Nitrification inhibition by common plants in New Zealand's agricultural landscapes

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Nitrification, the production of nitrate by the bacterial oxidation of ammonium, is an important economic and environmental issue in New Zealand. Nitrification can lead to high levels of fertiliser nitrogen loss from soil through nitrate leaching. This leaching can result in nitrate contamination of surface and ground water, and may be exacabated by New Zealand's recent ban of the nitrification inibitor dicyandiamide (DCD). Thus there is an imperative to investigate alternative methods of reducing nitrate contamination from NZ's agricultural systems.

Some plants, known as Biological Nitrification Inhibitors (BNIs), can decrease nitrification rates through production of phytochemicals. There are no studies investigaing whether NZ-native plants are BNIs. However, the phytochemical profiles of manuka (*Leptospermum scoparium*) and kanuka (*Kunzea ericoides*) indicate that they may have BNI properties. Other species commonly found in the New Zealand agricultural landscape, namely macrocarpa (*Cupressus macrocarpa),* monterey pine *(Pinus radiata*), and *Eucalyptus spp.* may also inhibit nitrification. Strategic plantings of such trees on farms may reduce the risk posed by nitrate leaching. This study aimed to determine the extent that these common species inhibied nitrification using plant extracts in a rapid biological assay.

A bioassay was developed using a *Nitrosospira* enriched cell culture and each assay ran for 48 hours. Plant extracts were produced for the assay by grinding leaves suspended in liquid nitrogen with a mortar and pestle, followed by extraction with water for half an hour. Aliquots of filtered supernatant were then added to the cell culture. Nitrate measurements were taken at the end of the assay, and species were compared to *L. perenne* and un-amended cells to determine the extent of nitrification inhibition.

The bioassay was used to test the aforementioned species, as well as an un-amended cell solution negative control and two species that were not be expected to be BNIs, namely kowhai (*Sophora tetraptera)* and perennial ryegrass (*Lolium perenne*). DCD was used as a positive control.

Results indicated that *L. scoparium*, *P. radiata* and *C. macrocarpa* decreased nitrate production relative to the control, *L. perenne* and *S. tetraptera* extracts. *P.radiata, L. scoparium* and *C. macrocarpa* decreased nitrate produced relative to *L. perenne* by 60%, 67% and 84%, respectively. Unpublished results of a recent lysimeter trial supported the findings that these species are BNI's. These results indicate that these species are potentially effective inhibitors of nitrification and could be used for mitigation of nitrogen loss in agricultural systems, which is of benefit to both the environment and agriculture.

Keywords: Nitrification, BNI, manuka, *Leptospermum scoparium*, kanuka, *Kunzea ericoides*, nitrate, *Pinus radiata, Cupressus macrocarpa*, *Lolium perenne, Sophora tetraptera, Eucalyptus nitens*

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Chapter 1

Introduction

1.1 Nitrogen losses from soil through nitrification

In New Zealand agricultural systems, large amounts of nitrogen (N) are applied in the form of urea. Agricultural systems are highly nitrifying environments, which transform the ammonium (NH_4^+) from urea, to nitrate (NO_3^-). Nitrate is vulnerable to being lost from the soil through leaching or denitrification (Figure 2.1). Leaching occurs because nitrate is negatively charged and therefore is not held in soil by cation exchange as ammonium is (Krantz *et al.*, 1944). This makes nitrate relatively mobile. Nitrate leaching into ground water causes health concerns for drinking water and eutrophication in surface water (Smith, 2003; World Health Organization, 2004). Denitrification of nitrate to NO, N_2O and N_2 occurs under anaerobic or partially anaerobic conditions (Bremner & Blackmer, 1978). This results in loss of N from the soil and N_2O is also a potent greenhouse gas. Furthermore assimilating nitrate results in direct N_2O emissions from crops (Smart & Bloom, 2001).

Nitrification inhibitors such as dicyandiamide (DCD) can be applied to the soil to decrease nitrate production (Amberger, 1989; Di & Cameron, 2002). However, in January 2013, New Zealand banned DCD application due to trace levels detected in milk. This means that research into alternative techniques for N management is required. This may be possible through investigating how plants manage N in natural ecosystems. In these systems N usually limits plant growth (Vitousek & Howarth, 1991) and plants that have evolved mechanisms to assimilate more N than their competitors are more successful.

1.2 Biological nitrification inhibition

Nitrification is controlled by environmental factors, such as substrate (NH_4^+) availability, temperature, soil pH, aeration, water content and texture (Tietema *et al.*, 1992), and by the phytochemicals of some plants (Paavolainen *et al.*, 1998; Rice & Pancholy, 1973; White, 1986, 1991).

Control of nitrification by phytochemicals is termed ‘biological nitrification inhibition’ (BNI). This occurs either through direct inhibition of the enzyme ammonia monooxygenase (AMO) or toxicity to nitrifying populations (Erickson *et al.*, 2000; Lodhi & Killingbeck, 1980; Paavolainen *et al.*, 1998; Rice & Pancholy, 1973; White, 1991). Phytochemicals that inhibit nitrification decrease nitrogen losses from leaching and denitrification due to lower nitrate levels present in the soil.

1.2.1 Candidate species

Plants that inhibit nitrification may have more available nitrogen in low N environments as less N is lost through leaching and denitrification of nitrate. Therefore, plants which occur on low fertility soils, such as manuka (*Leptospermum scoparium* J.R.Forst. & G.Forst.) and kanuka (*Kunzea ericoides* (A.Rich.) Joy Thomps.), may benefit from inhibiting nitrification. These species contain high concentrations of antimicrobial chemicals which may affect the nitrogen cycle (Porter & Wilkins, 1999). These include monoterpenes, triketones and sesquiterpenes.

Monoterpenes that inhibit nitrification do so via competition for the active site of ammonia monooxygenase (Ward *et al.*, 1997; White, 1988). This makes molecular structure important in determining whether a particular monoterpene will cause inhibition. The monoterpene α-pinene is a relatively strong nitrification inhibitor (Paavolainen *et al.*, 1998; Ward *et al.*, 1997; White, 1991). *K. ericoides*, shining gum (*Eucalyptus nitens* H.Deane & Maiden) (both Myrtaceae), Monterey pine (*Pinus radiata* D.Don) and macrocarpa (*Cupressus macrocarpa* Hartw. ex Gordon) all have moderate to high concentrations of α-pinene. Most of these species also contain other compounds shown to inhibit nitrification such as limonene, γ-terpinene and myrcene. Of these species, only *P. radiata* extracts have been tested for nitrification inhibiting properties. *P. radiata* stands have been observed to inhibit nitrification in a New Zealand field site (Cooper, 1986) and leaf extracts decreased nitrification in soil incubation trials (Suescun *et al.*, 2012).

K. ericoides, *L. scoparium*, *E. nitens, P. radiata* and *C. macrocarpa* are all common in, and suited to the New Zealand agricultural landscape, this is important as this makes them easier to utilise in this environment. Waste material from these trees is commonly used as firewood. However, leaf material is of little value and could potentially be used as a source of alternative nitrification inhibition to DCD.

P. radiata is the main tree species planted in New Zealand, particularly for forestry. In 2012, 90% of planted production forests were *P. radiata* (Ministry for Primary Industries, 2012). *C. macrocarpa* is commonly used in shelter belts. Eucalyptus, a native to Australia, is also planted in shelter belts and to a small extent for forestry (Ministry for Primary Industries, 2012). *L. scoparium* and *K. ericoides* are considered weeds on farms, but their growth, particularly of *L. scoparium*, has been promoted in recent years due to the production of high value manuka honey, as well as essential oils from both species.

Plants that candidate BNIs could be contrasted with include kowhai, a group of woody legumes in the genus *Sophora* that are not expected to be BNIs, and perennial ryegrass (*Lolium perenne* L.), which makes up the majority of NZ pastures and has limited BNI properties (Subbarao, Rondon*, et al.*, 2007).

1.3 Aims

The aim of this study was to determine whether common trees in the New Zealand landscape, namely *P. radiata, L. scoparium, K. ericoides, E. nitens,* and *C. macrocarpa*, inhibited nitrification. As negative controls, un-amended cells, *L. perenne* and kowhai (*Sophora tetraptera* J.S.Mill.), which were not expected to exhibit nitrification inhibition, were tested. As a positive control, DCD was used. I sought to develop a rapid test to screen for species that could be used in field trials to reduce nitrogen losses. Eventually, such species could be prioritised in locations such as riparian strips or farm boundaries to decrease the environmental problems of nitrogen moving into rivers and drains.

Chapter 2

Literature Review

2.1 Introduction

The nitrogen cycle is the conversion of nitrogen from one form to another in soil, plants, animals and the atmosphere (McLaren & Cameron, 1996). Some phytochemicals can directly alter the nitrogen cycle processes nitrification, immobilisation and mineralisation and some may also directly affect denitrification (Figure 2.1). These effects are typically through toxicity to microorganisms, inhibition of enzymes or supply of substrate to heterotrophic bacteria. This review describes process of the nitrogen cycle that phytochemicals can affect, and discusses the current knowledge of species, and phytochemicals they produce, which alter these processes, and the mechanisms by which this occurs.

Through identification of phytochemicals that have been shown to alter N cycling common trees in New Zealand's agricultural landscape, that are likely to affect the N cycle, can be selected via their chemical profiles. These plants can then be analysed in future experiments in order to determine their potential for providing environmental protection in agricultural systems. Species shown to inhibit nitrification could be prioritised in locations such as riparian strips or farm boundaries to decrease the environmental problems of nitrogen moving into rivers and drains.

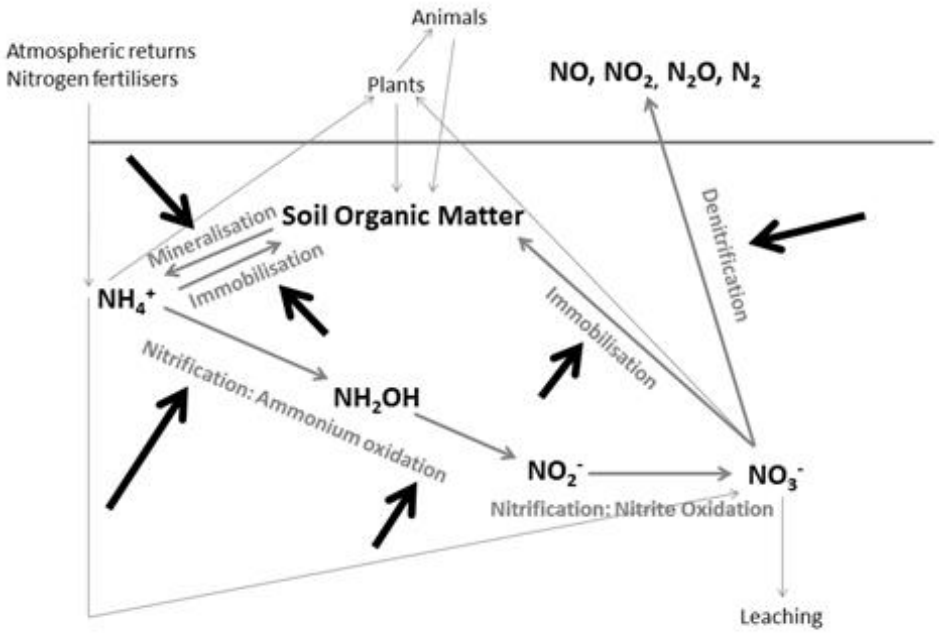

Figure 2.1: The nitrogen cycle. Large arrows show processes phytochemicals could directly alter.

2.2 Nitrogen cycle processes that could be affected by phytochemicals

2.2.1 Nitrification

Nitrification is the biological oxidation of ammonium to nitrate, via nitrite (NO_2^-). Nitrification is an important economic and environmental issue. It can lead to high levels of nitrogen loss from soil as it results in relatively immobile ammonium being oxidised to highly mobile nitrate.

Ammonium oxidation is the first step in the nitrification pathway and is also rate limiting. Ammonium oxidation is performed by the enzyme ammonia monooxygenase (AMO) in bacteria such as *Nitrosomonas* and *Nitrosospira* spp., and some archaea. This enzyme oxidises ammonia (NH_3) to NH_2OH. NH_2OH is then oxidised by hydroxylamine oxidoreductase (HAO) to produce nitrite. Nitrite is rapidly oxidised to nitrate by *Nitrobacter* species.

2.2.2 Mineralisation and immobilisation

Mineralisation of nitrogen is the conversion of organic compounds into inorganic (or mineral) N (McLaren & Cameron, 1996). This involves the breakdown of proteins into amino acids, followed by ammonification of these compounds into ammonia, which is then hydrolysed to plant available ammonium. Immobilisation is when microorganisms and plants take up mineral nitrogen, converting it into organic compounds.

When greater amounts of mineral N are released through mineralisation than is required by the organisms, mineral N levels increase in the soil meaning that net mineralisation occurs. Net mineralisation occurs when organic matter being decomposed has a relatively high N content (e.g. 3%), and therefore low C:N ratio (<25:1) (McLaren & Cameron, 1996). The microorganisms only need a certain amount of N relative to C and so excess N is released into the soil.

When more mineral N is taken up than produced through mineralisation net immobilisation occurs. When organic matter has a low N content, and therefore high C:N ratio the microorganisms must take up mineral N from the surrounding soil to breakdown the carbon rich organic compounds, therefore net immobilisation occurs (McLaren & Cameron, 1996).

2.2.3 Denitrification

Denitrification is the stepwise reduction of nitrate to N_2 (McLaren & Cameron, 1996). Denitrification occurs under anaerobic conditions where anaerobic bacteria can use nitrate as an electron acceptor instead of oxygen. Nitrate is reduced to N_2 via the nitrogen oxides [nitrite, nitric oxide (NO), and nitrous oxide (N_2O)]. Often N_2O escapes from the soil before it can be further reduced to N_2. In the

atmosphere, N_2O acts as a greenhouse gas having a global warming potential 300 times that of CO_2 (Rodhe, 1990).

2.3 Phytochemicals that alter the nitrogen cycle

2.3.1 Polyphenols

Polyphenols are commonly considered to cause nitrification inhibition through toxicity to nitrifiers (Castaldi *et al.*, 2009; Rice & Pancholy, 1973, 1974). Structures of polyphenols discussed in this review are shown in (Figure 2.2).

