Soil-Plant Interactions of New Zealand Native Vegetation Irrigated with Treated Municipal Wastewater

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A thesis

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in

Environmental Science

by

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Abstract

The reuse of treated municipal wastewater (TMW) for irrigation rather than disposal into waterways, has net positive effects on water quality. TMW could be irrigated onto native vegetation to create zones of enhanced ecological value, and possibly generate valuable native products. However, there is a lack of knowledge on how TMW irrigation will affect the performance of native plant species. The fluxes of nutrients and contaminants in such systems need to be assessed to determine the risks of soil degradation as well as ground- and surface water contamination.

Long-term field trials in Duvauchelle (Banks Peninsula) and Levin were used to study the soil-plant interactions of New Zealand native vegetation irrigated with TMW. The growth and survival of native species at the sites were monitored and the chemistry of the plants, as well as the underlying soil, was analysed. Results from both field sites showed that irrigation of TMW accelerated the growth of native vegetation. At Duvauchelle, TMW increased the overall average plant height by 10% after 3.5 years of irrigation. However, weed growth and competition was also accelerated by TMW irrigation. Weed control resulted in a ninefold increase in the survival of TMW irrigated plants at Levin. This shows that weed management is a critical success factor for the establishment of native ecosystems with TMW.

The application of TMW increased phosphorus, nitrogen, and sodium concentrations in the soil. Nitrogen fluxes were affected by plant species, and not all of the nitrogen applied with TMW could be accounted for. At Duvauchelle, soil nitrate concentrations were lower in the rootzone of *Coprosma robusta* than other species. TMW irrigation did not significantly increase soil nitrate concentrations at application rates of 1000 mm yr^{-1} (equivalent to approx. 200 kg N ha^{-1} yr^{-1}). In contrast, preliminary results from Levin showed that 28-38% of the applied nitrogen leached from the soil in the form of nitrate at TMW irrigation rates >4000 mm yr^{-1}, with no significant difference between *Kunzea robusta* and pasture.

A pot experiment was set up to quantify nitrifying and denitrifying microorganisms under native species compared to pasture. Ammonia oxidising bacteria were less abundant under monocotyledonous than dicotyledonous species. This was likely due to higher nitrogen acquisition by the monocotyledonous plants, which limited the nitrogen availability to the bacteria. The abundance of *nosZ*, the gene encoding nitrous oxide reductase that reduces nitrous oxide to dinitrogen during denitrification, differed between plant species and was significantly lower under *Coprosma robusta* than most other New Zealand native species and *Lolium perenne*. This reflects the importance of species selection to mitigate nitrogen losses from TMW irrigated soil.

If TMW irrigated plants were utilised to produce value from the land through native products, increased soil nutrient concentrations with TMW irrigation may affect the quality of products such as mānuka (*Leptospermum scoparium*) honey and essential oils. Data from a field study was analysed to determine the effects, if any, of soil properties on the quality of mānuka honey. Results showed that honey trace element and soil nitrate concentrations were negatively correlated with mānuka honey methylglyoxal concentration, which largely determines the honey's market value. It is possible that accelerated growth of exotic weeds in high fertility soil led to a dilution of mānuka honey. This is particularly relevant at sites with high nutrient application rates through TMW.

This research revealed that TMW could be beneficially reused to establish native ecosystems in New Zealand and elsewhere. Such multi-purpose restoration presents an opportunity to increase the percentage of TMW that is applied onto land. Globally, establishing native vegetation with TMW irrigation has the potential to improve water quality, increase biodiversity, and accelerate carbon sequestration.

Contribution and Outcomes

Much of this research was based on long-term field trials that were established by the biowastes research team at the University of Canterbury (UC), the Institute of Environmental Science and Research (ESR), and Lowe Environmental Impact (LEI).

Chapter 2

The field trial in Duvauchelle was set up by Lincoln University in 2015 and funded by the Christchurch City Council (CCC). I obtained additional funding from CCC at the value of NZ$ 29,000 for the experimental part described in this chapter. Sample collection and analysis were conducted together with Furong Li (Guangdong Academy of Agricultural Sciences, China). Data analysis was my sole responsibility. The results of this work were reported to CCC (Appendix B) and guided CCC for the decision and design of the current plans to irrigate the treated municipal wastewater from the Akaroa wastewater treatment plant onto 100 ha of native vegetation in Robinsons Bay, which is a > NZ$ 60 M investment. This chapter was submitted for publication as follows:

Meister, A., Li, F., Gutierrez-Gines, M. J., Gaw, S., Dickinson, N., Bourke, M., & Robinson, B. Interactions of treated municipal wastewater with native ecosystems. Submitted to *Ecological Engineering*.

Chapter 3

The field trial in Levin was set up in 2018 by LEI, ESR, and UC. Funding for the trial was from the Horowhenua District Council (HDC) and the Ministry for the Environment Freshwater Improvement Fund. Total funding for the project was NZ$ 1,297,000. Site management was by LEI. Trees for the preliminary trial, and the weed management trial were planted by LEI. The plant biomass in the preliminary plant growth trial was determined by Seinalyn Villanueva (ESR). I measured plant growth in all the trials over the years, analysed the data, interpreted the results, and wrote the client reports and this chapter in full. The results of this research are informing LEI and HDC on how to revegetate the whole site. Similarly, the results are informing the transformation of TMW irrigated harvested forestry plantations into native ecosystems in other parts of New Zealand, such as Wainui (Banks Peninsula) and Amberley (Canterbury).

Chapter 4

Sampling at the field site was conducted by me, together with Maria Jesus Gutierrez-Gines (ESR) and Izzie Alderton (ESR). I installed the lysimeters with Brett Robinson (UC) and Maria Jesus Gutierrez-Gines. Downer Group NZ assisted with excavation of the pits for the lysimeters. Kristin Bohm (ESR) and Richard Dean (ESR) helped with tree planting on the lysimeters and installation of the dataloggers.

Eise Venter (LEI) assisted with emptying of the lysimeters. Data analysis and writing was my sole responsibility.

Chapter 5

I designed and conducted the greenhouse experiment and was solely responsible for sample analysis, data analysis, and writing of the manuscript. The methods for the DNA extraction and quantification were developed by Kristin Bohm.

Chapter 6

Soil and plant samples were collected by Aydin Maxfield (LEI). Honey samples were provided by Watson & Son Ltd. (now Oha Honey LP). Sample analyses were conducted by accredited laboratories. I conducted the data analysis and wrote the manuscript. This chapter was published as follows:

Meister, A., Gutierrez-Gines, M. J., Maxfield, A., Gaw, S., Dickinson, N., Horswell, J., & Robinson, B. (2021). Chemical elements and the quality of mānuka (*Leptospermum scoparium*) honey. *Foods* 10, 1670. https://doi.org/10.3390/foods10071670

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Name: *Brett Robinson* Signature:  Date: *11/01/2022*

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List of Plant Species

New Zealand native species

Scientific name*	Family	Vernacular
Austroderia richardii (Endl.) N.P.Barker et H.P.Linder	Poaceae	Toetoe
Carex pumila Thunb.	Cyperaceae	Sand sedge
Carex secta Boott	Cyperaceae	Pukio, Purei
Coprosma propinqua A.Cunn. var. propinqua	Rubiaceae	Mingimingi
Coprosma repens A.Rich.	Rubiaceae	Taupata, mirror plant
Coprosma robusta Raoul	Rubiaceae	Karamu
Cordyline australis (Forst.f.) Endl.	Asparagaceae	Tī kōuka, cabbage tree
Corynocarpus laevigatus J.R.Forst. et G.Forst.	Cornyocarpaceae	Karaka, kopi
Dodonaea viscosa Jacq.	Sapindaceae	Akeake
Griselinia littoralis Raoul	Griseliniaceae	Kapuka, broadleaf
Juncus pallidus R.Br.	Juncaceae	Giant/leafless rush
Kunzea robusta de Lange et Toelken	Myrtaceae	Kānuka
Leptospermum scoparium J.R.Forst. et G.Forst. var. scoparium	Myrtaceae	Mānuka
Melicytus ramiflorus J.R.Forst et G.Forst	Violaceae	Mahoe, whitey wood
Metrosideros umbellata Cav.	Myrtaceae	Southern Rātā
Myoporum laetum G.Forst.	Scrophulariaceae	Ngaio
Olearia paniculata (J.R.Forst et G.Forst) Druce	Asteraceae	Akiraho, golden akeake
Phormium tenax J.R.Forst et G.Forst	Xanthorrhoeaceae	Harakeke, flax
Phormium cookianum Le Jol. subsp. cookianum	Xanthorrhoeaceae	Whararikī, mountain flax
Pittosporum eugenioides A.Cunn.	Pittosporaceae	Tarata, lemonwood
Plagianthus regius (Poit.) Hochr. subsp. regius	Malvaceae	Manatu, ribbonwood
Poa cita Edgar	Poaceae	Silver tussock
Podocarpus laetus Hooibr. ex Endl.	Podocarpaceae	Mountain/Hall's totara
Pseudopanax arboreus (L.f.) Allan	Araliaceae	Fivefinger, whauwhaupaku
Pseudowintera colorata (Raoul) Dandy	Winteraceae	Red/mountain horopito, alpine peppertree
Veronica stricta Benth. var.stricta	Plantaginaceae	Koromiko

Exotic species and weeds

Scientific name*	Family	Vernacular
Dactylis glomerata	Poaceae	Cocksfoot
Holcus lanatus L.	Poaceae	Yorkshire fog
Lolium perenne L.	Poaceae	Perennial ryegrass
Phytolacca octandra	Phytolaccaceae	Inkweed
Pinus radiata D.Don	Pinaceae	Monterey/radiata pine
Solanum chenopodioides Lam.	Solanaceae	Velvety nightshade
Solanum nigrum L.	Solanaceae	Black nightshade

* Botanical information retrieved from https://www.nzpcn.org.nz/ on 01 December 2021.

Abbreviations

AOA	Ammonia oxidising archaea
AOB	Ammonia oxidising bacteria
BNI	Biological nitrification inhibitor
BOD	Biochemical oxygen demand
CO_2	Carbon dioxide
DHA	Dihydroxyacetone
EC	Electrical conductivity
EOCs	Emerging organic contaminants
ETS	Emission Trading Scheme
HPLC	High performance liquid chromatography
ICP-MS	Inductively coupled plasma-mass spectrometry
ICP-OES	Inductively coupled plasma-optical emission spectrometry
MGO	Methylglyoxal
N_2	Dinitrogen
N_2O	Nitrous oxide
NH_3	Ammonia
NH_4^+	Ammonium
NO	Nitric oxide
NO_3^-	Nitrate
NO_2^-	Nitrite
NPA	Non-peroxide antimicrobial activity
NZ	New Zealand
PCA	Principal component analysis
qPCR	Quantitative polymerase chain reaction
SAR	Sodium Adsorption Ratio
TA	Total archaea
TB	Total bacteria
TMW	Treated municipal wastewater
TSS	Total suspended solids
WWTP	Wastewater treatment plant

All chemical elements are abbreviated without introduction, according to the periodic table.

Chapter 1

Introduction and Background

1.1 General Introduction

The disposal of treated municipal wastewater (TMW) into waterways or the ocean results in widespread degradation of these environments (Jarvie et al., 2006). The plant nutrients in TMW can cause eutrophication of freshwater bodies and exacerbate toxic algal blooms in marine environments (Lapointe et al., 2015; McDowell et al., 2009). Pathogens in TMW can cause widespread human illness (McBride & Ball, 2004). Disposal into waterways represents the squandering of the economic value of water that could be used for irrigation and nutrients that could offset fertiliser costs (Tsadilas & Vakalis, 2003).

Application of TMW to land can capture value from the TMW and mitigate some of the negative environmental outcomes that would otherwise occur in if the TMW were discharged into waterways. Land application of TMW is consistent with the concept of a circular economy (Geissdoerfer et al., 2017), where the consumption of resources and the production of wastes is minimised (Smol et al., 2020). However, there are challenges associated with the land application of TMW. The TMW can contain pathogens and emerging organic contaminants that may pollute local environments and render food production unsafe (García et al., 2020; Norton-Brandão et al., 2013). Nutrients in TMW applied to land may run off into surface waters or leach into groundwater (Jarvie et al., 2006; Sparling et al., 2006). High rates of N application may result in increased leaching of nitrate (NO_3^-) and, combined with semi-saturated soil, may lead to increased emissions of nitrous oxide (N_2O), a potent greenhouse gas (Philibert et al., 2013). Some components of the TMW, such as P and K, may accumulate in soil and cause nutrient imbalances in plants (Hanjra et al., 2012). Elevated concentrations of Na and K may result in degradation of the soil structure, reducing infiltration and aeration (Farahani et al., 2018). In addition, there are socio-cultural issues associated with the land application of TMW, particularly onto food crops (Pauling & Ataria, 2010).

Some of the challenges associated with TMW application onto land may be circumvented if the TMW is used to establish valuable native ecosystems. The value in such ecosystems may be ecological, for example increased biodiversity, or economic, with revenue generated through the creation of native (non-food) products (Seyedalikhani et al., 2019) and the maintenance of a positive environmental image in export markets or as tourist destination (Bain & Dandachi, 2015). Native products, for example essential oils, manufactured from indigenous plant species cannot be easily copied by

overseas competitors (Essien et al., 2019). Globally, the development of native forest systems receiving TMW may provide an important C sink (Evans et al., 2015).

Most research on TMW application has focused on food and energy crops, pasture, or timber species (Carlander et al., 2009; Christou et al., 2017; Gutierrez-Gines et al., 2020; Trichet et al., 2018). There is a lacuna of knowledge on how native species will interact with TMW both in New Zealand (NZ) and overseas. Many NZ native species are adapted to low fertility environments (Wardle, 1985), and may not benefit from TMW application. The goal of this thesis is to elucidate the critical factors affecting the interactions of native species with TMW. While the thesis uses pot and field trials in NZ, the findings will be relevant to systems globally where TMW may be used to establish native vegetation.

1.2 Background

1.2.1 Generation, treatment, and discharge of municipal wastewater

The production of wastewater is increasing globally with population growth and economic development (Morris et al., 2017). About 65-80% of global freshwater withdrawals are released back into the environment as wastewater, with lower percentages in arid areas (Liu & Lipták, 2000). Jones et al. (2021) estimated that 359 B m^3 yr^{-1} of wastewater is produced globally. Management of wastewater is a major social and environmental challenge (WWAP, 2017). Wastewater is a complex mixture of water, organic matter, nutrients, salts, metals, microorganisms, and xenobiotics (Henze & Comeau, 2008). Whether wastewater is treated prior to release into the environment greatly depends on the financial resources of a country. Low- and high-income countries are treating 8% and 70% of their wastewater, respectively (Sato et al., 2013). Treatment reduces concentrations of contaminants in wastewater prior to its release into the environment (Norton-Brandão et al., 2013).

Municipal wastewater originates from domestic, commercial, institutional, and industrial sources within human settlements (WWAP, 2017). It can include infiltration and inflow into sewer lines and stormwater runoff (Liu & Lipták, 2000). Municipal wastewater generally consists of 99.9% water and 0.1% solids (Karia & Christian, 2013). Municipal wastewater may be treated in septic tank systems or centrally through sewerage and redirection to wastewater treatment plants (WWTPs) (Jones et al., 2021). WWTPs process the inflowing wastewater in multiple steps, which continually reduce concentrations of various contaminants (Crini & Lichtfouse, 2019). The preliminary treatment aims to remove solids that could cause mechanical wear and damage or lead to clogging, and primary treatment further removes settable, suspended, and floatable materials through filtration and sedimentation (Spellman, 2014). The solids removed from the wastewater during treatment form the sludge, which is usually landfilled, incinerated, or applied to land (Raheem et al., 2018). Biological

processes in secondary wastewater treatment transform dissolved, suspended, and colloidal organic wastes into more stable solids (Spellman, 2014). Trickling filters, activated sludge, and oxidation ponds are common biological treatment units (Karia & Christian, 2013). Secondary treatment removes 85-95% of biochemical oxygen demand (BOD) and total suspended solids (TSS) from the wastewater, resulting in approximately 30 mg L^{-1} BOD and TSS (Spellman, 2014). The removal of N and P during secondary treatment is not significant, and wastewater typically contains 20-60 mg N L^{-1} and 6-16 mg P L^{-1} after secondary treatment (FAO, 2003). Additional treatment steps, referred to as advanced or tertiary treatment, can be added to further reduce BOD and TSS, and remove more than 99% of pollutants from raw wastewater (Spellman, 2014). Such treatment improves the quality of TMW to enable reuse for specific purposes (Smol et al., 2020). Examples of tertiary treatment processes include nitrifying trickling filters, P removal with metal salts, and UV disinfection to reduce pathogens (Beca et al., 2020; van der Graaf et al., 2005).

Most wastewater, treated or not, is directly released into aquatic environments (WWAP, 2017). Sato et al. (2013) estimated that only 3.8% of treated TMW in North America is reused. In Australia and New Zealand, about 86% and 91%, respectively, of treated wastewater is discharged into rivers and coastal areas (Water New Zealand, 2021; Watkinson et al., 2007). Depending on the level of treatment received, TMW still contains significant amounts of nutrients and contaminants. When TMW is discharged into waterbodies, these nutrients, primarily N and P, can cause eutrophication and toxic algal blooms (Davies-Colley, 2013; McDowell et al., 2009). Additionally, direct toxic effects of high ammonium (NH_4^+) concentrations on aquatic plants are possible (Cabaço et al., 2008). Discharge of TMW can significantly increase the BOD and the abundance of coliforms around wastewater outfall pipes in the ocean (Mossa, 2006). The release of TMW is a major point-source pollution of waterbodies (Gluckman, 2017). Advanced wastewater treatment can reduce the concentrations of nutrients to levels below drinking water standards, but such treatment can double wastewater treatment costs (Spellman, 2014). An alternative management option is the land application of wastewater, where the soil-plant system may lead to better environmental and economic outcomes (Tzanakakis et al., 2009).

1.2.2 Land Application of treated municipal wastewater: risks and opportunities

The land application of wastewater has occurred since the time of ancient Greece civilizations (ca. 3400-1200 B.C.) to protect public health (Angelakis et al., 2005), but has declined with the development of modern WWTPs (Tzanakakis et al., 2007). Recently, growing pressure on water resources is shifting wastewater management from a linear use-treat-dispose approach to a more circular approach (Figure 1-1). This has the potential to improve outcomes for human and environmental health (Smol et al., 2020). In a circular economy (Geissdoerfer et al., 2017), the water

and raw materials in wastewater can be regarded as valuable resource (Gebrezgabher et al., 2015). The recovery and reuse of these resources is essential to achieve a "waste-free" economy. (Chrispim et al., 2019; Otoo, Drechsel, et al., 2015). However, adopting circular economy frameworks in wastewater management requires technological, organisational, and societal changes as well as a fresh view on the meaning of wastes (Smol et al., 2020).

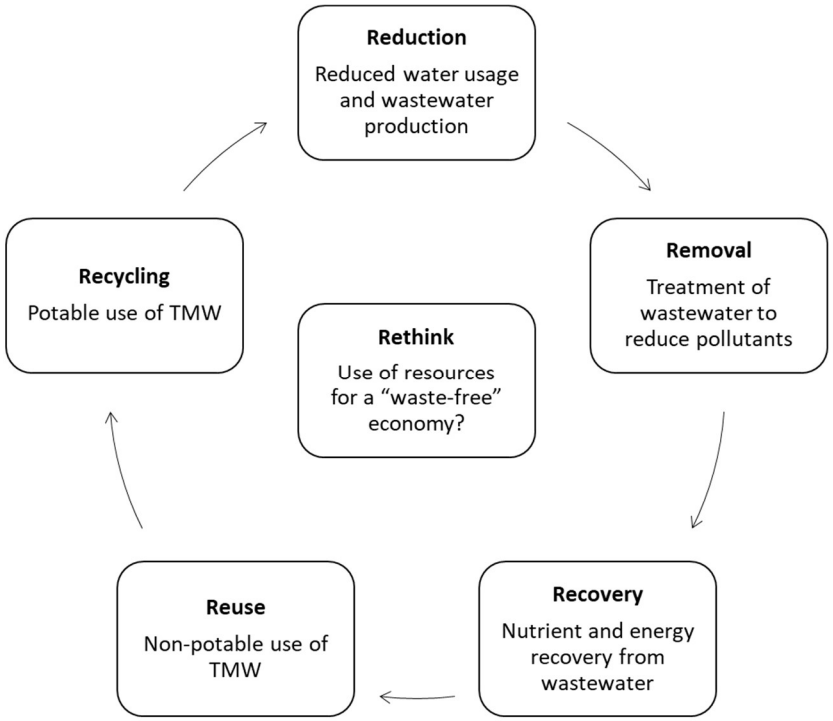

Figure 1-1 Circular management of treated municipal wastewater (TMW). Visualised after Smol et al. (2020).

The motivation for a circular approach to TMW management differs depending on the water stress that a region is experiencing. While the public awareness of environmental quality greatly drives the change to circular TMW management in humid areas, land application is further motivated by its potential for improved agricultural production in (semi-)arid areas (WWAP, 2017). The land application of TMW can improve plant growth while reducing the need for freshwater irrigation and fertilisation. Agriculture accounts for 70% of the global water retrieval (Wada et al., 2013), and recycling TMW has the potential to reduce the pressure on freshwater resources. Although not usually the main driver for TMW reuse, the concentration of plant nutrients in TMW can reduce the need for fertilisers, some of which are non-renewable resources, such as P (Pedrero et al., 2010). TMW is currently utilised to accelerate the production of vegetables, vineyards and other horticultural products, as well as cereals and fibre crops such as cotton (Cirelli et al., 2012; Hamilton et al., 2005;

Papadopoulos et al., 2009; Paranychianakis et al., 2006; Tsadilas & Vakalis, 2003). However, combining TMW reuse with food production can be restricted by cultural and societal views on wastes as well as industry perceptions and limitations (Ataria et al., 2016).

There are numerous management options to utilise TMW in combination with the production of non-food products. In Europe, TMW reuse is widely combined with the production of woody biomass as a renewable energy source (Hall, 2013), such as *Salix* spp. in Northern Europe (Dimitriou & Aronsson, 2004; Gonzalez-Garcia et al., 2012). Elsewhere, *Eucalyptus* spp., *Populus* spp., and *Acacia* spp. are used for short rotation coppice (Sims et al., 2001). Short rotation coppice can be utilised to produce ethanol for fuel and lignin for biopolymer production (Snowdon et al., 2008). The plants in such systems have a high nutrient removal potential from TMW irrigation (Carlander et al., 2009). Globally, TMW is used to accelerate the production of timber, such as in *Pinus radiata* plantations (Cromer, 1980; Evett et al., 2011). Landscaping and environmental purposes are increasingly explored as an option for the land application of TMW (WWAP, 2017). Examples include the irrigation of golf courses, parks, and gardens (Qian & Mecham, 2005). Furthermore, TMW is utilised for the restoration of wetlands and the creation of artificial lakes (Kivaisi, 2001). While such systems do not generate direct value from the land, the economic benefit can derive from averted health risks from the use of cleaner waterbodies, ecosystem services, and earnings from recreational and touristic activities (Otoo, Mateo-Sagasta, et al., 2015).

The quality of the irrigated TMW and the prevalent soil properties determine the irrigation rate at which TMW does not present a risk to the receiving soil-plant system and the local ground- and surface waters (ANZECC, 2000; Gutierrez-Gines et al., 2020). Biological, chemical, and physical soil properties affect the residing time of TMW in the soil (Houlbrooke et al., 2004). This, in combination with the ability of plants to accumulate nutrients, determines the overall nutrient retention capacity of a TMW land application system (Barton et al., 2005). Where the application of nutrients exceeds the retention capacity, there is a risk of leaching or gaseous losses of nutrients. Applied N can be lost through NO_3^- leaching and gaseous emissions of N_2O from the soil (Dimitriou & Aronsson, 2004; Li et al., 2015). Phosphorus is generally immobile in soils and typically accumulates in the soil profile (Monaghan et al., 2007). However, it can be lost through preferential flow pathways and surface runoff (Gupta et al., 1999).

A common risk for TMW land treatment schemes is the effect of applied Na on the structural stability of the soil (Menneer et al., 2001). Na is usually weakly bound and not accumulating in soils (Blume et al., 2016). However, Na concentrations are typically higher in (semi-)arid soils, and TMW irrigation can lead to sodicity and salinity (Farahani et al., 2018). For TMW, the relative concentration of Na

compared to Ca and Mg is commonly expressed as sodium adsorption ratio (SAR), which is used as an indicator for TMW suitability for irrigation (ANZECC, 2000). High concentrations of Na in the soil can lead to aggregate instability through swelling and dispersion of clay (Farahani et al., 2018). This can block soil pores and reduce the permeability and hydraulic conductivity of the soil (Menneer et al., 2001). As a consequence, erosion and surface runoff can be increased (Farahani et al., 2018). Sodicity and salinity can impair plant growth and health and be detrimental for microbial communities in the soil (Maathuis, 2014; Rietz & Haynes, 2003). Plant species that are tolerant to sodicity and salinity may therefore have an advantage in TMW irrigation schemes (Qian & Mecham, 2005).

Other compounds in TMW that could be harmful to the receiving environment are trace elements (Norton-Brandão et al., 2013). However, they are usually associated with the solid fraction and therefore removed from wastewater during secondary treatment (Kunhikrishnan et al., 2012). Nevertheless, it was demonstrated that for some trace elements (Cd, Cr, Pb, Fe, Ni, and Zn) 47-63% remained in the TMW after secondary treatment (Karvelas et al., 2003). The concentration of heavy metals can be higher with increased contribution of industrial sources to TMW (Norton-Brandão et al., 2013). Therefore, irrigation of TMW can increase the concentration of trace elements in soil, as demonstrated for Cr, Cu, and Zn by Irshad et al. (2015). In addition, TMW irrigation can lead to increased bioavailability of trace elements through its effects on soil pH, which can to some extent be offset by binding of trace elements to added soil organic matter (Kunhikrishnan et al., 2012). The availability of trace elements in soil is primarily determined by soil pH, with cations and anions increasingly available at low and high pH, respectively (Antoniadis et al., 2017). Where trace elements accumulate in plants, this can impair the health of the plant itself or negatively affect the associated food chains (Pedrero et al., 2010).

Conventional wastewater treatment does not significantly decrease the concentration of emerging organic contaminants (EOCs), such as pharmaceuticals and personal care products, pesticides, hormones, surfactants, flame retardants and plasticisers (García et al., 2020). Wastewater discharge can be a source of EOCs in ground- and surface water (Lapworth et al., 2012). It was reported that vegetation filters remove more than 90% of most EOCs from irrigated TMW (Martínez-Hernández et al., 2018). This indicates that land treatment of TMW may mitigate EOC pollution in the environment. In addition, TMW is a source of microplastics (Ruffell et al., 2021) and its irrigation was reported to increase the abundance of microplastics in soil (Ragoobur et al., 2021). The addition of microplastic to soils can affect the soil structure and hydraulic properties, as well as soil microbial activity (de Souza Machado et al., 2018). Similarly, microplastics can impair the performance of plants (de Souza Machado et al., 2019).

1.2.3 Land application of treated municipal wastewater in New Zealand

The discharge of TMW into waterways is culturally offensive to Māori, the indigenous people of NZ. Degradation of waterways from wastewater discharge can impair cultural identity and affect traditional water-based activities (Hughes et al., 2021). The cultural health of waterways is determined by tangata whenua (people of the land), and indigenous views on environmental management need to be respected under NZ legislation and government policy originating from the Treaty of Waitangi (Morgan, 2006; Tipa & Teirney, 2006). Central to tangata whenua views on the environment is the concept of mauri, which is "the essence or life force that provides life to all living things" (Morgan, 2004). The mauri of waterways is harmed by disposal of human waste such as TMW, independent of the treatment it received (Pauling & Ataria, 2010). Instead, the application of TMW to Papatūānuku (the earth mother) utilises the inherent purifying potential thereof and avoids destroying the mauri of waterways (Morgan, 2006).

Generally, indigenous people are often most vulnerable to waterway degradation from TMW discharge, particularly where they rely on the collection of food from the local environment (Neudorf et al., 2017). Full participation of indigenous people in wastewater management is a non-negotiable to meet the right to self-determination of indigenous people that is recognised by the United Nations (Black & McBean, 2017; UNGA, 2007). The relationship of Māori with waterways is one of the main drivers to increase the land application of TMW in NZ (Bristow & Prieto-Curiel, 2002). Nevertheless, TMW land application in NZ remains low to this date (Figure 1-2 a), and 73% of TMW is discharged into marine environments (Water New Zealand, 2021).

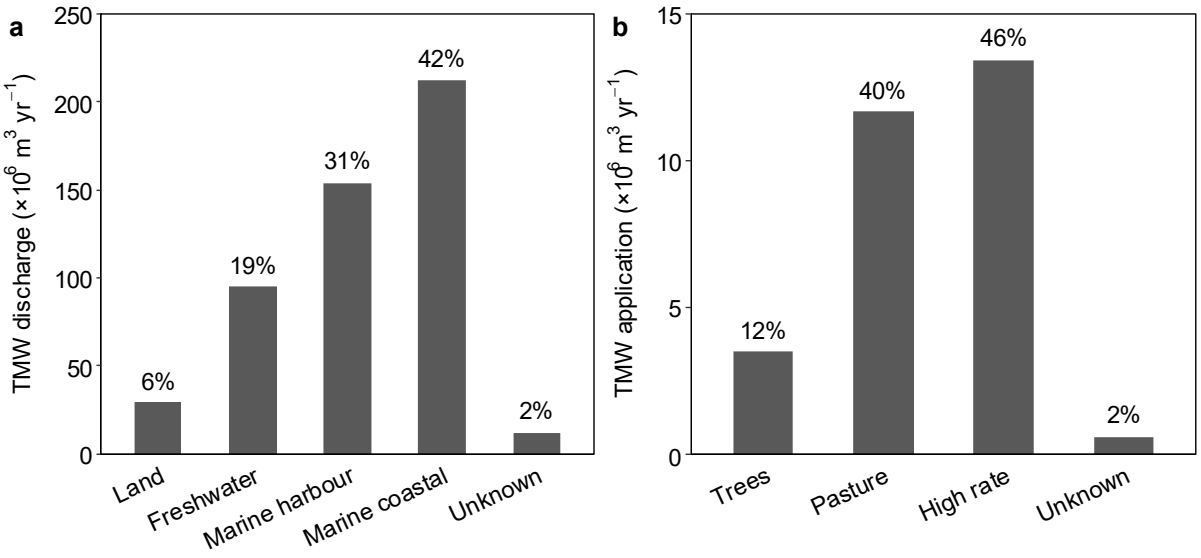

Figure 1-2 (**a**) Discharge of treated municipal wastewater (TMW) in New Zealand, calculated from Water New Zealand (2021), and (**b**) estimated volumes applied to different land treatment systems, based on percentages from Cass and Lowe (2016) and total volume of land application from Water New Zealand (2021). High rate application is mainly to wetlands and drainage trenches (Cass & Lowe, 2016).

New Zealand's Resource Management Act 1991 is the primary environmental legislation, but it does not provide specific guidance on TMW management (Bristow & Prieto-Curiel, 2002). However, its overarching goal of protecting natural resources can best be met when TMW is applied to land rather than discharged into waterways (Bristow & Prieto-Curiel, 2002). Tangata whenua views on TMW management require strict separation of waste streams and food chains (Pauling & Ataria, 2010), requiring non-agricultural land application options. Furthermore, in NZ it is not permitted to apply waste of human origin to paddocks with grazing milking animals, and the use of irrigated pasture to feed dairy cattle is strongly restricted (MPI, 2017). Being the largest exporter of dairy products in the world, NZ is exporting 95% of its dairy production, creating export revenues of approximately NZ$ 13.2 B yr^{-1} (Ballingall & Pambudi, 2017; Fernandez-Perez et al., 2021). Therefore, such industry restrictions are limiting TMW reuse options. Currently in NZ, most of the TMW land application is by high rate discharge to wetlands and soakage trenches, as well as for irrigation of pasture and trees (Figure 1-2 b) (Cass & Lowe, 2016; Water New Zealand, 2021).

The disposal of biowastes, including TMW, could be combined with ecological restoration through plantings of native vegetation (Simcock et al., 2019). Multi-purpose restoration may increase the public support for both restoration plantings and TMW land application (Van Diggelen et al., 2001). Thereby, it may provide an opportunity to increase the share of TMW that is reused on land rather than disposed into waterbodies. While TMW is used to irrigate forestry plantations in many countries,

including NZ (Barton et al. 2005; Evett et al., 2011), there is a paucity of knowledge on the possible use of NZ native species for TMW irrigation in both forestry and non-forestry contexts.

1.2.4 Benefits of New Zealand native ecosystems

Native vegetation has strongly declined in NZ for hundreds of years. It is estimated that native forest covered 82% of land prior to human arrival to NZ (Ewers et al., 2006). Following Polynesian and European settlement in NZ, anthropogenic activities have reduced native forest land cover to about 23% (Blackwell et al., 2008; Ewers et al., 2006). However, the loss of native land cover is still ongoing. It was estimated that 71,000 ha of native land cover was lost from 1996 to 2012, thereof 44% tussock grassland, 34% native shrubland, and 22% native forest (MfE & Stats NZ, 2018). Within high intensity agricultural landscape native vegetation now covers as little land as 0.5% in the Canterbury Plains (Franklin et al., 2015).

Native ecosystems and biodiversity significantly contribute to the wellbeing of all New Zealanders through the provision of ecosystem services (Ausseil et al., 2013; Mark et al., 2013), which are defined as "direct and indirect contributions of ecosystems to human well-being" (Kumar, 2011). Ecosystem services are split into provisioning, regulating, cultural, and supporting services (Table 1-1). It was estimated that NZ's terrestrial ecosystems contribute NZ$ 57 B yr^{-1} to human welfare (Patterson & Cole, 2013). Dymond et al. (2013) found that more than 2 M ha of pasturelands in NZ have a high biodiversity benefit over cost ratio and are therefore suitable for native restoration plantings. Similarly, forestry land can be transformed into permanent native vegetation after clear-felling, as such land is often prone to erosion (Simcock et al., 2019). Native biodiversity is closely linked to the provision of ecosystem services (McAlpine & Wotton, 2009).

Table 1-1 Ecosystem services, adapted from Millenium Ecosystem Assessment (2005) and van den Belt and Blake (2014).

Service	Description	Examples
Provisioning	Products obtained from ecosystems	Food, water, fibre, fuel, biochemicals, genetic resources
Regulating	Benefits obtained from regulation of ecosystems	Air quality, climate, water flow, pollination, pest and disease control, erosion control
Cultural	Non-material benefits obtained from ecosystems	Aesthetic, recreational, educational
Supporting	Services needed for the production of all other ecosystem services	Primary production, soil formation, nutrient cycling

Nutrient cycling

Many NZ native species are adapted to low fertility soils (Wardle, 1985). However, their use in agricultural landscapes and other high nutrient environments has recently gained attention (Franklin et al., 2015; Simcock et al., 2019). Franklin et al. (2015) found no effects of N application on native plant biomass. In contrast, positive growth responses of *Leptospermum scoparium* to biosolids application were reported by Reis et al. (2017) and Gutierrez-Gines et al. (2019). A suite of other NZ native species showed a biomass increase following the application of biosolids (Gutierrez-Gines et al., 2017). Native species have the potential to accumulate significant amounts of N in their biomass. Esperschuetz, Balaine, et al. (2017) reported that *L. scoparium* and *Kunzea robusta* accumulated 100 kg N ha^{-1} in their stem biomass, compared to 35 kg N ha^{-1} in *P. radiata.* Franklin et al. (2015) found luxury accumulation of N in NZ native species with application of urea.

There is evidence that some NZ native species have the potential to distinctively affect the biogeochemical cycling of N in the soil (Figure 1-3) and reduce N losses. Esperschuetz, Balaine, et al. (2017) found that the myrtaceous species *L. scoparium* and *K. robusta* showed significantly lower NO_3^- leaching (2 kg NO_3^--N ha^{-1}) following the application of urea compared to *P. radiata* (53 kg NO_3^--N ha^{-1}). Their research included a nitrification assay that indicated that this was a result of the plants suppressing soil nitrification (Esperschuetz, Balaine, et al., 2017). Nitrification is a microbially mediated process and plant root exudates that inhibit enzymes involved in nitrification are summarised as biological nitrification inhibitors (BNIs) (Subbarao et al., 2012). However, the mechanisms by which *L. scoparium* and *K. robusta* affect the N cycle are unknown, and there is a lack of knowledge on their root exudate composition. Alternatively, nitrification inhibition in the soil may derive from leaf compounds through litter fall and incorporation (Subbarao et al., 2006). The leaves of both species contain essential oils with antimicrobial properties, associated with triketones and terpenes in *L. scoparium* and α-pinene in *K. robusta* (Douglas et al., 2004; Porter & Wilkins, 1999). It was reported that some plants with antimicrobial properties can inhibit nitrification in soil (Tahir et al., 2021), although specific mechanisms were not described. There is evidence of nitrification and denitrification inhibition by some NZ native plants. Halford et al. (2021) reported that NO_3^- concentrations were significantly lower in the rootzone of *L. scoparium* than pasture. Franklin et al. (2017) measured significantly reduced N_2O emissions from soil under *K. robusta* compared to bare soil following the application of dairy shed effluent. The authors listed suppression of denitrification and differences in water filled pore spaces as possible explanations (Franklin et al., 2017). This is consistent with results of a study by Esperschuetz, Balaine, et al. (2017), whereby N_2O emissions were lower from soil under *K. robusta* than pasture following urea application.

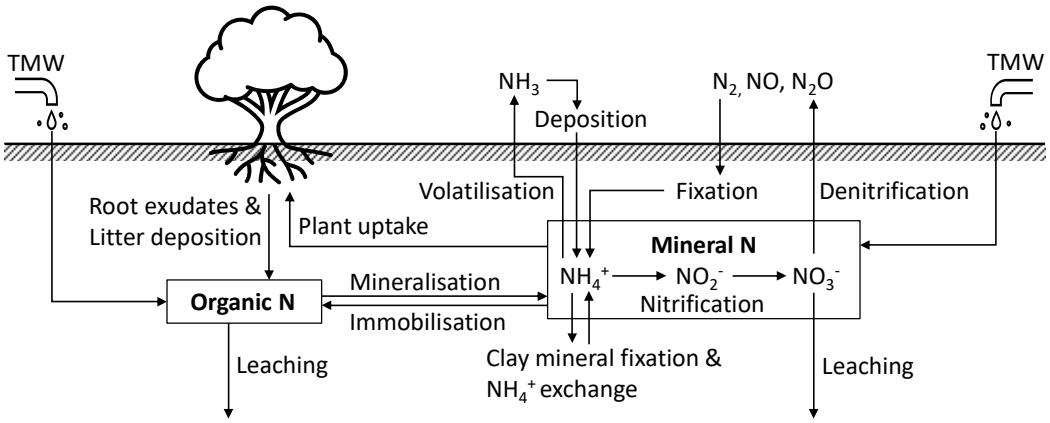

Figure 1-3 The biogeochemical N cycle with N inputs from irrigation of treated municipal wastewater (TMW). Adapted from Cameron et al. (2013).

While there are some studies on N cycling in NZ native vegetation, the knowledge on interactions of native species with P is limited. Lense (2018) found an increased concentration of foliar P in *K. robusta* with application of 100 kg ha^{-1} KH_2PO_4-P, while the P concentration in *L. scoparium* foliage did not differ from a non-fertilised control treatment. However, *K. robusta* growth was significantly reduced when this species was fertilised (Lense, 2018). This is in contrast with positive growth responses following biosolids application equivalent to 297 kg P ha^{-1} reported by Esperschuetz, Anderson, et al. (2017). P is relatively immobile in soils and the main pathway of P contamination in waterways is through surface runoff, which is often related to critical erosion event such as storms (Pionke et al., 2000; Uusi-Kampaa et al., 2000). It was estimated that 49% of P enters waterways diffusely with sediments (Elliott et al., 2005). However, subsurface runoff can also contribute significantly to P loading, depending on the soil hydraulic conductivity and (limited) sorption capacity, and preferential flow paths (Mittelstet et al., 2011). Therefore, indirect effects of plants on nutrient cycles can derive from root system effects on physical soil properties and infiltration rates (Bharati et al., 2002; Neilen et al., 2017). Growing native vegetation on marginal land and steep slopes provides erosion control and soil conversation (Ross et al., 2009). Where a variety of native species is planted, the diverse root morphology among the species can significantly reduce erosion (Marden et al., 2018). While erosion control is a regulating ecosystem service itself (Table 1-1), it is closely linked to the P cycle.

