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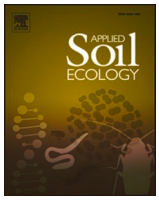

Effects of native plants on nitrogen cycling microorganisms in soil

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ABSTRACT

Plants modify the nitrogen (N) cycle in soil through plant N uptake, root exudation, and nitrification inhibition resulting from root exudates and changes to the physicochemical properties of soil. Plants with specific traits can be selected to manage N fluxes in the soil following the land application of N-rich wastes thereby reducing losses of N from these systems into the atmosphere (via nitrous oxide) and waterbodies (via nitrate). Previous work has shown that some New Zealand native myrtaceous species can reduce such losses more than other species, but the underlying mechanisms are unknown. It was hypothesized that lower N losses may result from the inhibition of nitrification and denitrification. We aimed to determine the effect of New Zealand native plant species on the abundance of nitrifying and denitrifying microorganisms in the soil using a pot experiment with five native plant species (*Carex secta*, *Coprosma robusta*, *Kunzea robusta*, *Leptospermum scoparium*, and *Metrosideros umbellata*) and exotic pasture (*Lolium perenne*). Half of the pots received fertilisation with N (urea), phosphorus, potassium, and sulphur, while the remainder were unfertilised controls. We quantified the abundance of bacteria, archaea, and the functional genes encoding nitrite reductase (*nirK*, *nirS*), nitrous oxide reductase (*nosZ*), and bacterial and archaeal ammonia monooxygenase (*amoA*) in the rhizosphere of these plants. Results indicated that plant species had a significant effect on the abundance of *nosZ* and bacterial *amoA*. In fertilised soil, the abundance of bacterial *amoA* was lower under mono- than dicotyledonous species. Similarly, the chemical properties of the soil differed between these groups. Monocotyledonous species took up more N and had lower concentrations of mineral N in the rhizosphere. This indicates that the increased competition for N likely reduced the abundance of *amoA* and therefore nitrification and nitrate losses. The results support the utilisation of species selection to reduce N losses. In particular, monocotyledonous species may be planted in high-fertility environments to mitigate N contamination of ground- and surface water. Future work should determine the mechanisms of plant specific interaction with ammonia-oxidising bacteria, although plant uptake can explain some of the observed differences.

1. Introduction

Nitrogen (N) pollution is a global environmental issue (Kanter et al., 2020). Galloway et al. (2003) calculated that 88 % of N inputs into agroecosystems are lost into air, soil, or water prior to reaching humans, totalling 149 Tg yr^{-1} worldwide. 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 and Groffman, 2015). Inputs of NO_3^- to surface waters can lead to eutrophication (Smith and Schindler, 2009). Groundwater with elevated NO_3^- concentrations can pose a public health risk where it is utilised for drinking water, since concentrations >1 mg NO_3^- L^{-1} in drinking water can increase the risk of colorectal cancer (Richards et al., 2022). Furthermore, high NO_3^- concentrations in drinking water have been linked with methemoglobinemia in infants, commonly known as blue baby syndrome (Brender, 2020).

Transformation processes of N in the soil are largely microbially mediated (Robertson and Groffman, 2015). Denitrification is the stepwise reduction of NO_3^- to nitrite (NO_2^-), NO, N_2O, and N_2 (Cameron

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et al., 2013). It mainly occurs under anaerobic conditions, when heterotrophic denitrifying bacteria use NO_3^- as terminal electron acceptor during respiration (Robertson and 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). There are two types of NO_2^- reductases, cytochrome *cd1* NO_2^- reductase (encoded by *nirS*) and copper-containing NO_2^- reductase (encoded by *nirK*). N_2O reductase is encoded by *nosZ* (Kandeler et al., 2006). However, archaea and fungi can also play a role in denitrification, with distinct underlying mechanisms (Hayatsu et al., 2008).

In contrast, nitrification is the two-step microbial oxidation of NH_3 and ammonium (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, NH_4^+ is oxidised to 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 and Groffman, 2015). The second step of nitrification is the oxidation of NO_2^- to NO_3^- by nitrite oxidoreductase associated with nitrite-oxidising bacteria (Robertson and Groffman, 2015). There is evidence of complete nitrification by *Nitrospira* spp. (Daims et al., 2015), as well as heterotrophic nitrification at low soil pH (Zhang et al., 2014).