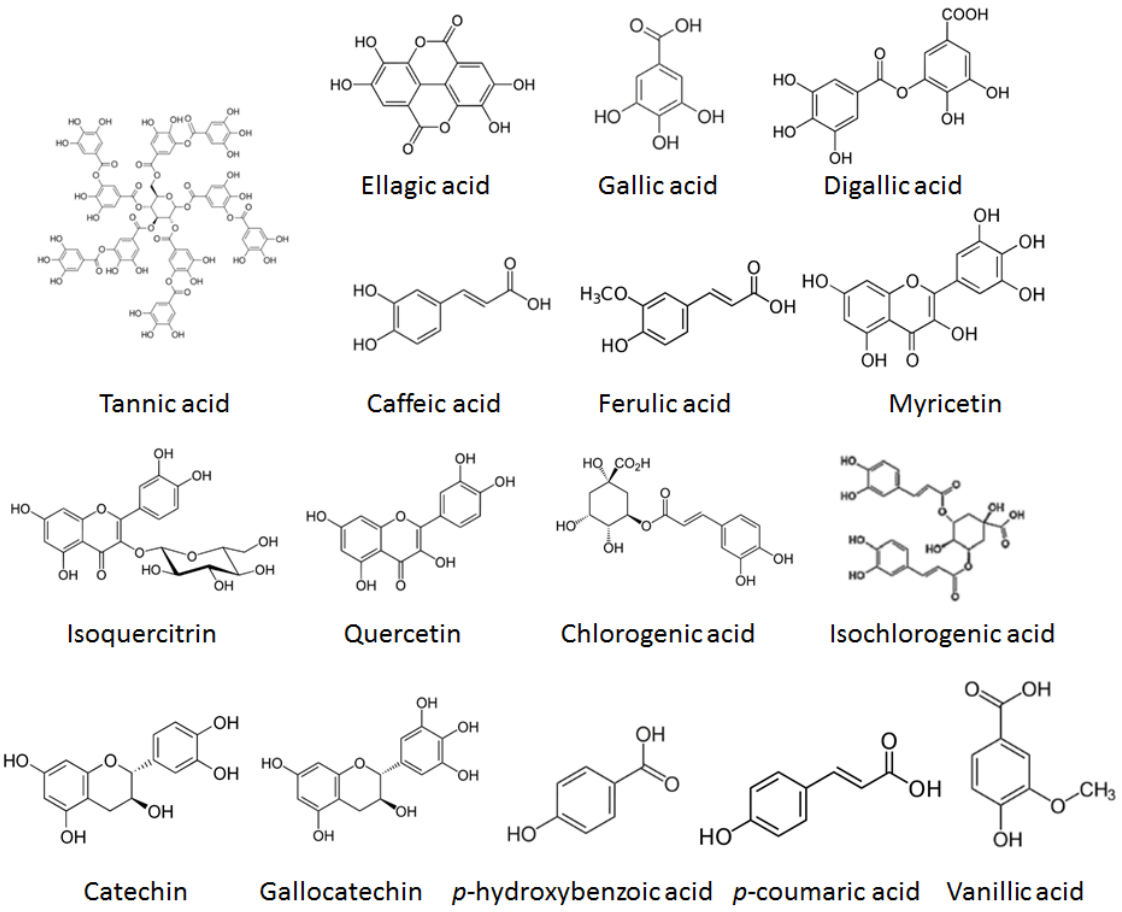

Figure 2.2: Polyphenolic compounds that have been tested for nitrification inhibiting properties. Isocholorogenic acid image retrieved from Ma *et al.* (2011). Remaining images retrieved from Wikimedia Commons (2013).

Rice and Pancholy (1973, 1974) measured nitrification inhibition by *Nitrosomonas* and *Nitrobacter* through measurement of change in nitrite concentration. In each experiment, nitrite appearance was measured for *Nitrosomonas* and nitrite disappearance for *Nitrobacter*.

Rice and Pancholy (1973) found that tannic acid and tannin derivatives ellagic, gallic, and digallic acids strongly inhibited ammonium oxidation when added to soil solutions from oak-blackjack oak forest soil or oak-pine forest soil at lower concentrations than naturally present in soil from their research plots (Table 2.1).

Rice and Pancholy (1974) found that the phenolic compounds caffeic acid, chlorogenic acid, ferulic acid, isochlorogenic acid, isoquercitrin, myricetin and quercetin all completely inhibited ammonium oxidation by *Nitrosomonas* in a 10^{-1} soil suspension. The concentration required to completely inhibit nitrification varied with the compound and assay soil (Table 2.1).

Ferulic, gallic, ellagic and tannic acid also inhibited nitrite oxidation by *Nitrobacter* but the others did not at the highest concentrations trialled (Rice & Pancholy, 1973, 1974).

Table 2.1: Most dilute concentration (mol L^{-1}) which completely inhibited nitrification for three week in a 10^{-1} soil suspension (Rice & Pancholy, 1973, 1974). Letters indicate no inhibition at highest concentration tested. 'A' is not inhibited completely at 10^{-3}; 'B' at 10^{-4}, 'C' at 10^{-5} mol L^{-1}).

Compound	*Nitrosomonas*		*Nitrobacter*	
Caffeic acid	10^{-6}		A	
Chlorogenic acid	10^{-7}		A	
Ferulic acid	10^{-8}		10^{-4}	
Isochlorogenic acid	10^{-7}		A	
Isoquercitrin	10^{-6}		B	
Myricetin	10^{-6}		B	
Quercetin	10^{-6}		A	
Tannins and tannin derivatives:	Oak-blackjack oak forest soil	Oak-pine forest soil	Oak-blackjack oak forest soil	Oak-pine forest soil
Tannic Acid	10^{-5}	10^{-7}	C	10^{-5}
Digallic Acid	10^{-8}	10^{-7}	C	C
Ellagic Acid	10^{-5}	10^{-7}	10^{-5}	C
Gallic Acid	10^{-8}	10^{-8}	10^{-5}	10^{-5}

Castaldi *et al*. (2009) also showed that polyphenols inhibit nitrification. Plant extracts of *Arbutus unedo* L. which were high in the flavan-3-ols gallocatechin and catechin resulted in inhibition of nitrification in incubated soil samples. Castaldi *et al*. also found that ammonium was the only mineral N form present in soils of an *A. unedo* Mediterranean woodland.

Some studies have disagreed as to whether or not nitrification inhibition is caused by these compounds. McCarty and Bremner (1986) disagreed with Rice and Pancholy (1973,1974)'s methodology due to nitrite appearance being measured for *Nitrosomonas* and nitrite disappearance for *Nitrobacter,* instead of net ammonium and nitrate, despite a nutrient medium inoculated with soil being used. This means that nitrite was likely being rapidly oxidised to nitrate.

McCarty and Bremner (1986) tested nitrification inhibition by various concentrations of caffeic acid, chlorogenic acid, *p*-coumaric acid, ellagic acid, ferulic acid, gallic acid, *p*-hydroxybenzoic acid and vanillic acid. They measured both nitrate and nitrite. They found little, if any, inhibitory effect on nitrification in four soils studied with rates up to 250 mg phenolic kg^{-1} soil. This was greater than phenolic levels in soil profiles under climax stands tested by Rice and Pancholy (1973) which ranged from 8 to 93 mg kg^{-1} soil.

McCarty *et al.* (1991) tested the effect of caffeic acid, *p*-coumaric acid, and ferulic acid at higher concentrations than found in soil on inhibition of nitrite production by three pure cultures of nitrifiers (*Nitrosomonas, Nitrosospira, or Nitrosolobus*). They found no inhibitory effect on ammonium oxidation with rates of 10^{-6} or 10^{-5} M (McCarty *et al.*, 1991). Slight inhibition was observed at 10^{-4} M and ferulic acid did not inhibit nitrification when its concentration was 10^{-3} M.

Effect of removing polyphenols

Removal of protein binding polyphenols from forest floor extracts by binding with casein has been shown to remove nitrification inhibition from soil, increasing ammonia oxidation (Baldwin *et al.*, 1983). Charcoal has also been shown to have a similar affect (DeLuca *et al.*, 2006). Addition of charcoal to a Ponderosa pine *(Pinus ponderosa* Douglas ex C.Lawson)/ Douglas Fir (Pseudotsuga menziesii (Mirb.) Franco) forest soil with an understory of *Centaurea maculosa* Lam. increased net nitrification and decreased the soil concentration of the phenolic allelochemical catechin (±). Addition of charcoal to a grassland site, with high nitrification rates, had no effect. This suggests that inhibitory phenolics were not present in the grassland but were present in the forest soil. DeLuca *et al.* (2006) suggested that immobilisation of phenols by charcoal may be reducing their inhibiting effects on nitrifiers. Further study would be required to determine which removed phenols were causing the inhibition, and if charcoal and casein were also removing other non-phenolic potential inhibitors.

Howard and Howard (1991), cited in White (1994), also tested nitrification inhibition with and without polyphenols removed. Conifer extracts from a range of trees were used and polyphenols were removed with PVP (polyvinylpyrrolidone). After removal of polyphenols the inhibitory activity was unchanged or in some cases increased, for all species except Norway spruce *(Picea abies L.)* which was slightly less inhibited. This may indicate that compounds other than polyphenols were responsible for inhibition or that PVP was not reducing the inhibiting effects of polyphenols on nitrifiers.

Impacts of phenols on succession

Rice and Pancholy (1973) proposed that ecosystem succession is characterised by decreasing N-fixation and soil nitrogen levels, and increasing inhibition of nitrification. This increased inhibition of nitrification may be controlled by phenolics. Nitrification was strongest in mature climax ecosystems. Climax ecosystems exhibiting nitrification inhibition had higher levels of ammonium, higher concentrations of polyphenols and lower levels of nitrate than ecosystems in earlier stages of succession. High polyphenol production could be a mechanism used to both limit N loss through nitrate leaching and give one species a competitive advantage over another.

Preference for ammonium and nitrate is species specific. Nitrification inhibition has been suggested to exclude competitors that require relatively high nitrate concentrations for growth and reproduction, are inefficient at using ammonium, or have low tolerance for intracellular ammonium toxicity (Rice & Pancholy, 1973).

Douglas fir, for example, had high production with a nitrate source and intolerance to ammonium resulting in low root weight. Lodgepole pine (*Pinus contorta* Dougl.) and Engelmann spruce (*Picea engelmannii* Parry) did not grow well with only nitrate (Bigg & Daniel, 1978; Krajina *et al.*, 1973). Christersson (1972) found that Scots pine (*Pinus sylvestris* L.) seedlings had twice the needle production and 1.8 times higher shoot weight when grown with ammonium than when grown with nitrate.

Chandler and Goosem (1982) found that nitrification increased with time in a rainforest succession despite soil phenols also accumulating. Chandler (1985), using low fertility hill and lowland soils with dipterocarp climax vegetation found that nitrification was not inhibited by the polyphenol tannic acid even at polyphenol levels higher than naturally found.

Polyphenols increased immobilisation of N into organic forms in soil under *Pinus muricata* D.Don (Northup *et al.*, 1995) and balsam poplar (*Populus balsamifera*) (Schimel *et al.*, 1996) as opposed to inhibiting nitrification. Northup *et al.* (1995) showed that the *P. muricata* mycorrhizae had a

competitive advantage in utilising the organic N. Polyphenol control over N-cycling could explain the convergent evolution of tannin-rich plant communities on highly leached soils (Northup *et al.*, 1995).

Inhibition mechanism

The mechanism behind polyphenol nitrification inhibition may be toxic effects on nitrifiers (Lodhi & Killingbeck, 1980; Rice & Pancholy, 1973). Addition of ponderosa pine needles and bark or soil extracts from ponderosa stands decreased populations of nitrifiers (Table 2.2) (Lodhi & Killingbeck, 1980).

Castaldi *et al.* (2009) showed that soil polyphenol concentration under stands of *A. unedo* was greater than the annual input via leaf litter. This meant that the concentration accumulated over time and indicated that inhibition may be exerted throughout the whole year.

Table 2.2: Number of *Nitrosomonas* relative to control and percent inhibition in control and treatment cultures where inhibition in control is 0. * indicates significant difference from control group (Lodhi & Killingbeck, 1980)

Extract treatment	Relative number of *Nitrosomonas*	% Inhibition
Control	1	0
Needles-water	0.069	93
Needles-ether	0.32	68
Bark-acetone	0.086	91
Soil-acetone	0.12	88
Soil-hydrolysis	0.75	93

2.3.2 Monoterpenoids

Monoterpenoids are produced by plants and have been shown to inhibit nitrification. Monoterpenoids include monoterpenes, which are unsaturated hydrocarbons consisting of two isoprene units and the formula $C_{10}H_{16}$, and oxygenated derivatives of monoterpenes such as ketones, alcohols and carboxylic acids. These derivatives are more toxic due to higher water solubility leading to greater bioavailability (Weidenhamer *et al.*, 1993). Their alcohol and ketone groups can also react with and damage proteins. Despite monoterpenes being hydrocarbons they have been shown to be slightly water soluble and in many cases biologically active at concentrations lower that their solubility limits (Fischer *et al.*, 1994; Weidenhamer *et al.*, 1993).

Monoterpenoids provide plants with increased thermal tolerance (Loreto *et al.*, 1998) and protection against herbivores (Gershenzon & Croteau, 1991). They also have antimicrobial properties (Chalchat

et al., 2000; Himejima *et al.*, 1992; Stotzky & Schenck, 1976). Their release into soil via root exudates and excised plant matter, such as leaf-litter, can affect soil microbial processes, inhibiting growth and activity of some microorganisms but also increasing activity of others. A number of studies have tested monoterpenes for their nitrification inhibiting properties and these are discussed below. Structures of discussed monoterpenes are shown in Figure 2.3.

