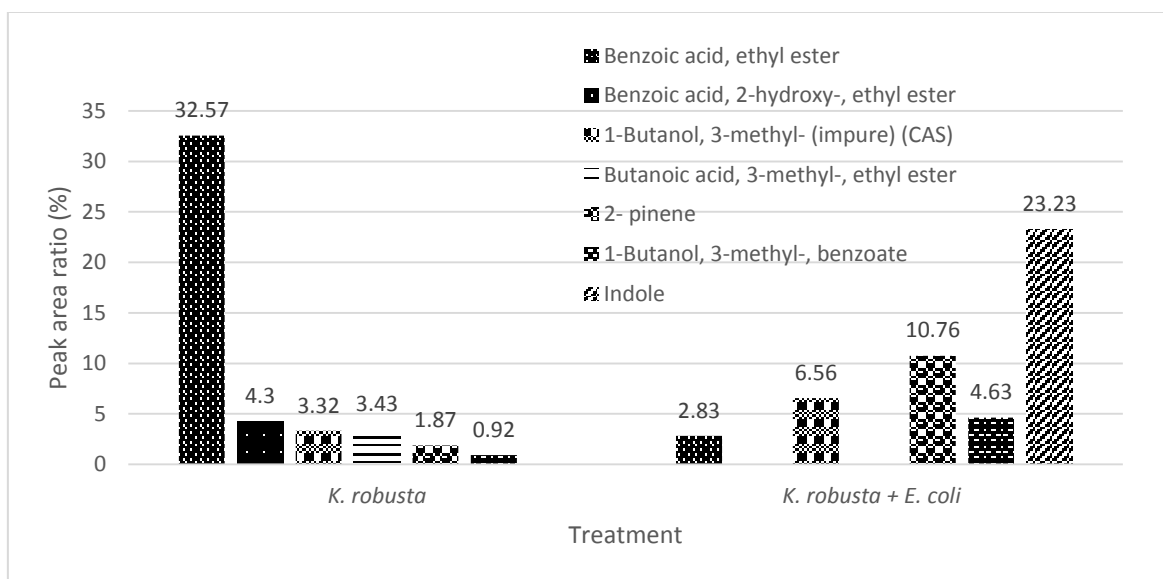


Figure. A.4. Shows *K. robusta* VOCs and the effect of plant interaction with *E. coli* volatiles. Each column represents the peak area ratio percentage of each component.

A.4 Concluding points

The interaction between *E. coli* and *L. scoparium* VOCs resulted in the inhibition of bacterial indole production, an important component for bacterial physiology and life processes (Lee et al., 2009; Teale et al., 2006). However, *K. robusta* volatiles had no effect on bacterial indole production, although it partially inhibited some other bacterial VOCs but overall showing lower inhibitory activity than *L. scoparium*. The regular VOCs blend of both plants was also affected during the interaction with *E. coli* with maximum inhibition of the Benzoic acid production. Both plants and bacteria have shown a change in their regular VOCs secretions and also the production of some new volatile components during the interaction (Broek et al., 2005). Bacterial regrowth after exposure to the plants VOCs was not tested hence, the type of inhibitory effect (bacteriostatic or bacteriocidal) cannot be stated although inhibition of bacterial Indole when exposed to the plants confirmed the inhibitory activities of the VOCs released by both *L. scoparium* and *K. robusta* against *E. coli* which can be a possible mechanism for bacterial growth inhibition by these native species, which needs further investigations.

Appendix B

Testing the effective antibacterial activities of other NZ native plants used in Māori medicines.

B.1 Aim

This aim of this study was to investigate the potential of a variety of NZ native plants extracts, traditionally used in Māori medicines, against a range of human and animal pathogenic bacterial species commonly found in animal wastes. It was hypothesised that similar to *L. scoparium* and *K. robusta*, the sterile water extracts of other native NZ plants species will inhibit pathogenic growth.

B.2 Methods

B.2.1 Selection of plants species and extracts preparation

A total of fifteen NZ native plants were selected (Table B.1) based on their traditional use in Māori medicines (Bloor, 1995; Kellam et al., 1992; Wharemate, 2003) and were sourced from both the South and North Island. Two to three-year-old plants were sourced from Taupo nursery (38°40'05''S, 176°06'18''E) and Forest flora nursery (37°39'46''S, 175°08'15''E) North Island, and from the Department of Conservation nursery, Motukarara, South Island (43°43'47''S, 172°34'45''E). Only leaf extracts of all plants were prepared following the method used in Chapter 3 (Section 3.3.2). Extracts of *L. perenne* were used as negative control, since they do not have any known antimicrobial properties (Prosser, 2011). All extracts were stored at -20°C until used. A subsample of the extracts selected after biolux test below was used for the total organic carbon (TOC) and pH analysis. However, results are only presented for the extracts showing the growth inhibition of tested bacterial species. Streptomycin was used as a positive control with *E. coli* only.

Table B.1. Shows the New Zealand native plant species used in the experiment. Names are checked in March 2018 from (NZ Flora, 2018).

Species	Family	Vernacular name
<i>Leptospermum scoparium</i> J.R.Forst. & G.Forst.	Myrtaceae	Mānuka, tea tree
<i>Leptospermum scoparium</i>	Myrtaceae	Swamp mānuka
<i>Kunzea ericoides</i> Rchb.	Myrtaceae	kānuka
<i>Myoporum laetum</i> G. Forst.	Scrophulariaceae	Ngaio
<i>Phormium tenax</i> J.R. Forst. & G. Forst	Hemerocallidaceae	Flax
<i>Pseudowintera colorata</i>	Winteraceae	Horopito
<i>Macropiper excelsum</i>	Piperaceae	Kawakawa
<i>Metrosideros robusta</i>	Myrtaceae	Rakapika
<i>Veronica stricta</i>	Plantaginaceae	Koromiko
<i>Aciphylla aurea</i>	Umbelliferae	Spaniard
<i>Aciphylla subflabellata</i>	Umbelliferae	Speargrass
<i>Olearia paniculata</i> (J.R. Forst. & G. Forst.) Druce	Compositae	Akiraho, golden akeake

B.2.2 The Biolux screening of the extracts

All sixteen plants extracts were first screened to identify their antibacterial activities against *E. coli* lux biosensor in a rapid acute toxicity test. This screening method was adapted from Horswell and Aislabie (2006) and was applied as described by Prosser (2011). The *E. coli* biosensor used produces high luminescence when immersed in favourable conditions and vice versa. Biosensor was exposed to plant extracts diluted to 10% extract concentration in sterile water. Each 1 ml test tube was composed of 100 µl of extract at 10% dilution, 800 µl sterile water, and 100 µl biolux *E. coli* suspension. Negative controls substituted 100 µl of extract for 100 µl sterile water. The fluorescence was tested after 15 and 30 minutes of biolux exposure to 10% extracts and sterile water. Luminescence intensity was measured using a Luminoskan TLequipment. This test was repeated twice. A quality control test was also performed twice with the commercial antiseptic product called TCP (Omega Pharma, United Kingdom).

Inhibition of fluorescence was calculated comparing the fluorescence of the tubes with plant extracts with the fluorescence of sterile water. Fluorescence of *L. perenne* (negative plant control) was between 80% - 90% of the sterile water fluorescence. For this reason, it was considered 40% fluorescence

compared with sterile water as a limit for selecting potential extracts for the following tests against the pathogenic bacteria. A subsample of the extracts selected for the following tests was analysed for total organic carbon (TOC).

B.2.3 Testing extracts pH interference with fluorescence inhibition

A pH lower than 5 affects the results of the biolux test (Speir et al., 2004). Hence, the pH of the extracts producing luminescence lower than 40% compared with sterile water were measured. If the extracts pH was lower than 5, it was adjusted to pH 5 or higher using NaOH, and the biolux test was repeated. Swamp *L. scoparium* was included as an internal control (with no pH adjustment) and no difference in the fluorescence inhibition was observed.

As the antimicrobial activities of the North Island *L. scoparium* and *K. robusta* were already established by Prosser (2011) these two plants were not tested any further.

B.2.4 Selection of bacterial species

The microorganisms were chosen based on their known presence in DSE (Fyfe et al., 2016; Hashsham et al., 2004) and resistance against many clinically important antibiotics: a non-pathogenic strain of *Escherichia coli* ATCC13706, *Staphylococcus aureus* 87, *Pseudomonas aeruginosa* 3007, *Burkholderia cepacia* 2768 and *Campylobacter jejuni* 2607. Each bacterial species were obtained from The New Zealand Reference Culture Collection, at The Institute of Environmental Science and Research Ltd. (ESR), Wellington. Other than *E. coli*, all pathogens were tested in the PC2 laboratory facilities at ESR, Wellington.

B.2.5 Bacterial Culturing

Escherichia coli was cultured following the method described in Section 3.3 of Chapter 3. *Escherichia coli* was resuscitated from glycerol stock on nutrient agar (NA) plates at 30°C for 24 hours. Two fresh *E. coli* colonies were then transferred onto 100 ml sterile Nutrient broth (NB) and the media was incubated in a shaking incubator at 30°C at 120 rpm (Section 3.3).

Staphylococcus aureus, *P. aeruginosa* and *B. cepacia* were resuscitated from the glycerol stock into Tryptic soy agar (Fort Richard) and then into Tryptic Soy Broth (Fort Richard). The inoculated broth vials were incubated in a shaking incubator at 37°C at 180 rpm. *Campylobacter jejuni* was maintained in Mueller - Hinton Broth (Oxoid CM0405) at 37°C at 180 rpm, inside an air tight container with Oxoid™ CampyGen™ sachet to create microaerobic conditions.

Following a few preliminary trials, the optimum dilution of the bacterial seed stock to use in the assay was pre-determined by assessing bacterial growth curves at various dilutions. The dilution of the

bacterial stock selected was chosen such that the full growth curve would be visible over the incubation period. The chosen dilution for all bacterial species was 10^{-2} .

B.2.6 Testing *E. coli* inhibition

The test with *E. coli* bacteria was carried out using the method outlined in Chapter 3, reading results a) after 24 hours of 10% extracts exposure (Fig. B.1) and b) after 2, 4, 6, 8, 10, 22 and 24 hours of 10% extracts exposure (Fig. B.2). The inhibition test was repeated twice in triplicates. Optical density (OD) of each bacterial suspension was measured at 600 nm using the Genesys 10S UV-Vis spectrophotometer. Average OD was later converted into cfu/ml using *E. coli* regression curve formula (Appendix C.5).

B.2.7 Testing the inhibition of *S. aureus*, *P. aeruginosa* and *B. cepacia*

Staphylococcus aureus, *P. aeruginosa* and *B. cepacia* were tested using 96 well microplate bioassay adapted from Prosser (2011). All bacterial cultures were prepared and incubated at 37°C to achieve optimum growth just before plating (tested in preliminary tests). Serial dilutions of each plant extract were prepared in sterile water for obtaining final concentration in the test wells of 50%, 25%, 12.5%, 6.25%, 3.12%, 1.56%, 0.39%, 0.10% and 0% extract concentrations. Each test well contained 100 µl of plant extract at decreasing concentration, and 100 µl of bacterial suspension, giving a total volume of 200 µl in each well. Prepared plates (containing extracts and bacteria) were then incubated in the automated absorbance plate reader (FLUOstar Optima) 37°C for 24 to 26 hours, reading OD at 595 nm every 30 minutes, with a previous 15 s orbital shaking. Tests were repeated in four replicates and two blanks containing no bacteria and an average of tests were used as the final OD. Measurement was stopped when the growth curve reached the stationary phase. The final OD results were calculated after subtracting the blanks OD value from the tests.

Plant extracts of *M. robusta*, swamp *L. scoparium* and *P. colorata* generated turbidity when mixed with the bacterial cultures in the 96 well plates and hence, OD couldn't be measured. For these extracts, maximum extract concentration was reduced to 20% (160 µl bacterial suspension + 40 µl extracts) to avoid the interference with OD reading. Serial dilutions in these extracts generated final concentrations in test wells of 20%, 10%, 5%, 2.5%, 1.25%, 0.625%, 0.3% and 0% in each 200 µl well.

B.2.8 Testing the inhibition of *C. jejuni*

Campylobacter jejuni was only tested against the extracts which produced inhibition with other bacterial species i.e. *M. robusta*, *P. colorata*, and swamp *L. scoparium*. Maximum concentration of plant extracts in test tube was 20%, as outlined in Section B.2.7.

Due to the microaerobic growth conditions required, *C. jejuni* could not be incubated in the FLUOstar Optima equipment. Four ml of test mixture was prepared in 15 ml centrifuge tubes by mixing 1 ml of diluted plant extract with 3 ml of bacterial suspension. The test mixture was then divided into Eppendorf tubes (700 µl each) in four replicates including two blanks (no bacteria). Eppendorf tubes were placed in an air tight container with the Oxoid™ CampyGen™ sachets, and were incubated for 24 and 48 hours in a shaking incubator at 37°C at 180 rpm. After 24 and 48 hours, the Eppendorf tubes were centrifuged at 3219 x *g* for 20 minutes. Supernatant was removed and pellet was re-suspended in 700 µl Ringers solution and vortexed. A 200 µl aliquot of the bacterial suspension was placed in 96-well plate and the OD was read at 595 nm in the FLUOstar Optima equipment.

Any of the selected bacteria showing an abnormally low growth curves in a particular extract concentration were randomly tested for their regrowth in agar plates at 37°C for 24 hours. Any regrowth of *C. jejuni* was tested in Sheep Blood Agar plates (Fort Richard) for 48 hours at 37°C in an air tight container with the Oxoid™ CampyGen™ sachets. Colony Forming Units (cfu) were counted in the dilutions where the cfu were >1 in the three replicates and less than 30 (results not shown).

B.2.9 Statistics

B.2.9.1. *Escherichia coli*

Microsoft excel (Version 2013) was used to calculate cfu/ml using the regression formula obtained from the *E. coli* linear regression curve of OD versus cfu (Appendix C.5). When required, data were transformed into log₁₀ and then analysed using one-way analysis of variance (ANOVA) followed by comparison of individual values of the treatments using Fishers Unprotected LSD Analysis. Bacterial count below detection limit i.e. 0 cfu/ml, was considered 1 cfu/ml when transforming cfu values to the log₁₀. All data analysis was carried out in GenStat 16th edition (VSN International Ltd.)

B.2.9.2. Biolux *E. coli*

The results of Lux bioassay are expressed as percentage of luminescence of an extract compared with water used as control.

B.2.9.3. Microplate assay

Data were analysed using Microsoft Excel (Version 2013). The results of the microplate bioassay are presented as the average OD of the four replicates per dilution of extract. The final test ODs were presented after subtracting the OD of the blanks for each extract dilution to the OD of each of the four replicates of bacterial suspension exposed to the extract.

B.3 Results

B.3.1. The biolux screening of the extracts

The inhibition of Biolux luminescence by plants extracts (compared to sterile water) is presented in Fig. B.1. Before pH adjustment (Fig. B.1.a) six plant species i.e. *L. scoparium*, *K. robusta*, *P. colorata*, *M. robusta*, swamp *L. scoparium* (all from North Island) and *O. paniculata* (South Island) reduced biolux luminescence to 40% or lower with maximum inhibition obtained by the extracts of *P. colorata* followed by *M. robusta*. When the pH of the extracts was adjusted to higher than 5 (Fig. B.1.b), the reduction of luminescence was less pronounced, with only *P. colorata* reducing the luminescence to 50%. Swamp *L. scoparium* used as an internal control (with no pH adjustment) presented no difference in the Biolux fluorescence inhibition.

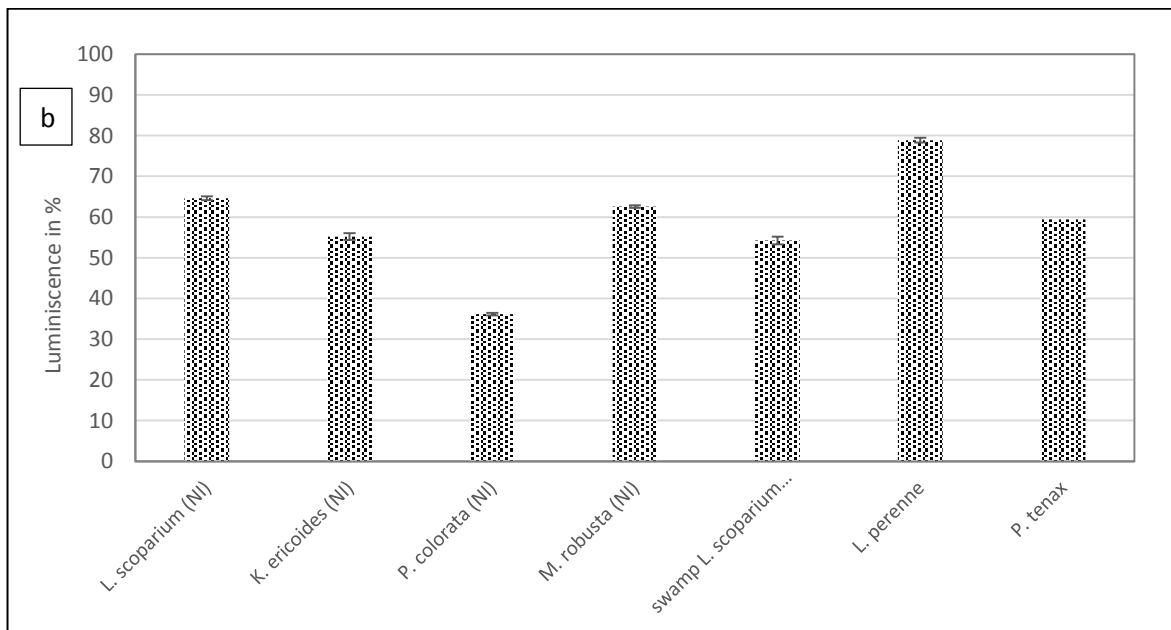
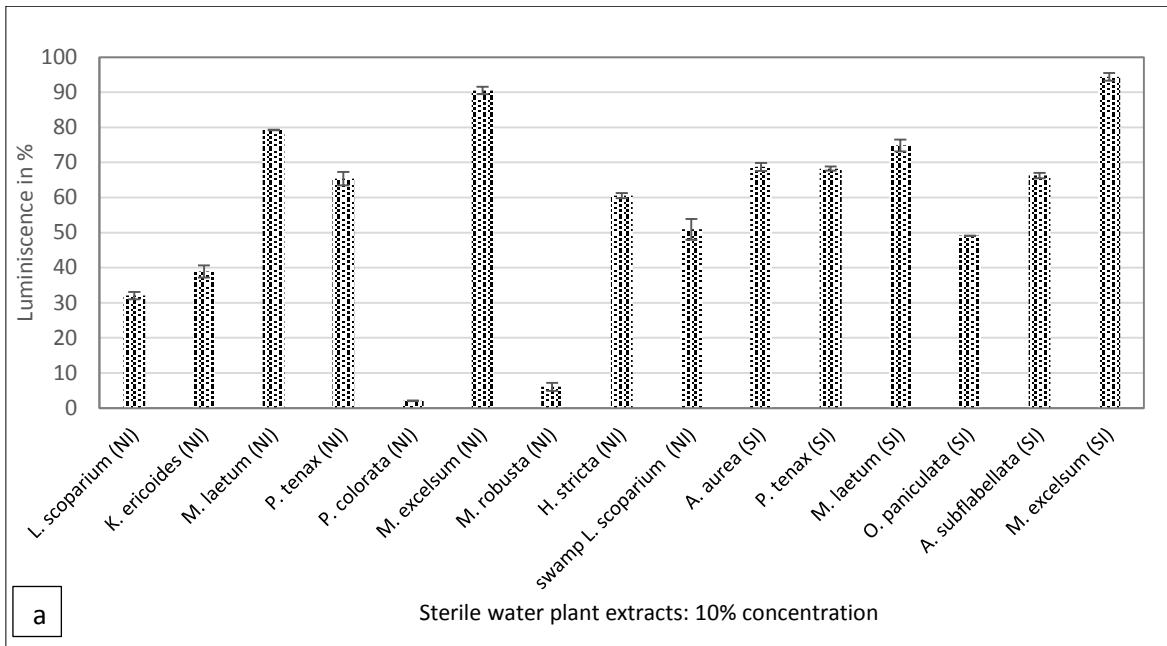


Figure B.1. The inhibition of bioluminescence of *E. coli* (in percentage) compared to water by the sterile water extracts of selected native NZ plants species **a)** before and **b)** after the pH adjustment. Error bars show standard error of the average value (n = 3). NI = North Island, SI = South Island.

8.1.2 Testing inhibition of *Escherichia coli*

Figure B.2 shows *E. coli* growth after 24 hours exposure to the 10% extracts of selected natives. All native species increased *E. coli* growth compared to sterile water. *Pseudowintera colorata* has generated a maximum increase of 142% in *E. coli* growth as compared to the other species. Minimum

growth was accelerated by the South Island *P. tenax* generating 11% more bacterial population than control followed by North Island *P. tenax* with 22% more bacterial growth.

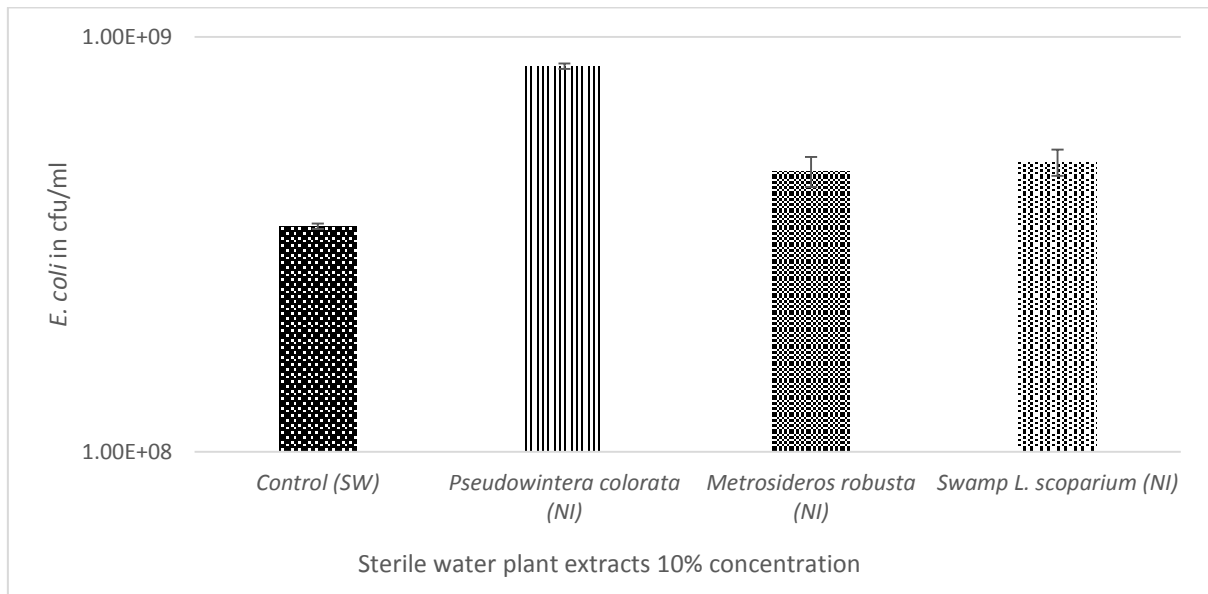


Figure B.2. Average *Escherichia coli* growth after 24 hours in Nutrient broth amended sterile water extracts of *P. colorata*, *M. robusta* and swamp *L. scoparium*. Control consisted of sterile water replacing the extracts and referred as the negative control. Error bars represent standard error of average value (n = 4). NI = North Island, SI = South Island, SW = Sterile water.

Fig. B.3 shows *E. coli* growth curve over 24 hours of exposure to the extracts of selected natives. Similar to Fig. B.2, *P. colorata* increased the growth of *E. coli* to the maximum of 8.2×10^8 cfu/ml by 24 hours showing a 98% increase from the control. However *M. robusta* delayed the bacterial growth rate with growth being below the limit of detection (1 cfu/ml), after ten hours of incubation it reached to the bacterial growth of 3.1×10^8 cfu/ml showing a 22% less bacterial growth than the control. None of the species completely inhibited the growth of *E. coli* over 24 hours. Streptomycin, used as a positive control, as expected generated bacterial growth below detection limit throughout the experiment.

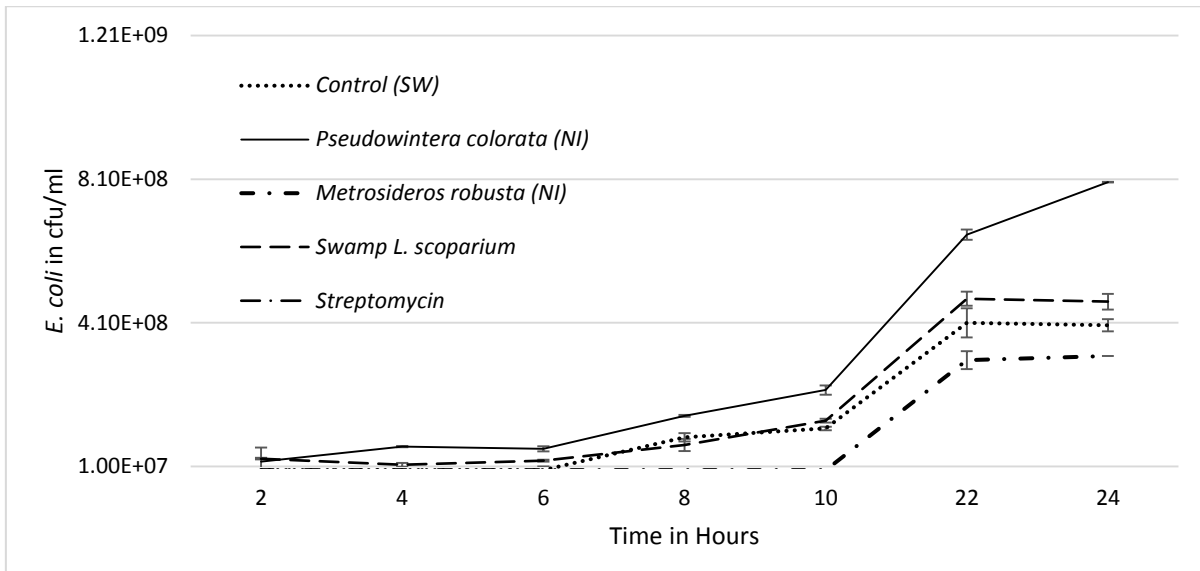


Figure B.3. Average *Escherichia coli* growth over 24 hours in Nutrient broth amended with sterile water extracts of *P. colorata*, *M. robusta* and swamp *L. scoparium*. Control consisted of sterile water replacing the extracts referred to as the negative control. Error bars represent standard error of average value (n = 3). NI = North Island, SI = South island, SW = Sterile water.

The TOC concentration of the extracts of *M. robusta*, Swamp *L. scoparium* and *P. colorata* are presented in Fig. B. 4. The TOC concentration range was between 412 to 740 mg/l. *Pseudowintera colorata* showing maximum *E. coli* growth had the highest TOC concentration of 740 mg/l.

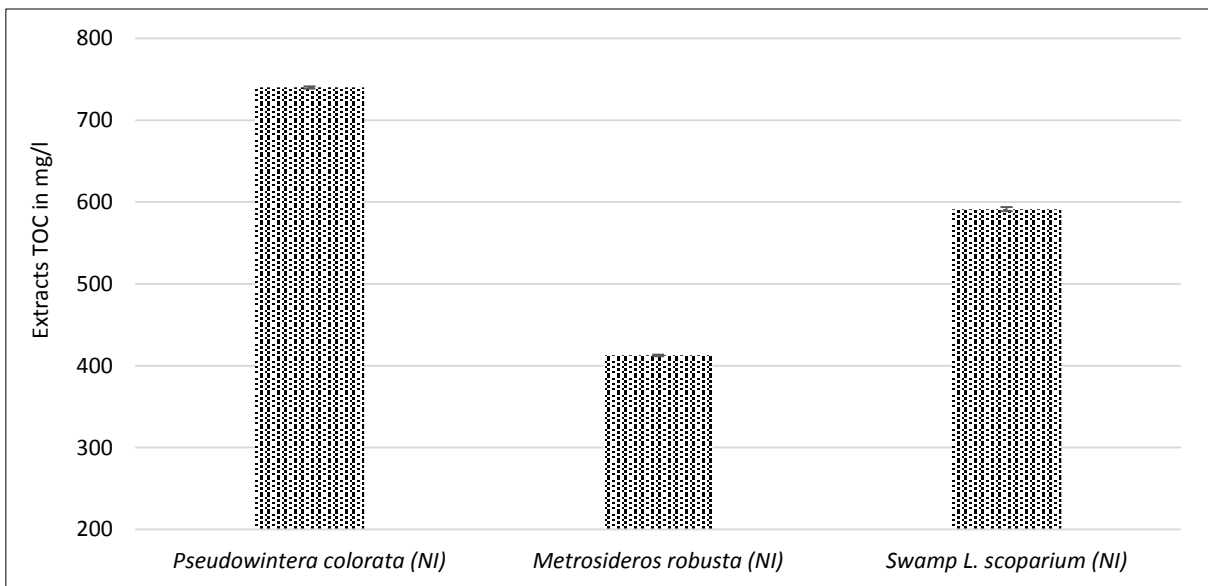


Figure B.4. Average total organic carbon concentrations of *P. colorata*, *M. robusta*, swamp *L. scoparium* and *P. tenax* extracts to which *E. coli* was exposed to in the 10% extract concentration. NI = North Island. Error bars represent standard error of the average value (n = 3).

Extracts pH is presented in Fig. B.5. Other than *M. robusta*, the pH of other two plant extracts was in the range of *E. coli* survival as tested in Chapter 3 (Fig. 3.6).

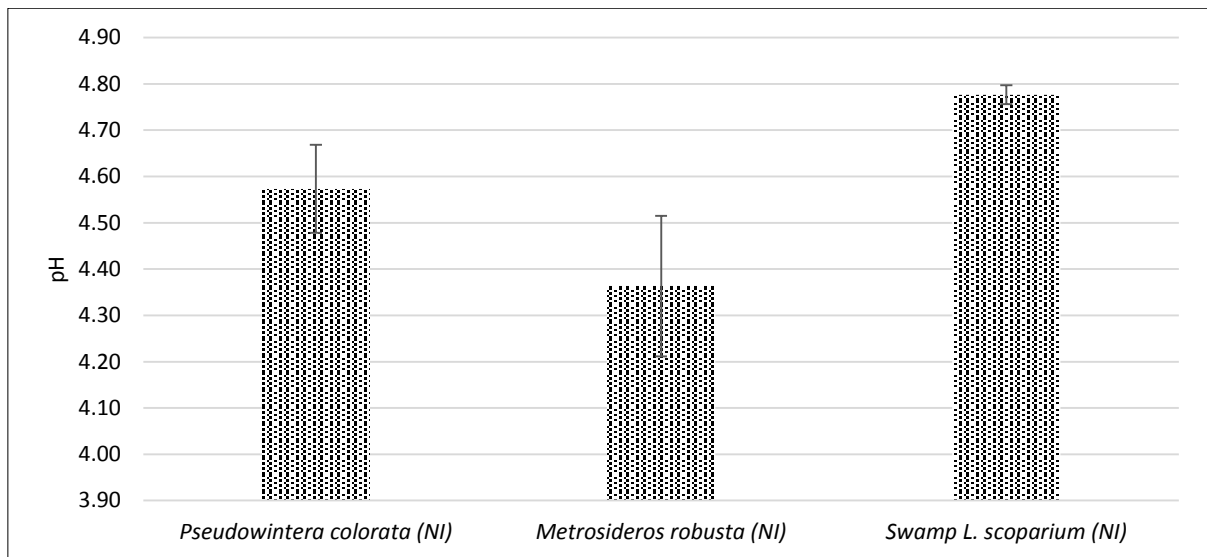


Figure B.5. Average pH of *P. colorata*, *M. robusta*, swamp *L. scoparium* and *P. tenax* extracts to which *E. coli* was exposed to. NI = North Island. Error bars represent standard error of average value (n = 3).

8.1.3 Testing inhibition of *P. aeruginosa*, *B. cepacia* and *S. aureus*

The growth of *P. aeruginosa* was inhibited by both the extracts of *P. colorata* (Fig. B.6.a) and *M. robusta* (Fig. B.6.b). *Pseudowintera colorata* reduced the growth of *P. aeruginosa* at 10% and 20% extract concentrations only and this reduction in growth was 50% of the maximum OD measured (EC50). In contrast, *M. robusta* extracts showed an inhibition of growth from 1.25% concentration, and was more pronounced when the concentration was higher. A 50% reduction in the OD compared with the maximum value measured was reached at a concentration of 2.5%.