Plants can significantly 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). These factors can affect the abundance and activity of (de) nitrifying microorganisms (Bowatte et al., 2016). Some plants can release compounds that directly inhibit nitrification, 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). The associated nitrification inhibition derives from a direct inhibiting effect of BNIs on ammonia monooxygenase or hydroxylamine oxidoreductase (Coskun et al., 2017). The use of plants with potential to suppress nitrification could reduce N losses from agricultural land and other high-nutrient environments.

Some New Zealand (NZ) native species in the Myrtaceae family have the potential to reduce NO_3^- leaching and N_2O emissions more than other species (Esperschuetz et al., 2017; Franklin et al., 2017; Halford et al., 2021). No underlying mechanisms for these observations have been described. However, Esperschuetz et al. (2017) reported 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. Furthermore, *K. robusta*, *L. scoparium*, *Metrosideros robusta*, and *Pseudowintera colorata* 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 the synthetic nitrification inhibitor dicyandiamide (Tahir et al., 2021). The antimicrobial properties in the essential oil of *K. robusta* are associated with α-pinene (Porter and 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).

We hypothesized that N speciation and N fluxes in the soil will be affected by plant species, through both direct and indirect effects of plants on the abundance of N-cycling microorganisms. This study aimed to determine whether microorganisms involved in nitrification and denitrification differed 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.

2. Materials and methods

2.1. Pot experiment

2.1.1. 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") using 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 a NPKS-fertiliser treatment, equivalent to 200 kg N ha^{-1}). *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 (Meister et al., 2022). *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 ryegrass *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 the 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 physicochemical properties. 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). 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 of NZ. 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 was used (Specialty Seeds, 2019). Within the greenhouse, pots were arranged in a completely randomised design and were newly randomised every two weeks. Pots were watered to field capacity every 2–3 days with tap water.

2.1.2. Fertilisation

After 26 weeks, half of the pots received NPKS fertilisation (Table S-1). Nitrogen was applied in the form of urea, equivalent to 200 kg N ha^{-1} (Table S-1). Furthermore, P (30 kg ha^{-1} equiv.), K (224 kg ha^{-1} equiv.), and S (61 kg ha^{-1} equiv.) were added because these plant nutrients are present in biowastes (Gutierrez-Gines et al., 2019) and can affect microbial activity (Lejoly et al., 2020). All compounds were dissolved in water and a total of 40 mL of solution was applied to each pot in 10 mL increments.

2.1.3. 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-like 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.

2.2. Chemical analysis of soils and plants

A subsample of 10–20 g thawed soil was dried at 105 °C for 24 h to determine the moisture content. For analysis of NO_3^- and NH_4^+, 4 g of thawed soil was extracted with 20 mL of 2 M KCl (Clough et al., 2001). 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). Soil pH was determined in deionised water in a 1:2.5 soil:water extract (Blakemore et al., 1987) using a HQ 440d Multi-Parameter Meter with pH probe PHC735 (HACH, Loveland, CO, USA). Total C and N concentrations in the soils and plants were determined by dry combustion using a LECO CN828 CN analyser (LECO, St. Joseph, MI, USA).

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 3 M 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 (Thermo-Fisher Scientific, Waltham, MA, USA).

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 NO_2^- reductase), *nirK* (encoding copper-containing NO_2^- reductase) and *nosZ* (encoding N_2O 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 the functional genes was adapted from Lourenço et al. (2018). The quantification of archaeal 16S rRNA was adapted from Siles and Margesin (2016). 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. Details on primers, standards, reaction details, and thermal cycler conditions are included in the supplementary material (Tables S-2, S-3, S-4). 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 regression to relate the cycle threshold value to the known copy numbers of the standards (Siles and Margesin, 2016). The reaction efficiency ranged from 85 % to 100 % and R^2 was >0.98. Quantification results were used to calculate gene copy numbers per gram of soil (on a dry weight basis).

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. 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. The package *factoextra* (Kassambara and Mundt, 2016) was used to conduct Principal Component Analysis (PCA) of soil data. All data were analysed using R (R Core Team, 2020).