Pathogen mitigation

Some NZ native plants have antimicrobial properties that affect pathogenic bacteria in the soil. Prosser et al. (2014) found that *L. scoparium* leaf and root extracts have inhibitory effects on certain bacteria, namely *Salmonella typhimurium*, *Escherichia coli*, *Clostridium perfringins*, *Campylobacter jejuni*, and *Listeria monocytogenes*. Leaf extracts showed the strongest inhibitory potential, indicating that effects on pathogens in soil may derive from plant litter incorporation (Prosser et al., 2014). A different

antimicrobial screening study found antimicrobial effects of leaf extracts from these species, *Pseudowintera colorata*, and *Metrosideros robusta* against *E. coli*, *Pseudomonas aeruginosa*, *Staphylococcus aureus*, and *Burkholderia cepacia* (Gutierrez-Gines et al., 2021). Similar results were found in a pot trial, where decimal reduction times of *E. coli* were significantly lower under *L. scoparium* and *K. robusta* (8 and 5 days, respectively) compared to *Lolium perenne* (93 days) (Prosser et al., 2016). This is consistent with results of Gutierrez-Gines et al. (2021), showing that the 90% reduction time of *E. coli* in soil was significantly shorter under *L. scoparium* and *M. robusta* compared to *L. perenne*. It is possible that this was due to a lower pH under these native species compared to *L. perenne* (Gutierrez-Gines et al., 2021). However, underlying mechanisms have not been described to date. It was suggested that *L. scoparium* may release leptospermone from their roots, which was described in another myrtaceous species *Callistemon citrinus* (Crimson bottlebrush) to suppress weeds and has antimicrobial properties (Cornes, 2005; Prosser et al., 2014).

Carbon sequestration

Globally, deforestation results in 1-2 Gt C yr^{-1}, which is equivalent to 17-25% of anthropogenic greenhouse gas emissions (Strassburg et al., 2009). Land use practices influence the C equilibrium, and it was estimated that C losses from NZ dairy pasture soils average to 0.73 t C ha^{-1} yr^{-1} (Schipper et al., 2010). Native vegetation acts as a carbon sink, with *L. scoparium* and *K. robusta* showing a net C accumulation rate of 1.9-2.5 t C ha^{-1} yr^{-1} over an active growth phase of 40 years (Trotter et al., 2005). Similarly, Whitehead et al. (2004) found that the net primary productivity of native vegetation dominated by *L. scoparium* and *Kunzea ericoides* (closely related to *K. robusta*) was 2.2 t C m^{-2} yr^{-1}. While the growth rate and C sequestration in native forests is slower than exotic species, overall C sequestration does not differ in the long-term (Bergin & Kimberley, 2011). While C sequestration is limited once a forest reaches maturity, it can store C if it remains undisturbed (Aimers et al., 2021). Carbon sequestration by native vegetation has the potential to generate an additional income for landowners through C trading (Bagrie et al., 2015). Native vegetation that was planted after 1989 and fulfils the criteria of a 'carbon forest' can be included in NZ's Emission Trading Scheme (ETS), which generates direct economic profit from an ecosystem service (Aimers et al., 2021). The ETS encourages the plantings of native vegetation and about 333,000 ha of land were registered with the ETS in 2021 (Te Uru Rākau, 2021).

Habitat provision

Native trees can create favourable conditions and shelter for other native species to grow in their understorey, such as ferns under dense *K. robusta* canopies (Ecroyd & Brockerhoff, 2005). *K. robusta* and *L. scoparium* are early successional species and their stands show a large native plant diversity compared to succession in exotic gorse (*Ulex europaeus*) (Sullivan et al., 2007). Native plants provide

a habitat for native birds, arthropods, and mammals (McAlpine & Wotton, 2009; Molloy et al., 1995). The presence of native vegetation correlates with an increased abundance of native birds (Moller et al., 2008; L. Roberts et al., 2015). Native plants are a source of nectar and pollen for arthropods such as native bees, predators, and parasitoids, which in term ensures crop pollination and can potentially control pests, reducing the need for pesticide use (Isaacs et al., 2009; Shields et al., 2016).

Cultural value

Indigenous views on the value of ecosystems are often associated with cultural heritage, a sense of place and kinship, and stewardship over the land (Lyver, Timoti, Gormley, et al., 2017). According to the Māori term Te Ao Mārama (a world of light and opening) all life within the earth, sky, air, and water is connected through whakapapa (genealogy) (Harmsworth & Awatere, 2013). This allows for a more integrated view on ecosystem services, where people are caring for ecosystems (manaaki whenua) and ecosystems are caring for people (manaaki tangata), as described by Dymond et al. (2014). Typical culturally relevant themes associated with native ecosystems by NZ Māori are mahinga kai (procurement of food), rongoā (traditional healing), and weaving (Lyver, Timoti, Jones, et al., 2017). The loss of native forest and the associated fauna, food, and traditional way of life can impair the cultural well-being of Māori (Lyver, Timoti, Gormley, et al., 2017). A survey by Pauling and Ataria (2010) on culturally acceptable TMW land application systems among Māori registered with the Ngāi Tahu Whakapapa Unit revealed that 89% of respondents approved with the use of TMW irrigated land for native restoration.

Aesthetic value and overseas perception

The value of aesthetic landscapes is difficult to quantify, but mountains, natural waterways, and forests are usually preferred landscape features (Swaffield & McWilliam, 2013). Aesthetic landscapes are essential for the image that is portrayed of NZ overseas. Prior to the global Covid-19 pandemic, tourism was NZ's largest export industry, contributing 20.7% (NZ$ 14.5 B) to NZ's total exports of goods and services in 2016 (Stats NZ, 2016). Since 1999, NZ has been promoting a successful tourism brand "100% Pure New Zealand" that builds on a green and clean perception of NZ overseas (Bain & Dandachi, 2015). In 2001, the Ministry for the Environment estimated annual losses of NZ$ 389-530 M in case of a negative perception of NZ's environment by tourists (MfE, 2001). Native vegetation contributes to the aesthetic value of the landscape and can mitigate nutrient contamination of waterbodies (McAlpine & Wotton, 2009), and therefore enhance the clean green image of NZ.

Valuable native products

New Zealand native plants can be used to create a range of valuable native products. *L. scoparium* is used for the production of mānuka honey, which can provide returns of up to $128 kg^{-1} bulk honey

(MPI, 2018). It is marketed globally, and its exports generated a revenue of NZ$255 M in 2019 (MPI, 2020). Mānuka honey is different from other honeys due to its non-peroxide antimicrobial activity (NPA), that primarily derives from the compound methylglyoxal (MGO) (Mavric et al., 2008). Mānuka honeys with a high NPA are in high demand, but their production is limited and temporally variable (Millner et al., 2016). The Ministry of Primary Industries (MPI), Mānuka Research Partnership (NZ) Ltd., and Comvita Ltd. teamed up to create the High Performance Mānuka Plantations PGP programme, aiming to increase the value of the mānuka honey to NZ$ 1.2 B yr^{-1} by 2028 (MPI, 2014; MPI, 2016). While *L. scoparium* and *K. robusta* are closely related and their honeys have a similar phenolic composition, MGO appears to be unique to *L. scoparium* honey (Stephens et al., 2010). Other NZ native plants that are used to generate common monofloral honeys are *Weinmannia racemosa* (kāmahi), *Metrosideros umbellata* (Southern rātā), *Knightia excelsa* (rewarewa), and *Ixerba brexioides* (tāwari) honeys (Vanhanen et al., 2011).

L. scoparium and *K. robusta* are used for the production of essential oils (Perry, Brennan, et al., 1997; Perry, Van Klink, et al., 1997). They have oil sacs (schizogenous secretory cavities) in their leaves and the oil is harvested by extraction through distillation (Crop & Food Research, 2000). The antimicrobial properties of the essential oil derive from triketones and terpenes in *L. scoparium* , and from α-pinene in *K. robusta* (Porter & Wilkins, 1999). Both oils were historically used for medicinal purposes and are now widely utilised for their anti-inflammatory and antispasmodic modes of action (Maddocks-Jennings et al., 2005). *L. scoparium* oil has higher antimicrobial activity than *K. robusta*, primarily due to its high β-triketone content (Essien et al., 2019), and it shows anthelmintic, insecticidal and herbicidal properties (Dayan et al., 2011; Douglas et al., 2001). Wholesale prices for generic *L. scoparium* oil are NZ$ 500-600 kg^{-1} (Boffa Miskell, 2017). There is a global market for NZ native essential oils, with 80% of *L. scoparium* essential oil being exported (Porter, 2003). While the demand for *L. scoparium* oil is higher than for *K. robusta* oil, the latter typically has a higher oil yield from its foliage (Essien et al., 2019). Seyedalikhani et al. (2019) reported that the application of biowastes can augment the oil production of both species.

Several native plant species could be used for timber production, such as *Agathis australis*, *Dacrydium cupressinum*, and *Podocarpus totara* (Bergin & Gea, 2007; Pizzirani et al., 2019). The demand and import of hardwood timber has recently increased and is valued at > NZ$ 50 M yr^{-1}, which could potentially be (partially) substituted by native species such as *Sophora* spp. (Nguyen et al., 2021) or *Lophozonia menziesii* (formerly *Nothofagus menziesii*) (Olsen, 2004). Numerous native plants that have long been utilised in a rongoā (traditional Māori healing) context (Mark et al., 2019) are now used for commercial pharmaceutical and cosmetic products. Examples include (i) *Piper excelsum* that contains the active compounds myristicin and cadinenes that can be used in pharmaceuticals

(Awatere et al., 2018), (ii) *Pomaderris kumeraho* that is used in shower gel and shampoo (Dodd & Ritchie, 2007), (iii) *Phormium tenax* that contains an antiseptic gel which can be used to treat wounds and skin conditions (McGruddy, 2006), and (iv) native *Podocarpus* spp. that can be used for the extraction of Totarol™, an aromatic diterpenoid with antibiotic properties (Wharemate, 2003). Fibres from *Phormium* spp., *Cordyline australis*, and other species have been used for centuries for the production of sails, mats, clothing, containers, and hunting and fishing tools (PCE, 2001). The use of *P. tenax* fibres is again gaining attention as alternative to synthetic fibres for textile and non-textile (pulp, paper, packaging, car interiors, pots, etc.) products (McGruddy, 2006).

1.2.5 Summary and knowledge gaps

The application of TMW to land, rather than discharge into waterbodies, is consistent with a circular economy approach to wastewater management. Short-term pot trials with application of biowastes to NZ native plants revealed that most species either showed no growth response or an increase in biomass. This indicates that TMW may be beneficially reused to accelerate the establishment of native vegetation. However, there is a lack of knowledge on the growth response of NZ native plant species to TMW application over the medium to long term. Due to the controlled conditions of previous experiments, it is unknown how TMW irrigation affects the establishment of native vegetation in the field, where there are a plethora of environmental factors and other organisms, such as weeds and herbivores. Long-term application of TMW can result in the accumulation of TMW compounds in the soil. While native plants can take up significant amounts of nutrients, it is unclear how TMW irrigation would affect fluxes of nutrients and contaminants in the soil-plant system. It was demonstrated that some NZ native myrtaceous species can reduce NO_3^- leaching and N_2O emissions more than other species, but underlying mechanisms are unknown. Biowastes can accelerate the generation of valuable native products. However, the effects of nutrient application and soil chemistry on the quality of native products, such honey and essential oils, is poorly understood.

1.3 Hypotheses

From the literature, the following testable hypotheses can be drawn:

1. TMW can be beneficially irrigated onto some NZ native species. Species will have contrasting responses to the TMW depending on the environment that they are adapted to.

2. TMW will increase the growth of exotic weeds that are adapted to high-fertility environments.

3. TMW will increase the fluxes of nutrients through the soil profile and may result in soil degradation due to the accumulation of P and Na.

4. The fluxes of nutrients and contaminants in the soil profile will be affected by the plant species, through accumulation and plant-induced changes to soil microbiological activity.

5. TMW can be used for the generation of NZ-native products that do not contain excessive concentrations of trace element contaminants.

1.4 Research aim and objectives

Based on the above hypotheses, this research aimed to assess the viability of TMW irrigation onto plantings of NZ native species. Specifically, this thesis sought to investigate:

1. The effect of TMW irrigation on the survival and growth of NZ native species at low (1000 mm yr^{-1}) and high (>4000 mm yr^{-1}) application rates (Chapters 2 and 3).

2. Critical management strategies and considerations for the establishment of native plantings with high TMW application rates (Chapter 3).

3. The effect of NZ native species on the distribution and speciation of TMW associated nutrients and contaminants in the soil-plant system (Chapters 2 and 4).

4. The effect of NZ native species on the abundance of soil nitrifying and denitrifying microorganisms. (Chapter 5).

5. How the chemical composition of the soil affects the quality of mānuka honey, with a view to determining whether TMW irrigation would increase or reduce honey quality (Chapter 6).

1.5 Thesis outline

This thesis is presented in seven chapters. The introduction (Chapter 1) is followed by five experimental chapters (Chapters 2-6) that address the thesis objectives, and a synthesis of all findings (Chapter 7). The experimental Chapters 2, 5, and 6 are presented as stand-alone manuscripts for publication in peer-reviewed journals, while Chapters 3 and 4 will be combined for publication. Therefore, there is some repetition between chapters.

Chapter 2 is based on a long-term field trial that was established in Duvauchelle on Banks Peninsula in 2015, with TMW irrigated onto NZ native vegetation at a rate of 1000 mm yr^{-1}. The growth of native species with and without TMW irrigation, their chemical composition, as well as irrigation effects on the distribution and speciation of nutrients in the soil under five NZ native species were analysed.

Chapter 3 investigates the growth and survival of native vegetation with irrigation of TMW at high application rates (>4000 mm yr^{-1}). It is based on large-scale field trial that was established in Levin in 2018. Weed development was measured at the site and weed management strategies were investigated.

Chapter 4 is also based on the Levin field trial. It investigates the effect of high irrigation rates on the growth and chemical composition of the NZ native species *L. scoparium* and *K. robusta* compared to exotic pasture. Contemporaneously, the effect of increasing TMW irrigation rates on the chemistry of the soil underneath these species was analysed. An *in situ* lysimeter experiment compared NO_3^- leaching under *K. robusta* and pasture.

Chapter 5 describes a pot experiment that was set up to test whether NZ native plants affect the abundance of soil nitrifying and denitrifying microorganisms. DNA was extracted from the soil to quantify the abundance of total bacteria and archaea, as well as functional genes involved in nitrification and denitrification.

Chapter 6 is based on results of chemical analysis of soil, plant, and mānuka honey samples from the Wairarapa region of New Zealand. These were used to test effects of soil and plant chemistry on the chemical composition and quality of mānuka honey.

Chapter 7 is a general discussion of all results of the experimental Chapters 2-6. This chapter revises the research objectives, draws conclusions, and recommends future research objectives.

Chapter 2

Interactions of Treated Municipal Wastewater with Native Ecosystems

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Submitted to *Ecological Engineering*

2.1 Abstract

Over 90% of Treated Municipal Wastewater (TMW) in New Zealand (NZ) is discharged into marine and freshwater ecosystems. High levels of nutrients, pathogens and other contaminants from this source potentially degrade water quality. Land application of TMW could reduce the nutrient load in water bodies and increase the productivity of terrestrial vegetation. Using TMW to re-establish indigenous ecosystems could create zones of ecological value in NZ and elsewhere. However, there is a paucity of knowledge about the response of NZ native species to irrigation with TMW. We aimed to determine the distribution and speciation of nutrients in the soil-plant system following application of TMW onto 11 species of native plants in a long-term field trial on Banks Peninsula, NZ. TMW was irrigated at a rate of 1000 mm per annum, equivalent to Na, N, and P loading rates of 950, 194, and 110 kg ha yr^{-1}, respectively. There was no evidence of impaired soil structure following TMW irrigation. Nitrogen did not accumulate in the soil, and it is likely to have been taken up by plants or lost through denitrification and nitrate leaching. The accumulation rate of P indicated that soil P concentrations will remain within the range found in NZ agricultural soils for 30 years. Average plant height increased by 10% with TMW irrigation relative to the unirrigated control. Plant species significantly affected the concentrations of total C, total N, nitrate (NO_3^-), and Na in the soil. TMW application had negligible effects on the elemental composition of plant foliage. The trial demonstrated the feasibility of using TMW to establish NZ native vegetation to increase local biodiversity, support ecological restoration, and improve environmental outcomes.

2.2 Introduction

United Nations Sustainable Development Goals (SDGs), Target 6.3, calls for increased wastewater recycling and reuse to improve the water quality in aquatic ecosystems (WWAP, 2017). When discharged into waterways, wastewater can exacerbate eutrophication (Carey & Migliaccio, 2009). Its origin and level of treatment determine the concentrations of sodium, nutrients, pathogens, and trace elements in wastewater (Norton-Brandão et al., 2013). Applying Treated Municipal Wastewater

(TMW) to land may reduce waterway degradation and mitigate the public health risks (Hamilton et al., 2007). This practice is particularly valuable in arid and semiarid areas where the pressure on available water is high due to the irrigation of agricultural land (Pedrero et al., 2010). The nutrients in TMW reduce the need for synthetic fertilisers (Cirelli et al., 2012). However, the application of wastewater onto productive agricultural land is not appropriate where insufficient treatment poses a public health risk (Sato et al., 2013). Additionally, public and industry perceptions can hinder the use of TMW for agricultural production (Lowe, 2009; Simcock et al., 2019). Many indigenous peoples, including Māori in New Zealand (NZ), do not allow the combination of waste from human origin with food production chains (Pauling & Ataria, 2010).

Non-agricultural practices for the land application of TMW include the creation of artificial wetlands (Kivaisi, 2001), the production of energy crops, such as *Salix* spp. (Gonzalez-Garcia et al., 2012), and the irrigation of public and private green spaces, including golf courses (Qian & Mecham, 2005). Many of these systems consist of monocultures and plants that are exotic to the local area. Alternatively, native plants could be used in wastewater land treatment systems to mitigate contaminants. Native hyperaccumulators are used for the phytoremediation of land contaminated with trace elements from irrigation with municipal and industrial wastewater (Hasan et al., 2021; Irshad et al., 2015; Sharma et al., 2021). In artificial wetlands, native species are used to support local biodiversity and to prevent the naturalisation and spread of exotic species (Maschinski et al., 1999; Quadros et al., 2017). Combining the land application of TMW with restoration projects may not only provide an opportunity to increase native biodiversity (Cunningham & Gharipour, 2018), but may also accelerate public support for both concepts (Van Diggelen et al., 2001).

The value of native ecosystems is increasingly recognised worldwide, and the interest in rehabilitation and restoration of native forests and degraded lands is growing (Thomas et al., 2014). In New Zealand (NZ), native land cover has decreased by 72% since the arrival of humans and eventual agricultural intensification (Ewers et al., 2006). Within intensive agricultural landscapes, native vegetation now covers as little as 0.5% of the land (Franklin et al., 2015). Native vegetation increases local biodiversity by providing habitats for indigenous birds, arthropods, mammals, and other successional plant species, and helps to reduce greenhouse gas emission through carbon sequestration (Dymond et al., 2013). Many native ecosystems are adapted low fertility soils (Wardle, 1985). It is therefore unclear how native species would respond to high-nutrient TMW irrigation. Mohammadi et al. (2021) found that a desert plant native to Iran (*Niteria schoberi*) responded positively to TMW application. TMW irrigation could, therefore, accelerate the revegetation of native plants with beneficial effects on local economies and environments (Abdullah et al., 2022). Franklin et al. (2015) reported that some NZ native species tolerated high application rates of N but did not show an increase in plant biomass.

However, Reis et al. (2017) and Gutierrez-Gines et al. (2019) measured large increases of *L. scoparium* biomass following the application of biosolids. A positive growth response to biosolids application was described in various, but not all, native species by Gutierrez-Gines et al. (2017). Therefore, it is unknown how the addition of water and nutrients from TMW irrigation would affect the growth of different species. Nevertheless, if biomass is not harvested for native products, there is no removal of nutrients from a TMW land application site. This might result in nutrient imbalances in the soil or increased leaching and runoff. The application of TMW to land is largely limited by N, P, sodicity and heavy metals (Barton et al., 2005; Houlbrooke et al., 2011; Tzanakakis et al., 2009), and the fluxes of the fate of these compounds when TMW is applied to native vegetation require investigation to determine the sustainability of such TM land application systems.

We hypothesised that the land application of TMW combined with the re-establishment of native ecosystems on marginal land can increase native biodiversity, without unacceptable accumulation or loss of inorganic contaminants. This research aimed to quantify responses of NZ native vegetation to irrigation with TMW. Specifically, we sought to determine (i) changes in the distribution and speciation of nutrients and inorganic contaminants in the soil-plant system, and (ii) the establishment and growth of NZ native species under TMW irrigation.

2.3 Materials and Methods

2.3.1 Field site

A field trial was set up in July 2015 in Duvauchelle (NZ, 43°45'9"S, 172°56'36"E), Banks Peninsula, on the east coast of the South Island of NZ (Figure 2-1). The soil at the site has a short period of summer moisture deficit and is defined as a Pawson silt loam (46% sand, 29% silt, 25% clay) that develops brown earth characteristics (Molloy, 1988). The annual rainfall measured in the nearby town of Akaroa is 969 mm (Macara, 2016). The trial included eleven NZ native species divided into three different vegetation types (Table 2-1). Vegetation type 1 included the most common early successional species in NZ (Sullivan et al., 2007), type 2 contained species that are well adapted to wet environments (Czernin & Phillips, 2005), and type 3 contained species that are naturally regenerating on Banks Peninsula (Wilson, 1994). Seedlings were 2 years old when they were planted into established *Dactylis glomerata* (cocksfoot) at the site, which was used for extensive sheep grazing prior to the experiment. Tree guards were used to protect the plants from weed competition. The species used were of local provenance (Department of Conservation, 2021). Some 1350 trees were planted in 24 blocks of 5 m × 5 m. A high density of 2 plants m^{-2} was chosen to achieve fast canopy closure and data collection. There were four replicates of each vegetation type per control and irrigated treatment (Figure A-1).

The irrigated blocks received TMW from the local wastewater treatment plant (WWTP) at a rate of 1000 mm yr^{-1} through surface drip irrigation, which started in January 2016. TMW irrigation occurred daily and was applied evenly throughout the year. The control treatment did not receive any form of irrigation. Table 2-2 shows the properties of the TMW and annual application rates of the components.

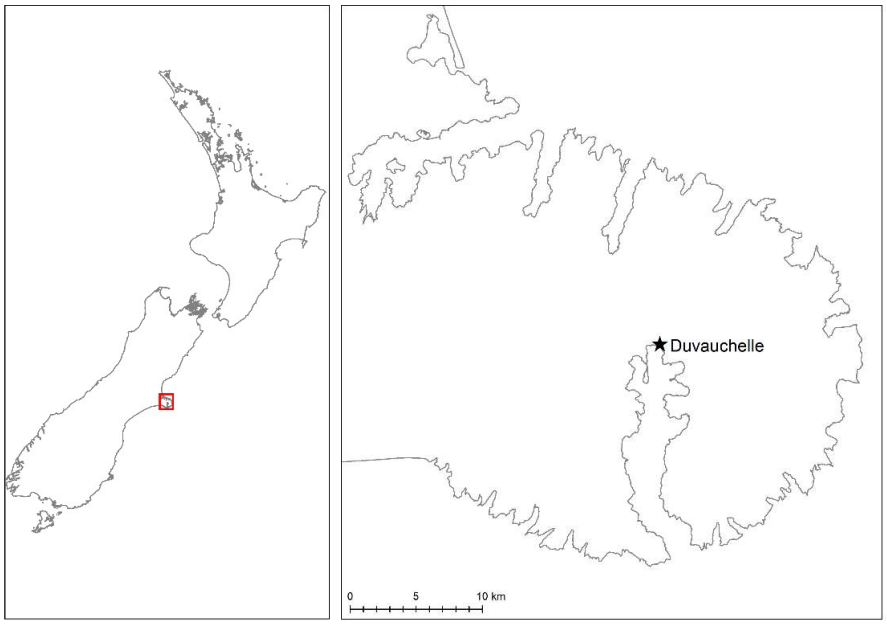

Figure 2-1 Location of the field site in Duvauchelle, Banks Peninsula, on the east coast of the South Island of New Zealand.

Table 2-1 Eleven New Zealand native plant species planted at the field site in Duvauchelle, divided into three vegetation types.

Vegetation type 1		Vegetation type 2		Vegetation type 3	
Mānuka	*Leptospermum scoparium* J.R.Forst. & G.Forst.	Akiraho	*Olearia paniculata* (J.R.Forst. & G.Forst) Druce	Kapuka	*Griselinia littoralis* (Raoul) Raoul
Kānuka	*Kunzea robusta* de Lange & Toelken	Puahou	*Pseudopanax arboreus* (L.f.) K.Koch	Tarata	*Pittosporum eugenioides* A.Cunn.
		Karamu	*Coprosma robusta* Raoul	Tī kōuka	*Cordyline australis* (G.Forst.) Endl.
		Hall's totara	*Podocarpus laetus* Hooibr. ex Endl.	Harakeke	*Phormium tenax* J.R.Forst. & G.Forst.
				Whaririki	*Phormium cookianum* Le Jol.

Māori names and scientific names are shown. Scientific names were reviewed on 27 August 2021 according to https://nzflora.landcareresearch.co.nz/.

Table 2-2 Characteristics of the treated municipal wastewater (TMW) used at the field site in Duvauchelle and application rate in the TMW irrigated treatment, receiving 1000 mm yr^{-1}. Adapted from Gutierrez-Gines et al. (2020).

Variable	Concentration	Application rate (kg ha^{-1} yr^{-1})
pH	7.5	-
EC (µS cm^{-1})	423 (40)	-
Total suspended solids	32	320
NH_4^+-N	0.49 (0.15-0.80)[a]	4.9
NO_3^--N	18 (7.5)	180
NO_2^--N	0.86 (0.09)	8.6
Al	0.43 (0.11-1.7)[a]	4.3
B	0.10 (0.04)	1
Ca	59 (12)	590
Cd	<0.001	-
Cu	0.04 (0.03)	0.4
Fe	0.96 (0.25-3.6)[a]	9.6
K	22 (5.0)	220
Mg	19 (5.5)	190
Mn	0.06 (0.03)	0.6
Na	95 (21)	950
P	11 (5.0)	110
S	25 (11)	250
Zn	0.17 (0.11)	1.7
Sodium Adsorption Ratio (SAR)[b]	2.75	-

Mean ± standard deviation of the mean ([a] geometric mean and standard deviation range) with *n*=54, except for trace elements *n*=14. Values are in mg L^{-1} unless otherwise indicated.
[b] Relative concentration of Na to Ca and Mg, calculated according to Ayers and Westcot (1985)

2.3.2 Plant monitoring

The number of dead plants was recorded in October 2015, November 2015, December 2015, and June 2019 to calculate survival rates. The height of each plant was measured in June 2019, using a measuring tape.

2.3.3 Soil and plant sample collection

Soil samples were collected in October-November 2018, from underneath five species: *L. scoparium*, *K. robusta*, *Coprosma robusta*, *Cordyline australis* and *Phormium tenax.* These species were chosen because they are commonly used in restoration plantings throughout NZ and are adapted to wet environments that can result from TMW irrigation (Franklin et al., 2019). We dug a total of 24 soil pits, four per vegetation type and treatment combination. The pits in vegetation types 1 and 3 were dug in a place that allowed to sample both targeted species on either side of each pit. Soil pits were dug to a depth of 65 cm and had a width of 60 cm × 60 cm. The locations of the pits within each plot were chosen randomly. A hand trowel was used to access densely rooted soil (rhizosphere) directly underneath the plant, where soil samples were taken at five different depths: 0-5 cm, 10-20 cm, 25-35 cm, 40-45 cm, and 55-65 cm. This ultimately resulted in a total of 200 soil samples that were transported to the laboratory on ice, where they were kept at 4 ˚C overnight until further analysis.

In June 2019 the foliage of the 40 plants where soil samples were taken from was sampled. For each plant, 10 branches or leaves of different age and aspect were cut by secateurs and combined to generate a representative sample. For *P. tenax* and *C. australis*, the entire leaf was cut at its base. For *C. robusta*, *K. robusta*, and *L. scoparium* branches with a diameter <10 mm were selected and cut off at the stem.

2.3.4 Soil and plant analysis

Soil NO_3^- and exchangeable ammonium (NH_4^+) were extracted from 4 g of fresh soil with 2 M KCl (Blakemore et al., 1987). Colorimetric methods were used to determine NO_3^- (Miranda et al., 2001) and NH_4^+ concentrations (Mulvaney, 1996) in the extract, using a Cary 100 Bio UV-visible spectrophotometer (Agilent Technologies, Santa Clara, CA, USA).

Soil moisture content was determined by drying a subsample of fresh soil at 105 °C for 24 hours (Blakemore et al., 1987). The rest of the soil was dried at 40 °C for 4 days and sieved to <2 mm. Plant samples were washed with deionised water before being dried at 60 °C until a constant weight was achieved (4 days). Leaves of *C. robusta*, *K. robusta*, and *L. scoparium* were then separated from the stems. Dried plant leaves and soils were ground with a Rocklabs Bench Top Ring Mill (Scott, Dunedin, New Zealand).

A Vario-Max CN Elemental Analyser (Elementar, Langenselbold, Germany) was used to determine total C and N in the ground soil samples. A LECO CN828 Carbon/Nitrogen analyser (LECO, St. Joseph, MI, USA) was used to determine total C and N in the ground plant samples. Soil pH was determined in deionised water using a 1: 2.5 soil: water ratio with a HQ 440d Multi-Parameter Meter with pH probe

PHC735 (HACH, Loveland, CO, USA). In all, 0.2 g of ground soil and plant samples were digested in 5 mL 69% HNO_3. Samples were left to pre-digest overnight prior to digestion in a ultraWAVE microwave digester (Milestone Srl, Sorisole, Italy) at 220 °C and 110 bar. Elemental concentrations in the soil digests were analysed by ICP-OES (Varian 720-ES, Agilent Technologies, Santa Clara, CA, USA). Elemental concentrations in the plant digests were analysed by ICP-MS (7500cx, Agilent Technologies, Santa Clara, CA, USA). To determine phytoavailable concentrations of elements, 5 g of soils were extracted with 0.05 M $Ca(NO_3)_2$ (Gray et al., 1999). Elemental concentrations in the extracts were analysed by ICP-MS (Agilent 7500cx). Plant-available phosphorus (Olsen P) was determined in a 0.5 M $NaHCO_3$ extract, using a Cary 100 Bio UV-visible spectrophotometer for colorimetric analysis (Olsen et al., 1954). Certified reference materials were included for soil and plant digestions (SRM 2710a Montana I Soil and SRM1573a Tomato Leaves, National Institute of Standards and Technology, U.S. Department of Commerce). Recoveries ranged from 9 (for Na) to 110% for soil digests and from 92 to 125% for plant digests. While recoveries were low for soil digests, they agreed with results reported by other laboratories that used USEPA SW-846 Method 3050B (NIST, 2018). Analysis of a subset of samples for Na by an accredited laboratory correlated with our results, with y=1.0x at r=0.95 and $p \leq 0.001$ (Figure A-2).

2.3.5 Statistical analysis

Data were analysed with R (R Core Team, 2021). Three-way analysis of variance (ANOVA) was carried out for soil parameters. Depth, species, and irrigation were used as independent variables with interactions. The residuals were plotted to test the assumptions of normality and homoscedasticity. Data were log10 or sqrt transformed where the assumptions were not met. Tukey's honestly significant difference (HSD) post-hoc test was used where significant effects were found, using the package *agricolae* (de Mendiburu, 2021). A two-tailed unpaired t-test was used to compare element concentration at each depth individually after combining results from all plant species. Two-way ANOVA was carried out for plant parameters, using species and irrigation as independent variables with interaction effects. The package *multcomp* (Hothorn et al., 2021) was used for Tukey's HSD post-hoc test where significant effects were found. Assumptions for ANOVA were tested as described for soil parameters. Plant heights in the TMW and control blocks were compared for each species individually by two-tailed unpaired t-test. The significance level for all results was at $p \leq 0.05$. The package *factoextra* (Kassambara & Mundt, 2016) was used to perform a principal component analysis (PCA) of plant variables.

2.4 Results and Discussion

2.4.1 Elemental concentrations in soils

The Sodium Adsorption Ratio (SAR) of the TMW was 2.75, which was within the limit of 6 that is used as a guideline for effluent irrigation in Australia (Hanjra et al., 2012). However, irrigation of TMW with this SAR and an electrical conductivity (EC) of 423 µS cm^{-1} (Table 2-2) can lead to the degradation of soil structure through clay dispersion (ANZECC, 2000; Mojid & Wyseure, 2013). There were no visual signs of ponding or runoff following the irrigation of TMW at the site that would have indicated that infiltration was impaired. This is consistent with findings by McIntyre (2018), whereby infiltration rates of local soils were not impaired by TMW irrigation with similar Na loading rates. Na significantly increased throughout the soil profile, except at 40-50 cm depth (Figure 2-2). Nevertheless, only 735 kg Na ha^{-1} was recovered in the soil following TMW irrigation equivalent to 2700 kg Na ha^{-1} over the experimental period. This reflects the mobility of Na in the soil and indicates that the majority of applied Na leached through the soil profile. Our results were consistent those of Gutierrez-Gines et al. (2020), who reported that Na accumulation in TMW irrigated silt loams from the same region was not proportional to Na application. While the accumulation of Na can increase soil pH (Blume et al., 2016), we did not find significant differences between treatments. The average pH in the topsoil at 0-5 and 10-20 cm was 5.7 and 5.6, respectively. This is within the optimal range for plant growth (Neina, 2019).

High application rates of Na compared to Ca and Mg can lead to increased leaching of these cations and K^+ from the soil (Chahal et al., 2011). This was not observed at our field site, with a trend of all these elements to increase with TMW irrigation (Figure 2-2). K was significantly higher in the subsoil (40-50 and 55-65 cm) of TMW irrigated plots. Ca and Mg showed an overall (0-65 cm) increase of 5.5% and 4.6% respectively following TMW irrigation. Ca and Mg can offset possible negative effects of Na on the soil structure (Gutierrez-Gines et al., 2020). Our results indicated that TMW had no negative effects on the equilibrium of cations in the soil and that there was a low risk of Na impairing soil structure and plant growth.

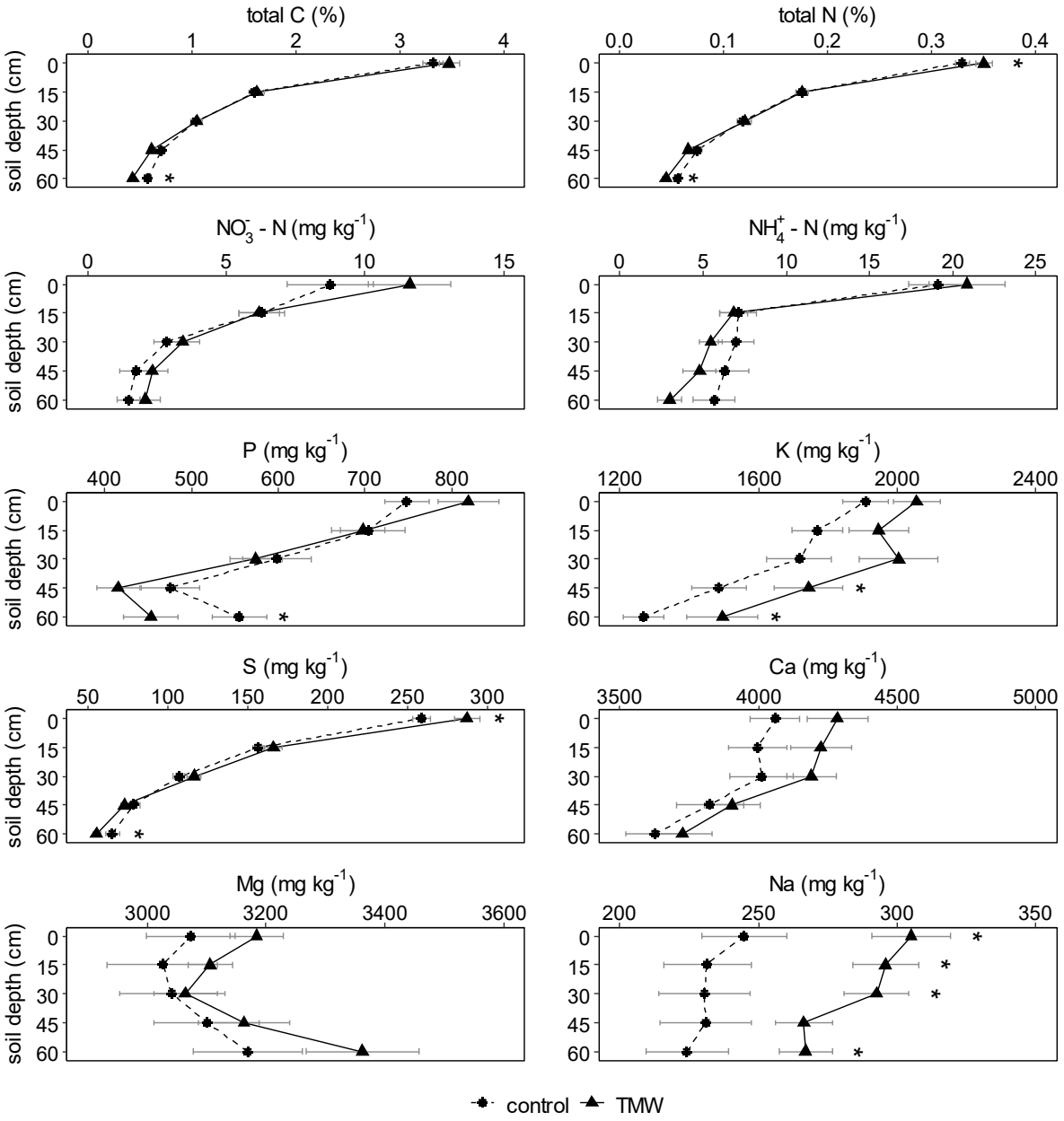

Figure 2-2 Concentrations of elements in the soil profile (0-60 cm) at the Duvauchelle field site in November 2018, comparing TMW irrigated plots and non-irrigated control plots. Values shown are means and standard errors (*n*=4). Asterisks (*) indicate significant differences between treatments at $p \leq 0.05$ according to two-tailed unpaired t-test.

TMW irrigation significantly increased the N concentration in the topsoil (0-5 cm) by 6% compared to the control. In contrast, the N concentration in the subsoil (55-65 cm) was 21% lower in the TMW irrigated plots than in the control plots (Figure 2-2). The application rate of N at the site was 194 kg N ha^{-1} yr^{-1}, but total N throughout the soil profile only increased by 5.6 mg kg^{-1} over the experimental period, equalling to an increase of 17 kg N ha^{-1} yr^{-1}. Possible pathways of N losses are through plant uptake, NO_3^- leaching, or emissions of N_2 or N_2O following denitrification. The latter can be elevated

with increased soil moisture and elevated pH in the TMW irrigated plots (Clough et al., 2004; Šlmek & Cooper, 2002). Van der Weerden et al. (2016) reported that just 1% of N applied with TMW is emitted as N_2O. Assuming such losses we could expect 2 kg N_2O-N ha^{-1} yr^{-1} to be emitted from the irrigated plots at our site. Leaching could account for a larger proportion of N losses, as Sparling et al. (2006) reported that up to 22% of applied TMW-N leached from soil after 4 years of irrigation. However, Gutierrez-Gines et al. (2020) found that NO_3^- leaching from a local Silt Loam irrigated with TMW from the same WWTP at double N loading rates equalled to just 2 kg N ha^{-1} over 17 months. Soil NO_3^- concentrations were unaffected by TMW irrigation. This is consistent with results of Sparling et al. (2006) who found no increased NO_3^- in the soil after 4 years of TMW irrigation onto four different NZ soils. Given the high mobility of NO_3^- in soil (Cameron et al., 2013), it is likely that NO_3^- that was not taken up by plants was leached from the soil. Soil NH_4^+ was not significantly different in the TMW irrigated plots but tended to be reduced under TMW irrigation, where NO_3^- was increased. This could indicate accelerated nitrification with TMW irrigation and increased NH_4^+ supply (Robertson & Groffman, 2015).

The lowest concentration of NO_3^- was under *C. robusta* (3.1 mg kg^{-1}, Table A-1) and it was significantly higher under *P. tenax* (4.7 mg kg^{-1}) and *K. robusta* (6.2 mg kg^{-1}). The results are consistent with those of Franklin et al. (2015), whereby the selection of plant species affects N fluxes in the soil profile. However, unlike reported by Esperschuetz, Balaine, et al. (2017) and Halford et al. (2021), NO_3^- concentrations in the soil under the myrtaceous species *L. scoparium* and *K. robusta* were not lower than under other species. Low NO_3^- concentrations under *C. robusta* were not reported previously, and underlying mechanisms are unknown. NZ native plant species have diverse root morphologies (Franklin et al., 2019), which can affect fluxes of waters through impacts on preferential flow and hydraulic conductivity (Clothier et al., 2007). In addition, distinct exudation from plant roots can affect the mobility of elements and the rates of biogeochemical nutrient cycling, as is the case for N (Carlton et al., 2019). Due to the high mobility of NO_3^- (Di & Cameron, 2002), lower concentrations indicate reduced likelihood of NO_3^- leaching and therefore ground- and surface water contamination and associated public health risk (McDowell et al., 2009).