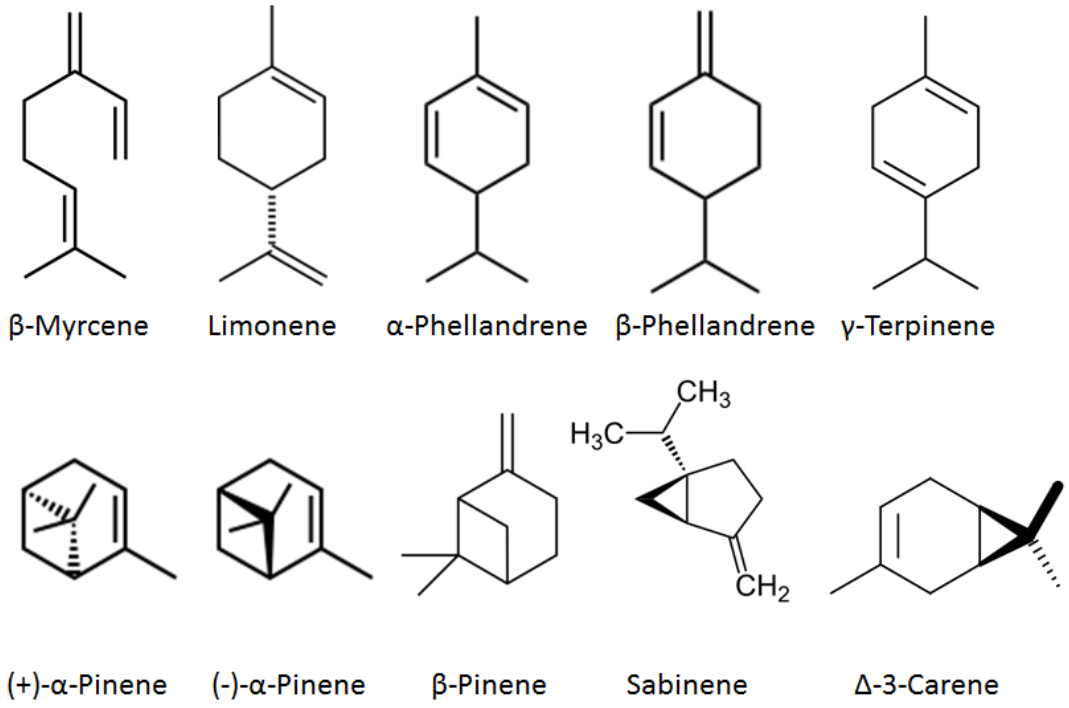

Figure 2.3: Monoterpenes that have been tested for nitrification inhibiting properties. Images retrieved from Wikimedia Commons (2013)

Nitrification inhibition

Inhibition of nitrification by monoterpenes was first suggested by White (1986). He found that both volatile organics produced by ponderosa pine and volatiles released from a terpenoid mix containing equal parts of limonene, myrcene, α-pinene, β-pinene and β-phellandrene, resulted in low levels of nitrate and high levels of ammonium, relative to the control in assay soil (Figure 2.4). White (1986) showed that the ponderosa pine and terpenoid mix inhibited nitrification 87% and 100%, respectively, by inhibiting AMO. It also resulted in net immobilisation of nitrate, as total inorganic N decreased. White (1986) did not determine the chemical composition of the volatiles produced by ponderosa pine or examine the effects of individual monoterpenes.

White (1991) proposed that the soil's response to monoterpenes depended on the inherent rate of nitrification in soil. White found that in soil with inherently low nitrification rates, 3 µL monoterpene additions of limonene, α-phellandrene, Δ-3-carene, α-pinene or β-pinene to 20 g fresh weight

mineral soil, inhibited nitrification and net mineralisation. When nitrification was inherently high monoterpenes did not inhibit nitrification

Nitrification inhibition was also dependent on the initial ammonium concentration (White, 1991). With a low initial concentration (2 μg NH_4^+ g^{-1}), nitrate production did not occur with 10 or 20 μL additions of limonene or α-pinene. When ammonium concentration was higher (25 μg NH_4^+ g^{-1}) most was converted to nitrate with the same monoterpene additions. The inverse relationship was suggested by White (1991) to be due to competitive inhibition of AMO by monoterpenes at the active site. A higher substrate concentration is better able to compete with the inhibitor.

Studies of monoterpene inhibition have shown that many inhibit nitrification; however, there is a large amount of variation between studies. This is likely due in part to the methodology and different assay soils used. Ward *et al*. (1997) tested inhibition of *Nitrosomonas europaea* nitrite production by 1 and 10 μg mL^{-1} of the five most abundant monoterpenes (limonene, sabinene, α-pinene, mycene and γ-terpinene) in coastal redwood needles (*Sequoia sempervirens* (D. Don) Endl.), and β-pinene, compared to 1 μg mL^{-1} of the commercial nitrification inhibitor nitrapyrin. Myrcene was the strongest inhibitor at 1 μg mL^{-1} with nitrite production at 56% of the negative control, α-pinene and limonene were the strongest inhibitors at 10 μg mL^{-1} with nitrite production rates 11% and 35% of the control respectively (Figure 2.5). In contrast to α-pinene, β-pinene did not inhibit nitrite production at 1 or 10 μg mL^{-1}.

Paavolainen *et al.* (1998) compared net production of (NO_2^- + NO_3^-)-N after two weeks incubation of a soil suspension in a mineral solution containing ammonium with 250 μg mL^{-1} α- or β-pinene and 0.1 g (NH_2SO_4) added every three days. They showed that β-pinene had the greater nitrification inhibition (98±1%) than α-pinene (90±2%). The concentration of monoterpenes in this experiment was greater than in other studies.

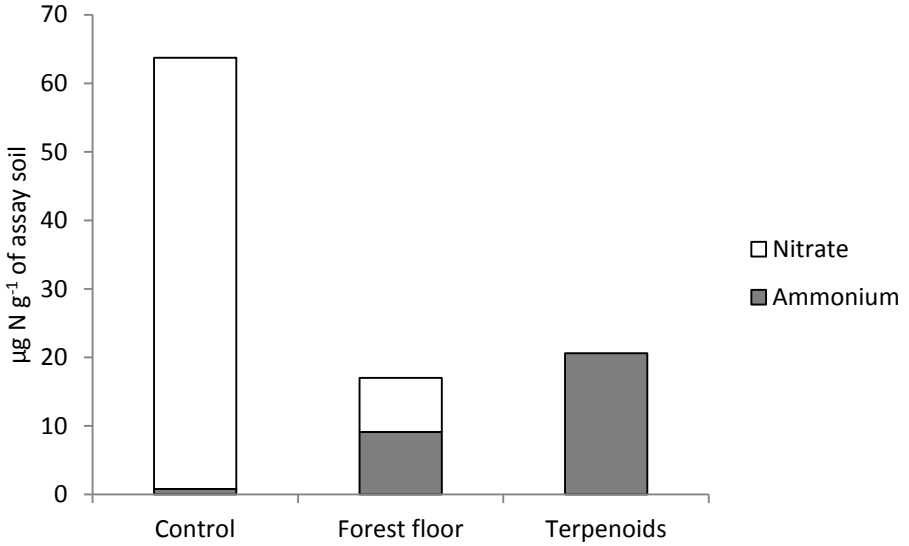

Figure 2.4: Mean inorganic nitrogen levels (µg N g^{-1}) after 10 week incubation at 20°C. Vials containing forest floor, terpenoid mixture or nothing were placed in jars with assay soil. Figure adapted from White (1986).

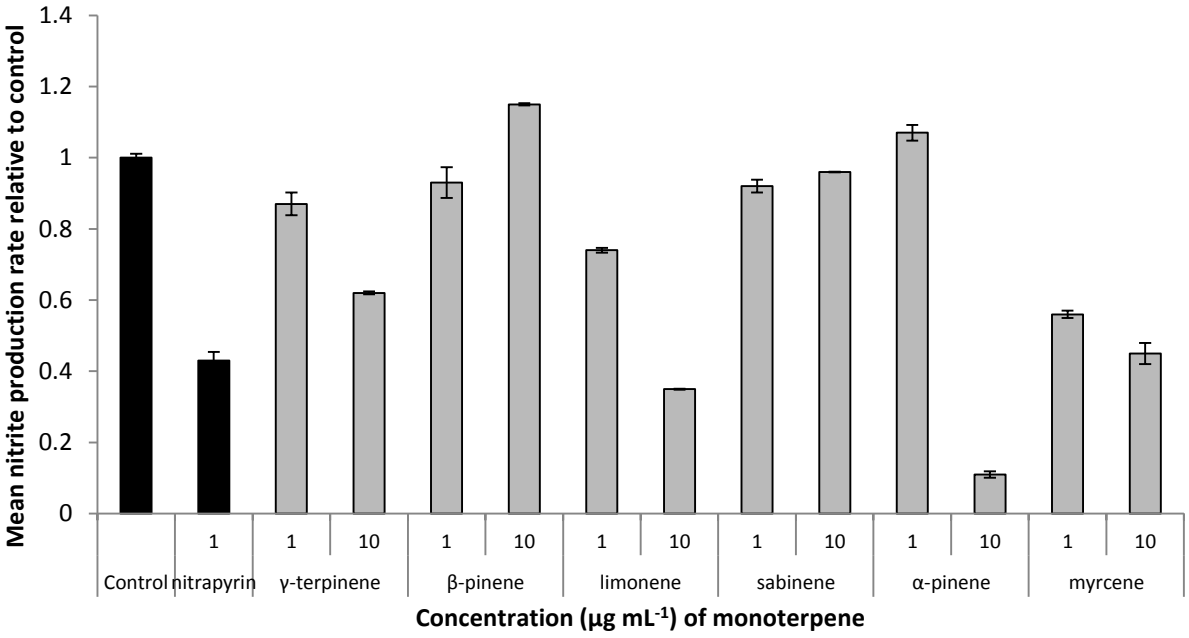

Figure 2.5: Mean nitrite production rate at 1 or 10 µg mL^{-1} of monoterpene relative to control with no monoterpene addition, and compared to commercial nitrification inhibitor nitrapyrin. Error bars are standard deviation of two or three replicates. Figures adapted from Ward *et al.* (1997)

Bremner & McCarty (1988) showed that addition of monoterpenes caused immobilisation of N with no inhibition of ammonium oxidation. White (1990) agreed that their findings indicated immobilisation rather than inhibition, however he did not agree that his hypothesis was invalidated, due to the high ammonium status and high inherent nitrification rate of the cropping soils studied by

McCarty and Bremner. Monoterpene additions to soil that is N-rich results in increased CO_2 production and decreased inorganic N levels (NH_4^+ and NO_3^-) (White, 1991). This is because although the monoterpenes inhibit AMO of nitrifiers they can be used as a carbon source in heterotrophic bacteria (Paavolainen *et al.*, 1998).

The forest soils in White (1986)'s study were N limited, with five to ten times less ammonium than Bremner & McCarty's study. In N-limited soils addition of carbon had little effect on the heterotrophic community allowing the monoterpenes to persist in the soils and inhibit nitrifying organisms (White, 1994).

White (1991) showed that net immobilisation occurred with high monoterpenes additions. He found that 3 μL additions of monoterpenes to 20 g fresh weight mineral soil inhibited nitrification and lowered net mineralisation. This resulted in lower inorganic N and nitrate levels than the control, but no net immobilisation as inorganic N was higher than the initial level indicating there was still net mineralisation. Net immobilisation occurred for all monoterpenes except myrcene at 6 and 12 μL. At 24 μL all monoterpenes tested caused immobilisation. Monoterpene additions of 6, 12 and 24 μL also inhibited nitrification as almost no nitrate was produced and ammonium was the only, or main, form of inorganic N at the end of incubation. In the soils examined by Bremner and McCarty (1988) only nitrate was present at the end of incubation. White (1990) suggested this may be due to aeration of samples by Bremner and McCarty every second day leading to removal of volatiles, which would decrease inhibition and cause nitrate production.

Inhibition mechanism

The mechanism behind nitrification inhibition by monoterpenes is thought to be competition for the nitrifier enzyme ammonia monooxygenase (AMO) (Ward *et al.*, 1997; White, 1988). This inhibition mechanism has also been shown with other inhibitors such as nitrapyrin [2-chloro-6-(trichloromethyl) pyridine] and acetylene (Bédard & Knowles, 1989; Hynes & Knowles, 1982).

The amount of inhibition varied with molecular structure but was not dependent on position of unsaturated bonds or cyclicity (Ward *et al.*, 1997). Ward suggested this may mean more than one mode of inhibition occurs.

Other terpenoids

Subbarao *et al*. (2009) showed using bioluminescence assays that the cyclic diterpene brachialactone (Figure 2.6) was a nitrification inhibitor. This compound is released from the tropical forage grass *Brachiaria humidicola* (Rendle) Schweick. Unlike nitrapyrin, which only affects AMO, brachialactone inhibited both AMO and hydroxylamine oxidoreductase (HAO) in *Nitrosomonas*. The release of this

compound was plant regulated and was caused by ammonium availability in the root zone (Subbarao *et al.*, 2009; Subbarao, Wang*, et al.*, 2007). Plants grown with only nitrate did not release BNIs. The release only occurred in roots directly exposed to ammonium. The inhibition of nitrification was also found to decrease N_2O emission by up to 90%.

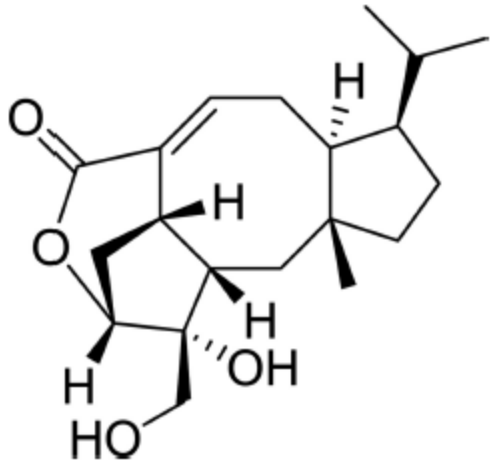

Figure 2.6: Chemical structure of brachialactone isolated from root exudates of *B. humidicola*. Figure retrieved from Subbarao *et al.* (2009)

No studies have been done on nitrification inhibition by oxygenated derivatives of monoterpenes which are considered more toxic than monoterpenes due to higher water solubility (Weidenhamer *et al.*, 1993). α-pinene oxide and (R)-(+)-limonene oxide have been shown to cause less inhibition of the enzyme for methane oxidation, methane monooxygenase (MMO), than their hydrocarbon forms (Amaral *et al.*, 1998). AMO and MMO are similar enzymes (Holmes *et al.*, 1995) and share many inhibitors such as nitrapyrin [2-Chloro-6-(Trichloromethyl) Pyridine] , α-pinene and limonene (Amaral *et al.*, 1998; Amaral & Knowles, 1998; Salvas & Taylor, 1984; Topp & Knowles, 1984).

Denitrification

Denitrification is decreased when nitrification inhibitors are present as less nitrate is available to be reduced. It has also been suggested that some monoterpenes may have a direct effect on denitrifying bacteria (Amaral *et al.*, 1998).

Amaral *et al.* (1998) tested three monoterpenes, (-)-a-pinene, γ-terpinene, and β-myrcene, on isolates from a polluted sediment and a swamp soil to determine whether any had a direct effect on denitrifiers. Acetylene was applied to prevent transformation from N_2O to N_2. Denitrification by the polluted isolate was not inhibited by monoterpenes applied with acetylene. In contrast a swamp soil isolate showed no denitrification occurred with (-)-α-pinene present**.** Partial N_2O production occurred with γ-terpinene, and β-myrcene did not decrease denitrification. Amaral *et al.* (1998) could not conclude that monoterpenes are specific inhibitors of denitrification but suggested they inhibit a

subset of denitrifiers. Lack of inhibition in polluted sediment could be due to tolerance towards cyclic organic compounds.

