Applied Soil Ecology xxx (2016) xxx–xxx

Contents lists available at ScienceDirect

Applied Soil Ecology

journal homepage: www.elsevier.com/locate/apsoil

Interactions between earthworm burrowing, growth of a leguminous shrub and nitrogen cycling in a former agricultural soil

Young-Nam Kim[a], Brett Robinson[b], Keum-Ah Lee[a,c], Stephane Boyer[a,d], Nicholas Dickinson[a,*]

[a] Department of Ecology, Lincoln University, Lincoln 7647, Christchurch, New Zealand
[b] Department of Soil and Physical Sciences, Lincoln University, Lincoln 7647, Christchurch, New Zealand
[c] School of Biological Science, University of Canterbury, Christchurch 8041, New Zealand
[d] Department of Natural Sciences, Unitec Institute of Technology, Auckland 1025, New Zealand

ARTICLE INFO

Article history:
Received 29 June 2016
Received in revised form 11 October 2016
Accepted 23 October 2016
Available online xxx

Keywords:
Soil quality
Legume
Nitrogen
Greenhouse gas
Root nodulation
Ecosystem services

ABSTRACT

Attempts to restore native biodiversity into agricultural landscapes in New Zealand appear to be compromised both by soil nitrogen enrichment from farming and N-leakage to the wider environment. We investigated whether interactions between native earthworms and a native rhizobium-inoculated leguminous shrub (*Sophora microphylla*) have a measurable effect on the mobility of nitrogen in an agricultural soil that has been nitrogen-enriched and colonised by exotic earthworms. Plants grew better in the presence of both native and exotic soil-burrowing earthworms. Rates of root nodulation were considerably enhanced in the presence of the native megascolecid anecic earthworm *Maoridrilus transalpinus*. This species consumed more organic matter in the presence of inoculated plants whilst marginally lowering soil pH and enhancing critical concentrations of nitrate, but also reducing nitrous oxide emissions. Earthworms raised dehydrogenase enzyme activity and microbial activity in soil, but this was not commensurate with rates of nodulation. Our results show that some combination of earthworm-mediated soil aeration, modification of moisture conditions in the rhizosphere and drilosphere, and comminution of organic matter, modify microbial communities and significantly impact the N cycle.

1. Introduction

Conservation of highly endemic flora and fauna in lowland New Zealand depends on our ability to construct novel native ecosystems on soils that have been profoundly modified from their natural state by agriculture. Ecological restoration attempts to achieve some meaningful semblance of an historic naturalistic vegetation on these soils, and to provide habitat for above- and below-ground faunal biodiversity (Tongway and Ludwig, 2011; Dickinson et al., 2015). This may be reliant on an erroneous assumption of the suitability of modified soils to support the desired species (Smith et al., 2016). Even if agricutural soils provide suitable habitat, little is known of how the soils may be further modified by native biota. However, it is essential that land management and conservation incorporate soil biodiversity as an important criterion to benefit ecosystem functioning, service provision and human health (Bardgett and Wardle, 2010). This may be especially challenging in situations where land use changes have substantially modified soil structure (Franklin et al., 2015).

Little is known of the requisite underlying environmental conditions to optimise the restoration trajectory within agricultural landscapes in New Zealand. This creates real challenges for restoration practitioners; re-introduction takes place in the presence of exotic weeds and animal pests, including mammals that were formerly absent from this landmass. The most troublesome invasive plants in New Zealand are often legumes, including gorse (*Ulex europaeus*), European brooms and American lupins, although gorse often also plays an important role assisting the recovery of native vegetation on former stock-grazed pasture (Wilson, 2013). Leguminosae are poorly represented amongst the native flora, both in number of species and abundance; 4 genera and 34 species represent 1.4% of the vascular flora (Given and Meurk, 2000), compared to 8% worldwide (Yahara et al., 2013) and native legumes compete poorly with introduced gorse and brooms, particularly in human-modified landscapes (Wardle, 2002). One concern is the elucidation of the role played by native species of

* Corresponding author.
E-mail address: nicholas.dickinson@lincoln.ac.nz (N. Dickinson).

http://dx.doi.org/10.1016/j.apsoil.2016.10.011
0929-1393/

nitrogen-fixing plants in vegetation recovery. Among the New Zealand native Leguminosae, eight species of *Sophora* are shrubs or small trees (Heenan et al., 2001; Thomas and Spurway, 2001), which could have a particularly important role in global legume diversity assessment (Yahara et al., 2013).