As with total N, total C was significantly lower in the subsoil (55-65 cm) following TMW irrigation, showing a 25% decrease. It is possible that the high input of N and C with TMW irrigation led to increased microbial activity, referred to as the priming effect (Kuzyakov et al., 2000). Jueschke et al. (2008) reported that while total soil organic C decreased in the subsoil under long-term TMW irrigation, total C in the topsoil significantly increased. At our site total C concentrations did not change at any other soil depths. An initial decrease of soil C can be expected following the transition of grazed pasture into native vegetation at restoration sites, independent of irrigation (Scott et al., 2006). This

likely had a larger effect on total soil C concentration than TMW irrigation. However, soil C is typically increasing from 30 years after afforestation of grassland (Paul et al., 2002).

Phosphorus was not significantly affected by TMW irrigation, except for a 18% decrease in the subsoil (55-65 cm). P is generally immobile in soils and vertical movement in the soil profile occurs through macropores and preferential flow (Gupta et al., 1999). However, due to its immobility, P is mainly lost through surface runoff from a TMW irrigated area, and there were no signs of erosion and runoff at the site. The diverse morphology of native plant species can stabilise the soil and reduce the risk of erosion (Franklin et al., 2019). It is therefore expected that P losses from the site will be small. Gutierrez-Gines et al. (2020) reported that TMW irrigation onto a local silt loam at a loading rates of 75 kg P ha^{-1} yr^{-1} would not exceed concentrations of P that are common in NZ productive soils for at least 50 years. This indicates that the field site will not reach critical P levels for at least 30 years. Olsen P in the TMW irrigated soils was not significantly increased. The Olsen P in the control and TMW irrigated plots was 14 and 17 mg kg^{-1} respectively, which is below recommended values for productive soils and indicates that native plants have low P requirements (Gutierrez-Gines et al., 2020).

Sulphur significantly increased in the topsoil (0-5 cm) and decreased in the subsoil (55-65 cm). While 250 kg S ha^{-1} yr^{-1} were applied with TMW, we observed no overall increase in the soil profile. This is consistent with the high mobility of S in soil and typical leaching losses of 20-120 kg S ha^{-1} yr^{-1} even from non-irrigated soil (Blume et al., 2016). However, while S can lead to acidification of soils, it is not associated with eutrophication (Posch et al., 2015)

None of the soil trace element concentrations were significantly affected by plant species, but the concentration of As, Cr, Li, and Pb was significantly increased by 6-14% following TMW irrigation (Table A-2). However, trace element concentrations did not exceed background concentrations in local soils (Percival et al., 1996). The application of trace elements with TMW was low, and results by Smith et al. (1996) suggested that it will take 50 to 100 years for trace elements to reach values of environmental concern. However, in contrast to their results, we did not observe increases of extractable element concentrations (Table A-3) and found significantly decreased concentrations of extractable Al and Cd in the topsoil (0-5 cm). Our results indicate that the risk of trace element accumulation in soil is lower from TMW irrigation than the application of mineral fertilisers, which can contain trace element contamination (Alloway, 2013).

Depth had the strongest effect on soil parameters (Table 2-3), which was expected due to the physico-chemical changes within the soil profile (Blume et al., 2016). The plant species affected the soil concentration of total C, total N, NO_3^-, and Na, and to a lesser extent S and Mg. Irrigation affected the

concentration of Na and K the most, and to a lesser extent total C, total N, Ca, and Mg. Interaction effects of species and irrigation were strong for NH_4^+ and Na concentrations.

Table 2-3 Results of three-way ANOVA for soil parameters with significant depth, interaction, species, or interaction effects (*** $p \leq 0.001$, ** $p \leq 0.01$, * $p \leq 0.05$). Variables with a significant depth effect only are not included.

Soil parameter & data transformation	Depth	Irrigation	Species	Depth × Irrigation	Depth × Species	Irrigation × Species	Depth × Irrigation × Species
$Log_{10}(C)$	***	**	**	**			
$Log_{10}(N)$	***	**	**	**			
$Log_{10}(NO_3^-\text{-}N)$	***		**		*		
$Log_{10}(NH_4^+\text{-}N)$	***			*		***	
P	***			*			
K	***	***				*	
$Log_{10}(S)$	***		*	***			
Ca	***	*					
Mg	***	*	*				
$Na^{1/2}$	*	***	**			***	
$Log_{10}(As)$	***	***				*	
$Log_{10}(Cr)$		**				***	
$Log_{10}(Li)$	***	***					
$Log_{10}(Pb)$	***	*		*	*		

The results demonstrate that the selection of plant species influences the fate of TMW applied nutrients and contaminants. Species differ in their biomass production and nutrient concentration, affecting the amount of nutrients taken up (Tzanakakis et al., 2009). Plant roots further influence the properties of the rhizosphere (Neumann et al., 2009): distinct root morphologies and rhizosphere processes affect the fate of nutrients and contaminants in the soil (Franklin et al., 2019). These properties need to be considered when selecting plants for TMW land application schemes.

2.4.2 Plant response to TMW irrigation

Following the transplantation of seedlings in July 2015, overall plant survival decreased from 97% in October 2015 to 87% in December 2015 before irrigation began in January 2016. The initial decline in plant survival was likely due to the high temperature and low rainfall during that period. In June 2019,

3.5 years after onset of irrigation, there were a total of 815 surviving plants at the site, equalling to an overall survival rate of 68%. The total survival rate of TMW irrigated plants did not differ from the control. However, plants in the TMW irrigated plots have begun to self-thin, a phenomenon where increased biomass production results in higher mortality in even-aged high-density plantings (Westoby, 1984). Across all species, the average height of the native vegetation receiving TMW was 2.1 m, significantly higher than the unirrigated plants in the control treatment at 1.9 m. Figure 2-3 shows the heights of the individual species. There was no significant decrease in height in any species. Seven species showed a significant increase in plant height when irrigated with TMW. The largest increase was observed in *Griselinia littoralis*, where the plant height was 42% higher with TMW irrigation.

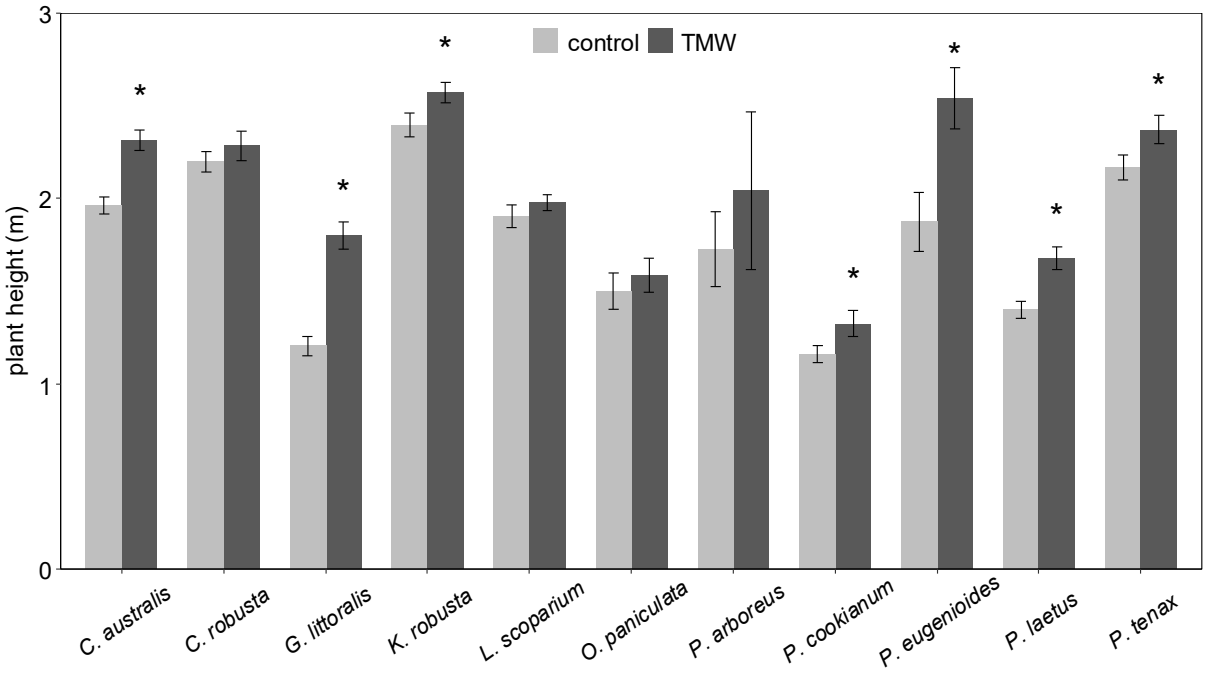

Figure 2-3 Plant height by species four years after planting, comparing plants growing in TMW irrigated plots and non-irrigated control plots. Values shown are means and standard errors (*n*=7-83). Asterisks (*) indicate significant differences ($p \leq 0.05$) between treatments according to two-tailed unpaired t-test.

Our findings are consistent with accelerated plant growth following the application of other biowastes onto NZ native vegetation such as biosolids (Gutierrez-Gines et al., 2017) and vermi-compost (Xue et al., 2016). Species that showed no growth response appeared to do so for different reasons. *Pseudopanax arboreus* was not well adapted to the local environment at the site and showed signs of stress in both treatments. Its survival rate dropped to 57% before irrigation started and was at 21% in June 2019. Sooty mould (*Capnodium walteri*) was observed on all *L. scoparium* plants, which is likely

a result of honeydew production by *Acanthococcus campbelli* and *Acanthococcus laptospermi*, the two common scale insects associated with *L. scoparium* (Bohórquez et al., 2019). *Olearia paniculata* showed evidence of stress and chlorosis in its leaves, which can indicate a nutrient imbalance (Sharma et al., 2021). *C. robusta* grew vigorously in both treatments, and there was no significant difference in plant height.

2.4.3 Elemental concentrations in plants

The concentrations of the essential elements in the plant foliage were not affected by TMW irrigation, except for Zn and Mn (Table 2-4). Following TMW irrigation, foliar concentrations of Mn in *K. robusta* and Zn in *P. tenax* decreased by 47% and 28%, respectively. This indicates dilution following increased biomass production with TMW irrigation (Jarrell & Beverly, 1981). Unlike previously reported with NZ native species (Franklin et al., 2015), there was no luxury uptake of elements with TMW irrigation. It appears that a combination of factors may have limited plant growth at the site (Marschner, 1995). However, other stressing factors such as light and temperature may have influenced the concentration of elements in the plant foliage (Güsewell & Koerselman, 2002). Not all species adapted to low-fertility conditions take up nutrients beyond their growth requirements when fertilised (Iversen et al., 2010). Concentrations of As, Cd, Cr, and Pb (Table A-4) were all within normal ranges for plant foliage (Chaney, 1989). Na, which was significantly increased in the soil with TMW irrigation, did not differ in the foliage between treatments. The small differences in elemental composition between treatments indicates that TMW did not impair the nutrition of NZ native plants.

Table 2-4 Concentrations of elements in plant foliage at the site in June 2019 by species and treatment; Irrigated with treated municipal wastewater (TMW) and non-irrigated (control).

	C. australis		*C. robusta*		*K. robusta*		*L. scoparium*		*P. tenax*	
	control	TMW	control	TMW	control	TMW	control	TMW	control	TMW
N (%)	1.3	1.5	1.6	1.6	1.9	1.8	1.6	1.6	1.4	1.5
	±0.02	±0.11	±0.05	±0.07	±0.09	±0.09	±0.07	±0.05	±0.05	±0.07
Na	696	690	671	729	2,767	2,765	2,503	2,336	2,031	1,541
	±135	±97	±41	±116	±179	±251	±136	±241	±224	±188
Mg	3,247	3,478	4,143	4,439	2,434	1,841	2,492	2,497	1,546	1,606
	±242	±194	±620	±486	±286	±253	±343	±67	±70	±277
P	1,651	1,687	2,226	2,151	1,710	1,576	1,191	1,114	1,871	2,145
	±73	±142	±54	±211	±279	±176	±98	±64	±24	±241
K (%)	0.81	0.72	1.2	1.2	0.66	0.66	0.67	0.51	1.3	1.6
	±0.08	±0.10	±0.10	±0.21	±0.04	±0.05	±0.07	±0.04	±0.03	±0.12
Ca (%)	1.8	1.5	2.1	2.3	0.59	0.46	0.81	0.84	0.39	0.34
	±0.23	±0.12	±0.12	±0.09	±0.11	±0.04	±0.08	±0.06	±0.03	±0.05
Zn	121	126	51	50	36	24	11	9.3	**32**	**25**
	±15	±121	±4.2	±6.4	±2.8	±4.7	±0.57	±0.28	**±1.5**	**±1.3***
Mn	794	946	149	162	**691**	**368**	289	127	135	181
	±172	±196	±26	±36	**±71**	**±58***	±67	±24	±17	±55
Fe	52	63	141	157	252	283	296	575	54	56
	±2.2	±6.0	±15	±42	±17	±59	±34	±136	±3.1	±4.6
Cu	5.6	5.0	6.5	5.7	4.2	3.6	3.7	2.5	4.3	4.2
	±0.53	±0.82	±0.27	±0.45	±0.31	±0.94	±0.64	±0.39	±0.08	±0.27

Mean ± standard error (n=5). Values are in mg kg^{-1} unless otherwise indicated. Significant differences between treatments at $p \leq 0.05$ according to Tukey's HSD post-hoc test are indicated in bold followed by asterisk (*).

PCA revealed that species were grouped based on their elemental composition (Figure 2-4). PC1 (explaining 36.5% of variation) divided species into Myrtaceae (*L. scoparium* and *K. robusta*) and non-Myrtaceae species, while PC2 (explaining 18.2% of variation) divided species into mono- and dicotyledons. This is consistent with results of Hahner et al. (2014), who also found distinct elemental concentrations between native mono- and dicotyledons. Here, PC1 was mainly weighted by Na, C, Cd, and Pb, while PC2 was heavily weighted by Al, Co, and Mg.

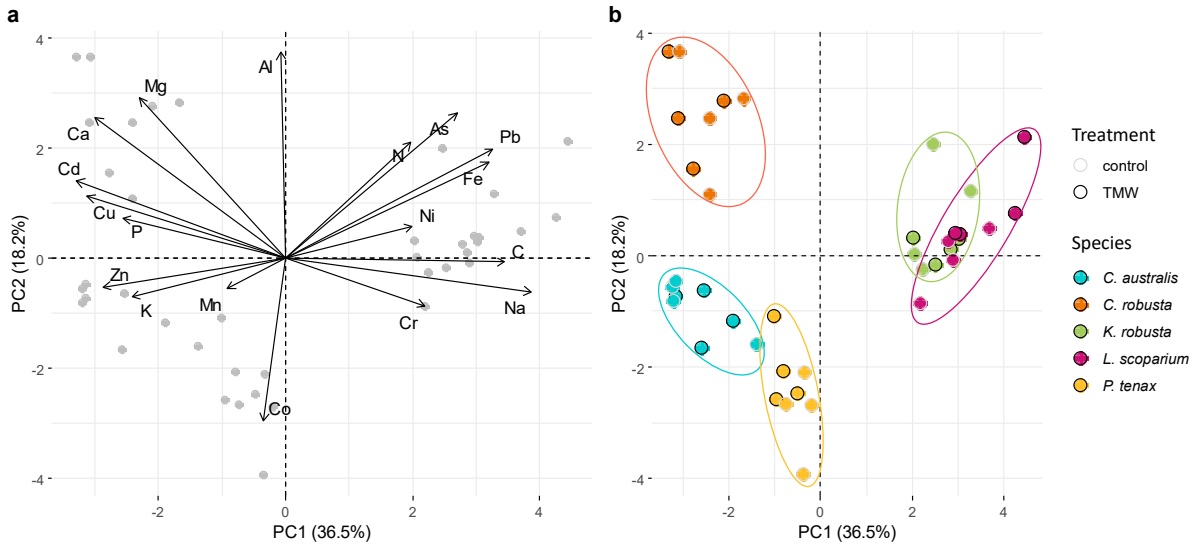

Figure 2-4 Principal component analysis (PCA) of plant composition at the Duvauchelle field site: (**a**) loading plot and (**b**) score plot.

The physiological traits of plants differ as a consequence of genetic influences, resulting in differential nutrient uptake among species (Dickinson et al., 2015). The selection of species with higher nutrient accumulation potential may mitigate elements associated with TMW (Tzanakakis et al., 2009). For example, we found that Na concentrations were four times higher in *K. robusta* than *C. australis*. However, the biomass of these species would need to be known to determine total plant uptake. Furthermore, elements that are taken up by plants will eventually return to the soil through litter, unless biomass is removed from the site. Possible uses for the biomass of NZ native plants at the field site include the production of essential oils from *L. scoparium* and *K. robusta* (Seyedalikhani et al., 2019), fibres from *P. tenax*, or timber from *Podocarpus laetus*. Additionally, some species could be used as a fodder supplement for livestock (Dickinson et al., 2015), particularly *G. littoralis* which was the most responsive species in the present study.

2.5 Conclusions

Application of TMW onto NZ native vegetation had negligible effects on the soil chemistry after nearly 3 years of irrigation. Soil Na concentrations were increased, but the majority of applied Na was readily leached from the soil profile. A small but significant fall in concentrations of soil P below 30 cm requires further investigation of TMW effects on the movement of P in the soil profile. P is not expected to accumulate beyond the range found in NZ agricultural soils for at least 30 years. Despite the application of 194 kg N ha^{-1} yr^{-1}, there was only a small increase of N in the soil profile. Plant species significantly affected the soil concentrations of C, N, NO_3^-, and Na. TMW irrigation did not impair the

growth of NZ native plant species. The overall height of irrigated species was significantly increased. However, selection of species that are well adapted to the local environment is essential for their performance. The chemical composition of the plant foliage was not adversely affected by TMW irrigation. Our results suggest that TMW could be used to support the establishment of native vegetation in NZ and elsewhere. Future research should investigate gaseous N losses and N leaching from TMW irrigated native vegetation to quantify the risk of environmental degradation.

Chapter 3

Challenges Associated with Establishing New Zealand Native Vegetation Irrigated with Treated Municipal Wastewater

3.1 Introduction

About 12% of the treated municipal wastewater (TMW) irrigated onto land in New Zealand (NZ) is applied to trees (Cass & Lowe, 2016). At many of these sites, *Pinus radiata*, the most commonly grown timber species in NZ, is receiving TMW irrigation (Speir, 2002). In Rotorua and Levin, secondary treated TMW has been applied to *P. radiata* since 1991 and 1986, respectively (Hu et al., 2007; Vogeler, 2009). The trees at these sites are reaching maturation and being felled, with rotations of about 28 years in *P. radiata* plantations (Pizzirani et al., 2019). There is hesitancy to re-establish *P. radiata* in these areas because biowastes can impair the health of the trees and reduce timber quality (LEI, 2017; Wilks & Wang, 2009). There is public pressure to increase NZ native plantings, with restrictions for plantation forestry on land that is vulnerable to erosion (Simcock et al., 2019). Esperschuetz, Balaine, et al. (2017) reported lower levels of NO_3^- leaching under some NZ native species (*Leptospermum scoparium* and *Kunzea robusta*) compared to *P. radiata* following the addition of biowastes to soils. Moreover, some NZ native species have the potential to reduce microbial pathogens in the soil (Gutierrez-Gines et al., 2021; Prosser et al., 2016). This indicates that replacing *P. radiata* in TMW land application systems with native vegetation may reduce nutrient and pathogen inputs into ground- and surface waters. However, there is a lacuna of information on the establishment of native plants on TMW irrigated land, unlike *P. radiata*, which is well studied (Hu et al., 2007; Magesan et al., 1998; Schipper et al., 1996).

Many NZ native plant species are adapted to low fertility soils (Wardle, 1985). For most natives, there is limited knowledge on their growth in high nutrient environments Franklin et al. (2015) showed that *Phormium tenax* and *Austroderia richardii* aerial biomass was not reduced by application of urea up to 1600 kg N ha^{-1}. The growth of *Dodonaea viscosa* was not negatively affected by biosolids application, but its aerial biomass was reduced by biochar application (Dickinson et al., 2015). Similarly, Reis et al. (2017) and Esperschuetz, Anderson, et al. (2017) reported that biosolids increased the biomass of *L. scoparium* and *K. robusta* significantly. Furthermore, many native species, such as *Cordyline australis*, *Coprosma robusta*, and *Carex* spp. thrive in riparian areas within agricultural landscapes, where nutrient concentrations are typically high (Marden et al., 2005; McKergow et al., 2016). Results from a field trial in Duvauchelle (Chapter 2) showed that NZ native vegetation was not adversely affected by irrigation of TMW at a rate of 1000 mm yr^{-1}. After 3.5 years, the average plant

height was 10% higher with TMW irrigation compared to a non-irrigated control. Potentially, native plants could be established with TMW at other locations throughout New Zealand (NZ). This is supported by findings of Speir et al. (1999), who showed that native trees and tree-ferns were naturally replacing the exotic species gorse (*Ulex europaeus*) when shrubby vegetation was irrigated with TMW. However, distinct local environments, land uses, and irrigation regimes require for sites to be assessed individually.

Where exotic plantation forest is clear-felled, the disturbed open site is susceptible to the growth of weeds (Bergin & Gea, 2007). Many weeds are able to establish on disturbed soil faster than native species (Porteous, 1993). Furthermore, weed abundance increases with soil fertility (Timmins & Williams, 1991). This indicates that weeds may have an advantage growing in TMW irrigated areas. Trichet et al. (2018) showed that TMW irrigation increased the growth of herbaceous species in *P. radiata* plantation forests. Similarly, raised water tables due to irrigation of land can increase the growth of exotic woody species (Meurk et al., 1995). At Duvauchelle (Chapter 2), weeds were mostly grasses and were controlled with a lawnmower and weedeater. These weed management options are less effective for native plantings into former *P. radiata* plantations where wood-waste creates impediments.

At the TMW land application site in Levin, the irrigation rate is nearly fivefold higher than at Duvauchelle (Chapter 2), and the edaphic and climatic conditions are distinct. Gutierrez-Gines et al. (2019) and Seyedalikhani et al. (2019) demonstrated that an excess of biosolids application was deleterious for *K. robusta* and *L. scoparium* growth. It is unknown whether the high TWM irrigation rates in Levin would result in impaired plant health compared with Duvauchelle. However, plant growth accelerating effects of TMW irrigation may be particularly important in Levin, where hot summers and the low water holding capacity of the sandy soil create water stress (Vogeler, 2009).

It was hypothesised that TMW irrigation will accelerate the growth of both NZ natives and exotic weeds. The aim was to measure the survival and growth rates of NZ native vegetation and exotic weeds under high rates (>4000 mm yr^{-1}) of TMW irrigation in a coastal dune environment. Additionally, critical management strategies to improve the establishment of NZ native species on former *P. radiata* plantation forest soils, especially to control weeds, were investigated.

3.2 Field trial

3.2.1 Site characteristics

The Horowhenua District council was the first to build a pressurised spray irrigation system for TMW in NZ. The system, locally referred to as "The Pot", is located 5 km from the township of Levin on the North Island of NZ (40°37'27''S 175°10'48''E, Figure 3-1). The Pot was set up in 1986 to manage TMW from the Levin wastewater treatment plant (WWTP). The Levin WWTP has an output of >2 M m^3 TMW per annum, which is transferred to a 7 ha pond at The Pot (GHD, 2018). The unlined pond can hold 425,000 m^3 of wastewater and allows the irrigation of controlled volumes of TMW onto land, while leaching about 19% of the TMW influx into groundwater (Horowhenua District Council, 2018). From the pond, TMW is irrigated onto 40.5 ha of land through overhead sprinkler irrigation (GHD, 2018). Since The Pot was established, TMW was irrigated into *P. radiata* plantation forest.

Figure 3-1 Location of "The Pot" (red star) near Levin, on the west coast of the North Island of New Zealand.

At the site, parabolic dunes are running east to west on the sand plains of the Manawatu (Boffa Miskell, 2018). Within The Pot, >75% of the areas consists of well-drained sand dunes and sand plains. The rest consists of imperfectly to very poorly drained sandy and organic soils in inter-dune areas (McLeod, 2018). The latter are not considered for irrigation (LEI, 2017). Soils in the experimental area are well-drained Sandy Recent soils that were provisionally correlated with the Foxton-Omanuka association and Pukepuke-Foxton association after Cowie et al. (1967) (McLeod, 2018; Manaaki Whenua, 2020). Prior to human modification, the vegetation at the site was likely podocarp forest in the lower lying and inter-dune areas, and predominantly *Dacrycarpus dacrydioides*, *Prumnopitys taxifolia*, *Beilschmiedia tawa*, and *Melicytus ramiflorus* in the dry areas of the dunes (Boffa Miskell,

2016). The median annual temperature at the site is 13.5 ˚C, and the annual rainfall is 1163 mm, which is distributed relatively evenly throughout the year (Table 3-1) (Chappell, 2015).

Table 3-1 Average monthly rainfall and average monthly rain days (≥0.1 mm)in Levin. Adapted from Chappell (2015).

	Jan	Feb	Mar	Apr	May	Jun	Jul	Aug	Sep	Oct	Nov	Dec	Annual
Rainfall (mm)	69	85	114	83	90	114	106	91	99	111	99	104	1163
Rain days	11	10	11	12	14	18	18	18	17	16	15	16	176

The land at The Pot is split into four land titles, with two being owned by the Horowhenua District Council (HDC) and the other two by Muaūpoko Lands Trust (LEI, 2017). The experiments described here were conducted on the land titles owned by the HDC. Iwi consultation was conducted by Lowe Environmental Impact Ltd. (Palmerston North, NZ) and site management was in accordance with iwi management values.

3.2.2 Wastewater irrigation

The average TMW irrigation rate at The Pot is 4667 mm yr^{-1} (GHD, 2018). Table 3-2 shows the concentration of elements and contaminants in the TMW and associated application rates. The application of N at The Pot exceeds the rate of 200 kg N ha^{-1} yr^{-1} that is common on productive agricultural land irrigated with farm effluents (Longhurst et al., 2000). Compared to Duvauchelle (Chapter 2), annual application rates of total N and P are about 7 and 3 times higher, respectively. The overhead sprinkler irrigation system is operating over-night, and irrigation of each plot occurs 1-3 times per week, depending on the TMW level in the pond.

Table 3-2 Concentrations of nutrients and contaminants in treated municipal wastewater (TWM) irrigated onto land at The Pot.

Parameter	Concentration in TMW (mg L^{-3})[a]	Application (kg ha^{-1} yr^{-1})
NO_3^--N	10 ± 1.1	468
NO_2^--N	0.04 ± 0.01	1.9
NH_4^+-N	8.0 ± 1.1	373
Total N	33 ± 9.5	1540
PO_4^{3-}-P	1.2 ± 0.19	56
Total P	6.6 ± 0.91	308
Na	61 ± 3.2	2847
K	25 ± 1.9	1167
Ca	12 ± 0.19	560
Mg	3.2 ± 0.13	149
B	0.16 ± 0.01	7.5
Chloride	86 ± 15	4014
Total suspended solids (TSS)	13 ± 6.3	607
Electrical conductivity (EC)	74 ± 5.1 (mS m^{-1})	-
Sodium adsorption ratio (SAR)[b]	4.0	-
E. coli	3408 ± 1767 (cfu 100 mL^{-1})	-
As, Cd, Cr, Cu, Pb, Hg, Ni	<0.01	<0.47

Mean ± standard error (*n*=4-148). Data was provided by Lowe Environmental Impact (LEI) Ltd.
[a] unless otherwise indicated
[b] Application rates derive from TMW irrigation of 4667 mm yr^{-1}.
[c] The SAR shows the concentration of Na relative to Ca and Mg, calculated after Ayers and Westcot (1985).

3.3 Native plantings

3.3.1 Site preparation

In 2018, all *P. radiata* plantation forest (approx. 40 ha) at The Pot were removed from the site. The remaining slash was pushed into windrows. However, stumps and logs were left throughout the site. In some areas of The Pot there are mature stands of native plants that were left in place. This included 4.2 ha of *K. robusta* forest as well as 2.0 ha of *Carex spp.* wetland, both considered regionally threatened habitat types (LEI, 2017).

3.3.2 Species selection

Following the harvest of *P. radiata* at The Pot, 10 ha were planted in native vegetation for experimental purposes in 2018. In addition, native species were planted along water features (approx. 5 ha), while *P. radiata* was planted in the remaining area of The Pot. The native planting occurred at a density of 10,000 stems ha^{-1}. The plants were purchased from Kauri Park Nurseries (Palmerston North). The seedlings were raised in rootrainers and were 30-50 cm tall at planting, depending on species. In total, 60% of the planted seedlings were *K. robusta* and *L. scoparium*, both members of the Myrtaceae family. These two species grow naturally in coastal dunelands. *K. robusta* typically occurs in the dry upper dunes, while *L. scoparium* can tolerate the wet conditions and waterlogging in inter-dunes (KCDC, 1999). Both species are able to establish on disturbed sites, where they are early species in the succession to forest (Stephens et al., 2005; Wilson, 1994), providing a suitable habitat for other native species (Evans, 1983). *K. robusta* and *L. scoparium* previously showed a positive growth response to biosolids application (Esperschuetz, Anderson, et al., 2017) and were not impaired by TMW irrigation at rates of 1000 mm yr^{-1} at Duvauchelle (Chapter 2).

A site visit to the nearby Lake Papaitonga allowed the identification of other species adapted to the local dune environment. Table 3-3 shows the composition of the planted vegetation at The Pot. All species that were chosen grow naturally in different parts of dune systems (KCDC, 1999). The diverse root morphology among the chosen native species was expected to increase the capacity of the vegetation to retain water and nutrients and enhance the stability of the soil (Czernin & Phillips, 2005; Phillips et al., 2011). To achieve an even distribution of the selected species throughout the experimental area, seedlings were planted in rows, with three rows of *K. robusta* and three rows of mixed species alternating.

Table 3-3 New Zealand (NZ) native species planted at The Pot, adapted from ESR & UC (2018).

Species [a]	Vernacular	% [b]	Native habitat within dunelands (KCDC, 1999)
Kunzea robusta	Kānuka	49.1	Dry duneland (on younger dunes & dry sand plains)
Leptospermum scoparium	Mānuka	11.0	Duneland wetlands (damp raised ground or occasional waterlogging)
Coprosma spp. *Coprosma propinqua* *Coprosma robusta* *Coprosma repens*	 Mingimingi Karamu Taupata	9.5	Foredunes (seaward side & in the lee), dry duneland (on younger dunes & dry sand plains), duneland wetlands (damp raised ground or occasional waterlogging)
Carex spp. *Carex pumila* *Carex secta*	 Sand sedge Pukio	5.7	Foredunes (seaward side), dry duneland (moist sand plains & hollows), duneland wetlands (wet or damp edges & hollows)
Cordyline australis	Tī kōuka	4.6	Dry dunelands (moist sand plains & hollows), duneland wetlands (damp edges & hollows, damp raised grounds or occasional waterlogging)
Phormium tenax	Harakeke	4.0	Dry duneland (moist sand plains & hollows), dune wetlands (damp edges & hollows)
Melicytus ramiflorus	Mahoe	3.9	Dry duneland (older dunes with soils)
Dodonaea viscosa	Akeake	3.5	Foredunes (in the lee), dry duneland (on younger dunes & dry sand plains)
Veronica stricta	Koromiko	3.1	Duneland wetland (damp raised ground or occasional waterlogging), banks of duneland streams
Myoporum laetum	Ngaio	2.1	Foredunes (in the lee), dry duneland (on younger dunes & dry sand plains), banks of duneland streams
Austroderia richardii	Toetoe	2.0	Dry duneland (moist sand plains & hollows), banks of duneland streams, duneland wetlands (damp raised ground or occasional waterlogging)
Corynocarpus laevigatus	Karaka	0.5	Dry duneland (moist sand plains & hollows)
Juncus pallidus	Giant rush	0.5	Coastal to lowland. Usually in damp swampy hollows, on the margins of wetlands and lakes, in open shrubland on damp ground, or near saltmarshes in places that can be flooded by king tides [c]
Plagianthus regius	Manatu	0.5	Coastal to lower montane, often a prominent tree in lowland alluvial forest [c]

[a] Scientific names were reviewed on 20 September 2021 according to https://nzflora.landcareresearch.co.nz/.
[b] % of trees of this species in the experiment at The Pot.
[c] Information obtained from http://nzpcn.org.nz on 20 September 2021.

3.3.3 Ecotypes

Selecting local provenances and ecotypes for native plantings is referred to as eco-sourcing (Norton et al., 2018) and is now common in restoration projects (Stevens et al., 2015). Local ecotypes are usually best adapted to the local environment (Clarkson & McQueen, 2004). However, a variety of ecotypes of *K. robusta* was planted at The Pot. They originated from Levin, North Canterbury, Kaipara, Foxton, Eastern Bays, Tutamoahoe, Horowhenua, Rodney, South Branch, Eastern Bays, and Waikanae. The environment at The Pot is strongly modified through the irrigation of TMW and it is expected that a mixture of ecotypes would result in better overall survival of plants. The chemical composition of *K. robusta* and *L. scoparium* differs between NZ regions, and their growth is typically not limited by location (Essien et al., 2019).

Eco-sourcing can limit the genetic variation within a population (Stevens et al., 2015). As a large area was planted predominantly with myrtaceous species, it was hoped that a large gene-pool would reduce the risk of complete eradication by Myrtle rust. Myrtle rust (*Austropuccinia psidii*) is currently spreading throughout NZ, infecting various Myrtaceae *spp.* including *L. scoparium* and *K. robusta* (Toome-Heller et al., 2020). However, some resistance has been found in these species, with seed family and provenances differing in their resistance (Smith et al., 2020). Similarly, using non-local ecotypes could buffer plantings against climate change effects, but the initial survival of non-local ecotypes might be impaired (Norton et al., 2018). The temperatures at The Pot are anticipated to be up to 1.1 ˚C and 3.1 ˚C warmer by 2040 and 2090, respectively (Holland et al., 2019).

3.4 Materials and Methods

3.4.1 Preliminary plant growth trial

To determine whether selected native species could tolerate high TMW spray irrigation rates >4000 mm yr^{-1}, a preliminary trial was established in one area of The Pot. Table 3-4 shows the nine native species that were included in this trial. Irrigated plants were arranged at equal distance around a sprinkler and received overnight TMW irrigation once per week. The amount of rain and irrigation was measured weekly with rain gauges. For a non-irrigated control, plants were planted outside the reach of the same sprinkler. A total of 138 seedlings were planted on 07 May 2018 and grown for about 15 weeks prior to destructive sampling on 22 August 2018. At sampling, the height of the plants was measured, and plants were cut 20 mm above ground. The plants were dried at 35 ˚C for 15 days before they were weighed.

Table 3-4 NZ native species planted in the preliminary trial and number of plants used per treatment (unirrigated control and TMW irrigated).

Plant species	*n* control	*n* TMW
Cordyline australis	5	5
Coprosma robusta	5	5
Coprosma repens	5	5
Corynocarpus laevigatus	4	4
Kunzea robusta	18	19
Leptospermum scoparium	17	17
Melicytus ramiflorus	5	5
Myoporum laetum	5	5
Veronica stricta	4	5
Total	68	70

3.4.2 Weed control trial

A weed control trial was set up to determine the growth of *L. scoparium* seedlings with different weed control treatments. The trial was split into three plots, with 64 plants each, that were arranged East to West (Figure 3-2). Each plot was subdivided into four areas of different weed control treatments with 16 plants each: (a) no weed control, (b) wool mulch mats, (c) a combination of wool mulch mats and CombiGuards, and (d) black polypropylene weed mats. Figure 3-3 shows treatments a-c after the experiment was installed. Seedlings of *L. scoparium* were planted on 26 August 2020. The plots were located at equal distances from the sprinkler heads to ensure even TMW irrigation.

a **b**

Figure 3-2 (**a**) Experimental area at The Pot, highlighted in yellow, and (**b**) location of the weed control trial plots (green rectangles) within the experimental area. The red dots indicate the location of the lysimeters, which pertain to the experiment reported in Chapter 4.

a **b** **c**

Figure 3-3 Treatments in the weed control trial; (**a**) black polypropylene weed mats, (**b**) wool mulch mats, and (**c**) a combination of wool mulch mats and CombiGuards. The control treatment without weed control is not shown.

After 12 weeks of growth, the survival, height, and health of each *L. scoparium* seedling was determined. Plant health was determined using a semi-quantitative visual assessment, rating plants from 1 (heavily chlorotic or necrotic) to 5 (healthy). If a plant was clearly dead, this was a 0 on the health index. Plants that could not be found were assumed to be dead and recorded as 0.

3.4.3 Plant survival and growth

Plant survival, plant height, and weed cover at The Pot were measured in May 2019 and May 2021. Transects of 25m^2 (12.5 m × 2 m) were chosen randomly in different areas of the experimental plots. Plants within 1 m from a 12.5 m tape were recorded and their height was measured with a measurement stick. The coverage of weeds was visually estimated based on a percentage of total ground area per weed species. In May 2019 (approx. one year after plant establishment), transects were conducted in four different vegetation management types; (1) non-irrigated, (2) irrigated with weed management, (3) irrigated without weed management, and (4) irrigated with natives planted into grass. Weed management included manual weeding and herbicide spot spraying. A minimum of three transects was recorded per vegetation management type. In May 2021, 24 transects were conducted in plots of vegetation management type 3. The other types could not be assessed due to additional planting of native seedlings in these areas after 2019.

3.4.4 Statistical analysis

Data were analysed and visualised using R (R Core Team, 2021). For the preliminary growth trial, a two-tailed unpaired t-test was used to determine significant above-ground biomass differences

between control and irrigated plants for each species. For the weed control trial, two-way analysis of variance (ANOVA) with Tukey's honestly significant difference (HSD) post-hoc test was used to determine significant effects of treatments and blocks using the package *multcomp* (Hothorn et al., 2021). Assumptions of homoscedasticity and normality were tested by plotting the residuals against the fitted values and quantiles of the normal distribution, respectively. Data were log transformed where the assumptions were not met.

3.5 Results and Discussion

3.5.1 Preliminary plant growth trial

Over the 15 weeks of plant growth the irrigated treatment received a total of 1008 mm of TMW. Additionally, plants received a cumulative rainfall of 365 mm. With average N and P concentrations of 33 mg L^{-1} and 6.6 mg L^{-1} respectively (Table 3-2) the irrigated treatment received 333 kg N ha^{-1} and 67 kg P ha^{-1} during the experimental period. This equalled to a yearly application rate of 232 kg P ha^{-1} and 1154 kg N ha^{-1} which was nearly six times higher than 200 kg N ha^{-1} yr^{-1} that is commonly applied to productive agricultural land in NZ (Wang et al., 2004). The irrigation in the trial was equivalent to a TMW irrigation rate of 3494 mm yr^{-1}, which was 25% lower than the overall average irrigation rate at The Pot.

Plant survival was 97% and unaffected by TMW irrigation. None of the planted species showed a significant decrease in aerial biomass following TMW irrigation (Figure 3-4) and there were no visible signs of toxicity in TMW irrigated plants. Salinity can impair plant growth, and the electrical conductivity (EC) of the TMW at The Pot indicates that slight to moderate usage restrictions would be required (Table 3-2, FAO, 2003). However, the soil at the site was well-drained and precipitation rates were high (Table 3-1), which likely resulted in rapid downward movement of salts in the soil (Pedrero et al., 2010). Chloride, Na, and B can cause specific ion toxicity in plants. However, these ions did not exceed critical values for sprinkler irrigation of TMW (Table 3-2, Pedrero et al., 2010). Overall, plant biomass was 39% higher with TMW irrigation compared to the non-irrigated control. *Coprosma repens* nearly doubled its biomass with TMW irrigation, which was the highest increase observed. The biomass of *C. australis*, *C. robusta*, *Melicytus ramiflorus*, and *Veronica stricta* increased between 28% and 70%. Most *Myoporum laetum* plants in the control treatment were missing a large proportion of their foliage, which appeared to be due to grazing by rabbits. Consequently, the difference in biomass between the TMW-irrigated and control treatments was affected more by animal browsing than TMW irrigation. There were no signs of animal browsing in any of the TMW irrigated plants.

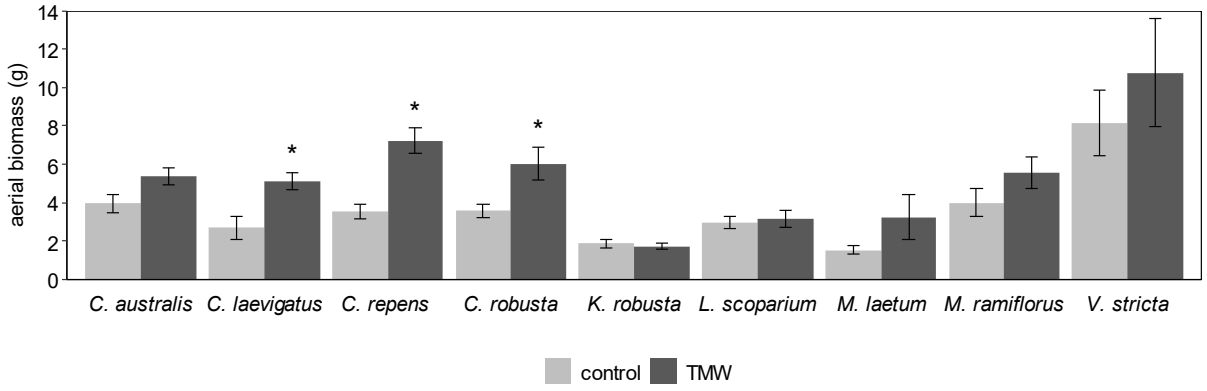

Figure 3-4 Above-ground biomass (g) of the NZ native species grown in the preliminary trial with TMW irrigation (TMW) and without irrigation (control) after 12 weeks of growth. Values shown are means and standard errors. Significant differences between treatments at $p \leq 0.05$ according to two-tailed t-test are indicated by asterisks (*).