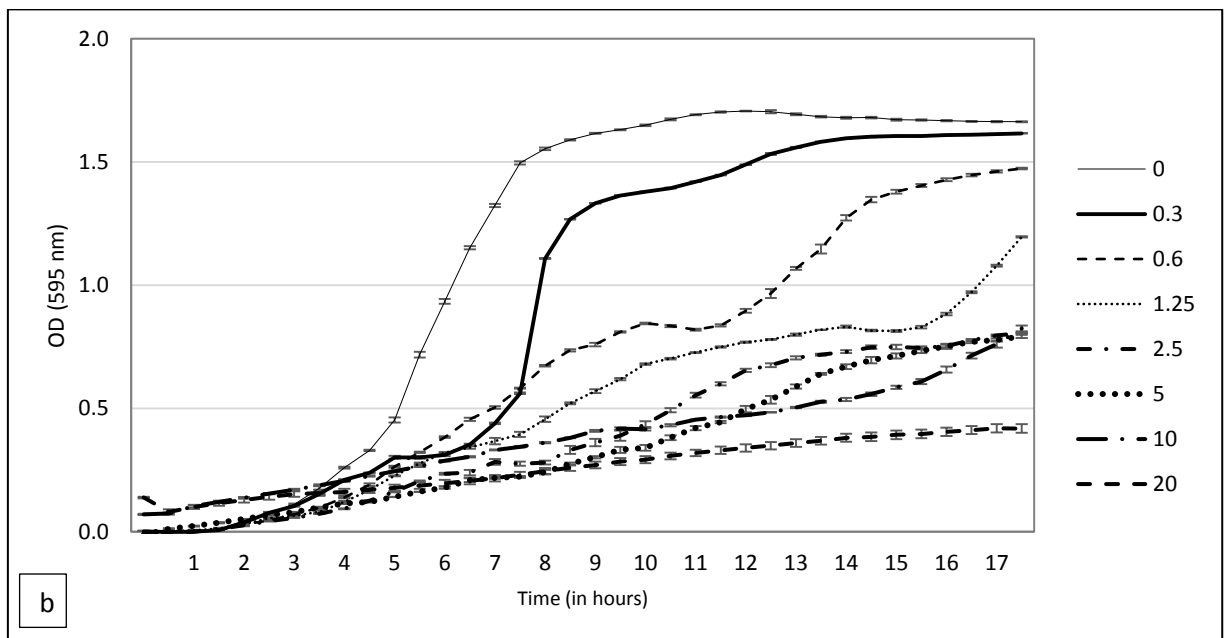
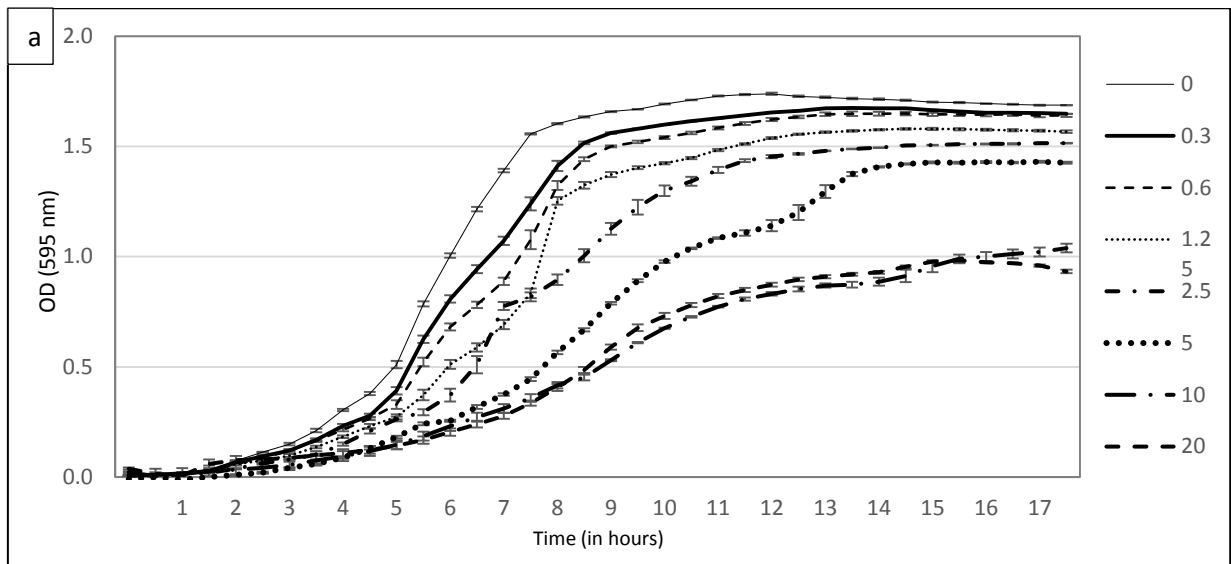
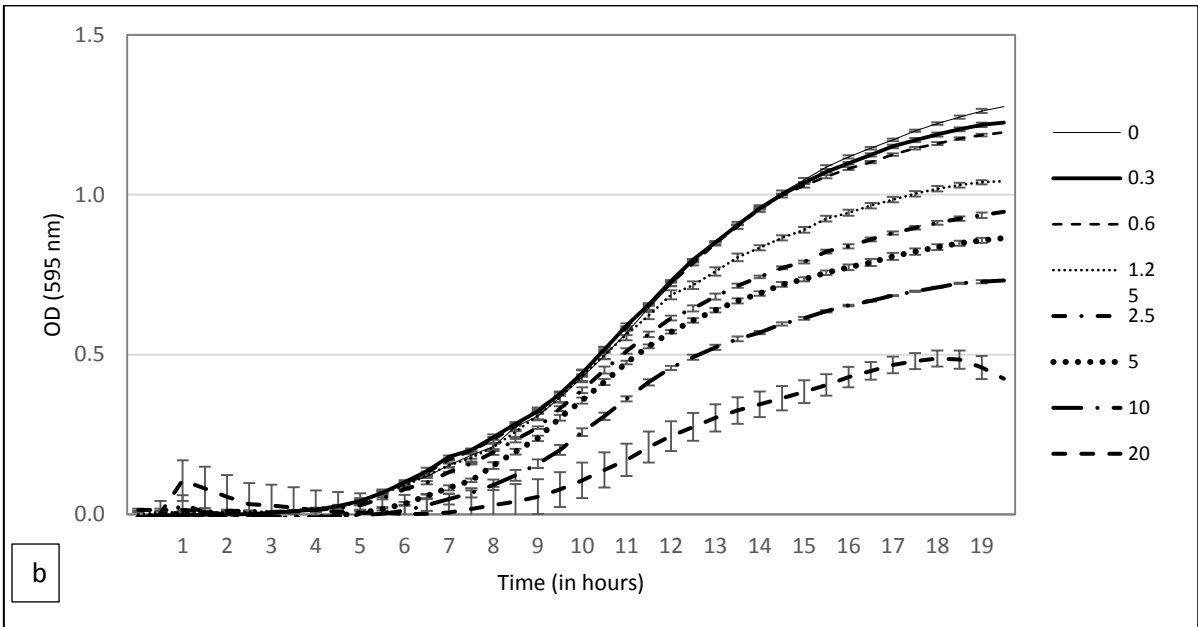
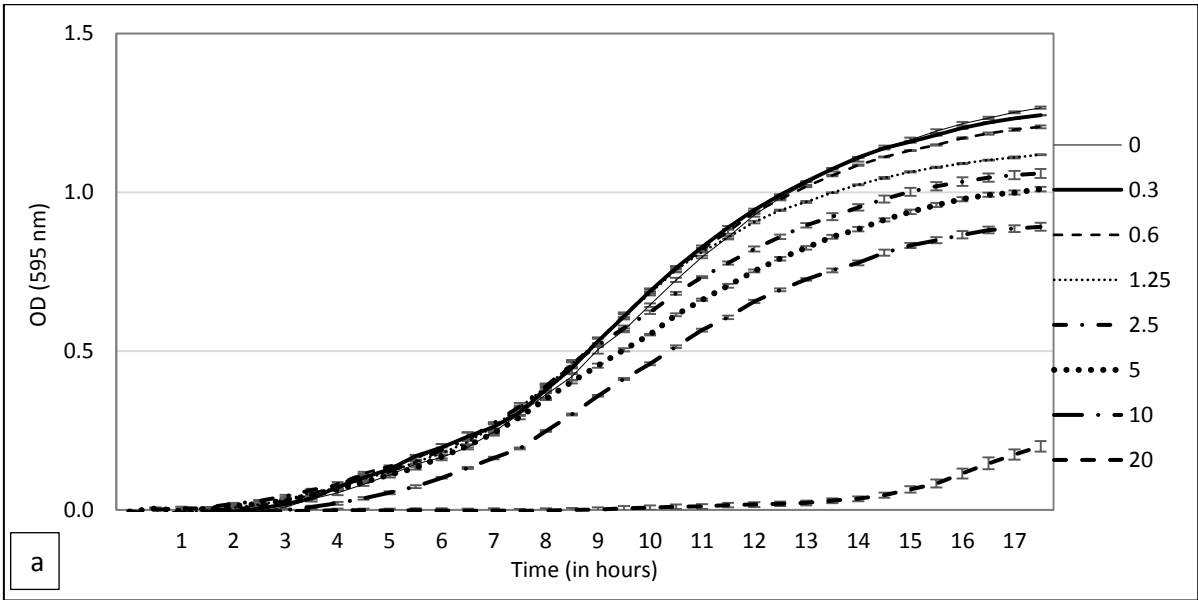


Figure: B.6. Growth curve of *P. aeruginosa* exposed to the eight different concentration of sterile water extract of **a)** *P. colorata* and **b)** *M. robusta*.

The growth of *B. cepacia* was inhibited by extracts of the three native species i.e. swamp *L. scoparium* (Fig. B.7.a), *P. colorata* (Fig. B.7.b) and *M. robusta* (Fig. B.7.c). Only swamp *L. scoparium* extract at 20% concentration reduced the OD by more than a half compared with the highest OD measured. *Pseudowintera colorata* extract inhibited the growth of *B. cepacia* with increasing extract concentration. The concentration that reduced the OD to half (EC50) was 10%. *Metrosideros robusta* extract reduced the OD to 50% of the highest OD measured at 0.6% concentration.



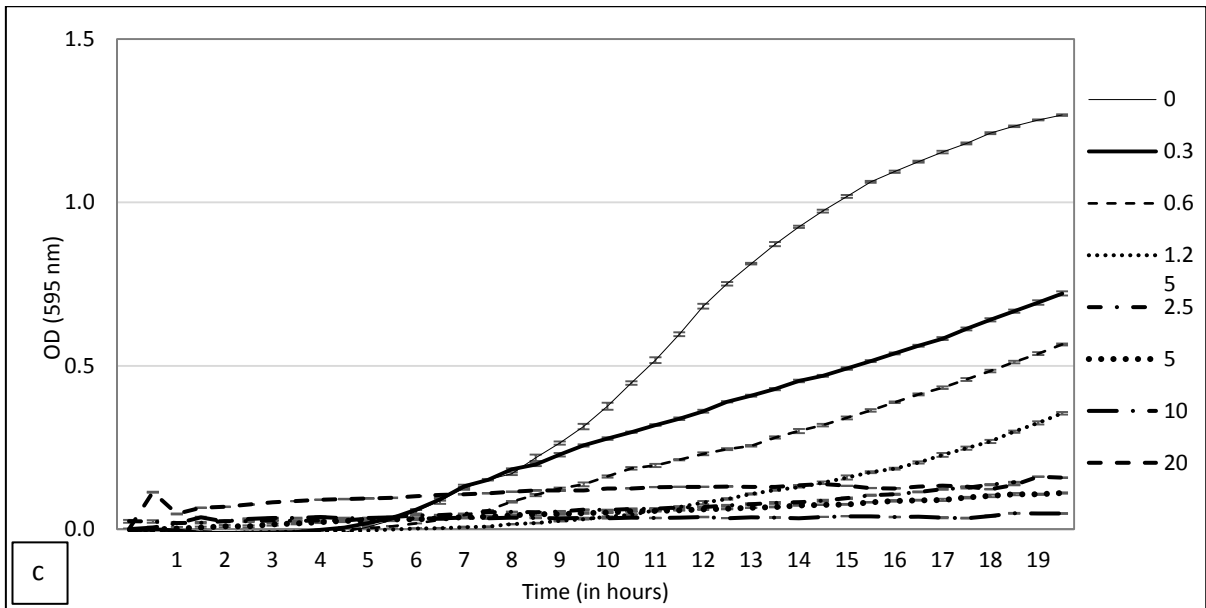
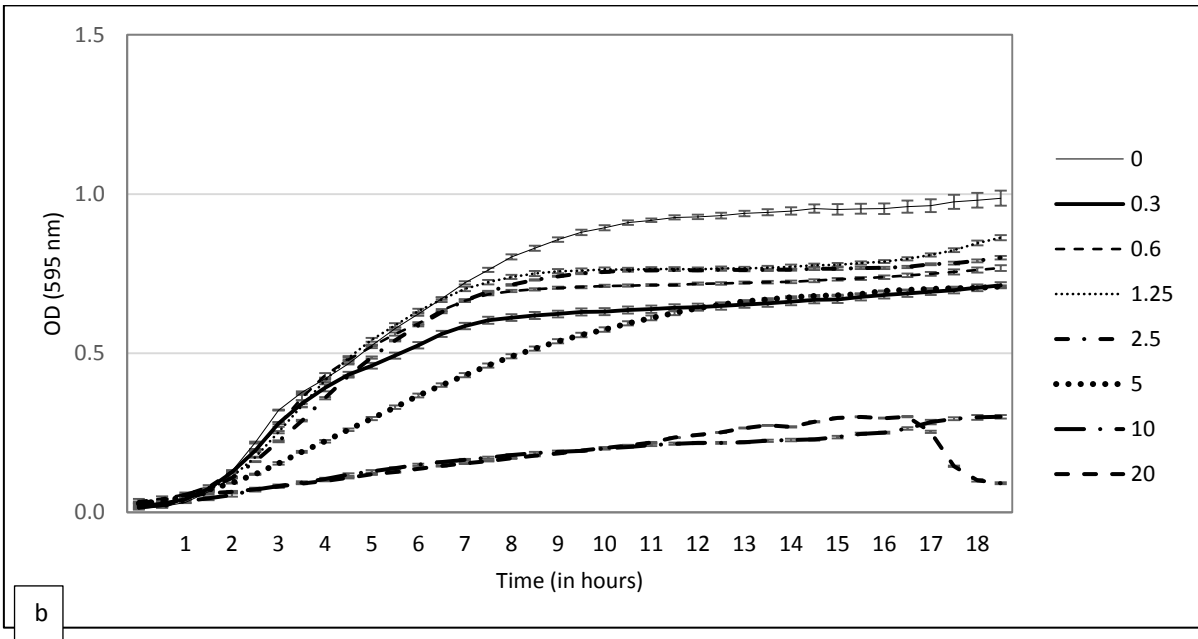
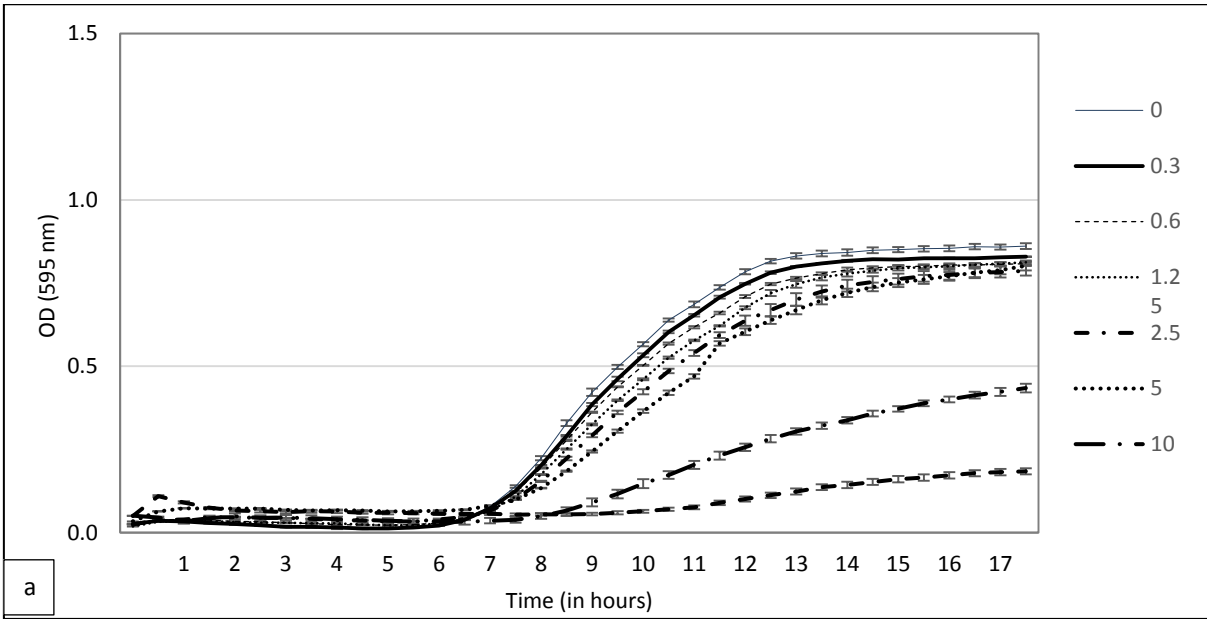


Figure: B.7. Growth curve of *B. cepacia* exposed to the varying concentrations of sterile water extracts of **a)** swamp *L. scoparium* **b)** *P. colorata* and **c)** *M. robusta*.

The growth of *S. aureus* was inhibited by the swamp *L. scoparium* extract at 10% and 20% extract concentrations (Fig. B.8.a). Similarly, *M. robusta* also inhibited *S. aureus* growth rate at 10% and 20% extract concentrations only (Fig. B.8.b). However, increasing concentrations of *P. colorata* extract resulted in a corresponding decrease in bacterial growth, although the OD was not reduced to half of the maximum measured until exposed to a concentration higher than 10% (Fig. B.8.c).



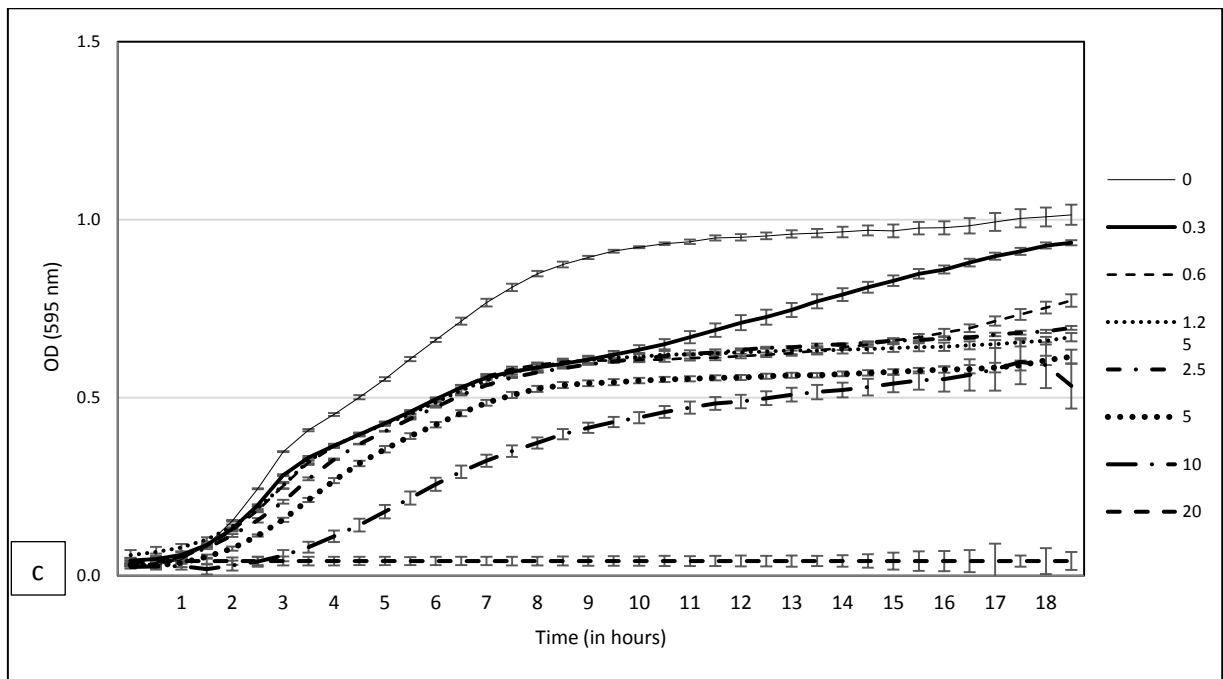
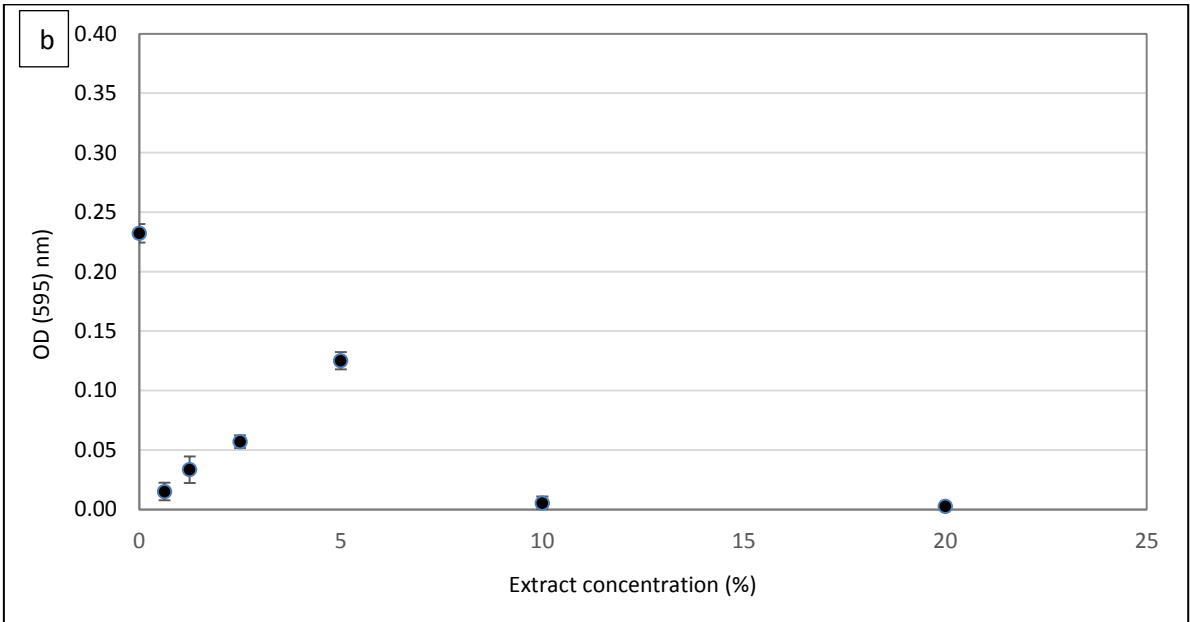
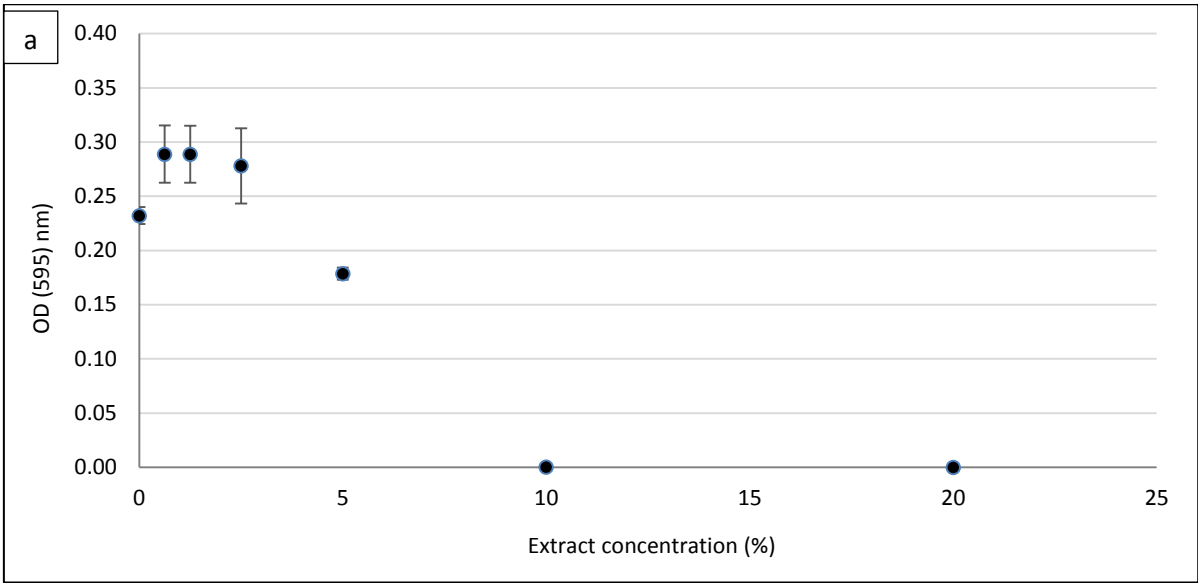


Figure: B.8. Growth curve of *S. aureus* exposed to eight different concentrations of sterile water extracts of **a)** swamp *L. scoparium* **b)** *M. robusta* and **c)** *P. colorata*.

8.1.4 Testing the inhibition of *C. jejuni*

The extracts of *P. colorata* (Fig. B.9.a), *M. robusta* (Fig. B.9.b) and swamp *L. scoparium* (Fig. B.9.c) presented a strong growth inhibition of *C. jejuni* below 10% extract concentration. The *M. robusta* extracts had a bactericide effect on *C. jejuni* with no colonies recovered when these extracts/*C. jejuni* suspension were plated. Although cfu measurements of the bacterial suspension after 48 hours of incubation with *P. colorata* and swamp *L. scoparium* indicated a bacteriostatic effect (results not shown).



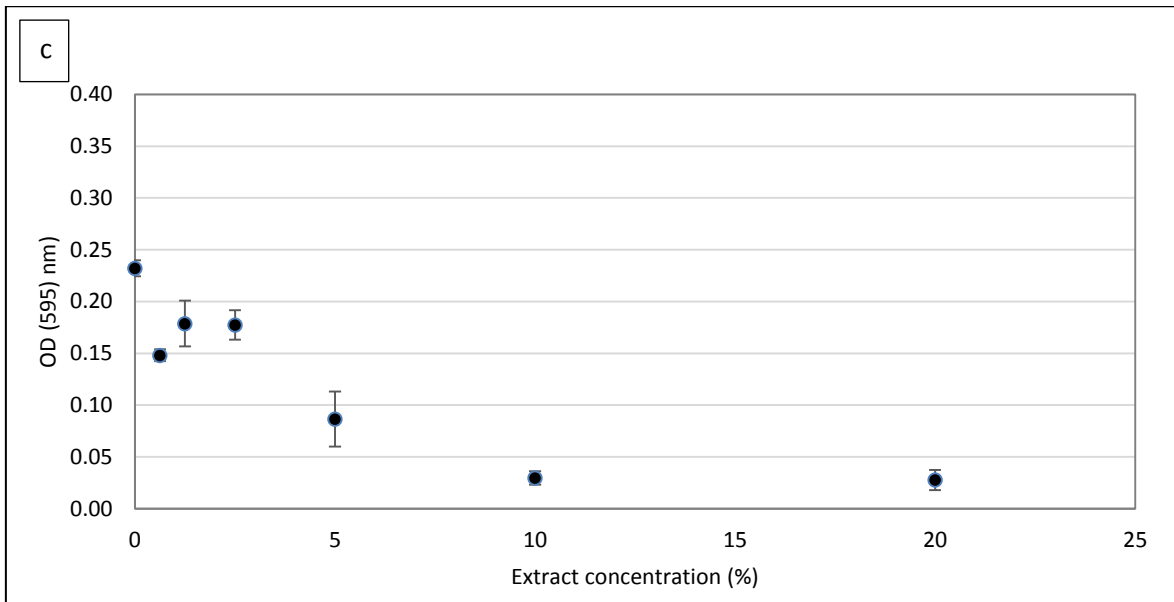


Figure. B.9. Growth inhibition of *C. jejuni* after 48 hours of exposure to eight different concentrations of sterile water extracts of a) *P. colorata* b) *M. robusta* and c) swamp *L. scoparium*.

Appendix C

General Methods

C.1 Glycerol stock preparation of *Escherichia coli*

1. Prepare 80% concentration of glycerol with water and autoclave.
2. Grow the *E. coli* culture in sterile Nutrient broth (NB; Difco-bacto NB) for 24 hours at 30°C in a shaking incubator.
3. Add 2 ml of sterile glycerol solution (80%) to 10 ml of bacterial culture growing in NB (to make final concentration of glycerol ~15% as recommended for storing bacterial culture collections (Park, 1976)) in a sterile falcon tube and vortex for 5 seconds to give a uniform mix.
4. Divide this glycerol and bacterial mix into several Eppendorf tubes or cryotubes (2- 5 ml in each). If contamination, mutation or loss of viability occurs the untouched seed stock can then be used.
5. Store the Eppendorf tubes or cryotubes in a freezer at -50°C or below.

C.2 Resuscitation of glycerol frozen stock cultures of *Escherichia coli*

The methods was adopted and modified from (Prosser, 2011).

1. Remove the bacterial culture stocks (in 15% glycerol) from the freezer and keep aside for 2-3 minutes.
2. In a laminar flow inoculate a loop full (~1 µl) of the bacterial stock into three falcon tubes filled with NB.
3. Incubate the falcon tubes in a shaking incubator at 30°C for 24 hours.
4. Vortex the falcon tubes for nearly 5 seconds to give a uniform mix and then centrifuge at 700 *g* for 10 minutes at 4°C.
5. Remove the supernatant using a sterile pipette and re-suspend bacterial culture into 3 ml Ringers solution to wash.

6. Centrifuge at 4175 x *g* for 2 minutes at 4°C and remove supernatant with a sterile pipette.
7. Re-suspend bacterial pellet in 3 ml NB and store at 4°C. At this stage bacteria from NB can also be cultured in Nutrient Agar (NA; Oxoid CM0003) plates at 30°C for 24 hours.
8. Use the NA or NB cultured colonies for three to four weeks while maintaining them at 4°C and then use a fresh frozen (-50°C) stock, or as required.

C.3 Total organic carbon (TOC) analysis method

The Total organic carbon (TOC) of all the extracts was analysed using an Elementar Vario TOC Cube after preparing 1: 10 dilution of prepared extracts with sterile water (SW). The method was adopted from the laboratory manual for the Department of Soil and Physical Sciences (unpublished by Roger Cresswell and Leanne Hassall).

1. Measurement of Total Carbon (TC)

The sample is injected into a combustion tube filled with oxidation catalyst at 850°C. The TC component of the sample is combusted or decomposed to become CO₂. CO₂ is detected via a non-dispersive infra-red gas analyser (NDIR).

2. Measurement of Inorganic Carbon (IC)

The sample is acidified and injected into an IC reactor vessel which has ultra zero grade air bubbling through. The IC is decomposed to CO₂ which in turn is detected by the NDIR.

3. Calculation of Total Organic Carbon (TOC)

Total TOC is calculated by subtracting the inorganic fraction from total carbon concentration i.e.

$$\text{Total C} = \text{Inorganic C} + \text{TOC}$$

C.4 pH analysis method for all samples

The method was adopted from the laboratory manual for the Department of Soil and Physical Sciences (unpublished by Roger Cresswell and Leanne Hassall).

- Weigh 10 g air dried soil into a vial and add 25 ml of deionised water. Stir and leave to stabilise overnight.
- Read on pH meters using buffers of pH 4 and pH 7 for calibration.

- For liquid samples, directly measure on pH meters using buffers of pH 4 and pH 7 for calibration.

C.5 The linear regression curve of *E. coli*

Escherichia coli from the glycerol stock was resuscitated and cultured on NA plates. One colony from this NA plate was inoculated into each of 3 x 25 ml sterile NB in 50 ml Schott bottles. The inoculated broth was incubated at 30°C overnight in a shaking incubator at 120 rpm. Sterile NB (without *E. coli*) was used as a blank and was incubated in similar manner. After 24 hours all three bacterial broth cultures were aseptically mixed together in a 1l sterile Schott bottle and divided into 50 ml sterile falcon tubes under laminar flow. Falcon tubes with *E. coli* were centrifuged at 4175 g for 10 minutes at 4°C. Supernatant was discarded and each bacterial pellet was re-suspended in 5 ml sterile Ringers solution. The blank was treated in similar manner. Sterile Ringers solution was used to adjust blank in spectrophotometer. Optical density (OD) of bacterial culture was adjusted between 0.1 and 0.9 using Ringers solution, at 600 nm using Genesys 10S UV-Vis spectrophotometer. These *E. coli* cultures adjusted to different OD's, were then serially diluted to 10⁻⁹ dilutions and plated on NA plates in triplicates and incubated for 24 hours at 30°C. Bacterial colonies were counted from plates growing 30 to 300 colony forming units (cfu), and average cfu/ml were calculated to perform linear regression in Microsoft Excel. Linear regression of *E. coli* fits the straight-line equation (n=3) (Fig. C.1).

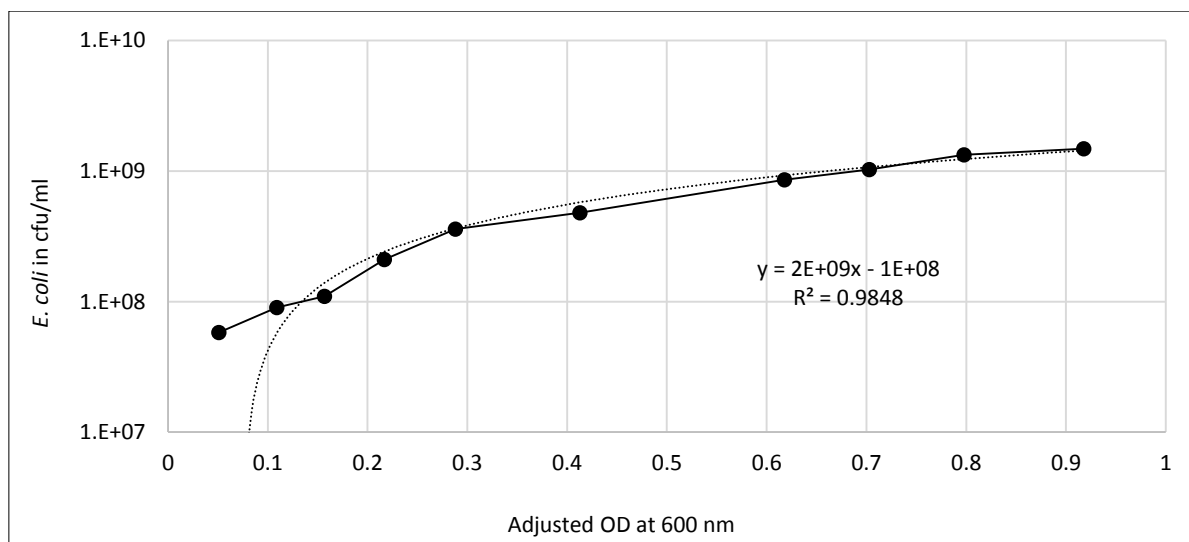


Figure: C.1. Linear regression of *E. coli* growth at different OD at 600 nm.

C.6 *Escherichia coli* exposed to 480 µl sterile water plants extracts (EXT-2) in NB

With EXT-2 (Chapter 3), the effect on *E. coli* survival was similar to that observed with 10% extract concentrations (Fig. 3.1). Root and leaf extracts of both *L. scoparium* and *K. robusta* inhibited *E. coli* growth as compared to the control, with a significant difference. Root extracts of *K. robusta* were active against *E. coli* showing 99% growth inhibition. However, *L. scoparium* root extracts inhibited 95% of *E. coli* growth, as compared to the control. Root extracts of both species were more active against bacterial growth than their leaf extracts showing significant difference between root and leaf extracts inhibition rates. Although, the error rates were higher for the root extracts. Leaf extracts of *L. scoparium* and *K. robusta* generated no significant difference in their activity, showing 18% and 16% of total *E. coli* growth, respectively, as compared to the control. Compared to the control, *L. perenne* extracts significantly increased the growth of *E. coli* by 10%. While with the positive controls i.e. essential oils of *L. scoparium* and *K. robusta* and streptomycin, *E. coli* growth was below detection limit.

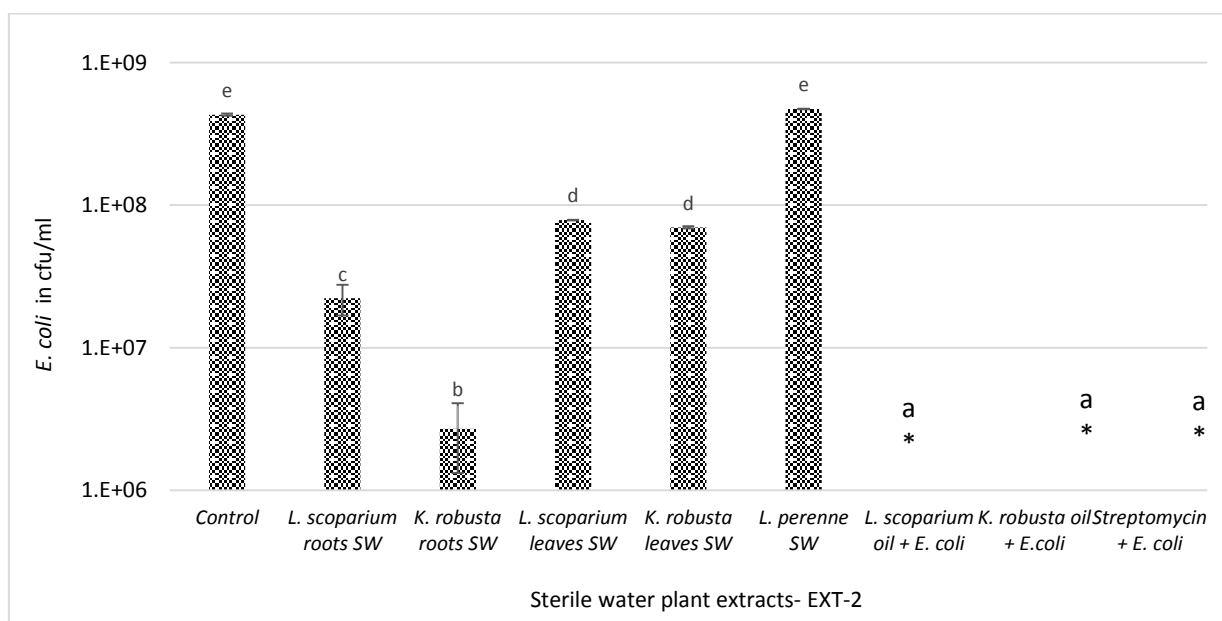


Figure. C.2. Average *Escherichia coli* growth in Nutrient broth amended with sterile water extracts of *L. scoparium* and *K. robusta* at 48% concentration. *Lolium perenne* was used as a plant control. Control consisted of sterile water replacing the 10% plant extracts referred to as the negative control. Bars with the same letters do not differ significantly ($p < 0.05$) according to Fishers unprotected LSD analysis ($n = 3$). Error bars represent standard error of average value. SW = Sterile water.

