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Can manuka (*Leptospermum scoparium*) antimicrobial properties be utilised in the remediation of pathogen contaminated land?

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ABSTRACT

Society is increasingly conscious of waste reduction and contaminant control. The application of biosolids to land (re-cycling) is one means of waste disposal that has been shown to improve various soil properties whilst re-using valuable nutrients. However, there is concern that land application of biosolids can introduce microbial contaminants to soil. The aim of this research was to investigate the potential use of manuka (*Leptospermum scoparium*), a shrub with known antimicrobial properties, to reduce microbial contaminants from land applied biosolids. We evaluated the efficacy of water extracted manuka components against five bacterial pathogens potentially found in biosolids. Water extracted manuka components significantly inhibited an *Escherichia coli* lux biosensor, as well as inhibited the growth of *Salmonella typhimurium*, *E. coli* 0157, *Clostridium perfringins*, *Campylobacter jejuni* and *Listeria monocytogenes*. EC_{50} calculations for these pathogens showed that 50% inhibition of growth occurred at relatively low manuka extract concentrations (0.07–27.9% v/v). For C. *jejuni*, complete inhibition of growth was observed at extract concentrations of 0.78%. These results indicate potential for the use of manuka in the rehabilitation of microbial contaminated sites.

1. Introduction

Intensive land use world-wide has led to carbon loss and nutrient depletion from soil. Such soil can be described as "degraded" and one option for rehabilitation is amendment with organic material such as biosolids. This has been shown to improve the physical, chemical and biochemical properties of soil, whilst reducing the need for inorganic fertilisers (Smith, 1996; Alvarenga et al., 2009). Recycling of biosolids to land is considered a beneficial means of re-using an otherwise unwanted waste product, and has been implemented with success under forestry and plantation land use (Henry et al., 1994; Henry and Cole, 1997; Magesan and Wang, 2003; Kimberley et al., 2004).

However, land application of biosolids is not without its drawbacks as there are social and cultural aspects that must be considered (Goven et al., 2012), particularly where public perception is not favourable. In addition there is potential to introduce microbial and chemical contaminants into the environment (Pepper et al., 2006; Elving et al., 2009; Sindhu and Toze, 2009). For this reason, recycling of biosolids to land is restricted and highly regulated in most countries (NZWWA, 2003; Horswell et al., 2009). In New Zealand, the use of Grade B biosolids (those containing greater than 100 colony forming units (cfu) of *Escherichia coli* per g dry biosolids) for land application is restricted, to minimise environmental and human health risks (NZWWA, 2003). Restrictions include: where it can be applied, subsequent land use, length of time between application, and the security of the land (i.e. degree of access by people and livestock) (NZWWA, 2003; Pepper et al., 2006; Elving et al., 2009). Sufficient time must be given for natural attenuation of pathogens before the land can be used for cropping or public access.

It is accepted that pathogen numbers are reduced during sewage processing, however, it is unlikely that they can be completely eliminated (Carrington, 2001; Garrec et al., 2003; Lemunier et al., 2005; Horswell and Aislabie, 2006; Horswell et al., 2009). In addition, some pathogenic organisms can survive in soil for considerable lengths of time after land application (Avery et al., 2004; Nicholson et al., 2005; Eamens et al., 2006; Horswell and Aislabie, 2006), and studies have shown apparent regrowth of pathogenic bacteria in a range of organic wastes some months after reporting complete die-off (Vasseur et al., 1996; Zaleski et al., 2005; Eamens et al., 2006; Elving et al., 2010). The perceived and real risks present a major barrier to land application of biosolids.

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Integrating a natural antiseptic component into the system of disposal may enhance public confidence in current restrictions, or reduce the need for such stringent restrictions.

Phytoremediation has become a popular technique to control soil contaminants and to remediate degraded land (Robinson et al., 2003; Ghosh and Singh, 2005; Santibanez et al., 2008; Kim and Owen, 2010; Bolan et al., 2011; Evangelou et al., 2012). In particular, phytostabilisation is considered an effective means of immobilising contaminants. Through the establishment of plant cover, contaminant mobility is reduced and thus the movement of contaminants off-site is reduced (Bolan et al., 2011). Whilst this process is usually applied to metal or chemical contaminants, it may also apply to microbial contaminants such as those present in biosolids.