Paavolainen *et al.* (1998) found 2 g dry weight soil samples, adjusted to 100% of the water holding capacity (WHC), exposed to a 0.5 mL monoterpene mixture (α-pinene 38%, β-pinene 38%, limonene 6%, myrcene 6%, camphene 6%, 3-carene 4% and β-phellandrene 2%) had no direct effect on N_2O in the assay soil.

Residence time and profile distribution

There is a large difference in volatile organic compound production between soil with a living root system, soil with excised plant material, and soil where plant material has been removed (Paavolainen, 1998). Forest soil in situ under 66 year old Norway Spruce produced approximately 1600 times more monoterpenes than soil samples, with plant material removed, measured in the laboratory. These soil samples without plant material also did not inhibit nitrification in assay soil, resulting in increased nitrate and decreased ammonium levels. This indicates that the volatiles have a short residence time in soil and require plant material to be released into the soil. Volatiles from a monoterpene mixture at levels similar to those measured by micro-air passive diffusive samplers had strong inhibition resulting in little change in ammonium, nitrate or net inorganic N from the initial levels. Studies have shown that leaf litter contains the highest levels of monoterpenes (Wilt *et al.*, 1993) but roots may also contribute (White, 1991).

There can be large decline in monoterpenes concentration between horizons. For example in the ponderosa pine stand used by White (1986; 1991) monoterpenes levels were about 1400 μg g^{-1} in fresh litter, 150 μg g^{-1} in year-old litter, 30 μg g^{-1} in the F-H horizon and 0.6 in the 0-10 cm-depth mineral soil horizon (White, 1994).

2.4 Plants present in New Zealand with potential to alter the nitrogen cycle

2.4.1 Kanuka and manuka

Kanuka (*Kunzea ericoides*) and manuka (*Leptospermum scoparium*) may have potential for inhibition of nitrification. *K. ericoides* and *L. scoparium* were shown to have inhibitory effects on a range of enzymes such as trypsin, β-galactosidase and α-amylase, although AMO was untested (Kellam *et al.*, 1992).

In *L. scoparium* essential oil, monoterpenes were present at low levels (<3%), while sesquiterpenes were predominant (>60%) and oxygenated sesquiterpenes and triketones were also present (<30%) (Porter & Wilkins, 1999). The triketones are reported to be what gives *L. scoparium* its antimicrobial

properties. The major triketones were flavesone, *iso*-leptospermone and leptospermone (Figure 2.7), however this varied with region and these triketones were not detected in *L. scoparium* from some regions. Research into whether these compounds will cause nitrification inhibition is limited. Phytochemical composition of *L. scoparium* also varies considerably between species, population, age and seasons (Porter *et al.*, 1998; Porter & Wilkins, 1999).

K. ericoides oil, on the other hand, had high levels of the monoterpene, α-pinene (>50%), and low levels (<10%) of sesquiterpenes, and none of the three major triketones of *L. scoparium* were detected (leptospermone, isoleptospermone or flavesone) (Porter & Wilkins, 1999). *K. ericoides* had lower antimicrobial properties than *L. scoparium*; however α-pinene is a known nitrification inhibitor and directly competes for AMO's active site (Ward *et al.*, 1997) so inhibition should be expected under *K. ericoides* stands.

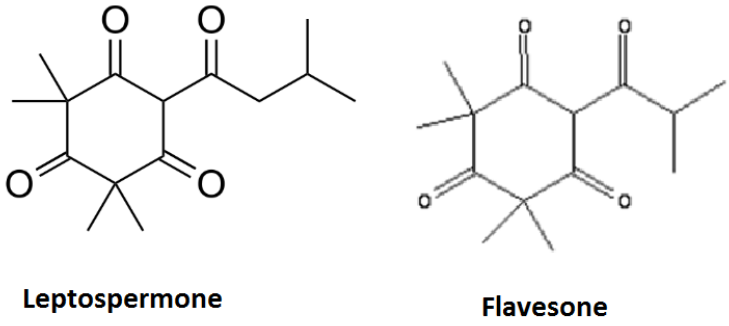

Figure 2.7: Triketones present in *L. scoparium*. Leptospemone image retrieved from Wikimedia Commons (2013); flavesone image retrieved from http://webbook.nist.gov (2013)

K. ericoides and *L. scoparium* are both primary successors. If *K. ericoides* and/or *L. scoparium* are found to inhibit nitrification, this will be in contrast to Rice and Pancholy's hypothesis that nitrification inhibition increases with succession.

2.4.2 Eucalyptus

Eucalyptus spp. is in the same family of plants as *L. scoparium* and *K. ericoides* (Myrtaceae) and contains compounds with potential nitrifying effects. The phytochemical composition is variable between species, such as, for *E. nitens,* the average composition of oil dry weight for adults across 13 locations in Australia was 1,8-cineole (eucalyptol) (37.6%), α-pinene (20.9%), *p*-cymene (6.6%) as well as other monoterpenes at lower concentrations (Li *et al.*, 1994). For *Eucalyptus denticulata* I.O.Cook & Ladiges, the average composition for adults from two locations was *p*-cymene (30%), γ-terpinene (18-22%) and lower amounts of 1,8-cineole (11-15%) and a-pinene (0.5%) (Li *et al.*, 1994). The effects of 1,8-cineole and *p*-cymene on nitrification are unknown.

Studies have shown ammonium to be the predominant form of mineral N in a range of eucalypt soils and climatic conditions, and nitrification to be low. Only trace amounts of nitrate was detected in leachate over ten weeks following addition of 200 μg N g^{-1} (Ellis & Pennington, 1989). The reasons for low nitrification under eucalyptus stands are not well understood and could include low ammonium availability, inhibition by phytochemicals, low populations of autotrophic nitrifiers and soil pH (Ellis & Pennington, 1989). Including a species of eucalyptus in a nitrification inhibiting trial may allow the reason behind the low nitrification inhibition to be better understood.

2.4.3 Macrocarpa

Macrocarpa (*Cupressus macrocarpa*) is commonly grown on New Zealand farms for shelter belts and hedging. The main monoterpenes in *C. macrocarpa* are α-pinene, myrcene, sabinene and terpinen-4-ol (Briggs & Sutherland, 1942; Malizia *et al.*, 2000; Zavarin *et al.*, 1971) (Figure 2.8). Myrcene and α-pinene have been shown to inhibit nitrification but sabinene has not. The monoterpenoid alcohol terpinen-4-ol has not been tested but has high antimicrobial activity (Carson & Riley, 1995). This is likely due to greater solubility than similar hydrocarbons. It may therefore have an inhibiting effect on nitrifiers. Other monoterpenes which are present in low concentrations may also potentially inhibit nitrification. *C. macrocarpa* also produces the bicyclic diterpenoid isocupressic acid; this compound can cause abnormal birth or abortion in cattle (Parton *et al.*, 1996; Sloss & Brady, 1983)

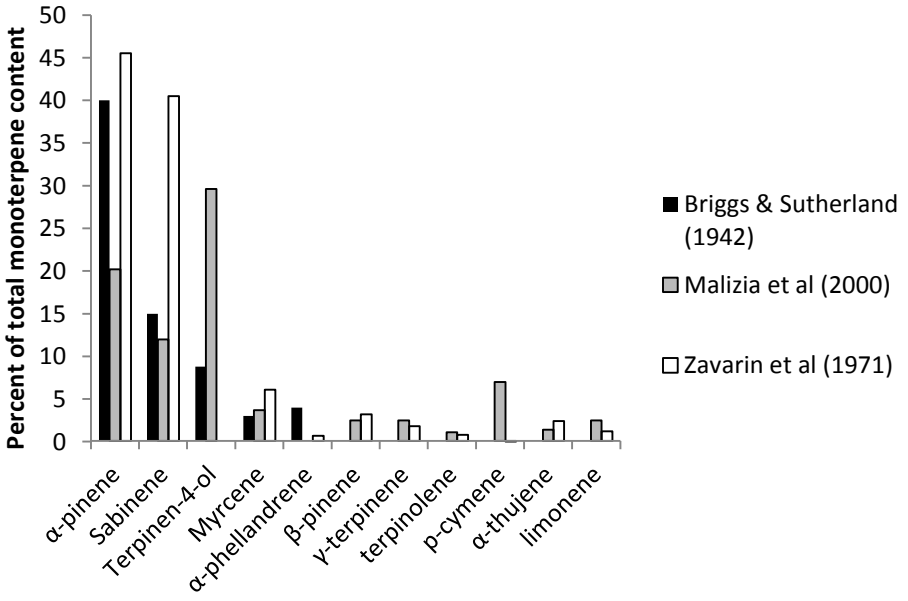

Figure 2.8: Proportion of total monoterpenoid content in essential oil from macrocarpa (Data retrieved from Briggs & Sutherland (1942), Malizia *et al.* (2000) & Zavarin *et al.* (1971).

2.4.4 Monterey pine

Pinus radiata is the most common tree grown in New Zealand (Ministry for Primary Industries, 2012). It is widely used in forestry and is also present on many farms. *P. radiata* is in the same genus as the ponderosa pine which can inhibit nitrification (Lodhi & Killingbeck, 1980; White, 1986, 1991).

Cooper (1986) measured soil inorganic N and nitrate loss to surface water each month between May 1982 and May 1983 on New Zealand's Central Volcanic Plateau. Nitrate losses to rivers draining *P. radiata* and pasture watersheds were 0.6 kg ha^{-1} y^{-1} and 7.6 kg ha^{-1} y^{-1}, respectively. There was little difference in the ammonium levels between the two sites but mean nitrate concentration in the pine watershed was lower throughout the year. The nitrifying potential of pasture soils was higher than pine soils on all sampling occasions. The average nitrifying potentials were 0.330 µg N g^{-1} ha^{-1} and 0.115 µg N g^{-1} ha^{-1} for pasture and pine soils, respectively.

Cooper (1986) did not find this difference in nitrifying potential in laboratory studies. It was suggested that this could have been due to phenolic compounds being bound by allophanic clays present in the soils. This could prevent them from inhibiting nitrifiers and meant that a living root system was required to continuously produce inhibiting compounds. They further concluded that a live root system would limit ammonium supply to nitrifiers through competition or by slowing mineralisation rates.

P. radiata bark extracted with ethanol, and leaves extracted with water, had high levels of phenols and inhibited nitrification and mineralisation. The bark extracts also decreased soil respiration and microbial biomass (Suescun *et al.*, 2012).

P. radiata wood has been shown to have high contents of α-pinene and β-pinene (54 and 149 mg kg^{-1} wood, respectively) (McDonald *et al.*, 1999). Other main monoterpenoids were myrcene, limonene, terpinolene, 1,4-terpineol and α-terpineol (McDonald *et al.*, 1999). Valenzuela *et al.* (1966) showed that limonene was high in needles and shoot tips (21.6% and 24.7% of total monoterpenes, respectively) of seedlings grown in Chile (Valenzuela *et al.*, 1966). α-Pinene and limonene have both been shown to be inhibitory and are present in large quantities. The other monoterpenoids are also likely to cause inhibition.

Chapter 3

Materials and Methods

Most of the time during this project was spent developing the following method. Appendix A shows the procedures, critical steps and trial data obtained during this method development.

This study used a bacterial bioassay to determine the nitrification inhibition potential of *Pinus radiata* D.Don*, Cupressus macrocarpa* Hartw. ex Gordon*, Eucalyptus nitens* H.Deane & Maiden*, Leptospermum scoparium* J.R.Forst. & G.Forst.*, Kunzea ericoides* (A.Rich) Joy Thomps., and *Sophora tetraptera* J.S.Mill. compared to perennial ryegrass (*Lolium perenne* L.). The bacterial culture used in this study was obtained from Plant and Food Research and contained *Nitrosospira* spp. enriched from a Lincoln, New Zealand soil. The *Nitrosospira* sp. in the enrichment has been shown to fall within the 3a2 group of *Nitrosospira*, based on ammonium monooxygenase (AMO) gene phylogeny (Bulman, pers. comm.). This culture has been proposed as a bioassay to determine nitrification (Bulman, pers. comm.). *Nitrosospira* cultures were grown at 27°C in an ammonium buffer solution (Recipe in Appendix B). Progress of the cultures was monitored by the colour change in the phenol red pH indicator. Cultures were typically grown for two days after being refreshed, for establishing bioassays. 1.8 L of cultures was centrifuged at 10,000 rpm for ten minutes to concentrate the cells and remove nitrate and nitrite. Cells were re-suspended in 400 mL of fresh buffer through agitation and then incubated at 18°C for 16 hours. Low cell density prevented an accurate determination of the optical density (Bulman, pers. comm.).

The suspension contained excess ammonium, which is the only energy source for the nitrifying bacteria contained in the broth. This was important for ensuring changes in ammonium pool, such as through volatilisation or immobilisation, did not affect nitrate production rate (i.e. ammonium was not rate limiting). The pH of all the solutions used in these experiments was 7.8. A buffer (0.5 mmol K_2HPO_4 L^{-1}) ensured that the plant extracts did not significantly alter the pH of the solutions. Change in pH not only affects rates of nitrification, but may result in significant ammonia volatilisation, the sole energy source for the bacteria, at high pH values.

The plant extracts also contained N. Although this was not determined, the N added in the extracts can be estimated using the C concentration in the final extract solution (max 5 mmol L^{-1}), and a C:N ratio of 10 (e.g. a high-N pasture containing 5% N and 50% C). In this case, the extract would have added just 0.5 mmol of N, which is insignificant compared to the 20 mmol N present in the initial solution. Given that the nitrate concentrations in the solutions immediately following the addition of

the plant extracts were below detection limits (1.6 µmol L^{-1}), and nitrite was not significantly different between treatments, the plant extracts did not add significant amounts of (NO_2^-; NO_3^-)-N to the system.

3.1.1 Plant material

Plant material was collected from Lincoln University, Canterbury, New Zealand during May (late autumn) and stored frozen for a month prior to the trials (Figure 3.1). There were three replicates of each species taken from different individuals in the vicinity of Lincoln University. Plant material was washed thoroughly in deionised (DI) water and excess water removed using paper towels.