C.7 *Escherichia coli* growth in pH adjusted NA

Fig. C.3 shows *E. coli* growth in the pH adjusted NA. The pH of the NA was adjusted between 2.5 to 9.5 using NaOH or HCl (0.1 M) prior to autoclaving as described in Section 3.3.3.3. The NA was then poured into the Petri plates and a sterile loop was used to inoculate the NA plates with an *E. coli* suspension

prepared in Ringers (Section 3.3.3.3) at equidistant positions and at 1 cm from the edge of the Petri plate. Three plates were set up for each pH treatment and arranged in a completely randomised design. All plates were incubated at 30°C for 24 hours and the presence/absence of *E. coli* was assessed at the inoculation points after incubation. All plates were photographed to compare the growth rates. The experiment was repeated two times.

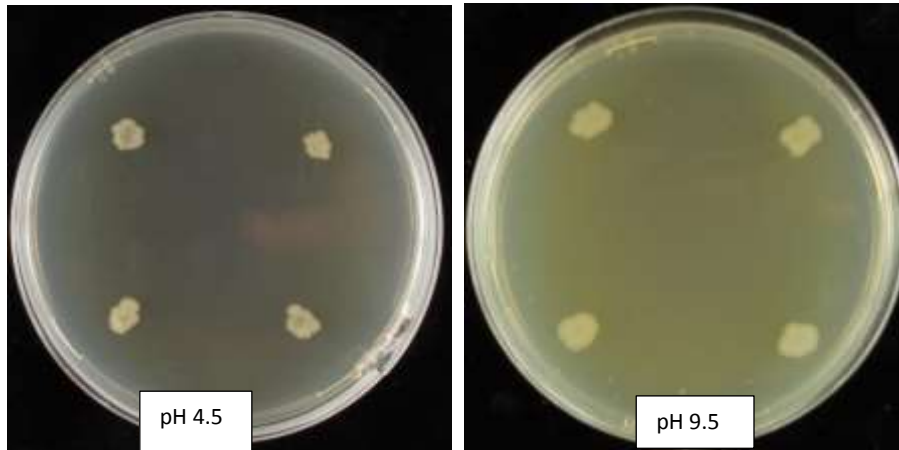


Figure. C.3. Shows *Escherichia coli* growth on pH adjusted NA at 4.5 and 9.5. Bacterial growth was not observed below pH 4.5.

C.8 Assay of bacterial treatment with extracts in NA plates

Fig. C. 4 shows the number of *E. coli* colonies on NA plates amended with sterile water extracts (roots and leaves) of *L. perenne* and *K. robusta*. Details of the method is described in Section 3.4.1.2. The numbers were too high to be counted.

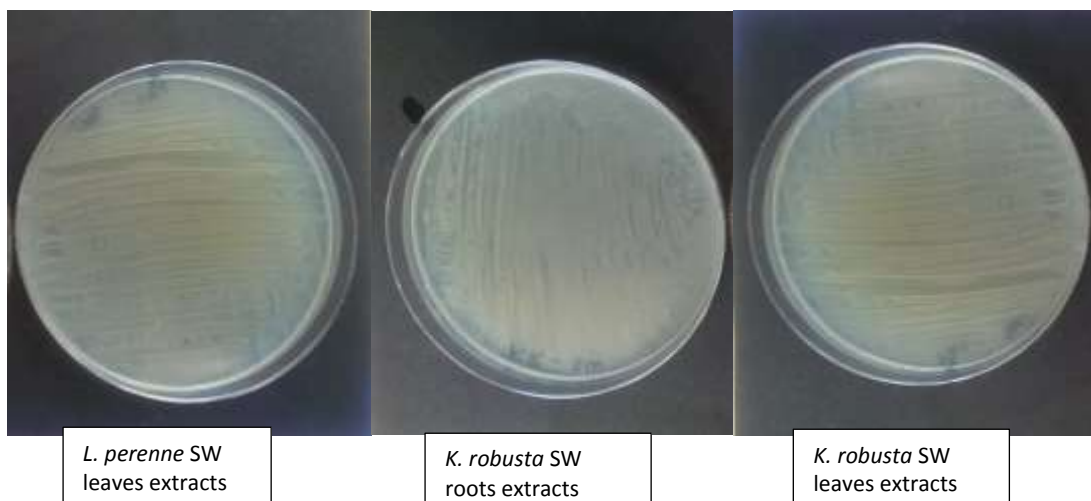


Figure. C.4. Shows *E. coli* growth in NA plates after their exposure with sterile water extracts of *K. robusta* and *L. perenne*. SW = Sterile water used as control.

C.9 *E. coli* survival at increasing concentrations of NaBr

Fig. C.5.a, b and c shows *E. coli* survival at increasing concentrations of NaBr in solid i.e. NA and liquid media i.e. NB. To trace the path followed by *E. coli* in the soil, NaBr was chosen as a tracer and its toxicity against *E. coli* was tested in both NA (Fig. C.5.a) and NB (Fig. C.5.b and C.5.c). For the NA trial, 100 ml of NA was mixed with 0% to 6% NaBr and the agar was autoclaved. Four Petri plates were established for each concentration and *E. coli* dots were introduced to these plates, from a fresh culture of *E. coli* at equidistant positions and at 1 cm from the edge of the Petri plate. Plates were incubated at 30°C for 24 hours and growth was monitored the next day. The 3% NaBr concentration was the limit of *E. coli* survival in both media. Up to 3% salt concentration, had no effect on the growth rate of *E. coli*. However, as soon as the concentration reached 4% or higher, bacterial survival was critically affected with no growth observed at 4% NaBr concentration in both media.

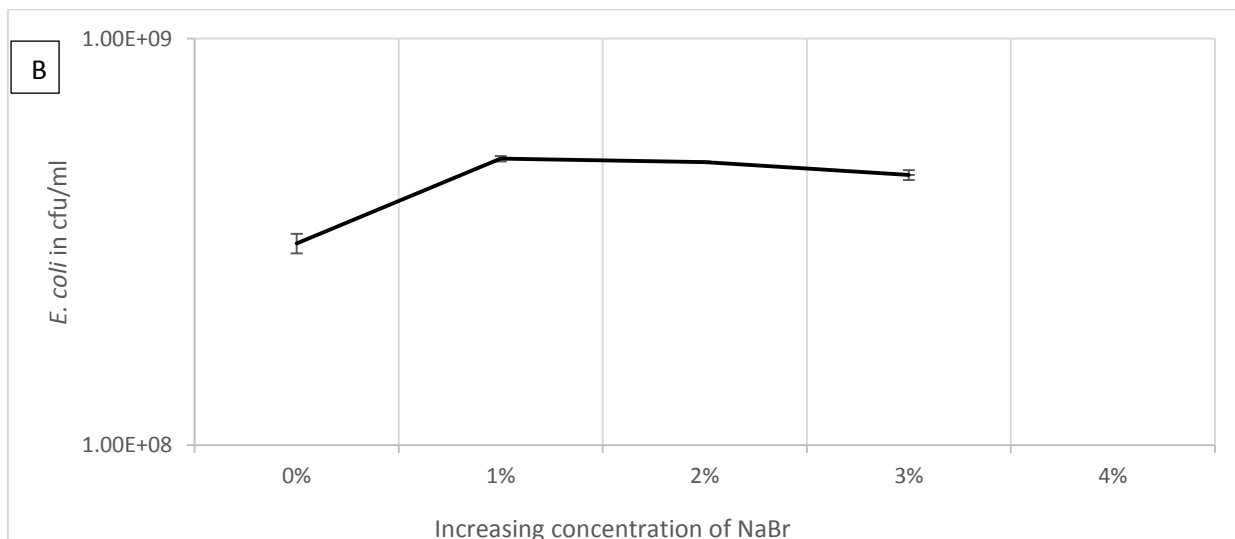
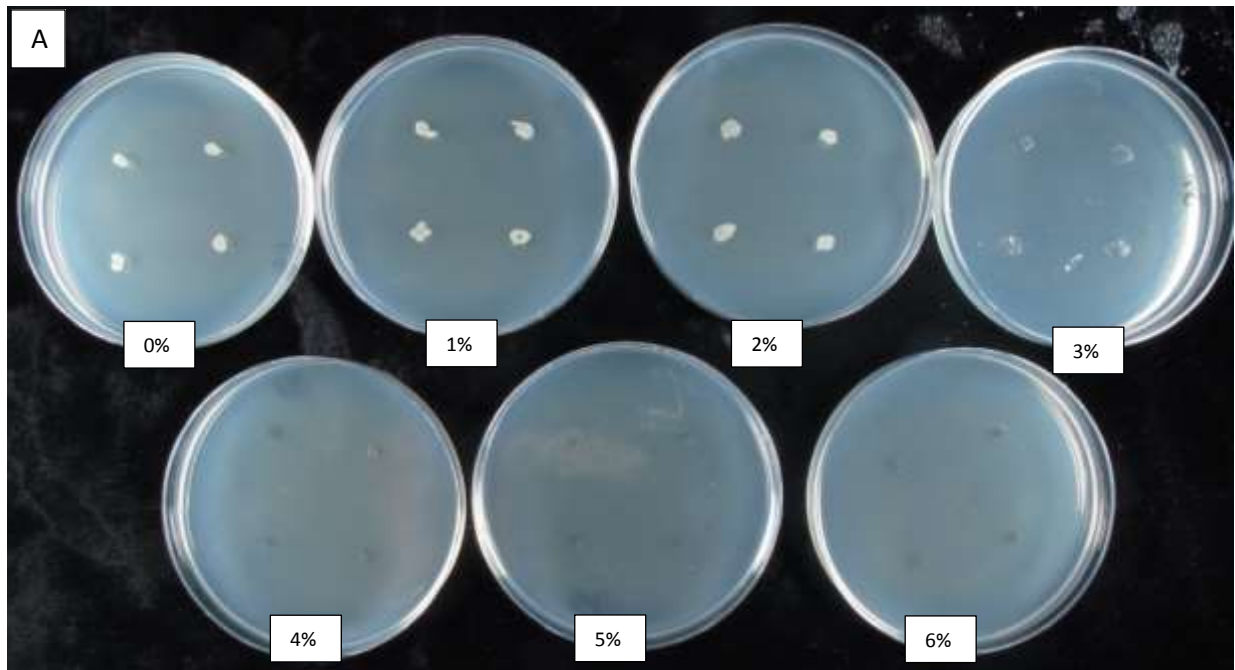




Figure. C.5. Shows *E. coli* survival at increasing concentrations of NaBr (0% to 6%) in **A)** Nutrient Agar and **B)** and **C)** Nutrient broth.

C.10 The lysimeter harvest of Chapter 4

Fig. C.6 shows the harvested soil columns of *L. scoparium*, *K. robusta* and *L. perenne* during the harvesting preparation.



Figure. C.6. Show the soil columns harvesting process after completion of glasshouse experiment of Chapter

C.11 Murashige and Skoog's media recipe

One-tenth strength of Murashige and Skoog (MS) medium without any organics or sugars was prepared following (Murashige & Skoog, 1962). The details of the ingredients are provided in Table C.1.

Table. C.1. Shows the list of ingredients and their concentration used to prepare Murashige and Skoog (MS) media.

Compound	Concentration (mg l ⁻¹)
NH ₄ NO ₃	165
KNO ₃	190
CaCl ₂ .2H ₂ O	44
MgSO ₄ .7H ₂ O	37
KH ₂ PO ₄	17
KI	0.083
H ₃ BO ₄	0.62
MnSO ₄ .H ₂ O	2.23
ZnSO ₄ .7H ₂ O	0.86
Na ₂ MO ₄ .2H ₂ O	0.025
CuSO ₄ .5H ₂ O	0.0025
CoCl ₂ .6H ₂ O	0.0025
Na ₂ EDTA	3.73
FeSO ₄ .7H ₂ O	2.78

C.12 Soil bacteria from Chapter 4

Fig. C. 7 shows the total *E. coli* population recovered from the Akaroa soil (0 – 10 cm) systems of *L. scoparium*, *K. robusta* and *L. perenne* after the completion of glasshouse trial of Chapter 4. Minimum bacterial population were observed under *L. scoparium* and *K. robusta* soil systems lowering down to of 8.5 to 1.7 x 10³ cfu of total *E. coli* by the end of the experiment. However, *L. perenne* maintained a significant high bacterial number with 6.6 x 10⁴ cfu of total *E. coli* recovered at the end of the experiment.

As this bacterial analysis in soil was a preliminary analysis and performed using Colilert-18, which is a method for *E. coli* enumeration in water samples, this data are not fully reliable unless a second enumeration using MPN is performed.

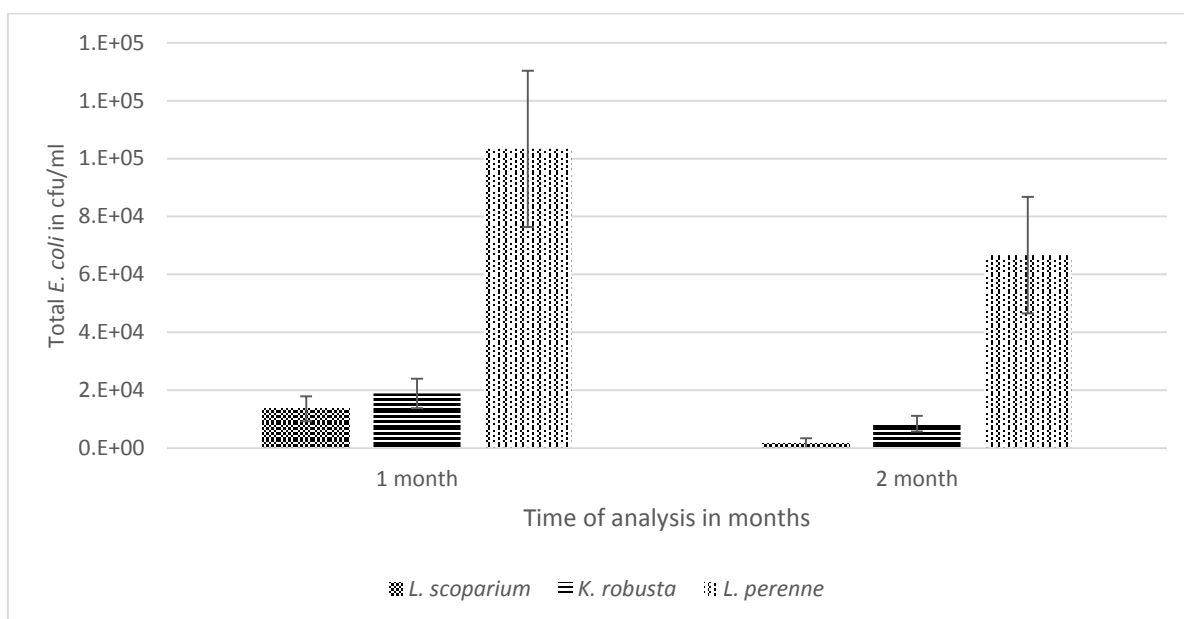


Figure C.7. Shows total *E. coli* population residing in the soil system of *L. scoparium*, *K. robusta* and *L. perenne*. Error bars represent standard error of the mean value (n = 5).

C.13 Fresh and dry biomass of EXT-1

Table C.2. The Fresh and dry biomass of the roots and leaves of *L. scoparium*, *K. robusta* and *L. perenne* used for EXT-1 preparation.

Plants species	Fresh biomass in (g)	Dry biomass in (g)
<i>L. scoparium</i> leaves	10	6.3
<i>L. scoparium</i> roots	10	8.81
<i>K. robusta</i> leaves	10	6.12
<i>K. robusta</i> roots	10	8.24
<i>L. perenne</i>	10	2.31

Appendix D

Methods Development

D.1 Investigating the antibacterial activities of *Leptospermum scoparium* and *Kunzea robusta* extracts against *E. coli* (Method development). (September 2015- January 2016).

1. **AIM:** This Appendix presents the method development for Chapter 3. The aim here was to develop the best method to assess antibacterial (ANB) activities of two NZ native plants *Leptospermum scoparium* and *Kunzea robusta* against the lab strain *Escherichia coli* ATCC13706.
2. **HYPOTHESIS:** It was hypothesised that the leaf and root extracts of *L. scoparium* and *K. robusta* will inhibit the growth of *E. coli* as a result of their antibacterial properties, representing their potential against dairy shed effluent (DSE) pathogens.

3. MATERIALS AND METHODS

Details of the methods and materials are provided in Chapter 3 (Section 3.3). A set of 9 months old *L. scoparium* plants were obtained from Comvita Limited, North Island (NI) later in the method development (Method 10). These plants were stored at 4°C during delivery and until being transplanted into soil. Some of these plants were immediately used to obtain sterile water (SW) extracts (before being transplanted into the soil) while others were grown in both Eyrewell and Akaroa soil in 3 l pots to be used later if required (Fig. D. 1). All the chemicals and media were obtained from the laboratory facilities of Department of Soil and Physical Sciences or Bio-protection Research Centre (BPRC) at Lincoln University.



Figure D.1. Shows the North Island *L. scoparium* growing in two different soils in a gravelled area at the horticultural amenity area.

4. METHOD OPTIMISATION: Each method described below was repeated two to three times with different sets of *L. scoparium* and *K. robusta* plants.

4.1. Method 1: Liquid media protocol in Luria Bertani agar (LB)

The methods used were adopted and modified from (Prosser, 2011) LB agar was used for *E. coli* growth. Fifteen months old *L. scoparium* and *K. robusta* plants, grown for a glasshouse study by another student (all controls with no inoculation) (Fig. D.2), were first used to obtain the extracts.



Figure. D.2. Shows the experimental set up (in 2014-15) from which *L. scoparium* and *K. robusta* plants were harvested for extracts preparation.

Leaf and root harvest

Plants leaves were harvested first and stored at 4°C until used (4 hours). Immediately after the leaves were harvest roots were destructively harvested from the lysimeters. Both the leaves and roots were weighed fresh within four hours of harvesting (Table D.1) and no dry biomass was collected for this trial. The plant material were thoroughly washed three times with tap water with a final rinse with deionised (DI) water to remove any dirt. Washed plant materials were allowed to air dry for three hours on clean paper towels. Leaves and roots were crushed using separate pestle and mortars for 30 to 45 minutes with sterile water (SW) to obtain extracts in 1:4 (w/v) dilution. The paste obtained after crushing the leaves/roots was pressed using a clean spatula to separate the liquid from the crushed plant material. The liquid obtained was then filtered using 0.45 µm filter paper and was allowed to filter until a clear solution was collected in the sterile vials (Fig. D.3). Collected extracts were further sterilised used 0.45 µm cellulose acetate syringe filters under sterile conditions in a laminar flow hood. All extracts were immediately transferred to 4°C to be used within 24 hours and were frozen after that. A part of the leaf extract (5 ml) was used to measure pH and TOC (Total organic carbon) (Table D.2).

Leaf extracts of *L. perenne* were not tested during the first two trials with LB media. Commercially obtained 2 cm streptomycin discs (10 µg/disc) were used as a positive control and sterilised water as negative control. Essential oils of *L. scoparium* and *K. robusta* were also plated as positive controls both neat and in 50% dilution (emulsion) with SW.



Figure D.3. Shows collected leaf extracts of *L. scoparium* and *K. robusta*.

Table D.1. Total biomass obtained from *K. robusta* and *L. scoparium* plants.

Samples	Fresh weight (g)	Dry weight (g)
<i>L. scoparium</i> leaves	39.3	19.5
<i>L. scoparium</i> roots	58.9	39.6
<i>K. robusta</i> leaves	61.5	30.2
<i>K. robusta</i> roots	69.8	43.4

Testing bacterial survival

A fresh *E. coli* culture was prepared in Ringers solution after culturing in NB. Two methods i.e. **1)** zone of inhibition or well diffusion method and **2)** agar disc diffusion, were used to test the antibacterial properties of the extracts. One hundred µl of bacterial suspension was spread plated on each LB agar plate and twenty minutes later 100 µl (in well diffusion) or 20 µl (in disc diffusion) of one of the extracts were added using 2 cm plain paper discs. Discs were first placed on the inoculated agar and then 20 µl of the extract were pipetted onto the discs. The plates were allowed to sit on bench for 20 to 30 minutes and then incubated at 34°C ± 0.5°C, for 24 hours (or 48 hours if required) in dark. To assure sterility, *E. coli* culture (prepared in Ringers solution) was streak plated and incubated in similar manner.

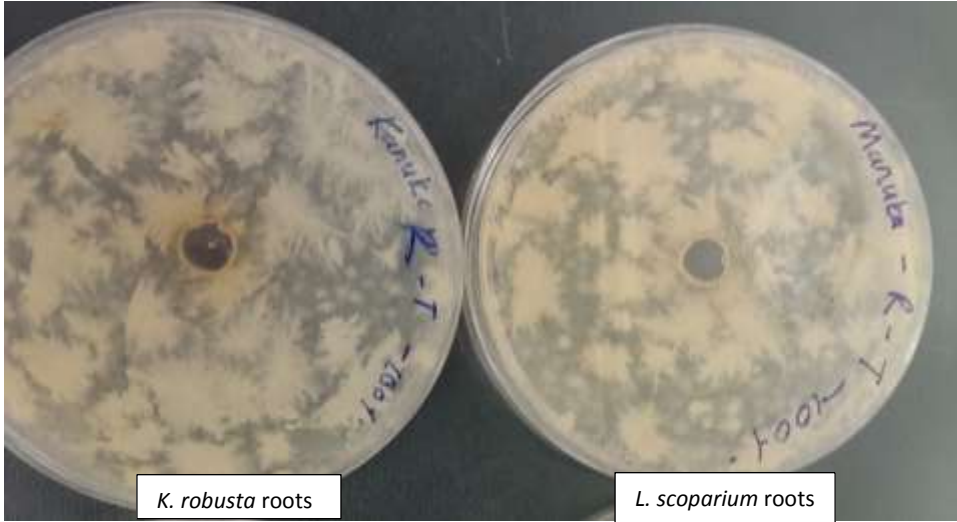
4.1.1. Results of method 1

Table D.2. pH and Total organic carbon of *L. scoparium* and *K. robusta* extracts. N.d. = Not detected.

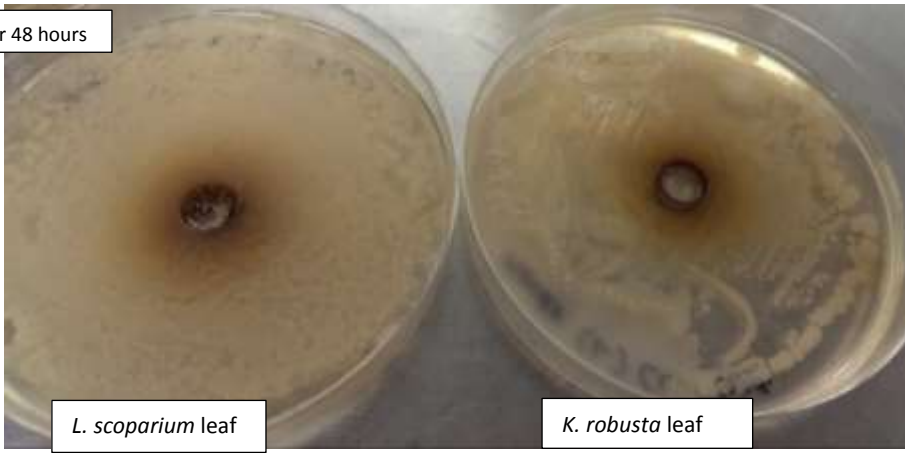
Extracts	pH	Total organic carbon in ppm or mg/l (no dilution)
<i>L. scoparium</i> leaf	4.62	4717
<i>L. scoparium</i> root	5.18	n.d.
<i>K. robusta</i> leaf	4.94	4933
<i>K. robusta</i> root	5.21	n.d.

No inhibition zone was observed after 24 hours of bacterial exposure to any extract i.e. leaf or roots of either *L. scoparium* or *K. robusta*, in the well or disc diffusion method (Fig. D.4.a). After a further 24 hour incubation, a variety of contaminating microbial colonies were observed to grow on the plates (Fig. D.4.b) with no inhibition exhibited by the extracts. All extracts diffused their colour into the media. Inhibition zones of less than 10 mm was considered ineffective (Lense, 2011). Interestingly, no inhibition of *E. coli* growth was also seen with the streptomycin discs even after 48 hours (Fig. D.4.c). Commercially obtained essential oils (EOs) of *L. scoparium* and *K. robusta* presented inconclusive antimicrobial activity against a few of the contaminating microbial colonies only (Fig.D.4.d). The prepared *E. coli* stock and the blank LB media plate incubated at the same temperature, represented no contamination (Fig. D.4.e).

A: After 24 hours

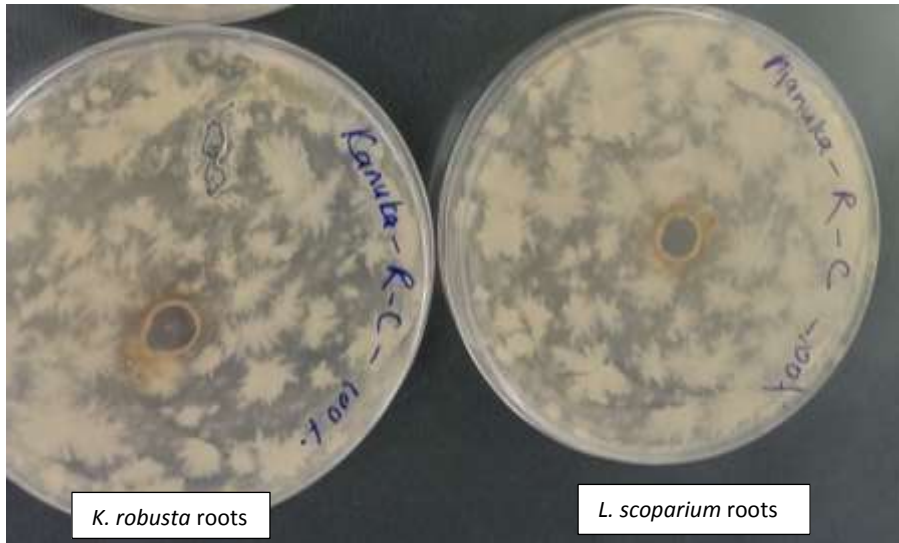


B) After 48 hours



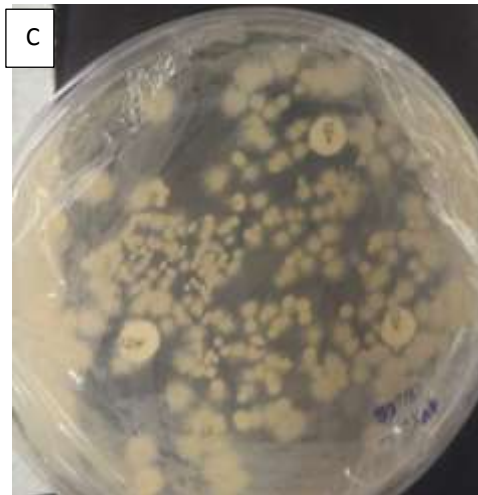
L. scoparium leaf

K. robusta leaf



K. robusta roots

L. scoparium roots



C

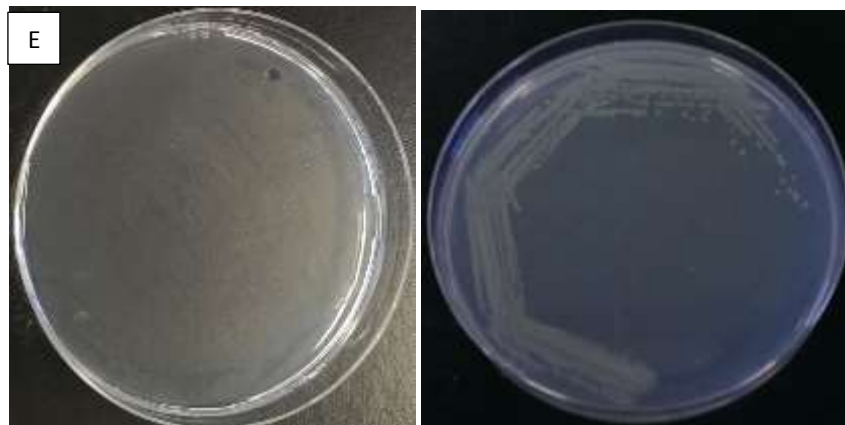
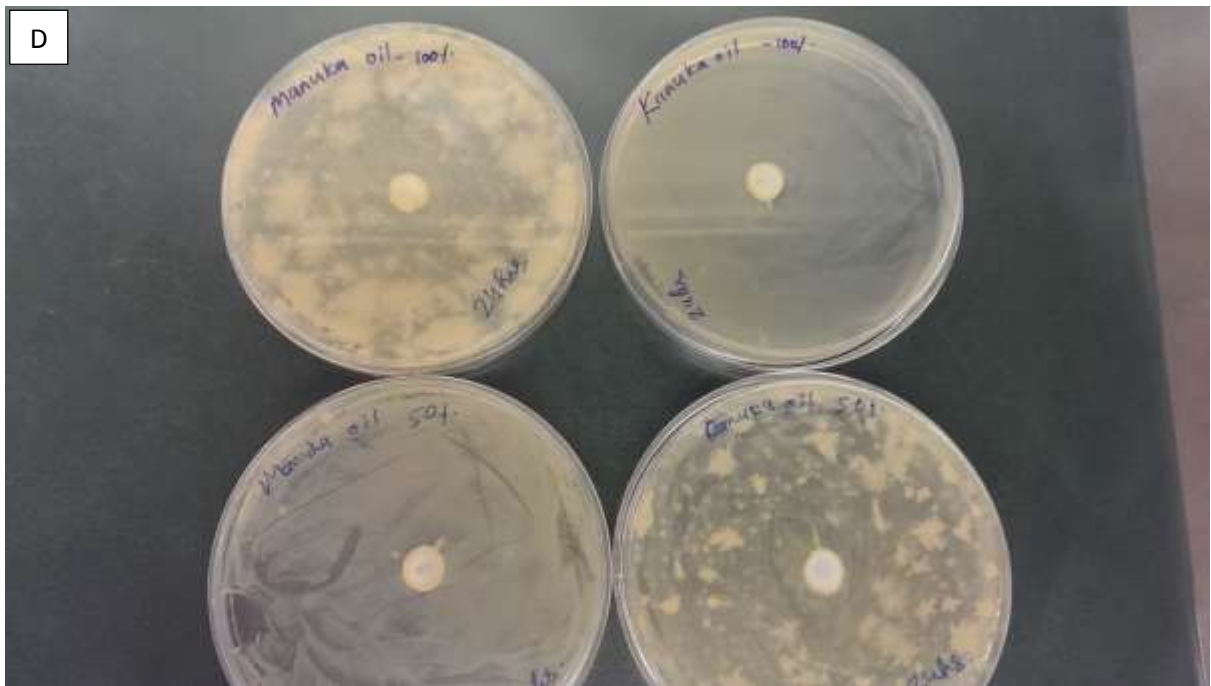


Figure. D.4. No *E. coli* inhibition was observed with the sterile water leaf or root extracts of *L. scoparium* or *K. robusta* after **a)** 24 hours and **b)** 48 hours in LB media. Streptomycin discs **(c)** exhibited no bacterial inhibition while essential oils of both *L. scoparium* and *K. robusta* **(d)** generated varying levels of bacterial inhibition at the two different concentrations used. No contamination appeared in the *E. coli* stock culture prepared or in the blank LB media plates **(e)**. Contaminating microbial colonies appeared in all plates after 48 hours.

4.1.2. Conclusion from method 1

No *E. coli* inhibition was exhibited by any of the extract at any concentration used. All LB plates indicated contamination with unwanted microbial colonies. No contamination in the blank media plate and the *E. coli* culture plate assured that the agar media and the *E. coli* cultures were not contaminated, but indicated that the extracts and essential oils were not sterile. The possible source of contamination could also be the transfer of the cultured media plates between buildings i.e. Biotron to Burns, as the

Health and safety approval was awaited and the required temperature incubator were not available into the building. No inhibition by the streptomycin discs and varying level of inhibition by the EO's against some unwanted colonies was unexplained. Although the no inhibition of the microbial growth by the streptomycin discs could be because of its specific bactericidal nature against gram negative bacteria and the unwanted colonies growing on the plates may not be gram negative and even not bacteria. The method was repeated thrice although no change in the results was observed. As the media was showing contamination, another media should be tested in further trials.