Leptospermum scoparium (manuka) is a native New Zealand shrub that is known to be hardy and tolerant to varying soil and climatic conditions (including elevated heavy metals), and is commonly used in land restoration projects (Bergin et al., 1995; Perry et al., 1997a,b; Perry et al., 1997a, b; Scott et al., 2000; Stephens et al., 2005). Manuka has a shallow intricate root system that acts to prevent water movement and to de-water surface soil layers (Watson and O'Loughlin, 1985; Bergin et al., 1995). In addition, manuka components such as bark, leaves and sap have been used in medicinal preparations in New Zealand for centuries (Porter et al., 1998). Many studies have confirmed the antibacterial activity of manuka honey and oil (Porter and Wilkins, 1999; Lusby et al., 2005) and manuka products are sold widely throughout the world as natural remedies for minor infections and aliments (Lusby et al., 2005; Visavadia et al., 2008).

Despite the reported antibacterial activity of manuka components, the potential in situ antibacterial effect of manuka growing on pathogen-contaminated soil has not yet been investigated. The components responsible for the antimicrobial activity of manuka oils have previously been associated with the b-triketone content of the oil, particularly leptospermone (Perry et al., 1997a,b; Porter et al., 1998; Christoph et al., 2000; Porter and Wilkins, 1999). These manuka antibacterial agents may end up in the soil via a number of pathways; in particular through rhizodeposition from roots or continual leaf fall and subsequent degradation.

Preliminary work carried out by Prosser (2011) sought to test the antibacterial efficacy of various manuka products; manuka water extracts, manuka honey (obtained from a speciality honey store, 5 + active) and commercially-produced pure manuka oil (certified 100%), on a range of human pathogens. Initial experiments using agar plates spread with the outlined manuka products and either pure cultures of pathogenic bacterial strains or serial dilutions of sewage biosolids indicated the bacteria were negatively affected by the manuka oil and the manuka leaf extracts, and to a lesser extent the manuka root extracts (Prosser, 2011). The current study was designed to build upon the findings of this preliminary research by assessing the effect of laboratory extracted manuka components on a range of bacterial pathogens commonly found in biosolids (Sindhu and Toze, 2009). Both root and leaf extracts are assessed to investigate whether potential antimicrobial ability is more likely to come from root exudation or degrading biomass. The overall aim of our research was to investigate the potential use of manuka as a remediation species for contaminated land with specific regard to its antimicrobial properties.

2. Methods

2.1. Preparation of plant extracts

Manuka seedlings were obtained from Matatoa Trees and Shrubs plant nursery, Shannon, New Zealand. For manuka leaf samples, herbage was removed from all aerial portions from the base of the stem, whilst manuka root samples included all plant matter from the base of the stem downwards. Plant components were washed with deionised water to remove attached soil. Ryegrass was used as a control species as it has no known antimicrobial activity, and biosolids are frequently recycled to pasture in many countries (Joshua et al., 1998). For ryegrass (*Lolium perenne*), 20 g of herbage was taken from a ryegrass field by random grab-sampling and transported to the laboratory, where any herbage other than ryegrass was removed. Each of the three samples (manuka leaf, manuka root and ryegrass) was processed independently, but identically as follows. The samples were cut into 1 cm pieces and homogenised by quartering. A 5 g subsample was mixed with 20 ml deionised water in a blender, and then further ground using a mortar and pestle. The ground paste was pressed to obtain a liquid which was filtered using Whatman #1 filter paper and sterilised by passing through Millipore syringe filters (0.2 μm).

2.2. Bacterial bioluminescence-based bioassay

A bacterial bioluminescence-based bioassay (*Escherichia coli* lux biosensor), adapted from Horswell et al. (2006) was used to investigate the microbial toxicity of the water-soluble plant extracts as well as a commercially produced pure manuka oil. The biosensor (supplied by the University of Aberdeen, UK) produces luminescence when immersed in favourable conditions, and exhibits reduced luminescence when in less favourable conditions. The biosensor consists of lyophilised cells of *E. coli* HB101 genetically modified with the luxCDABE genes, originally isolated from *Vibrio fischeri*, using the multicopy plasmid pUCD607 (Rattray et al., 1990; Sousa et al., 1998; Horswell et al., 2006). The bioassay was performed as described in Horswell et al. (2006). All of the water-soluble plant extracts were diluted 1:10 with sterile deionised water before use. Bioluminescence was measured in a LumiSkan TL (Labsystems, Finland) after a 15 min exposure to each sample. The assay was carried out in triplicate with sterilised water used as a blank. Data were expressed as average luminescence.