The increased biomass with TMW irrigation in some species is consistent with observations of increased plant height following TMW irrigation onto natives at Duvauchelle (Chapter 2). However, the results do not indicate whether the biomass increase was due to irrigation or a fertilisation effect of added nutrients, such as N and P. Positive growth responses to N fertilisation were observed by Esperschuetz, Balaine, et al. (2017) in *L. scoparium* and *K. robusta* following urea application. Similarly, Reis et al. (2017) and Esperschuetz, Anderson, et al. (2017) found significant aboveground biomass increases in *L. scoparium* and *K. robusta* following biosolids application. Results of Gutierrez-Gines et al. (2017) extend these findings to a range of other native species, including *C. robusta*, *P. tenax*, and *C. australis*. Given that the rainfall was relatively constant during the 15 weeks of the experiment, and that the experimental period was during late autumn, it is unlikely that un-irrigated plants were experiencing water-stress. Therefore, the results indicate that the nutrients in TMW were beneficial for the growth of native species at The Pot and that the observed biomass increase was not solely an irrigation effect.

K. robusta biomass was unaffected by TMW irrigation. As this was the main species that was planted at The Pot, its survival and growth would be essential for the outcome of the 10 ha main planting. Findings from the preliminary trial were not consistent with those from a field trial in Duvauchelle (Chapter 2), whereby *K. robusta* plant height significantly increased following TMW irrigation. Application rates of P and N in the preliminary trial at The Pot were approximately two and five times higher, respectively, than at Duvauchelle. The additional irrigation and nutrients were not beneficial for *K. robusta* in the short term. However, it is possible that *K. robusta* roots were disturbed at

planting, which can negatively impact the seedling's performance (Boffa Miskell, 2017) and may therefore limit any possible positive growth effects of TMW in the early stages.

3.5.2 Weed control trial

The black weed mats significantly increased plant height compared to the control, while the other treatments showed no effect (Figure 3-5 a). Plant health and survival were significantly higher in the plants that had a combination of wool mulch mats and CombiGuards than all other treatments (Figures 3-5 b and c). The survival in all other treatments was below 60%. Findings are consistent with those of Dollery et al. (2018), who showed that the use of CombiGuards in native plantings could reduce overall costs of native plantings due to reduced mortality of seedlings. Lai and Wong (2005) demonstrated that native vegetation in Hong Kong did not benefit from weed mats alone but showed significantly higher survival and growth with a combination of weed mats and tree guards.

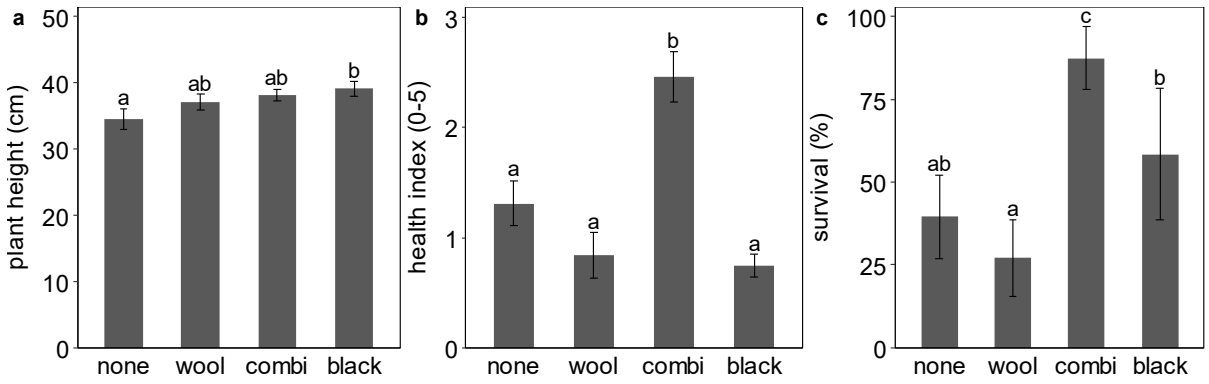

Figure 3-5 Effect of weed treatments on; (**a**) plant height (cm), (**b**) health index (0-5), and (**c**) survival (%) of L. scoparium seedlings after 12 weeks of growth with TMW irrigation. Values shown are means and standard errors, *n*(a & b)=48 and *n*(c)=3. None: no weed control, wool: wool mulch mats, combi: wool mulch mats and CombiGuards, black: black polypropylene weed mats.

Independent of treatment, the growth of pasture in all plots was high and many *L. scoparium* seedlings were not found. Pasture densely over-growing young seedlings can limit the amount of light that reaches the *L. scoparium* seedling (Figure 3-6). The competition for light can be detrimental for the seedling (Williams & West, 2000). However, pasture species (predominantly *Holcus lanatus*) were the only weeds observed in the trial, and other pasture species would likely have differing levels of competition with *L. scoparium* seedlings.

Figure 3-6 Pasture grass overgrowing a *Leptospermum scoparium* seedling at The Pot

3.5.3 Plant survival and growth

In May 2019, plant survival was affected by weed management. Irrigation increased the growth of both native plants (Figure 3-7 a) and weeds (Figure 3-7 b). However, the survival rate in the TMW irrigated plots with and without weed control was lower (27% and 2.2%, respectively) than in the non-irrigated plots (65%). This was likely due to the lower weed coverage in the non-irrigated plots. Where native seedlings were planted into unmanaged pasture, the survival rate was about 70%, based on visual assessment.

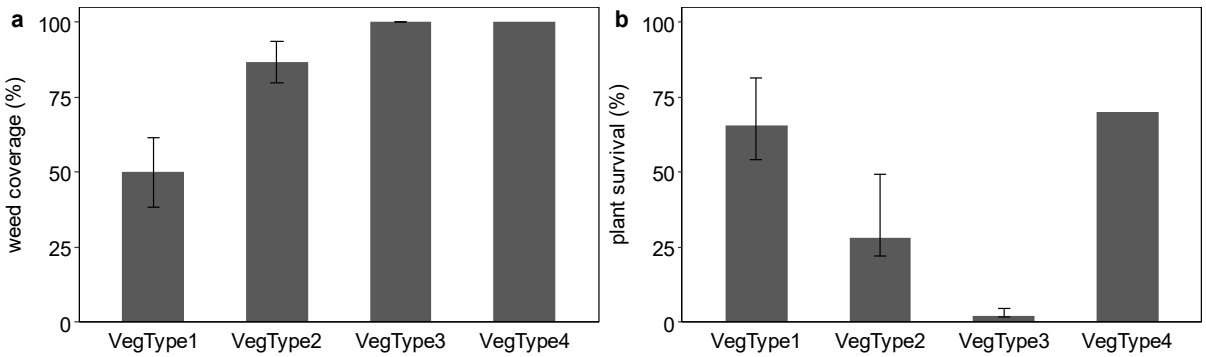

Figure 3-7 Weed coverage (**a**) and plant survival (**b**) in native plantings at The Pot in May 2019, approx. one year after planting. VegType1: non-irrigated, VegType2: irrigated with weed management, VegType3: irrigated without weed management, and VegType4: irrigated with natives planted into unmanaged pasture. Values for VegType4 are estimate-based.

The main weeds observed at the site were *Solanum* spp. (nightshade, Figure 3-8 a), mostly *Solanum nigrum* (black nightshade) and *Solanum chenopodioids* (velvety nightshade), as well as *Phytolacca octandra* (inkweed, Figure 3-8 b). *Solanum* spp. are annual to perennial herbs, that can regenerate after frost and smother other plants (Healy, 1974). They grow well in fertile soils and under high moisture conditions (Mwai et al., 2007). Opiyo (2004) reported that N fertilisation increased the vegetative growth of *S. nigra*. This indicates that TMW likely benefited the growth of this weed at The Pot. *Solanum mauritanium* (woolly nightshade) was found to suppress seed germination of *Hebe stricta* (now *Veronica Stricta*) (van den Bosch et al., 2004). It is therefore possible that *Solanum* spp., if not managed, may suppress the natural regeneration of native species at The Pot. *P. octandra* is a perennial shrub that grows up to 1.5 m high and typically grows in coastal sand dune environments (Duncan, 1962). The growth of *P. octandra* can be accelerated by fertilisation, as it typically grows on fertile soils. Once it is densely established, it is hard to control *P. octandra* with herbicides, and pulling plants can be difficult due to their strong taproot (Duncan, 1962).

a **b**

Figure 3-8 Weeds at The Pot; (**a**) *Solanum* spp. (nightshade) overgrowing native plants, and (**b**) *Phytolacca octandra* (inkweed) with characteristic purple fleshy seeds.

In the transects recorded in May 2019, the height of the native plants differed between non-irrigated controls and TMW irrigated plants (Figure 3-9). Plants were higher when they received TMW and weed management (Type 2) compared to a non-irrigated control (Type 1). *K. robusta* was the only species found in irrigated areas without weed control. Its average plant height was higher than the non-irrigated control. *K. robusta* is known to establish on disturbed sites as an early successional species (Aimers et al., 2021) and was planted at the highest percentage (49%) in this field trial. Due to the high

mortality of the plants and the high weed coverage, it was not possible to identify *K. robusta* ecotypes when transects were recorded.

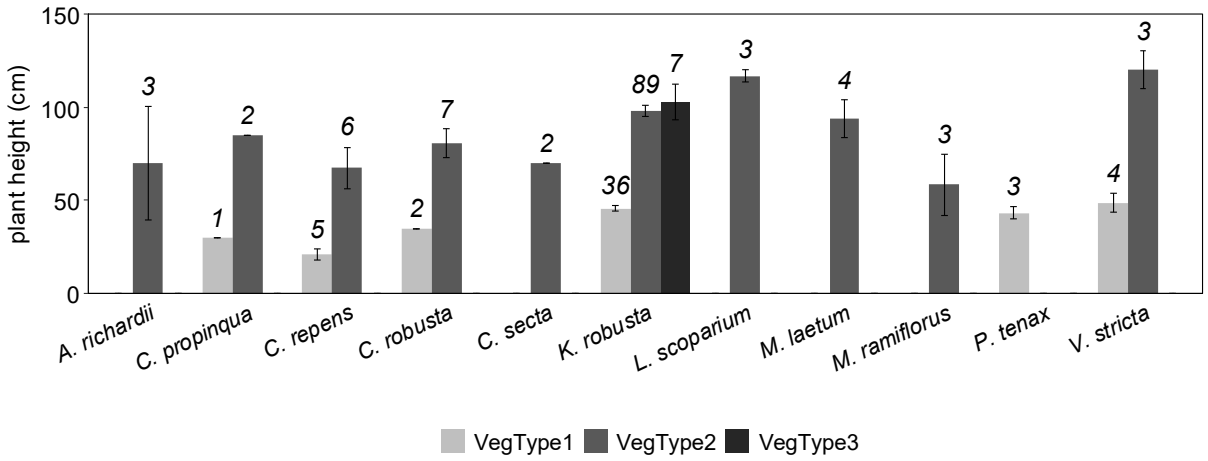

Figure 3-9 Plant height of individual native species in different vegetation management types (VegTypes) at The Pot in May 2019, approx. one year after planting. VegType1: non-irrigated, VegType2: irrigated with weed management, VegType3: irrigated without weed management. Values are means ± standard errors, with *n* shown above error bars.

Figure 3-10 shows the height of the plants in transects recorded in May 2021. Some species had significantly increased in height since 2019, for example *C. repens* (53% height increase) and *M. laetum* (107% height increase). In contrast, the average height of *K. robusta* and *V. stricta* only increased marginally (10% and 17%, respectively). Results are in contrast to those of Bergin and Kimberley (2011), who reported that *K. robusta* had a height growth rate higher than other shrubs and small trees. At The Pot, this was only observed in the early stages of plant growth, when *K. robusta* was one of the tallest plants measured in May 2019.

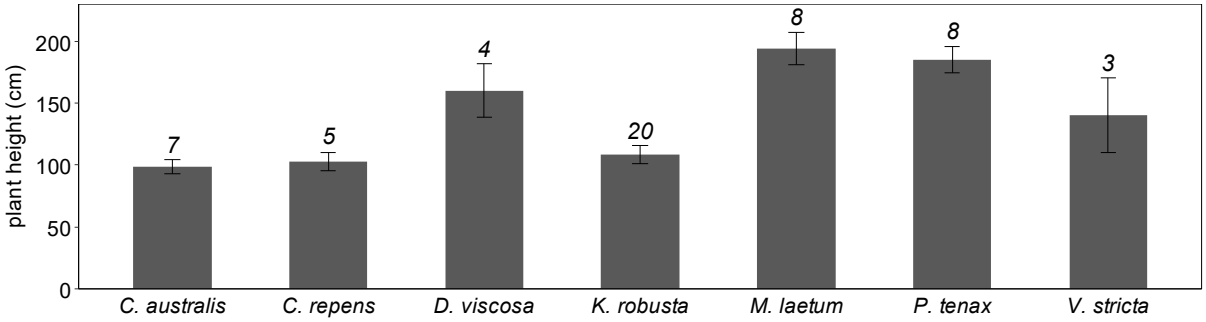

Figure 3-10 Plant height of native species recorded in transects at The Pot in May 2021, approx. three years after planting. All transects were taken in VegType3 (irrigated without weed management). Values above bars show the number of plants recorded (*n*).

The distribution of plants along all transects taken in May 2021 is shown in Figure 3-11. There was at least one plant for every 20 m section of the transects, with an average of 3.7 plants per 20 m and a variance of 8.7. The distribution of plants along the transects can therefore be classified as moderately clumped (Fakhar Izadi & Keshtkar, 2020). It is expected that regeneration to native vegetation will occur naturally, as colonisation of sites with or close to existing native vegetation follows seed dispersal (Sullivan et al., 2009).

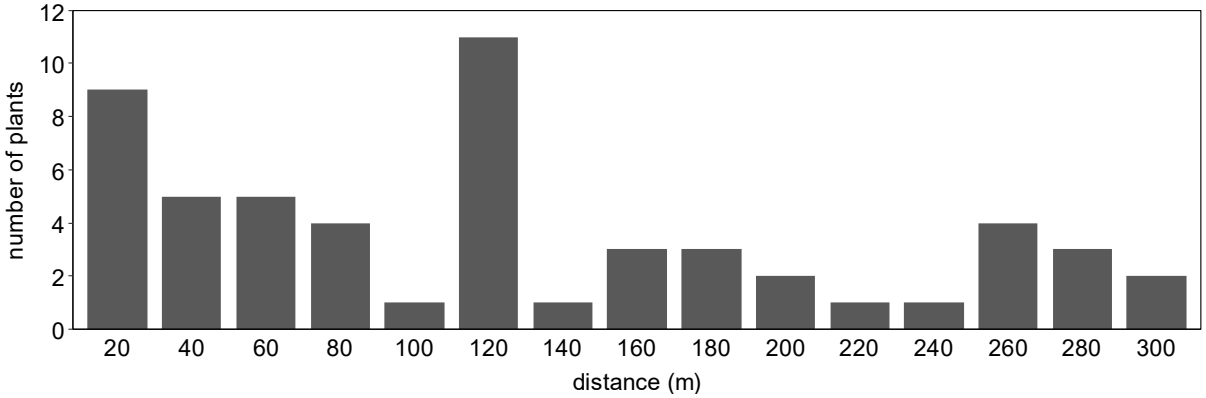

Figure 3-11 Distribution of native plants along all transects recorded at The Pot in May 2021, approx. three years after planting, in VegType3 (irrigated without weed management).

Both native plants and weeds can act as nurse species for developing natives (Sullivan et al., 2007). However, the high density and humidity under *Solanum* spp. and *P. octandra* at The Pot may be unfavourable for such developments. It is expected that the regeneration will be slow and take at least 15-20 years, assuming canopy closure is achieved to suppress weed growth (Bergin & Gea, 2007). The small patches of mature native bush in some areas of The Pot could act as seed bank for potential

regeneration. Replacing dead plants is recommended in native plantings 2 years after the initial planting, commonly referred to as blanking (Meurk & Bergin, 2011). If fast growing species that are performing well at the site such as *M. laetum*, *D. viscosa,* and *V. stricta*, were used for blanking, this would likely accelerate canopy closure and natural regeneration.

3.6 Synthesis

Weed growth impaired the development of native vegetation at The Pot. It appeared that native species responded differently to weed growth, as species composition changed. Transect monitoring of the site in 2021 showed that 36% of surviving plants were *K. robusta*. This indicates that this species performed relatively poorly compared to the other species, as it initially contributed 49% to all plants at the site. Similarly, *L. scoparium* was not present in any of the transects conducted in 2021, indicating that this species was not establishing well at The Pot. However, during the preliminary plant growth trial these species lost a lot of their leaves in both the control and TMW irrigated treatments. It is possible that transplanting the seedlings affected the initial growth of these species, more than irrigation and weed growth, as they are sensitive to root disturbance (Boffa Miskell, 2017).

Several factors at The Pot may have affected native plant growth between 2019 and 2021. There was an invasion of snails in the irrigated area of The Pot, indicating that they were thriving in the wet environment that was created by TMW irrigation in combination with the dense weed cover (Figures 3-12 a & b). Furthermore, there was damage by browsing mammals, such as deer. This, likely in combination with animals rubbing against the plants, resulted in branches of trees being broken off and dying off (Figure 3-12 c). In addition, there was damage to some plants that appeared to derive from a suspected pesticide drift from areas that have undergone chemical weed treatment (Figures 3-12 d & e). Whereas snails caused widespread damage to the plants, the damage from browsing animals and pesticide drift were more localised.

a **b** **c** **d** **e**

Figure 3-12 Damage to native plants at The Pot caused by snails (**a** & **b**), animal browsing (**c**), and suspected pesticide drift (**d** & **e**).

A combination of snail and mammal herbivory on plants can be problematic, as these groups target different areas of the plant foliage (Grime et al., 1968). Some species are more palatable to pest species than others, which can affect forest composition (Allen et al., 1984). Large leaved species such as *Melicytus ramiflorus* and *Coprosma* spp. are preferred by mammals, while small leaved species are less palatable (Smale et al., 1995). Bee et al. (2007) reported that palatable species tend to disappear from forests if browsing pressure is high, while non-palatable species can spread. This can result in near-eradication of palatable native species from forests (Bellingham et al., 2010). The most widespread herbivore pests in NZ are red deer (*Cervus elaphus scoticus*), goats (*Capra hircus*) and the Australian brushtail possum (*Trichosurus vulpecula*) (Nugent et al., 2001). Fencing and conventional lethal means of pest control such as shooting and poisoning can be used to exclude and eradicate pests from native plantings (Clapperton & Day, 2001). Pest control measures at The Pot included the shooting of deer and possums. Furthermore, there was evidence from the preliminary growth trial that TMW may prevent animal browsing, but underlying causes are not known.

The growth of weeds was the main factor limiting the establishment of native vegetation at The Pot. Clear-felling the *P. radiata* disturbed the soil, and likely accelerated the establishment of weeds (Porteous, 1993). The overall plant survival in 2021, approx. three years after planting, was 6.1% in TMW irrigated plots without weed management. This was slightly higher than the 3.3% survival rate measured in these plots in 2019. The results showed that the highest mortality in plants can be expected in the first year after planting. The seedlings were small in the first year and competed with weeds for light (Williams & West, 2000). Once seedlings were higher than weeds, their chance of survival increased. The apparent increase in plant survival observed between 2019 and 2021 is likely

due to the dense weed cover in 2019. The high density of the weeds made it hard to locate native plants that were overgrown. This likely resulted in the under-recording of native plants. In addition, it is possible that some heterogeneity in planting occurred, which meant that some areas were planted more densely than others. This may have affected the results since calculations were based on homogeneous plantings (1 plant m^{-2}). Nevertheless, the loss of native plants since planting was high and the competition with exotic weeds was accelerated in TMW irrigated areas. This is consistent with an increase in exotic species with higher soil nutrient levels as reported by Prober and Wiehl (2012). This highlights the importance of controlling weeds early to increase native plant survival, particularly perennials that grow higher than seedlings, such as *Solanum* spp. and *P. octandra*. While herbicide spraying can be used to control some weeds, it should be considered that TMW irrigation may increase the mobility of the applied herbicide (Müller et al., 2007), and if not properly applied, herbicides may cause native plant causalities.

Results from the weed trial showed that tree guards can be used to protect plants from competing weeds. CombiGuards were used for subsequent inter-planting at The Pot (Figure 3-13). Nevertheless, weed control remains important if weeds persist under taller natives, as dense weed cover can suppress the germination of native seeds and eventually change the composition of the vegetation (Porteous, 1993). Shading by the native vegetation will ultimately reduce weed growth in the understorey, and higher density plantings accelerate canopy closure (Bergin & Gea, 2007). Blanking, the replacement of native plants one to two years after the initial establishment, is a way to maintain the original density of the planting. The time until canopy closure furthermore varies with differing crown spread among native species. The use of species with high crown spread, such as *D. viscosa* (2.5 m crown spread), *L. scoparium* (2.1 m crown spread) and *K. robusta* (2.0 m crown spread) achieve faster canopy closure than species with a medium crown spread such as *C. robusta* (1.8 m crown spread) (Bergin & Gea, 2007).

Figure 3-13 Blanking; NZ native seedlings that were planted one and two years after the initial planting at The Pot. CombiGuards and wool mulch mats were used in some areas to support plant establishment.

While planting density is closely related with canopy closure and weed suppression, it also determines the costs of native plantings. It is common practice for the establishment of NZ native vegetation to transplant nursery grown seedlings, which is more expensive than direct seeding of plants (Douglas et al., 2007). Table 3-5 shows a comparison between costs for establishing low- and high-density native shrubs, in comparison to a *P. radiata* plantation.

Table 3-5 Comparison of establishing costs (NZ$ ha^{-1}) for high- and low-density NZ native shrubs versus *Pinus radiata* plantations, adapted from Bergin and Gea (2007) and Pizzirani et al. (2019).

Initial stocking	Native shrubs, high-density (10,000 stems ha^{-1})	Native shrubs, low-density (2,500 stems ha^{-1})	*Pinus radiata* [a] 833 stems ha^{-1}
Site preparation	$5,000	$1,250	$183
Planting costs [b]	$20,000-40,000 for purchase	$5,000-10,000 for purchase	$584 for purchase
	$10,000 for planting	$2,500 for planting	$324 for planting
Thinning	-	-	$320
Weed control [c]	$10,600	$3,700	$136-324 [d]
Harvesting costs [e]	-	-	$27,881
Total	$45,600-65,600	$12,450-17,450	$29,561

[a] Cost estimation based on flat land.
[b] For native plantings, price is based on NZ$2 for shrub species and NZ$4 for tree species.
[c] This may increase when trees are included in the planting, as canopy closure is slower in trees.
[d] For spot spraying with herbicide (Richardson et al., 2019).
[e] This includes felling, extraction, skid site, and transport.

There is a significant price difference between low- and high-density native plantings. Costs of purchasing seedlings and transplanting them are the main contributor to overall costs of native plantings (Bergin & Gea, 2007). Investments in weed management will likely increase the survival of native plants, and therefore reduce the overall costs. Dollery et al. (2018) reported that the savings from using tree guards exceeded the initial installation costs of the guards and reduced the final costs for each surviving plant. This was reflected at The Pot, where areas that received weed management had a higher plant survival rate, reducing the overall costs per surviving plant. Similarly, other weed management strategies such as localised spraying are expected to reduce the overall costs of establishing native vegetation.

The difference in costs between native plantings and *P. radiata* mainly derives from the low price of *P. radiata* seedlings, as well as rapid planting and effective weed management with herbicides (Rolando et al., 2017). If methods from commercial forestry could be applied to planting and maintaining native vegetation, this may reduce establishment costs of the latter. Furthermore, *P. radiata* is planted to harvest its timber and will generate revenue when the trees are harvested. Native vegetation typically remains unharvested and does not produce any monetary value from the land, unless it is utilised for C trading (Bagrie et al., 2015). In addition, there is potential to use some NZ native species for valuable products, such as timber and fibre (PCE, 2001), as well as essential oil and honey from *L. scoparium* and *K. robusta* (Essien et al., 2019). However, the production of food might not be a practicable option at The Pot, as the pathogen load in the TMW with the current treatment at the Levin WWTP is high. Nevertheless, while some native species may not generate a direct financial profit from the land, their ecological value and benefit to the environment are significant (Daigneault et al., 2017).

3.7 Conclusions

The survival and growth of NZ native vegetation at The Pot was impaired by the accelerated growth of *P. octandra* and *Solanum* spp. caused by TMW irrigation. Weed management increased the survival and growth of native species. The native plants were not directly impaired by high rates (>4,000 mm yr^{-1}) of TMW irrigation. Planting native species into established pasture reduced the occurrence of (semi)woody weeds and resulted in a higher survival rate. There was evidence for the use of tree guards and weed mats to improve the survival of native seedlings. However, high growing weeds such as *P. octandra* and *Solanum* spp. require early intervention and spraying. Further research is required to determine the most suitable control methods against weeds in native plantings, including the effect of various herbicides on native species. Early weed management interventions can reduce the number of plants required for replacement plantings and can therefore reduce overall establishment costs, as

seedling purchase and planting are the largest costs in native plantings. Furthermore, pest control measures at The Pot and elsewhere are essential to minimise browsing from mammal herbivores such as deer and rabbits. Overall, replacing *P. radiata* plantation in TMW land irrigation schemes with native vegetation may be a viable option to include more native biodiversity in the landscape at The Pot and elsewhere in NZ.

Chapter 4

Effects of Selected Species of Myrtaceae on Nutrient Fluxes with Irrigation of Treated Municipal Wastewater

4.1 Introduction

The irrigation of treated municipal wastewater (TMW) at "The Pot" (Chapter 3) is affecting the water quality of the nearby Waiwiri Stream (Allen et al., 2012). Nutrients enter the Waiwiri Stream from The Pot through drains and subsurface flows (LEI, 2017). The sandy soils at The Pot are well drained, and such soils are leaching more of the TMW applied nutrients than other soil types (Sparling et al., 2006). However, the low water quality in the Waiwiri Stream is associated with multiple anthropogenic activities in its catchment (Allen et al., 2012). The Waiwiri Stream originates from Lake Papaitonga, which has elevated levels of N and P itself (Allen et al., 2012), and crosses 4.8 km of dunes and farmland on the southern margin of The Pot prior to entering the ocean (Horowhenua District Council, 2011). Nutrient losses from agricultural land are a major diffuse source of waterway N and P pollution in New Zealand (NZ) (Elliott et al., 2005). The influx of nutrients and contaminants from The Pot and pastoral farmland degrades the water quality of the Waiwiri Stream and exacerbates the loss of biodiversity in both the stream and the coast near the stream outflow (Allen et al., 2012).

The land around Lake Papaitonga and Waiwiri Stream is of ecological and cultural significance (Allen et al., 2012). It provides a habitat to endangered native land snails (*Powelliphanta spp*.), rare leafless mistletoe (*Korothalsella salicornioides*), brown mudfish (*Neochanna apoda*), and longfin eel (*Anguilla dieffenbachia*) (Horowhenua District Council, 2011; NIWA, 2021). Both fish species are under the conservation status 'Declining' (Dunn et al., 2018). The poor water quality in the Waiwiri stream is a significant issue for local iwi, who have noted the disappearance of eel and shellfish from the stream and the coast surrounding the stream outlet (Allen et al., 2012). The Waiwiri Stream is a historic fishing site and embedded in a highly valued ancestral landscape (Horowhenua District Council, 2011). Local iwi wish to rehabilitate the Waiwiri Stream to once again harvest eel from the stream and shellfish along the coastline (Allen et al., 2012).

There are significant differences between plant species in their ability to reduce N losses from the soil. Esperschuetz, Balaine, et al. (2017) showed that nitrate (NO_3^-) leaching and nitrous oxide (N_2O) emissions were reduced under *Kunzea robusta* and *Leptospermum scoparium* compared to the exotic *Pinus radiata*. Furthermore, it was reported that myrtaceous species have the potential to reduce microbial pathogens in the soil (Gutierrez-Gines et al., 2021; Prosser et al., 2016). In 2018, 10 ha of native vegetation, dominated by *K. robusta* and *L. scoparium*, was established at The Pot (Chapter 3)

to replace the harvested *P. radiata* plantation forest. Maximizing the retention of nutrients and pathogens in the TMW irrigated soil-plant system will minimise the masses of nutrients and contaminants lost (Tzanakakis et al., 2009) and entering ground- and surface water, such as the Waiwiri Stream.

This study aimed to determine the distribution of nutrients in the soil-plant system following irrigation of TMW at rates >4000 mm yr^{-1}. Specifically, it sought to (1) quantify nutrient concentrations in the soil-plant system in *K. robusta* and *L. scoparium* compared to pasture, and (2) determine differences in NO_3^- leaching under *K. robusta* compared to pasture.

4.2 Materials and Methods

4.2.1 Soil and plant chemistry

This research was carried out at The Pot (Chapter 3) in November 2020, in a TMW irrigated plot that had received intensive weed management (including manual weeding and herbicide spot spraying). Seventeen specimens each of *K. robusta* and *L. scoparium*, which were planted approximately 2.5 years prior, as well as 17 areas of pasture (dominated by *Holcus lanatus* and *Lolium perenne*) were selected at varying distances from central sprinklers. Figure 4-1 shows the location of the selected specimens and areas. A rain gauge was placed besides each specimen to record the relative TMW irrigation rate during one run of overnight irrigation. There was no rainfall during this period. The height of *K. robusta* and *L. scoparium* specimens was measured with a measuring tape. Foliage was sampled from each specimen by cutting 10 individual branches of varying age and aspect using secateurs. All branches had a diameter of <5 mm and were cut near the trunk. To collect pasture samples, a rectangle of 0.2 × 0.2 m was placed on the ground and all pasture within that rectangle was cut 10 mm above the soil surface.

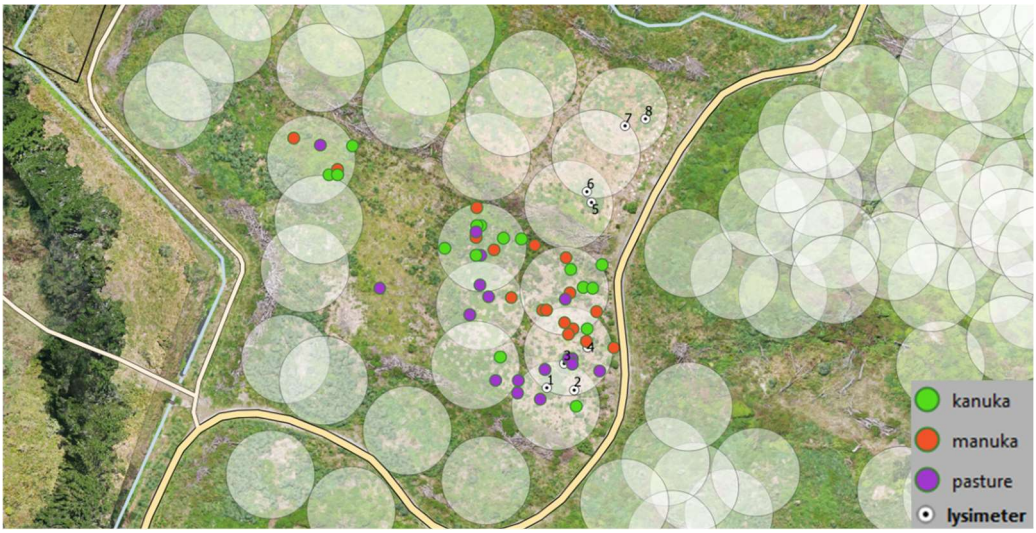

Figure 4-1 Location of sampled specimens of *Kunzea robusta* (green), *Leptospermum scoparium* (orange), and pasture (purple) at The Pot. The white circles show TMW irrigated areas surrounding central sprinklers. Lysimeters are numbered.

Soil samples were collected from underneath each sampled specimen, within 0.3 m from the trunk. Samples were taken at two depths, using a bucket soil sampler (23 mm diameter) to collect samples from the topsoil (0-10 cm depth, Figure 4-2 a) and a soil auger (25 mm diameter) to collect samples from the subsoil (30-45 cm depth, Figure 4-2 b). Soils were stored in polyethene bags, cooled with ice packs, and transported to the lab in insulated containers, where they were immediately frozen until analysis.

a **b**

Figure 4-2 Soil sampling; (**a**) a bucket soil sampler to collect samples from 0-10 cm depth, and (**b**) a soil auger to obtain samples from 30-45 cm depth.

Soil NO_3^- and exchangeable ammonium (NH_4^+) were extracted from the soil with 2 M KCl (Blakemore et al., 1987). In brief, 40 mL of 2M KCl was added to 4 g fresh soil, shaken for 1 hour at 20 rpm in an end-over-end shaker, and filtered through Whatman No. 42 filter paper. Colorimetric methods were used to determine NO_3^--N (Miranda et al., 2001) and NH_4^+-N (Mulvaney, 1996) in the extract, using a Cary 100 Bio UV-visible spectrophotometer (Agilent Technologies, Santa Clara, CA, USA).

The soil moisture content was determined by drying a subsample of 10-20 g fresh soil at 105 °C for 24 hours. The soil weight was recorded before and after drying and the difference used to determine the moisture content (Blakemore et al., 1987).

The remaining soils were spread on aluminium trays, dried at 40 °C for 4 days and sieved to <2 mm. Plant samples were washed with deionised water before being dried at 60 °C until a constant weight was obtained (4 days). Leaves of *K. robusta*, and *L. scoparium* were separated from the stems. There were no flowers or fruits on the sampled foliage. Dried plant leaves and soils were ground with a Rocklabs Bench Top Ring Mill (Scott, Dunedin, New Zealand). A LECO CN828 Carbon/Nitrogen analyser (LECO, St. Joseph, MI, USA) was used to determine total carbon and nitrogen contents in the ground soil and plant samples.

Soil pH and electrical conductivity (EC) were determined in deionised water in a 1:5 soil: water extract. 10 g dry soil sieved to <2 mm was mixed with deionised water. The samples were shaken vigorously and left to equilibrate overnight. The pH and EC were determined using a HQ 440d Multi-Parameter Meter with pH probe PHC735 and EC probe CDC40101 (HACH, Loveland, CO, USA).

Soil and plant samples were digested in HNO_3 to determine total element concentrations. 0.2 g of ground soil and plant material was digested with 5 mL ultrapure HNO_3. Samples were left to pre-digest overnight and were then digested on an ultraWAVE microwave digester (Milestone Srl, Sorisole, Italy). Samples were diluted 21 times with ultrapure water. Element concentrations in the digests were determined by ICP-MS (7500cx, Agilent Technologies, Santa Clara, CA, USA). Certified reference materials were included for soil and plant digestions (SRM 2710a – Montana I Soil and SRM1573a – Tomato Leaves, National Institute of Standards and Technology (NIST), U.S. Department of Commerce). Recoveries ranged from 2 to 100 % in soils and 73 to 142 % in plants. However, the 2% recovery of Na was likely due to a different analytical method used by NIST (Chapter 2).

$Ca(NO_3)_2$ was used to extract phytoavailable metals from the soil (Gray et al., 1999). 5.0 g of soil (air-dried, sieved to <2mm) was shaken with 30 mL of 0.05 M $Ca(NO_3)_2$ for 120 min at 15 rpm in an end-over-end shaker, followed by centrifugation at 10,000 rpm for 10 min. Extracts were filtered through

Whatman No. 42 filter paper. Extracts were diluted 21 times with ultrapure water and element concentrations analysed by ICP-MS (Agilent 7500cx).

To determine plant-available phosphorus (Olsen P), 1.0 g of soil (air dried, <2mm) was extracted with 20 mL 0.5 M $NaHCO_3$ extractant (Blakemore et al., 1987). Samples were shaken for 30 min in an end-over-end shaker at 50 rpm and centrifuged at 2,000 rpm for 10 min. The extract was filtered through Whatman No. 42 filter paper. The P concentration in the extract was determined colorimetric using a Cary 100 Bio UV-visible spectrophotometer (Olsen et al., 1954).

4.2.2 Lysimeters

Eight lysimeters (Drain Gauge G3, METER Environment, Pullman, WA, USA) were installed at The Pot in May 2019 (Figure 4-1 and Table A-5). The soil at the site is a well-drained Sandy Recent soil that was provisionally correlated Pukepuke-Foxton association after Cowie et al. (1967) (McLeod, 2018; Manaaki Whenua, 2020; Table A-6). A digger was used to excavate pits and the lysimeters were installed below the rootzone, with the top of the divergence control tube located 0.3 m below the surface (Figure 4-3). The lysimeters were re-filled with the excavated soil. The dimensions of the divergence control tube were 254 mm inner diameter and 635 mm length. Pressure sensors measured the depth of the water in the drainage reservoir, allowing the calculation of the leachate volume. Two months after lysimeter installation, 4 m^2 areas surrounding the centre of four lysimeters were planted with 25 seedlings of *K. robusta* each at a spacing of 0.5 m × 0.5 m. Seedlings were 2 years old at planting and grown in rootrainers. The remaining four lysimeters were planted in pasture species (predominantly *Holcus lanatus* and *Lolium perenne*). One lysimeter each of pasture and *K. robusta* showed signs of groundwater ingression, as they refilled rapidly after they were emptied. These two lysimeters were excluded from the experiment.

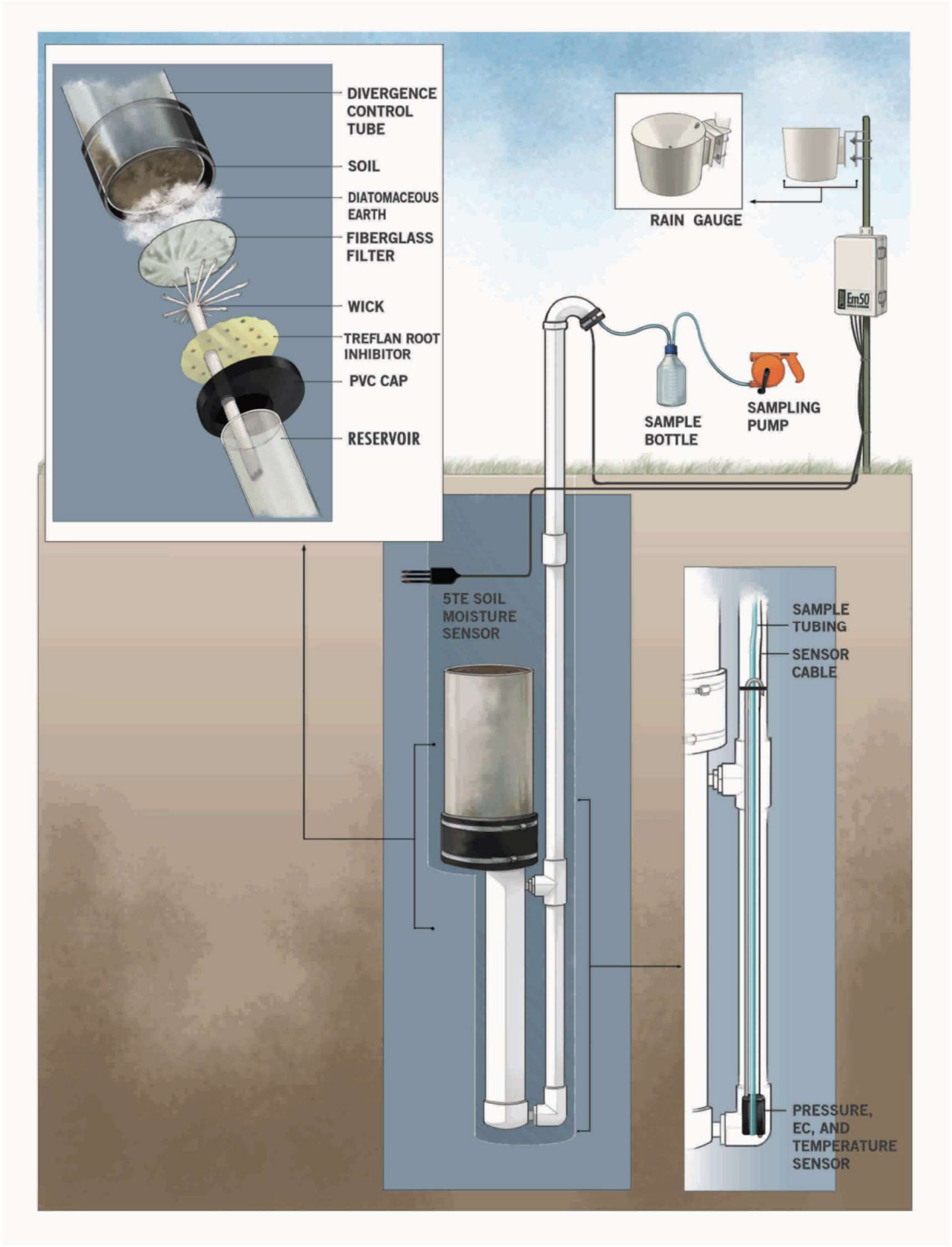

Figure 4-3 Drain Gauge G3 (METER Environment, Pullman, WA, USA). Soil moisture probes and rain gauges were not included in this experiment. Reprinted from Decagon Devices Inc. (2018), with permission from METER Group, Inc.