3. Results

3.1. Plant biomass and chemistry

Fertilisation significantly increased aerial biomass of *C. robusta*, *C. secta,* and *L. perenne*, but not the myrtaceous species (Table 1). The N concentration in the foliage ranged from 0.62 % to 2.8 % and was significantly increased by fertilisation in all species. Concentrations of N in the fertilised plants were significantly higher in *L. perenne*, *L. scoparium*, *K. robusta*, and *C. robusta* than in *C. secta* and *M. umbellata*. There were significant interspecific differences in the total mass of N extracted by plants. In the fertilised treatment, *C. secta* extracted the most N, while *C. robusta* extracted the least (Table 1). Assuming a root: shoot ratio of 1 and a root N concentration equivalent to half of the shoot N concentration (Wayman et al., 2014), *C. secta* took up more N (approx. 390 mg) than was added with the fertilisation (353 mg) whereas *L. perenne* took up nearly as much N as was applied (approx. 300 mg).

3.2. Soil chemistry

The soil in this study was slightly acidic, and NPKS fertilisation resulted in significant acidification under *C. robusta*, *L. scoparium*, and *M. umbellata* (Table 2). Soil pH was strongly negatively correlated ($r = -0.92$, $p \leq 0.001$) with soil NO_3^- concentration. Fertilisation increased soil NO_3^- concentrations in the dicotyledonous native species *C. robusta*, *L. scoparium*, *K. robusta*, and *M. umbellata*, but not in the monocotyledonous species *L. perenne* and *C. secta*. In contrast, fertilisation increased NH_4^+ concentrations in *L. perenne*, as well as in *K. robusta*, *L. scoparium*, and *M. umbellata* (Table 2).

3.3. Microbial abundance

Bacterial 16S rRNA (total bacteria, TB) ranged from $(1.63 \pm 0.23) \times 10^{10}$ to $(9.60 \pm 1.74) \times 10^{10}$ gene copies g^{-1} soil (Table S-6) and was 1000 times more abundant in the soil than archaeal 16S rRNA (total

Table 1
Aerial plant biomass, plant carbon and nitrogen concentrations, and total nitrogen uptake per pot in each of the plant and fertiliser treatments (control = no added fertiliser, NPKS = fertilised with nitrogen, phosphorus, potassium, and sulphur).

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

Values are means ± standard errors ($n = 6$). Different letters indicate significant differences between means ($p \leq 0.05$) according to Tukey's HSD post-hoc test.
† For *C. robusta*, *K. robusta*, *L. scoparium*, and *M. umbellata* total C and N were measured in the leaves, stems were excluded.
‡ Assuming equal N concentrations in the stems and leaves for *C. robusta*, *K. robusta*, *L. scoparium and M. umbellata*.

Table 2
Soil chemical properties in each of the plant and fertiliser treatments (control; no added fertiliser, NPKS; fertilised with nitrogen, phosphorus, potassium, and sulphur).

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}

Values are means ± standard errors ($n = 6$). Different letters indicate significant differences between means ($p \leq 0.05$) according to Tukey's HSD post-hoc test. nd: not detectable (<0.25 mg/kg NO_3^--N)

Table 3
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*

archaea, TA), which ranged from (1.51 ± 0.17) × 10^7 to (8.90 ± 1.36) × 10^7 gene copies g^{-1} soil. The abundance of TB was not significantly affected by fertilisation and did not differ between plant species (Table 3). The abundance of TA was also unaffected by fertilisation, but in contrast to TB there were significant differences between plant species and a significant interaction effect (Table 3).

For the denitrifying bacteria, the genes encoding nitrite reductase (*nirK*, *nirS*) and nitrous oxide reductase (*nosZ*), were similarly abundant, in the range of 10^7–10^8 gene copies g^{-1} soil (Fig. 1, Table S-6). None of these genes were affected by the fertiliser application (Table 3). However, *nirS* and *nosZ* were affected by plant species (Table 3). For the nitrifying microorganisms, AOB *amoA* was more abundant than AOA *amoA* (Table S-5). 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. 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). Fertilisation increased the abundance of AOB *amoA* above the detection limit in all species (Fig. 2).

Plant species affected the abundance of AOB *amoA* in the fertilised soil (Fig. 2). The abundance of AOB *amoA* differed between monocotyledonous and dicotyledonous species. *L. scoparium* and *M. umbellata* showed significantly higher AOB *amoA* abundances than *L. perenne* and *C. secta* (Fig. 2). Soil NO_3^- and NH_4^+ concentrations were positively correlated with the AOB *amoA*: bacterial 16S rRNA ratio (Fig. 3).

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 (Fig. 4). In the fertilised soil, variation along PC1 (31.4 % of variation) was mainly explained by soil microbial abundance, primarily *nosZ*, *nirS*, and bacterial *16S rRNA*, while PC2 (25.1 % of variation) was mainly explained by soil chemical properties.