4.2. Method 2: Testing Difco™mfc media

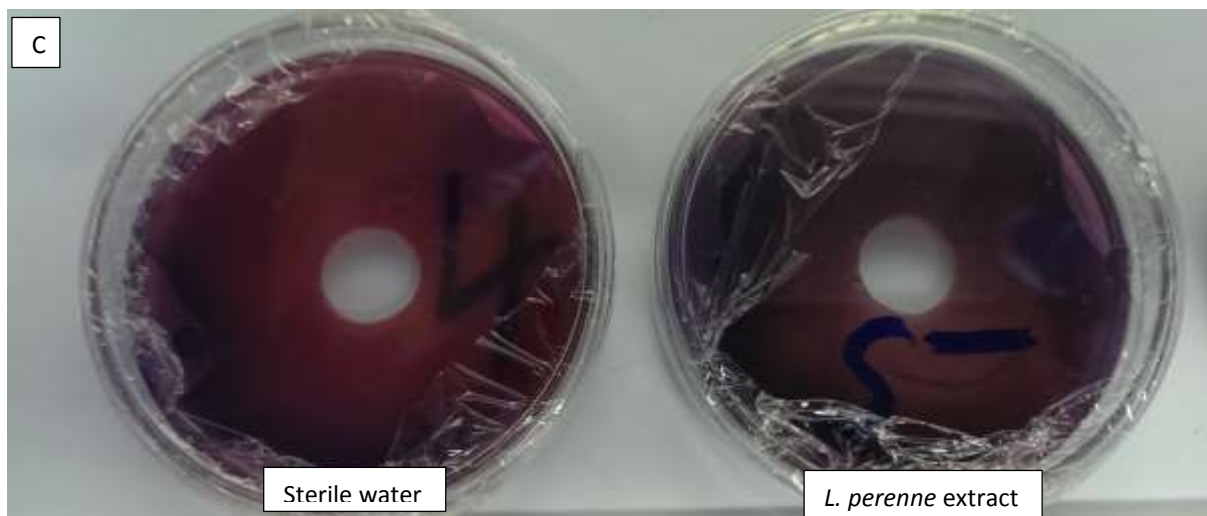
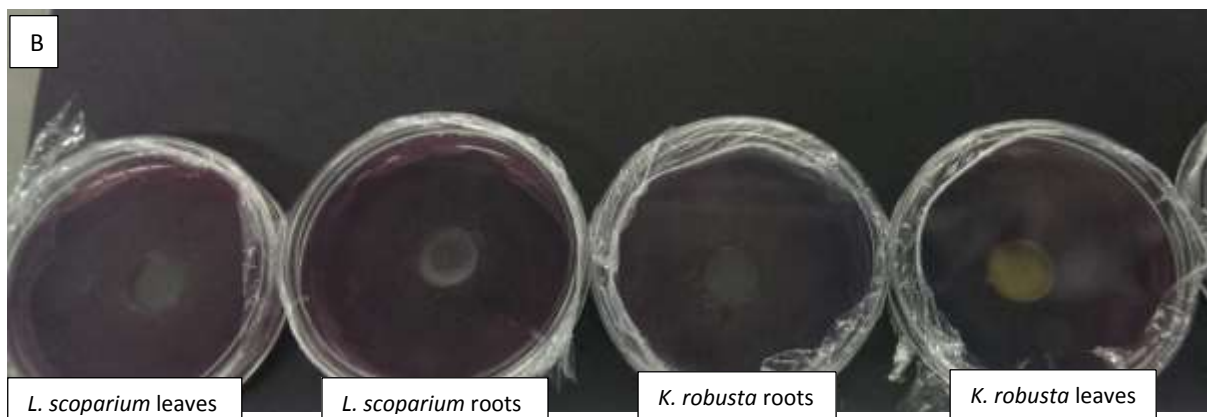
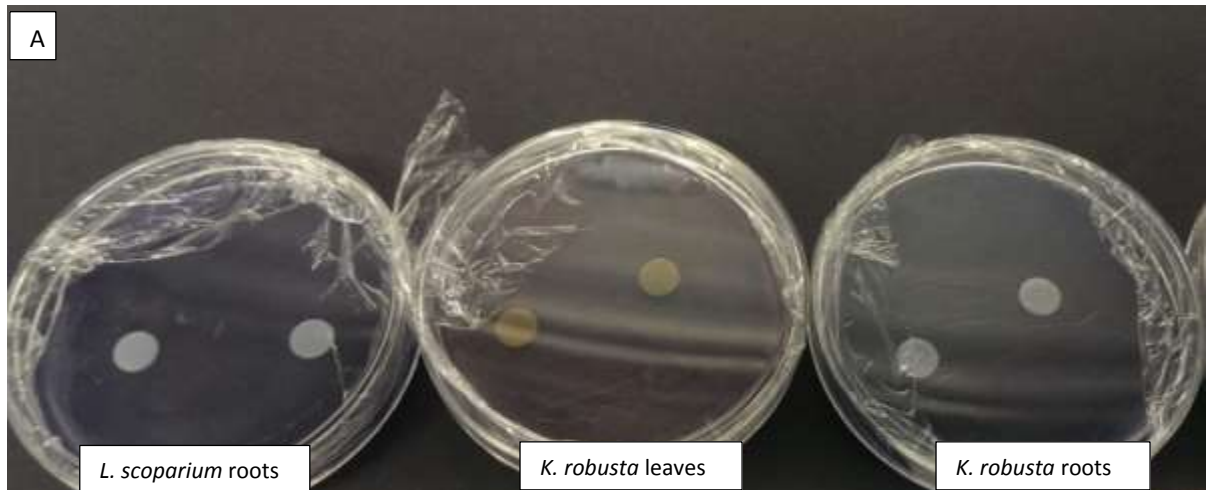
Lolium perenne leaves were collected from the Field Research Centre (FRC), Lincoln University. Method 1 for extract preparation was repeated for all extracts, with some modifications. After pressing leaves and roots to obtain extracts, samples were transferred into 30 ml centrifuge tubes and kept in a bench top shaker at 130 rpm for 4 hours. The leaf or root pellet was discarded and the supernatant obtained was separated using sterile tips in sterile vials under sterile conditions in a laminar flow hood and centrifuged at 1789 *g* for 10 minutes. The obtained supernatant was filtered and sterilised with 0.45 µm syringe filters and stored at 4°C if used within 24 hours or else frozen.

Both the well diffusion and disc diffusion methods were used for this trial. Because of no antimicrobial activity of Streptomycin discs used in method 1, another positive control i.e. Chloramphenicol (unknown concentration-obtained from the Bio-protection research Centre, Lincoln University) was tested in this method along with a Streptomycin solution prepared (1 g/µl). Negative control and the essential oils of *L. scoparium* and *K. robusta* were plated in similar manner as described in method 1. The same concentration and volume of extracts described in method 1 was used. This time the extracts were plated in medium to enumerate faecal coliforms (mfc) media plates (Difco™) obtained commercially prepared from Fort Richard Laboratories Ltd (Auckland, NZ). All plates were incubated at 25°C for 24 or 48 hours in dark.

4.2.1. Results of method 2

The mfc media did not support the growth of contaminating microorganisms like LB, but no inhibition zones from any extract was observed after 24 or 48 hours of incubation. The streptomycin treatment and essential oils resulted in bacterial inhibition (Fig. D.5). Commercially obtained streptomycin discs again did not result in any inhibitory zones as a few bacterial colonies were seen to grow around the discs (confirmed by Dr Hossein Alizadeh). No bacterial inhibition was seen with *L. perenne*, the negative control. The dark purple colour of the mfc media presented difficulty in accurately identifying or capturing the inhibition zones in pictures.

K. robusta oil was more effective with a mean inhibition zone of 13 mm compared with *L. scoparium* oil with an inhibition zone of 8 mm. The negative control (sterile water) supported full growth of purple coloured colonies which appear like *E. coli*, while the positive control chloramphenicol, a broad spectrum antibiotic, generated a 12 mm inhibition zone. However, as stated earlier, in the photographs the negative control looks similar to the positive control as the purple colour colonies were unidentifiable.



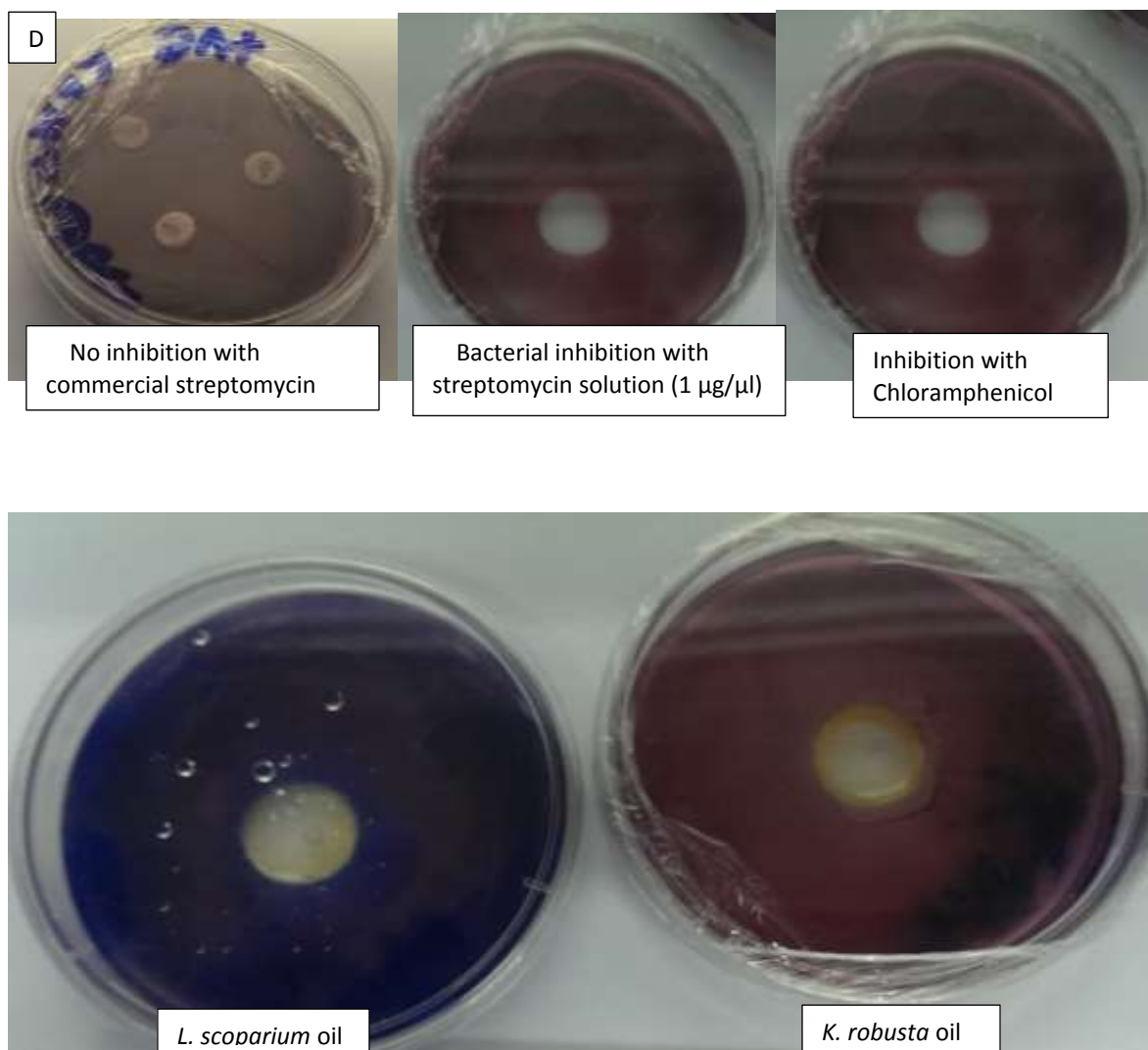


Figure D.5. No inhibition generated by the *L. scoparium* and *K. robusta* leaf or root extracts in the mfc media in both **a)** the disc diffusion method and **b)** the zone of inhibition method. No inhibition zone appeared with the *L. perenne* extract and sterile water **(c)** and **(d)** shows the inhibitory activities of all the positive controls.

4.2.2. Conclusion of method 2

No bacterial inhibition was observed with any of the extract. The mfc media did not allow the growth of any contaminating bacterial colonies, although identification of the inhibition zones in this purple media was difficult. Purple colour colonies growing in the negative controls (*L. perenne* and SW), confirmed the sterility of the *E. coli* culture. No growth inhibition by the streptomycin discs in this experiment confirms the results obtained in the LB media (method 1) and presents doubts about the activity of the discs. As both the chloramphenicol and streptomycin solutions prepared showed bacterial inhibition both were selected for use in the next trial to test the media. Some reasons for no bacterial inhibition observed with the extracts could be:-

- 1) The weak antimicrobial properties of the plants cannot be delivered using 100 µl of the extracts.

2) Water as a solvent which might not be able to effectively extract the compounds from the plant tissue. Gas chromatography (GC) study performed by another Ph.D. student (Saloomah Alikhani) has shown more effective chemical extraction using ethanol and dichloromethane solvents than water.

3) The high resistance of the leaves to grinding, might have lowered the efficiency in extraction from leaves and roots. The use of Liquid N for grinding the plant parts might help in improving the efficacy of the grinding.

Recommendations for modification for following experiments

Before testing different solvents, the selection of an appropriate media needs to be determined. Because LB media supported the growth of unwanted microbial species and the mfc media was not ideal for recognition of the inhibition zones because of its dark purple colour and also comes with a short expiry date, a search of another media was important. The use of 0.2 µm syringe filters was also selected for use in filtering the extracts for the next trials as these may eliminate any endophytes or surface attached microbes.

Working laboratory was changed before the third trial as the University health and safety committee determined the work could be carried out under PC1 (at BPRC) and general laboratory conditions with the non-pathogenic *E. coli* strain.

4.3. Method 3: Filtering extracts with 0.2 µm filters and testing Nutrient Agar (NA; Oxoid CM0003) media for *E. coli* growth

Nutrient agar (NA) was chosen for *E. coli* growth. For the positive controls, agar was prepared with and without the addition of the antibiotics, chloramphenicol and new prepared streptomycin solution (500 µl/500 ml), and then autoclaved. Commercially obtained streptomycin discs were also used separately in blank agar plates. All positive controls were inoculated using the bacterial lawn method. Interestingly, unlikely the previous two trials, the commercially obtained streptomycin discs were also active in this media and generated inhibition zones of 21 to 29 mm. Method 2 was repeated after filtering all the extracts used in the first two methods, using 0.2 µm syringe filters. *Escherichia coli* was exposed to the extracts in NA using the disc diffusion method (used in methods 1 and 2) and incubated at two different temperatures, 25°C and 30°C, in single replicate. No contaminating bacterial growth was observed after three days of incubation on any of the plates. Although there was no contamination, none of the extracts inhibited *E. coli* growth. The method was repeated two times using single replicates and as no contamination appeared as in methods 1 and 2, NA was chosen as the best media for this study.

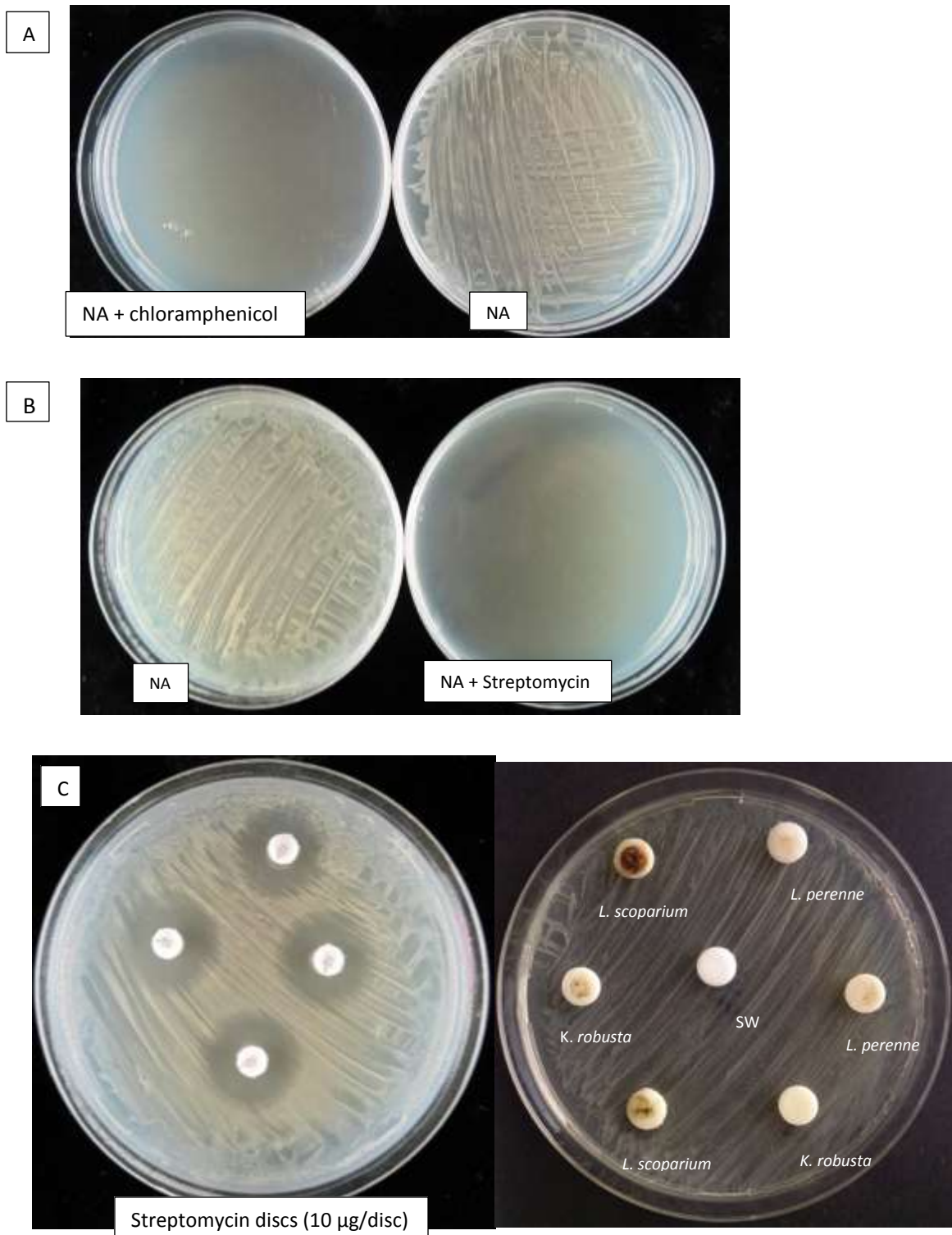


Figure D.6. *E. coli* inhibition in Nutrient Agar plates **A)** with and without chloramphenicol and **B)** with and without Streptomycin, **(C)** commercial streptomycin discs, and **(D)** sterile water extracts of *L. scoparium*, *K. robusta* and *L. perenne*.

4.3.1. Conclusion from method 3

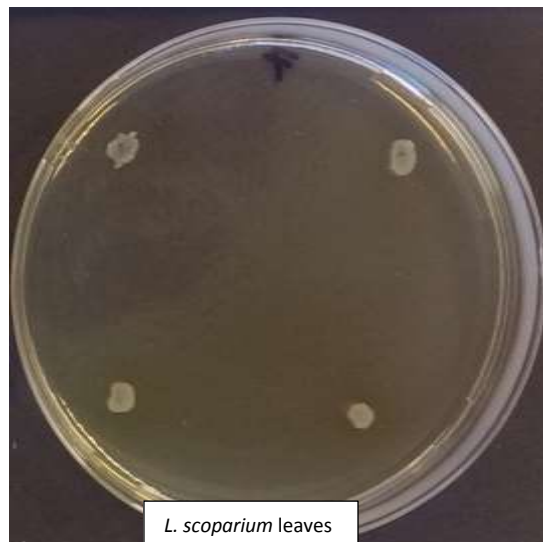
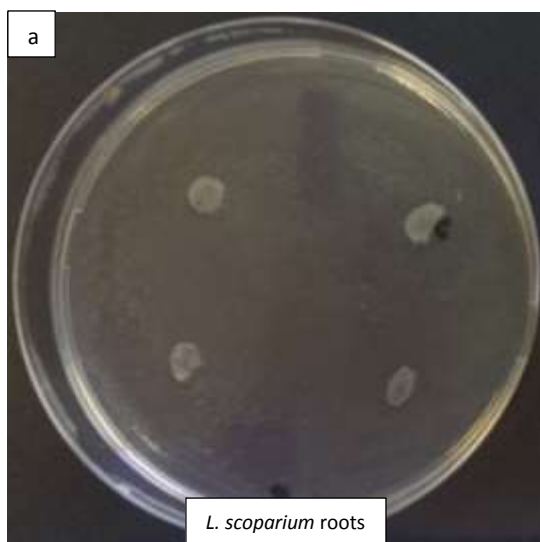
Nutrient agar is the best media for this study. In future trials all extracts should be filtered with 0.2 µm syringe filters only.

4.4. Method 4: Testing high extract concentrations against *E. coli* using pour plate method

To further test for any antimicrobial properties of the plant extracts, sterile water plant extracts were used in larger volumes i.e. 1 ml compared with the 100 μ l used previously, using the pour plate method. One ml of each extract was poured into the Petri plates. Liquid agar (cooled but not solidified) was poured over the extracts in the Petri plates and the plates were shaken gently for ten seconds to uniformly mix the extract and the agar and allowed to set for 2 hours. The negative controls, sterile water and *L. perenne* extract, were inoculated in similar manner. An uninoculated NA plate was used as a blank. The positive controls chloramphenicol and streptomycin were mixed with the agar before autoclaving (as in method 3) and once poured into the Petri plates were allowed to set for two hours. Four *E. coli* dots were inoculated equidistance, 1 cm from the edge of the Petri plates using a sterile loop and plates were incubated at 30°C for 24 hours. Essential oils were not used here, as their strong antibacterial activity had already been determined in the previous methods. This method was repeated twice in triplicates.

4.4.1. Results of method 4

Similar to the previous trials, none of the extracts of either *L. scoparium* or *K. robusta* inhibited the growth of *E. coli* in NA, being similar to the negative controls i.e. sterile water and *L. perenne*. The positive controls inhibited *E. coli* growth to below the detection limit i.e. no bacterial colonies appeared.



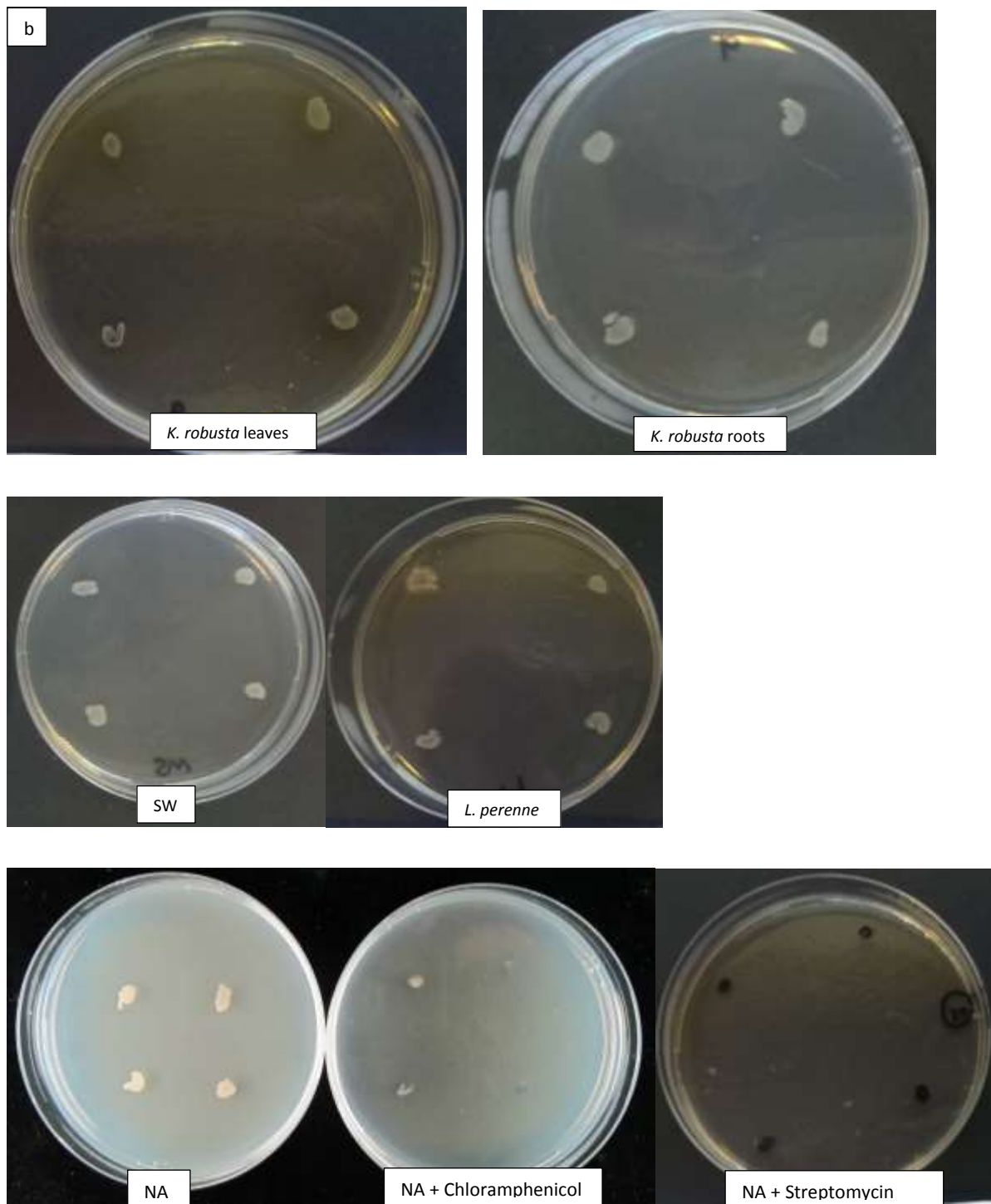


Figure D.7. *E. coli* growth exposed to the sterile water root and leaves extracts of **a)** *L. scoparium*, **b)** *K. robusta*, **c)** negative controls and **d)** blank (NA) and positive controls.

4.4.2. Conclusion from method 4 and objectives for next trial

The no inhibition by sterile water extracts of *L. scoparium*, *K. robusta* and *L. perenne* confirms that either these South Island plants have no or weak antibacterial properties or the effective antibacterial components of the plants could not be extracted with SW. Hence, extraction using different solvents

might present a better understanding of the antibacterial properties of the plants. Because leaves are resistant to grinding using a pestle and mortar, the use of liquid N for grinding the leaves and roots may be more effective in the production of a fine biomass powder.

4.5. Method 5: Ethanol extraction of leaves and roots

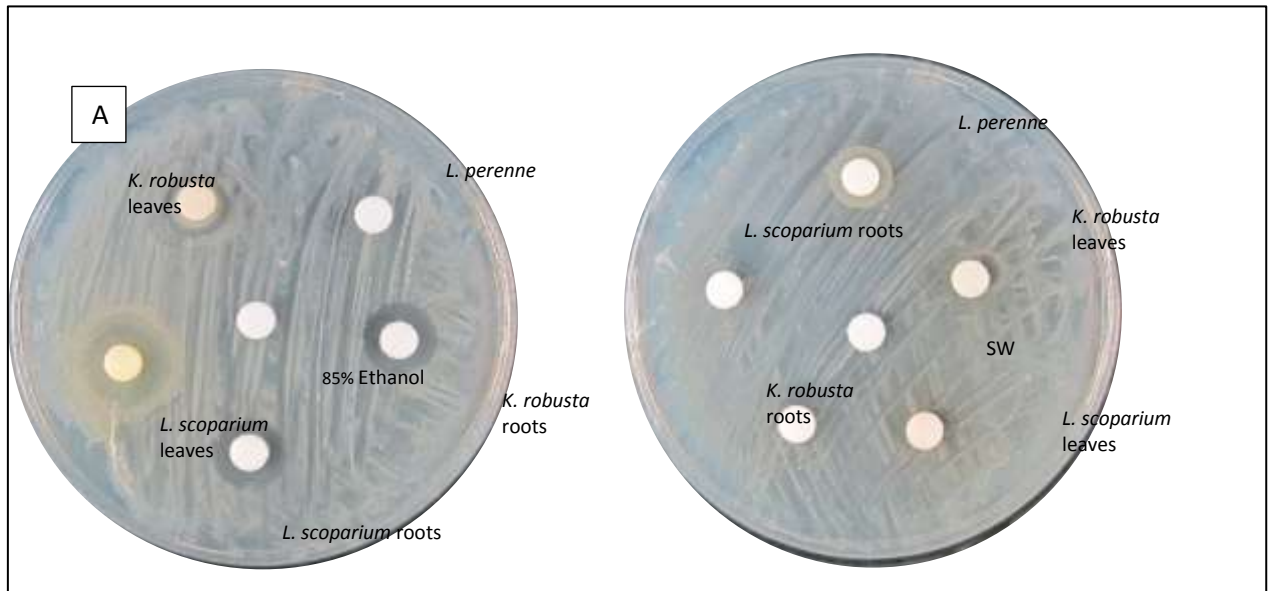
Methods 2 and 3 were repeated using 85% ethanol extracts, and a fresh batch of sterile water extracts from new plant material were also prepared. This experiment was repeated first, in single replicates (Fig.D.7.a) and later in triplicates (Fig. D.7. b and c). The new set of plants (13 months old) were harvested from the glasshouse (grown by another student as control) and leaves and roots were immediately sealed in zip-lock bags and in this case kept on ice until processed. Plant parts were washed thoroughly three times under tap water and finally with DI water and air dried for two hours on clean paper towels. Liquid N was used to grind the leaves and roots, and the powder obtained was immediately mixed with sterile water or 85% ethanol and maintained on ice until used within 24 hours (or frozen after 24 hours). All extracts were shaken in a flask shaker for 40 minutes at 350 oscillations per minute and centrifuged at $161 \times g$ at 4°C for 20 minutes. The supernatant was removed and placed in sterile vials using clean pipette tips and filtered sterilised using $0.2 \mu\text{m}$ syringe filters under sterile conditions in a laminar flow hood. Bacterial culture was prepared by mixing two *E. coli* colonies in 5 ml Ringers and vortexed for 2 minutes. A $100 \mu\text{l}$ bacterial aliquot was spread plated on NA plates and 6 mm sterile plain paper discs (single or triplicates per plate) were first placed onto the plates. Fifty μl of one of the extracts was then pipetted onto the discs and the plates were left undisturbed in the laminar flow for 30 minutes. Essential oils of both plants were included as the positive controls. All plates were sealed and incubated at 30°C for 24 hours in dark. After observing the results, all plates were further incubated for 3 days at 30°C to observe any changes (Fig. D.8.a and b).

4.5.1. Results of method 5

During the first run with single replicates (Fig. D.7.A) excellent bacterial growth inhibition was obtained with the 85% ethanol extracts. The ethanol extract of both *L. scoparium* and *K. robusta* inhibited *E. coli* growth and root extracts of both plants were more active than the corresponding leaf extracts. *Lolium perenne* and 85% ethanol (used as control) generated no inhibition. The sterile water extracts as with the previous trials resulted in no inhibition from any extract. Interestingly, sterile water extract of *L. perenne* generated a zone of increased bacterial growth.

Unlikely as in the first run (Fig.D.7.A), when the trial was repeated using triplicates (Fig. D.7.B and C) for each treatment, the diameter of the extract treatments were similar to the controls plates (SW or 85% ethanol). Ethanol and sterile water used as controls, also generated inhibition zones (Fig. D.7.B and C). Essential oil of *K. robusta* was more active against the tested *E. coli* strain than *L. scoparium*

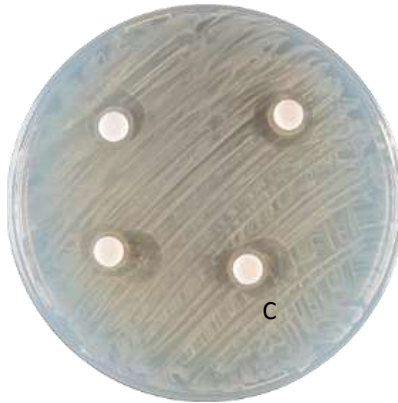
essential oil generating 17.0 and 11.6 mm of inhibition zones, respectively (Fig.D.7.D). Commercial streptomycin discs generated an inhibition zone of 19.1 mm while the prepared solution of streptomycin generated a 33.3 mm inhibition zone.



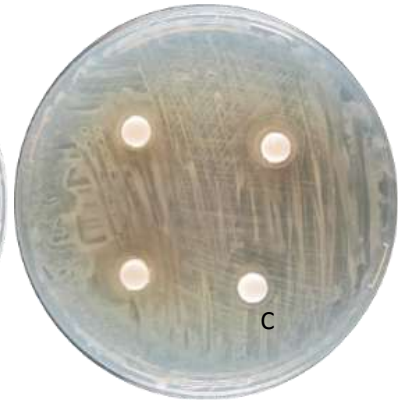
B. Sterile water extracts



L. scoparium leaves



L. scoparium roots



K. robusta leaves

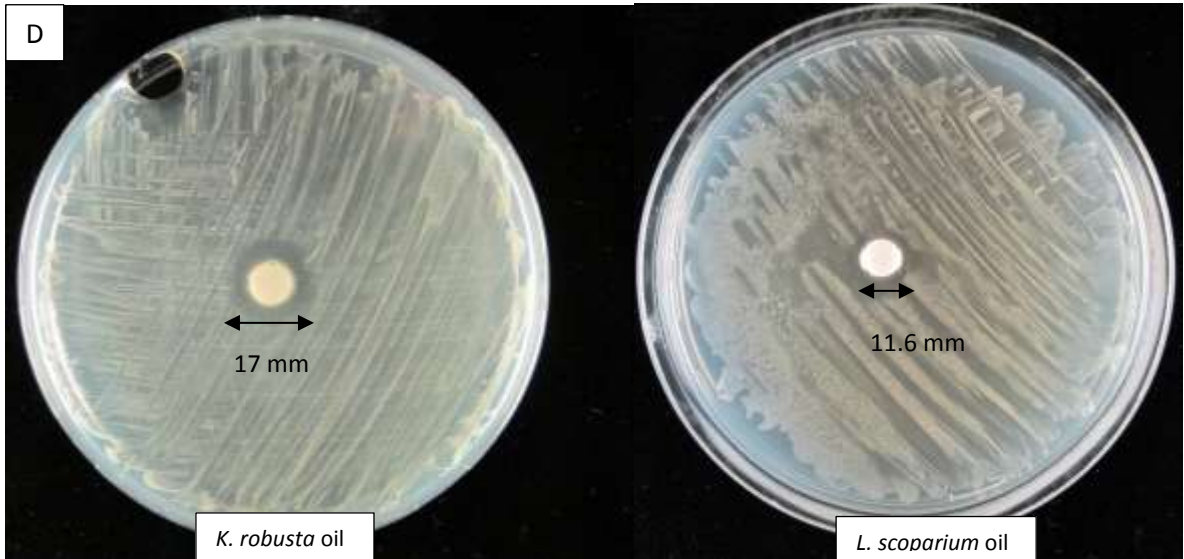
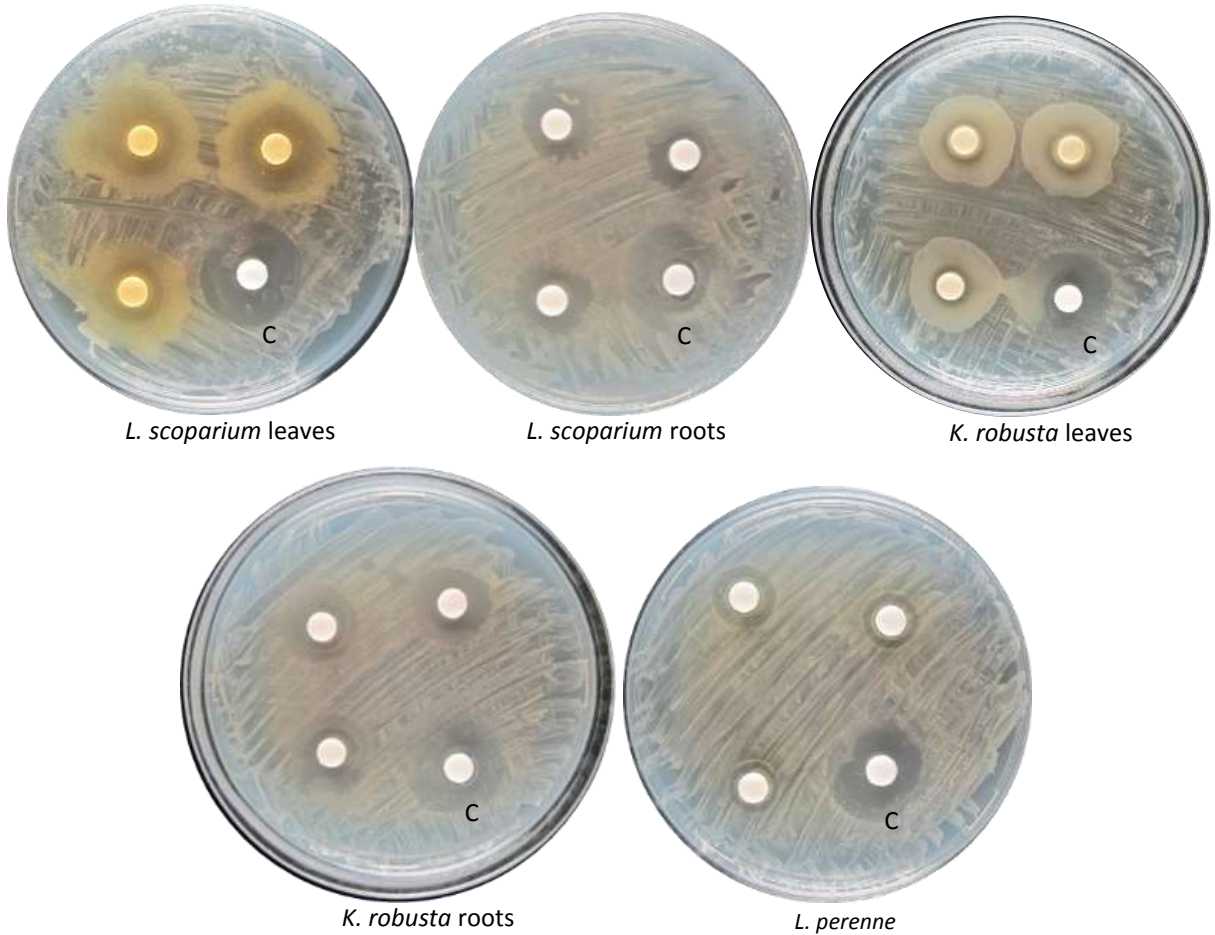


K. robusta roots



L. perenne

C. 85% Ethanol extracts



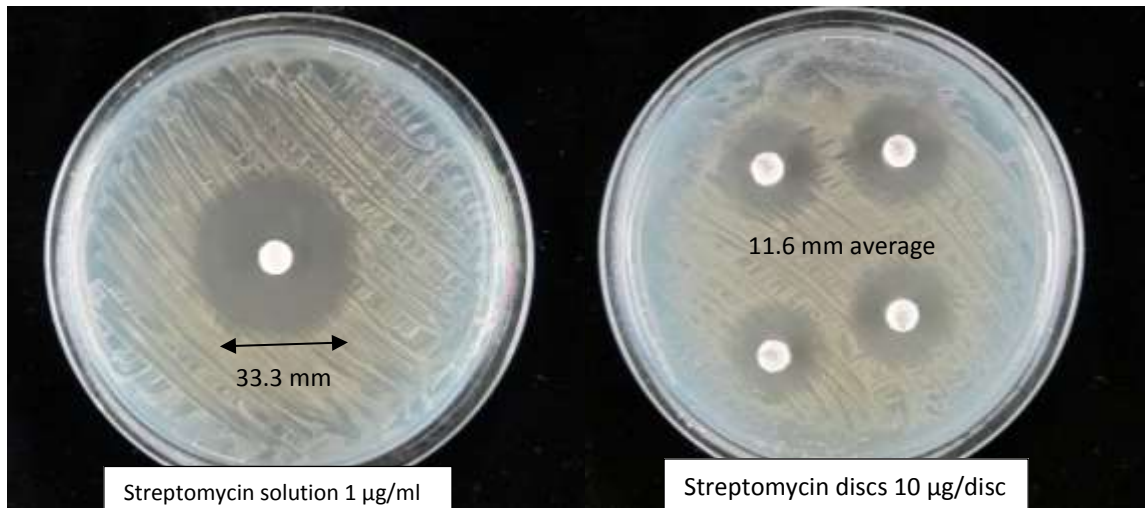


Figure D.7. Shows the *E. coli* inhibition zones obtained after plating 50 µl of **A)** 85% ethanol and sterile water extracts during the first run, **B)** sterile water extracts in second run, **C)** 85% ethanol extracts in second run, and **D)** all the positive controls. “C” represents solvents plated as control.