2.3. Microplate assay

Based on the results obtained from the *E. coli* lux biosensor, only the manuka leaf extract was chosen for further investigation, with ryegrass extract as a control. To further investigate the microbial toxicity of the manuka leaf extract it was tested using a spectrophotometric bioassay adapted from Patton et al. (2006) which has been previously used to determine microbial sensitivity to manuka honey. This assay was chosen based on its time and cost efficiency when compared to previous assays for the detection of antimicrobial activity (Smith et al., 2008), and because broth dilution methods have been found to be more easily reproducible in studies of essential oils than the commonly used agar plate diffusion techniques (Christoph et al., 2000). Each assay test sample contained a total volume of 200 μl, which consisted of 100 μl bacterial suspension, 50 μl of broth and 50 μl of plant extract. Serial dilutions (1:2) of manuka and ryegrass leaf extract were prepared in deionised water. The final extract concentrations in each 200 μl sample were 25, 12.5, 6.25, 3.13, 1.56, 0.78, 0.39, 0.20, 0.10, 0.05, 0.02, and 0.01%.

Five bacterial strains were chosen for analysis based on their potential presence in biosolids (Carrington, 2001; Pepper et al., 2006; Sindhu and Toze, 2009): *Salmonella typhimurium*, *E. coli* 0157, *Clostridium perfringins*, *Campylobacter jejuni* and *Listeria monocytogenes*.

Strains of *E. coli* and *Clostridium* sp. are considered representative of faecal indicator organisms (Eamens et al., 2006), whilst *S. typhimurium* and *C. jejuni* are enteric pathogens responsible for

Table 1
Optimum growing condition for five pathogenic bacterial strains.

Bacteria	Growth medium	Incubation temperature	Frequency of absorbance measurements
S. typhimurium	Luria-Bertani (LB) broth	30 °C	Read every hour for 24 h
L. monocytogenes	Tryptic soy broth	37 °C	Read every hour for 24 h
E. coli 0157	Luria-Bertani (LB) broth	30 °C	Read every hour for 24 h
C. jejuni	Brucella broth	37 °C	Plates were placed in aerojars with microaerobic gas-producing packs. Readings taken every 12 h for 48 h
C. perfringins	Cooked meat broth with the solid portions removed	37 °C	Plates were placed in aerojars with anaerobic gas producing packs (for *Clostridium* sp.) Readings taken every 2 h for 10 h.

high rates of gastroenteritis in New Zealand [reported incidence rates are 23.3 and 163.8 cases per 100 000 persons respectively (New Zealand Public Health Surveillance Report, 2012)]. *L. monocytogenes*, a pathogenic bacterium causing listeriosis, is widely present in the environment and in treated sewage sludge, and is a particular risk during pregnancy (Garrec et al., 2003; Lemunier et al., 2005).

Each bacterial strain was grown from freeze dried stock in the appropriate growth medium (Table 1). The optimum dilution of bacterial seed stock to use in the assay was pre-determined by assessing bacterial growth curves at various dilutions. The concentration of bacterial stock selected for each bacterium was chosen such that the full growth curve would be visible over the incubation period. Each strain was subsequently grown from the stock broth under optimum conditions in a 96-well plate, in the presence of either manuka leaf or ryegrass extracts (Fig. 1). The assay was carried out using four test replicates, two control replicates (no plant extract) and two blanks (no microbial strain). Inhibition of growth was determined by measurement of well optical density (OD) over time (595 nm). Prior to each OD measurement, the plate was shaken for 10 s, and OD of each well was measured using an automated absorbance plate reader (Optima). An increase in OD was taken to indicate growth of bacteria.