Lysimeters were connected to a cloud data logger (ZL6, METER Environment, Pullman, WA, USA). This allowed remote access of data. In May 2021, 2 years after installation, drainage was collected for chemical analysis. The drainage reservoirs of the lysimeters were completely emptied and TMW irrigation was increased to ca. 14 mm d^{-1} for three days. Irrigation volumes were measured for each

lysimeter using a rain-gauge. Drainage samples were collected from the drainage reservoirs of the lysimeters each day for three days after initial emptying. A peristaltic pump was used to pump the drainage from the lysimeter reservoir (Figure 4-4), and samples were collected in clean 50 mL falcon tubes. Samples were transported to the laboratory in insulated containers with ice packs and frozen immediately. Prior to analysis, the leachates were thawed and filtered through a 0.45 µm syringe filter. Samples were diluted 10 times with ultrapure water. The concentration of NO_3^- was measured by ion chromatography (Dionex ICS-2100, Thermo Fisher Scientific, Waltham, MA, USA). Nitrate leaching was calculated based on leachate volumes and measured NO_3^- concentration in the leachate.

a **b** **c**

Figure 4-4 Lysimeters; (**a**) *Kunzea robusta* on lysimeter 3, (**b**) pasture on lysimeter 5, and (**c**) emptying of drainage from the lysimeter with a peristaltic pump.

4.2.3 Statistical analysis

Data were analysed and visualised using R (R Core Team, 2021). Descriptive statistics included geometric means and standard error ranges for soil and plant variables, due to the range of relative irrigation received by the sampled specimens. Pearson's correlation coefficients were determined for correlations between individual soil and plant variables in each species. A two-way unpaired t-test was used to compare NO_3^- leaching under *K. robusta* and pasture.

4.3 Results and Discussion

4.3.1 Soil chemistry

Table 4-1 shows the chemical composition of the topsoil (0-10 cm depth) and Table 4-2 shows the chemical composition of the subsoil (30-45 cm depth). Soil pH was low, but still within the typical range for NZ soils (4.1 to 7.4) (Sparling & Schipper, 2002). Plantation forests and native vegetation typically show lower soil pH than other land uses in NZ, highlighting the low pH tolerance of these plants (Sparling & Schipper, 2002). Under pasture, the relative TMW irrigation rate was negatively correlated with soil pH in both the top- and subsoil. This was not observed under *K. robusta* and *L. scoparium*. The negative correlation is in contrast to other studies reporting pH increases in TMW irrigation systems (Sparling et al., 2006; Walker & Lin, 2008). Stewart et al. (1990) reported soil pH increases as high as 1.3 units after four years of TMW irrigation. However, soil acidification can be a result of leaching of basic cations (Ca^{2+}, K^{+}, Mg^{2+}) from the soil and the associated decrease in the soil's acid buffering capacity (Chahal et al., 2011; Werner et al., 2019). Na application at The Pot was 2847 kg Na ha^{-1} yr^{-1}, and the Sodium Adsorption Ratio (SAR) of the TMW was 4.0. The SAR expresses the relative concentration of Na compared to Mg and Ca (Ayers & Westcot, 1985). According to FAO (2003) TMW with a SAR of 4.0 and an EC of 74 mS m^{-1} (Table 3-2, Chapter 3) requires slight to moderate restriction on use to avoid impairment of soil stability and plant growth by Na. However, the soil at The Pot is sand, and the low clay content of 7% (Vogeler, 2009) reduces the likelihood of clay dispersion negatively affecting infiltration (Farahani et al., 2018).

There was a negative correlation between extractable Mg and TMW irrigation in the topsoil under *K. robusta* and pasture (Table 4-1). In the subsoil, extractable Mg was negatively correlated with TMW irrigation under *L. scoparium* and pasture (Table 4-2). This is consistent with results of Stewart et al. (1990), who reported significant decrease of soluble Ca, K, and Mg in the soil following four years of TMW irrigation. However, there was no correlation between extractable K and TMW irrigation in this study (data not shown). Extractable Ca could not be determined because $Ca(NO_3)_2$ was used as extractant. The total Na concentrations in the topsoil were positively correlated with relative TMW irrigation under *K robusta* and pasture, but not under *L. scoparium*. This is consistent with increased Na concentrations in TMW irrigated soils in a study of Gutierrez-Gines et al. (2020) and the experiment in Duvauchelle (Chapter 2). Total concentrations of Mg, Ca and K were unaffected by the TMW irrigation rate at both depths. This was likely due to the low application rate of these elements compared to their total concentration in the soil, as was also reported by Gutierrez-Gines et al. (2020) in an irrigated Fragic Pallic soil and a Recent Fluvial soil.

Table 4-1 Relative irrigation and soil chemistry at 0-10 cm depth under *K. robusta*, *L. scoparium*, and pasture.

0-10 cm	*Kunzea robusta*	*Leptospermum scoparium*	Pasture
Irrigation (mm day^{-1})	11 (2.5-50)	10 (2.4-43)	12 (2.6-81)
pH	4.7 (4.4 - 5.0)	4.7 (4.4 - 5.0)	4.8 (4.4 - 5.2) **[-S]**
EC (dS m^{-1})	149 (83 - 265)	116 (54 - 250)	118 (73 - 235)
Total C (%)	5.5 (3.4 - 8.7)	4.9 (2.6 - 9.0)	5.4 (3.5 - 10)
Total N (%)	0.27 (0.17 - 0.33)	0.25 (0.13 - 0.49)	0.28 (0.18 - 0.58)
NO_3^--N	7.5 (2.5 - 23)	7.0 (1.9 - 26)	4.9 (1.8 - 16) **[+S*]**
NH_4^+-N	19 (11 - 33)	16 (9. 1 - 30)	16 (9.7 - 30)
Total P	579 (500 - 671)	577 (491 - 678)	535 (456 - 634)
Olsen P	131 (105 - 163) **[+S]**	121 (66 - 222) **[+S*]**	122 (91 - 214)
Total Na	734 (616 - 876) **[+S]**	803 (689 - 936)	708 (589 - 878) **[+S*]**
Total K	2584 (1999 - 3088)	2506 (2080 - 3019)	2278 (2008 - 2736)
Total Ca	7208 (6643-7822)	7476 (6988-7997)	7233 (6746-7834)
Total Mg	2570 (2325-2842)	2580 (2299-2896)	2409 (2265-2631)
Extractable Mg	129 (62-269) **[-S*]**	158 (87-287)	99 (37-235) **[-S]**
Total As	2.2 (1.9 - 2.6)	2.3 (2.0 - 2.5)	2.0 (1.9 - 2.3) **[+S*]**
Total Cd (µg kg^{-1})	8.3 (5.6 - 12.3) **[-S*]**	6.5 (4.8 - 8.8) **[-S]**	5.5 (3.9 - 8.2) **[-S]**
Total Cu	5.0 (4.1 - 5.9)	4.7 (3.7 - 5.8) **[-S*]**	4.6 (3.7 - 5.8)
Total Pb	4.2 (3.4 - 5.3) **[-S]**	4.0 (3.6 - 4.5)	3.9 (3.5 - 4.8)

Values are geometric means and standard deviation ranges (n=17). Values are in mg kg^{-1} unless otherwise indicated. Variables that were significantly correlated with the relative irrigation are indicated in bold in square brackets; S: $p \leq 0.05$, S*: $p \leq 0.01$, S**: $p \leq 0.001$. Positive and negative correlations are indicated by + and -, respectively.

Table 4-2 Soil chemistry at 30-45 cm depth under *K. robusta*, *L. scoparium*, and pasture.

30-45 cm	*Kunzea robusta*	*Leptospermum scoparium*	Pasture
pH	5.3 (4.9 - 5.7)	5.5 (5.0 - 6.1)	5.3 (4.8 - 5.8) **[-S]**
EC (dS m^{-1})	30 (20 - 47)	27 (19 - 39)	22 (17 - 32) **[+S]**
Total C (%)	0.7 (0.5 - 1.0)	0.6 (0.5- 0.8)	0.7 (0.5 - 0.9)
Total N (%)	<0.05	<0.05	<0.05
NO_3^--N	2.0 (0.6 - 6.4)	1.9 (0.6 - 5.9)	1.1 (0.3 - 3.9)
NH_4^+-N	4.6 (2.5 - 8.5)	4.2 (2.3 - 7.5)	4.0 (2.0 - 7.2)
Total P	388 (307 - 490)	372 (299 - 464)	417 (325 - 524)
Olsen P	39 (22 - 66)	28 (12 - 62)	50 (27 - 110)
Total Na	734 (658 - 818)	712 (586 - 866)	680 (570 - 817)
Total K	2121 (1683 - 2676)	2416 (2163 - 2700)	2118 (1640 - 2423)
Total Ca	7418 (6771-8126)	7726 (7296-8182)	7562 (6487-8277)
Total Mg	2880 (2584-3209)	3058 (2821-3315)	2882 (2407-3198)
Extractable Mg	30 (18-52)	32 (19-54) **[-S]**	26 (14-44) **[-S]**
Total As	2.5 (2.2 - 2.9) **[+S]**	2.6 (2.4 - 2.9)	2.6 (2.2 - 3.0)
Total Cd (µg kg^{-1})	5.2 (3.5 - 7.9)	6.5 (5.3 - 4.1)	5.1 (3.5 - 7.7)
Total Cu	3.7 (3.4 - 3.9)	3.7 (3.3 - 4.1)	3.7 (3.2 - 4.0)
Total Pb	3.7 (3.4 - 4.2)	3.8 (3.6 - 4.1)	3.8 (3.4 - 4.2)

Values are geometric means and standard deviation ranges (n=17). Values are in mg kg^{-1} unless otherwise indicated. Variables that were significantly correlated with the relative irrigation (Table 4-1) are indicated in bold in square brackets; S: $p \leq 0.05$, S*: $p \leq 0.01$, S**: $p \leq 0.001$. Positive and negative correlations are indicated by + and -, respectively.

Total C and N concentrations were ca. 10-fold lower than those found in pastoral soils (Reiser et al., 2014). This is typical for a sandy soil as its ability to store organic matter is low due to the low specific surface area of sand (McLaren & Cameron, 1996). There was no correlation between soil total C and N concentrations and relative TMW irrigation rate. However, there were strong positive correlations between total C and N in the topsoil under all species (r=0.97, $p \leq 0.01$), which is consistent with most of the soil N being present as organic N. About 45% of the N applied with TMW was applied as organic N (Table 3-2, Chapter 3). Organic N can readily leach from Recent soils (Barton et al., 2005) and would therefore not have accumulated in the soil.

Soil NO_3^- and NH_4^+ concentrations were similar to those found in Duvauchelle after 2.8 years of TMW irrigation (Chapter 2). Concentrations of NO_3^- were also similar to those reported by Barton et al.

(1999), with 8.5 mg N kg^{-1} after 12 months of TMW irrigation, compared to 2.4 mg kg^{-1} in an unirrigated control. Given the high mobility of NO_3^- in soil (Di & Cameron, 2002), the NO_3^- concentration likely rose with the onset of TMW irrigation but did not continue to increase over time due to leaching. Soil pH was negatively correlated with NO_3^- in the topsoil under pasture (r=-0.58, $p \leq 0.05$). Nitrification is a major source of soil acidity (Robertson & Groffman, 2015). Inputs of N through TMW irrigation likely provided sufficient substrate for high rates of nitrification (Cameron et al., 2013). The optimum pH range for nitrification is between 4.5 and 7.4 (Cameron et al., 2013) , which is consistent with the soil pH measured at The Pot.

Similar to N, total P concentrations in the soil were just 50% of what is typically found in NZ pastoral soils (McDowell & Condron, 2004; Reiser et al., 2014). However, plant-available P in the topsoil, as indicated by Olsen P (~125 mg kg^{-1}), was manifold higher than suggested target ranges of 5-50 mg kg^{-1} in NZ soils (Mackay et al., 2013) and comparable with values reported in market gardens (Drewry et al., 2021). Olsen P in the topsoil was positively correlated with relative irrigation under *K. robusta* (r=0.60, $p \leq 0.05$) and *L. scoparium* (r=0.64, $p \leq 0.01$). While P is mostly entering streams through surface runoff (Pionke et al., 2000), leaching losses of P can be high with TMW irrigation. Sparling et al. (2006) reported that P leaching losses from a Recent soil (well drained sand) during four years of TMW irrigation were 8 % of the applied P, while leaching was negligible in Pumice and Allophanic soils. Olsen P correlates with dissolved reactive phosphorus (DRP), and values above 50 mg kg^{-1} can result in high P losses even via leaching from flat land, which can lead to environmental degradation (Drewry et al., 2021; Taylor et al., 2016). Given the high Olsen P content at The Pot and the relatively high hydraulic conductivity of sandy soils (LEI, 2017; McLaren & Cameron, 1996), subsurface flow can be expected to contribute to P fluxes into groundwater and the Waiwiri Stream (Mittelstet et al., 2011).

Soil concentrations of Na were only 2-3 times higher than those measured in TMW irrigated soil in Duvauchelle (Chapter 2), despite the high application rate of Na (2847 kg ha^{-1} yr^{-1}) and TMW irrigation for over 30 years. This is consistent with findings by Gutierrez-Gines et al. (2020), whereby Na accumulation is not proportional to Na application and reflects the high mobility of Na in soil (Blume et al., 2016). Soil concentrations of trace elements were similar to or below levels found in NZ pastoral soils (Reiser et al., 2014). Concentrations of As, Cd, Cr, Cu, Pb, Hg, Ni in the TMW were below detection limit (<0.01 mg L^{-1}), within the recommended limits for continuous TMW irrigation (FAO, 2003) and not expected to accumulate in the soil. However, there was a positive correlation between TMW irrigation rate and As in the topsoil under pasture, and in the subsoil under *K. robusta*. In the topsoil, Cd was negatively correlated with TMW irrigation under all species, and the same was true for Cu under *L. scoparium* and Pb under *K. robusta*. Soil pH is the major factor controlling the solubility of

trace elements in soil (Bradl, 2004). The low pH at The Pot likely resulted in high solubility of trace elements, and therefore a reduction in the topsoil with high TMW irrigation.

4.3.2 Plant chemistry

Table 4-3 shows the chemical composition of the plants. The application rate of the major plant nutrients (N, P, K) exceeded plant requirements (FAO, 2003). Concentrations of these elements in pasture were higher than average values reported by Reiser et al. (2014). In *L. scoparium* and *K. robusta* the concentrations of N, P, and K were higher than in other unamended soils (Dickinson et al., 2015) and in soils with biosolids application (Esperschuetz, Anderson, et al., 2017; Gutierrez-Gines et al., 2019; Reis et al., 2017).

Table 4-3 Plant height, biomass (dry weight), and elemental composition of *K. robusta*, *L. scoparium*, and pasture.

Parameter	*Kunzea robusta*	*Leptospermum scoparium*	Pasture
Irrigation (mm day^{-1})	11 (2.5-50)	10 (2.4-43)	12 (2.6-81)
Plant height (cm)	164 (140-194)	157 (137-180)	n.d.
Plant biomass (g m^{-2})	n.d.	n.d.	725 (525-835)
C (%)	51 (51-52)	52 (52-53)	43 (42-44)
N (%)	2.0 (1.7-2.4)	2.2 (1.9-2.5)	2.8 (2.2-3.2) **[+S**]**
K (%)	0.6 (0.5-0.7)	0.6 (0.5-0.7) **[-S]**	3.7 (3.0-4.4)
P (mg kg^{-1})	2695 (2272-3196) **[+S]**	2398 (2009-2864)	4386 (3926-5314)
Na (mg kg^{-1})	4991 (4097-6082)	4575 (3745-5588) **[+S]**	5480 (3229-7013) **[+S**]**
Zn (mg kg^{-1})	51 (39-66)	30 (23-39) **[-S]**	35 (28-54) **[+S*]**
Mn (mg kg^{-1})	1341 (907-1983)	651 (411-1032) **[-S]**	177 (108-338) **[-S]**
Cu (mg kg^{-1})	3.1 (1.9-5.0)	3.3 (2.4-4.5) **[-S*]**	4.2 (3.1-6.1)
Cr (mg kg^{-1})	0.43 (0.22-0.84) **[+S]**	0.33 (0.18-0.59) **[+S]**	0.57 (0.42-1.3)
As (µg kg^{-1})	57 (43-77) **[+S]**	67 (49-91)	22 (14-30)
Cd (µg kg^{-1})	17 (10-29)	12 (7.4-19)	2.4 (0.9-4.1)
Pb (µg kg^{-1})	82 (55-121) **[+S*]**	83 (54-127)	14 (5.3-21) **[+S]**

Values are geometric means and standard deviation ranges (n=17). Variables that were significantly correlated with relative TMW irrigation (Table 4-1) are indicated in bold and square brackets; S: $p \leq 0.05$, S*: $p \leq 0.01$, S**: $p \leq 0.001$.

The chemical composition of *K. robusta* and *L. scoparium* differed from pasture. The pasture contained higher concentrations of macronutrients and lower concentrations of micronutrients than the

myrtaceous species. There was no correlation between relative irrigation and *L. scoparium* and *K. robusta* height. This is consistent with findings from the preliminary growth trial (Chapter 3) and Duvauchelle (Chapter 2). Similarly, there was no correlation between relative TMW irrigation rates and pasture biomass. These results are in contrast with a study by Gutierrez-Gines et al. (2020) that showed a positive growth response of pasture to TMW irrigation. However, pasture in these studies was regularly harvested, while pasture at The Pot remained unharvested. It is likely is that the soil already contained appropriate levels of nutrients for pasture growth, since the site had been irrigated for >30 years prior to this experiment.

There was a significant ($p \leq 0.01$) negative correlation between the height of *K. robusta* and *L. scoparium* and their foliar Na concentrations (Figure 4-5). Na concentrations in *L. scoparium* were 2-3 times higher than those reported in unplanted and established stands of *L. scoparium* (Chapter 6) and 2-8 times higher than results from pot experiments (Esperschuetz, Anderson, et al., 2017; Reis et al., 2017). Similarly, *K. robusta* Na concentrations were 5 times higher than those reported by Esperschuetz, Anderson, et al. (2017). For pasture, there was no correlation between biomass and Na concentrations, which is in contrast with findings of Gutierrez-Gines et al. (2020). Pasture Na concentrations were 30% higher than those in TMW irrigated pasture reported by Gutierrez-Gines et al. (2020). While Na concentrations in the TMW were high (61 mg L^{-1}), it is possible that sea spray also had an effect on plant Na concentrations, as sea spray can increase the concentration of Na in plants within 50 km from the sea, with pasture Na concentrations correlating with distance from the sea (Jensen et al., 2019). The distance between the experimental area at The Pot and the sea is about 1 km and strong westerly winds are common in this area, increasing sea spray (KCDC, 1999).

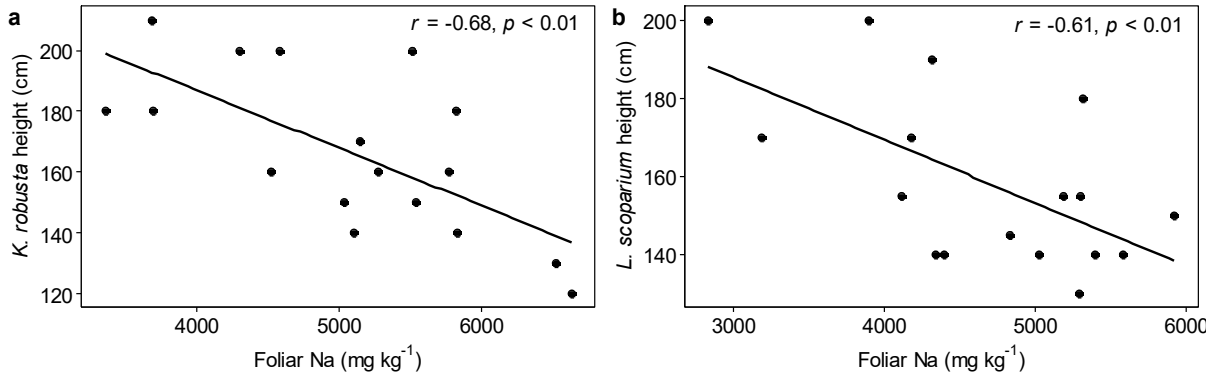

Figure 4-5 Foliar Na versus plant height of (**a**) *Kunzea robusta* and (**b**) *Leptospermum scoparium*.

Sodium is not an essential element for most terrestrial plants (Maathuis, 2014). It can be directly toxic to plants, resulting in leaf burn, with woody species being most susceptible (Bernstein, 1975). However, no signs of brown lesions indicating such toxicity were visually observed in any of the sampled *K. robusta* and *L. scoparium* plants. Trace elements, that may have had a negative effect on plant growth, were similar or lower than found elsewhere in *L. scoparium* (Chapter 6), *K. robusta* (Hahner et al., 2014), and pasture (Reiser et al., 2014).

4.3.3 Lysimeters

The recorded irrigation of TMW during the three days of monitoring was 14 ± 2.3 mm d^{-1}. There was no significant difference in irrigation between lysimeters with *K. robusta* and pasture. The average drainage in the *K. robusta* and pasture lysimeters was 1.0 mm d^{-1} and 1.4 mm d^{-1}, respectively (Table A-7). As irrigation was not measured underneath the canopy, the lower leaching under *K. robusta* was likely affected by the "umbrella effect" (Mertens et al., 2005) where the irrigated TMW directly re-evaporated from the *K. robusta* canopy.

There was no significant difference in NO_3^- leaching between *K. robusta* and pasture (Table A-7). The results are not consistent with those of Esperschuetz, Balaine, et al. (2017), who reported that *K. robusta* may inhibit nitrification and reduce NO_3^- leaching more than other species. However, potential inhibiting effects of *K. robusta* on nitrification may have been offset by the high TMW irrigation rate, which limits any potential plant effect on N cycling. NO_3^- leaching was equivalent to 28% and 38% of the applied N under *K. robusta* and pasture, respectively. In a study by Barton et al. (2005), 22% of applied N was lost through leaching, although 87% thereof was leaching as organic N. With approximately 45% of N at The Pot applied as organic N (Table 3-2, Chapter 3), it can be expected that organic N leaching significantly contributes to total N leaching.

4.4 Conclusions

Application of TMW at a rate of >4000 mm yr^{-1} had no negative effect on the growth and health of *L. scoparium*, *K. robusta*, and pasture, as measured by plant height, biomass, and trace element concentrations after three years of irrigation. However, there was evidence that growth benefits of the water and nutrients in TMW were offset by Na toxicity in *L. scoparium* and *K. robusta*, as indicated by negative correlations between plant height and foliar Na concentrations. The application rates of water and nutrients were higher than what plants, both pasture and myrtaceous species, could uptake or manage, resulting in NO_3^- leaching of about one third of the applied N. There was no significant difference between NO_3^- leaching under *K. robusta* and pasture. It is therefore likely that NO_3^- leaching into groundwater and the adjacent Waiwiri Stream occurs independent of vegetation type at high

TMW irrigation rates. Future research should consider effects of different irrigation schedules, as more frequent irrigation of smaller volumes may increase the capacity of the vegetation to manage nutrients and reduce losses thereof. Further monitoring of the lysimeters will allow to quantify annual leaching of NO_3^- and other nutrients.

Chapter 5

Nitrifying and Denitrifying Microorganisms in the Rhizosphere of New Zealand Native Plants

5.1 Introduction

The land application of N-rich wastes, such as treated municipal wastewater, dairy shed effluent, biosolids, and meat industry effluent, can result in environmental degradation from losses of N into waterways and the atmosphere (Julian et al., 2017; Wilcock et al., 2009). Typically, N is lost from soil through nitrate (NO_3^-) leaching and gaseous emissions of nitrous oxide (N_2O), nitric oxide (NO), and dinitrogen (N_2), as well as through volatilised ammonia (NH_3) at high soil pH (Cameron et al., 2013). The global warming potential of N_2O is 298 times higher than that of CO_2, making it a potent greenhouse gas (Philibert et al., 2013). NO_3^- is highly mobile in soils due to its negative charge and readily leaches when precipitation exceeds evapotranspiration (Robertson & Groffman, 2015). Inputs of NO_3^- to waterways can lead to their eutrophication, accelerating the growth of photo- and heterotrophic organisms and thereby reducing the oxygen content of the water (Subbarao et al., 2006). Furthermore, NO_3^- is toxic to fish, amphibians, and aquatic invertebrates (Camargo et al., 2005). Groundwater with elevated NO_3^- concentrations can pose a public health risk where it is utilised for drinking water. It was estimated that the drinking water of 14% of New Zealanders exceeds 1 mg NO_3^--N L^{-1}, which has been linked to an increased risk of colorectal cancer (Richards et al., 2021). Harmful losses of N can be minimised when it is taken up by plants or converted into N_2 through complete denitrification.

Nitrification in soil is the two-step microbial oxidation of NH_3/NH_4^+ to NO_3^-, mediated by autotrophic ammonia-oxidising bacteria and archaea (AOB and AOA, respectively) and nitrite oxidising bacteria (Li et al., 2018). In the first step, ammonium (NH_4^+) is oxidised to nitrite (NO_2^-) by ammonia monooxygenase, which is encoded by *amoA,* and hydroxylamine oxidoreductase, which is encoded by *hao* (Francis et al., 2005; Sayavedra-Soto et al., 1994). These enzymes are associated with AOA and AOB, such as *Nitrosospira* spp. and *Nitrosomonas* spp. (Robertson & Groffman, 2015). The relative abundance of AOB and AOA depends on the conditions of the soil and the availability of N (Rütting et al., 2021). AOB usually outcompete AOA in soils with high concentrations of NH_4^+, while AOA are more abundant in soils with low concentrations of NH_4^+ (Di et al., 2010). The second step of nitrification is the oxidation of NO_2^- to NO_3^- by nitrite oxidoreductase associated with nitrite-oxidising bacteria (Robertson & Groffman, 2015). There is evidence of complete nitrification, including both steps, by

Nitrospira spp. (Daims et al., 2015), as well as heterotrophic nitrification at low soil pH (Zhang et al., 2014).

The reverse process, denitrification, is the stepwise reduction of NO_3^- to NO_2^-, NO, N_2O, and N_2 (Cameron et al., 2013). Denitrification mainly occurs under anaerobic conditions, when heterotrophic denitrifying bacteria use NO_3^- as terminal electron acceptor during respiration (Robertson & Groffman, 2015). The reduction steps are enzymatically controlled by NO_3^- reductase, NO_2^- reductase, NO reductase and N_2O reductase, which are associated with most denitrifying bacteria (Cameron et al., 2013). Two types of NO_2^- reductases are cytochrome *cd1* (encoded by *nirS*) and Cu-containing enzymes (encoded by *nirK*). N_2O reductase is encoded by the *nosZ* gene (Kandeler et al., 2006). However, archaea and fungi can also play a role in denitrification, with distinct underlaying mechanisms (Hayatsu et al., 2008).

Plant-microbe interactions are complex, and plants influence the abundance and community composition of microorganisms in the rhizosphere (Bais et al., 2006; Wardle et al., 2004). Nitrification and denitrification are indirectly controlled by plant species specific effects on NO_3^- and NH_4^+ availability, organic C availability, pH, soil moisture, and oxygen availability (Laffite et al., 2020). Plant compounds that directly inhibit nitrification are termed biological nitrification inhibitors (BNIs) (Subbarao et al., 2012). They are produced in the plant tissue and enter the soil through litter decomposition or root exudation (Subbarao et al., 2006). However, only five root released compounds have been identified (Coskun et al., 2017b). The associated nitrification inhibition derives from a direct inhibiting effect of BNIs on ammonia monooxygenase or hydroxylamine oxidoreductase (Coskun et al., 2017a). Synthetic nitrification inhibitors are applied to agricultural land to reduce N losses from agricultural land (Di & Cameron, 2012). Dicyandiamide is an effective synthetic nitrification inhibitor, mitigating NO_3^- and N_2O losses, but it is no longer used in New Zealand (NZ) after its residues were detected in milk (Cameron et al., 2014; Leahy et al., 2019). Therefore, the use of plants with BNI potential could reduce losses from agricultural land and high nutrient environments in general.

Some NZ native Myrtaceae have the potential to reduce NO_3^- leaching and N_2O emissions more than other species (Esperschuetz, Balaine, et al., 2017; Franklin et al., 2017). To date no underlying mechanisms for these observations have been described. However, Esperschuetz, Balaine, et al. (2017) found higher concentrations of NH_4^+ in a nitrification assay with *Leptospermum scoparium* and *Kunzea robusta* compared to *Pinus radiata*, indicating an inhibition of nitrification under these species. Similarly, Halford et al. (2021) reported that NO_3^- concentrations in the field were significantly lower under *L. scoparium* than pasture, which was likely a consequence of plant effects on soil N cycling. These and other plant species may be utilised for the land application of N-rich wastes to minimise

N_2O emissions and NO_3^- leaching, as N losses can limit the sustainability of such systems (Bond, 1998). Indirect effects of plant species on N cycling in the rhizosphere are possible through root exudation of organic compounds that increase microbial activity and N immobilisation (Leptin et al., 2021). In addition, alteration of the N cycle may be due to the antimicrobial properties of some NZ-native species such as *K. robusta*, *L. scoparium*, *Metrosideros robusta*, and *Pseudowintera colorata*. These plant species were found to suppress pathogenic bacteria in soil (Gutierrez-Gines et al., 2021; Prosser et al., 2016), and may potentially also affect microorganisms involved in nitrification. A study from Pakistan showed that local medicinal plants with antimicrobial properties can inhibit nitrification in soil more than dicyandiamide (Tahir et al., 2021). The leaves of *L. scoparium* and *K. robusta* contain essential oils with antimicrobial properties, associated with triketones and terpenes in *L. scoparium* and α-pinene in *K. robusta* (Douglas et al., 2004; Porter & Wilkins, 1999). Previous studies reported that α-pinene directly competes for ammonia monooxygenase's active site (Ward et al., 1997) and suppresses nitrification in forest soil (Paavolainen et al., 1998). Inhibiting effects of *K. robusta* and *L. scoparium* on other enzymes such as trypsin, β-galactosidase, α-glucosidase, and α-amylase were previously demonstrated by Kellam et al. (1992).

It was hypothesised that N fluxes in the soil will be affected by the plant species, through direct and indirect effects of plants on the abundance of N-cycling microorganisms. This study aimed to determine whether there were differences in the abundance of microorganisms involved in nitrification and denitrification in the rhizosphere of NZ native plants, including the myrtaceous species *K. robusta* and *L. scoparium*. It sought to quantify the abundance of total bacteria and archaea, as well as the functional genes encoding nitrifying and denitrifying enzymes in the rhizosphere of five NZ native species compared to the exotic pasture species *Lolium perenne* with and without the application of fertiliser. Additionally, the speciation and concentration of N in the rhizosphere and plants was determined.

5.2 Materials and Methods

5.2.1 Pot experiment

Experimental setup

A pot experiment was set up in a greenhouse at the University of Canterbury in Christchurch, NZ (S 43° 31' 24", E 172° 35' 15"). A cross factorial design with six plant treatments (*L. scoparium*, *K. robusta*, *Coprosma robusta*, *Metrosideros umbellata*, *Carex secta*, and *L. perenne*) and two fertilisation treatments (non-fertilised control and NPKS fertilised treatment equivalent to 200 kg N ha^{-1}) was chosen. *L. scoparium*, *K. robusta*, and *M. umbellata* were selected as they have

previously shown antimicrobial activity in soil (Gutierrez-Gines et al., 2021; Prosser et al., 2016). *C. robusta* was included because of the low NO_3^- concentration in the soil under this species compared to others (Chapter 2). *C. secta* was selected because it is widely used in riparian areas to mitigate N losses from agricultural land throughout NZ (McKergow et al., 2016). The exotic species *L. perenne* was used as control because (i) it was demonstrated to show low BNI capacity (Subbarao et al., 2007), and (ii) it is a typical pasture species in NZ (Kirkman et al., 1994). With six replicates per treatment combination the experiment included 72 pots in total. The soil used in this experiment was a Typic Allophanic Brown Soil (Hewitt, 2010), commonly known as Craigieburn silt loam (Gutierrez-Gines et al., 2019). The soil was collected between Lake Lyndon and Lake Coleridge in the South Island of NZ (S 43° 20' 35", E 171° 36' 59"). The same soil was used by Gutierrez-Gines et al. (2019), who analysed its physico-chemical properties (Table 5-1). The site was not previously cultivated or fertilised and vegetation was dominated by *Dracophyllum longifolium*, *L. scoparium*, and *K. robusta* (Gutierrez-Gines et al., 2019).

Table 5-1 Composition of the soil used for the pot experiment, adapted from Gutierrez-Gines et al. (2019).

Parameter	Horizon Ah
pH	5.6 ± 0.00
EC (µS cm^{-1})	36 ± 1.0
C (%)	1.5 ± 0.18
N (%)	0.24 ± 0.00
NH_4^+-N (mg kg^{-1})	<0.01
NO_3^--N (mg kg^{-1})	0.04 ± 0.02
Olsen P (mg kg^{-1})	15 ± 0.37

Value are means ± standard error (*n*=5).

The vegetation was removed from the collection site and a spade was used to collect soil from the Ah horizon (0-15 cm depth). All stones, vegetation and roots >2 mm diameter were removed from the soil and the soil was thoroughly mixed to achieve maximal homogeneity. 1.7 kg of fresh soil (equivalent to 1.06 kg dry weight) was weighed into each pot before native seedlings were transplanted and *L. perenne* was sown. The native seedlings were two years old at the beginning of the experiment and 12-25 cm tall. Seedlings were native to the Canterbury region. The sowing density of *L. perenne* was equivalent to the recommended sowing rate of 25 kg ha^{-1} (Specialty Seeds, 2019). However, 60 days after seeding the sowing density of *L. perenne* was increased to an equivalent of 75 kg ha^{-1} to ensure sufficient growth. The diploid variety Mega Rich (Specialty Seeds, 2019) was used. Within the

greenhouse, pots were arranged in a completely randomised design and were newly randomised every fortnight. Pots were watered to field capacity every 2-3 days with tap water.

Fertilisation

26 weeks after experiment setup, half of the pots received NPKS fertilisation. Nitrogen was applied in the form of urea, equivalent to 200 kg N ha^{-1}. Phosphorus, K, and S were added because these plant nutrients are present in biowastes (Gutierrez-Gines et al., 2019) and can affect microbial activity (Lejoly et al., 2020). Table 5-2 shows the mass of nutrients applied per pot and on a per hectare basis. A total of 40 mL of solution was applied to each pot in 10 mL increments.

Table 5-2 Fertilisation in the pot experiment (NPKS treatment).

Nutrient	Compound	Fertilisation (mg pot^{-1})	Fertilisation equivalent (kg ha^{-1})
N	CH_4N_2O	353	200
P	K_2HPO_4	52	30
K	K_2HPO_4, K_2SO_4	395	224
S	K_2SO_4	108	61

Harvest and sample preparation

Pots were harvested 8 weeks after fertilisation. At that point the roots of all but one species had occupied the pots sufficiently to classify the entire soil as rhizosphere soil. *C. robusta* was growing more slowly than the other species and its root system did not fully occupy the pot at the time of harvest. Plants were cut 5 mm above the soil surface. Roots were removed from the soil and the soil was mixed to achieve homogeneity. Soils were split into three parts: (1) frozen at -20 ˚C and used for moisture determination and mineral N analysis within one week, (2) dried at 40 ˚C for 4 days and sieved to <2 mm for further chemical analysis, and (3) frozen at -80 ˚C for nucleic acid extraction.

5.2.2 Chemical analysis of soils and plants

A subsample of 10-20 g fresh soil was dried at 105 ˚C for 24 hours to determine the moisture content. For analysis of NO_3^- and NH_4^+, 4 g of fresh soil was weighed into a 50 mL falcon tube and 20 mL of 2 M KCl was added for extraction (Clough et al., 2001). The tubes were shaken in an end-over-end shaker for 1 hour at 20 rpm prior to filtration through Whatman No. 42 filter paper. Colorimetric methods were used to determine concentrations of NO_3^--N (Miranda et al., 2001) and NH_4^+-N (Mulvaney, 1996) in the extracts with a Cary 100 Bio UV-visible spectrophotometer (Agilent Technologies, Santa Clara, CA, USA). To measure soil pH, 10 g of dried soil (<2 mm) was weighed into

a falcon tube and 25 mL of ultrapure water was added (Blakemore et al., 1987). The tubes were shaken vigorously, and extracts were left to equilibrate overnight. The pH was measured using a HQ 440d Multi-Parameter Meter with pH probe PHC735 (HACH, Loveland, CO, USA). Dry combustion was used to determine the total C and N concentrations in the soils and plants. 0.1 g of dried material was weighed into tin foil cups and analysed with a LECO CN828 Carbon/Nitrogen analyser (LECO, St. Joseph, MI, USA).

5.2.3 DNA extraction

The extraction of DNA from soil was modified from Lever et al. (2015) and Lim et al. (2016). In brief, 0.6-0.8 g of fresh soil was added to a lysing matrix E tube. 100 µL 100 mM dNTPs (1:1:1:1 mixture of dATP, dGTP, dCTP, and dTTP), 500 µL 5% cetyltrimethyl ammonium bromide (CTAB) extraction buffer (consistent of 10 g CTAB and 4.09 g NaCl mixed 1:1 with 240 mM $NaPO_4$ buffer of pH 8), and 50 µL of 4% sodium dodecyl sulphate (SDS) were added. Samples were frozen at -80 °C for 15 min prior to heating at 50 °C and 1000 rpm for 15 min on a thermomixer (ThermoMixer® C, Eppendorf, Hamburg, Germany). 400 µL phenol-chloroform-isoamyl alcohol (25:24:1) was added. Samples were lysed in two rounds of 30 s at 5.5 m s^{-1} using a FastPrep-24 tissue homogenizer (MP Biomedicals, Irvine, CA, USA). Samples were centrifuged (14,000 × *g*) at 4 °C for 7 min. The aqueous phase was transferred into a new 2 mL tube and 1 volume of chloroform-isoamyl alcohol (24:1) was added, followed by centrifugation (14,000 × *g*) at 4 °C for 3 min. The aqueous phase was transferred into a new 2 mL tube and 1 volume isopropanol, 0.1 volume 3M sodium acetate, and 1 µL 20 mg/mL glycogen were added. Samples were centrifuged (14,000 × *g*) at 12 °C for 20 min to precipitate the nucleic acids. The supernatant was removed. 500 µL ice cold 70% (vol/vol) ethanol was used to wash the pelleted nucleic acids. This step was repeated twice. The washed pelleted nucleic acid was air-dried (speed-vac at 40 °C for 2-5 min) and resuspended in 100 µL DNase/RNase-free water. The QIAGEN DNeasy® PowerClean® Pro Cleanup Kit was subsequently used for secondary purification of extracted DNA following the manufacturer's instructions. Extracted DNA was quantified with a NanoDrop spectrophotometer (ThermoFisher Scientific, Waltham, MA, USA).

5.2.4 Quantitative real-time PCR

The abundance of bacterial and archaeal 16S ribosomal RNA (16S rRNA) and the functional genes *amoA* (encoding the subunit A of ammonia monooxygenase, both bacterial and archaeal), *nirS* (encoding cytochrome cd_1 nitrite reductase), *nirK* (encoding Cu-containing nitrite reductase) and *nosZ* (encoding nitrous oxide reductase) was determined in triplicates by quantitative real-time PCR (qPCR) in a 96-well plate using a CFX Connect Real-Time PCR System (Bio-Rad, Hercules, CA, USA). The quantification of bacterial 16S rRNA and functional genes was adapted from Lourenço et al. (2018).

The quantification of archaeal 16S rRNA was adapted from Siles and Margesin (2016). The primers used are shown in Table 5-3. The total reaction volume was 15 µL for each assay, containing 7.5 µL of SYBR® Green Supermix (Bio-Rad, Hercules, CA, USA) and 5 µL of DNA. Reaction details and thermal cycler conditions are listed in Tables A-8 and A-9. Dilution series of extracted DNA were performed to test inhibition by humic acids. Final DNA concentrations used for reactions were 0.5 ng μL^{-1} for bacterial and archaeal 16S, 1-2 ng μL^{-1} for *nirK*, *nirS*, and *nosZ*, and 10-20 ng μL^{-1} for bacterial and archaeal *amoA*. Melting curve analysis was performed after each assay to ensure that only targeted genes were amplified. Target genes were synthesised into plasmid containing vectors by Integrated DNA Technologies (IDT™, Coralville, IA, USA) and used as standards. Standard curves were performed using 10-fold dilution series from 10^{-2} to 10^{-8} gene copies μL^{-1} and using regression to relate the cycle threshold value to the known copy numbers of the standards (Siles & Margesin, 2016). The reaction efficiency ranged from 85% to 100% and R^2 was above 0.98. Quantification results were used to calculate gene copy numbers per gram of soil (on a dry weight basis).