4. Discussion

The results support our hypothesis that plant species affect the concentrations of N in the soil, as well as the abundance of microorganisms involved in the N cycle. There were strong species-dependent effects on the abundance of TA, *nirS*, and *nosZ*. However, 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 including nitrifying or denitrifying bacteria in this study. Furthermore, against expectations, TB and TA abundance did not significantly increase with NPKS application. The soil C:N ratio in this experiment ranged from 11 to 15, which indicates that the soil bacteria and archaea were more likely limited by C than N (Bengtsson et al., 2003). There were significant differences between plant species for TA abundance, with TA being significantly lower in *C. robusta* than *M. umbellata* and *C. secta*. This is consistent with a higher sensitivity of archaea to plant variation compared to bacteria (Yarwood et al., 2016).

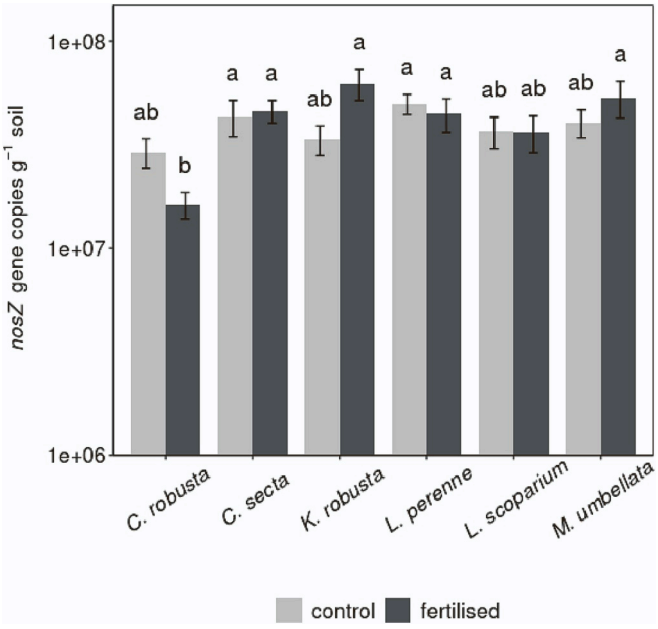

Fig. 1. Abundance of *nosZ* in the control and fertilised (NPKS) treatments by plant species. Values show means ± standard errors ($n = 6$). Different letters indicate significant differences between species and treatment combinations at $p \leq 0.05$ according to Tukey's HSD post-hoc test.

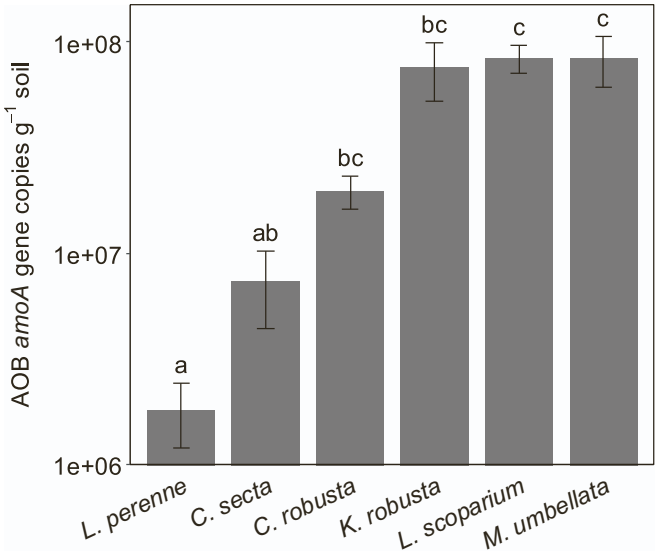

Fig. 2. Abundance of ammonia-oxidising bacteria (AOB) *amoA* in the fertilised (NPKS) treatment by plant species. Values show means ± standard errors ($n = 6$). Different letters indicate significant differences between species at $p \leq 0.05$ according to Tukey's HSD post-hoc test.

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). With the smaller root systems developed in *C. robusta* than other species, it is possible that lower availability of C exudates limited the growth of archaea.