2.4. Statistical analysis

The microplate data were first normalised by subtracting the optical density (OD) value at time zero from all subsequent values. Growth curves were then obtained by averaging the replicate well OD values at each time point and subtracting the respective extract blank values. This was to account for any absorbance caused by the presence of extracts which exhibited some colour. Extinction curves were obtained by plotting the total growth at the endpoint of the assay (maximum growth), calculated as a percentage of the control (Patton et al., 2006), against the concentration of manuka or ryegrass extract in the well. GraphPad prism (v5.04) was used to plot extinction curves, these graphs were subsequently used to determine environmental effect concentrations (EC_{50} and EC_{20}). Means and standard errors were calculated using Excel 2012. Analysis of variance was performed using IBM SPSS statistics 21, and the significance of difference assessed using Tukey post-hoc tests at a 5% confidence level ($p \leq 0.05$).

3. Results

3.1. E. coli lux biosensor

The *E. coli* lux biosensor showed a significant reduction ($p < 0.05$) of luminescence in the presence of all three extracts when compared to a water control, implying some microbial toxicity (Fig. 2). Luminescence was reduced by 49 and 89% in manuka root and leaf extracts respectively when compared to the water control. Both manuka treatments were found to have a significantly lower luminescence than the ryegrass control in the order of: manuka root extract > manuka leaf extract > commercial oil (17, 83, and 96 percent reduction in luminescence respectively, $p < 0.05$; Fig. 2). Although the *E. coli* lux biosensor is affected by solution pH, the bioluminescence response has been found to be stable across pH 4.5–8 (Sousa et al., 1998). The pH of each extract solution was measured (Table 2), and although a difference was observed, this was not considered to be enough to cause an effect when the extracts were diluted at the level used in the assay (100 µl

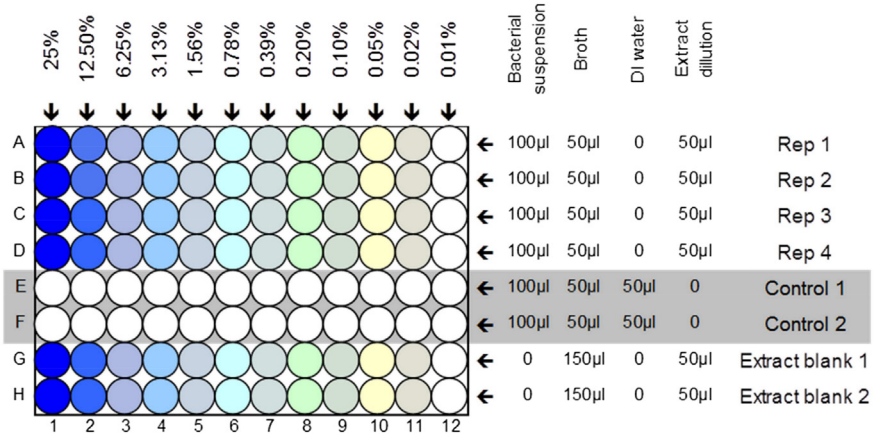

Fig. 1. The layout of each 96 well plate. Plant extracts were the last to be added and were diluted in a 1:2 serial dilution with deionised water. Each extract and bacteria combination was run on a separate 96 well plate.

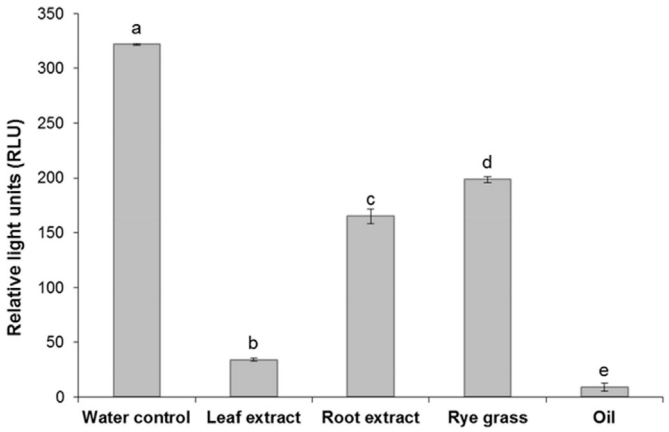

Fig. 2. Luminescence of the *E. coli* lux bacterial biosensor in the presence of manuka root and leaf extracts. Letters indicate significant difference ($p < 0.05$).

with 800 μl DI water). The observed reduction in luminescence shows some level of microbial toxicity of the extracts, particularly with reference to manuka leaf components.