Table 5-3 Primers and standards for the quantification of bacterial and archaeal 16S rRNA and functional genes encoding nitrifying enzymes (AOB *amoA* and AOA *amoA*) and denitrifying enzymes (*nirS*, *nirK*, and *nosZ*). Adapted from Lourenço et al. (2018).

Primers[a]	Fragment size (bp)	Sequence[b] (5'→ 3')	Gene target	Model strain (NCBI accession number)[c]	Reference
Eub338 Eub518	200	ACTCCTACGGGAGGCAGCAG ATTACCGCGGCTGCTGG	Bacterial 16S rRNA	*Pseudomonas sp.* (DQ778036)	Fierer et al. (2005)
Arch-967F Arch-1060R	137	AATTGGCGGGGGAGCAC GGCCATGCACCWCCTCTC	Archaeal 16S rRNA	*Thaumarchaeota archaeon* (NZ_QLTN01000004.1)	Cadillo-Quiroz et al. (2006)
amoA1F amoA2R	491	GGGGTTTCTACTGGTGGT CCCCTCKGSAAAGCCTTCTTC	Subunit A of bacterial ammonia monooxygenase (AOB *amoA*)	*Nitrosospira multiformis* (CP000103.1)	Rotthauwe et al. (1997)
Arch-amoAF Arch-amoAR	635	STAATGGTCTGGCTTAGACG GCGGCCATCCATCTGTATGT	Subunit A of archaeal ammonia monooxygenase (AOA *amoA*)	*Nitrososphaera viennensis* (FR773159.1)	Francis et al. (2005)
nirScd3aF nirSR3cd	410	GTSAACGTSAAGGARACSGG GASTTCGGRTGSGTCTTGA	Cytochrome cd_1-containing nitrite reductase (*nirS*)	*Nitrososphaera viennensis* (AXRC01000008)	Throbäck et al. (2004)
nirK876 nirK1040	164	ATYGGCGGVCAYGGCGA GCCTCGATCAGRTTRTGGTT	Copper-containing nitrite reductase (*nirK*)	*Pseudomonas aeruginosa* (AE006469.1)	Henry et al. (2004)
nosZ2F nosZ2R	267	CGCRACGGCAASAAGGTSMSSGT CAKRTGCAKSGCRTGGCAGAA	Nitrous oxide reductase (*nosZ*)	*Sinorhizobium meliloti* (AE006469.1)	Henry et al. (2006)

[a] Forward primers are indicated by F and reverse primers are indicated by R.
[b] M=A/C, R=A/G, W=A/T, K=G/T, Y=C/T, S=G/C according to IUBMB, International Union of Biochemistry and Molecular Biology, Cornish-Bowden (1985).
[c] NCBI is the National Center for Biotechnology Information (https://www.ncbi.nlm.nih.gov/).

5.2.5 Statistical analysis

Analysis of variance (ANOVA) with Tukey's honestly significant difference (HSD) post-hoc test was used to determine differences between species and fertilisation treatments. The package *multcomp* (Hothorn et al., 2021) was used for Tukey's HSD. The assumptions of homogeneity of variance and normality were tested by plotting the residuals against the fitted values and quantiles of the normal distribution, respectively. Soil and plant parameters were *log* transformed where the assumptions were not met. For chemical soil variables, the lowest standard was set as half the detection limit for statistical analysis (McDowell et al., 2017). All AOA *amoA* results and the results of AOB *amoA* in the control soil were excluded from analysis because >50% of values were below the cycle threshold value of the lowest standard. All data were analysed using R (R Core Team, 2021).

5.3 Results and Discussion

5.3.1 Plant biomass and chemistry

Plant biomass was significantly increased by fertilisation in *C. robusta*, *C. secta,* and *L. perenne*, but not in the myrtaceous species (Table 5-4). This is consistent with results of Franklin et al. (2015), whereby *Carex virgata* responded to NPS fertilisation with a biomass increase, while *K. robusta* and *L. scoparium* did not. The large biomass increases upon NPKS fertilisation is consistent with one or more of these elements limiting plant growth (Güsewell & Koerselman, 2002). Fertilisation significantly increased the N concentration in the foliage of all species (Table 5-4). Concentrations of N in the fertilised plants did not differ between *L. perenne*, *L. scoparium*, *K. robusta*, and *C. robusta*. This contrasts with the results reported by Franklin et al. (2015), who measured higher N concentrations in *L. perenne* than NZ native species. The lack of difference in this study was likely due to the low N availability in the Craigieburn silt loam, as fertilisation may not have been sufficient to meet plant requirements. Kirkby (2012) reported that the average concentration of N in plant shoot dry matter was 1.5%, which was significantly higher than the N concentrations of *C. secta* in the unfertilised treatment (0.61%). Similarly, *M. umbellata* N concentrations were just 0.66% without fertilisation. The N concentrations in both species doubled with NPKS application. Many NZ native species do not require high levels of N, as they are adapted to low fertility soils (Wardle, 1985). However, the increase in N concentrations in natives with fertilisation indicates that native plants can take up more N than required for optimal growth, a phenomenon known as luxury uptake (Iversen et al., 2010). This was also reported by Franklin et al. (2015) for *K. robusta* and *L. scoparium* grown in a low fertility soil after N fertilisation.

Table 5-4 Plant biomass and chemistry (N concentration, C concentration, and C/N ratio) of the plant foliage.

Species	Treatment	Biomass (g)	Total N [a] (%)	Total C [a] (%)	C/N ratio	N uptake [b] (mg)
C. robusta	control	1.3 ± 0.19^{g}	1.9 ± 0.11^{bc}	44 ± 0.13^{a}	33 ± 1.9^{bf}	25 ± 4.4^{a}
	NPKS	2.9 ± 0.27^{df}	2.7 ± 0.12^{e}	44 ± 0.23^{a}	77 ± 8.0^{d}	80 ± 6.7^{bc}
C. secta	control	9.9 ± 1.2^{ab}	0.61 ± 0.07^{a}	44 ± 0.12^{a}	16 ± 0.59^{a}	57 ± 3.2^{c}
	NPKS	19 ± 1.0^{h}	1.4 ± 0.07^{d}	45 ± 0.05^{a}	23 ± 1.5^{ce}	260 ± 5.2^{d}
K. robusta	control	4.1 ± 0.28^{cde}	1.9 ± 0.07^{bc}	49 ± 0.26^{a}	18 ± 0.29^{a}	77 ± 5.5^{bc}
	NPKS	5.5 ± 0.17^{bce}	2.8 ± 0.05^{e}	50 ± 0.16^{a}	26 ± 0.96^{cef}	152 ± 5.9^{def}
L. scoparium	control	3.1 ± 0.23^{cdf}	2.0 ± 0.10^{c}	49 ± 0.45^{a}	16 ± 0.57^{a}	62 ± 6.0^{bc}
	NPKS	4.2 ± 0.34^{cde}	2.5 ± 0.10^{e}	50 ± 0.29^{a}	27 ± 0.98^{ef}	106 ± 11^{bce}
M. umbellata	control	14 ± 2.6^{ah}	0.66 ± 0.06^{a}	48 ± 0.24^{a}	20 ± 0.71^{ac}	86 ± 13^{bc}
	NPKS	9.5 ± 1.7^{ab}	1.3 ± 0.08^{d}	48 ± 0.16^{a}	25 ± 1.3^{ce}	123 ± 24^{bef}
L. perenne	control	1.7 ± 0.12^{fg}	1.6 ± 0.06^{bd}	43 ± 0.13^{a}	39 ± 2.7^{b}	27 ± 1.9^{a}
	NPKS	7.1 ± 0.31^{abe}	2.8 ± 0.12^{e}	44 ± 0.09^{a}	74 ± 5.8^{d}	198 ± 7.0^{df}

Mean ± standard error (n=6).
[a] For C. robusta, K. robusta, L. scoparium, and M. umbellata this is in the leaves, stems were excluded.
[b] Assuming equal N concentrations in the stems and leaves for C. robusta, K. robusta, L. scoparium and *M. umbellata*.

The total amount of N accumulated by the plants differed between species. In the fertilised treatment, *C. secta* showed the highest N uptake, while *C. robusta* showed the lowest N uptake (Table 5-4). At a root: shoot ratio of 1 and assuming that the root N concentration was half of the shoot N concentration (Wayman et al., 2014), *C. secta* took up more N (approx. 390 mg) than added with the fertilisation (353 mg) and *L. perenne* took up nearly as much N as applied (approx. 300 mg). This may explain why, in contrast to results of Franklin et al. (2015), the N concentration in *L. perenne* was not higher than that of native dicotyledonous plants.

5.3.2 Rhizosphere chemistry

The soil was slightly acidic (Table 5-5), which is consistent with typical NZ grassland soils (McDowell & Condron, 2004) and NPKS fertilisation led to acidification under *C. robusta*, *L. scoparium*, and *M. umbellata*. This was likely due to the release of protons during nitrification following the hydrolysis of urea (Rodriguez et al., 2008). Nitrification is a major source of acidity in soil and can result in the mobilization of trace elements and loss of base cations (Robertson & Groffman, 2015). Soil NO_3^- concentration was strongly negatively correlated (r=-0.92, p≤0.001) with soil pH. Furthermore, the

uptake of NH_4^+ by plant roots is associated with the efflux of protons from the roots (Paterson, 2003), adding to the acidification of the rhizosphere. A two-way ANOVA showed that the concentration of mineral N (NO_3^- and NH_4^+) and soil pH was significantly affected by plant species (all $p \leq 0.001$). Fertilisation increased soil NO_3^- concentrations in the dicotyledonous native species, but not in *L. perenne* and *C. secta*. This is consistent with the higher N uptake by these species, which likely reduced the concentration of mineral N in the soil, as plants usually take up N in the form of NO_3^- and NH_4^+ (Britto & Kronzucker, 2013).

Table 5-5 Soil chemical properties at harvesting by plant species and treatment.

Species	Treatment	pH	Total C (%)	Total N (%)	NO_3^-N (mg/kg)	NH_4^+-N (mg/kg)
C. robusta	control	5.6 ± 0.03^{ab}	5.7 ± 0.04^{a}	0.44 ± 0.01^{a}	2.2 ± 1.0^{a}	39 ± 3.7^{def}
	NPKS	5.2 ± 0.08^{c}	5.8 ± 0.02^{a}	0.47 ± 0.01^{a}	67 ± 7.7^{c}	48 ± 1.5^{e}
C. secta	control	5.6 ± 0.01^{ab}	5.7 ± 0.04^{a}	0.45 ± 0.00^{a}	nd.	10 ± 0.40^{a}
	NPKS	5.5 ± 0.02^{ab}	5.7 ± 0.03^{a}	0.49 ± 0.01^{a}	nd	15 ± 0.33^{ab}
K. robusta	control	5.6 ± 0.02^{ab}	5.8 ± 0.03^{a}	0.42 ± 0.01^{a}	nd	18 ± 0.93^{bc}
	NPKS	5.6 ± 0.03^{ab}	5.8 ± 0.03^{a}	0.46 ± 0.01^{a}	16 ± 4.4^{b}	42 ± 0.93^{def}
L. scoparium	control	5.6 ± 0.04^{a}	5.8 ± 0.04^{a}	0.40 ± 0.01^{a}	nd	30 ± 4.4^{d}
	NPKS	5.4 ± 0.11^{bd}	5.8 ± 0.02^{a}	0.43 ± 0.01^{a}	43 ± 12^{bc}	46 ± 0.84^{ef}
M. umbellata	control	5.6 ± 0.03^{ab}	5.8 ± 0.04^{a}	0.40 ± 0.00^{a}	2.4 ± 1.8^{a}	14 ± 0.66^{ab}
	NPKS	5.3 ± 0.07^{cd}	5.8 ± 0.04^{a}	0.40 ± 0.01^{a}	46 ± 8.7^{bc}	33 ± 5.5^{df}
L. perenne	control	5.6 ± 0.02^{ab}	5.7 ± 0.02^{a}	0.41 ± 0.00^{a}	1.0 ± 0.34^{a}	14 ± 1.8^{ab}
	NPKS	5.6 ± 0.02^{ab}	5.8 ± 0.02^{a}	0.42 ± 0.01^{a}	0.99 ± 0.37^{a}	28 ± 3.2^{cd}

Mean ± standard error (n=6). Different letters indicate significant differences between sites ($p \leq 0.05$) according to Tukey's HSD test.
nd = not detectable (<0.25 mg kg^{-1} NO_3^--N)

5.3.3 Microbial abundance in the rhizosphere

Bacterial 16S rRNA (total bacteria, TB) was 1000 times more abundant in the soil than archaeal 16S rRNA (total archaea, TA). The abundance of TB was not significantly affected by fertilisation and did not differ between plant species (Tables 5-6 and 5-7). While some NZ native Myrtaceae can reduce pathogenic bacteria in soil (Gutierrez-Gines et al., 2021), *K. robusta*, *L. scoparium* and *M. umbellata* did not affect the abundance of TB in this study. The soil C:N ratio in this experiment ranged from 11 to 15, which indicates that bacteria were more likely limited by C than N (Bengtsson et al., 2003). This

may explain why TB abundance did not significantly increase with NPKS application, despite a trend of increased TB abundance with fertilisation. The abundance of TA was unaffected by fertilisation, but there were significant differences between plant species. This is consistent with results of Yarwood et al. (2016), whereby the TA but not TB abundance differed between lineages of *Phragmites australis*, highlighting the higher sensitivity of archaea to plant variation compared to bacteria. The contrasting abundance of TA between plant species likely derived from differing availability of energy in the form of labile C from root exudates (Karlsson et al., 2012; Valentine, 2007).

Table 5-6 Results of two-way ANOVA for total archaea (TA), total bacteria (TB) and the functional genes encoding nitrite reductase (*nirK*, *nirS*) and nitrous oxide reductase (*nosZ*). Asterisks indicate significant effects (* $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$). Log-normally distributed data were log transformed.

	TA	TB	*nirK*	*nirS*	*nosZ*
Transformation	log	none	none	log	log
Treatment	0.999	0.064	0.752	0.195	0.644
Species	0.001***	0.113	0.110	0.015*	0.001**
Interaction	0.033*	0.350	0.582	0.332	0.040*

Table 5-7 Abundance of archaeal 16S rRNA (total archaea), bacterial 16s rRNA (total bacteria), and functional genes encoding nitrite reductase (*nirS* and *nirK*) and nitrous oxide reductase (*nosZ*).

		gene copies g^{-1} soil (dry weight basis)				
Species	Treatment	Total archaea	Total bacteria	*nirK*	*nirS*	*nosZ*
C. robusta	control	$(5.12 \pm 0.58) \times 10^7$ *ab*	$(3.79 \pm 0.76) \times 10^{10}$	$(6.88 \pm 0.90) \times 10^7$	$(1.91 \pm 0.35) \times 10^7$	$(2.90 \pm 0.27) \times 10^7$ *ab*
	NPKS	$(1.51 \pm 0.17) \times 10^7$ *b*	$(1.63 \pm 0.23) \times 10^{10}$	$(3.18 \pm 0.47) \times 10^7$	$(1.11 \pm 0.21) \times 10^7$	$(1.62 \pm 0.13) \times 10^7$ *b*
C. secta	control	$(8.78 \pm 0.96) \times 10^7$ *a*	$(6.23 \pm 1.33) \times 10^{10}$	$(7.91 \pm 1.07) \times 10^7$	$(1.32 \pm 0.27) \times 10^7$	$(4.31 \pm 0.53) \times 10^7$ *a*
	NPKS	$(8.09 \pm 0.78) \times 10^7$ *a*	$(7.91 \pm 0.85) \times 10^{10}$	$(9.77 \pm 1.49) \times 10^7$	$(1.51 \pm 0.30) \times 10^7$	$(4.59 \pm 0.35) \times 10^7$ *a*
K. robusta	control	$(5.42 \pm 0.81) \times 10^7$ *ab*	$(4.11 \pm 0.88) \times 10^{10}$	$(9.15 \pm 1.02) \times 10^7$	$(1.39 \pm 0.31) \times 10^7$	$(3.35 \pm 0.32) \times 10^7$ *ab*
	NPKS	$(8.90 \pm 1.36) \times 10^7$ *a*	$(9.60 \pm 1.74) \times 10^{10}$	$(1.26 \pm 0.19) \times 10^8$	$(2.35 \pm 0.73) \times 10^7$	$(6.23 \pm 0.65) \times 10^7$ *a*
L. scoparium	control	$(4.12 \pm 0.94) \times 10^7$ *ab*	$(2.98 \pm 0.58) \times 10^{10}$	$(8.44 \pm 1.24) \times 10^7$	$(1.79 \pm 0.38) \times 10^7$	$(3.66 \pm 0.39) \times 10^7$ *ab*
	NPKS	$(5.61 \pm 0.79) \times 10^7$ *ab*	$(6.21 \pm 1.05) \times 10^{10}$	$(7.63 \pm 1.27) \times 10^7$	$(1.50 \pm 0.37) \times 10^7$	$(3.62 \pm 0.43) \times 10^7$ *ab*
M. umbellata	control	$(8.46 \pm 1.62) \times 10^7$ *a*	$(4.65 \pm 0.97) \times 10^{10}$	$(8.76 \pm 0.99) \times 10^7$	$(3.64 \pm 0.61) \times 10^7$	$(4.03 \pm 0.36) \times 10^7$ *ab*
	NPKS	$(1.76 \pm 0.36) \times 10^8$ a	$(5.94 \pm 0.85) \times 10^{10}$	$(8.45 \pm 1.30) \times 10^7$	$(3.67 \pm 0.72) \times 10^7$	$(5.33 \pm 0.62) \times 10^7$ *a*
L. perenne	control	$(7.45 \pm 0.95) \times 10^7$ *a*	$(5.21 \pm 0.90) \times 10^{10}$	$(9.03 \pm 1.07) \times 10^7$	$(5.53 \pm 1.05) \times 10^7$	$(4.98 \pm 0.32) \times 10^7$ *a*
	NPKS	$(7.19 \pm 1.07) \times 10^7$ *ab*	$(6.58 \pm 1.06) \times 10^{10}$	$(1.07 \pm 0.19) \times 10^8$	$(1.78 \pm 0.30) \times 10^7$	$(4.44 \pm 0.49) \times 10^7$ *a*

Mean ± standard error (n= 18, 6 experimental and 3 technical replicates). Significant differences ($p \leq 0.05$) according to Tukey's HSD are indicated by letters in italics.

For the denitrifying bacteria, the genes encoding nitrite reductase (*nirK*, *nirS*) and nitrous oxide reductase (*nosZ*) were unaffected by the application of NPKS (Table 5-6). This contrasts with a study by Kastl et al. (2015), in which fertilisation of 200 kg urea (NH_4NO_3) ha^{-1} halved the abundance of denitrifying microbes. However, Fischer et al. (2013) showed that the abundance of denitrifiers was more limited by low levels of dissolved organic C than NO_3^-. The abundance of *nirS* and *nosZ* differed between plant species, while the abundance of *nirK* did not. This may be a result of differing root exudation of organic compounds among species. As *nosZ* is responsible for the transformation of N_2O to N_2 (Kandeler et al., 2006), it is likely that a higher abundance and activity would increase the rate of N_2 emissions compared to the greenhouse gas N_2O. With plant species having the strongest effect on *nosZ*, species selection may be critical to favour complete denitrification in the soil.

For the nitrifying microorganisms, AOB *amoA* was more abundant than AOA *amoA*. AOB *amoA* abundance in the fertilised soil ranged from 10^6 to 10^8 gene copies g^{-1} soil, while AOA *amoA* was below the detection limit (<10^4 gene copies g^{-1} soil) in 50% of samples. The percentage of samples where AOA *amoA* was detected was unaffected by fertilisation and did not differ between plant species. This is consistent with findings by Kastl et al. (2015) and Adair and Schwartz (2008), who reported that AOA abundance was not affected by N fertilisation or plant species, nor by soil C:N ratio and environmental parameters. However, the results contrast with several studies whereby AOA are more abundant in soil than AOB (Adair & Schwartz, 2008; Di et al., 2009; Leptin et al., 2021). A Luvisolic soil studied by Fischer et al. (2013) contained 10^7-10^9 AOA *amoA* copies g^{-1} dry soil compared to 10^4-10^7 AOB *amoA* copies g^{-1} dry soil. It was demonstrated that AOA and AOB occupy different niches, with AOB being predominant in neutral or alkaline N-rich soils with high levels of ammonia (Di et al., 2010; Shen et al., 2012). However, the Craigieburn silt loam used in this study had low N concentrations and a pH of 5.6, indicating that AOA would dominate, which was not the case.

The abundance of AOB *amoA* was below the detection limit (<10^4 gene copies g^{-1} soil) in the control treatment, except for *C. robusta* ((2.68 ± 0.72) × 10^6 gene copies g^{-1} soil). The detection of AOB *amoA* in the control treatment with *C. robusta* was likely due to the insignificant root system and slow plant growth in this species and reduced plant-microbe competition. Fertilisation increased the abundance of AOB *amoA* above the detection limit in all species. The low abundance of AOB *amoA* in the control treatment was likely due to competition between microbes and plants for organic and inorganic N in the soil (Kaye & Hart, 1997). Plants are superior to AOB when competing for NH_4^+ and the reduced mobility of AOB limits their ability to utilise NH_4^+ (Skiba et al., 2011). AOB therefore require higher concentrations of NH_4^+ in the soil, which is reflected in their strong response to NPKS application. Results are consistent with those of Kastl et al. (2015) and Okano et al. (2004), who reported that the abundance of AOB was significantly increased by ammonium nitrate (NH_4NO_3) and ammonium

sulphate ($(NH_4)_2SO_4$) application, respectively. Furthermore, Di et al. (2009) reported that AOB gene copy numbers increased 3-10 times with addition of urine equivalent to 1000 kg N ha^{-1}.

5.3.4 Species effects on ammonia oxidising bacteria

Plant species affected the abundance of AOB *amoA* in the fertilised soil. Results indicate that AOB *amoA* abundance differs between monocotyledonous and dicotyledonous species. *L. scoparium* and *M umbellata* showed significantly higher AOB *amoA* abundances than *L. perenne* and *C. secta* (Figure 5-1). Typically, plants affect nitrification through (i) direct competition for N, (ii) the release of BNIs, and (iii) physico-chemical changes in the rhizosphere that affect the microbial activity and community composition (Bowatte et al., 2016). The higher uptake of N by mono- than dicotyledonous species (Table 5-4), and the associated lower concentrations of mineral N, is likely the main explanation for the lower abundance of AOB under these species. In addition, heterotrophic bacteria are more competitive for NH_4^+ than nitrifying bacteria under low N and sufficient organic C conditions (Verhagen et al., 1992). Differential root exudation by monocotyledonous and dicotyledonous species may also explain the differences in AOB between the rhizospheres of the two groups (Chai & Schachtman, 2022; Oburger & Jones, 2018). AOB were shown to negatively correlate with C inputs from roots (Ollivier et al., 2011). This can be explained by an accelerated growth of heterotrophic bacteria in response to C inputs, increasing their N use and reducing N that is available to AOB (Leptin et al., 2021).

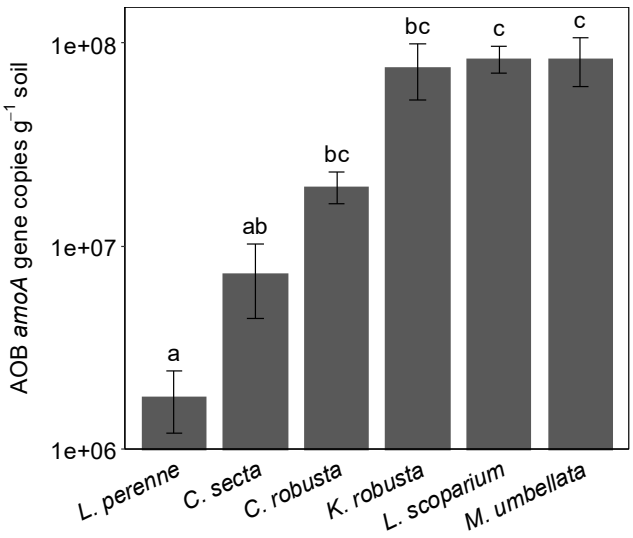

Figure 5-1 Abundance of AOB *amoA* (gene copy no. g^{-1} soil) in the fertilised treatment, separated by species. Values shown are means and standard errors (*n*=6). Different letters indicate significant differences between species at $p \leq 0.05$ according to Tukey's HSD post-hoc test.

It was previously hypothesised that *L. scoparium* and *K. robusta* may suppress nitrification in soil more than other species (Esperschuetz, Balaine, et al., 2017; Franklin et al., 2017), but the high abundance of AOB *amoA* under these species indicates that this was not the case here. However, it was not possible to assess inhibitory effects of myrtaceous species on nitrification and potential BNI activity, as enzyme activity was not analysed. While measuring the abundance of functional genes in soil allows the quantification of its genetic potential for a particular turnover process (Fischer et al., 2013), it does not necessarily correlate with the activity of the enzymes they are encoding (Laffite et al., 2020), nor with actual turnover rates (Fischer et al., 2013). Nevertheless, Ouyang et al. (2018) found that functional gene abundance was correlated with corresponding enzyme activity, but the variation in enzyme activity was explained better by soil chemical properties than functional gene abundance. Furthermore, Di et al. (2009), found a correlation between the growth of AOB and nitrification. In the present study, soil NO_3^- and NH_4^+ concentrations were positively correlated with the AOB *amoA* : bacterial 16S rRNA ratio (Figures 5-2 a and b). This provides evidence that AOB *amoA* abundance is correlated with nitrification rates, as previously reported by Nicol et al. (2008). Further analysis would be required to quantify enzyme activity.

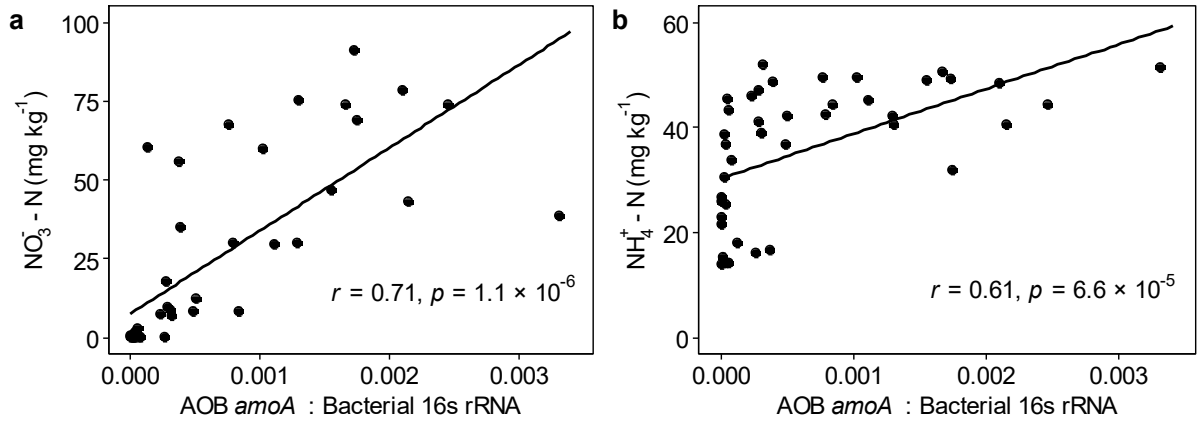

Figure 5-2 (**a**) Soil NO_3^--N concentration vs. AOB *amoA* : Bacterial 16s rRNA in the fertilised soil, and (**b**) and soil NH_4^+-N concentration vs. AOB *amoA* : Bacterial 16S rRNA in the fertilised soil. The black lines show the linear regression. The *r* values are Pearson's correlation coefficients.

Mono- and dicotyledonous species not only affected the abundance of AOB *amoA*, but also the chemical properties of the soil. PCA showed that fertilisation resulted in the separation of mono- and dicotyledons in the fertilised soil (Figures 5-3 and 5-4). Variation along PC1 was explained by soil microbial abundance, primarily *nosZ*, *nirS*, and bacterial *16S rRNA*, while PC2 was mainly explained by soil chemical properties. This reflects that plant-soil-microbe interactions in the rhizosphere are highly

complex and closely linked to the chemical and physical properties of the soil, as well as root characteristics (Pathan et al., 2019). Species affect soil properties and ecosystem processes through their distinct architectural, morphological, physiological, and biotic root traits (Bardgett et al., 2014).

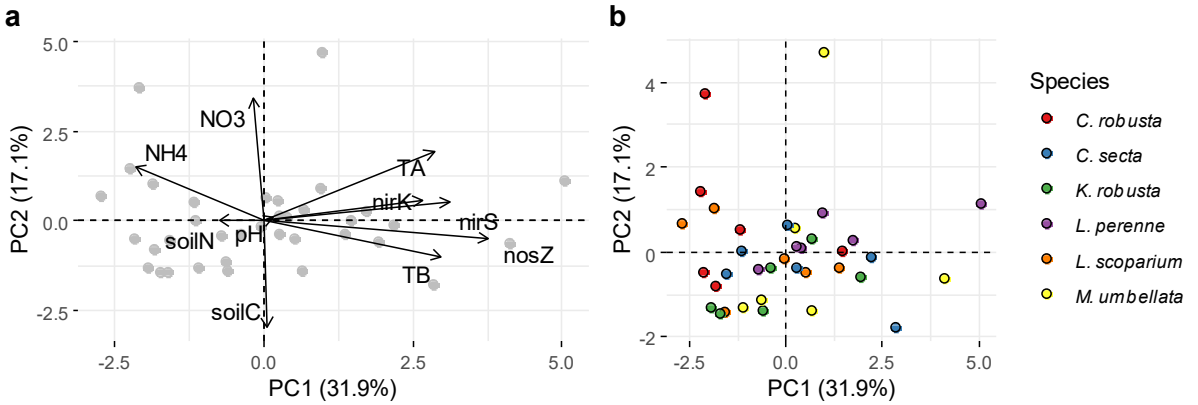

Figure 5-3 Principal component analysis (PCA) of chemical and microbial soil parameters in the control treatment; (**a**) loading plot and (**b**) score plot. TB: total bacteria, TA: total archaea, *nirK*/*nirS*/*nosZ*: denitrification genes.

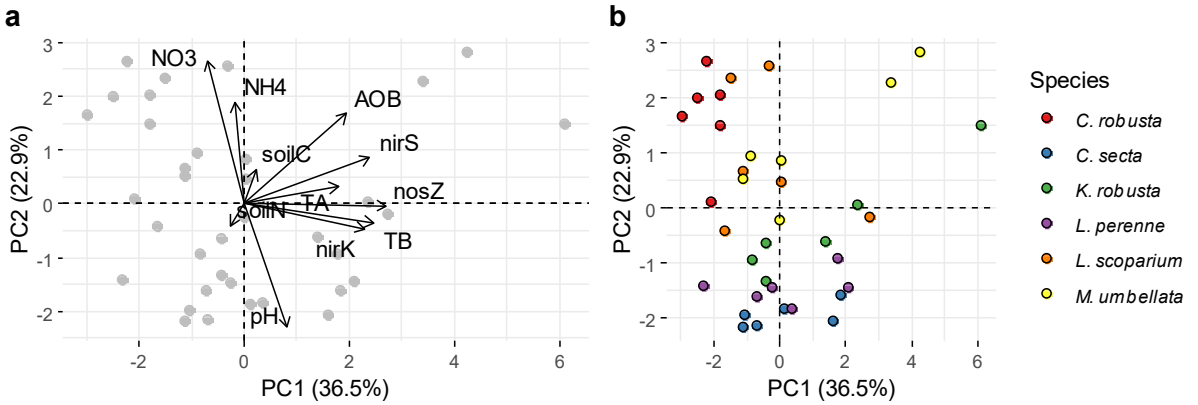

Figure 5-4 Principal component analysis (PCA) of chemical and microbial soil parameters in the fertilised (NPKS) treatment; (**a**) loading plot and (**b**) score plot. TB: total bacteria, TA: total archaea, *nirK*/*nirS*/*nosZ*: denitrification genes, AOB: ammonia oxidising bacteria.

Root activity can affect the pH in the rhizosphere through proton influx and/or efflux related to nutrient uptake (Hinsinger et al., 2009) and the release of organic anions (Hinsinger et al., 2003). Soil pH can affect microbial processes in the rhizosphere such as nitrification and denitrification (Levy-

Booth et al., 2014). In this study, there was a negative correlation (r=-0.60, p≤0.001) between soil pH and the abundance of AOB *amoA* relative to bacterial 16S rRNA. Preferential uptake of one mineral N species over the other can affect the pH of the rhizosphere, as uptake of NO_3^- and NH_4^+ is associated with alkalinization and acidification, respectively (Hinsinger et al., 2009). However, while N uptake mechanisms of NZ native plants are mostly unknown, there is evidence that uptake of mineral N by *L. scoparium* and *K. robusta* is non-preferential (Esperschuetz, Balaine, et al., 2017).

The species used in this experiment have distinct root morphologies, which can affect the fate of water and nutrients, and therefore nutrient cycling and microbial activity in the rhizosphere (Franklin et al., 2019). Monocotyledons have more fibrous root systems (Franklin et al., 2015), indicating that preferential flow is less likely under these species compared to dicotyledons. Mishra (2018) showed that preferential flow was significant under *L. scoparium* and *K. robusta* due to the tap root system of these species. Preferential flow pathways can lead to nutrient losses under these species and result in a heterogeneous distribution of water and nutrients in the soil (Clothier et al., 2007; Gutierrez-Gines et al., 2021). The results indicate that monocotyledonous species take up more N and have a lower potential for nitrification in their rootzone.

5.4 Conclusions

There are interspecific differences between NZ native monocotyledonous and dicotyledonous plant species with respect to N-cycling in high fertility environments. The abundance of AOB *amoA* was affected by plant species and was lower under mono- than dicotyledonous species. There was no evidence of an inhibitory effect of NZ native Myrtaceae on AOB. Further work should determine the mechanisms of plant specific interaction with AOB, although plant uptake can explain some of the observed differences. The use of monocotyledonous native species for the land application of N-rich wastes, in NZ and elsewhere, may reduce NO_3^- leaching from the system through plant uptake of N and competition with AOB. In addition, there was a strong species effect on the abundance of *nosZ*. This indicates that the targeted selection of plant species may reduce emissions of the potent greenhouse gas N_2O through complete denitrification.

Chapter 6

Chemical Elements and the Quality of Mānuka (*Leptospermum scoparium*) Honey

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6.1 Abstract

Soil properties in the foraging range of honeybees influence honey composition. We aimed to determine relationships between the antimicrobial properties of New Zealand mānuka (*Leptospermum scoparium*) honey and elemental concentrations in the honey, plants, and soils. We analysed soils, plants, and fresh mānuka honey samples from the Wairarapa region of New Zealand for the chemical elements and the antimicrobial activity of the honey as indicated by methylglyoxal (MGO) and dihydroxyacetone (DHA). There were significant negative correlations between honey MGO and the concentrations of Mn, Cu, Mg, S, Na, Ba, K, Zn, and Al. These elements may provide a low-cost means of assessing mānuka honey quality. For individual elements, except for K, there were no correlations between the honeys, plants, and soils. Soil nitrate concentrations were negatively correlated with concentrations of MGO and DHA in the honey, which implies that soil fertility may be a determiner of mānuka honey quality.

6.2 Introduction

Leptospermum scoparium J.R. et G. Forst. is the most widespread indigenous shrub species in New Zealand and is commonly known as mānuka or tea tree (Stephens et al., 2005). It is a member of the Myrtaceae family and one of 13 species in the *Leptospermum myrtifolium* subgroup (Thompson, 1989). Economically, *L. scoparium* is important due to production of its essential oil and mānuka honey. Most of the 8065 tons of honey exported from New Zealand in 2019, which created a revenue of NZD 355 M (approximately USD 250 M), was mono- or multi-floral mānuka honey (MPI, 2020).

Honey is naturally antiseptic because it is osmotically unfavourable to microbial growth and has a low pH (Molan, 1992). Whilst honeys typically contain the antimicrobial compound hydrogen peroxide (H_2O_2), mānuka honey is unusual due to its non-peroxide antimicrobial activity (NPA) (Adams et al., 2008). The dominant component responsible for NPA in mānuka honey is methylglyoxal (MGO) (Mavric et al., 2008). Other compounds, including leptosin and various phenolics, synergistically

modulate mānuka honey NPA (Carter et al., 2016). MGO is formed in the honey due to non-enzymatic dehydration of dihydroxyacetone (DHA) from *L. scoparium* nectar (Adams et al., 2009; Norton et al., 2015). Therefore, the concentration of MGO increases simultaneously with a decrease in DHA during maturation and storage of mānuka honey in warm temperatures (Atrott et al., 2012). Mānuka honey can inhibit a range of pathogenic bacteria genera, including *Enterococcus*, *Pseudomonas*, *Staphylococcus*, and *Streptococcus*, among others (Carter et al., 2016). Antimicrobial action may occur due to the disruption of regular cell division, impairment of cellular integrity, and reduction in cellular motility (A. E. L. Roberts et al., 2015). Its distinct antimicrobial characteristics mean that the market value of mānuka honey is primarily determined by its NPA, which is often commercially expressed as Unique Mānuka Factor (UMF™), though other marketing terms exist (Mavric et al., 2008). DHA concentration in *L. scoparium* nectar is affected by a plethora of genetic and environmental factors (Clearwater et al., 2018). Although these have yet to be quantified, they may include the concentrations of the chemical elements in the nectar.

Soil is the ultimate source of many elements in the floral nectar (Czipa et al., 2017; Xun et al., 2017). The concentration of elements in honey is affected by soil characteristics, and honey composition can be used for geographical discrimination or as a soil element indicator (Acquarone et al., 2007; Baroni et al., 2015; Czipa et al., 2017; Gonzalez-Porto et al., 2016). The response of *L. scoparium* to soil properties is cultivar-dependent (Nickless et al., 2017). However, Williams et al. (2014) found that soil properties do not affect the concentration of DHA in *L. scoparium* nectar. This is consistent with other studies which show that genetic factors and provenances are more relevant for *L. scoparium* nectar DHA (Hamilton et al., 2013; Millner et al., 2016). Noe et al. (2019), however, reported that *L. scoparium* nectar DHA varies more among plants than among sites. It is unclear how the environment affects the composition of *L. scoparium* nectar and, subsequently, mānuka honey.

There are no reports of the effect of soil and plant elemental concentrations on the elemental composition and NPA of mānuka honey. *L. scoparium* is typically found growing on low fertility soils (Stephens, 2006), and increased soil fertility accelerates growth of the plant (Esperschuetz, Anderson, et al., 2017). Nickless et al. (2017) showed that increased soil nutrient concentration also improved floral density of *L. scoparium*. The link appears to be missing between soil parameters and mānuka honey MGO. Although DHA contents of *L. scoparium* plants within 1000 m from the apiary correlate well with MGO in honey (Gardner, 2014), actual honey MGO contents are typically lower than nectar-DHA-based estimates (Williams, 2019). Mānuka honey is rarely collected from 100% *L. scoparium* nectar, so it is vital for beekeepers to increase the availability of DHA-containing nectar to honeybees in order to achieve high MGO mānuka honeys (Williams, 2019). Higher concentrations

of soil nutrients within the foraging range of honeybees might therefore result in increased availability of *L. scoparium* nectar by increasing the floral density.

We aimed to determine the effect, if any, of the elemental composition of soils, plants, and honey on the quality of mānuka honey as indicated by MGO and DHA. Additionally, we sought to compare the chemical composition of mānuka honey from different sites in the Wairarapa region of New Zealand.

6.3 Materials and Methods

6.3.1 Sample collection

Soil, plant foliage, and honey samples were collected from five sites in the Wairarapa region in the lower North Island of New Zealand (Figure 6-1). Soil and foliage samples were collected in April 2014. Five *L. scoparium* plants were sampled per hive location. Plants were between 1.5 and 3 m tall. Foliage was sampled at 2 m above ground where possible. A representative sample was taken by combining 10 individual twigs per tree. A soil sample was taken at the base of each sampled plant, within 0.5 m from the base. All sampling sites were within 1 km from the hive. This is within the foraging range of the honeybee *Apis mellifera* (Pohl et al., 2009). Soil and plant samples were immediately sent to the laboratory for further processing. Soils were kept cold in insulated containers with ice packs. Raw honey samples were extracted by Watson & Son Ltd. (Masterton, New Zealand, now Oha Honey LP) in January-February 2014. Honey samples from locations B, C, and D are composite samples, as multiple hives were within 1 km from each other at these sites.