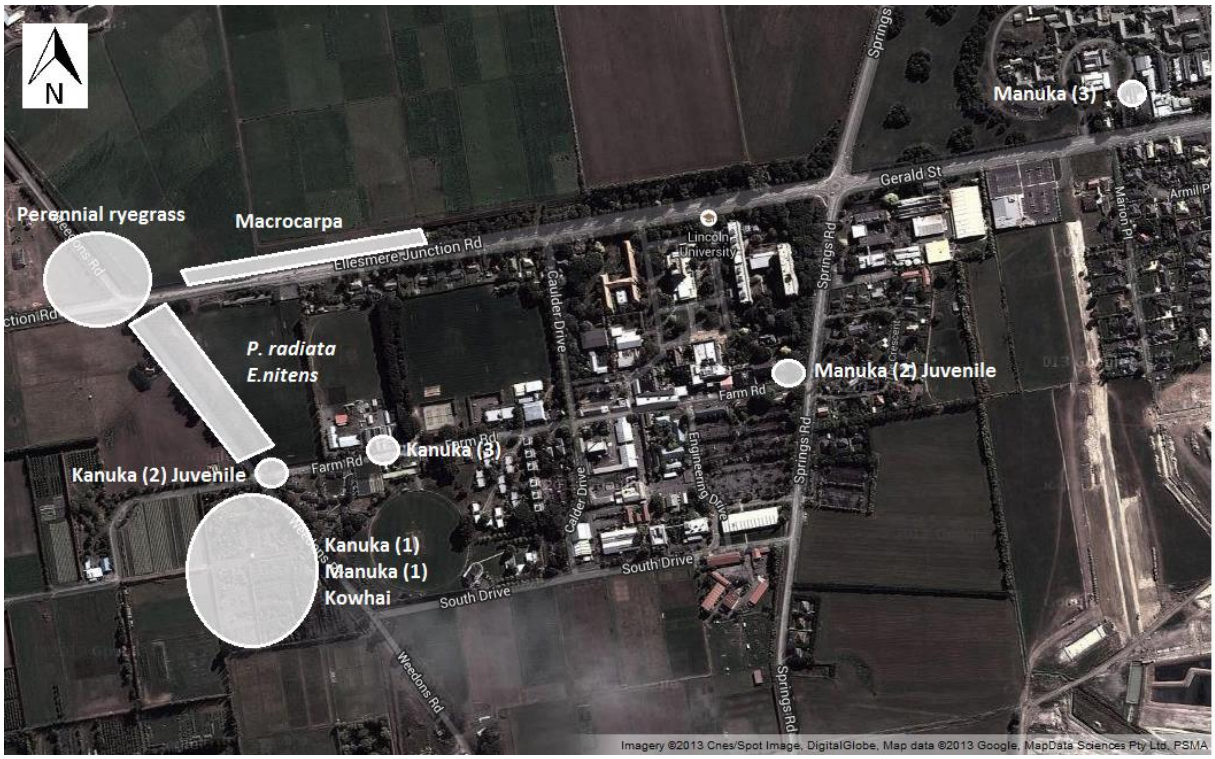

Figure 3.1: Map of Lincoln University campus and surrounds where plant material was collected.

A mortar and pestle was used to grind 2.0 g samples of frozen leaf material suspended in liquid nitrogen. This material was then extracted with 25 mL of water for half an hour before filtering through Whatman 1 filter paper.

3.1.2 *Nitrosospira* cell culture bioassays

From the prepared cells, 44 mL was added to conical flasks with 1 mL of plant extract. This resulted in extract concentrations equivalent to the extraction of 1.8 g of plant material per L of cell solution. Leftover plant extract was frozen for TOC analysis. For DCD, 0.145, 0.29, 0.58 or 1.16 mmol of DCD was added to 45 mL of cell culture to act as a positive control for nitrification inhibition. In addition, 45 mL of un-amended cells was used as a negative control and 45 mL of un-amended buffer solution

to indicate the absence of nitrification in the buffer. Conical flasks were covered with aluminium foil, agitated and incubated at 27°C for 48 hours. Method development (Appendix A) indicated that significant nitrification only occurred at 48 hours. Data for the 0 and 24 hour treatments are given in Appendix A.5.

3.1.3 Chemical analyses

After 48 hours of incubation, 10 mL of solution was removed from each sample and frozen. These were later analysed by a Flow Injection Analysis (FIA) system- ALPKEM Model 3000, for nitrate, nitrite and ammonium concentrations. The detection limits were 1.6 µmol L^{-1} for nitrate, 0.43 µmol L^{-1} for nitrite and 0.01 mmol L^{-1} for ammonium. Nitrite and ammonium values can be found in Appendix B. Plant extracts were measured by Total Organic Carbon Analyser (Shimadzu TOC-5000A) to determine soluble carbon in solution.

3.1.4 Statistical analysis

Statistical analysis was carried out using Minitab 16.0. Results were analysed with one and two-way ANOVA. A two-way ANOVA was used to compare the interaction between species and time on inorganic N concentrations while a one-way ANOVA was used to compare species at a specific time interval. The *post hoc* test used to identify significantly different groups of data was Fisher's method (95% confidence interval). Standard errors of the mean were calculated using the standard deviation divided by the square root of the number of samples.

Chapter 4

Results

The negative control treatments, namely the un-amended cells and *L. perenne* extracts had the highest nitrate concentrations in both trial one (41 and 61 µmol L^{-1}, respectively) and trial two (119 and 90 µmol L^{-1}, respectively). There were no significant differences between the nitrate concentrations of the un-amended cells and the *L. perenne* treatment in either trial. In both trials, the positive control, DCD, had nitrate concentrations below detection limits (1.6 µmol L^{-1}) with a DCD concentration in solution of just 0.145 mmol L^{-1}.

Nitrate concentration with *S. tetraptera*, *E. nitens* and *K. ericoides* were not significantly different from that of *L. perenne*. This indicates that these species do not exhibit significant nitrification inhibition. In contrast, *P. radiata, L. scoparium* and *C. macrocarpa* had significantly lower nitrate concentration than *L. perenne* ($P<0.001$).

In trial one (Figure 4.1-A), *P. radiata* and *C. macrocarpa* decreased nitrate produced by 90% and 83%, respectively, compared to *L. perenne.* In trial two (Figure 4.1-B), *P. radiata* and *L. scoparium* decreased nitrate produced by 60% and 67%, relative to *L. perenne*. Results for *K. ericoides* in our study were variable with a mean of 62 µmol L^{-1}, but a high standard error (20 µmol L^{-1}). One specimen decreased nitrate produced by 75% relative to *L. perenne*, but the other two had no effect on nitrate concentration.

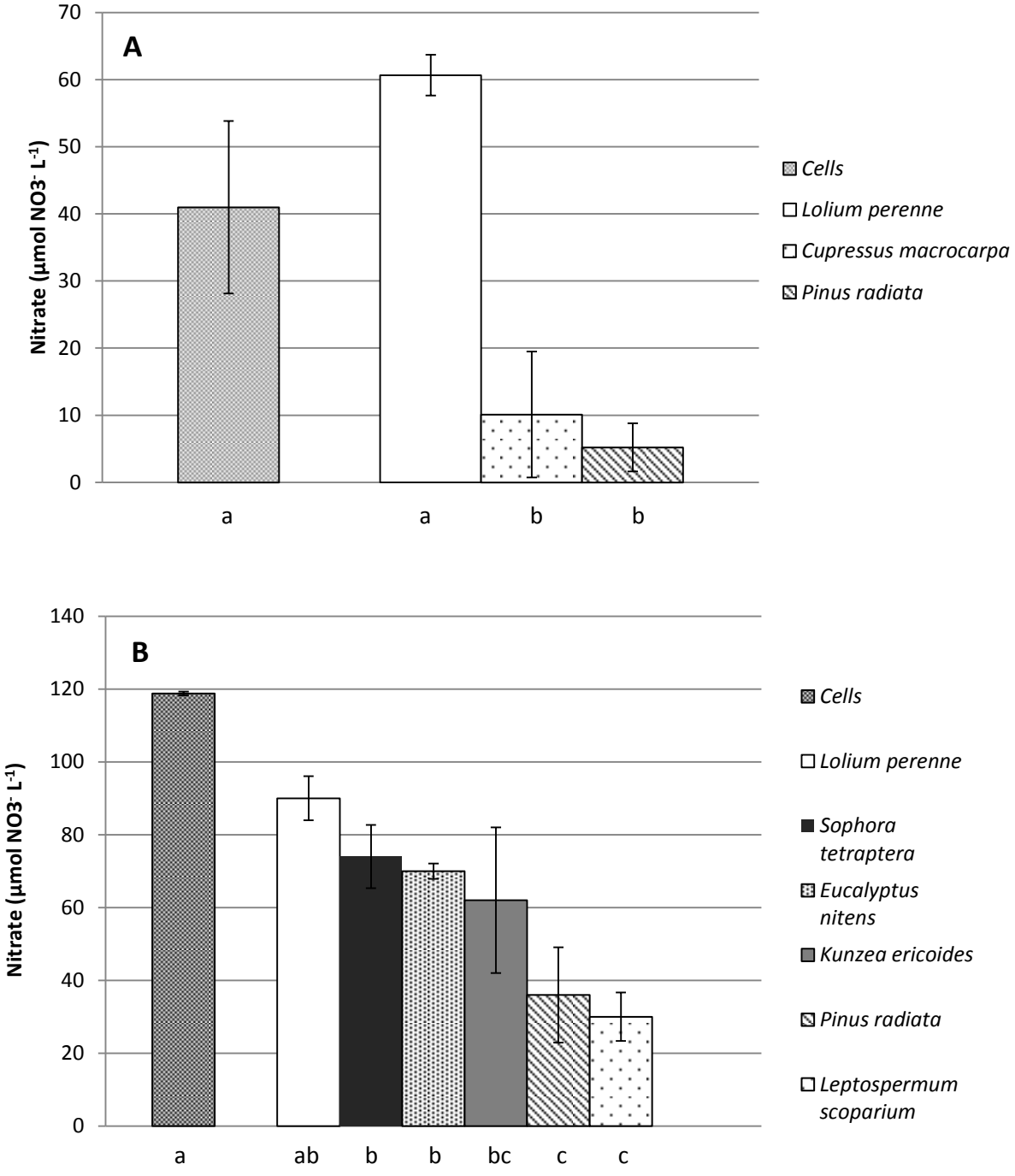

Figure 4.1: Nitrate produced 48 hours after addition of 1.8 g of plant material extracted per L of cell solution, in the first (A) and second (B) trials. Initial nitrate concentrations were below detection limit of 1.6 µmol L^{-1}, and below detection limits with DCD at each time point, therefore not shown. Error bars are standard error of the mean (n=3); cells (n=2). Letters below graphs indicate significant difference (P=0.05).

Table 4.1 shows the organic C concentrations in the cell cultures that resulted from the addition of the plant extracts. The concentration of any nitrification-inhibiting compound will be proportional to this concentration. However, the proportions of nitrification-inhibiting compounds, or indeed any compounds, in individual extractions were not determined. Carbon concentrations for all plant extracts were greater than DCD (0.145 mmol L^{-1}). However, DCD is a pure nitrification inhibitor, whereas the plant extracts were mixtures of a suite of compounds.

There was no significant difference between carbon concentrations of plant extracts in trial one. Carbon concentrations were significantly higher in *L. scoparium*, *S. tetraptera,* and *E. nitens* than other treatments in trial two (Table 4.1). There was no correlation between soluble carbon from plant material and nitrate concentration at the plant extract concentrations trialled. Soluble carbon in cell solution ranged from 2.3- 5.0 mmol C L^{-1} (Table 4.1).

Table 4.1: Mean carbon concentration (mmol C L^{-1}) of plant extract in cell solution. Values in brackets represent the standard error of the mean (n=3). Letters following a mean indicate significantly different groupings (P<0.05). Note that concentrations in *Trial 1* and *Trial 2* are not statistically compared.

Species	Carbon concentration (mmol C L^{-1})
	Trial 1
L. perenne	2.9 (0.3)a
C. macrocarpa	3.2 (0.4)a
P. radiata	3.3 (0.4)a
	Trial 2
L. perenne	2.7 (0.1)a
K. ericoides	2.6 (0.1)a
P. radiata	3.3 (0.3)a
L. scoparium	4.1 (0.2)b
S. tetraptera	4.2 (0.4)b
E. nitens	4.3 (0.4)b

There was no significant difference between ammonium concentration with potential BNIs and ammonium concentration with *L. perenne* (Appendix B) and amount of nitrate produced was insignificant compared to the ammonium concentration present. Final ammonium concentrations ranged from 12, 000-20,000 µmol L^{-1} and final nitrate concentrations up to 120 µmol L^{-1}. N_2O production and N_2 production were not determined in this study.

Based on these results, the leaves of *P. radiata*, *L. scoparium* and *C. macrocarpa* contain chemicals that inhibit nitrification in cell solution. These findings were not due to differences in carbon

concentration as there was no correlation with nitrate concentration; or due to ammonium concentration (Appendix B).

Chapter 5

Discussion

5.1 Potential of plants for biological nitrification inhibition

That *L. perenne* did not inhibit nitrification was expected as previous studies have shown only weak BNI properties (Subbarao, Rondon*, et al.*, 2007). The lack of significant difference, between the un-amended cells and *L. perenne,* confirms *L. perenne* is a suitable species for comparison with potential BNIs. This is important as comparing potential BNIs to un-amendened cells neglects the effect of carbon addition.

P. radiata had previously been tested for its BNI properties, which showed it to be a nitrification inhibitor (Cooper, 1986; Suescun *et al.*, 2012). Findings in this study were consistent with those study. Current lysimeter research by Esperschuetz (unpublished) gives further evidence that *P. radiata* inhibits nitrification. Trials showed a decrease in nitrate leaching with *P. radiata,* compared to *L. perenne* under low N and high N treatments (Figure 5.1). Compounds causing nitrification inhibition could include monoterpenoids, such as limonene, α-pinene, myrcene, and/or phenolics.

This study found that *C. macrocarpa* inhibited nitrification in cell solution. Compounds that could be the cause of decreased nitrate production in *C. macrocarpa* include a-pinene and myrcene, which have both shown BNI effects (Paavolainen *et al.*, 1998; Ward *et al.*, 1997; White, 1991), and/or the monoterpenoid alcohol terpinen-4-ol, which has not been tested as a BNI but has high antimicrobial activity (Carson & Riley, 1995).

L. scoparium also inhibited nitrification in cell solution. Current lysimeter research by Esperschuetz (unpublished) is consistent with this finding. Trials showed a decrease in nitrate leaching with *L. scoparium,* compared to *L. perenne* under low N and high N treatments (Figure 5.1). The inhibition by *L. scoparium* indicates that triketones such as flavesone, *iso*-leptospermone and leptospermone, and/or sesquiterpenes could have potential in inhibiting nitrification. The triketones give *L. scoparium* its antimicrobial properties (Porter & Wilkins, 1999).