Understanding the functionality, interactions and combined influence of native legumes and soil fauna on ecosystem development presents further challenges (Bardgett and Wardle, 2010; Blouin et al., 2013). In New Zealand, the role of native earthworms in particular requires more attention (Kim et al., 2015). There are more than 200 species of native Megascolecid earthworms in New Zealand (Lee, 1959a,b; Boyer et al., 2011) that are almost entirely unrepresented on agricultural land, even though several species of exotic Lumbricid earthworms are commonly found in farm paddocks (Lee, 1961; Fraser et al., 1996; Springett et al., 1998).

Agricultural landscape matrices in New Zealand are depauperate in native flora and fauna (Winterbourne et al., 2008) where remnants of natural and re-planted vegetation are only represented as little more than refugia in riparian zones, along fence lines and on the borders of agricultural land (Bowie et al., 2016). These natural remnants are now significantly expanding though renewed interest in native species and through modern intensive agricultural systems that are integrating restoration of biodiversity into farm planning (Dickinson et al., 2015; Franklin et al., 2015). The broad aim of our current research is to understand the interactions between native species of plants, soil fauna and soil physicochemistry. The objectives of the mesocosm experiment reported in this paper were to investigate whether we could demonstrate significant integration of the role of a native species of nitrogen-fixing plant and earthworms in the context of nitrogen cycling and soil quality.

2. Materials and methods

2.1. Establishment of pot experiment

Surface soils (0–15 cm) were collected from the Lincoln University commercial dairy farm for use in this mesocosm experiment. The dairy farm soil (Templeton silt loam) is intensively managed, irrigated and fertilised, and is representive of intermediate terraces in the Province of Canterbury on South Island (Molloy, 1988). The soil was sieved and uniformly mixed prior to planting. Four species of earthworms representing different ecological groups and burrowing behaviours were selected for this study. Two native anecic species, *Maoridrilus transalpinus* and *Maoridrilus* sp.2 were collected respectively from a nature reserve (Ahuriri Reserve, Banks Peninsula) and beneath a mature stand of exotic *Quercus ilex* trees on the university campus. The earthworms were identified using DNA barcoding (16S and COI), which showed that *Maoridrilus* sp.2 has not been previously recorded and may be new to science (Kim, 2016). An exotic endogeic species (*Octolasion cyaneum*) was also sampled from the Ahuriri Reserve; we have observed that both *Maoridrilus* and *Octolasion* commonly coexist in Banks Peninsula forests. The exotic epigeic, *Eisenia fetida*, was purchased from a local vermicomposting company. One-year-old single plants of *Sophora microphylla* (Kowhai) of uniform size were purchased from a nursery, acclimated to glasshouse growth conditions for 4 weeks, then transplanted into the dairy farm soil in 55 plastic plant pots (5 l volume), and maintained for a further 7 days before the addition of rhizobial and then earthworm inocula (Fig. 1).