The genes involved in denitrification, *nirK*, *nirS*, and *nosZ*, were unaffected by the application of NPKS (Table 3). This contrasts with a study by Kastl et al. (2015), in which fertilisation of 200 kg 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. *C. robusta* had a significantly lower abundance of *nosZ* than all other species, except *L. scoparium*. This may be a result of differing root exudation of organic compounds among species, which can increase microbial activity (Leptin et al., 2021). As *nosZ* is responsible for the reduction 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 relative to N_2O. With plant species having the strongest effect on *nosZ*, species selection may be critical to favour complete denitrification in the soil and reduce emissions of N_2O.

The abundance of bacterial *amoA* was higher than that of archaeal *amoA* in the present study, which is consistent with TB being more abundant than TA. However, this contrasts with several studies whereby AOA are more abundant in soil than AOB (Adair and Schwartz, 2008; Di et al., 2009; Fischer et al., 2013; Leptin et al., 2021). 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*, which was likely due to the insignificant root system and slow plant growth in this species and reduced plant-microbe competition. 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 and 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 required higher concentrations of NH_4^+ in the soil, which was reflected in their strong response to NPKS application. Fertilisation increased the abundance of AOB *amoA* above the detection limit in all species. 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 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}.

Plant species affected the abundance of AOB *amoA* in the fertilised soil. The 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* (Fig. 2). Typically, plants affect nitrification through (i) direct competition for N, (ii) the release of BNIs, and (iii) physicochemical 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 1), 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 and Schachtman, 2022; Oburger and 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).

Mono- and dicotyledonous species were separated in the PCA by chemical and microbial properties, which reflects that different plant species affect soil properties through their distinct architectural, morphological, physiological, and biotic root traits (Bardgett et al., 2014). Monocotyledonous species have more fibrous root systems, which likely affected nutrient cycling, microbial activity, and preferential flow differently compared to the dicotyledonous species (Franklin et al., 2015; Mishra, 2018). An increase in in soil NO_3^- following fertilisation under dicotyledons but not monocotyledons is consistent with

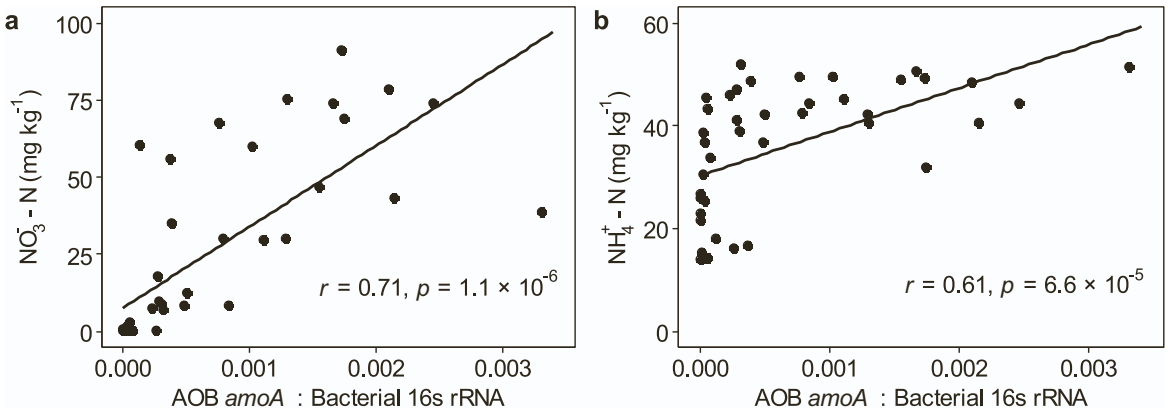

Fig. 3. (**a**) Soil NO_3^--N concentration vs. AOB *amoA*: Bacterial 16S rRNA ratio in the fertilised (NPKS) treatment, and (**b**) soil NH_4^+-N concentration vs. AOB *amoA*: Bacterial 16S rRNA ratio in the fertilised (NPKS) treatment. The black lines are linear regression lines. *R* values are Pearson's correlation coefficients.