3.2. Microplate assay

The growth of all five bacterial strains was significantly impacted by the manuka and ryegrass extracts. A significant inhibition of growth of *S. typhimurium, E. coli* 0157, *C. perfringins*, *C. jejuni* and *L. monocytogenes* was observed in the presence of the manuka leaf extract. Environmental effect concentrations (EC_{50} and EC_{20}) are a commonly reported threshold used for assessing the environmental impact of contaminants on biological parameters (Speir, 2008). In the current experiment, EC_{50} and EC_{20} were assessed, representing the concentration of our plant extract required to inhibit pathogen growth by 50 and 20 percent respectively. EC_{50} and EC_{20} data showed a 50% and 20% inhibition in growth at relatively low manuka extract concentrations for all five strains (Fig. 3), with the lowest EC_{50} values observed for *C. perfringins* (EC_{50} = 0.070% manuka leaf extract concentration) and *C. jejuni*. (EC_{50} = 0.597% manuka leaf extract concentration). These figures equate to the active component extracted from just 40 μg and 300 μg of fresh manuka herbage (respectively). This is in contrast to the effect of the ryegrass extract which significantly increased the growth of *S. typhimurium, E. coli* 0157, *C. jejuni* and *L. monocytogenes* (Fig. 4). Conversely, the presence of ryegrass extract inhibited the growth of *C. perfringins* (EC_{50} 33.4%, Fig. 5.), although not to the extent of the manuka extract.

4. Discussion

4.1. Explanation of antimicrobial activity

Pure manuka oil has been found to have activity against many pathogenic bacteria, including Staphylococcus, Listeria and Enterococcus species (Douglas et al., 2001), but predominantly against gram-positive strains (Christoph et al., 2000; Maddocks-Jennings et al., 2005). This is in partial agreement with our findings. We observed inhibition of the gram positive species *C. perfringins* and *L. monocytogenes* in the presence of our manuka extracts. However, we also observed inhibition of *S. typhimurium, E. coli* 0157 and *C. jejuni*, which are all gram-negative. Although some research has shown effects of manuka oil on gram-negative bacteria (Christoph et al., 2000) the extent of the inhibition observed towards these pathogenic strains with our water extracts was unexpected. A survey of the literature provides no previous evidence for manuka extracts having such a strong impact against these bacterial strains. This is a significant finding and to the best of our knowledge is the first reported observance of such strong antimicrobial properties of manuka components on gram-negative species.

The outer membrane of gram-negative bacteria restricts the diffusion of lipophilic/hydrophobic compounds (Burt, 2004) but does not protect it from hydrophilic compounds as efficiently. This may present a possible explanation for the observed effect of our extracts on gram-negative species which were prepared using water, and were not lipid based as most essential oils are. However, this would not explain the observed impact on gram-positive bacteria. In previous experiments, this was thought to be due to the degradation of peptidoglycan in cell walls caused by the lipophilicity of manuka oil (Christoph et al., 2000).

Water was chosen as an extractant in the current experiment to more closely mimic conditions present in soil when herbage decomposes. Previous studies have used an array of solvents when extracting plant products to isolate antimicrobial components (Eloff, 1998), and although solvent extracts have been shown to enhance antibacterial activity, water extracts have been shown to be particularly effective against some bacteria (Eloff, 1998; Hassan et al., 2009). For the purpose of our research, water was determined to be sufficiently effective, and more relevant, and its use would avoid any potential inherent toxicity of the solvents themselves.

The determination of environmental effect concentrations (EC_{50} and EC_{20}) is a widely used method of determining thresholds for environmental contaminants (Moreno et al., 2001; Horswell et al., 2006; Speir, 2008; Speir et al., 2007). Based on this approach, all five bacterial species assayed showed significant reduction in growth, and in some cases complete inhibition of growth, in the presence of water extracts of manuka. This can be attributed to components present in the manuka extracts, as the control ryegrass extract increased the growth of all but one bacterial species. This is a surprising result, as the manuka component reported to be responsible for the antimicrobial activity of manuka oils (leptospermone) is not thought to be soluble in water (Weston et al., 2000). We are unsure what components of the extracts have caused the observed inhibition, and whether the components responsible would persist in soil once herbage decay is underway. Further experiments identifying the chemicals responsible would be highly valuable to this research.