Novel *Mesorhizobium* sp. cultures (Strain ICMP 19535; Tan et al., 2015) were obtained from the International Collection of Microorganisms from Plants at Landcare Research (Auckland, NZ) and incubated into Yeast Mannitol Broth (YMB) at 25 °C in the dark for a week to derive liquid cultures which were poured in 50 ml aliquots into 25 pots, on two occasions 7 days apart. This strain is known to be effective on *S. microphylla*. Autoclaved YMB was added to 5 reference pots in the same amounts and at the same time as a control. One week later, four adult earthworms of a single species were added to each pot, with 5 replicates, and an additional 5 pots without earthworms. Weight of all species of earthworm were recorded prior to inoculation. The drainage holes and upper surface of the pots were covered with gauze to prevent earthworms escaping during the experiment. Pots were randomized and maintained for 8 weeks in natural light at 20 ± 5 °C. There were 5 replicates of each treatment, providing a total of 45 pots (4 species of earthworms, with and without rhizobial inoculation, plus 5 pots with neither earthworms nor inocula). These mesocosms were lightly watered every 3 days for 8 weeks, adding the same volume of water to each pot to maintain likely optimal moisture conditions of 25–30% (Wever et al., 2001; Eriksen-Hamel and Whalen, 2006).

Fig. 1. Establishment of *S. microphylla* mesocosms in the glasshouse. Photographs inset show the gauze covers (top inset) and gas sampling cylinders (bottom inset).

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2.2. Plant and soil analyses

Plant height was measured after 8 weeks, then plants were harvested and root nodules were counted by hand sorting after gently removing roots from the soil. Roots and shoots were separated and oven-dried (65 °C, 3 days) prior to measurement of dry weight. Earthworms were collected from each pot, then visually assessed for health and individually weighed.

About 1 kg of fresh soil was collected from each pot and stored at 4 °C for less than one week prior to soil analyses. Following extraction in 2 M KCl, samples of the fresh soil were analysed for N (NH_4 and NO_3) using a FIA star 5000 triple channel analyser (Foss Tecator AB, Sweden) (Clough et al., 2001). For soil dehydrogenase enzyme activity (DHA), 2 g of fresh soil was incubated with 2 ml of 2,3,5-triphenyltetrazolium chloride (TTC) solution for 24 h at 25 °C in darkness. After extraction with 10 ml of methanol, the supernatant was measured at an absorption of 475 nm using a UV 160A spectrophotometer (Shimadzu, Japan) (Casida et al., 1964). Air-dried soil samples (20 °C, 5 days) were sieved (< 2 mm) prior to determination of pH and electrical conductivity (EC). Following 0.5 M $NaHCO_3$ extraction (1 g soil: 20 ml extractant), plant available-P was measured as Olsen P, spectroscopically at a wavelength of 880 nm using a UV 160A spectrophotometer (Shimadzu, Japan) (Blakemore, 1987). Following oven-drying (100 °C), Total Organic Matter (TOM) was analysed as Loss on Ignition (LOI) in a muffle furnace at 500 °C. Total N and C were analysed using a Vario-Max CN elemental analyser (Elementar GmbH, Germany).

2.3. Gas sampling

Gas measurement for nitrous oxide was carried out after 55 days. A plastic chamber (0.5 l) was installed on the soil surface. Gas (10 ml) was collected from the headspace, 0, 20, and 40 min after sealing. Gas samples were stored at 16 °C in darkness for less than one week. Nitrous oxide (N_2O) was analysed using a gas chromatograph (GC) (SRI 8610 GC, CA, USA) with a ^{63}Ni electron capture detector (ECD) and a flame ionisation detector (FID) linked to an auto-sampler (Gilson 222 XL, USA). Methods follow those described by Clough et al. (2006).

2.4. Statistical analyses

All data analyses used Minitab 16 (Minitab Inc., State College, Pennsylvania, USA), with One- and Two-Way ANOVA and post-hoc Fisher's LSD tests employed to identify differences between soil properties, plant growth, nodulation and N_2O emissions. Mean and standard error of soil pH values were calculated by conversion to the equivalent hydrogen ion concentrations and back calculation to the pH.

3. Results

3.1. Earthworms growth and mortality

After 8 weeks, mortality rates of earthworms were less than 10% for all species, except for *M. transalpinus* (60% mortality with rhizobial inoculation, 75% mortality without rhizobial inoculation). The amount of organic matter consumed was calculated crudely from the change in soil TOM over the duration of the experiment (Fig. 2). *M. transalpinus* consumed more organic matter than other species, compared to the control pots, despite poor survivorship and a 31% weight loss of surviving earthworms. There was less weight loss with rhizobial inoculation, where soil organic matter consumption was even higher (data not shown). An overall weight gain was recorded only for *Maoridrilus* sp.2. Differences were only small, but comminution of OM significantly differed in relation to earthworm species ($p < 0.001$), rhizobial inoculation ($p < 0.01$) with a significant interaction ($p < 0.001$).