When all the plates were incubated for a further three days, on top of the initial one day incubation, inhibition zones around the ethanol discs, used as a control, started to be covered with bacterial growth while slight inhibition zones were still observed around the *K. robusta* extract discs. No inhibition zones were observed for any of the sterile water extracts.

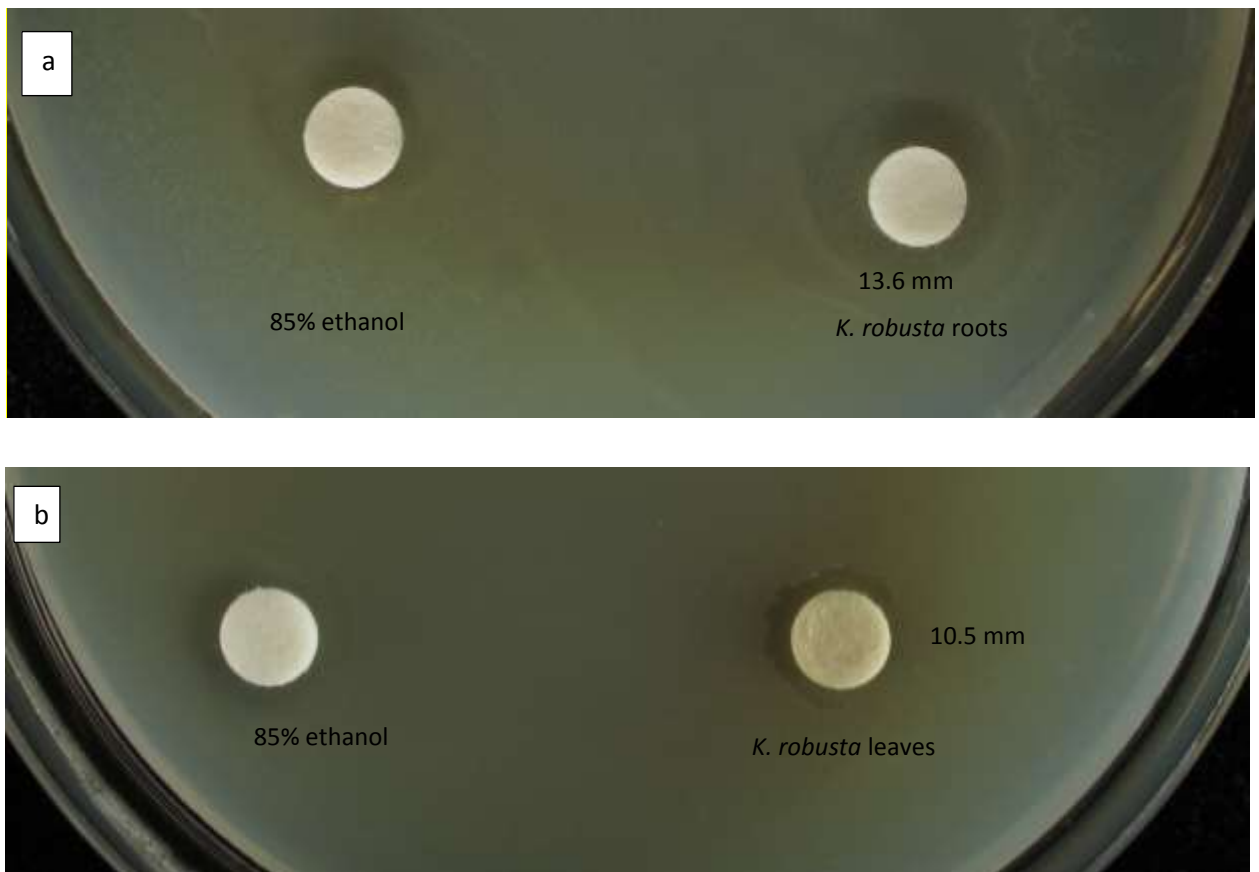


Figure D.8. Shows coverage of the inhibition zones of *E. coli* obtained from 85% ethanol used as control and the active zone of inhibition around *K. robusta* root **(a)** and **(b)** leaf extracts respectively after three days (on top of the initial one day incubation).

4.5.2. Conclusions from method 5 and objectives for next trial

When 85% ethanol extracts were plated in single replicates, the ethanol control generated no inhibition zone while all extracts inhibited *E. coli* growth showing effective extraction of antibacterial compounds with ethanol. When this experiment was repeated in triplicates the inhibition by the controls was observed. Because the sterile water (control) also generated a zone of inhibition, it was concluded that the zones obtained might be a result of the washing of the bacterial inoculum from the areas immediately surrounding the discs by the relatively high volume of extracts applied onto the discs. As the ethanol itself inhibited *E. coli* growth, any inhibition obtained from extracts could not be considered true.

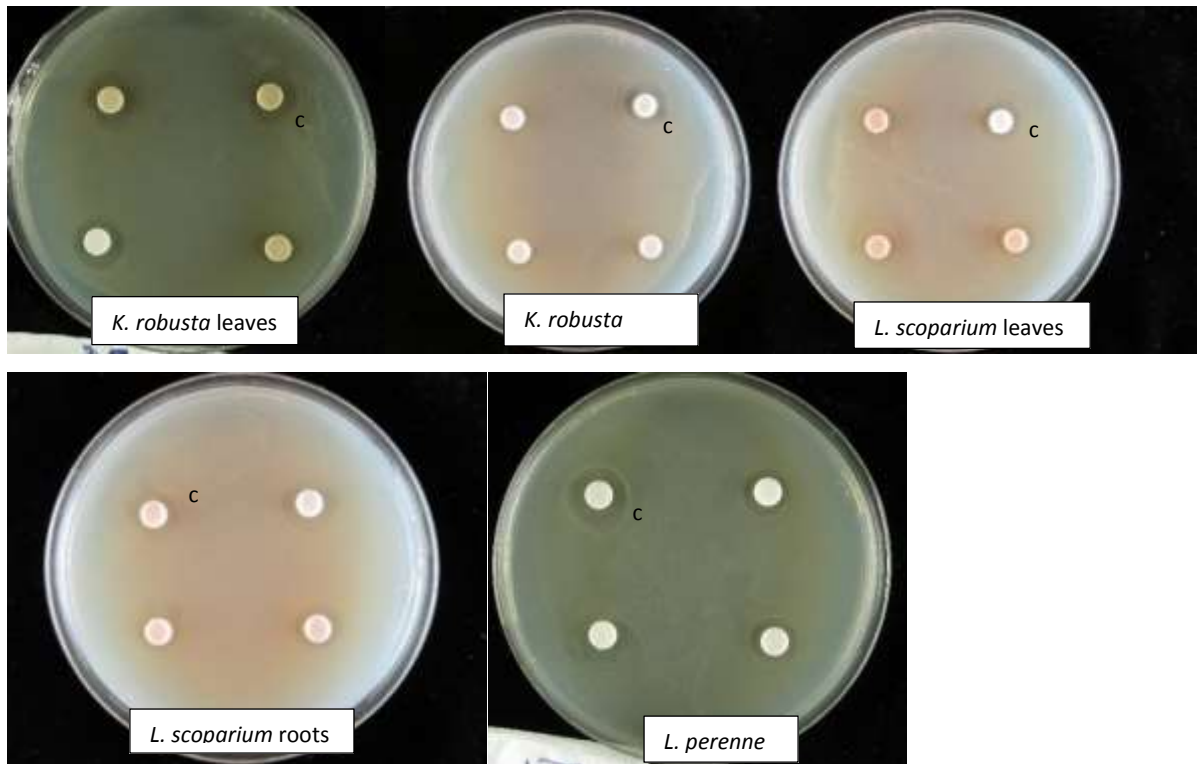
The appearance of colonies within the inhibition zones around the 85% ethanol (control) after 3 days of incubation could be due to the evaporation of the ethanol during this time allowing the regrowth of *E. coli*. The small zone of inhibition around both *K. robusta* root and leaf extracts demonstrates low antibacterial activity of *K. robusta* extracts and its high activity at inhibiting *E. coli* growth compared

with *L. scoparium*. The results of this trial confirms the effectiveness of ethanol as a solvent, although its toxicity against *E. coli* does not support its use as a control or solvent. A lower volume of 25 μ l extract was considered for next trial.

4.6. Method 6: Repetition of method 5 with 25 μ l extracts

Although the 25 μ l volume plated (Fig. D.9) eliminated any washing effect of the bacterial colonies obtained with either ethanol or sterile water in Fig. D.7 B and C. Sterile water extracts did not inhibited *E. coli* growth (D.9.A). 85% ethanol used as control inhibited the growth of *E. coli*, indicating it lack of suitability as a solvent for extraction (Fig. D.9.B).

A. Ethanol extracts plated in 25 μ l



B. Sterile water extracts

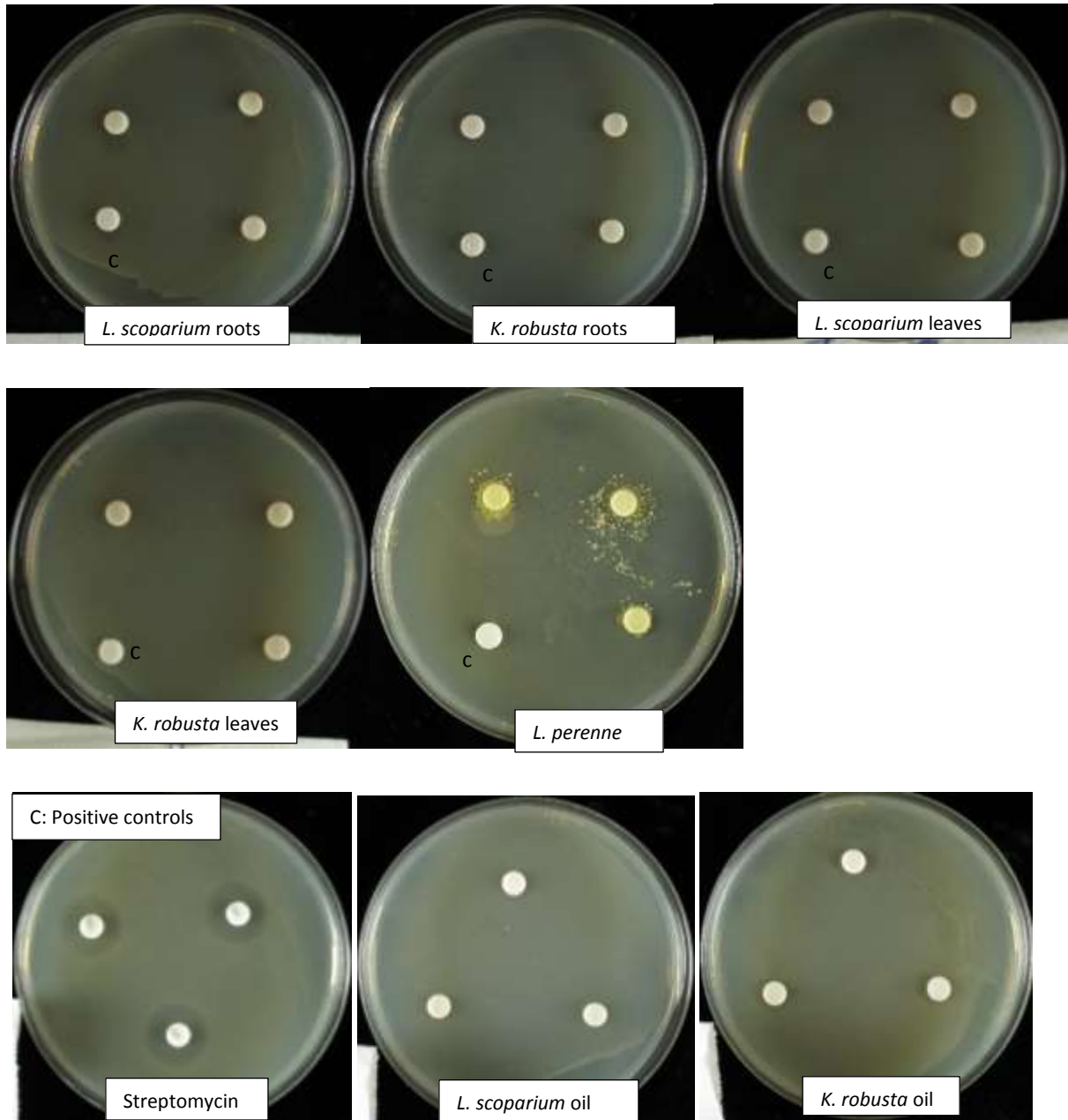


Figure D.9. *E. coli* inhibition zones obtained with 25 μ l **A)** sterile water extracts **B)** 85% ethanol extracts and **C)** positive controls. C= sterile water or 85% ethanol negative controls.

4.6.1. Conclusion from method 6 and objectives for next trial

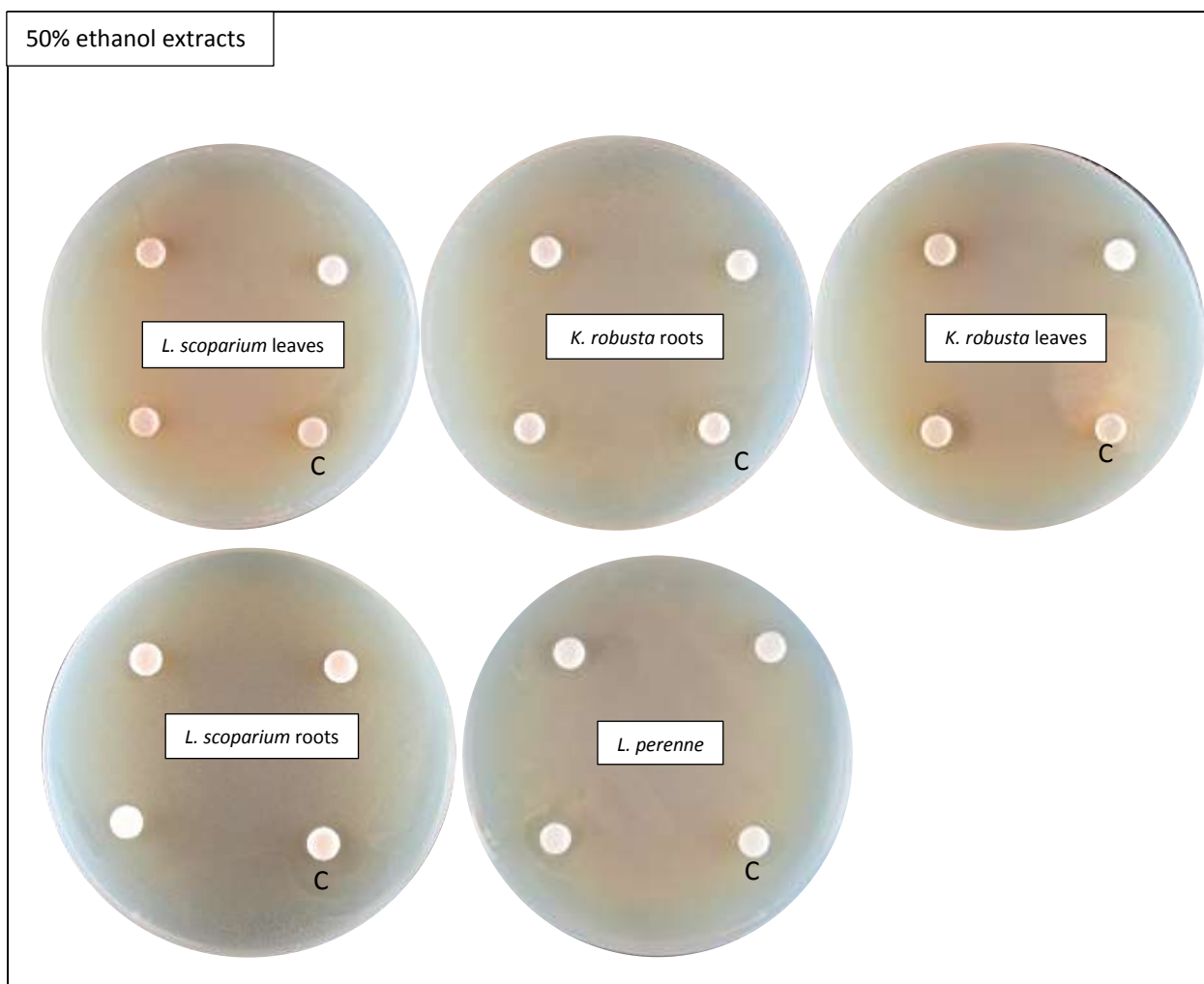
The 25 μ l volume eliminated the washing off of the *E. coli* by the plated extracts although 85% ethanol inhibited *E. coli* growth. None of the sterile water extracts inhibited *E. coli* growth. Lower ethanol concentrations should be used tested for extract preparation to reduce its toxicity against *E. coli*.

4.7. Method 7: Extract preparation with 50% ethanol

Extracts were prepared using 50% ethanol following method 5 description. All extracts were plated at 25 μ l extract volume on 8 mm discs and plates were left under a laminar flow hood for 20 to 30 minutes to allow the ethanol to evaporate and then incubated at 30°C for 24 hours in the dark. This method was repeated twice in triplicates.

4.7.1. Results from method 7

Inhibition zones (a few mm's) were obtained with the 50% ethanol control, and the extracts had inhibition zones equal to the control (Fig. D.9). The positive control, Streptomycin discs were active generating an average inhibition zone of 17.4 mm followed by essential oils of *L. scoparium* and *K. robusta* generating an average inhibition zone of 9.5 mm and 10.5 mm, respectively.



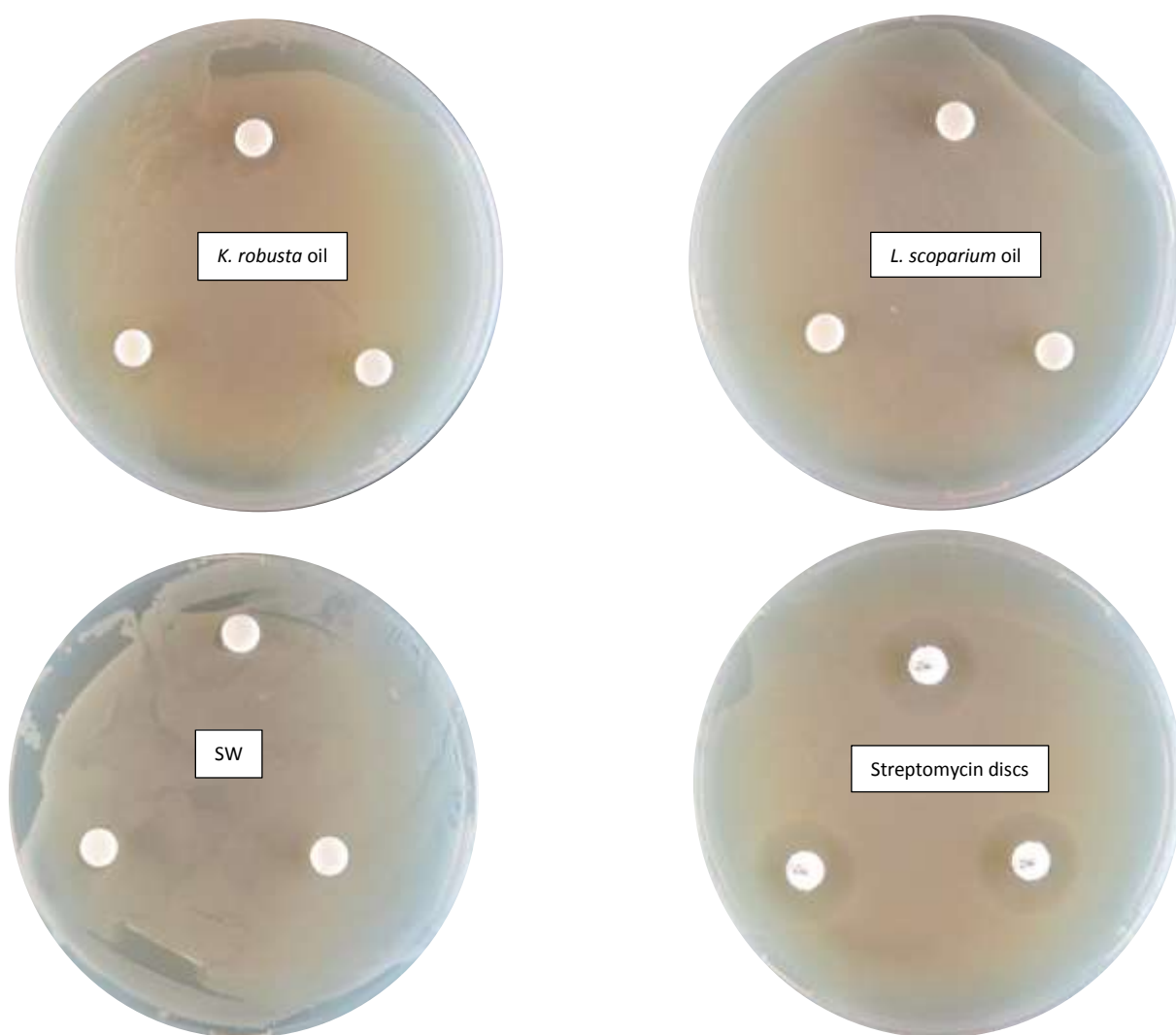


Figure D.9. *E. coli* inhibition zones obtained with the 50% ethanol extracts and the positive and negative controls. C = Control.

4.7.2. Conclusion from method 7 and objectives for next trial

The inhibition zones obtained with ethanol suggests that it cannot be used as a solvent for extraction. The inhibition zones produced by the extracts might be because of the toxicity of ethanol against *E. coli* rather than their antibacterial activity. Other solvents were tested in next trials.

4.8. Method 8: Extraction using different polar solvents

As only the EO's of both plants effectively inhibited *E. coli* growth, solvents were chosen on the basis of oil and solvent mixtures so that the antimicrobial compounds present in the EO's can be extracted effectively from leaves and roots.

4.8.1. Solvent selection

Sterile water, ethanol (85%), 100% dichloromethane (DCM), 100% methanol, Tris buffer 10 mM and Tween 40 were chosen and the EO's of both *L. scoparium* and *K. robusta* were mixed with these solvents at a 1:1 (v/v) ratio and vortexed for one minute. Each mixture was allowed to settle for five minutes and the phases were carefully observed (Fig. D.10). Sterile water, 85% ethanol and Tween 80 generated two separate phases (or no mixing with the oils) in the mixture and hence, ethanol and Tween 80 were excluded from further trials. Sterile water was used even though it also generated two phases with the oil because it is likely in the soil that the plant active compounds will be eluted in water, and therefore was relevant for this research and from environmental perspectives. Hundred percent ethanol was also suggested to be used, hence, it was also used for extract preparation.

All extracts were obtained following the method outlined in method 5 as a 1:8 dilution (w/v) and later as a 1:2 dilution (w/v). Extracts were plated both on 6 mm and 8 mm paper discs in single replicates and were incubated at 30°C.

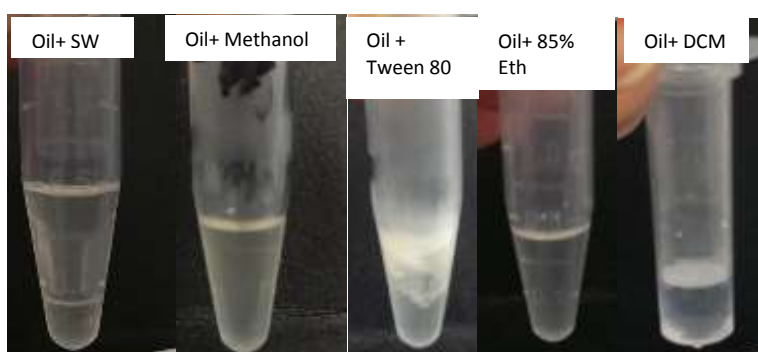


Figure D.10. Phases obtained with different solvents mixed with *L. scoparium* essential oil at a 1:1 ratio.

4.8.2. Results of method 8

Results obtained from both concentrations of the extracts i.e. 1:4 dilution or 1:8 dilution with the solvent, were similar. Inhibition zones of less than 10 mm diameter were considered ineffective. Sterile water and Tris buffer extracts generated negative antibacterial activity. Ethanol, methanol and DCM alone inhibited bacterial growth. Similar to the results of method 5, inhibition zones were slowly recolonised by the bacterial colonies around the ethanol control after three to five days but a 7 mm to 9 mm inhibition zone around the *K. robusta* extracts (root and leaf) showed the *K. robusta* extracts to have weak antibacterial activity against *E. coli* (not shown).

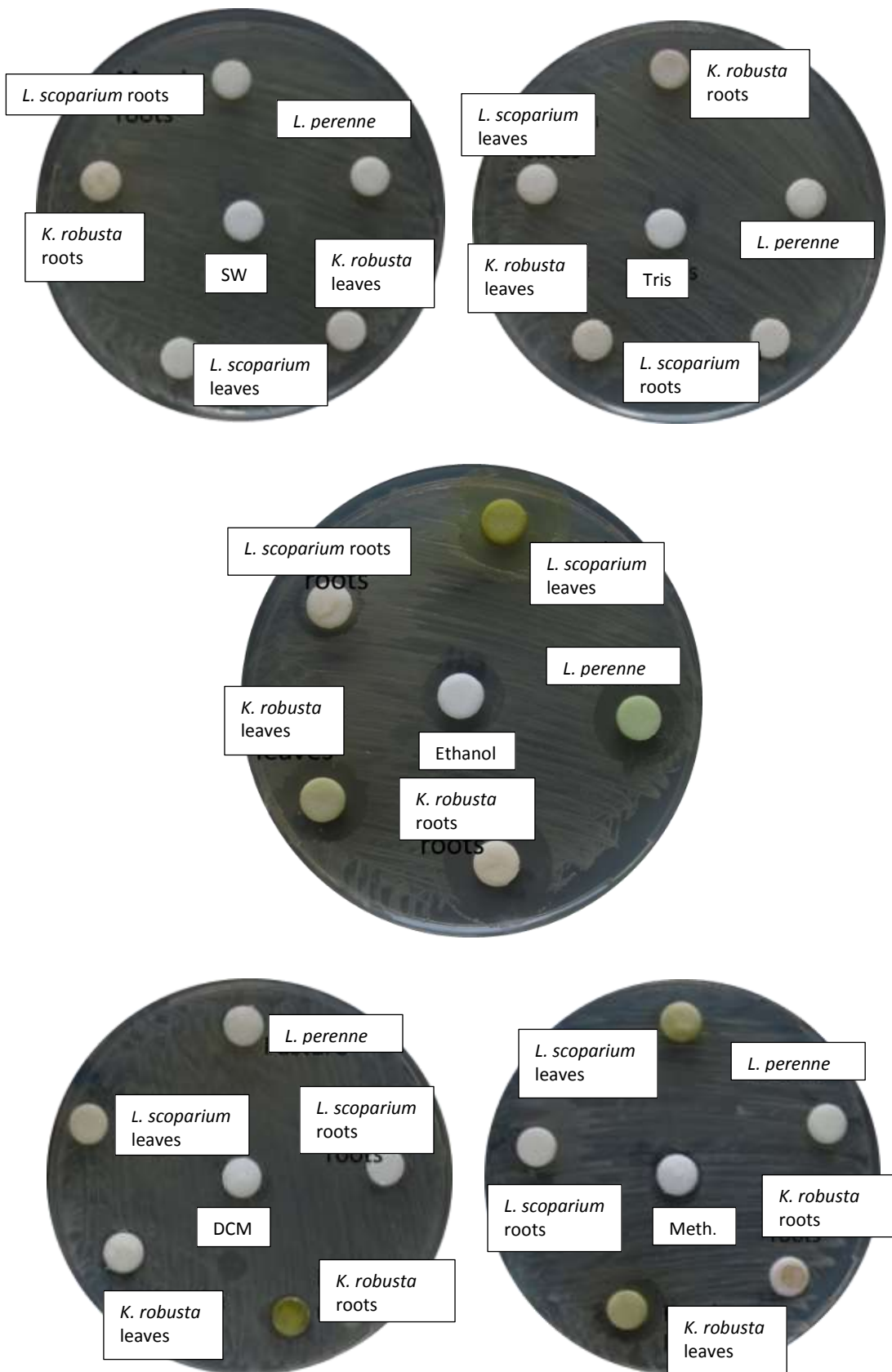


Figure D.11. *E. coli* Inhibition zones obtained with sterile water, 10 mM Tris buffer, 100% ethanol, dichloromethane and 100% methanol extracts of *L. scoparium*, *K. robusta* and *L. perenne*, after 24 hours of exposure. SW = Sterile water, Meth = methanol, DCM = dichloromethane controls.

4.8.3. Conclusion of method 8 and objectives for next trial

Methanol, ethanol and DCM alone resulted in inhibition of *E. coli* and hence, cannot be used as solvents. Tris buffer and sterile water extracts resulted in no inhibition against *E.coli* hence would not be effective solvents for extracting from the plant material.

To avoid inhibition by the controls, dipping the sterile paper discs into the extracts using a sterile tweezer and allowing to air dry for two minutes before placing onto the *E. coli* inoculated NA plates is recommended. The pour plate method, used in method 4, would increase the evaporation of the solvents for the controls as well as using higher concentrations of the extracts.

4.9. Method 9: Eliminating solvent effect

The pour plated method was performed with 100% ethanol, 100% DCM and Ethanol: DCM (1:1) mixture extracts. Extracts were prepared with ethanol, DCM or ethanol: DCM mixture following the methods outlined in method 5. One ml of the extracts were used for amending the agar using the pour plate method with the remainder of the extracts were placed in 100 ml wide mouth sterile vials in a fume hood and the solvents were allowed to evaporate i.e. DCM (3 hours), ethanol (overnight). The pellets obtained after solvent evaporation was divided (approximately) into three parts into sterile Eppendorf tubes and each part was mixed with either 50 µl SW, 85% ethanol or DCM. The pellet and solvent mixture was vortexed for 2 minutes and extracts were plated following the **1)** paper disc method and **2)** paper discs dipped into the extracts or controls and allowed to air dry for 2 minutes before plating onto the NA plates inoculated with *E. coli* in duplicates. Plates were incubated at 30°C.

4.9.1. Results from method 9

4.9.1.1. Pour plate method

All ethanol: DCM extracts (1:1 volume) and the respective controls resulted in corrosion of the plates. No inhibition was obtained by any of the ethanol: DCM solvent extracts (Fig. D.12.A) or the 100% ethanol extracts (results not shown). As expected from the previous trials, the ethanol controls generated inhibition (Fig. D.12.B).

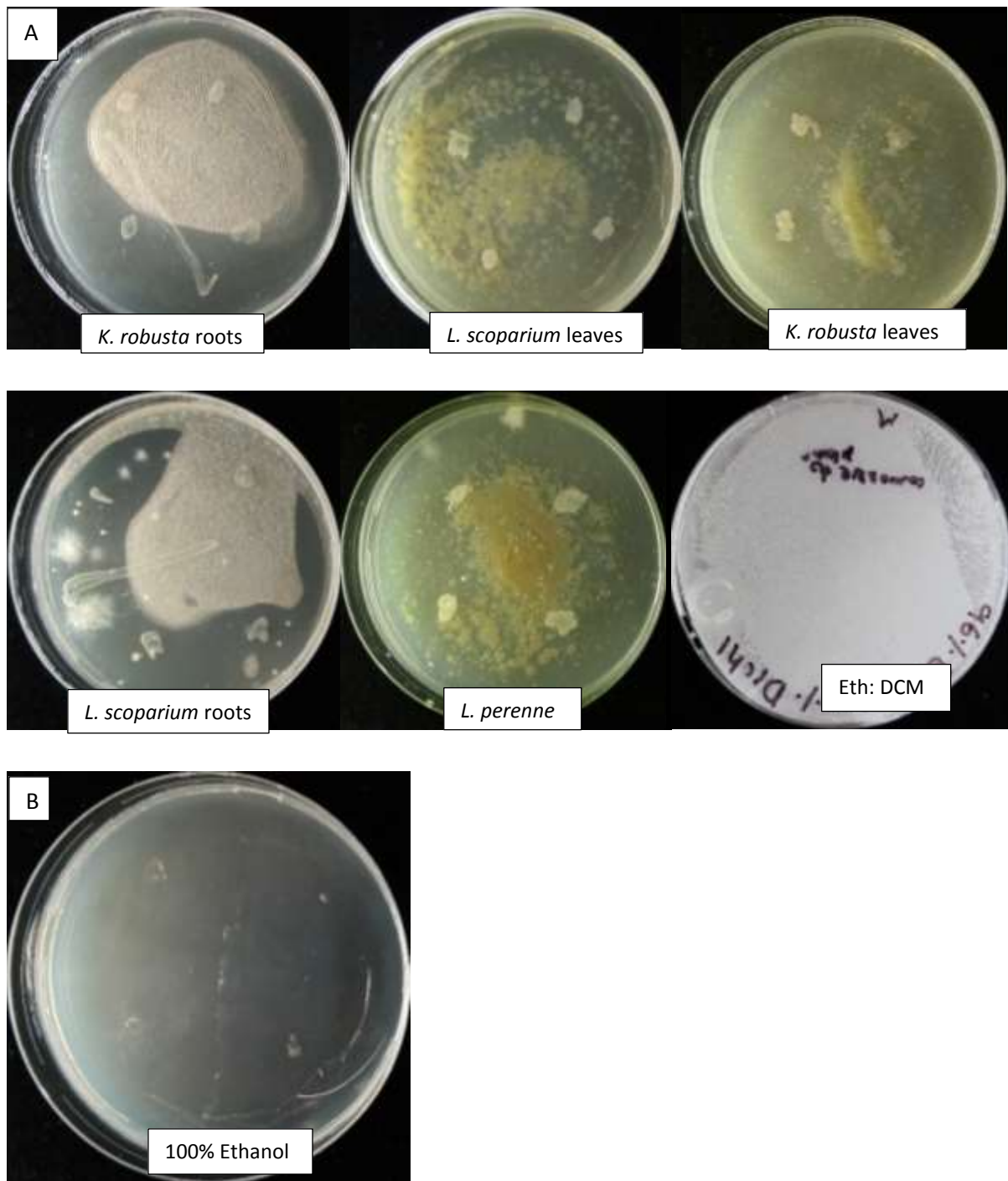


Figure D.12. Corrosion of the plates after pour plating ethanol: DCM extracts (A) and *E. coli* growth inhibition observed after pour plating 100% ethanol as control (B).