4.2. Potential pathways to the soil environment

Manuka antimicrobial agents may end up in the soil environment via a number of pathways. The two potential pathways highlighted in the current research are rhizodeposition and degradation of plant material. It was thought that rhizodeposition would play an important role in the transfer of antimicrobial agents to soil. Through the release of carbon sources/organic compounds (organic acids, amino acids) and protons, plant roots are able to alter soil pH, and select for different bacterial populations and enzymatic activity (Cheng et al., 2004; Yang et al., 2007; Castaldi et al., 2009; Quartacci et al., 2009). Studies have found that root exudates can remain stable in soils for significant lengths of time

Table 2
pH of ryegrass, manuka leaf and manuka root extracts before and after dilution with DI water.

		Manuka leaf extract	Manuka root extract	Ryegrass extract
pH	Neat	4.88	5.13	5.63
	Diluted 1:8	5.64	5.99	5.79

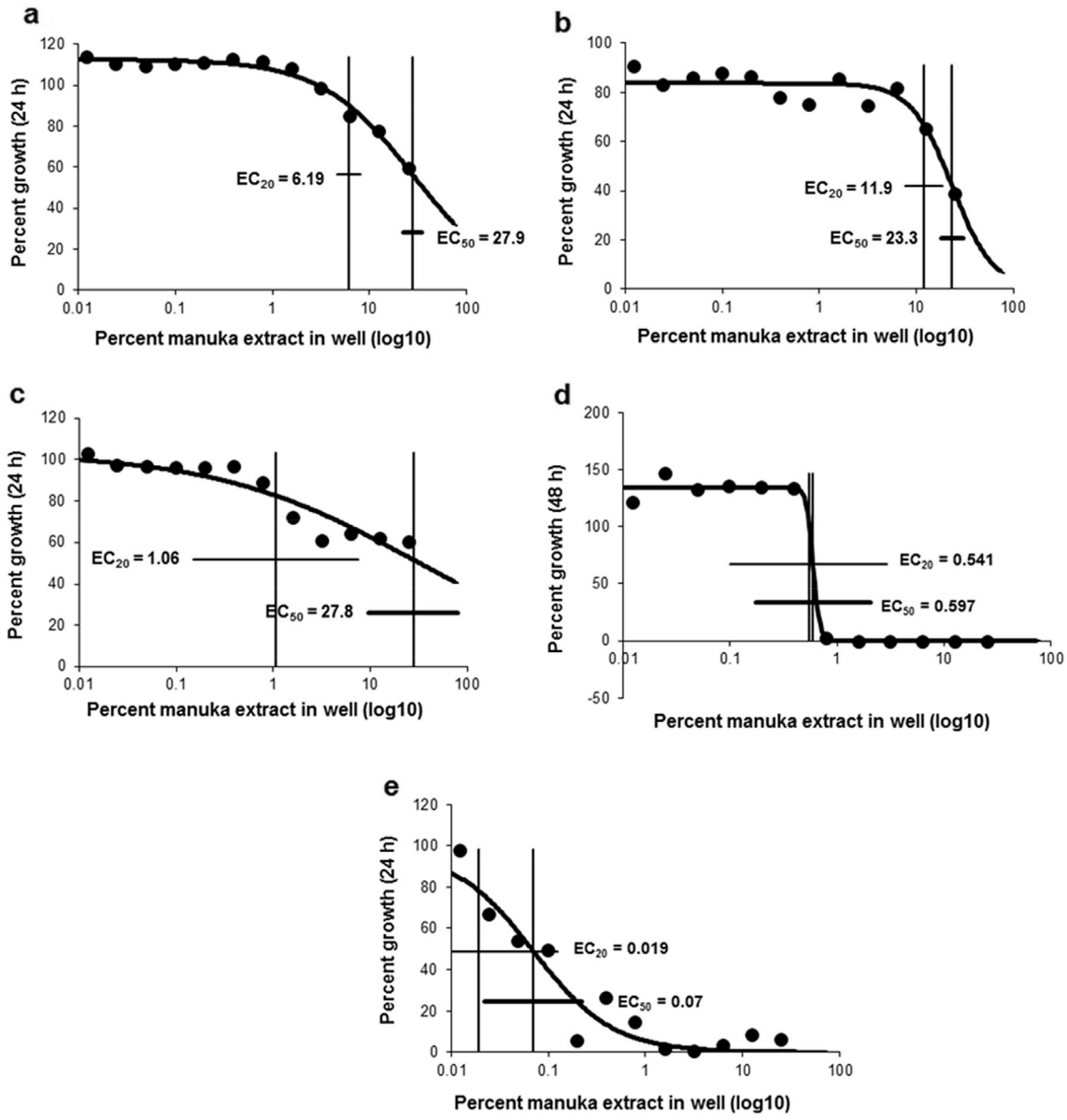

Fig. 3. The effect of increasing manuka extract concentration on the growth of (a) *S. typhimurium*, (b) *L. Monocytogenes*, (c) *E. coli 0157*, (d) *C. Jejuni*, and (e) *C. perfringins*.

(Quartacci et al., 2009). Cornes (2005) found that leptospermone (the component thought to be responsible for antimicrobial activity in manuka oil) produced by the roots of bottle brush (*Callistemon citrinus*) suppressed weeds around the plant's base. Roots of manuka have not been investigated for leptospermone content, but this may be similar to that found in the bottle brush. If this is the case, leptospermone released by manuka roots may also inhibit bacterial populations in soil, including pathogenic