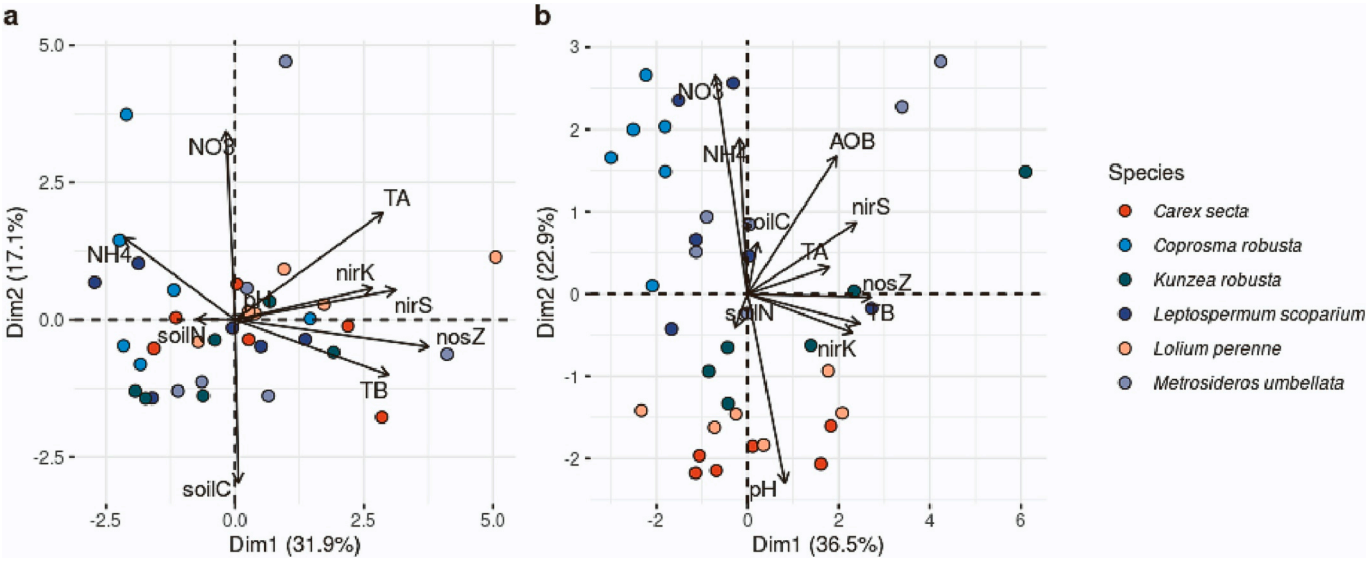

Fig. 4. Principal component analysis (PCA) of chemical and microbial soil parameters; (**a**) control treatment and (**b**) fertilised (NPKS) treatment. TB: total bacteria, TA: total archaea, *nirK/nirS/nosZ*: denitrification genes.

the higher N uptake by the latter 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 and Kronzucker, 2013). The large increase in biomass following NPKS fertilisation is consistent with one or more of these elements limiting plant growth (Güsewell and Koerselman, 2002). No difference in N concentrations between species 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 our 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. N concentrations in the foliage of the unfertilised *C. secta* and *M. umbellata*, were only 40–44 % of the average concentration of N in plant shoot dry matter of 1.5 % (Kirkby, 2012). However, 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.

Despite previous reports that *L. scoparium* and *K. robusta* may suppress nitrification in soil more than other species (Esperschuetz et al., 2017; Franklin et al., 2017), the relatively high abundance of AOB *amoA* under these species indicates that this was not the case in the present study. 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, 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. Moreover, 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. 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.

Although not part of this research, it is important to highlight the role of fungi in nitrification and denitrification. Fungi can dominate heterotrophic nitrification (Zhu et al., 2015) and are capable of denitrification and other NO_3^-/NO_2^- reduction processes (Aldossari and Ishii, 2021). However, their relative contribution to these processes in high-nutrient environments is not clear, as it was demonstrated that long-

term fertiliser inputs can reduce fungal biomass in soil (Schon et al., 2023). To obtain a complete unerstanding of soil N cycling under native plants in high-nutrient environments, future research should include fungal N transformation processes.

5. Conclusions

We have shown that there were interspecific differences between NZ native monocotyledonous and dicotyledonous plant species with respect to N-cycling in fertilised soil. AOB were limited by N and could only be quantified in soils that were fertilised. There, 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. *L. perenne* and *C. secta* took up significantly more N than the dicotyledonous species. The use of monocotyledonous native species in riparian zones and farm margins, as well as for the land application of N-rich wastes, in NZ and elsewhere, may reduce NO_3^- leaching from the system through plant uptake 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 also reduce emissions of the potent greenhouse gas N_2O through complete denitrification. Further research is required to elucidate species specific mechanisms of interaction between plant roots and AOB and denitrifying microorganisms, although plant uptake can explain some of the observed differences. Overall, the results of this study demonstrated that species selection may be used to reduce N losses from high-fertility environments.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.apsoil.2023.105031.

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