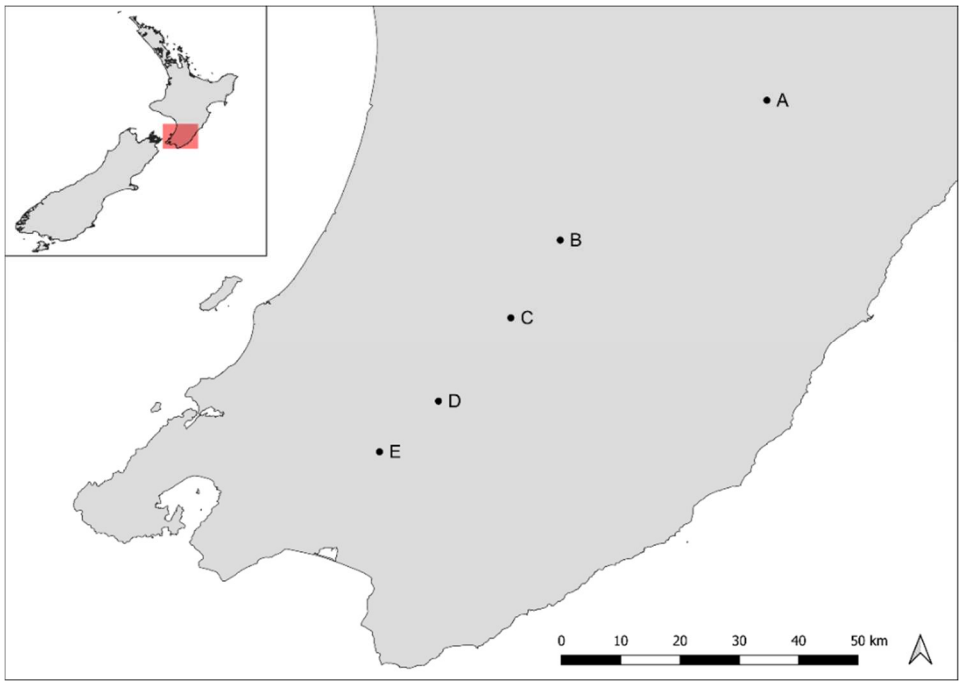

Figure 6-1 Sampling sites (A-E) in the Wairarapa region of New Zealand.

6.3.2 Honey analysis

Honey samples (0.5 g) were digested in 8 mL ARISTAR® 69% HNO_3 with a microwave (MARS Xpress, CEM Corporation, Matthews, NC, USA). Total concentrations of Al, As, B, Ba, Ca, Cd, Cr, Cu, Fe, K, Mg, Mn, Na, P, Pb, S, and Zn were determined by ICP-OES (Varian 720-ES, Agilent Technologies, Santa Clara, CA, USA). Concentrations of honey DHA and MGO were simultaneously determined by HPLC at Watson & Son Ltd. (Masterton, New Zealand, now Oha Honey LP) following the method described by Windsor et al. (2012). The HPLC system consisted of a Dionex ACC-3000 autosampler, a Dionex LPG-3400SD quaternary pump, and a Dionex VWD-3100 detector (λ = 263 nm) (Thermo Fisher Scientific, Waltham, MA, USA). A Phenomenex Synergi Fusion 75 mm × 4.6 mm, 4 µm, 80Å reversed-phase column was used with a Phenomenex Synergi 4 mm × 3 mm guard column (Phenomenex, Torrance, CA, USA).

6.3.3 Soil and Plant analysis

NH_4^+ and NO_3^- were extracted from fresh soil with 2 M KCl and analysed by a flow injection analyser (FIAstar 5000, FOSS, Hillerød, Denmark) (Esperschuetz et al., 2016). Soils were dried at room temperature until a constant weight was achieved and then were sieved to 2 mm. Soil pH was determined in a 1:2.5 water extract (Blakemore et al., 1987). In all, 0.5 g of soil was digested by microwave (CEM MARS Xpress) in 5 mL ARISTAR® 69% HNO_3 and 1 mL ARISTAR® 30% H_2O_2. Pseudo-total Al, As, B, Ca, Cd, Cr, Cu, Fe, K, Li, Mg, Mn, Na, Ni, P, Pb, S, Sr, and Zn concentrations in the digest

were analysed by ICP-OES (Varian 720-ES). The exchangeable element fraction was determined in a 0.05 M $Ca(NO_3)_2$ extract using ICP-OES (Varian 720-ES) (Reiser et al., 2014). Total C and N were determined using an Elementar Vario Max CN elemental analyser (Elementar, Langenselbold, Germany).

Foliage samples were rinsed with deionised water and dried at 65 °C until a constant weight was achieved. Leaves were separated from the twigs and ground using a Retch ZM200 mill. In all, 0.5 g of ground foliage was digested in 8 mL ARISTAR® 69% HNO_3 by microwave (CEM MARS Xpress). Total Al, As, B, Ca, Cd, Co, Cr, Cu, Fe, K, Li, Mg, Mn, Na, Ni, P, Pb, S, Sr, and Zn concentrations in the digest were determined by ICP-OES (Varian 720-ES) (Reiser et al., 2014). Total C and N concentrations were determined using an Elementar Vario Max CN elemental analyser.

6.3.4 Quality Control

Wageningen Evaluating Programmes for Analytical Laboratories (WEPAL) certified reference materials ISE 921 and IPE 100 were used for quality assurance in soil and plant digestions. Recoveries ranged from 91% to 108% of certified values. Analytical blanks were included in all analyses.

6.3.5 Statistical Analysis

Data were analysed using R version 4.0.5. (R Core Team, 2021). One-way analysis of variance (ANOVA) followed by Tukey's honestly significant difference (HSD) test was used to assess site differences using the package *multcomp*. Data were log-transformed where the assumption of normality was not met. The significance level for all statistical analyses was $p \leq 0.05$. A principal component analysis (PCA) was carried out for honey variables using the package *factoextra*. The packages ggplot2 and *ggpubr* were used to visualise results of correlation analysis. No statistical analyses were performed on honey Cd, As, and Pb concentrations as these were below detection limits (<0.001 mg kg^{-1}).

6.4 Results and Discussion

6.4.1 Soil and Plants

The soils were typically yellow-brown loams (Cowie, 1982). Soil pH at all sites was moderately to strongly acidic (Table A-10) and lower than typical soil pH under New Zealand pasture, which ranges from 4.8 to 6.9 (Reiser et al., 2014). *L. scoparium* is commonly found growing on acidic soils (Berninger, 1992). At low soil pH, some trace element cations, including Al, Cu, Fe, Mn, and Zn, are more soluble and more plant-available (Blume et al., 2016). We found soil pH was negatively correlated with some extractable elements (Table A-11), which included Al (r=-0.71, $p \leq 0.001$), Cd (r=-0.49, $p \leq 0.005$), Cr

(r=-0.51, p≤0.001), and Zn (r=-0.45, p≤0.005). Soils were generally low in P (452-878 mg kg^{-1}) when compared with New Zealand pasture soil but had similar concentrations of N and C (Reiser et al., 2014).

Plant samples were particularly high in Mn and Ni (Table A-12) when compared with average plant shoots (Kirkby, 2012). In contrast, the concentration of some of the P, K, and Mg was lower than average for plant dry matter concentrations (Kirkby, 2012). Most plant parameters did not differ significantly between sites (C, N, C/N, As, B, Ca, Cd, Cu, Ni, and Zn). Site C differed from all other sites as it had significantly higher K and significantly lower Mn foliage concentrations.

Plant Mn in this study ranged from 53 to 1309 mg kg^{-1} with a median of 194 mg kg^{-1}. This is comparable to studies by Gutierrez-Gines et al. (2019) and Reis et al. (2017), who reported 185-292 and 186-331 mg Mn kg^{-1} in non-fertilised *L. scoparium*, respectively. Both of these studies measured increased leaf Mn concentrations following the application of biosolids, which potentially reach phytotoxic levels at >400 mg Mn kg^{-1} (Chaney, 1989). In the present study, sites A and E exceeded this threshold with an average Mn concentration of 874 and 448 mg kg^{-1}, respectively.

For the major nutrients N and P, higher soil concentrations that increase plant growth may result in a dilution of other elements in plant tissues (Jarrell & Beverly, 1981). However, we found significant positive correlations between extractable soil P and plant P (r=0.42, p≤0.005), extractable soil Mg and plant Mg (r=0.40, p≤0.01), and extractable soil Mn and plant Mn (r=0.34, p≤0.05).

6.4.2 Honey

Table 6-1 reports honey MGO, DHA, and elemental concentrations. The DHA concentrations in this study were similar to those reported in fresh mānuka honey by Atrott et al. (2012) and Adams et al. (2009). MGO concentrations were in the low range, lower than those in fresh mānuka honeys (309-658 mg kg^{-1}) reported by Stephens et al. (2010). There was a strong positive correlation (r=0.99, p≤0.001) between DHA and MGO, with MGO concentrations being on average 7% of DHA concentrations. Therefore, we henceforth report MGO as an indicator of mānuka honey quality.

Table 6-1 Methylglyoxal (MGO), dihydroxyacetone (DHA), and elemental concentrations in honeys from sites A-E. The locations of the sites are shown in Figure 6-1.

site	A			B			C			D	E
n=	*6*			*9*			*4*			*1*	*2*
MGO	266	±	25[a]	126	±	6.9[b]	77	±	18[b]	141	121
DHA	3246	±	221[a]	1856	±	71[b]	1293	±	226[b]	1983	1642
Al	6.5	±	0.91[a]	11	±	0.60[b]	9.1	±	0.34[ab]	5.1	5.5
B	2.8	±	0.18[a]	2.8	±	0.10[a]	2.5	±	0.12[a]	2.8	2.8
Ba	0.08	±	0.01[a]	0.09	±	0.00[a]	0.11	±	0.00[b]	0.09	0.09
Ca	61	±	2.9[a]	60	±	2.5[a]	61	±	1.9[a]	62	68
Cr	0.02	±	0.01[a]	0.02	±	0.00[a]	0.02	±	0.00[a]	0.02	0.03
Cu	0.13	±	0.01[a]	0.24	±	0.01[b]	0.30	±	0.02[c]	0.16	0.21
Fe	1.2	±	0.45[a]	1.1	±	0.09[a]	0.91	±	0.12[a]	0.72	0.95
K	487	±	14[a]	671	±	25[b]	1108	±	55[c]	463	465
Mg	11	±	0.68[a]	18	±	0.61[b]	21	±	0.35[c]	15	16
Mn	1.1	±	0.23[a]	2.7	±	0.15[b]	4.2	±	0.60[c]	2.9	3.2
Na	27	±	0.99[a]	34	±	0.99[b]	47	±	2.2[c]	38	40
P	54	±	0.99[a]	50	±	1.4[a]	65	±	4.4[b]	62	64
S	21	±	0.62[a]	28	±	0.85[b]	31	±	43[b]	24	25
Zn	0.32	±	0.02[a]	0.36	±	0.03[a]	0.47	±	0.03[b]	0.37	0.47

Mean ± standard error. Values are in mg kg^{-1}.

The elemental concentrations in the honey were comparable to analyses of other fresh mānuka honey in New Zealand (Vanhanen et al., 2011). However, Na and Al were four and seven times higher, respectively, than concentrations reported by Vanhanen et al. (2011). The elemental concentrations in our fresh honey samples were lower than those in commercially available New Zealand mānuka honeys reported by international studies (Alqarni et al., 2014; Deng et al., 2018; Kek et al., 2016; Moniruzzaman et al., 2014). The concentration of chemical elements in honey may increase following moisture reduction during honey processing (Singh & Singh, 2018).

The most abundant element in the tested honeys was K, followed by Ca and P. These were the overall most abundant elements in mono-floral New Zealand honeys analysed by Vanhanen et al. (2011). They were also the most prominent elements in various international honey types (Solayman et al., 2016). Mānuka honey in this study had higher concentrations of elements, particularly Na, Ca, Mg, P, and Mn, compared to other honey types (Bogdanov et al., 2008). In the case of Mn, mānuka honey was

shown to have higher concentrations than other New Zealand mono-floral honeys, with the exception of rewarewa honey (Vanhanen et al., 2011).

An increased elemental concentration as such can be beneficial for human nutrition (González-Miret et al., 2005), although, given the average daily consumption of honey (0.1-0.8 kg per annum), human health benefits from the elements contained in honey are negligible (Bogdanov et al., 2008; Machado De-Melo et al., 2017). Heavy metals such as, Cd, and Pb were below the detection limit (<0.001 mg kg^{-1}) in this study and therefore not of significance to human health.

A PCA was used to investigate similarities between mānuka honey quality parameters and elemental composition between site (Figure 6-2). The honeys can be separated into three distinct groups at sites A, B, and C, with honey DHA and MGO having a positive weighting in PC1 (explaining 53.9% of variance) and other elements, dominated by Mg, Mn, Cu, Ba, Na, Zn, and K, having negative weightings. PC2 (explaining 16.5% of variance) separated the sites mainly based on Cr, Fe, Ca, and Ba concentrations.

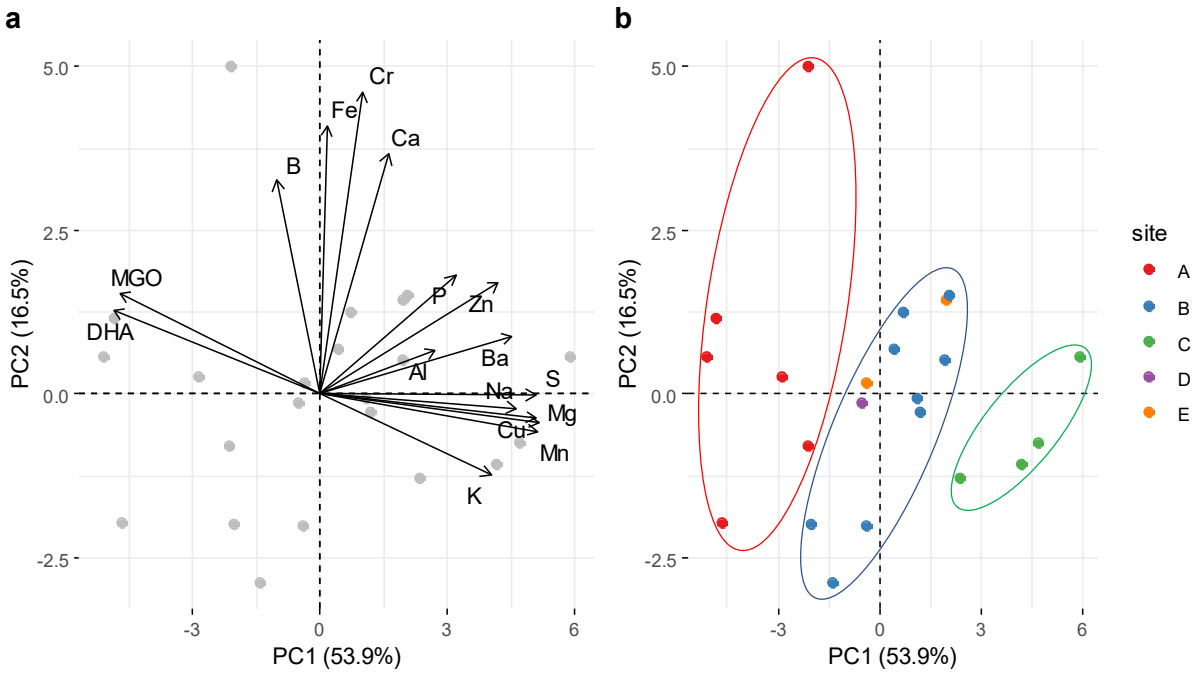

Figure 6-2 Principal component analysis (PCA) describing the variation of honey MGO, DHA, and elemental concentrations: (**a**) loading plot; (**b**) score plot. The ellipses are eye-guides to delineate the three distinct groups.

MGO concentrations were 89-245% higher in site A compared to sites B, C, D, and E. The spatial variation of honey MGO concentrations aligns with inter- and intra-regional variation in *L. scoparium* nectar DHA previously observed by Williams et al. (2014). This is likely a result of genetic and

environmental effects on nectar composition and yields (Nickless et al., 2017; Noe et al., 2019). Similarly, site A differed from other sites regarding the elemental composition of the honey. This is particularly true for Mn, which at site A was only 26-47% of the concentrations at sites B, C, D, and E. Furthermore, honey from site A had significantly lower Cu, K, Mg, Na, and S concentrations than sites B and C. Our findings are consistent with those of Grainger et al. (2021), who showed that concentrations of Ca, K, Mg, Mn, and Na in honeys could be used to differentiate between the regions in New Zealand where the honeys were produced.

Negative correlations between honey elements and honey MGO were most pronounced for Mn, Cu, Mg, and S (Figure 6-3) but were also found for Na (r=-0.69, p≤0.001), Ba (r=-0.61, p≤0.01), K (r=-0.57, p≤0.01), Zn (r=-0.57, p≤0.01), and Al (r=-0.51, p≤0.05). While there is no previous study correlating the concentration of mānuka honey elements and MGO, Alqarni et al. (2014) studied the elemental composition of honeys in Saudi Arabia and included two New Zealand mānuka honeys with differing UMF. The UMF 18 honey in their study had lower concentrations of Mg, Mn, K, and Zn than the UMF 10 honey, but a higher Na concentration. They did not report Cu, S, Ba, and Al concentrations (Alqarni et al., 2014).

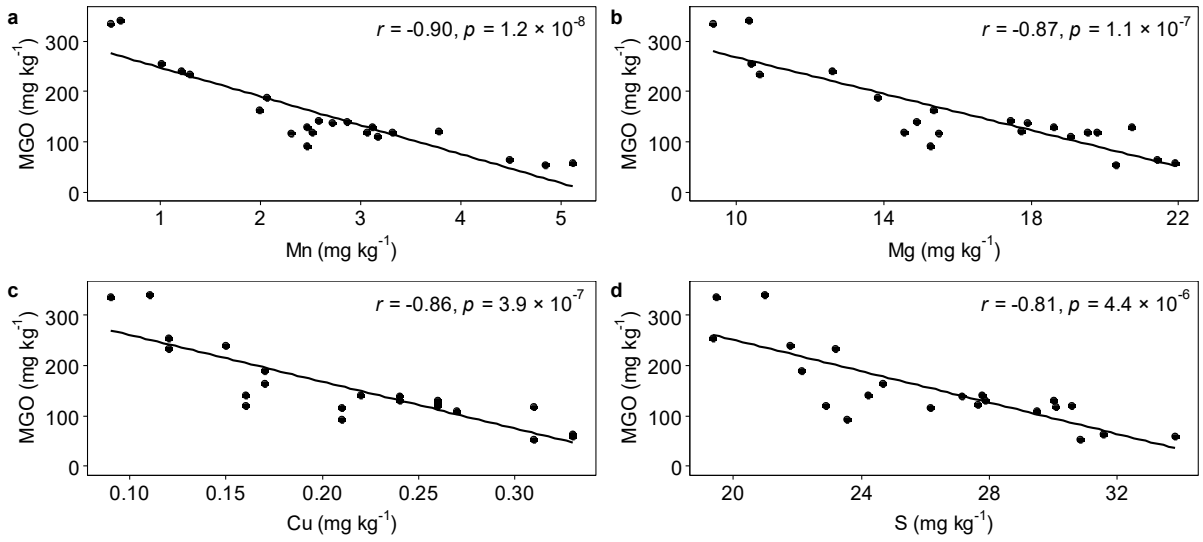

Figure 6-3 Honey MGO versus honey elemental concentrations: (**a**) Mn; (**b**) Mg; (**c**) Cu; (**d**) S. The black lines are linear regression lines. R values are Pearson's correlation coefficients.

6.4.3 Interactions

Contrary to studies on other honeys (Czipa et al., 2017; González-Miret et al., 2005), there were no correlations between the elemental composition of mānuka honey and elemental concentrations in

soils. The only soil factor that correlated with honey MGO was soil NO_3^- (r=-0.88, p≤0.05) (Figure 6-4). NO_3^- was shown to accelerate *L. scoparium* root growth (Gutierrez-Gines et al., 2019; Reis et al., 2017) and could increase the accumulation of Fe, Mn, Zn, and Cu (Klikocka & Marks, 2018). However, soil NO_3^- only correlated positively with plant Co (r=0.49, p≤0.005). It correlated negatively with plant Fe (r=-0.34, p≤0.05). This could indicate a dilution of elements in the plants (Jarrell & Beverly, 1981). There was a positive correlation between the concentrations of K in plants and honeys (r=0.91, p≤0.01). Unlike most other elements tested, K is highly mobile in the plant phloem (Hawkesford et al., 2012). Therefore, elevated K concentrations in the plants may result in higher concentrations in the nectar.

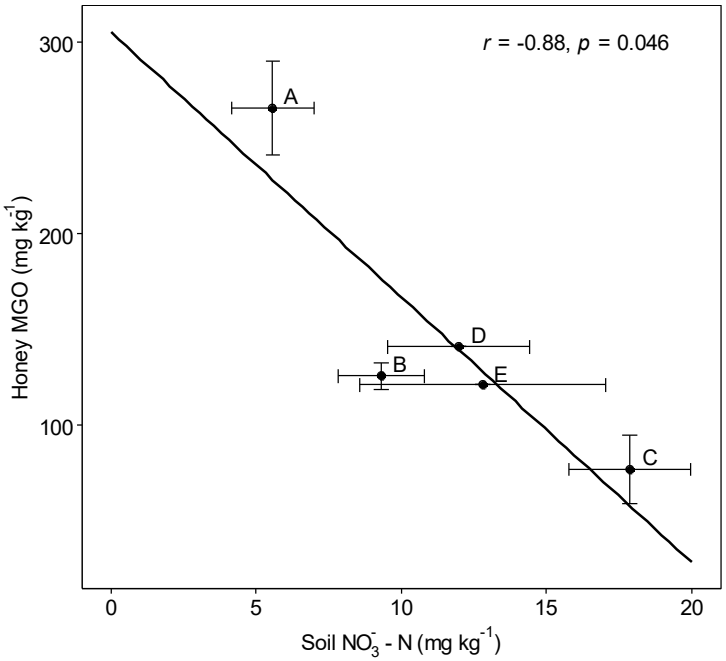

Figure 6-4 Honey MGO versus soil NO_3^--N. Values are means for sites A-E (honey n=1-9, soil n=5-14). Bars represent the standard error of the mean. The black line is a linear regression line. R is the Pearson's correlation coefficient.

The negative correlation between honey MGO and concentrations of Mn, Cu, Mg, S, Na, Ba, K, Zn, and Al does not necessarily indicate that these elements cause a reduction in honey antimicrobial activity. Both the honey MGO and elemental concentrations may correlate with another (unmeasured) factor. Direct effects of these elements in *L. scoparium* nectar on nectar DHA have not been described in the literature to date.

Smallfield et al. (2018) found that DHA is not present in the phloem of *L. scoparium*, which indicates that its production is linked to nectar metabolism. Williams (2019) suggested that DHA production might be associated with dihydroxyacetone phosphate production, which may occur in the floral nectaries (Wenzler et al., 2008). The involved fructose 1, 6-bisphosphate requires Mg, Mn, Zn, or Co for activity (Liang et al., 1993). In contrast, triosephosphate isomerase is inhibited by sulphate, phosphate, and arsenate (Lambeir et al., 1987). Furthermore, in the nectar of Nicotiana spp., manganese superoxide dismutase generates high concentrations of H_2O_2 (Carter & Thornburg, 2004). While H_2O_2 is only present at low levels in *L. scoparium* nectar (Brudzynski et al., 2011), it can react with DHA to glycolate (Maksimovic et al., 2006). This indicates that, while the nectar and DHA production in *L. scoparium* is not understood, element-associated shifts in enzymatic reactions might affect levels of DHA in the nectar.

High concentrations of certain elements in nectar can also negatively affect the foraging behaviour of honeybees. Xun et al. (2018) showed that flowers treated with Zn, Cu, Ni, and Pb reduced the time honeybees spent foraging on these flowers and the amounts of nectar removal. Similarly, Meindl et al. (2014) found that Ni-hyperaccumulation in plants reduced pollinator visitation. High concentrations of Mn and Cu in *L. scoparium* nectar might negatively affect the foraging behaviour of honeybees and lead to more visitation of other nectar sources, which would therefore result in a lower MGO mānuka honey.

Previous studies have highlighted the importance of the botanical origin of honey as the main factor determining its elemental concentrations (Bogdanov et al., 2007). The lack of correlation between mānuka honey and *L. scoparium* foliage composition in this study indicates that honey elemental concentrations might have been diluted with other nectar. When honeybees gather nectar from different floral sources, the composition of the mānuka honey is affected. While mānuka honey has a higher total elemental concentration than non-native New Zealand honeys such as clover, native kāmahi (*Weinmannia racemose*) and rewarewa (*Knightia excelsa*) honeys have particularly high elemental concentrations (Vanhanen et al., 2011). High concentrations of chemical elements in this study could therefore be an indication of mānuka honey contamination with other floral nectar sources. *K. excelsa* is a common nectar contaminant that can result in dilution of mānuka honey (Stephens et al., 2010). Similarly, honeydew honey has high elemental concentrations (Vanhanen et al., 2011). *L. scoparium* is often infested by honeydew-producing scale insects, which leads to sooty mould development (Porter et al., 1998). Sooty mould coverage was found to not directly affect the DHA concentration in *L. scoparium* nectar (Williams et al., 2014). However, a dilution of mānuka honey with collected honeydew might affect the honey's elemental composition. Furthermore, higher soil nutrient levels can be associated with increased exotic weed growth (Prober & Wiehl, 2012), which

might in turn result in a dilution effect of mānuka honey with nectar from exotic species with high nectar elemental concentrations. It is therefore possible that the negative correlation between soil NO_3^- and mānuka honey MGO in our study is a result of accelerated growth of other non-native vegetation.

It is also possible that the elements in our mānuka honey samples did not originate from floral nectar. Elements in honey can derive from environmental pollution, agrochemicals, or natural non-nectar sources that the bees are in contact with when foraging, including air, water, and soil (Grainger et al., 2021; Porrini et al., 2002; Scripca & Amariei, 2021). Elements can also be introduced during honey processing (Pohl et al., 2009). Mānuka honey has a low pH of about 3.5-4.5 (Alvarez-Suarez et al., 2014), which can result in contamination of honey with Zn from galvanised metal or Cr and Ni from stainless steel (Stoewsand et al., 1979; Tong et al., 1975). Furthermore, elemental concentrations in the honey may be changed when beekeepers use other sugar sources as bee feed (Pita-Calvo et al., 2017).

While the chemical elements in mānuka honey may not be causative of honey quality, they may provide a low-cost indication of MGO levels and may be used as a quality indicator for honey. The lack of correlation between plant chemistry and honey MGO concentrations may be due to the large number of plants whence honeybees forage. Future work should test the hypothesis that higher concentrations of Mn, Cu, Mg, S, Na, Ba, K, Zn, and Al are associated with a lower DHA concentration in *L. scoparium* nectar.

Chapter 7

General Discussion

7.1 Native plant survival and growth

Treated Municipal Wastewater (TMW) from New Zealand's wastewater treatment plants (WWTPs) can be irrigated onto native vegetation without impairing plant growth. Chapters 2-5 showed no detrimental effects of high nutrient concentrations on NZ native species. However, there was evidence that the beneficial effects of water and nutrients in the TMW were offset to some extent by species receiving >4000 mm yr^{-1} of TMW irrigation at The Pot (Chapters 3 and 4). Plant height of *Kunzea robusta* and *Leptospermum scoparium* was negatively correlated with foliage Na concentration (Chapter 4). While there were no visible signs of necrosis, this can indicate that Na was reaching growth restricting concentrations at high application rates (2800 kg Na ha^{-1} yr^{-1}). With lower TMW irrigation and Na loading rates (950 kg ha^{-1} yr^{-1}) at Duvauchelle, Na concentrations in the foliage were unaffected by TMW. The Na concentration in *L. scoparium* and *K. robusta* at The Pot was about two times higher than at Duvauchelle. This is consistent with reports of other authors, growing NZ native plants in biowastes, with the limiting factor being salinity or sodicity at high application rates of biosolids (Gutierrez-Gines et al., 2019; Gutierrez-Gines et al., 2017).

At Duvauchelle, the average plant height was 10% higher in TMW irrigated plants after 3.5 years of irrigation (Chapter 2), while at The Pot TMW irrigation increased plant biomass by 39% in a 15-week preliminary trial (Chapter 3). At both sites, dicotyledonous species with medium to large leaves, such as *Griselinia littoralis*, *Pittosporum eugenioides*, and *Coprosma repens* showed the largest growth response. The growth of the myrtaceous species *K. robusta* and *L. scoparium* was unaffected by TMW irrigation at The Pot. However, the growth of *K. robusta* was accelerated by TMW at Duvauchelle, where summers are dry, with 16% of annual rainfall falling from December to February (Macara, 2016). Irrigation with TMW eliminates drought stress, which can be beneficial for the establishment of NZ native vegetation. The normal winter planting window does not apply for TMW irrigated species and losses due to drought, which is becoming more common due to global climate change (Caloiero, 2017), are unlikely to occur. In contrast, high rainfall events, which may be more frequent in climate change scenarios, may result in increased risk of runoff, leaching and erosion in areas irrigated with TMW. However, the diverse root morphologies among NZ native species may reduce some of this risk (Franklin et al., 2019).

7.2 Weed management as critical success factor

While TMW generally improves the growth of NZ-native species, the growth of exotic weed species was increased to a similar or greater level. This is a critical factor in the establishment of NZ native plants in TMW irrigated areas. Fast-growing (semi)woody weeds such as *Phytolacca octandra* and *Solanum* spp. may outcompete and smother NZ native species. If weeding is insufficient, then plant survival may be limited. This was demonstrated at The Pot where survival rates three years after planting were reduced to 6.1% without weed control, which was likely due to accelerated growth of *P. octandra* and *Solanum* spp. (Chapter 3). The use of weed mats and tree guards improved the survival and growth of native seedlings and may facilitate weeding as it allows to easier locate and differentiate native plants.

It is evident from The Pot (Chapter 3) and elsewhere (Chia et al., 2016; Dollery et al., 2018; Smaill et al., 2011) that the use of weed mats and tree guards can significantly reduce the costs of native plantings. The improved survival and growth of native seedlings reduces the need to replace dead plants in subsequent planting seasons. Fast early growth shortens the time until canopy closure, which is a way to suppress the growth of weeds (Sullivan et al., 2009). Until canopy closure is achieved, the use of mechanical and chemical weed control methods can minimise weed competition. At Duvauchelle, weeds were removed every 2-3 weeks with a lawnmower and weed-eater in between native species, and there was no growth of (semi)woody weed species. However, at sites like The Pot, where woody debris from former *Pinus radiata* plantation forests cover the site, such strategies can be limited.

Planting native species into established pasture can be beneficial for the growth of native species, as pasture covers the soil and can reduce the germination of woody weed seeds. Results from The Pot showed that plant survival one year after planting was manifold higher when native seedlings were planted into unmanaged pasture compared to bare soil after *P. radiata* clear-felling (Chapter 3). However, the selection of species of pasture should also be taken into consideration. There were signs of *Holcus lanatus* overgrowing young native seedlings, restricting light reaching their foliage. Tree guards can prevent pasture overgrowth to some extent (Chapter 3). Elsewhere in NZ, nursery crops are used for native restoration projects, as they provide a suitable habitat for NZ natives to grow in their understorey. Even woody weed species such as *Salix* spp. can function as nursery or early successional species for native regeneration (McAlpine et al., 2018), with a rich diversity of NZ natives found in their understorey (McAlpine et al., 2021).

Chemical weed control, even if conducted carefully, possesses some risks. At The Pot (Chapter 3), it was observed that some native plants, mostly *Veronica stricta*, were likely poisoned by pesticide spray drift. The sudden death of some plants, even at distance from the sprayed areas, suggests that this species is particularly sensitive to chemical treatments. However, chemical weed control can be the most cost-effective weed management option (Harrington & Gregory, 2009) and further research is required to determine herbicide tolerance of native species. Alternatively, livestock could be used to control weeds in native plantings, where non-palatable species such as *K. robusta* are planted. However, scratching of animals on some plants at The Pot caused damage to plantings and may reduce plant survival (Chapter 3).

7.3 Contaminant uptake by native plants

The concentrations of N and P in the plant foliage was not affected by TMW irrigation at Duvauchelle (Chapter 2). This is in contrast with reports of luxury N uptake by NZ native species (Franklin et al., 2015). At The Pot (Chapter 4), where N and P loading rates were higher (1540 kg ha^{-1} and 380 kg ha^{-1}, respectively), concentrations of P in the foliage of *K. robusta* and *L. scoparium* were about twice as high as at Duvauchelle. In contrast, the difference was smaller for N, with 10-25% higher foliar N concentrations measured at The Pot compared to Duvauchelle. Elemental concentrations in the plant foliage differed between NZ native species. Results from Duvauchelle showed that myrtaceous and non-myrtaceous species can be separated based on the elemental composition of their foliage. In a pot experiment (Chapter 5), the monocotyledonous species took up significantly more N. Therefore, species selection can be an important tool for nutrient management at TMW irrigated sites.

The concentrations of non-essential trace elements in the plant foliage were within normal ranges (Kirkby, 2012). This is consistent with low application rates of these elements. However, at The Pot there were correlations between TMW irrigation rates and plant concentrations of Cr, As, and Pb in some species (Chapter 4). This demonstrates that native plants can counter the loss of trace element contaminants into the environment through increased uptake. However, nutrients and contaminants in the plant foliage are only removed from the site if plants are harvested. This could be the case where plants are used to generate valuable native products or to produce energy (Delplanque et al., 2013). Elsewhere, *Populus* spp. and *Salix* spp., for example, are well used to remediate trace elements due to the high accumulation thereof in their biomass (Delplanque et al., 2013; Madejón et al., 2004). However, native species with high concentrations of trace elements may not be suitable for TMW irrigation because plants with high trace element uptake may result in the surface contamination of the soil (Robinson et al., 2003). The risk of trace element contamination from TMW irrigation is increased where the contribution of industrial wastewater to TMW is higher (Henze & Comeau, 2008).

Native ecosystems, such as those established at Duvauchelle, support a native fauna of insects, birds, and other organisms (observed in the plots). Curtis et al. (2019) showed that native plantings within agricultural landscapes provide areas of increased abundance of native spiders as well as honeybees, parasitoid wasps, and hoverflies. The functioning of this fauna may be changed by the TMW, and future work could investigate the transfer of nutrients and contaminants from the TMW into native fauna. Plant uptake of contaminants may facilitate their entry into the food chain. While none of the species tested accumulated significant concentrations of non-essential trace elements, the uptake profile of new species should be determined before their use in TMW irrigated plots.

There are a range of contaminants in TMW that were not studied in this thesis, including pathogens, microplastics (Ragoobur et al., 2021), and emerging organic contaminants such as personal care products, pharmaceuticals, and agrichemicals (García et al., 2020), which may have significant impacts on the receiving environment. There is a need for further research in this area to understand possible interactions of such compounds with native ecosystems. Results of Chapter 5 indicate that the abundance of bacteria in the soil is affected by plant species more than fertilisation. The selection of pathogen suppressing species for TMW irrigation sites can reduce risks of pathogen contamination in the environment. Prosser et al. (2016) reported that *L. scoparium* and *K. robusta* can increase the die-off of pathogenic bacteria in soil. Gutierrez-Gines et al. (2021) found that swamp *L. scoparium* and *Metrosideros robusta* have a similar effect.

7.4 Environmental effects of treated municipal wastewater irrigation

At both Duvauchelle and The Pot, rates of P application with TMW irrigation were higher than what would occur on agricultural land (Chapters 2-4; Schipper et al., 2011; Tian et al., 2019). Even if the native vegetation were periodically harvested, the rate of P addition is greater than what would be removed by the plants. Unlike Na, there is limited downward mobility of P (Frossard et al., 2000), indicating that P will accumulate in the soil, which is often limiting long-term TMW irrigation (Hu et al., 2005). Calculations by Gutierrez-Gines et al. (2020) indicate that over a 50-year period, the concentration of P in Pawson silt loam (such as occur on Banks Peninsula, Chapter 2) irrigated with TMW at a rate of 500 mm yr^{-1} and 10 mg P L^{-1} in the TMW would increase to approx. 1349 mg kg^{-1}. This is still within the range found in NZ agricultural soil (McDowell & Condron, 2004). Over longer time periods, P will increase further, and this may result in either P entering waterways via runoff or even leaching (Lizarralde et al., 2021) as well as P toxicity of sensitive species, as demonstrated by some Australian members of the Myrtaceae family (Lambers et al., 2013). It should be noted that no P toxicity was observed for NZ native species, even when grown under high P condition (Olsen P >50 mg kg^{-1}, Chapter 4, Taylor et al. (2016)). Due to the strong adsorption of P in the soil, applications of P

fertilisers above plant requirements are necessary, which means that P is accumulating in agricultural soil worldwide (Simpson et al., 2011). Removal of excess P from TMW would reduce P accumulation in land application systems and potentially enable the P to be beneficially reused elsewhere (Chrispim et al., 2019).

Treated municipal wastewater was irrigated onto land without inducing severe soil degradation in the short-term. Na accumulation in the topsoil was limited and excess Na leached through the soil profile. This is consistent with results of Vogeler (2009), who showed that soil physical properties at The Pot and in a Silt Loam in Taupō were not impaired by TMW irrigation, and soil aggregate stability and infiltration rates were improved. Similarly, Menneer et al. (2001) reported that macropore flow at high moisture content can offset negative effects of high Na effluent irrigation on soil structure. In other countries, particularly drier regions, higher Na loadings in the TMW and low water fluxes through the soil caused by low rainfall and high evapotranspiration, can result in either clay dispersion, high soil Na concentrations, and high salinity (Qian & Mecham, 2005). In such situations, soil fertility can be (partially) restored by the occasional application of gypsum ($CaSO_4$) (Lizarralde et al., 2021). However, such situations are less likely to occur in NZ because of the relatively high rainfall: evapotranspiration ratio (Tanji, 1997). Nevertheless, regular soil testing would ensure that salinity and sodicity remain in an acceptable range.

Concentrations of other cations, namely Ca, Mg, and K, in the TWM are likely to have a negligible effect on soil stability in the systems tested. While K can have a similar effect to Na, replacing cations in clay minerals leading to aggregate instability, this is small compared to Na (Marchuk & Marchuk, 2018). At Duvauchelle, soil Ca and Mg concentrations were increased by TMW irrigation (Chapter 2), which can be beneficial in reducing the accumulation of Na in the soil. Trace element contaminants, such as Cd, Cu, Ni, Pb, and Zn, in the TMW of Duvauchelle and Levin were within the FAO and ANZECC limits for wastewater quality for irrigation (ANZECC, 2000; Pescod, 1992) and lower than drinking water standards (Ministry of Health, 2018). The application rates of these elements were lower than what would occur with the repeated application of superphosphate fertilisers or other agrichemicals (Alloway, 2013). Trace elements in wastewater are associated with suspended solids and usually accumulate in the sludge (Karvelas et al., 2003). The beneficial reuse of biosolids is therefore often limited by high trace element concentrations (Singh & Agrawal, 2007). However, the concentration of trace element contaminants is usually also higher from industrial wastewater (Sharma et al., 2021), which did not contribute much to the WWTPs in this study.

Nitrogen application rates in this study were 200-1500 kg N ha^{-1} yr^{-1}. Given that native vegetation is not removed, any N taken up by plants will be returned to the soil via leaf fall and plant senescence.

Therefore, excess N that is applied to soil will either accumulate in soil, leach into receiving waters, be volatilised as ammonia (NH_3) if ammonium (NH_4) is applied under high pH conditions, or be denitrified into nitrous oxide (N_2O) or dinitrogen (N_2) (Robertson & Groffman, 2015). The accumulation of N in TMW irrigated soil was small (Chapters 2 and 4) and did not account for all of the N applied. Moreover, Chapter 2 showed that below the rootzone, nitrate (NO_3^-) concentrations under some TMW irrigated species were similar or even lower than under unirrigated plants, indicating that NO_3^- leaching was limited. The concentration of NO_3^- in the soil was affected by plant species and was lower under *Coprosma robusta* than other species. The excess N may have been taken up by the plants, but total uptake could not be quantified because the plants were not harvested. In contrast, Chapter 4 showed that plant uptake could not account for the excess N added. The TMW irrigated soils leached significant amounts of NO_3^-, equivalent to 28-38% of the applied N, and there was no significant difference between *Kunzea robusta* and pasture. This is in contrast with a previous study (Esperschuetz, Balaine, et al., 2017), whereby NO_3^- leaching is lower under *K. robusta* and *L. scoparium* than other species. However, N application rates at The Pot were manifold higher than at Duvauchelle and the lower potential for N uptake, immobilisation or denitrification in the sandy soil likely affected NO_3^- leaching.