organisms. The current results indicate that whilst root extracts had a significant impact on the bacterial biosensor, the leaf extracts were far more active. Subsequently, only manuka leaf extracts were investigated in-depth. However, a closer look at manuka root exudates and rhizosphere soil may shed further light on the pathway described here.

Leaf-fall is very common in manuka all year, and is inferred in this work to be the most likely pathway for antimicrobial agents from manuka to accumulate in soil. Soils underneath long-standing manuka forests have been exposed to leaf-fall for considerable lengths of time, and fallen leaves that eventually degrade may release antibacterial agents. Chemicals from root exudates have been shown to stay in soil for considerable lengths of time (Quartacci et al., 2009), and therefore, it is likely that chemicals released through biomass degradation would behave in a similar way. However the potential influence of varying soil factors on the effectiveness of the active compounds should also be taken into account.

4.3. Practical application for manuka: remediation of pathogen-contaminated biosolids

We propose that the antimicrobial products released by degrading manuka biomass may serve as a means for reducing microbial contamination in soil. Our results indicate that there is potential for manuka to be used in the rehabilitation of microbial contaminated sites through the inhibition of growth and reduced survival of pathogenic microorganisms. One option may be the planting of manuka as a cover species on such sites, with aims of eventually regenerating native vegetation. There is also potential for the use of manuka alongside the application of organic products