4.9.1.2. Paper disc method and air drying of extracts dipped paper discs

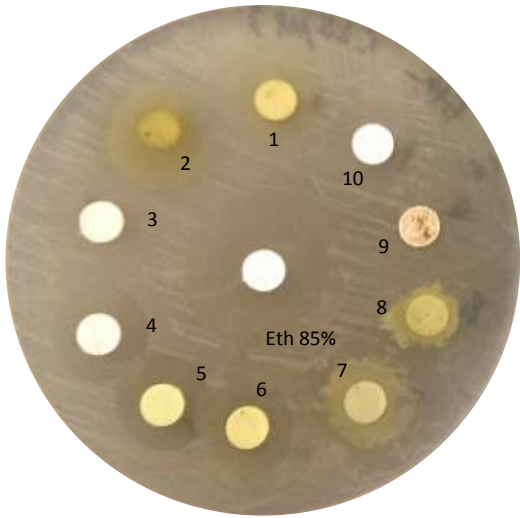
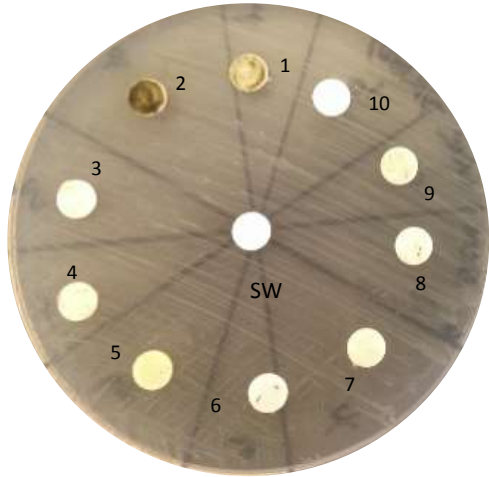
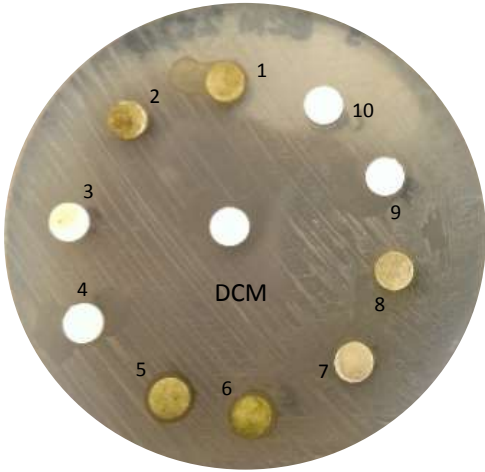
There was no difference in the results obtained with either direct plating (Fig. D13.A) or air drying of the extracts on the paper discs (Fig.D.13.B). Similar to the pour plate method, DCM extracts resulted in plate corrosion and no inhibition of *E. coli* including the DCM control. Ethanol: DCM extracted extracts were not tested with the paper disc method. Ethanol (85%) control again resulted in bacterial growth inhibition. Although similar to previous trials (Fig. D.8), the inhibition zone produced by the

ethanol plated as control was later (after three days) recolonised by *E. coli* although the ethanol extracted *K. robusta* extracts had a 10.9 mm inhibition zone active after three days, demonstrating antibacterial components extraction from *K. robusta* using ethanol (picture not shown).

Table D.2. Description of the extracts plated in Fig. D.13 (A and B) and the solvents used for extract preparation.

Sample ID	Extracts	Extracted with
1	<i>L. scoparium</i> leaves	100% Ethanol
2	<i>K. robusta</i> leaves	
3	<i>L. scoparium</i> roots	
4	<i>K. robusta</i> roots	
5	<i>L. perenne</i>	
6	<i>L. scoparium</i> leaves	Dichloromethane (DCM)
7	<i>K. robusta</i> leaves	
8	<i>L. scoparium</i> roots	
9	<i>K. robusta</i> roots	
10	<i>L. perenne</i>	

A: 25 μ l extracts onto discs



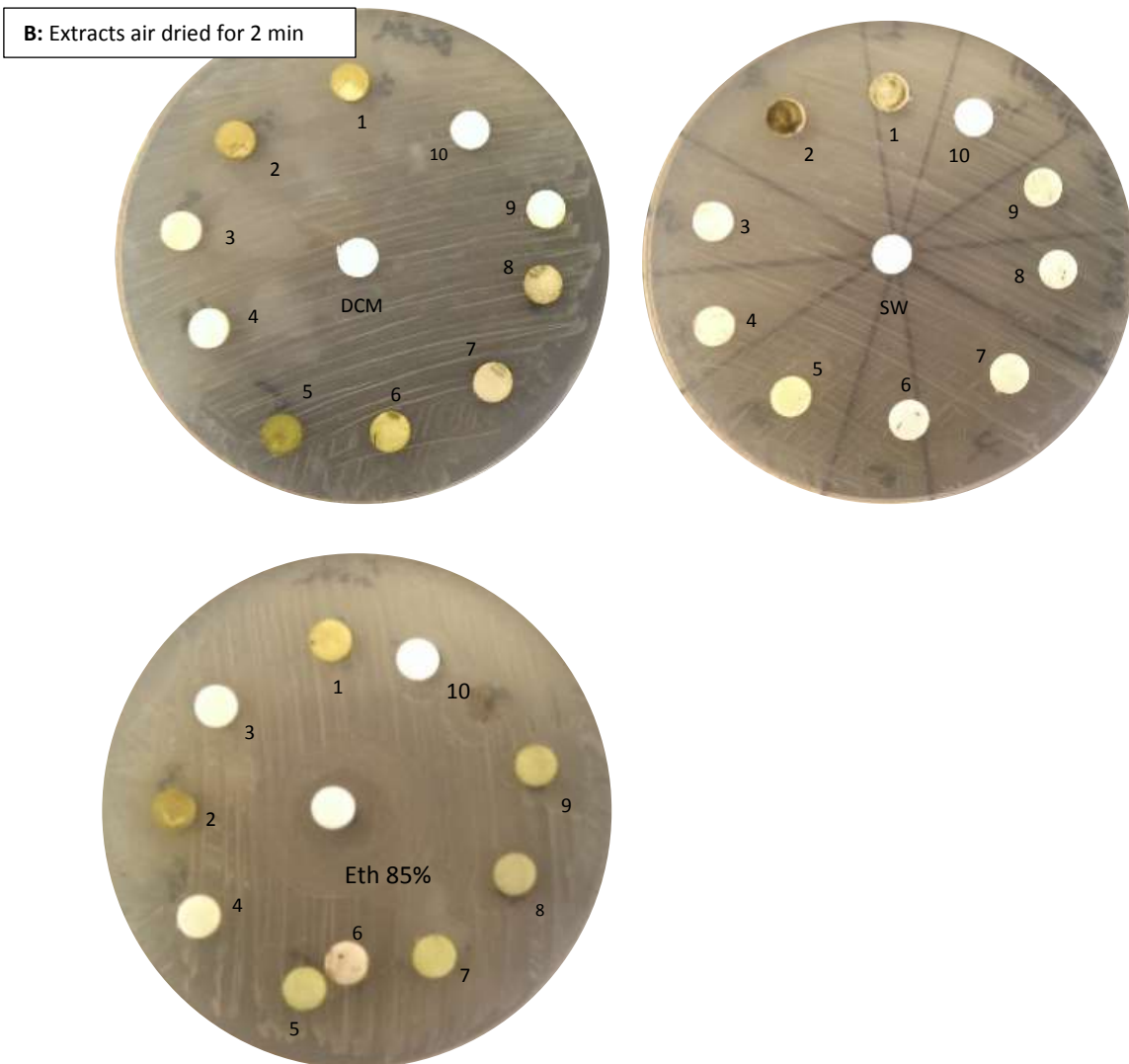


Figure D.13. *E. coli* inhibition zones obtained for the 100% ethanol or 100% dichloromethane extracts with the pellet re-dissolved with either sterile water, 85% ethanol or 100% DCM in **A**) extracts pipetted onto the paper discs **B**) paper discs dipped in the extracts and air dried for two minutes before plating. Eth = ethanol, SW = sterile water, DCM = dichloromethane.

4.9.2. Conclusion from method 8 and objectives for next trial

Corrosion of the plates by ethanol: DCM and bacterial inhibition obtained from 100% ethanol control in both the pour plate and disc diffusion methods, means that these solvents cannot be used in this study. Similar to Fig. D.8 above, the inhibition zones obtained after three days (result not shown) around ethanol *K. robusta* extracts (both leaves and roots) shows extraction of a low concentration of antibacterial components from *K. robusta* using ethanol, although an effective method to remove the ethanol inhibiting the bacterial growth could not be developed in this study. Although both sterile water and ethanol are polar solvents, ethanol can extract compound more effectively due to the presence of polar and nonpolar i.e. hydroxyl (-OH) and methyl (-CH₃) groups, respectively. Nutrient

agar contains sulphur group, which might have affected the effective diffusion of some inhibitory compounds (Jain et al., 2015) if extracted with sterile water.

Based on other studies (Douglas et al., 2004; Perry et al., 2007; Perry & Brennan, 1997; Perry et al., 1997a; Perry et al., 1997b) the plants from South Island used in this study might have low antibacterial activity as compared to North Island plants. This could possibly be a reason for the differences in the results of this study from that of (Prosser, 2011). Testing the antibacterial activities of North island plants might provide any explanation for the negative results.

4.10. Method 10: Comparison of the antibacterial activity with North Island plants

North Island *L. scoparium* plants were obtained from Comvita Limited (description in Section 3). These *L. scoparium* plants being young (9 months) had very little fine roots (less than 2 g in total) which were difficult to separate and clean from the potting mix they were growing in. Hence, only leaf extract was extracted using SW, Tris buffer 80 mM, ethanol, methanol and DCM each in 100% concentration and in 1:4 (w/v) dilution following method 5. All extracts were plated in 8 mm discs in duplicates at 30°C for 24 hours and the method was repeated twice.

4.10.1. Results of North Island plants

Only ethanol and DCM extracts generated inhibition zones (Fig. D. 14). From the results of the previous experiments, it was known that these two solvents themselves inhibit *E. coli*, therefore the inhibition zones observed may not be due to the extracts. Sterile water, buffer and methanol extracts generated no inhibition zones. The second repetition also showed similar results (not shown).

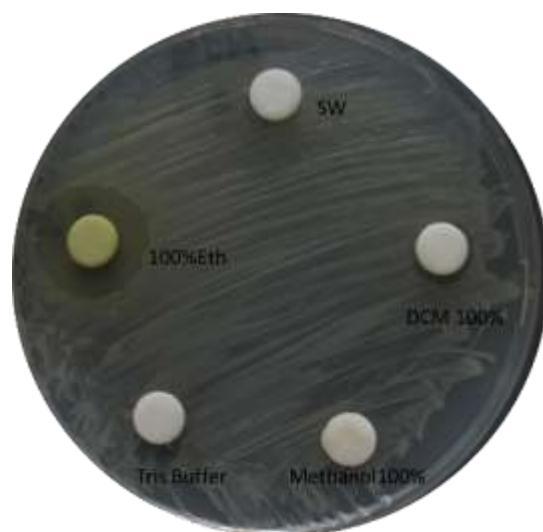


Figure D.14. Represents *E. coli* inhibition zones obtained from sterile water, Tris buffer, ethanol, methanol and dichloromethane extracts of North Island *L. scoparium* leaves. SW = sterile water. DCM = dichloromethane, Eth = ethanol.

4.10.2. Conclusion from method 10 and objective for next trial

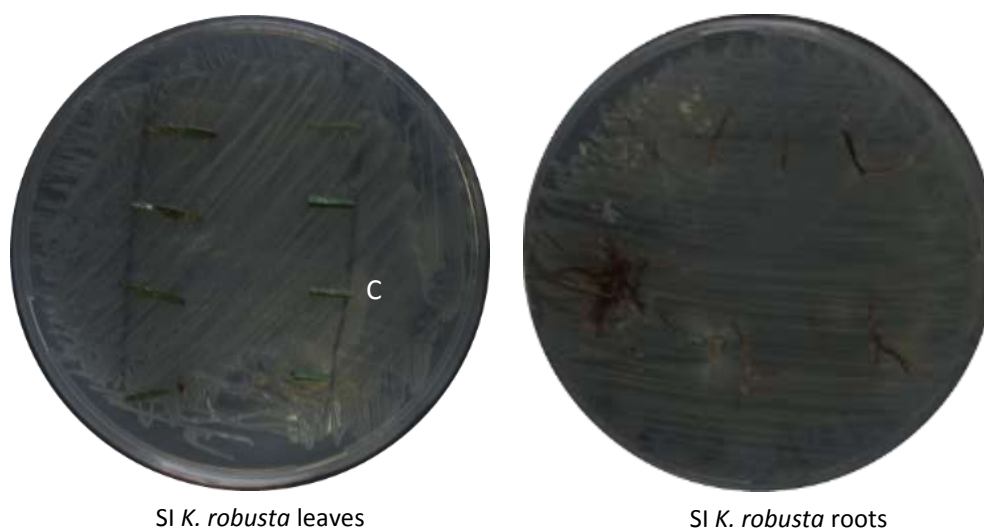
No inhibition zones were obtained with the SW, buffer and methanol extracts and those seen with the ethanol and DCM extracts could not be considered true. No inhibitory activity by the North Island plants could be because they were quite young and hence, the composition of the antimicrobial components may not be the same as those from the mature plants from the North Island. Collecting samples from North and South Island mature plants will be beneficial for comparison of the results obtained in previous trials. The rest of the North Island plants were grown in 3 l pots filled with the Eyrewell soil, Eyrewell soil + 20% sand and the Akaroa soil in natural conditions in a gravel area outside glasshouse (Fig. D.1) on November 2015 to be used later if required.

4.11. Method 11: Using plants parts (leaves and roots) directly against *E. coli*

To avoid the loss of any volatile antibacterial compounds, leaves and roots from South Island and only leaves from North Island plants were plated against *E. coli* without grinding. Leaves and roots (cut into small pieces) were first washed under running tap water then surface sterilised with 100% ethanol for 30 seconds and immediately washed with distilled water three times and allowed to air dry for few minutes under sterile conditions in a laminar flow hood. These air-dried leaves and roots were plated on NA plates inoculated with *E. coli* and slightly pressed into the surface of the agar. Small pieces of *L. perenne* leaves were plated as a control. All plates were incubated at 30°C for 24 hours.

4.11.1. Results of method 11

None of the North or South Island *L. scoparium* and *K. robusta* leaves and roots inhibited *E. coli* growth demonstrating no antibacterial components including volatiles released by the plant parts (Fig. D.15).



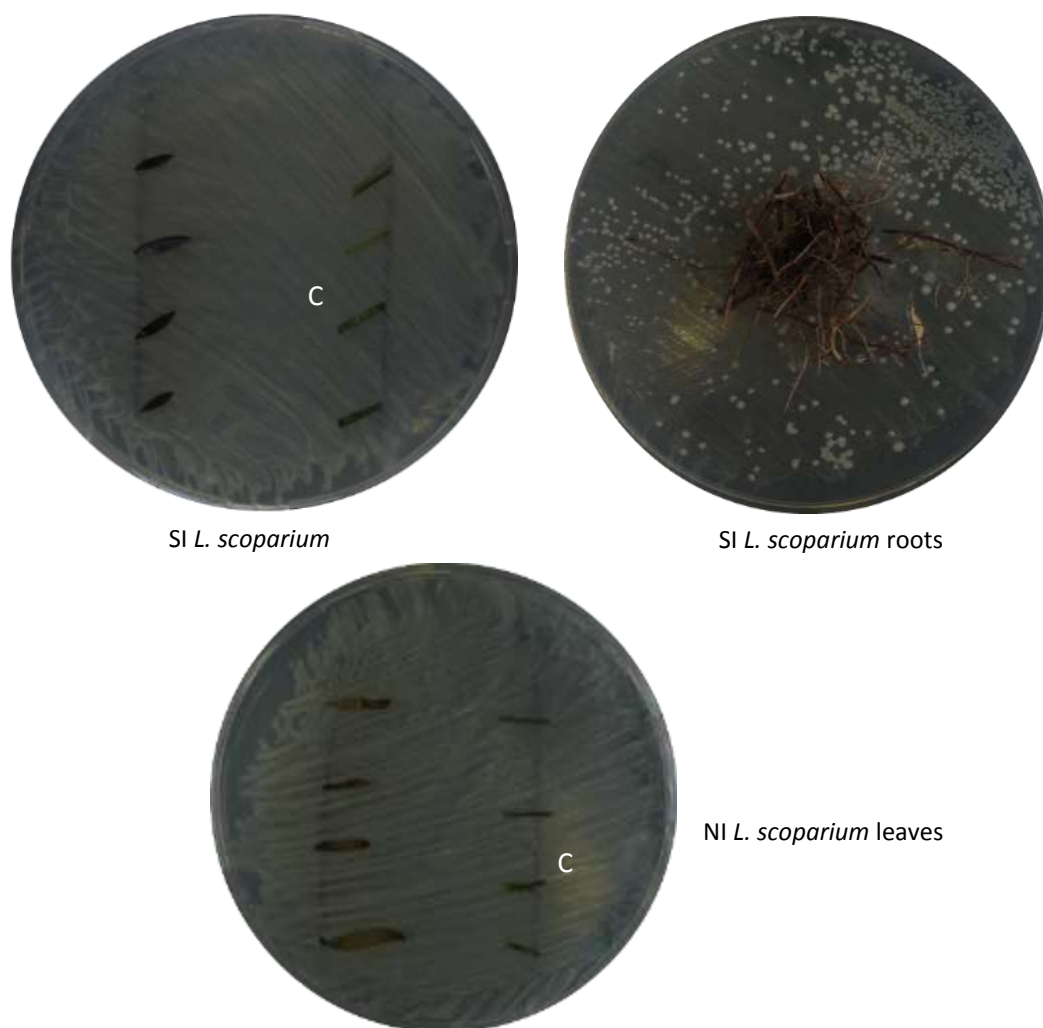


Figure D.15. No inhibition of *E. coli* obtained by plating whole leaves and pieces of roots from either North or South Island *L. scoparium* or *K. robusta*. "C" = *L. perenne* leaves plated as controls. SI = South Island, NI = North Island.

4.11.2. Conclusion from method 11

Separating the plant parts (from plant) or chopping them might result in the loss of any volatile antibacterial compounds released by the plants. Growing the NI plants for few months and then comparing the results and using older and more mature plants can be an option to test the activities.

4.12. Freeze drying of Essential oils

A method to extract essential oils from the plant parts and using freeze drying to check the inhibitory activities of the South Island plants being used in this study was tested. Commercially obtained essential oils of *L. scoparium* and *K. robusta* (1.5 ml each) were collected into Eppendorf tubes and freeze dried for 24 hours to understand the oil's properties. After the freeze drying, the remaining oil samples were plated on NA plates against *E. coli*.

4.12.1. Results from freeze drying

After 24 hours of freeze drying the, essential oils volume decreased to 290 μl for *L. scoparium* and 170 μl for *K. robusta* of the 1.5 ml used. When plated against *E. coli* the freeze dried samples of both oils, did not inhibited its growth (Fig. D.16).

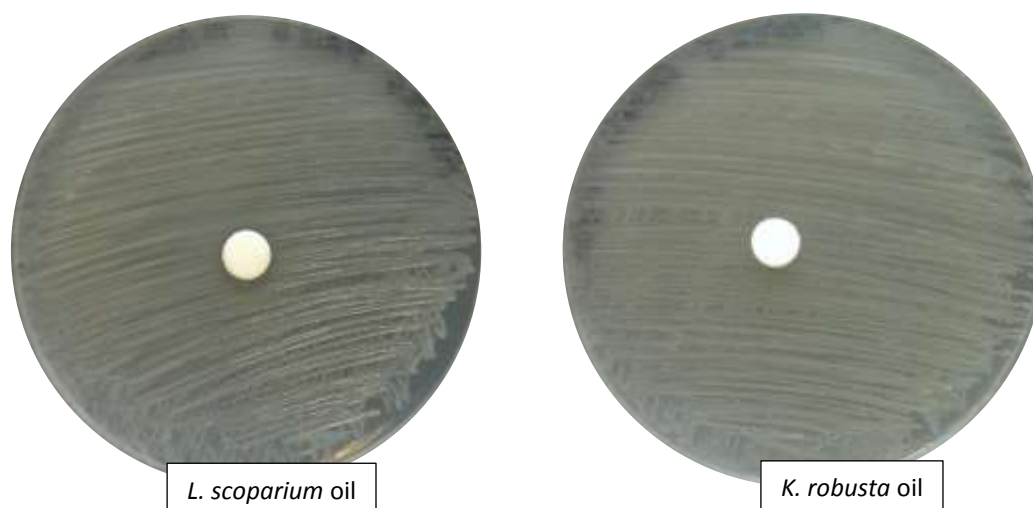


Figure D.16. No inhibition of *E. coli* observed for freeze dried samples of the commercial essential oils of both *L. scoparium* and *K. robusta*.

4.12.2. Conclusion of freeze drying of essential oils

No inhibition by the freeze dried essential oils demonstrates loss of the effective antibacterial compounds from the essential oils after freeze drying. This suggests that the antimicrobial or antibacterial compounds present in the essential oils and active against *E. coli* (in previous trials) are volatile in nature. In other words only the volatile component of the essential oil provides effective antimicrobial activity and the non-volatile compounds play no role in bacterial inhibition. Therefore testing the plant's VOC's against bacteria was carried out (Appendix A). It was concluded that the *K. robusta* essential oil had a higher volatile composition than *L. scoparium*. It was also concluded that it is not possible to extract essential oils (from leaves of the plants) using the freeze drying method due to the sublimation of the oils. Using Clevenger for essential oil extraction might be helpful to check the effective antibacterial compounds present in the South Island plants used during the trials.

4.13. Essential oil extraction using Clevenger

Essential oil was extracted from the two-year-old *L. scoparium* and *K. robusta* plants using Clevenger apparatus. The apparatus was arranged as shown in Fig. D.17 and the round bottle flask filled with the leaves and approximately three times by volume (by fresh weight) distilled water (Fig. D.17). The oil

extraction was performed for 3 hours and the oil and water mixture was collected every hour or as required. To separate the essential oil from the water-oil mixture, the complete mixture was dissolved in 100% DCM. As only the oil is 100% miscible with DCM (Fig. D.10), phase separation was obtained and the DCM phase, floating on the water surface was collected in sterile 50 ml centrifuge tubes. These centrifuged tubes were kept in a fume hood to allow slow DCM evaporation for 30 hours under careful observation. A little drop of oil ~10 or 20 μ l (identified by its colour and smell) was obtained at the end of the process. This oil was dissolved in 100 μ l of DCM and a paper disc was dipped to soak the oil-DCM mixture. This paper disc was held for 40 seconds to evaporate the DCM and then plated on NA inoculated with *E. coli*. Twenty and 100 μ l DCM was plated as controls on the same plate. The plate was left for 20 minutes under a laminar flow hood and incubated at 30°C for 24 hours.

The boiled leaf liquid obtained after the essential oil extraction (of *K. robusta* leaves only) was plated against *E. coli* using both the spread plate and disc diffusion method (Fig. D.18) to check if any non-protein compounds contribute to the antibacterial activities of these plants.



Figure D.17. The Clevenger set up for essential oil extraction.

4.13.1. Results of extracted essential oils plating against *E. coli*

The extracted essential oils produced an inhibition zone of 13.5 mm for *L. scoparium* and 33.5 mm for *K. robusta* essential oils (Fig. D.18A and B). Twenty μ l DCM did not produce any inhibition unlike 100

μl and confirms that the inhibition produced by the oils was not a result of any DCM activity. The boiled leaf liquid obtained after oil extraction and plated against *E. coli*, to test the presence of any non-protein compound, generated no inhibition of *E. coli* (Fig. D.19).

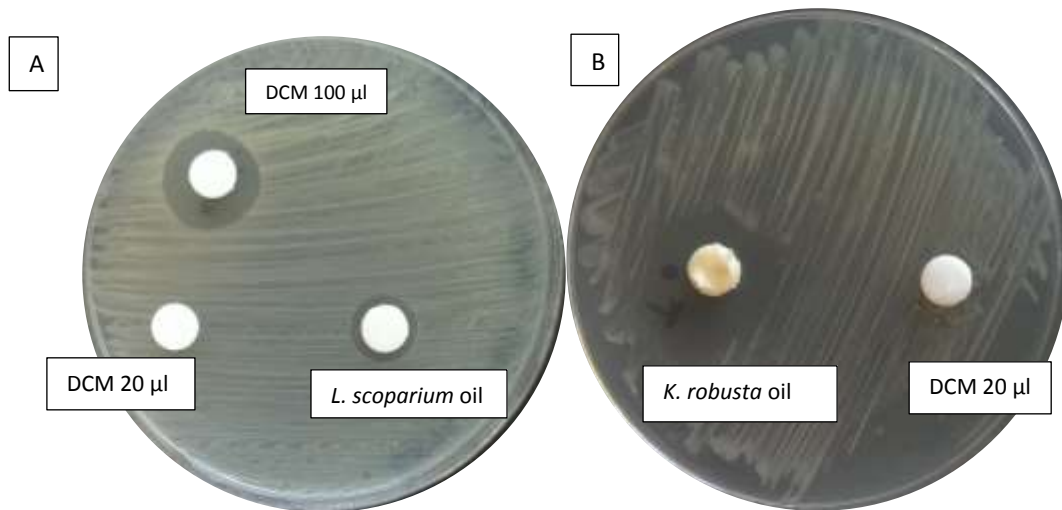


Figure D.18. *E. coli* inhibition zones obtained from Cleveger extracted **A)** *L. scoparium* (13.5 mm) and **B)** *K. robusta* (33.5 mm) essential oils. *K. robusta* oil represented higher antibacterial activity.

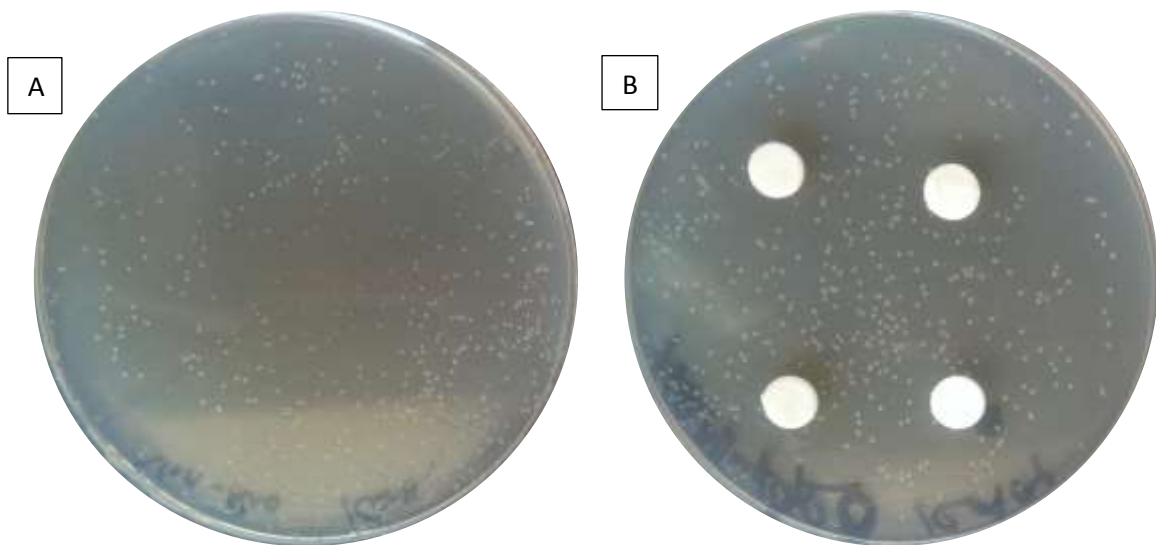


Figure D.19. *E. coli* inhibition obtained after plating the boiled leaf liquid onto the NA plates using the **A)** spread plating or **B)** disc diffusion methods.

4.13.2. Conclusion from essential oil extraction and objective for next trial

Essential oils from South Island plants used during the previous trials (obtained from the same glasshouse set up) were shown to have antibacterial properties against *E. coli*. *Kunzea robusta* essential oils had a higher antibacterial activity against *E. coli* than *L. scoparium* essential oils.

From the results obtained, it was concluded that the effective antibacterial compounds are only present in essential oils of *L. scoparium* and *K. robusta*. These antibacterial compounds are the volatile part of the essential oils or can be some non-polar compounds which cannot be extracted using sterile water. The other reason for the lack of antibacterial activities observed with sterile water extracts could be the NA media, as NA can resist the diffusion of the effective extracts into the agar due to the sulphur groups present in the agar which could bind a few chemicals. The use of liquid media might exclude the problem of any restricted diffusion of the antibacterial components and a clearer picture of the plants activity against *E. coli* can be obtained.

D.2 Monitoring *Escherichia coli* leaching under *Leptospermum scoparium* and *Kunzea robusta*. Method Development (January 2016- August 2016).

1. Aim

This aim of this study was to observe *E. coli* leaching under *L. scoparium* and *K. robusta* growing in two different soil types in the glasshouse, to illustrate the effect of antimicrobial rhizosphere activities of *L. scoparium* and *K. robusta* against *E. coli*.

2. Hypothesis

Based on the previous study by Prosser (2011) it was hypothesised that the antimicrobial rhizosphere activities under these native plants will decrease *E. coli* survival and hence their leaching/recovery into the leachate.

3. Materials and Method optimisation

3.1. Experimental set up

Details of the materials, experimental set up and irrigation maintenance are in Chapter 4 (Section 4.2.1.) and Chapter 5 (Section 5.2.1). Eyrewell plant set up was completed in late September 2015 and Akaroa in early October 2015 (Fig. D.20.). All plants were grown under daily spray irrigation (two times a day) to maintain soil moisture. Weeding was performed twice per week. Eyrewell soil was not mixed with any sand during the first few trials.

3.2. Optimisation of the leaching capacity

After four months of plants growth and stabilisation in soil, all plants i.e. *L. scoparium*, *K. robusta* and *L. perenne* were tested for their leaching potential. Plants were irrigated using a sprinkler to maintain uniform soil moisture and were left for 24 hours to drain any extra water. After 24 hours, lysimeters were connected using clean pipes to plastic containers (2.5 l) which had previously been cleaned with 20% bleach, washed and. All plants were irrigated using a measuring cylinder with equal quantity of water, 50 ml at a time. A gap of half an hour was maintained between each irrigation. Irrigation was maintained to receive 200 to 300 ml leachate daily or nearly 1.5 l weekly from each lysimeter. Once a constant amount of drainage started to leach from each lysimeter, this irrigation rate was maintained until bacterial spiking.



Figure D.20. Experimental set up in Eyrewell and Akaroa soil in the glasshouse in 2015.

3.3. Results of leachate optimisation

During the leachate optimisation, soil detachment from the sides of the lysimeters was observed in the Eyrewell soil and the gap was sealed by slightly pressing the soil every day (Fig. D.21).



Figure D.21. Soil was pressed slightly at the sides to avoid detachment from the pots during the method development.

The leaching capacity of each plant species is presented in Fig. D.22. *Leptospermum scoparium* and *K. robusta* leached nothing below the irrigation rate of 400 ml (~7 mm) per day in both soils. However, *L. perenne* started pooling at irrigation rates above 300 ml per day in both soils. As the study focused on

the outputs i.e. nearly equal leachate between species, the irrigation rate of 500 ml was selected for *L. scoparium* and *K. robusta* and 300 ml was selected for *L. perenne*.

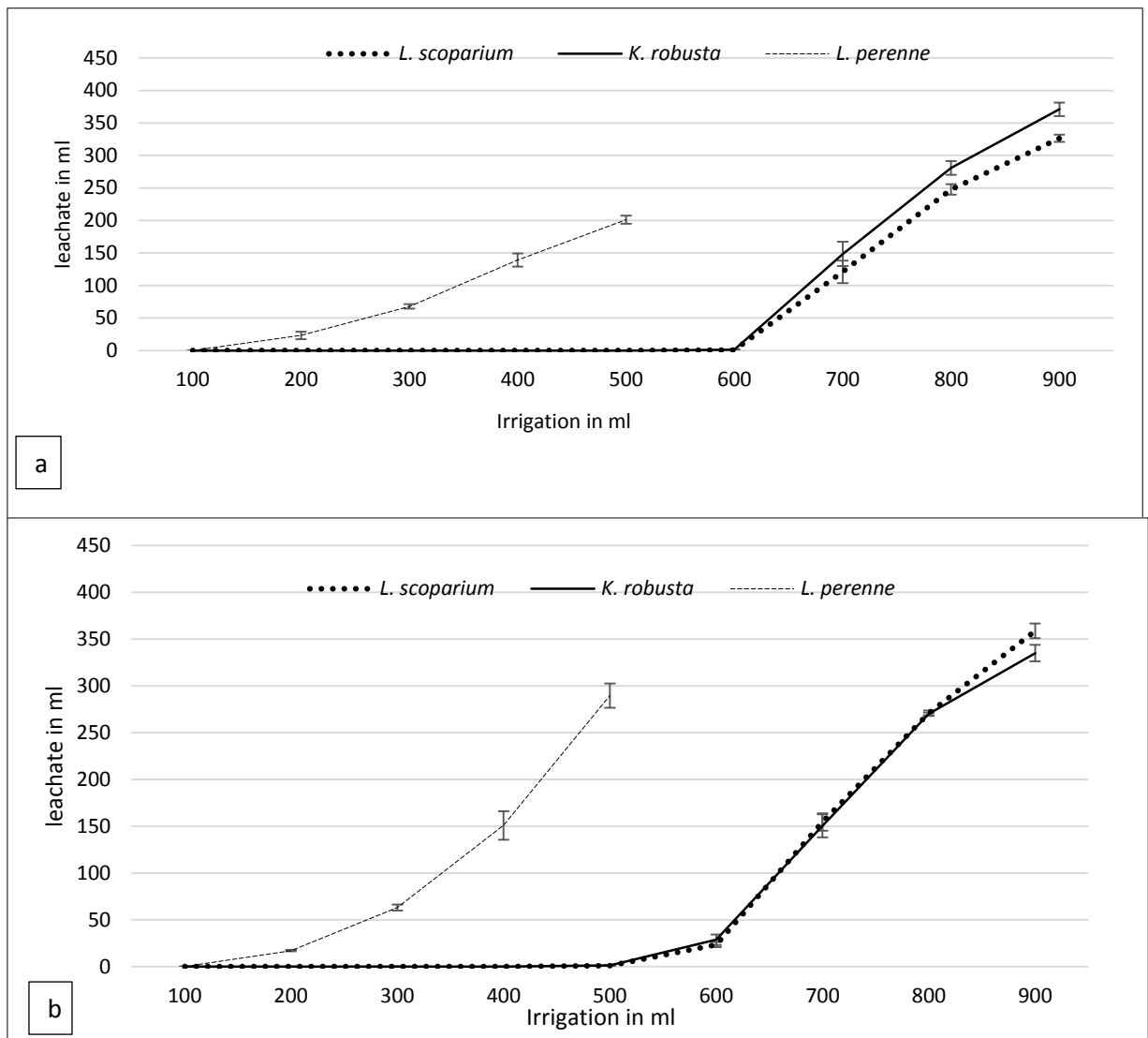


Figure D.22. Leaching potential of *L. scoparium*, *K. robusta* and *L. perenne* in **a)** the Akaroa soil and **b)** the Eyrewell soil.