The effect of *L. scoparium,* a pioneer species that thrives on disturbed sites, on nitrate concentration is of interest as it is in contrast to Rice and Pancholy's (1973) hypothesis that nitrification inhibition increases with succession. Unlike many primary successors, *L. scoparium* does not fix nitrogen. Therefore, to colonise low fertility soil, having a mechanism to limit nitrogen losses through nitrate leaching and denitrification, would be a competitive advantage, resulting in the evolution of BNI traits.

As a nitrogen fixer, *S. tetraptera* would have reduced benefits from BNI traits. However, *S. tetraptera* produces compounds that are toxic to mammals. Nitrification was not inhibited with *S. tetraptera,* and although phenolic constituents of *S. tetraptera* and *S. microphylla* are distinct (Markham & Godley, 1972), it is unlikely that the other common *Sophora* species in New Zealand, *S. microphylla*, is a potential BNI either, as neither *S. tetraptera* nor *S. microphylla* have general enzyme inhibiting properties (Kellam *et al.*, 1992).

The response of nitrate production to *K. ericoides* extracts measured in this trial was unexpected. *K. ericoides* contains high levels (>50% of dry oil composition) of α-pinene. In this trial, two specimens did not inhibit nitrification, but one reduced nitrate production relative to *L. perenne* by 75%. Esperschuetz findings for *K. ericoides* grown in lysimeters were less variable than the results for this study and all replicates reduced nitrate leaching from lysimeters relative to *L. perenne* (Figure 5.1). This indicates further research with this species is still required.

Interestingly, *E. nitens* leaves, which contain antiseptic compounds, did not inhibit nitrification. This was unexpected, as field sampling has shown nitrification to be low under eucalyptus stands in a range of soils and climatic conditions (Ellis & Pennington, 1989; Gomez-Rey *et al.*, 2010). Variation in monoterpenes between *E. nitens* species means that although nitrification was not inhibited by *E. nitens* other species may have an effect. For example, *E. nitens* was characterized by high levels of 1,8-cineole and α-pinene while *E. denticulata* had high levels of *p*-cymene and γ-terpinene (Li *et al.*, 1994). It was found that the oil yields from *E. nitens* juvenile leaves were significantly higher than that from the corresponding adult leaves for all source localities examined (Li *et al.*, 1994); the trees sampled in this trial were adults, so juveniles may differ from this study's results.

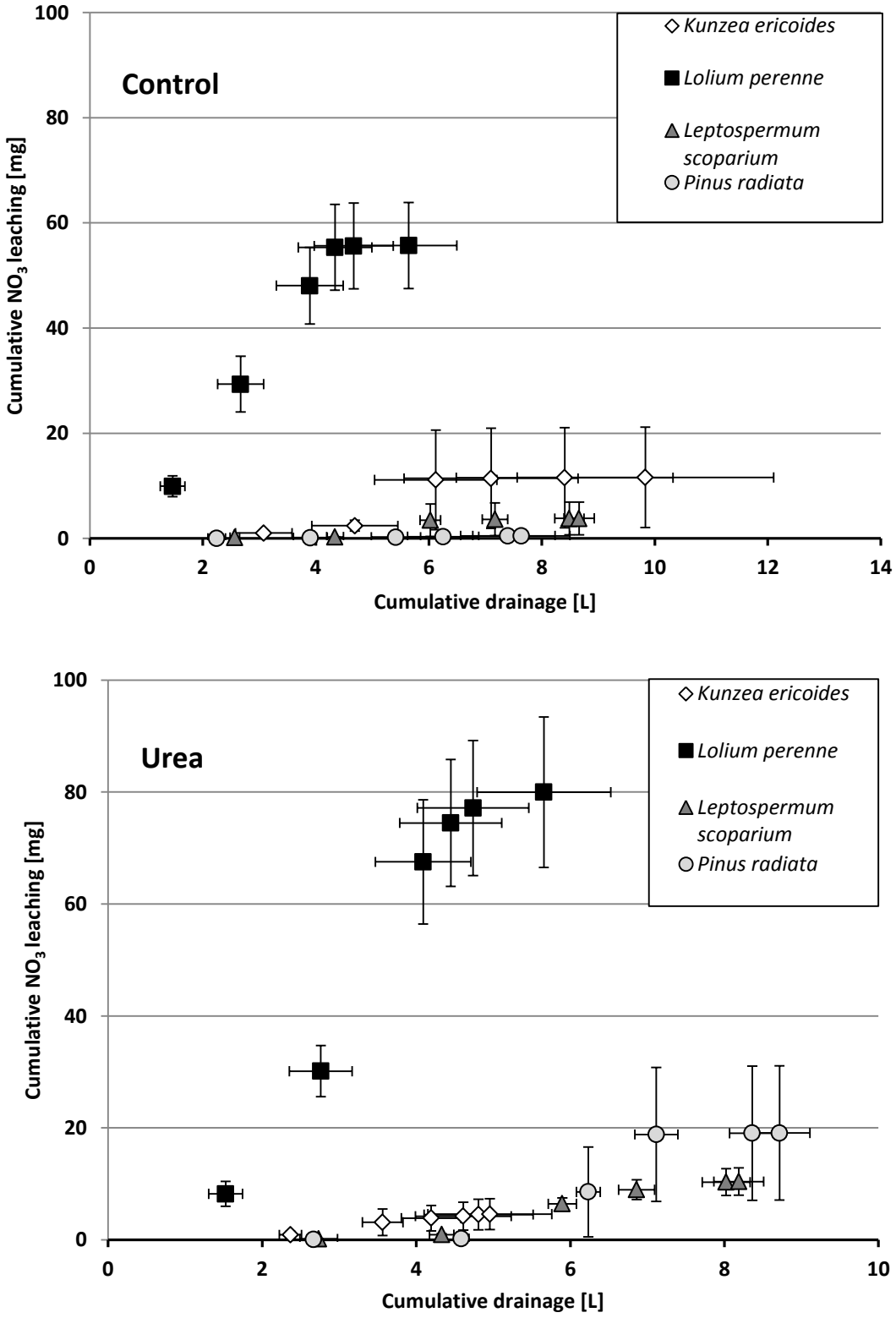

Figure 5.1: Cumulative NO_3^- leaching (mg) over time with cumulative drainage (L) from lysimeters for a control soil, with no amendments, and a urea amended soil (Esperschuetz, unpublished data).

As with previous work (Amberger, 1989; Di & Cameron, 2002), DCD completely inhibited nitrate production at the lowest concentration tested (0.29 mmol DCD-C L^{-1}), with nitrate levels below detection limits (<1.6 μmol L^{-1}). *L. scoparium, P. radiata,* and *C. macrocarpa* did not completely inhibit nitrification with higher carbon concentrations (2-5 mmol C L^{-1}). This carbon, however, is not all active chemical as it is also contained in sugars and structural compounds. The active compounds in the individuals of these species tested that caused BNI effects, are unknown and require further investigation. Previous studies indicated compounds likely to be present but this will vary based on genotype and environment. The decrease in nitrate concentrations observed with *L. scoparium*, *P. radiata* and *C. macrocarpa* extracts may have been due to either toxicity to the microorganisms or direct inhibition of AMO or HAO. Identification of active compounds, as well as plate counts of cell numbers, will allow the mechanism by which nitrification inhibition occurred to be determined. If inhibition is through toxicity, the effect on beneficial soil organisms should be examined.

This experiment occurred in cell solution containing a *Nitrosospira* enrichment. The bioassay was much more rapid and inexpensive than a lysimeter trial and allows a large number of samples to be screened. The use of cell culture allows many factors to be regulated that in soil would be difficult to control such as C:N ratio, pH, and distribution of plant extracts. In this system, total ammonium, nitrite and nitrate concentrations can be measured directly. Responses of these factors in cell culture are more likely to be directly due to the addition of phytochemicals rather than the abiotic environment, macro-organisms and non-nitrifier microorganisms.

However, a cell solution is not fully representative of soil processes and field conditions. The effects of a plant extract on nitrate concentration measured in cell solution may or may not be evident when the same extract is applied to soil. In soil there are many other variables and processes, and the BNIs will not reach soil nitrifiers at the same concentrations or against the same chemical background as cell solution. This means the potency of phytochemicals observed in cell solution is likely to differ when examined in soils. It is therefore important to verify species of interest identified from the bioassay in field or glasshouse trials.

The nitrifying bacteria culture used in these experiments was a New Zealand soil enrichment. The predominant species within this enrichment was a *Nitrosospira* species. Nevertheless, it is known that there were other bacteria species within the enrichment. These bacteria may affect other processes in the nitrogen cycle such as mineralisation, immobilisation and denitrification. This makes it harder to be certain whether changes in nitrate were due to nitrification inhibition or another factor, such as increased immobilisation of the nitrate as a response to carbon addition.

As ammonium concentration was in excess while nitrate concentration was low, it was assumed immobilisation of nitrate would be low, with predominant ammonium immobilisation. Mineralisation

is a process converting organic N to ammonium so should not influence nitrate by changing an already large ammonium pool. Volatilisation of ammonium would have occurred due to the pH 7.8 buffer solution, but again, as ammonium was in excess, this should not affect nitrification.

Results from this study assume denitrification is constant between all samples. However some studies have suggested denitrification can be directly inhibited by some compounds (Amaral & Knowles, 1998). This would result in increased nitrate. Future studies should measure N_2O and N_2.

Using an ammonium concentration that was in excess could have reduced the effectiveness of phytochemicals. High levels of ammonium means that ammonium is better able to compete with monoterpenes for the active site of AMO (White, 1991). This may explain why the α-pinene rich plants, *K. ericoides* and *E. nitens*, did not inhibit nitrification. Furthermore, monoterpene additions to soil that is N-rich results in increased CO_2 production and immobilisation of N (White, 1991). This is because the monoterpenes can be used as a carbon source by heterotrophic bacteria (Paavolainen *et al.*, 1998). In comparison, with N-limited soils, addition of carbon has little effect on the heterotrophic community allowing the monoterpenes to persist in the soils and inhibit nitrifying organisms (White, 1994). White (1991, 1994) and Paavolainen *et al*. (1998) used purified compounds in soil, while in this study BNI compounds were a component of a larger carbon pool containing compounds, such as carbohydrates, that are easier for the heterotrophic bacteria to utilise. This may have reduced decomposition of BNI chemicals.

Concentrations of the compounds that would be present in soil under these plants and whether they increase over time under the plant stands are unknown. The length of time they remain in the soil is also unknown and would depend on factors such as molecular structure, microbial activity and soil water content. White (1986) showed that volatiles, such as monoterpenes, had a short residence time in soil requiring plant material to maintain their concentrations. Opening containers each day to take out aliquots could have resulted in a loss of volatile compounds (White, 1990) This could decrease nitrification inhibition causing an increased nitrate production. These experiments were done over a 48 hour period, the effect of these compounds on nitrification after this time is unknown.

All of the plants were collected from Lincoln, New Zealand during May (late autumn). Chemical compounds are variable seasonally, for example plants have less active growth or are dormant during winter, which may affect nitrification inhibition. *L. scoparium* is genetically diverse between locations, with highly variable chemical profiles between and within populations (Porter *et al.*, 1998; Porter & Wilkins, 1999).

Addition of plant extracts would have introduced microorganisms. Future studies could use a filter that can remove bacterial cells from solution. However, such a filter may also remove colloidal material to which active phytochemicals are bound.

This study tested fresh leaves, whereas leaves present in leaf litter will likely differ due to senescence and decomposition processes. Given the short length (3 months) that Esperschuetz's lysimeters experiment has been running for, those results are more likely to have come from root exudates rather than from the decomposition of leaf litter.

5.2 Implications for New Zealand agriculture

L. scoparium, P. radiata, C. macrocarpa and *K. eriocoides* species could be planted as effluent disposal blocks, riparian strips or shelterbelts. Costs of planting *L. scoparium* may be offset by production of valuable manuka honey. *C. macrocarpa* is not recommended in areas where cattle could graze it as it contains isocupressic acid which causes abnormal birth and abortion.

Using nitrification inhibiting plants to produce mulch or oil extract, and applying directly to pastures, may have potential for reducing nitrate leaching. Addition of leaves directly to pasture would require a high application rate, exceeding that of pure DCD (approximately 20 kg ha^{-1} $year^{-1}$); this is likely to be costly both to harvest and spread, and difficult to incorporate into the soil, especially in established pastures. Identification of novel compounds produced by plants could allow selection of plants that produce greater than average concentrations, or, ideally, allow the production of purified active compound.

Chapter 6

Conclusions

This study showed that leaves from some common trees in New Zealand's agricultural landscapes, namely *L. scoparium*, *P. radiata* and *C. macrocarpa* have effective nitrification inhibiting properties making them potential alternatives to DCD. Leaves from other common trees, *E. nitens* and *S. tetraptera,* did not inhibit nitrification. Perennial ryegrass, *L. perenne,* did not inhibit nitrification and was a suitable negative control to compare candidate BNI species with.

The bioassay developed in this study was suitable for rapid, high throughput of plant samples, allowing effective screening of BNI potential. Preliminary and unpublished findings from a lysimeter trial at Lincoln University are consistent with the findings from these bioassays for *L. scoparium, P. radiata* and *L. perenne.* (Esperschuetz, unpublished data). Espershuetz's findings also indicated that *K. ericoides* had BNI properties, whereas results from this study were inconclusive about *K. ericoides*.

The environmental problem of nitrate moving into surface and ground water, which has become critical following the ban of nitrification inhibitor DCD, could potentially be decreased through use of *L. scoparium*, *P. radiata*, *C. macrocarpa* and *K. ericoides* in shelter belts, riparian and effluent plantings, or through application of leaf material or purified compounds from these plants to pastures.

Pioneer plants that are not nitrogen fixers could be further candidates for BNI research, potentially having evolved nitrification inhibiting properties to maintain nitrogen levels in the soil. *L. scoparium,* a common species on disturbed sites, was an effective nitrification inhibitor while a New Zealand native legume, *S. tetraptera,* was not a BNI.

Chapter 7

Further Research

Cell culture assays are an initial BNI screening technique. The main focus of this study was to identify potential biological nitrification inhibitors for field research and so the priority was on sampling a range of different species. From this screening *L. scoparium*, *P. radiata*, *C. macrocarpa* and *K. ericoides* were identified as requiring further research.

Further research should examine the effect of seasonal, environmental, genetic and developmental variation. Environmental factors could include N fertility, pH, soil texture, rainfall or temperature. These factors all influence nitrification (Tietema *et al.*, 1992). This study was focused on the BNI properties of fresh leaves. Decomposing senesced leaves and root exudates would also be of interest to study.

It is critical to determine the chemical profiles of plant extracts analysed that have BNI properties. Phytochemicals can be highly variable between genotypes, environments and developmental ages. Identifying compounds, such as through GC-MS, produced by these plants will enable a greater understanding behind the mechanisms of nitrification inhibition of *L. scoparium*, *P. radiata* and *C. macrocarpa* and may explain the variability between kanuka results.