7.5 Nitrogen cycling under native plants

The results of this research and previous studies (Esperschuetz, Balaine, et al., 2017; Franklin et al. 2017; Halford et al., 2021) demonstrate that plants affect N cycling in the soil. *Carex secta* and the exotic pasture species *Lolium perenne* had lower abundances of ammonia oxidising bacteria (AOB) in their rootzone than dicotyledonous NZ native species (Chapter 5). This was likely a result of higher N uptake by the monocotyledonous species and the competition with AOB for N (Kaye & Hart, 1997). The abundance of AOB can indicate the potential of a soil for nitrification (Fischer et al., 2013), which may affect leaching of NO_3^-. However, the abundance of AOB does not necessarily correlate with enzyme activity. Further research is required to understand underlying mechanism and enzyme activity as affected by NZ native plants. While the community composition was not analysed in this research, it is possible that when nutrients are applied to soil in the form of TMW, this may change the community composition of AOB. Oved et al. (2001) reported that AOB populations in wastewater irrigated soils were dominated by *Nitrosomonas*-like populations, while AOB populations in soil irrigated with fertiliser-amended water was dominated by *Nitrosospira*-like populations. This was despite equal application rates of N. It is possible that TMW irrigation changed the conditions in favour of one population over the other, or that such bacteria were part of the TMW.

Denitrification is another pathway by which N may have been lost from the system, but there is no information on denitrification from NZ native species. Soana et al. (2021), Castaldelli et al. (2020), and Hofstra and Bouwman (2005) reported that denitrification may account for up to 40% of N added to agricultural systems. However, denitrification rates in TMW irrigated soils show high temporal, spatial, and seasonal variability (Barton et al., 1999). Denitrification occurs at a greater rate under low redox potential (Mohn et al., 2000). Even in an aerobic soil, denitrification may occur from localised low-oxygen zones in the plant root zone (Stevens et al., 1997). The high TMW irrigation rate at The Pot (>4000 mm yr^{-1}) combined with the periodic irrigation (2-3 times per week) results in a low residence time of the N and other contaminants in the rootzone. Moreover, this irrigation pattern is less likely to result in low redox potential conditions than the daily irrigation at Duvauchelle onto a Pawson silt loam, which would limit the potential for dentrification. Similarly, the well-drained sandy soil at The Pot is less likely to have localised low redox potential conditions than the imperfectly drained Pawson silt loam at Duvauchelle (Blume et al., 2016). If denitrification accounts for the excess N added at Duvauchelle and The Pot, then it is critical to determine whether N is lost via benign N_2 or environmentally damaging N_2O. Given the contrasting morphologies and chemistry of NZ native species it is likely that there are significant interspecific differences in how these plants affect denitrification. As it was demonstrated in Chapter 5, plant species was the main factor affecting the abundance of *nirS* and *nosZ* in the soil, the functional genes that encode denitrifying enzymes. The results highlight the importance of species selection to mitigate N losses into the environment. A mass balance assessment of N in the soil-plant system of NZ native species irrigated with TMW would allow better understanding of the relevance of each N transformation process.

7.6 Potential use of native plants for valuable products

Increased growth of native species with nutrient application (Chapters 2-5) indicates that the production of valuable native products could be accelerated by irrigation of TMW. It was shown that the application of biosolids increases *K. robusta* and *L. scoparium* essential oils but does not affect their composition (Seyedalikhani et al., 2019). In contrast, Chapter 6 showed that honey methylglyoxal (MGO) concentration, an indicator for the quality of mānuka (*L. scoparium*) honey, was negatively correlated with soil NO_3^- concentrations. Mānuka honey can provide returns of \$10.80-128 kg^{-1} bulk honey (MPI, 2018), depending primarily on its MGO concentration, which indicates its potential to generate value form TMW irrigated land. However, it is not clear if the production of food from TMW irrigated land would be socially accepted. TMW irrigation can increase the concentration of soil NO_3^- (Chapter 4) and may therefore reduce the quality of mānuka honey where such is produced from TMW irrigated land. However, Williams et al. (2014) reported no effect of soil fertility on *L. scoparium* nectar

composition. There is evidence that increased soil fertility accelerates the growth of exotic weeds (Chapter 3, Prober and Wiehl (2012)), which can result in a dilution effect of mānuka honey with nectar from other floral sources (Stephens et al., 2010).

The growth of *Phormium tenax* and *Phormium cookianum* was accelerated by TMW irrigation (Chapter 2). *Phormium* spp. could be grown at TMW irrigation sites to produce fibres for textile and non-textile purposes (McGruddy, 2006). In addition, *P. tenax* may be utilised for medicinal purposes, as the exudate at the leaf base was historically used to treat wounds, burns, and other health conditions (Wehi & Clarkson, 2007). TMW also had growth accelerating effects on *Podocarpus laetus* (Chapter 2). This and other conifer species may be grown in TMW land treatment systems to produce valuable native timber (Dodd & Ritchie, 2007). Potentially, other native species, such as *Olearia paniculata*, *Coprosma robusta*, among others, may be used for stock fodder. There were significant interspecific differences in the elemental composition of the plant species (Chapters 2, 4, and 5), some of which may supplement essential micronutrients to stock (Dickinson et al., 2015).

7.7 Conclusions and Recommendations

- The irrigation of TMW onto NZ native plants at a rate of 1000 mm yr^{-1} improves plant survival during the establishment phase and has a positive or neutral effect on growth.

- TMW irrigation at rates >4000 mm yr^{-1} is likely to result in excessive weed growth and excessive nitrate leaching, regardless of the plant species. Tree guards can improve native plant survival and health but weed control will be required until canopy cover is achieved.

- Application of TMW up to 1000 mm yr^{-1} (approx. 200 kg ha^{-1} N equivalent) is unlikely to lead to excessive NO_3^- leaching or the unacceptable accumulation of P, Na, and trace elements in soil.

- There were significant differences in the fluxes of nutrients and contaminants between plant species. This indicates that location-based species can be selected to mitigate contaminant fluxes.

- Irrigation of 1000 mm yr^{-1} did not increase the concentration of nutrients and trace element contaminants in the plant tissue. At irrigation rates >4000 mm yr^{-1} foliar Na concentrations were negatively correlated with plant height of some myrtaceous species.

- Plant uptake of contaminants may facilitate their entry into the food chain. While none of the species tested accumulated significant concentrations of non-essential trace elements, the uptake profile of new species should be determined before their use in TMW irrigated plots.

- While plant uptake may account for a significant proportion of TMW-applied nutrients during the establishment phase, these nutrients will ultimately be returned to the soil during plant senescence. Nutrients could be removed by harvesting the biomass of the plants periodically. Nutrient losses from unmanaged native ecosystems will likely be greater than from cut-and-carry pasture.

- Land application of TMW may be used to enhance the abundance of native plant species in the landscape while mitigating contamination of surface and groundwaters that would occur if TMW is discharged directly into water.

- There is an opportunity to test the response of locally endangered species to TMW with a view to establish these in TMW irrigated native plantings.

- The production of non-food species, such as those that generate bioactive compounds, fibres, or timber, may be a viable option for the land application of TMW in countries where TMW irrigation on agricultural land is socially unacceptable.

- Internationally, the reuse of TMW may be manifold more valuable, particularly in arid regions that may otherwise be prone to desertification. In many countries the release of effluent into waterways causes widespread human illness. Were wastewater to be applied to native ecosystems, valuable native products may improve local economies.

- Potentially, much of the TMW generated in NZ and internationally could be beneficially reused to establish valuable native ecosystems. The next phase of this research will be to identify suitable areas for irrigation, considering land use, slope, and proximity to the WWTP.

Appendix A

Supplementary Material

Chapter 2

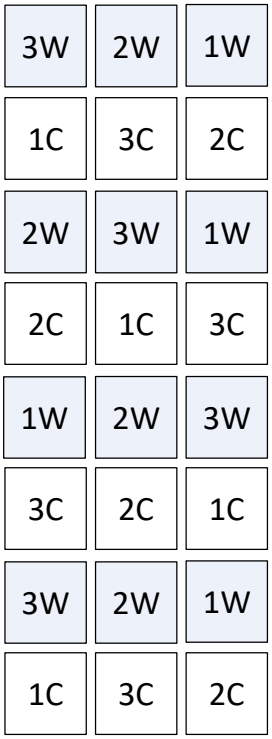

Figure A-1 Experimental design of the field trial in Duvauchelle. W: irrigated with treated municipal wastewater (TMW) at 1000 mm yr^{-1}. C: control, no irrigation. Eleven New Zealand native species were divided into three vegetation types: (1) *Leptospermum scoparium*, *Kunzea robusta,* (2) *Olearia paniculata*, *Pseudopanax arboreus*, *Coprosma robusta*, *Podocarpus laetus*, and (3) *Griselinia littoralis*, *Pittosporum eugenioides*, *Cordyline australis*, *Phormium tenax, Phormium cookianum.*

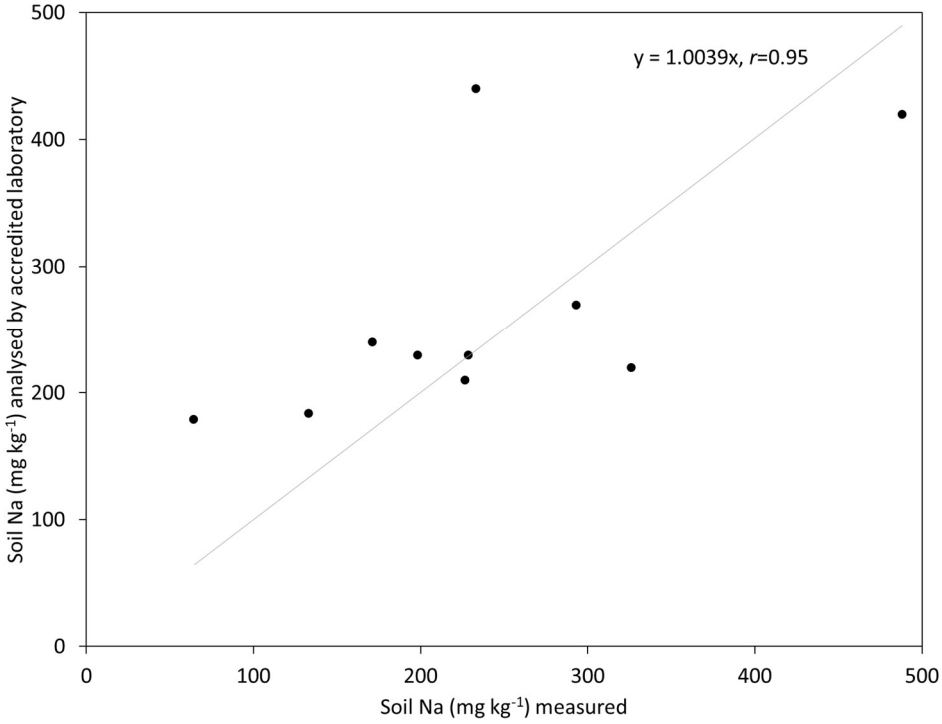

Figure A-2 Soil Na concentration measured vs. Soil Na concentration analysed by accredited laboratory. Linear regression and Pearson's correlation coefficient (*r*) are shown.

Table A-1 Concentrations of 2M KCl-extractable mineral N in the soil per species, for TMW irrigated and control plots combined.

Species	NO_3^--N (mg kg^{-1})	NH_4^+-N (mg kg^{-1})
Cordyline australis	4.3 ± 0.63^{ab}	8.3 ± 1.1^{a}
Coprosma robusta	3.1 ± 0.55^{a}	9.0 ± 1.3^{a}
Kunzea robusta	6.1 ± 1.1^{b}	9.0 ± 1.5^{a}
Leptospermum scoparium	4.8 ± 0.82^{ab}	7.0 ± 1.1^{a}
Phormium tenax	4.7 ± 0.66^{ab}	8.9 ± 1.2^{a}

Mean ± standard error (*n*=40). Different letters indicate significant differences between species at p≤0.05 according to Tukey's HSD test.

Table A-2 Soil pH and total elemental concentrations at five depths (mg kg^{-1} unless otherwise indicated).

	Treatment	0-5 cm	10-20 cm	25-35 cm	40-50 cm	55-65 cm
pH	Control	5.5 ± 0.04	5.6 ± 0.04	5.9 ± 0.04	6.1 ± 0.05	6.1 ± 0.06
	TMW	5.7 ± 0.05	5.7 ± 0.04	6 ± 0.05	6.1 ± 0.07	6.2 ± 0.08
Al	Control	16417 ± 336	16054 ± 394	16964 ± 413	17484 ± 499	17684 ± 562
	TMW	16289 ± 368	15714 ± 314	16546 ± 308	17212 ± 466	17789 ± 573
As	Control	**2.7 ± 0.07**	2.3 ± 0.11	**1.9 ± 0.07**	**1.6 ± 0.06**	1.7 ± 0.05
	TMW	**3.1 ± 0.09***	2.7 ± 0.15	**2.1 ± 0.09***	**1.9 ± 0.07***	1.8 ± 0.09
Ca	Control	4059 ± 90	3997 ± 105	4009 ± 115	3825 ± 121	3627 ± 106
	TMW	4284 ± 110	4224 ± 110	4190 ± 89	3903 ± 101	3725 ± 107
Cd	Control	14 ± 0.88	15 ± 1.3	16 ± 1.2	14 ± 1.1	16 ± 1.2
(µg kg^{-1})	TMW	13 ± 0.62	15 ± 0.74	13 ± 1.4	14 ± 1.6	14 ± 1.5
Cr	Control	12 ± 0.28	11 ± 0.26	12 ± 0.31	12 ± 0.3	11 ± 0.29
	TMW	12 ± 0.31	12 ± 0.34	12 ± 0.33	13 ± 0.29	12 ± 0.33
Cu	Control	5.8 ± 0.16	5.7 ± 0.2	5.1 ± 0.26	5.4 ± 0.25	5.8 ± 0.24
	TMW	6.9 ± 0.62	6.2 ± 0.39	4.8 ± 0.2	5.2 ± 0.25	5.7 ± 0.26
Fe	Control	14379 ± 503	15539 ± 633	16133 ± 704	16711 ± 778	17875 ± 824
	TMW	14701 ± 446	15494 ± 463	15447 ± 644	16858 ± 922	17422 ± 822
Li	Control	**17 ± 0.29**	**18 ± 0.32**	**20 ± 0.46**	**20 ± 0.47**	**20 ± 0.38**
	TMW	**18 ± 0.32***	**20 ± 0.51***	**23 ± 0.48***	**23 ± 0.46***	**22 ± 0.69***
Mn	Control	317 ± 13	439 ± 30	502 ± 45	252 ± 23	193 ± 15
	TMW	384 ± 32	493 ± 50	580 ± 49	345 ± 42	227 ± 23
Ni	Control	5.6 ± 0.15	5.9 ± 0.14	6.1 ± 0.18	6.2 ± 0.17	6.3 ± 0.19
	TMW	5.8 ± 0.12	6 ± 0.09	6.1 ± 0.12	6.4 ± 0.22	6.5 ± 0.21
Pb	Control	17 ± 1.4	13 ± 0.83	9.1 ± 0.35	**7.9 ± 0.19**	**7.4 ± 0.21**
	TMW	18 ± 0.99	14 ± 0.83	10 ± 0.53	**9.1 ± 0.22***	**8.3 ± 0.32***
Zn	Control	60 ± 2.7	59 ± 3.2	70 ± 16.3	56 ± 5.2	52 ± 2.1
	TMW	63 ± 1.9	59 ± 1.4	57 ± 1.6	53 ± 1.9	51 ± 1.9

Mean ± standard error (n=20). Values that differ significantly between treatments ($p \leq 0.05$) according to two-tailed unpaired t-test are indicated in bold and followed by asterisks (*).

Table A-3 Soil $Ca(NO_3)_2$ extractable element concentrations and Olsen P at five depths.

	Treatment	0-5 cm	10-20 cm	25-35 cm	40-50 cm	55-65 cm
Al	Control	**16851 ± 2277**	14172 ± 2294	5020 ± 977	3298 ± 742	4325 ± 1613
(mg kg^{-1})	TMW	**8870 ± 1909***	12271 ± 2272	6703 ± 2051	7638 ± 3494	7957 ± 3838
As	Control	8.9 ± 0.73	8.6 ± 0.87	4.6 ± 0.78	6.9 ± 1.9	9.7 ± 3.6
(µg kg^{-1})	TMW	8.0 ± 0.85	7.4 ± 0.96	5.4 ± 1.0	9.5 ± 3.4	14 ± 5.4
Cd	Control	**14 ± 1.0**	11 ± 1.2	6.7 ± 1.3	2.1 ± 0.42	1.3 ± 0.27
(µg kg^{-1})	TMW	**11 ± 1.1***	10 ± 0.65	6.2 ± 0.76	2.2 ± 0.30	0.9 ± 0.20
Co	Control	**152 ± 16**	74 ± 8.9	36 ± 7.0	32 ± 6.8	33 ± 5.2
(µg kg^{-1})	TMW	**93 ± 9.3***	74 ± 10	32 ± 8.7	22 ± 3.5	21 ± 5.3
Cr	Control	4.1 ± 1.0	3.6 ± 1.5	2.8 ± 1.1	2.3 ± 1.4	3.7 ± 2.3
(µg kg^{-1})	TMW	4.5 ± 1.9	5.8 ± 2.7	5.6 ± 2.1	4.6 ± 1.8	1.1 ± 0.37
Cu	Control	248 ± 73	529 ± 152	171 ± 48	336 ± 168	231 ± 71
(µg kg^{-1})	TMW	957 ± 399	226 ± 68	192 ± 60	249 ± 70	477 ± 148
Mn	Control	40 ± 3.3	22 ± 2.4	10 ± 1.8	3.1 ± 0.47	2.3 ± 0.35
(µg kg^{-1})	TMW	39 ± 4.1	25 ± 2.7	14 ± 1.9	5.0 ± 0.92	3.2 ± 0.79
Ni	Control	175 ± 13	218 ± 14	133 ± 12	63 ± 8.5	**46 ± 5.4**
(µg kg^{-1})	TMW	138 ± 16	201 ± 16	132 ± 16	52 ± 7.0	**30 ±3.2***
Pb	Control	12 ± 3.4	21 ± 6.0	7.6 ± 2.3	14 ± 5.8	12 ± 3.1
(µg kg^{-1})	TMW	25 ± 9.0	23 ± 14.7	8.4 ± 2.2	13 ± 4.1	22 ± 6.3
Zn	Control	2022 ± 248	**1565 ± 253**	1318 ± 833	663 ± 308	326 ± 71
(µg kg^{-1})	TMW	2221 ± 354	**996 ± 99***	498 ± 59	458 ± 171	406 ± 102
Olsen P	Control	14 ± 1.2	8.6 ± 1.6	8.6 ± 1.3	9.5 ± 1.3	18 ± 1.6
(mg kg^{-1})	TMW	17 ± 2.7	8.7 ± 1.3	6.8 ± 0.47	8.2 ± 0.80	15 ± 1.8

Mean ± standard error (n=20). Values that differ significantly between treatments ($p \leq 0.05$) according to two-tailed unpaired t-test are indicated in bold and followed by asterisks (*).

Table A-4 Elemental concentrations in the foliage of five plant species; *Cordyline australis*, *Coprosma robusta*, *Kunzea robusta*, *Leptospermum scoparium*, and *Phormium tenax*. Control: no irrigation, TMW: irrigation with treated municipal wastewater at 1000 mm yr^{-1}.

	C. australis		*C. robusta*		*K. robusta*		*L. scoparium*		*P. tenax*	
	Control	TMW	Control	TMW	control	TMW	control	TMW	control	TMW
C (%)	47	46	46	43	50	50	52	50	47	46
	± 2.4	± 0.48*	± 1.4	± 0.09	± 0.12	± 0.43	± 0.34	± 1.7	±0.16	± 0.65
Al	40	44	385	344	117	130	153	257	36	37
(mg kg^{-1})	± 4.6	± 3.1	±47	± 54	± 7.8	± 32	± 19	± 61	± 2.9	± 2.1
As	22	24	98	91	175	134	119	121	22	30
(µg kg^{-1})	± 3.1	± 4.2	± 10	± 16	± 43	± 12	± 17	± 10	± 3.5	± 8.3
Cd	288	254	205	317	22	11	10	7.7	33	47
(µg kg^{-1})	± 19	± 40	±57	± 118	± 5.4	± 2.2	± 3.3	± 0.95	± 2.7	± 13
Cd	288	254	205	317	22	11	10	7.7	33	47
(µg kg^{-1})	± 19	± 40	±57	± 118	± 5.4	± 2.2	± 3.3	± 0.95	± 2.7	± 13
Co	0.77	1.3	0.33	0.26	0.42	0.49	0.80	0.64	1.6	0.81
(mg kg^{-1})	± 0.04	± 0.19	±0.11	± 0.03	± 0.03	± 0.04	± 0.08	± 0.07	± 0.62	± 0.20
Cr	0.53	0.51	0.34	0.37	0.51	0.38	0.82	0.80	0.55	0.49
(mg kg^{-1})	± 0.06	± 0.03	± 0.07	± 0.03	± 0.11	± 0.02	± 0.15	± 0.07	± 0.07	± 0.04
Ni	1.1	1.0	0.41	0.53	3.1	3.1	1.2	1.0	0.39	0.30
(mg kg^{-1})	± 0.17	± 0.33	± 0.03	± 0.09	± 0.31	± 0.97	± 0.30	± 0.23	± 0.12	± 0.03
Ni	1.1	1.0	0.41	0.53	3.1	3.1	1.2	1.0	0.39	0.30
(mg kg^{-1})	± 0.17	± 0.33	± 0.03	± 0.09	± 0.31	± 0.97	± 0.30	± 0.23	± 0.12	± 0.03
Pb	20	22	36	34	57	47	70	87	18	17
(µg kg^{-1})	± 2.8	± 5.0	± 4.1	± 7.6	±11	± 5.7	± 12	± 8.0	± 1.8	± 4.9

Mean ± standard error (n=4). Values that differ significantly between treatments ($p \leq 0.05$) according to two-tailed unpaired t-test are in bold and followed by asterisks (*).

Chapter 4

Table A-5 Location of the lysimeters at The Pot.

Lysimeter	Species	Coordinates
1	Pasture	S 40º 37.729' E 175º 10.785'
2	*K. robusta*	S 40º 37.729' E 175º 10.792'
3	Pasture	S 40º 37.725' E 175º 10.789'
4	*K. robusta*	S 40º 37.723' E 175º 10.794'
5	Pasture	S 40º 37.699' E 175º 10.794'
6	*K. robusta*	S 40º 37.698' E 175º 10.793'
7	*K. robusta*	S 40º 37.686' E 175º 10.800'
8	Pasture	S 40º 37.685' E 175º 10.804'

Table A-6 Soil profile description for the lysimeter site at The Pot (sand plain, well drained with no mottles within 1 m), from McLeod (2018).

Ap	0-10 cm	Dark brown (10YR 3/3) sand; very friable; single grain structure; indistinct boundary
Bw	10-22 cm	Olive brown (2.5Y 4/4) sand; very friable; single grain structure; diffuse boundary
BC	22-60 cm	Olive brown (2.5Y 4/3) sand; very friable; single grain structure; diffuse boundary
C	60-100 cm	Light olive brown (2.5Y 5/3) sand; very friable; single grain structure

Table A-7 Drainage and nitrate leaching in the lysimeters planted with *K. robusta* and pasture.

	K. robusta	Pasture
Drainage (mm d^{-1})	1.0 ± 0.25	1.4 ± 0.35
Nitrate leaching (mg NO_3^--N d^{-1})	2.0 ± 0.78	2.7 ± 1.4

Mean ± standard error (*n*=6).

Chapter 5

Table A-8 qPCR-reaction mix (15 µL) for the quantification of total bacteria, total archaea, bacterial and archaeal *amoA*, *nirK*, *nirS*, and *nosZ*.

Reagent	Volume (µL)						
	Eub338/ Eub518	Arch-967F/ Arch-1060R	amoA1F/ amoA2R	Arch-amoAF/ Arch-amoAR	nirK876/ nirK1040	nirScd3aF/ nirSR3cd	nosZ2F/ nosZ2R
2x SsoAdvance Universal Inhibitor-Tolerant SYBR® Green Supermix (BioRad)[a]	7.5	7.5	7.5	7.5	7.5	7.5	7.5
BSA (5mg/mL)	0.3	0.3	0.3	0.3	0.3	0.3	0.3
Forward primer (10 pmol/µL)	0.25[b]	0.25	0.25	0.25	0.3[c]	0.3	0.3
Reverse primer (10 pmol/µL)	0.25	0.25	0.25	0.25	0.3	0.3	0.3
DNAse/RNAse free H_2O	1.7	1.7	1.7	1.7	1.6	1.6	1.6
Template DNA (1ng/µL)	5	5	5	5	5	5	5

[a] Contains antibody-mediated hot-start Sso7d fusion polymerase, SYBR green I dye, dNTPs, $MgCl_2$, enhancers, and stabilisers.
[b] equals 167 nM primer concentration in mix.
[c] equals 200 nM primer concentration in mix.

Table A-9 Thermal protocols for qPCR of bacterial and archaeal 16S rRNA, nitrification genes (AOB *amoA*, AOA *amoA*), and denitrification genes (*nirS*, *nirK*, *nosZ*).

PCR Step	Eub338/ Eub518	Arch-967F/ Arch-1060R	amoA1F/ amoA2R	Arch-amoAF/ Arch-amoAR	nirScd3aF/ nirSR3cd	nirK876/ nirK1040	nosZ2F/ nosZ2R
Initial denaturation	95°C/10 min	95°C/10 min	95°C/10 min	95°C/10 min	95°C/10 min	95°C/10 min	95°C/10 min
Denaturation	95°C /15 s	95°C /15 s	95°C /15 s	95°C /15 s	95°C /15 s	95°C /15 s	95°C /15 s
Annealing	59°C /20 s	60°C /25 s	63°C /25 s	64°C /10 s	60°C /20 s	61°C /10 s	61°C /25 s
Elongation and signal detection[a]	72°C /15 s	72°C /20 s	72°C /20 s	72°C /20 s	72°C /20 s	72°C /20 s	72°C /20 s
Number of cycles	35	38	40	40	45	45	45
Melting Curve[b]	59-95°C	60-95°C	63-95°C	64-95°C	59-95°C	63-95°C	63-95°C

[a] Fluorescence detection of SYBR green with CFX Connect Real-Time PCR System (Bio-Rad, Hercules, CA, USA).
[b] Increments of 1 °C

Chapter 6

Table A-10 Chemical characterization of soil at sites A-E.

site	A	B	C	D	E
n=	*10*	*14*	*5*	*5*	*5*
NH_4^+-N	11 ± 3.9^a	17 ± 6.7^a	2.6 ± 0.61^a	11 ± 1.8^a	15 ± 4.6^a
NO_3^--N	5.6 ± 1.42^a	9.3 ± 1.5^{ab}	18 ± 2.1^b	12 ± 2.5^{ab}	13 ± 4.26^{ab}
pH	4.5 ± 0.11^{ab}	4.5 ± 0.09^{ab}	5.0 ± 0.06^b	4.8 ± 0.37^{ab}	4.2 ± 0.05^a
N (%)	0.36 ± 0.06^a	0.69 ± 0.03^b	0.25 ± 0.01^a	0.65 ± 0.18^b	0.32 ± 0.05^a
C (%)	4.9 ± 0.95^{ab}	11 ± 0.74^c	2.6 ± 0.08^b	7.5 ± 1.9^{ac}	5.2 ± 0.57^a
C/N	13 ± 0.33^b	16 ± 0.5^a	11 ± 0.33^c	12 ± 0.35^{bc}	17 ± 0.85^a
Al	31559 ± 2339^a	51646 ± 2115^b	30685 ± 610^a	40267 ± 3888^{ab}	36919 ± 5195^a
As	3.6 ± 0.66^b	4.0 ± 0.17^b	4.2 ± 0.23^b	4.0 ± 0.55^b	2.1 ± 0.26^a
B	27 ± 0.7^a	35 ± 1.3^b	47 ± 1.0^c	42 ± 5.0^{bc}	22 ± 3.00^a
Ca	1854 ± 337^a	1617 ± 126^a	5171 ± 75^c	4036 ± 1193^{bc}	1848 ± 135^{ab}
Cd	0.08 ± 0.01^a	0.22 ± 0.02^b	0.18 ± 0.02^{bc}	0.11 ± 0.02^{ac}	nd
Cr	21 ± 0.72^a	31 ± 2.5^b	26 ± 0.45^{ab}	28 ± 2.2^{ab}	21 ± 1.6^a
Cu	5.8 ± 0.72^a	11 ± 1.3^b	13 ± 0.27^b	5.7 ± 0.90^a	5.2 ± 0.93^a
Fe	13064 ± 316^a	19799 ± 368^b	13249 ± 60.7^a	14339 ± 1138^a	12427 ± 1086^a
K	3168 ± 169^a	3339 ± 314^a	8870 ± 521^b	9057 ± 1410^b	4860 ± 161^a
Li	24 ± 1.7^{ab}	36 ± 1.9^c	35 ± 0.28^{bc}	80 ± 7.1^d	19 ± 3.6^a
Mg	3910 ± 250^{ab}	4699 ± 200^{bc}	5499 ± 43^c	4841 ± 338^{bc}	3501 ± 395^a
Mn	236 ± 28^a	274 ± 23^a	356 ± 6.2^a	386 ± 121^a	185 ± 36^a
Na	276 ± 17^{ab}	217 ± 5.8^a	267 ± 7.0^{ab}	318 ± 54^b	224 ± 20^{ab}
Ni	8.0 ± 0.38^a	7.2 ± 0.39^a	15 ± 0.23^b	8.2 ± 0.60^a	7.5 ± 1.2^a
P	452 ± 59^b	664 ± 49^c	560 ± 27^{bc}	878 ± 140^c	152 ± 16^a
Pb	12 ± 0.52^a	19 ± 0.45^b	22 ± 1.2^{bc}	26 ± 1.9^c	11 ± 1.9^a
S	404 ± 76^a	664 ± 42^c	204 ± 12^b	892 ± 274^c	311 ± 33^{ab}
Sr	19 ± 1.3^b	24 ± 1.5^b	24 ± 0.72^{ab}	43 ± 12^a	37 ± 1.1^a
Zn	44 ± 2.0^a	45 ± 2.5^a	81 ± 1.5^b	81 ± 8.2^b	33 ± 5.7^a

Mean ± standard error. Different letters indicate significant differences between sites ($p \leq 0.05$) according to Tukey's HSD test. Values are in mg kg^{-1} unless otherwise indicated.
nd=not detectable

Table A-11 Soil exchangeable element concentrations at sites A-E.

site	A	B	C	D	E
n=	*10*	*14*	*5*	*5*	*5*
Al	121 ± 18^{ab}	250 ± 37^{a}	21 ± 5.3^{c}	84 ± 37^{bc}	154 ± 16^{ab}
Cd	<0.01 ± 0.00	<0.02 ± 0.00	<0.01 ± 0.00	nd	nd
Co	0.19 ± 0.05^{ac}	0.08 ± 0.03^{b}	0.59 ± 0.07^{c}	0.39 ± 0.16^{ac}	0.13 ± 0.04^{ab}
Cr	<0.02 ± 0.00^{a}	0.02 ± 0.00^{a}	0.00 ± 0.00^{b}	0.02 ± 0.01^{a}	0.01 ± 0.00^{a}
Cu	<0.03 ± 0.01^{ab}	0.02 ± 0.00^{a}	0.05 ± 0.01^{b}	0.02 ± 0.00^{a}	0.01 ± 0.00^{a}
Fe	28 ± 8.6^{a}	96 ± 23^{b}	2.6 ± 0.46^{c}	14 ± 9.8^{ac}	43 ± 13^{ab}
Li	0.08 ± 0.02^{ab}	0.06 ± 0.01^{a}	0.18 ± 0.01^{bc}	0.31 ± 0.06^{c}	0.10 ± 0.02^{abc}
Mg	223 ± 60^{ab}	155 ± 18^{a}	179 ± 14^{ab}	460 ± 162^{b}	262 ± 44^{ab}
Mn	26 ± 5.6^{a}	22 ± 2.3^{a}	22 ± 3.4^{a}	66 ± 21^{a}	31 ± 11^{a}
Na	73 ± 12^{a}	59 ± 4.4^{a}	26 ± 3.0^{b}	105 ± 27^{a}	96 ± 19^{a}
Ni	0.19 ± 0.04^{ab}	0.12 ± 0.01^{a}	0.39 ± 0.06^{b}	0.13 ± 0.05^{a}	0.11 ± 0.01^{ab}
P	2.9 ± 1.1^{a}	2.4 ± 0.22^{ab}	1.3 ± 0.11^{a}	5.0 ± 1.1^{b}	1.4 ± 0.21^{a}
Zn	1.6 ± 0.60^{a}	1.7 ± 0.38^{a}	2.3 ± 0.13^{a}	1.5 ± 0.47^{b}	1.5 ± 0.30^{a}

Mean ± standard error. Different letters indicate significant differences between sites ($p \leq 0.05$) according to Tukey's HSD test. Values are in mg kg^{-1}.
nd=not detectable

Table A-12 *Leptospermum scoparium* foliage elemental concentrations at sites A-E.

site	A	B	C	D	E
n=	*10*	*15*	*5*	*5*	*5*
N (%)	1.0 ± 0.07[a]	0.99 ± 0.06[a]	1.03 ± 0.06[a]	0.97 ± 0.07[a]	1.1 ± 0.04[a]
C (%)	49 ± 0.31[a]	49 ± 0.24[a]	50 ± 0.39[a]	49 ± 0.33[a]	49 ± 0.30[a]
C/N	49 ± 3.4[a]	52 ± 2.9[a]	49 ± 2.4[a]	52 ± 4.2[a]	46 ± 2.1[a]
Al	212 ± 61[a]	105 ± 22[a]	26 ± 4.5[b]	132 ± 29[a]	72 ± 15[ab]
As	<0.16 ± 0.05[a]	<0.24 ± 0.04[a]	<0.12 ± 0.05[a]	0.29 ± 0.06[a]	0.21 ± 0.03[a]
B	24 ± 1.6[a]	21 ± 1.3[a]	19 ± 2.3[a]	19 ± 1.1[a]	22 ± 1.1[a]
Ca	4610 ± 466[a]	4584 ± 349[a]	4102 ± 365[a]	5640 ± 539[a]	4548 ± 524[a]
Cd	<0.05 ± 0.01[a]	<0.06 ± 0.02[a]	<0.04 ± 0.01[a]	<0.07 ± 0.02[a]	<0.03 ± 0.02[a]
Co	<0.05 ± 0.01[a]	<0.01 ± 0.00[b]	0.08 ± 0.02[a]	<0.04 ± 0.01[ab]	0.12 ± 0.05[a]
Cr	4.5 ± 2.2[a]	0.63 ± 0.08[b]	0.43 ± 0.16[b]	1.1 ± 0.28[ab]	0.81 ± 0.10[ab]
Cu	4.1 ± 0.50[a]	3.9 ± 0.32[a]	3.3 ± 0.42[a]	2.7 ± 0.25[a]	4.6 ± 0.60[a]
Fe	214 ± 57[c]	84 ± 16[ab]	36 ± 4.3[a]	128 ± 29[bc]	66 ± 12[ab]
K	3887 ± 168[bc]	3958 ± 124[b]	5015 ± 342[d]	3080 ± 159[ac]	3000 ± 289[a]
Li	0.29 ± 0.09[a]	0.08 ± 0.02[b]	0.22 ± 0.05[a]	0.39 ± 0.06[a]	0.14 ± 0.03[ab]
Mg	1009 ± 40[c]	927 ± 36[c]	1094 ± 106[ac]	1468 ± 102[b]	1397 ± 112[ab]
Mn	322 ± 53[bc]	169 ± 13[b]	74 ± 8.7[d]	448 ± 115[ac]	874 ± 165[a]
Na	2435 ± 212[ab]	2054 ± 154[abc]	1634 ± 153[bc]	1415 ± 163[c]	2819 ± 252[a]
Ni	4.3 ± 1.1[a]	1.8 ± 0.31[a]	2.6 ± 0.34[a]	3.7 ± 1.8[a]	4.1 ± 1.8[a]
P	601 ± 47[ab]	561 ± 30[a]	633 ± 48[ab]	771 ± 84[b]	429 ± 40[a]
S	934 ± 49[b]	926 ± 59[b]	685 ± 47[b]	814 ± 74[b]	1254 ± 103[a]
Sr	24 ± 3.0[b]	35 ± 2.5[ab]	21 ± 2.3[b]	37 ± 5.1[ab]	43 ± 9.8[a]
Zn	21 ± 1.8[a]	23 ± 1.7[a]	17 ± 1.9[a]	26 ± 5.5[a]	25 ± 2.1[a]

Mean ± standard error. Different letters indicate significant differences between sites ($p \leq 0.05$) according to Tukey's HSD test. Values are in mg kg^{-1} unless otherwise indicated.
nd=not detectable
< actual mean is lower due to sample concentrations being below detection limit

Appendix B

Report to the Christchurch City Council

Results from the Duvauchelle field site (Chapter 2) were reported to the Christchurch City Council. The following excerpt is the executive summary of the report. The full report is available online at http://www.kiwiscience.com/downloads/Copy%20of%20CCC_report_updated_Final23Sep20.pdf.

A field trial to determine the effect of the land application of treated municipal wastewater onto selected NZ-native plants on Banks Peninsula

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Executive Summary

- The application of Treated Municipal Wastewater (TMW) on NZ-native vegetation is a management option under consideration for towns on Banks Peninsula and elsewhere. There is little information on the effect of TMW on the growth of NZ-native plants or the fluxes of nutrients or contaminants in the underlying soil.
- In July 2015, 1350 native species were planted onto a 20 m x 55 m plot on Piper's Valley Road, Duvauchelle, Banks Peninsula. The plants were arranged into 27 blocks (4.5 m x 4 m), with 12 of the blocks receiving TMW. There were three NZ-native vegetation types tested: Type 1 (*Phormium tenax, Phormium colensoi, Cordyline australis, Griselinia littoralis, Pittosporum eugenioides*), Type 2 (*Leptospermum scoparium, Kunzea robusta*) and Type 3 (*Coprosma robusta, Pseudopanax arboreus, Podocarpus laetus, Olearia paniculata*). Irrigation with TMW at a rate of 1000 mm/yr started in January 2016.
- In October/November 2018 forty soil pits were opened and samples taken from five depths (0-5, 15, 30, 45 and 60 cm). From January 2016 to the time of sampling, the soils received a total of 3400 mm of TMW. Soils were analysed for pH, total elements, and soluble ('phytoavailable') fractions of key nutrients and contaminants (ammonium, nitrate, Olsen phosphorus, heavy metals).
- There was no visible evidence of changes in soil structure as a result of TMW application that have been reported to occur in other soils receiving TMW due to the accumulation of sodium. Nor was there any visible evidence of runoff.
- On average the Na concentrations in the topsoil (0-5 cm) was significantly higher in the TMW-irrigated plots compared to the control plots. This is only a 25% increase, despite a disproportionately large mass of Na that was added with the effluent. This indicates that Na is moving down the soil profile and not accumulating in the root-zone, where it may cause degradation of the soil structure.
- There was a significant (6%) increase in the total nitrogen concentration in the topsoil (0-5 cm) but at greater soil depths, the total nitrogen in the TMW-treated plots was not significantly greater than the control plots. There were no significant differences in ammonium in any of the soils. Nitrate was significantly higher in the surface soil but not deeper in the soil profiles. It is likely that most excess nitrogen added to the soil (200 kg/ha/yr) is either taken up into the vegetation, denitrified into N_2 and N_2O or leached.

- There was no evidence of phosphorus accumulation in the soil, probably because the amount of phosphorus added in the TMW (110 kg/ha/yr, total of 312 kg/ha) was small compared to the mass of P in the soil profile (7606 kg/ha). This is consistent with the findings of our previous report, modelling the accumulation of P in these soils. Available phosphorus (Olsen-P) was within the range (10 - 30 mg/kg) typically found on extensive farming systems, and well below concentrations reported on soils irrigated with high-P effluent.

- Soil concentrations of potentially toxic heavy metals, including copper, cadmium, lead, and zinc, were not affected by TMW application. The concentrations of these elements were similar to background values reported for Canterbury Soils.

- Plant survival and growth was monitored throughout the trial. Growth (biomass) was assessed initially by canopy volume, and following canopy closure, by plant height. Harvested biomass will be determined at the conclusion of the trial. Plant suitability for effluent application on Banks Peninsula was determined by survival and growth.

- The effluent had a negligible effect on the concentrations of nutrients and contaminants in the plant tissues. While the growth of all species was accelerated by the effluent, there was no indication of luxury uptake of plant nutrients or increased concentrations of elements that may be harmful. This indicates that TMW is unlikely to affect ecological food chains.

- This trial demonstrated the feasibility of establishing NZ-native vegetation using TMW. We recommend irrigation rates of 500 - 800 mm/yr. Further experimental plantings should be conducted with these species to explore the possibility of using TMW to re-establish rare or endangered plants that may significantly enhance the ecological value of the area. A critical success factor for the establishment of New Zealand native vegetation on Banks Peninsula that are to receive TMW is the control of exotic weeds. It is likely that some weeds will have a greater growth response to TMW than the native species. It is therefore critical that these weeds be suppressed as the native vegetation becomes established.

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