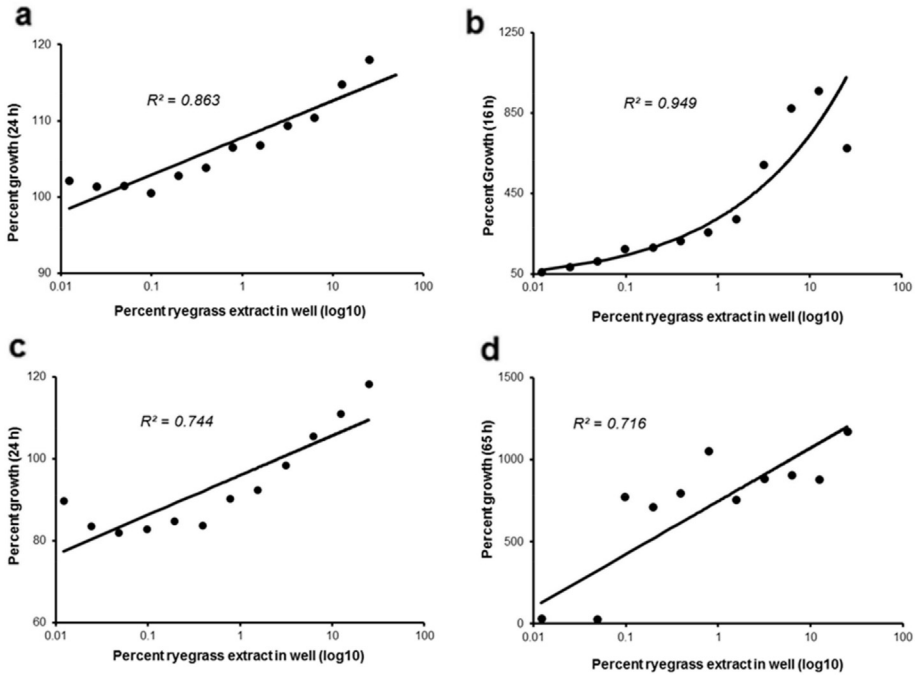

Fig. 4. The effect of increasing ryegrass extract concentration on the growth of (a) *S. typhimurium*, (b) *L. Monocytogenes*, (c) *E. coli 0157*, and (d) *C. Jejuni*.

such as biosolids to prevent contamination from pathogenic bacteria. This may serve to remove some of the perceived barriers to this potentially beneficial practice, eventually increasing public confidence in existing restrictions, and enhancing the economic viability and general appeal of recycling urban waste in this way.

Manuka is widely used in land restoration projects in New Zealand due to its hardy, tolerant nature. The species has the potential to act as a phytostabiliser in phytoremediation operations. It addition, manuka components have economic value through the commercial production of honey and essential oil-related products. This makes manuka plantations a viable agricultural industry, and if establishment can be achieved on otherwise unusable land (due to contamination or degradation), where biosolids or other organic wastes can be recycled, then this system has added potential to generate an economic return. In a scenario such as this, where manuka is planted for regeneration purposes, or as part of commercial production, the beneficial re-use of biosolids could become a sustainable option. This may be achieved through applying waste to established manuka stands or plantations which could act both as an aid to enhance growth of manuka, mitigating the need for commercial fertilisers, as well as a means of 'treating' bacterial contaminants in the biosolids. Continual degradation of leaf fall, or rotational cropping and mulching of manuka biomass, would aid in the attenuation of introduced bacteria, including pathogenic strains. There may be further potential to harvest manuka biomass to mix with organic wastes in co-composting systems. In the current climate where society is ever increasingly conscious of waste reduction and contaminant control, this remediation system may represent an economically, socially and environmentally acceptable solution to the ever-present question of what to do with biosolids.

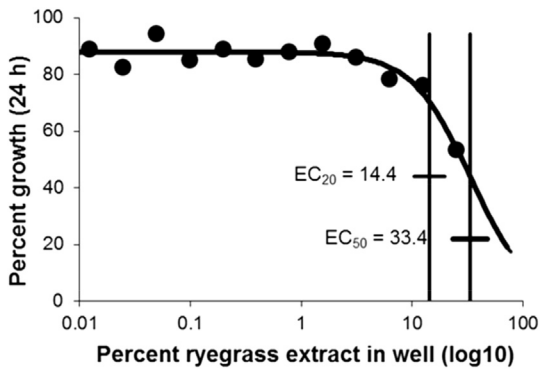

Fig. 5. The effect of increasing ryegrass extract concentration on the growth of *C. perfringins*.

5. Conclusion

Our study was undertaken to evaluate the potential of manuka as a remediation species for reducing microbial contaminants in biosolids-amended land. We found that water-soluble components of manuka caused an inhibitory response of the *E. coli* lux biosensor and inhibited growth of five pathogenic bacteria species. This was most evident for aqueous manuka leaf extracts, which significantly inhibited the growth of a number of pathogens of concern in biosolids. In the case of *C. Jejuni*, inhibition of growth was 100% at a leaf extract concentration of only 0.78% (equating to 390 μg of extracted fresh herbage). A survey of the literature found no previous evidence of such strong inhibition from manuka products. We suggest that further research into the potential use of manuka to manage levels of pathogenic soil microorganisms is warranted. Biosolids disposal systems could potentially be combined with manuka production to develop a sustainable system for pathogenic contaminant inhibition, with the added potential to return economic value through harvest of manuka-based commodities.

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