Future research should, in addition to the method used in this study, implement plate counts of cells, and measurement of CO_2, N_2O and N_2 emitted and organic nitrogen, over the runtime of the bioassay. This will allow quantification of denitrification, immobilisation and mineralisation as well as indicating whether nitrification inhibition is due to toxicity or inhibition of nitrifying enzymes.

Appendix A

Method Development

To test the potential of New Zealand natives and exotics on nitrification inhibition a reliable method was required. Many experiments use cell cultures of nitrifiers or soil isolates to indicate whether or not nitrification could potentially be inhibited in soils (Rice & Pancholy, 1974; Subbarao, Wang*, et al.*, 2007; Ward *et al.*, 1997). In the original method used by Plant and Food Research, based on Shaw *et al.* (2006), plant material was extracted with methanol, and nitrite was used as a proxy for nitrification inhibition. McCarty and Bremner (1986) showed nitrite to be an unreliable indicator; however, this study was done in soil. Under soil conditions nitrite has a very short half-life and is rapidly oxidised by *Nitrobacter*. Thus measuring nitrite in soil is inaccurate. The cell culture used in this study was enriched with *Nitrosospira* and its use in nitrification bioassays, with a nitrite measurement rather than nitrate, had been proposed. This was proposed due to the ease and inexpense of measuring nitrite through spectrophotometry Therefore, part of the experimental design was aimed at determining whether nitrite could be used with this enrichment as a proxy for nitrification. Other areas developed during the experimental design included extraction method, optimal run time of the experiment, and extract concentration.

A.1 Initial method

A mortar and pestle was used to grind 2.0 g samples of frozen leaf material with liquid nitrogen. This material was extracted with 35 mL methanol and centrifuged (8,000 rpm for ten minutes) before filtering through Whatman 1 filter paper. Filtrate were dried in a Labconco CentriVap Concentrator (T <60°C) overnight. The weight of the dried extract filtrate was recorded. The extract was then dissolved in 1.5 mL of deionised water.

From the cells, prepared as described in Chapter 3, 20 mL was added to conical flasks and 500 µL or 1 mL of extract or 26 mmol L^{-1} DCD was added. Extract concentrations were equivalent to the extraction of 26.7 g and 53.3 g of plant material per L of cell solution. DCD concentrations in cell solution were 0.63 and 1.24 mmol L^{-1}. Conical flasks were covered with aluminium foil, agitated and incubated at 27°C for 48 hours. Nitrite readings were taken at three, six, twenty four and twenty eight hours. To take a reading 50 µL of cell solution or nitrite standard was added to 25 µL of sulphanilamide (50 mg mL^{-1} in 2.4 M HCl). Next 25 µL of N-(1-Naphthyl)ethylenediamine (30mg mL^{-1} in 1.2 M HCl) was added. Tubes were vortexed ca. three seconds. A spectrophotometer (540 nm) was used to measure the concentration of nitrite in solution. Nitrite standards used were 1.7, 4.5, 46, 97 and 280 µmol L^{-1} (0.1, 0.2, 2.1, 4.5 and 13.0 ppm).

A.2 Extraction method

This trial was performed to determine the feasibility of the original method and compare water and methanol extracts.

Methanol can extract a greater quantity of phytochemicals than water; in particular polyphenols (Eloff, 1998). Methanol also removes microorganisms associated with the plant material. However, the drying out of plant extracts, used in the original method to remove the methanol, made it is likely that volatile compounds, that could potentially be nitrification inhibitors, were lost. The dried extracts were difficult to weigh, but would have been highly concentrated. This mades it difficult to determine how much compound was being added to the 1.5 mL of water. By using a water extract, and not drying it down, the total carbon concentration could be determined through TOC analysis.

This experiment showed no significant difference between water extraction and methanol extraction (Figure A.1), however, only nitrite concentration was measured and so this should be repeated with nitrate measurements.

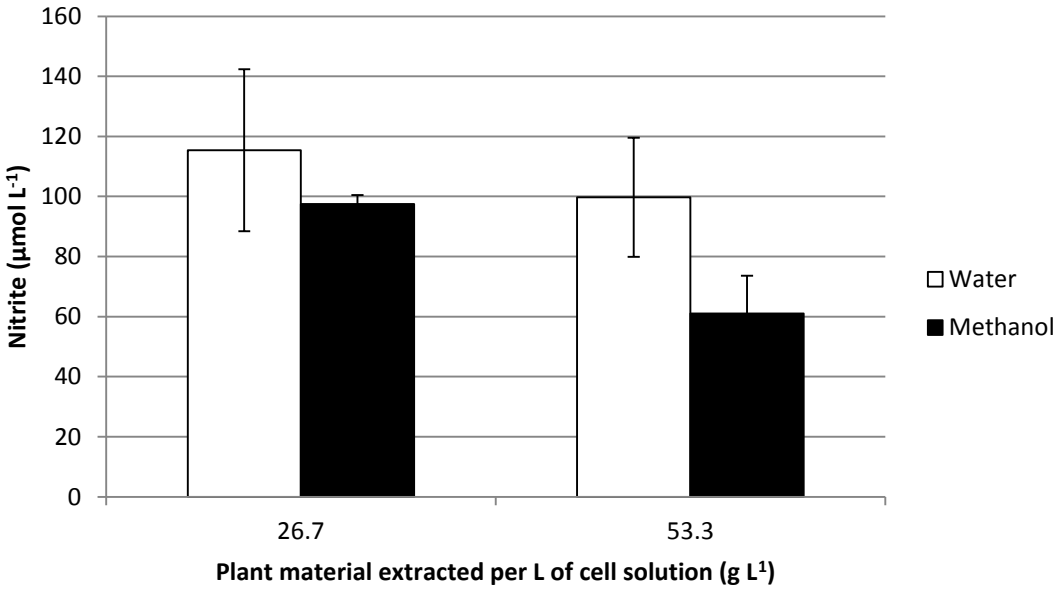

Figure A.1: Nitrite concentration, determined using FIA, after 28 hours at two concentrations of manuka extract and two extraction methods (2n). Error bars are standard error of the mean.

A.3 Extract concentration

Plant extracts (*L. perenne*, *P. radiata*, *C. macrocarpa*) were made using 2.00 g of leaves ground with liquid nitrogen and extracted with 25 mL of water and filtered with Whatman Grade 1 filter paper. In the first trial either 5 mL of plant extract or 1 mL of plant extract + 4 mL DI water was added to 40 mL of cell culture. This resulted in concentrations equivalent to the extraction of 8.9 g and 1.8 g of plant material per L of cell solution in trial one.

Table A.1 shows the change in nitrite concentration over time between species at high and low plant concentrations. With the high concentration extracts, there were no significant differences between species at 0, 24 or 48 hours. Nitrite concentration of perennial ryegrass was significantly lower at 24 hours than at 0 or 48 hours (P=0.024). There was no significant difference in nitrite concentration over time with macrocarpa or pine.

With the low concentration extracts, nitrite concentration increased over time (P<0.001). At 24 hours there was an apparent difference (P=0.054) in nitrite concentration, with 61.0 µmol L^{-1} for perennial ryegrass compared to 32.3 and 33.2 µmol L^{-1} for manuka and pine, respectively. At 0 and 48 hours there was no significant difference.

These results indicate that the 8.9 g L^{-1} plant extract concentration was too high as nitrite was not accumulating in the negative control (*L. perenne*). The lower nitrite concentration allowed net nitrite production to occur.

Table A.1: Mean nitrite concentration over time after the addition of high or low concentration plant extracts to cells (8.9 or 1.7 g of plant material extracted per L of cell solution).

Time (hours)	*Lolium perenne*	*Cupressus macrocarpa*	*Pinus radiata*
High concentration of plant extract (8.9 g L^{-1})			
0	5.2	6.4	5.9
24	2.3	3.0	1.5
48	6.3	5.3	11.0
Low concentration of plant extract (1.7 g L^{-1})			
0	5.4	5.0	4.7
24	61.0	32.3	33.2
48	68	75.5	70.8

A.4 Nitrite as a proxy for nitrification

Nitrite had been proposed as a measurement of nitrification inhibition for the cell culture used in this study. To determine whether it was a suitable alternative FIA readings were taken of nitrite and nitrate for treatments from both trial one and trial two, using the method described in Chapter 3. This section compares these results.

DCD reduced the presence of nitrite in a concentration dependent manner. There was a negative, linear correlation between DCD concentration and the change in nitrite concentration over 48 hours both trial one and two (R^2=0.81 and 0.94, respectively) (Figure A.2). This indicated nitrite levels were related to nitrification inhibition by DCD. However, nitrate was below detection limits with DCD and nitrite levels with the plant extracts were in the same range as nitrite with DCD, despite plant extracts not completely inhibiting nitrate.

Nitrite concentration did not increase with nitrate concentration with plant extract addition (Figure A.3). At low nitrate levels nitrite was completely independent of nitrate concentration. At higher nitrate concentrations (e.g. >8 μmol NO_3^- L^{-1}) there was an apparent negative correlation between nitrite and nitrate in trial two (R^2=0.419) but no linear correlation (R^2=0.258) for trial one. The overall results had a logarithmic correlation (R^2=0.648) for trial one but no logarithmic correlation (R^2= 0.288) for trial two.

The lack of positive correlation between nitrate and nitrite, and the fact considerable nitrate production is occurring, which means the cell cultures contain nitrite oxidising bacteria, indicates that nitrite cannot be used as an indicator of nitrification inhibition for these experiments. This agrees with McCarty and Bremner's (1986) findings that nitrite was too unstable for use as a proxy for nitrification inhibition. Based on these results an FIA analysis of nitrate is more appropriate than spectrophotometer measurement of nitrite, despite the ease of the spectrophotometer approach.

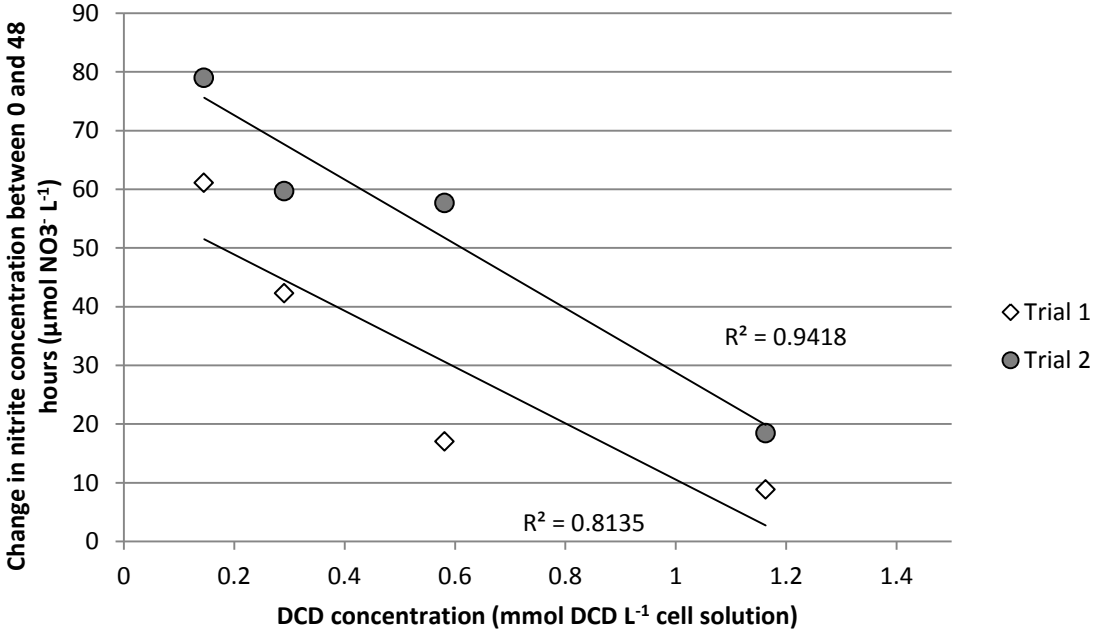

Figure A.2: Relationship between DCD concentration and the change in nitrite concentration over 48 hours in trials one and two.

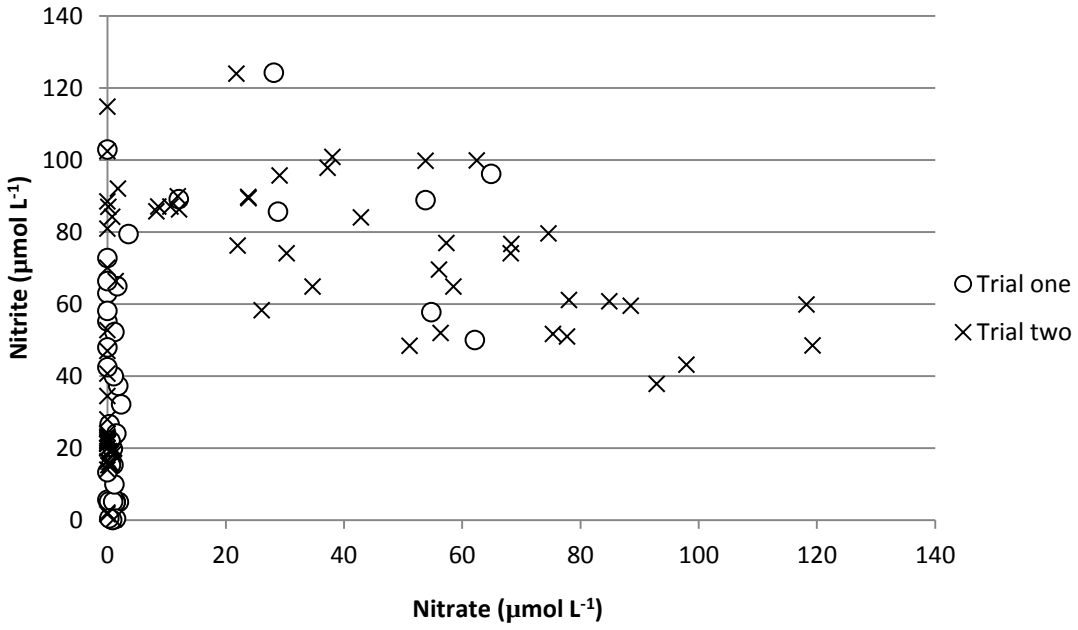

Figure A.3: Nitrite concentration (µmol NO_3^- L^{-1}) versus nitrate concentration (µmol NO_2^- L^{-1}) for trials one and two.

A.5 Experimental run time

Nitrate concentration was measured over time in trial one and trial two to determine the best time to take measurements of the bioassay with FIA. This section compares nitrate production between time points.