This leachate optimisation (with 500 ml for natives) was tested for one week prior to the main glasshouse trial i.e. Chapter 4, where maximum irrigation rates of 600 ml was chosen for *L. scoparium* and *K. robusta*, due to low leachate generation and maximum error rates between replicates. This change in the water use by plants could be because of the change in weather.

3.4. Testing the survival rate of *E. coli* ATCC13706

Before spiking plants with bacteria, the survival rate of *E. coli* ATCC13706 was tested in laboratory and a linear regression was performed using Microsoft Excel (Version 2013). Detail of the method and the graph is presented in Appendix C.5.

4. Glasshouse trial 1 (19/2/16 to 4/3/16)

4.1. *E. coli* spiking of plants

Based on the linear regression (Appendix C.5), the *E. coli* culture was prepared by adding two fresh bacterial colonies to each of 4 x 500 ml sterile Nutrient Broth (NB; Difco-bacto NB) in 1l Schott bottles. The inoculated broth was incubated overnight (18- 24 hours) in a shaking incubator at 30°C at 120 rpm. After 24 hours, broth cultures were transferred into 50 ml sterile Falcon tubes and centrifuged at 4025 x *g* for 10 minutes at 4°C. Bacterial pellet was washed two times in 25 ml Ringers solution and the supernatant was discarded. The bacterial pellets were suspended in sterile water (SW). Dilutions of this bacterial stock was prepared by adding 500 ml water each time and mixing vigorously for ten minutes to finally raise the volume (enough to inoculate each treatment with 100 ml bacterial stock) with 10⁷ cfu/ml. Three sub samples of the prepared stock were plated on NA (NA; Oxoid CM0003) for colony count following serial dilution (Fig. D.23).

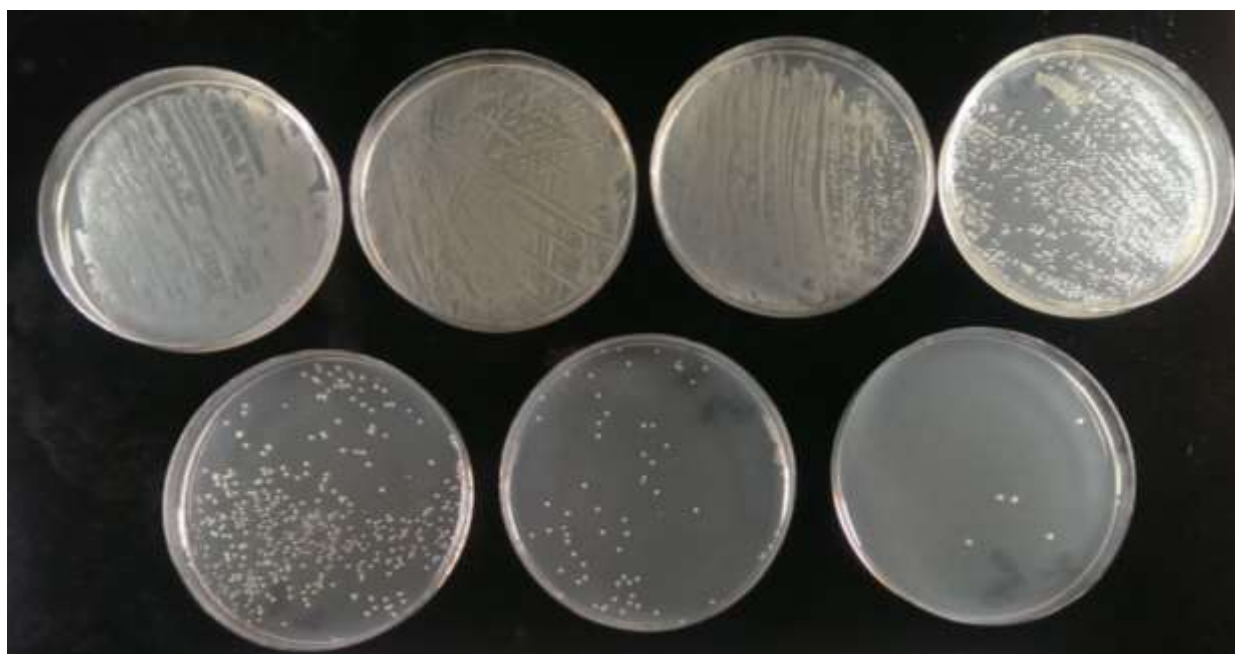


Figure D.23. The serial dilution ($10^{-1} - 10^{-7}$) of the bacterial stock ($68 \times 10^7/\text{ml}$) in sterile water added to plants during the glasshouse trial 1.

A 100 ml bacterial stock was added to each treatment at the rate of 50 ml/hour to avoid preferential flow (PF), using measuring cylinders. A 10 ml clean pipette was used to distribute *E. coli* in *L. perenne*

lysimeters to avoid error due to trapping of the bacteria by the plant leaves. Thirty minutes after bacterial stock addition, plants were irrigated with tap water (400 ml to natives and 200 ml to *L. perenne*). Control plants were irrigated with an equal amount of water only. After 24 hours, all plants were irrigated with tap water only and the collected leachates were tested for bacterial enumeration using Colilert-18 (Chapter 4, Section 4.2.4).

The set up for the leachate collection is shown in Fig. 4.1.b and description of the collection method is also provided in Chapter 4.

4.2. Results from the first glasshouse trial 1

The leachate collection was performed for fourteen days. None of the plant species showed positive results for *E. coli* leaching within fourteen days (Fig. D.24), indicating that either the bacteria added into the soil are dead under all plants (including *L. perenne*) or the bacteria are surviving in soil without leaching i.e. plants are holding the bacteria in the soil.

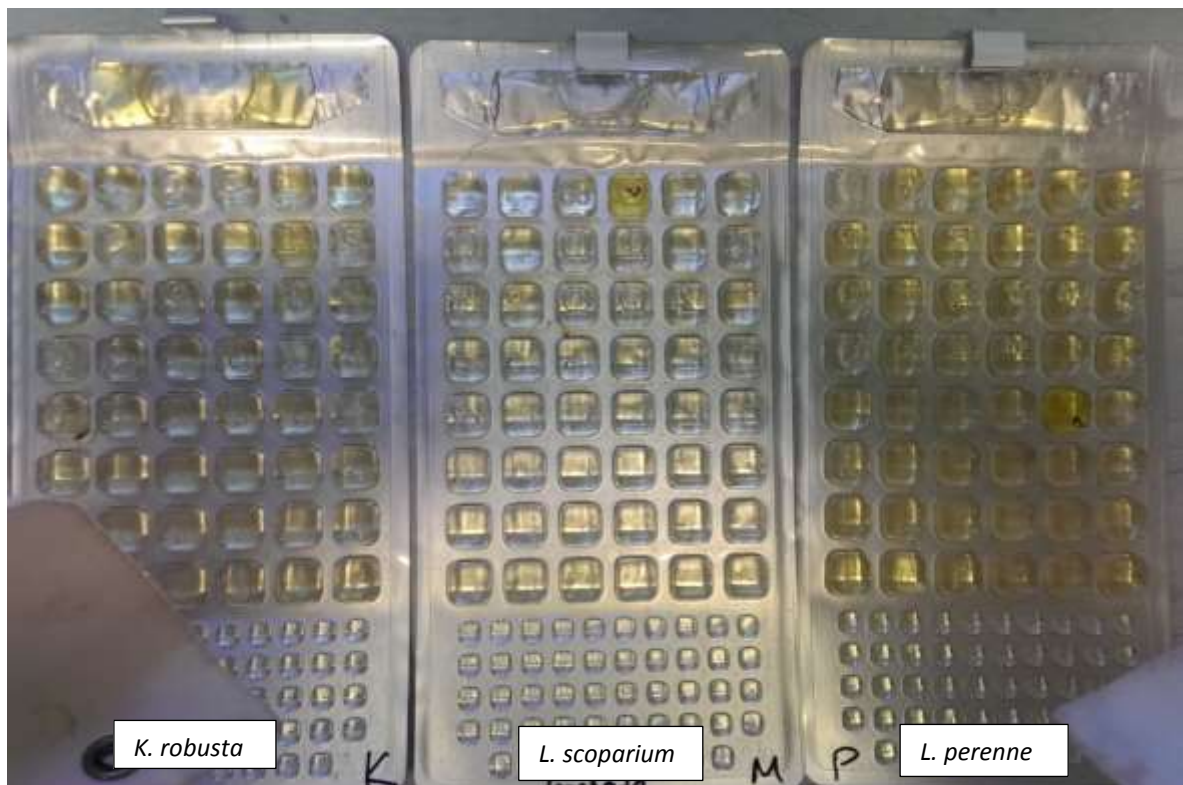


Figure D.24. Negative bacterial leaching in the collected leachate of *L. scoparium*, *K. robusta* and *L. perenne* tested with Colilert-18 during Glasshouse trial 1.

4.3. Conclusion from the first glasshouse trial

Colilert-18 showed no bacterial leaching under any plants species including *L. perenne* (plant control). This result leads to several hypothesis:-

Both *L. scoparium* and *K. robusta* have killed the inoculated bacteria in the soil and hence no bacteria is recovered in the leachate. However, *L. perenne* might have held the bacteria in its fibrous root structure rather than killing them.

- *Escherichia coli* is surviving within soil under all plants including *L. perenne* without leaching which indicates the use of no plant controls and soil sampling for bacterial enumeration would be beneficial.
- Kit or method is not working for *E. coli* enumeration.

5. Testing Colilert-18

Before making any cores into the lysimeters soil, Colilert-18 was tested with a fresh *E. coli* culture, suspended into sterile water.

5.1. Results for Colilert-18

All plates showed negative results with Colilert-18 at 44°C, i.e. no presence of *E. coli* in the sample.

5.2. Conclusion

The results suggest that either

- The bacterial stock culture is contaminated i.e. the culture used to inoculate the plants was not *E. coli* but a morphologically similar bacterial species picked from a contaminated culture plate, and hence Colilert-18 is generating negative results.
- Or Colilert-18 is not working.

6. Testing purity of the bacterial stock culture

To confirm the bacterial stock purity, the glycerol stock of *E. coli* and the stock used in the previous trial, were cultured separately on NA plates. A few bacterial colonies from each stock (glycerol and previously used) were added to 1 ml Ringers solution in sterile Eppendorf's tubes and vortexed to mix. Hundred µl of the bacterial stock was added into 100 ml sterile water and a mix, samples were sealed onto quantitrays. All quantitrays were incubated at 44°C for 24 hours.

6.1. Results

After 18 hours of incubation, both stock cultures i.e. the one used in previous glasshouse trial and fresh glycerol stock, appeared negative (no colour change was observed). This indicates that

- Either the incubator or method are not working

- Or the original stock i.e. the glycerol stock is contaminated.

To check the incubator conditions, same quantitrays were incubated in another incubator for the next 6 hours (in addition to the 18 hours of the previous incubation) at 44°C. After only six hours of incubation in this new incubator, sample colour changed to yellow i.e. positive for the presence of total coliforms) and when tested under UV light (500 nm) trays showed fluorescence i.e. positive for the presence of *E. coli* (Fig. D.25 and D.26). Both *E. coli* stocks (glycerol and one used in previous glasshouse trial) were tested again in both incubator and only the new incubator, generated positive results for bacteria indicating that the incubator used during the previous trial was faulty and also confirmed no contamination of either of the *E. coli* stocks.



Figure D.25. Difference in the yellow colour generation (positive indication for total coliforms) for the same bacterial sample between two incubators used. The two quantitrays are for glycerol and previous trial *E. coli* stock.

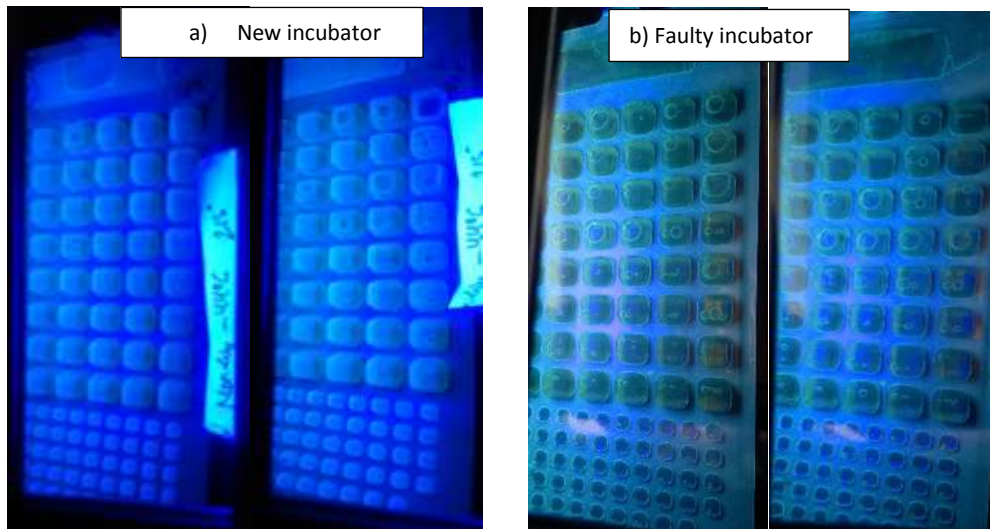


Figure D.26. Difference in the fluorescence obtained from the bacterial stock cultures incubated in **a)** new incubator and **b)** incubator used in previous glasshouse trial. The two quantitrays are for glycerol and previous trial *E. coli* stock.

Temperature log of the faulty incubator was monitored for 24 hours and the temperature was found stable throughout the incubation period (Fig. D.27).

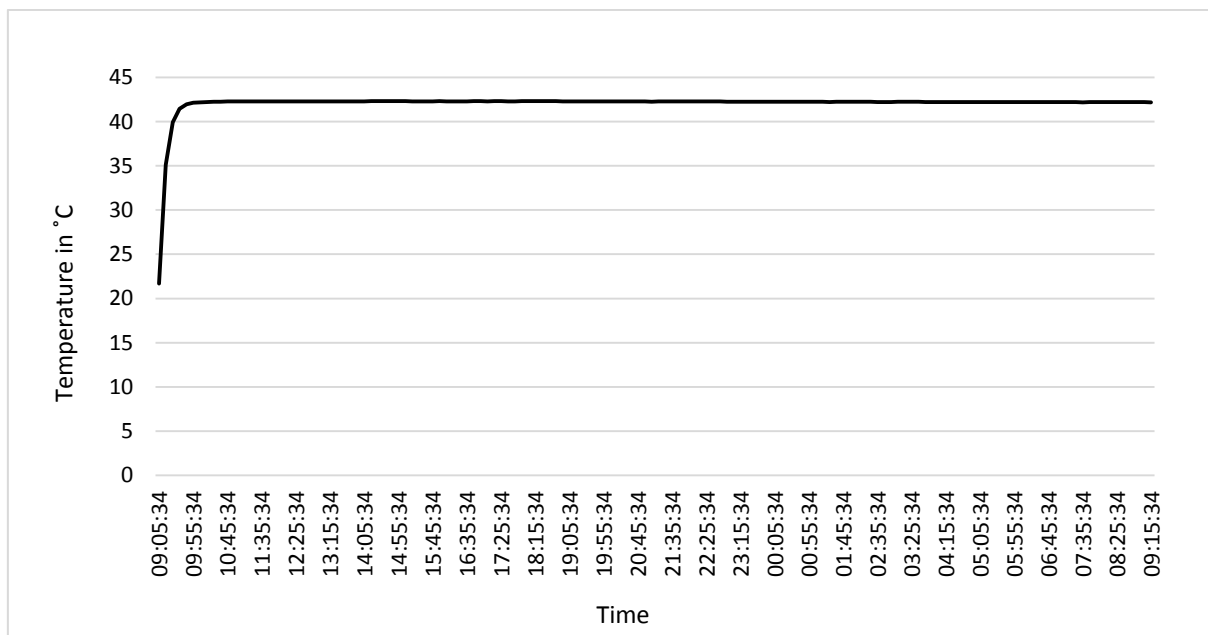


Figure D.27. Temperature log of the faulty incubator, used for the incubation of leachate samples of glasshouse trial 1.

6.2. Conclusion

The incubator used during the first glasshouse trial was faulty and no bacterial stock contamination exists. The reason for the negative results obtained from the faulty incubator was unknown as the temperature was stable throughout the time of experiment.

7. Testing all the methods before 2nd glasshouse trial

Based on the results of the 1st glasshouse trial and the problem with the incubator, it was now decided to use a tracer in the soil. Sodium bromide (NaBr) was decided to be used as a tracer along with a non-toxic dye i.e. brilliant blue. A concentration of NaBr was decided to be introduced to plants based on Jiang et al. (2010). Before their introduction to plants NaBr and dye toxicity was tested against *E. coli*.

7.1. Testing the toxicity of NaBr against *E. coli*

NaBr toxicity against *E. coli* was tested in both NA and NB and the method details and results are presented in Appendix C.9. The results obtained showed that the concentration of NaBr selected to be added to the plants i.e. 500 mg NaBr/plant (or 400 mg Br per plant) is non-toxic to the *E. coli* strain hence, any decline in *E. coli* number in the second trial will not be a result of NaBr toxicity to the bacteria.

7.2. Testing the toxicity of Brilliant blue dye against *E. coli*

Although Brilliant blue is a non-toxic dye, its toxicity was tested against our *E. coli* strain. A high dye concentration of 60 mg/100 ml was prepared using SW. The solution was filter sterilised using a 0.4 µm syringe filter. Dye solution was exposed to the bacteria using paper disc and bacterial dot methods. For the paper disc method 8 mm sterile discs were dipped into the dye solution and were held for 10 seconds to drain excess dye. These discs were then placed (one per plate) on *E. coli* inoculated NA plates along with a control disc dipped into SW. For the bacterial dot method, 100 µl dye solution was spread onto NA plates and allowed to dry for 5 minutes. Bacterial dots were then inoculated onto these plates using a sterile loop. Inoculated plates (triplicates) from both methods were incubated for 24 hours at 30°C. Plates were checked for any inhibition zones after 24 hours of incubation.

7.2.1. Results of *E. coli* survival under Brilliant blue dye

The dye showed no *E. coli* inhibition at the tested dye concentration (Fig. D.28) in either of the methods used.

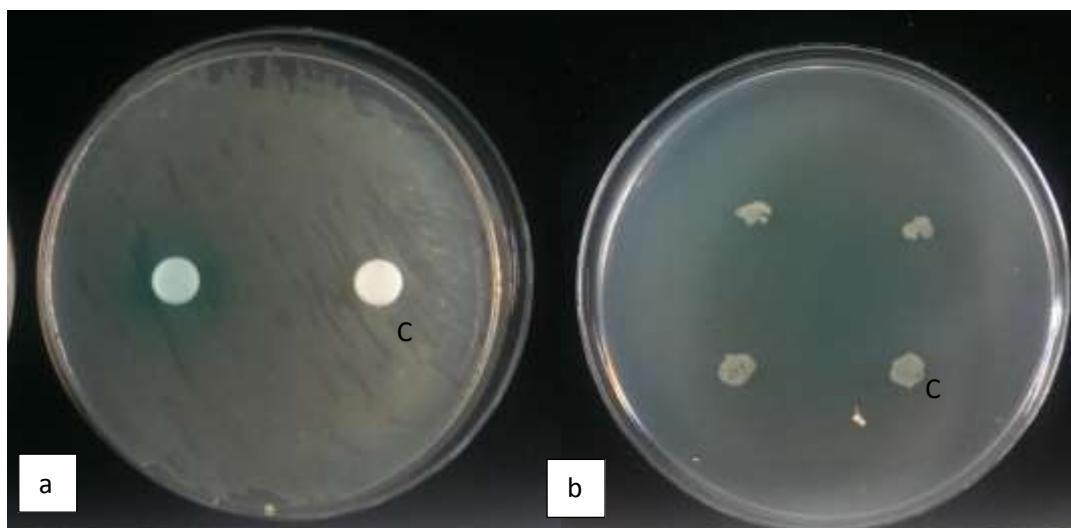


Figure D.28. No growth inhibition was observed for the *E. coli* exposed to 60 mg/100 ml brilliant blue dye solution in either **a)** paper disc method or **b)** dot method. C = sterile water used as control.

7.2.2. Conclusion

Brilliant blue dye is non-toxic to *E. coli* and will present no growth inhibition.

8. Leaching of brilliant blue from unplanted control lysimeters

Along with the toxicity, leaching and absorbance of brilliant blue dye was also tested in both soils (details in Chapter 4) using unplanted lysimeters. The lysimeters were irrigated at field capacity for a week to maintain a uniform soil moisture. Once the first leachate drained from both soils, the amount of water applied and the leachate obtained was noted and soils were again spray irrigated and left for 24 hours to drain any extra water. A 100 ml solution of 6 mg/l brilliant blue dye was slowly added to the lysimeters and left for an hour to be absorbed by soil. This concentration was chosen after testing the minimum concentration of dye generating visible blue colour after 10 times dilution (expecting dye absorbance by soil particles) (Fig. D.29). Once the dye was absorbed in both soils (~20 minutes), 400 ml tap water was further irrigated adding 100 ml at a time, with 30 minutes between each irrigation. Both lysimeters were left for two days to generate maximum leachate.

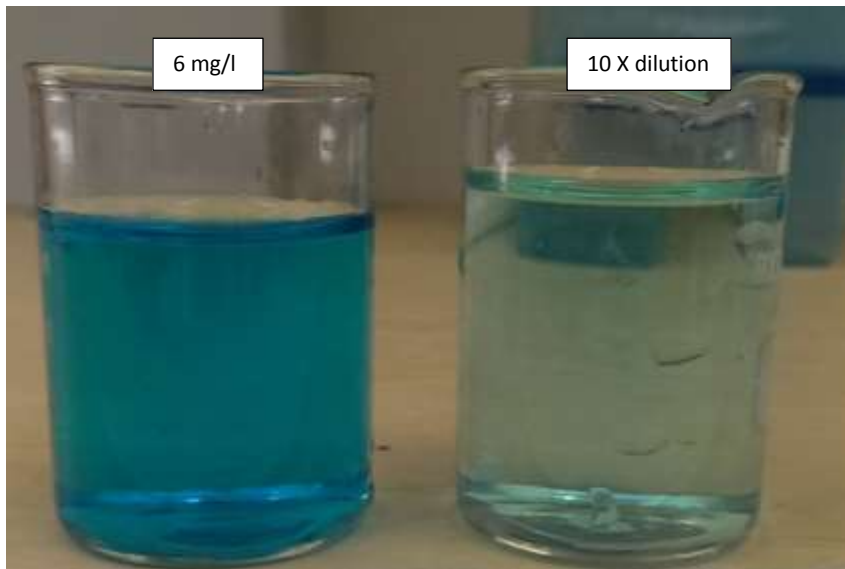


Figure D.29. The concentration of 6mg/l brilliant blue dye was still visible after 10 times dilution in water.

8.1. Results

No coloured leachate was obtained from any soil possibly due to the absorbance by the soil particles and the winter sheets (this is knitted cloth manufactured from high stitch monofilament which provides a shade level of 45 - 50%, purchased from Egmont Commercial) used at the base of the lysimeters during set up.

9. Testing dye absorbance by winter sheets in separate small pots

Before introducing a high dye concentration into the soil, dye absorbance by the winter sheets (used at the bottom of the lysimeters) was tested. The trial was performed using small pots filled with both soils with and without the winter sheets. Pots were filled up to the top with soil and irrigated with 200 ml water. After 24 hours of irrigation, a 100 ml dye solution (6 mg/l) was added to these pots. Three hours later, an irrigation using 100 ml water was performed and all pots were left with the collection trays (Fig. D.30).

9.1.1. Results

No coloured leachate was generated from any pot (with or without the winter sheets) in the collection trays (Fig. D.30).

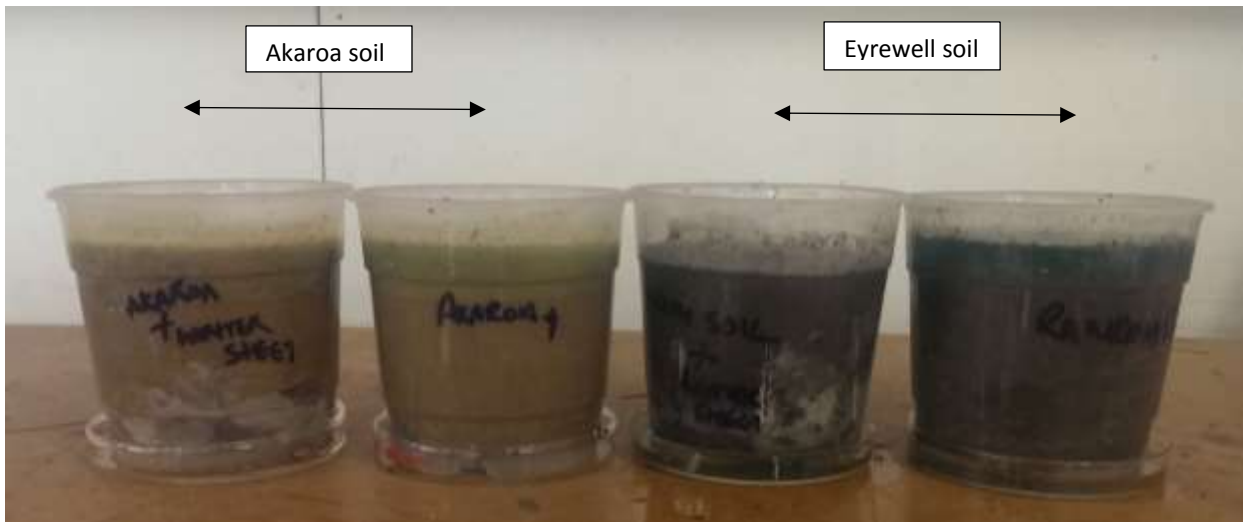


Figure D.30. No coloured leachate generated by either the soils with or without the winter sheets.

9.1.2. Conclusion

This trial shows no absorbance of the dye by the winter sheets used in the lysimeters set up.

10. Testing high dye concentrations

The dye concentration was increased to 20 mg/l and the trial was performed in the small pots used in Fig. D.30. The irrigation rates used in Section 9 above, were repeated here.

10.1. Results

Both soils now generated a blue coloured leachate (Fig. D.31).

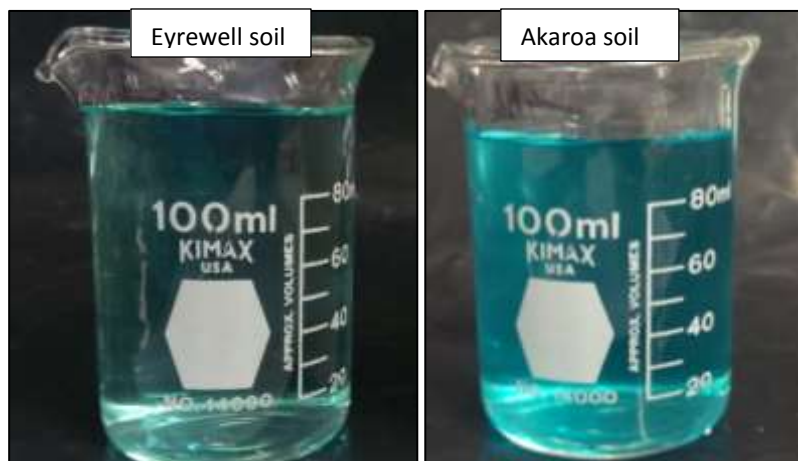


Figure D.31. Leachate obtained after applying 20 mg/l dye solution in the Akaroa and the Eyrewll soil.

10.2. Conclusion

The dye leaching results concluded that 20 mg/l brilliant blue dye solution is suitable to be used as a tracer in both soils.

11. Standard curve preparation for Colilert-18

To further confirm the method (Colilert-18) is working for *E. coli* analysis, a standard curve for *E. coli* enumeration was prepared using Colilert-18 and a known concentration of *E. coli* stock (enumerated in NA). Two colonies of freshly cultured *E. coli* were inoculated in 100 ml NB and incubated in a shaking incubator at 25°C overnight along with blank i.e. uncultured NB. After 24 hours, the broth was shaken vigorously and divided into 50 ml Falcon tubes in a laminar flow. Falcon tubes were centrifuged at 4025 x *g* for 10 minutes and the bacterial pellet was re-suspended in 15 ml of SW. Bacterial stock was vortexed until mixed and the OD of this bacterial stock was measured at 600 nm. Colony forming units of the stock were estimated based on the linear regression performed before. Dilutions of this stock were then prepared in sterile water to obtain 600, 1000, 1600 and 2100 cfu/ml count from the dilutions. This range was decided following the counting limitations or range of Colilert18 (<1 – 2419.6 cfu/ml). One ml of the OD adjusted *E. coli* stock was mixed with the required amount of sterile water to obtain the cfu counts stated above. The prepared dilution was kept in a shaker for 25 minutes and 1 ml from this dilution was then added to 99 ml sterile water (as quantitrays require 100 ml sample) and left in a shaker for another 25 minutes. This sample was then sealed in the quantitrays and incubated at 44°C for 18 hours. Similarly other dilutions were prepared (Table below). A 100 µl sample from the bacterial stock and its dilutions prepared above were plated on NA in triplicates (Fig. D.34). The original bacterial stock was also plated following serial dilution on NA to confirm the count (Fig. D.32). Plates were incubated at 30°C for 24 hours. Sterile water was used as the control. Calculations were checked with Dr Hossein Alizadeh and Dr Maria Jesus Gines. This method was repeated four times with sterile water (Fig. D.33) and three times with Ringers solution (Fig. D.35) in triplicates.

11.1. Results

The original bacterial stock plated in NA, confirmed the bacterial count of 66×10^7 cfu/ml *E. coli* (Fig. D.32).

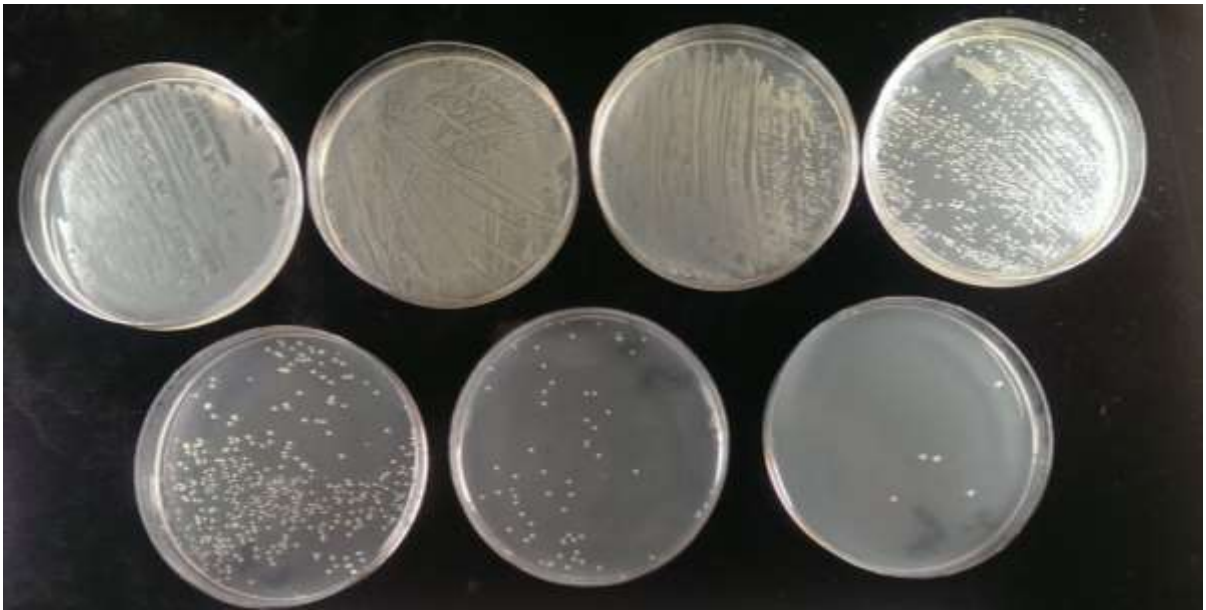


Figure D.32. Serial dilution ($10^{-1} - 10^{-7}$) of *Escherichia coli* stock ($66 \times 10^7/\text{ml}$) used for the standard curve preparation.

During bacterial stock mixing with SW, Colilert-18 showed lower *E. coli* counts or fluorescing blocks/columns in quantitrays. Standard curve prepared for these bacterial counts using Microsoft Excel also showed nearly 90% decrease in the bacterial numbers than what was expected (Fig. D.33). The bacterial stock dilutions plated on NA showed no bacterial growth (Fig. D.34).

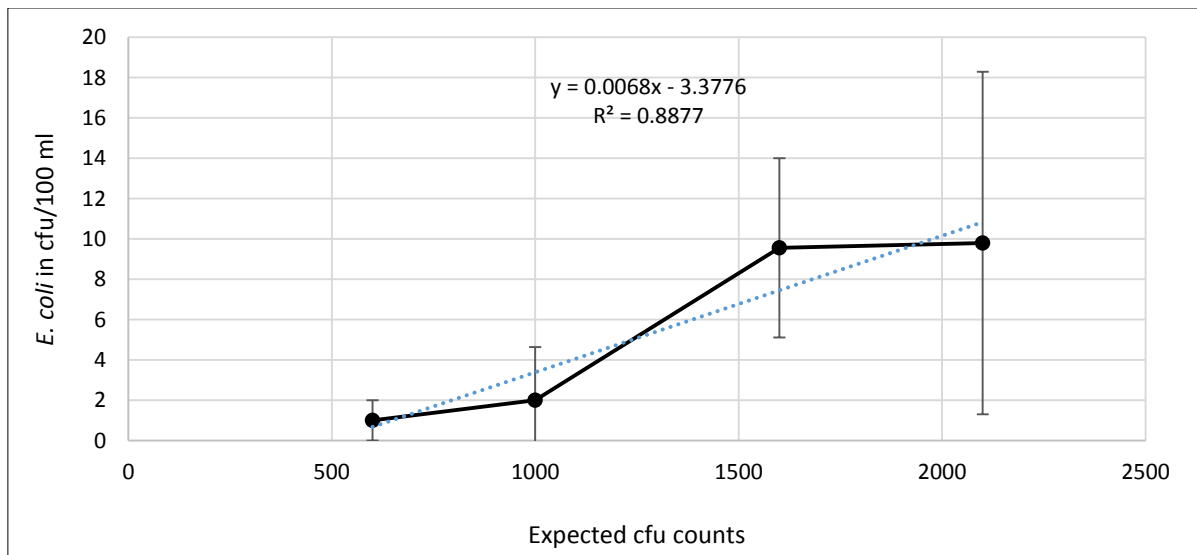


Figure D.33. Standard curve of *E. coli* bacterial stock diluted with sterile water and enumerated with Colilert-18. Error bars represent standard deviation of the mean value ($n = 3$).

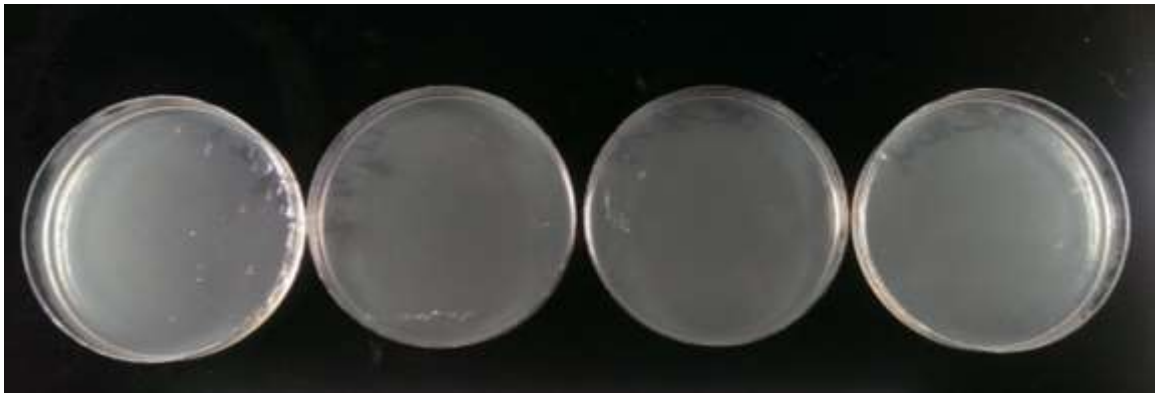


Figure D.34. The bacterial stock dilutions (ran through Colilert) when plated in NA, showed no bacterial growth.

When the bacterial stock was mixed with Ringers solution, instead of sterile water, a nice linear curve was obtained for the first three estimated numbers, however for the fourth dilution, the expected bacterial count was above the detection limit i.e. > 2419.6 cfu/100ml (Fig. D.35).

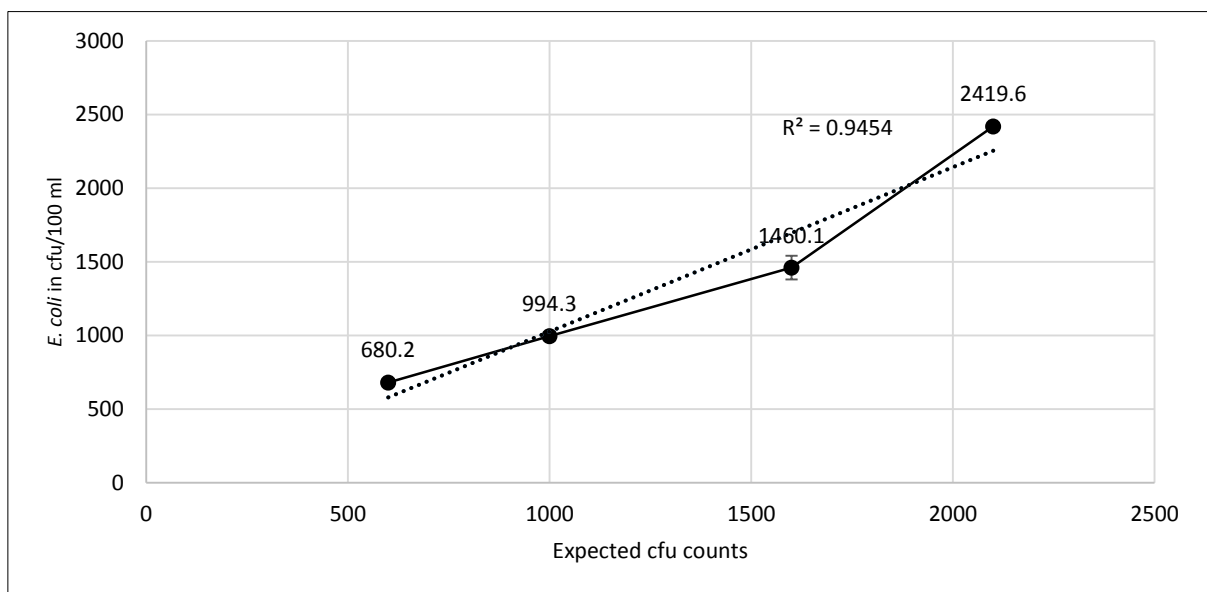


Figure: D.35: *E. coli* counts when the bacterial stock was dissolved in Ringers solution and enumerated with Colilert-18. Error bars represent standard deviation of the mean value (n = 3).

To further confirm the die-off of the *E. coli* in sterile water, a fresh bacterial stock was prepared by mixing two fresh bacterial colonies in 5 ml Ringers. A 100 μ l aliquot of this bacterial mixture was serially diluted in 900 μ l sterile water or Ringers. Each dilutions were plated in NA and plates were incubated at 30°C for 24 hours (Fig. D.36). The same bacterial stock generated two different counts of 61×10^7 /ml with Ringers and 76×10^5 /ml with SW.

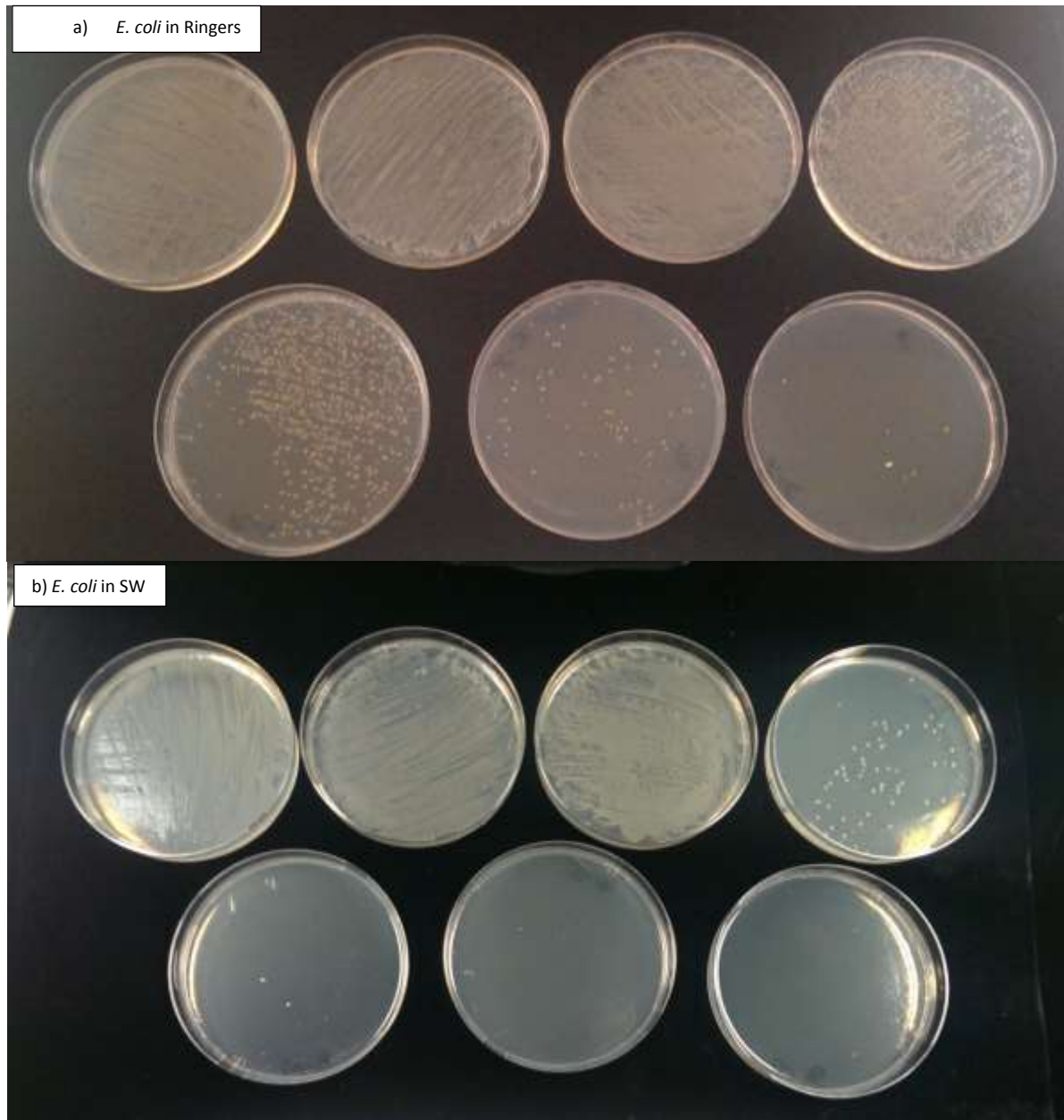


Figure D.36 The *E. coli* stock plated on NA after serial dilution in **a)** Ringers and **b)**sterile water generated variable bacterial counts of 61×10^7 cfu/ml and 76×10^5 cfu/ml, respectively. Sterile water = Sterile water.

11.2. Conclusion of standard curve preparation

The whole experiment confirmed bacterial die-off in sterile water and the exact reasons were unknown. However, osmotic shock could be possible reason for the same. Ringer's solution presented a nice linear curve with *E. coli* stock dilutions, representing working of Colilert-18 and the plating method. Results further confirm that the random results obtained during sterile water dilutions could be due to the osmotic pressure killing the bacterial cells in sterile water. This also shows that the bacterial dilutions prepared in sterile water during the first glasshouse trial, could also be the reason for the lack of bacterial recovery in the leachate. Although, it was also proven that the incubator used was faulty.

12. Testing purity of the bacterial culture

The purity of the *E. coli* bacterial stock was further tested using both the glycerol bacterial stock and the one used in previous trials above at both 44°C and 30°C. The temperature of 44°C was chosen as 44°C was expected to eliminate the growth of any other unwanted bacterial colonies. Bacterial colonies were growing similarly on both plates although, little stressed at 44°C (Fig. D.37). Bacterial colonies were further plated on Mfc media (specific media) and incubated at both temperatures (Fig. D.38). the growth of only purple colonies on the Mfc media confirmed the purity of the *E.coli* bacterial culture.

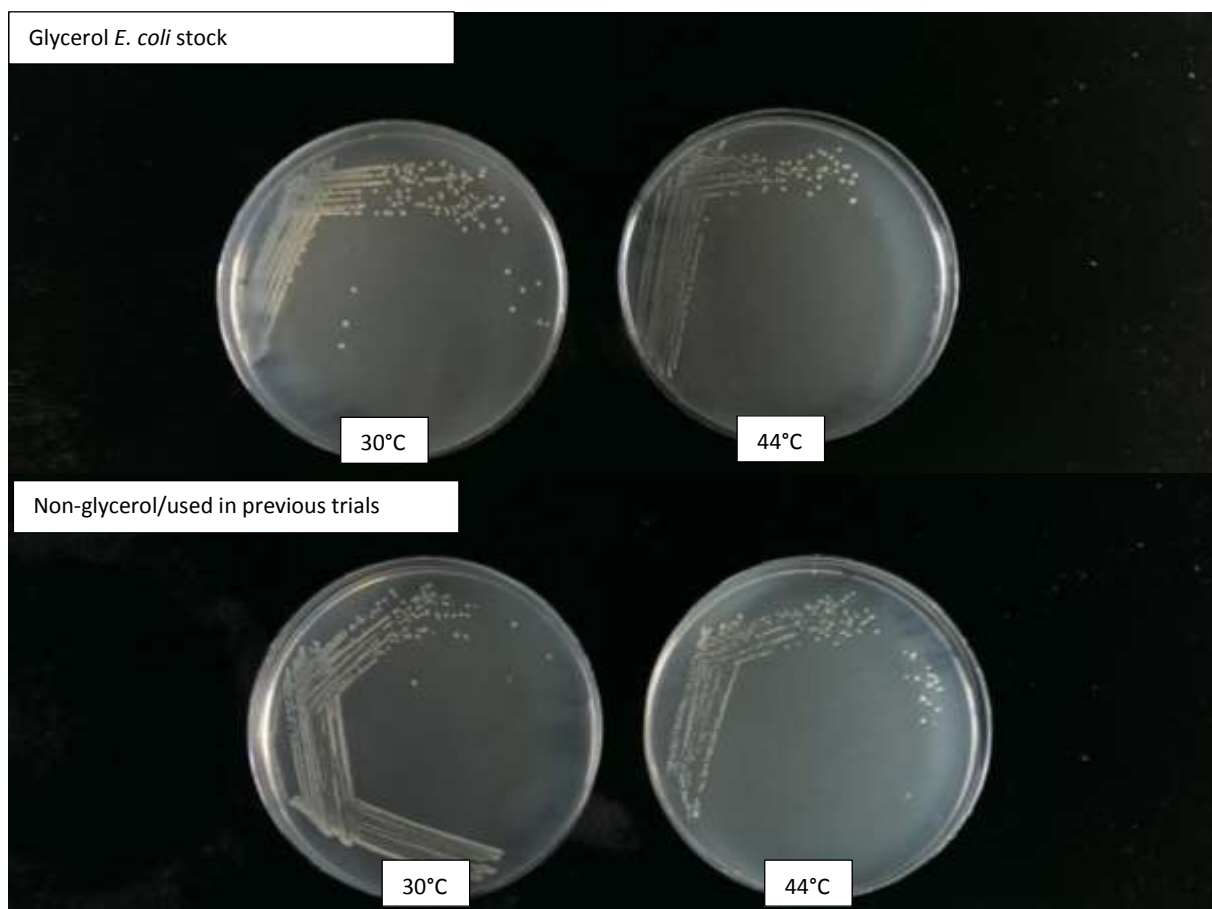


Figure D.37. Growth of glycerol and non-glycerol stocks of *E. coli* cultured at 44°C and at 30°C.

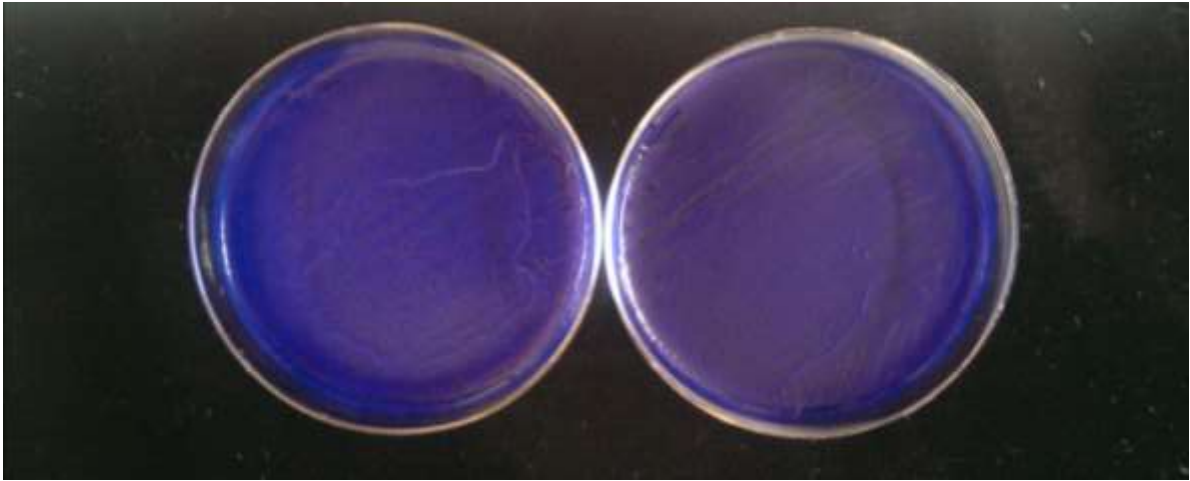


Figure D.38. Growth of purple coloured bacterial colonies on mfc media plates confirmed non-contamination of the *E. coli* stock.

13. Testing effect of brilliant blue on Colilert-18 results

Finally the effect of brilliant blue dye on the colour change and fluorescence of Colilert-18 positive results was tested. For this 20 mg/l and 10 mg/l dye solutions were prepared with and without NaBr (to make it similar to the glasshouse run) and 100 ml of these dye samples (with and without salt) were sealed in quantitrays after adding 100 μ l of fresh *E. coli* in one of the replicates. Trays were incubated at 36°C instead of 44°C (as suggested by Sarah Quaife, technician from ESR Wellington) for 18 hours.

13.1. Results

The blue colour of the dye was seen to have reacted with the yellow colour of the Colilert-18 and generated green blocks for total coliform confirmations (Fig. 39) with or without the salt. Fluorescence of *E. coli* was not affected by either the dye colour or the presence of NaBr (not-shown).



Figure D.39. Effect of Brilliant blue dye on the yellow colour production of Colilert at 36°C.

13.2. Conclusion

Neither the dye nor the salt affected the Colilert-18 readings.

14.The second glasshouse trial (27/4/16 – 18/5/16).

The lysimeters used for the first glasshouse trial were used here. Before starting the experiment, background *E. coli* was enumerated for all lysimeters using Colilert-18

14.1. *E. coli* stock preparation for plants inoculation

Details are described in Section 4.1 above (first glasshouse trial). All the dilutions were prepared in Ringers rather than sterile water and the new incubator tested above (Section 6.1) was used for all the sample incubations.

14.2. Dye and NaBr stock preparation

- As tested above, 20 mg/l of Brilliant blue dye solution was prepared in sterile Schott bottles with SW. In the same dye solution (4 l in total) 20 g of NaBr was added to give the final concentration of 500 mg NaBr/ 100 ml or 400 mg Br per pot.
- This dye and salt solution was mixed vigorously and sealed to be transferred to glasshouse.

14.3. Protocol of plants spiking with *E. coli*

- Before adding *E. coli*, plants were irrigated and allowed to drain for 24 hours. This drainage was used to obtain background *E. coli* numbers.
- Before adding the bacterial stock, 100 ml of the dye and salt solution was added to all the treatment pots by adding 50 ml at a time and maintaining a 30 minutes gap between each irrigation.
- After 30 minutes of adding the dye solution, 100 ml *E. coli* stock was added to the treatments applying 50 ml at a time and maintaining 30 minute gaps between each irrigation.
- 30 minutes after adding the bacterial stock, plants were irrigated with tap water adding 100 ml at one time. The native plants were irrigated with 400 ml tap water over the dye and bacterial solution however *L. perenne* was irrigated with 100 ml tap water only (leachate optimisation above).
- Plants were left overnight and after 24 hours, first leachate was collected.
- Leachate volume was measured using clean measuring cylinders and noted.
- A 100 ml leachate sample was collected for Colilert-18 and two replicates of each plant species were analysed within two hours of collection.
- A 30 ml leachate sample was collected for the dye and salt analysis. These samples were frozen within 2 hours of collection, until used.
- Leachate collection was performed for fourteen consecutive days.
- A positive control quantitray (inoculated with pure *E. coli* culture) was inoculated each time the leachate samples were incubated for the *E. coli* enumeration. This was done to assure the method was working and to avoid the previous problem of the faulty incubator.

14.4. Results of second glasshouse trial

The *E. coli* stock added to the plants had a bacterial count of 6.45×10^8 cfu/ml (Fig. D.40).

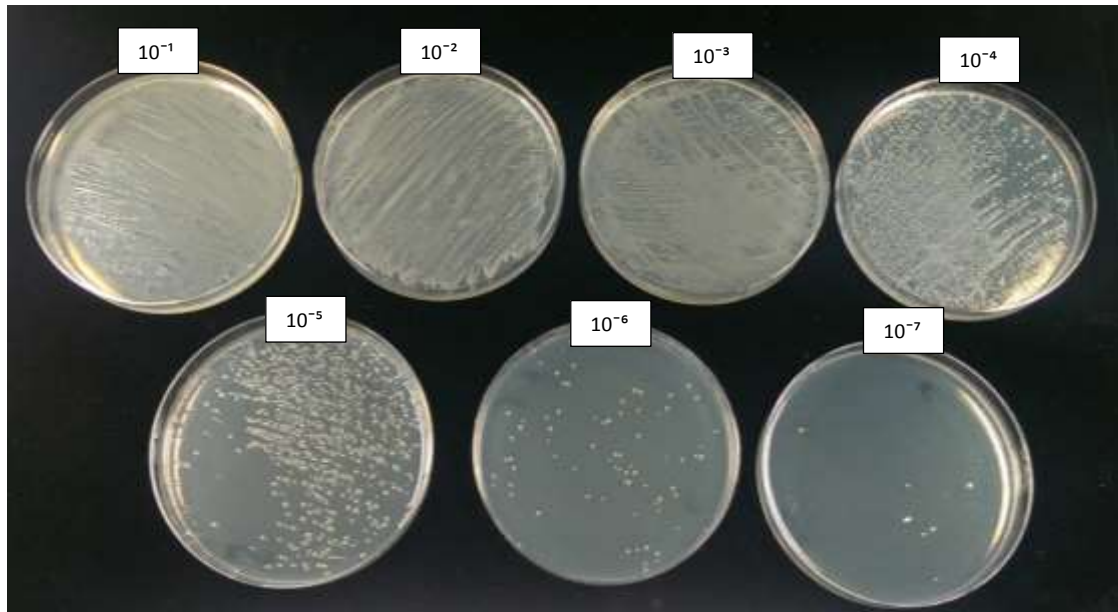


Figure D.40. Serial dilution of the *E. coli* stock added to the plants from (10^{-1} to 10^{-7} dilutions).

The highest bacterial leaching was observed between the second to third leachate collection from both soils (Fig. D. 41.a and b). The highest bacterial leaching occurred under *K. robusta* in both soils, however, in the Akaroa soil *K. robusta* leached maximum of 2.5×10^7 cfu/ml and in the Eyrewell soil it leached maximum of 9.2×10^8 cfu/ml. This indicates PF which could be a result of soil collapsing from the sides and resulting in an edge effect (Fig. D.21).

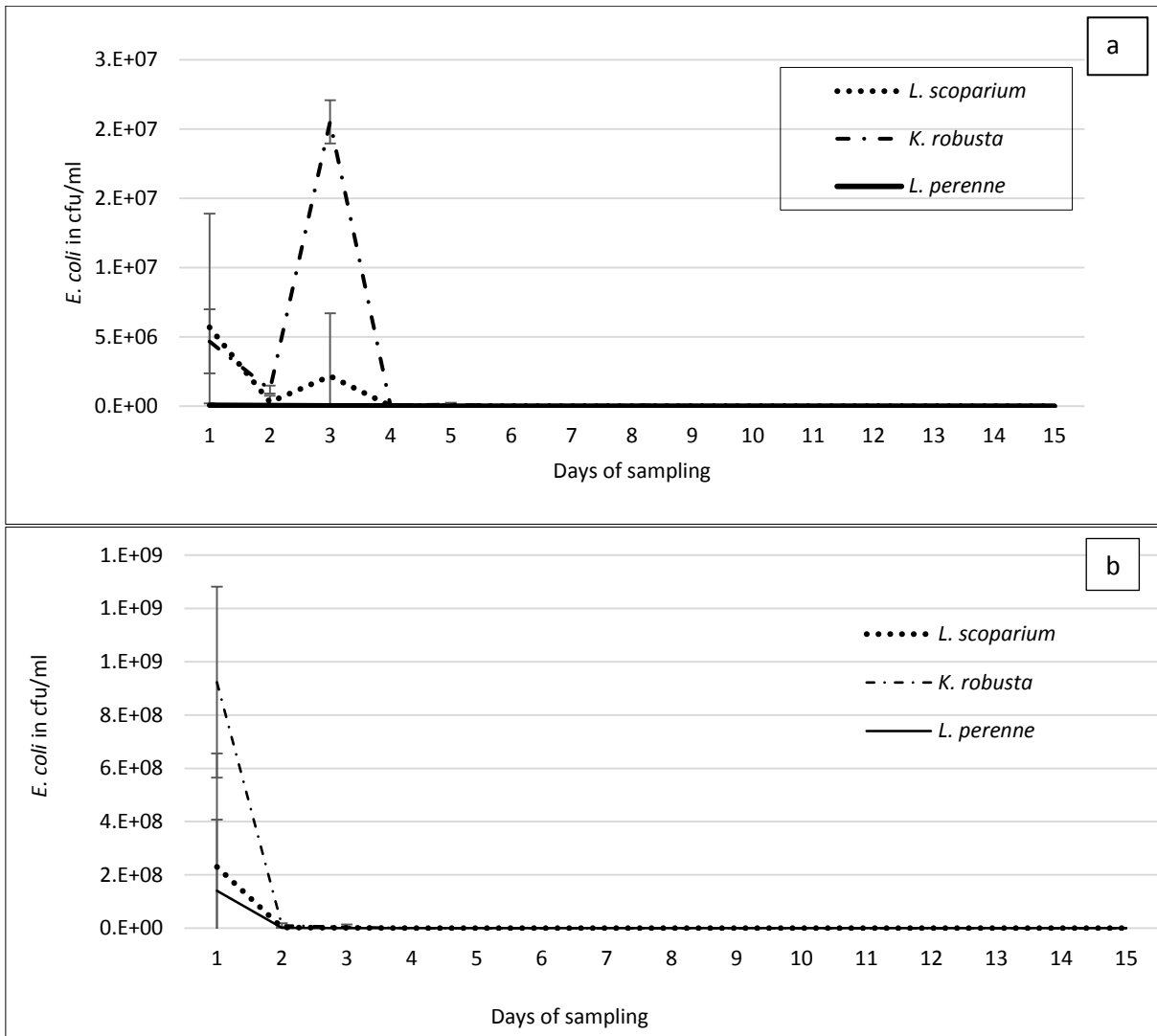


Figure D. 41. The Average total *E. coli* leaching from **a)** the Akaroa soil and **b)** the Eyrewell soil over a fifteen day time period. Error bars shows standard deviation of the mean value (n = 5).

14.5. Avoiding edge effect causing preferential flow

None of the plants were irrigated for two days, and the soil in all the lysimeters (especially in the Eyrewell soil) started to collapse from the sides. This few millimetre gap was sealed with petroleum jelly to avoid bacterial flow from the sides of the pots (Fig. D.42). Half an hour after the Vaseline application, plants were maintained back on field irrigation rates.



Figure D. 42. Petroleum jelly application on the sides of the lysimeters to seal the edges, avoiding preferential flow.

15. Re-confirming second glasshouse results using dye only

A re-confirmation of the second glasshouse trial was performed. Control plants were selected for a dye only trial. A solution of brilliant blue dye (20 mg/l) was prepared and 100 ml of this solution was added to the lysimeters (2 mg/pot). Two hours after the dye application, irrigation was continued as above, for leachate collection. Leachates were frozen after each collection and within 2 hours of collection. The experiment was continued for 17 days and at the end of the experiment, the first 4 days dye samples were defrosted together and measured, the next 4 days of samples were defrosted the next day and so on. Dye concentration was measured using a UV mini – 1240-UV-Vis spectrophotometer.

15.1. Results from dye trial and the conclusions

Leaching trend for the dye differed between measurements performed each day. Once all the leachates were measured and the results were analysed, most of the plants generated two peaks for the dye in both soils (Fig. D.43.a and b). To confirm any analytical errors in the measurements, the 17 days leachates for one replicate per species from each soil were de-frosted and measured together for the dye concentration. During this analysis, the trend obtained for the dye leaching was more or less similar with the first run, indicating no analytical error during the first dye measurements. However, the concentration obtained was lower than the first measurement. This indicates dye degradation and PF. A separate laboratory test was also performed with different dye concentrations and the results indicated dye degradation with time i.e. within 24 to 48 hours (results not shown). Senior Lecturer Henry Chao also confirmed the possibility of dye degradation with time and its absorbance at different rates in soil. This concluded that the Brilliant blue dye cannot be used as a tracer in the soil.

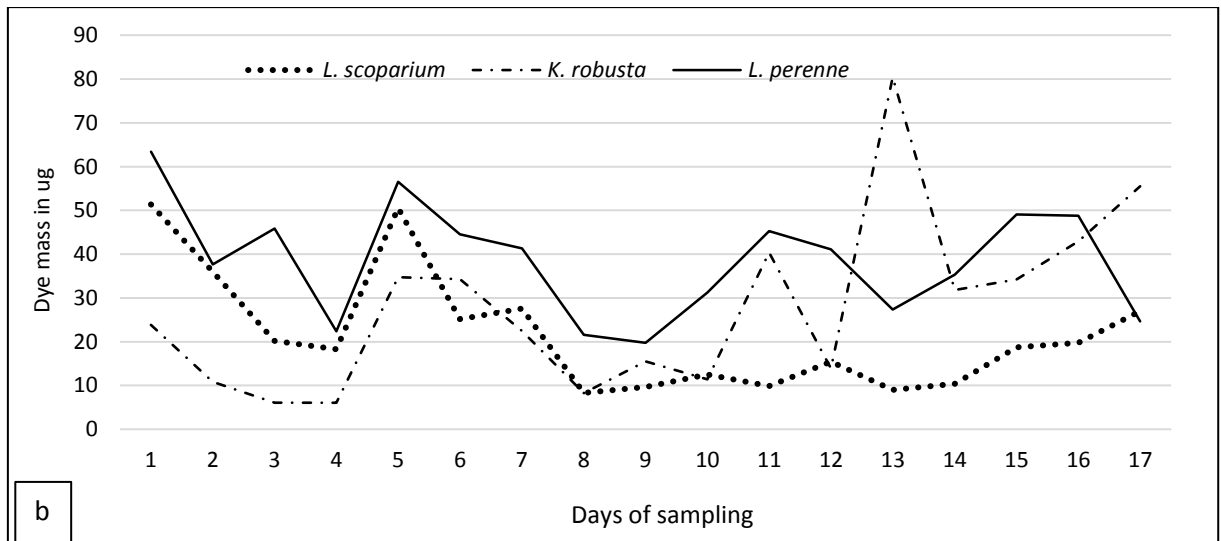
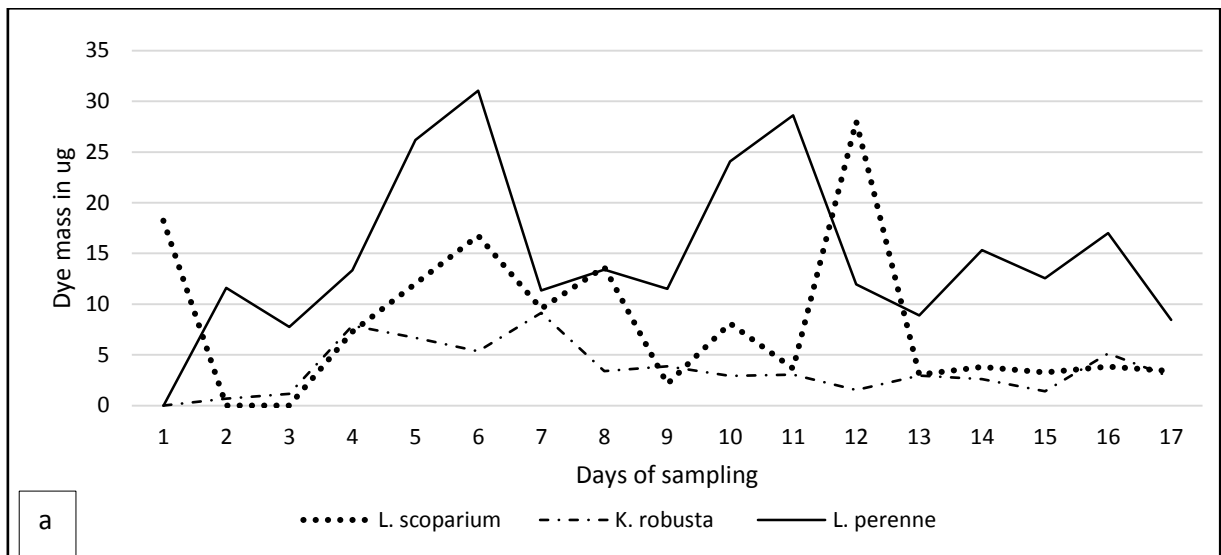


Figure D. 43. Brilliant blue dye concentration (μg) leached under *L. scoparium*, *K. robusta* and *L. perenne* ($n = 1$) from **a)** the Akaroa soil and **b)** the Eyrewell soil.

16. Testing NaBr stability in a quick glasshouse run

Based on the dye trial results above, one replicate per plant was selected from each soil for NaBr application. However, as HPLC is an expensive method, only the Akaroa leachates were analysed to test the working of the NaBr as a tracer (Fig. D.43). The application method is described in Section 14.2 above. Leachate collection was performed for 8 days.

16.1. Results of NaBr application

All plants showed a curve for NaBr leaching. However, PF was still present under *L. scoparium* and *K. robusta* (Fig. 43). As the peaks were nice and clear in the Akaroa soil, the Eyrewell soil samples were not tested.

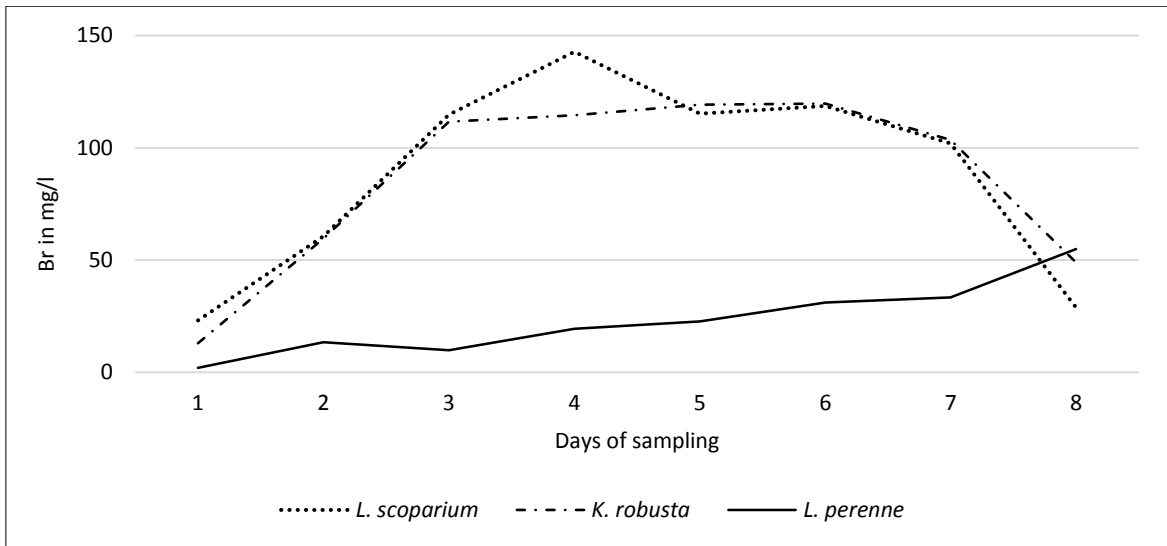


Figure. D. 43. Bromide leaching from *L. scoparium*, *K. robusta* and *L. perenne* (n= 1) in Akaroa soil.

16.2. Conclusion

NaBr can be used as a tracer for the glasshouse experiment, without the risk of degradation or toxicity to the *E. coli*.

Fig. D. 44 shows the taproot system of both *L. scoparium* and *K. robusta*.



Figure. D. 44. Shows the taproot system of *L. scoparium* and *K. robusta*.