In trial one (Figure A.4-A), at 0 and 24 hours there was no significant change in produced nitrate and no difference between species. At 48 hours, nitrate production was significantly different relative to 0 and 24 hours *(P*<0.001), and significantly different between species (*P*<0.001).

In trial two (Figure A.4-B), there was no significant difference between species at 0 hours. There was a significant increase in nitrate concentration between 0 and 24 hours (P<0.001) and a significant difference (*P*=0.011) between species at 24 hours. At 48 hours, nitrate production was significantly different relative to each of 0 and 24 hours (*P*<0.001), and significantly different between species (*P*=0.016).

The run time of the experiment was an important factor; this is likely to vary between studies. Taking measurements at a wide range of time points is recommended when using cell culture. The concentration of cells in solution will have a large role in the rapidness of nitrification. Based on the results of this study, the nitrate concentrations at 48 hours were discussed (Chapter 4; Chapter 5).

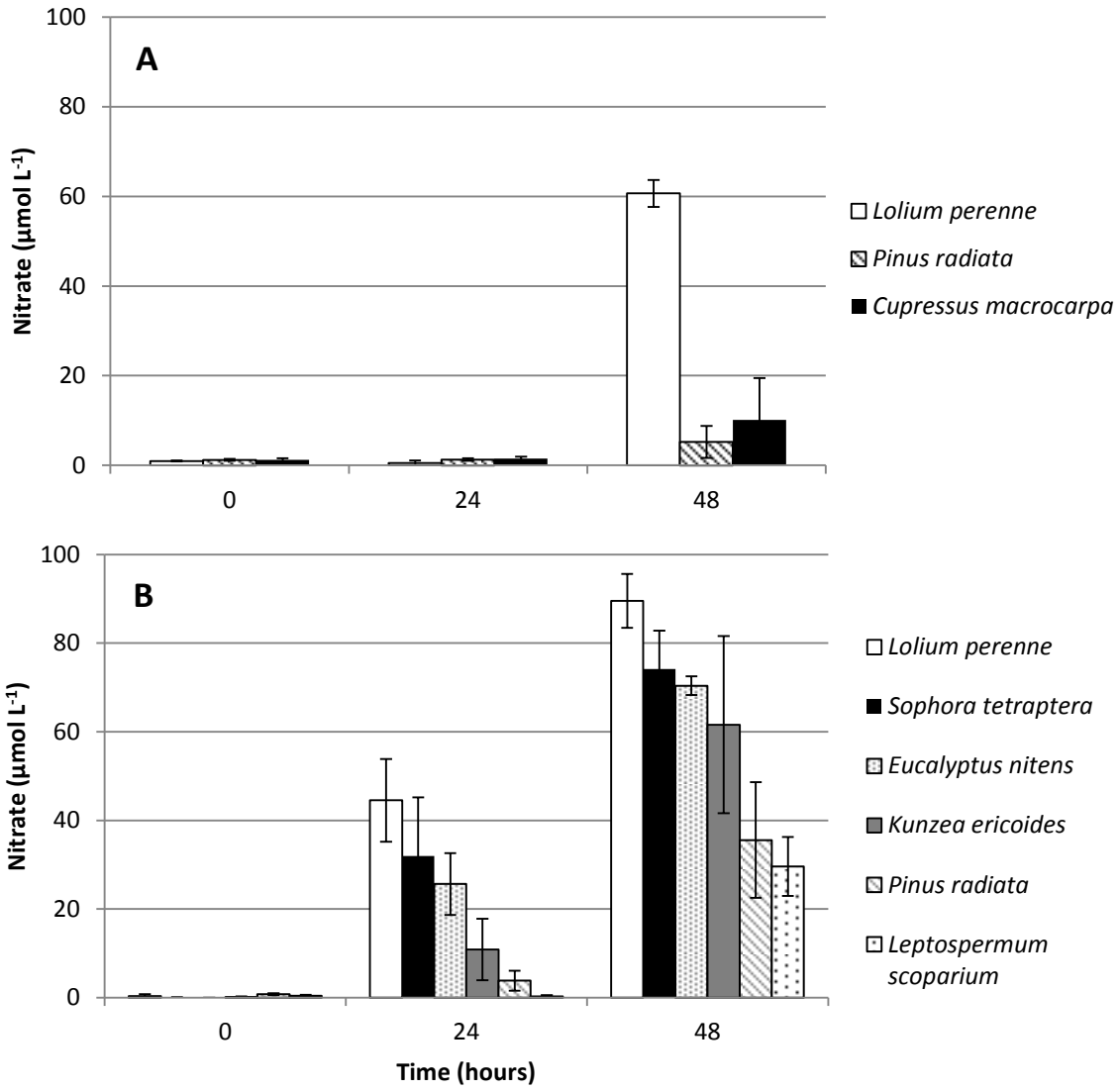

Figure A.4: Nitrate concentration (µmol L^{-1}) at 0, 24 and 48 hours in trial one (A) and trial two (B) following addition of 1.8 g plant material extracted per L of cell solution.

Appendix B

Ammonium and Nitrite Concentrations

B.1 Ammonium

Ammonium concentrations at 48 hours, compared to the initial level, for each species in trial one and trial two are shown in Figure B.1. Initial ammonium concentration was significantly greater (P=0.002) in the first trial than the second with a mean concentration at time zero of 19 mmol L^{-1} in trial one and 16 mmol L^{-1} in trial two. In trial one there was no significant difference in ammonium concentration between species at 48 hours. For trial two, at 48 hours, there was a significant difference (P=0.004) in mean ammonium concentration between kowhai (12.9 mmol NH_4^+ L^{-1}), and perennial ryegrass, kanuka, pine and eucalyptus with means ranging between 15.6 and 16.5 mmol NH_4^+ L^{-1}.

These results, show no difference in ammonium concentration between the non-BNI, *L. perenne,* and potential BNIs, *P. radiata*, *L. scoparium* and *C. macrocarpa,* this mean that changes in ammonium were not the cause of reduced nitrate concentration

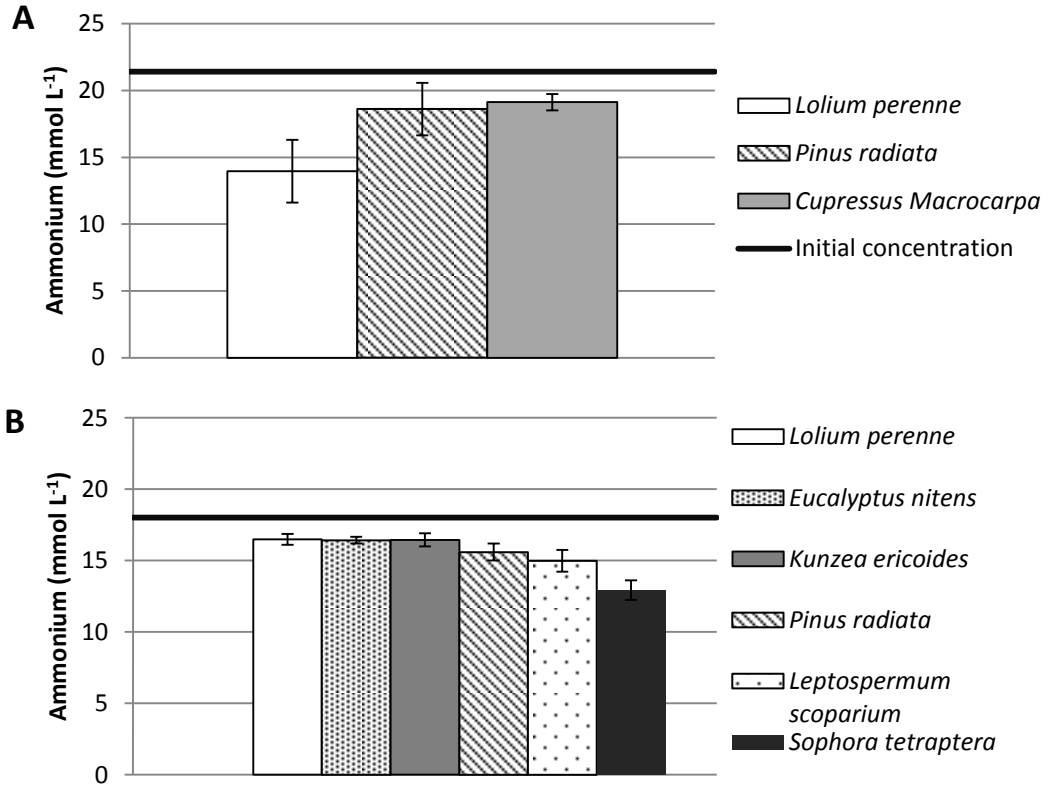

Figure B.1: Change in ammonium (NH_4^+) concentration 48 hours after addition of a plant extract to cell culture in the first (A) and second (B) trials, compared to initial ammonium concentration of cell solution. Error bars are standard error of the mean (n=3).

B.2 Nitrite

Nitrite concentration over time, for *L. perenne* and species that inhibited nitrate, is shown in Figure B.2.

In the first and second trials, mean nitrite concentration increased over time (P<0.001). There were no significant differences in nitrite concentration between species over time in trial one (P=0.453). In trial two there was a significant difference between species over time (P=0.023). At 0 and 24 hours for trial one and two there was no significant difference between species. At 48 hours there was no significant difference between species in trial one. In trial two *P. radiata, E. nitens* and *L. scoparium* had significantly higher nitrite concentrations than *L. perenne (*P<0.001).

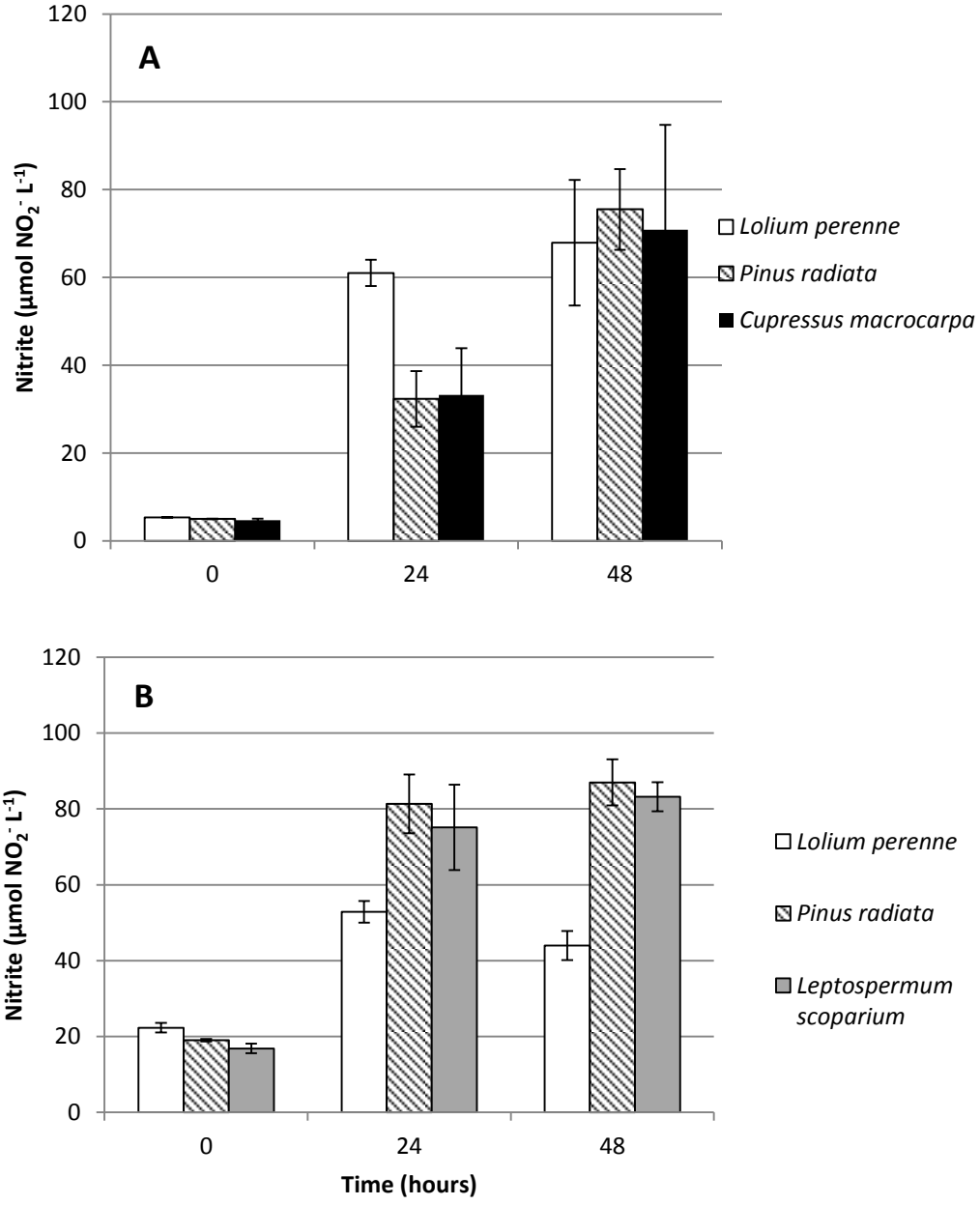

Figure B.2: Change in nitrite concentration over time after addition of a plant leaf extract to the cell culture. Error bars are standard error of the mean (n=3).

Appendix C

Growth Medium for *Nitrosomonas* and *Nitrosospira*

28 October 2008

From Margaret Roper

Stock solution 1

$(NH_4)_2SO_4$	1.7 g
$CaCl_2.2H_2O$	20 mg
$MgSO_4.7H_2O$	200 mg
K_2HPO_4	87 mg
Distilled water	1 L

Stock solution 2 (Trace elements)

$Na_2MoO_4.2H_2O$	10 mg
$MnCl_2.4H_2O$	20 mg
$ZnSO_4.7H_2O$	10 mg
$CoCl_2.6H_2O$	0.2 mg
$CuSO_4.5H_2O$	2 mg
Distilled water	100 ml

Stock solution 3 (Indicator solution)

Phenol red	50 mg
Distilled water	100 ml

Stock solution 4

FeNaEDTA	50 mg
$FeSO_4.7H_20$	50 mg
Distilled water	100 ml

To 1 L of stock solution 1, add 1 mL of stock solution 2, 1 mL of stock solution 3 and 1 mL of stock solution 4. Adjust the pH to 7.8 with 0.1M K_2CO_3 (indicator - pink).

The medium contains 20 mmol L^{-1} ammonium-N and is sterilised at 120^{o}C for 20 min.
The pH of the medium after autoclaving should be 7.8.

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