3.2. Plant growth

Two-way ANOVAs showed that earthworm species, rhizobia treatment, and their interaction significantly impacted the shoot length (Table 1). Plants generally grew better in terms of shoot length the presence of earthworms over the 8-week period. Both

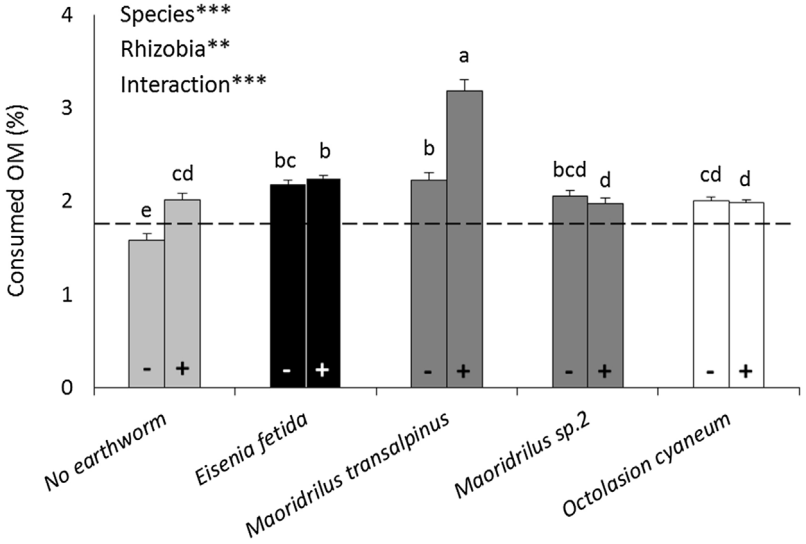

Fig. 2. Consumption of OM by earthworms after 8 weeks. Asterisks indicate two-way ANOVA analysis showing the effect of earthworm species, rhizobial inoculation, and interaction (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$). Symbols refer to absence (−) or presence (+) of rhizobial inoculation. Bars are means ± standard error (n = 5). Same letters indicate no significant difference (LSD, $p < 0.05$). Shading indicates different ecological groups of earthworms: epigeic (■), anecic (▩), endogeic (□) and control without earthworms (▩). Dashed line indicates YMB addition without rhizobial inoculant.

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Table 1
Plant growth in the mesocosm experiment after 8 weeks. Values in brackets represent standard error of the mean (n = 5). Same letters indicate no significant difference (LSD, $p < 0.05$). Two-way ANOVA analyses the effect of earthworm species, rhizobia addition, and their interaction are indicated with superscripts (ns for not significant, $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$).

Treatment		Shoot length	Dry weight		
		(cm)	Total (g)	Shoot (g)	Root (g)
Control	No inoculant[†]	50.4 (2.8)	10.0 (0.9)	7.8 (0.6)	2.5 (0.3)
No Rhizobia	No earthworm	50.4 (4.3)^{c}	10.0 (0.9)^{b}	7.6 (0.6)^{c}	2.4 (0.2)^{b}
	Eisenia fetida	61.8 (1.4)^{b}	12.0 (1.3)^{ab}	8.8 (0.8)^{bc}	3.2 (0.5)^{ab}
	Maoridrilus transalpinus	73.4 (3.0)^{a}	14.8 (0.6)^{a}	11.3 (0.5)^{a}	3.5 (0.2)^{a}
	Maoridrilus sp.2	70.6 (1.5)^{a}	13.9 (1.8)^{a}	10.7 (1.3)^{ab}	3.2 (0.4)^{ab}
	O. cyaneum	58.8 (2.8)^{b}	10.2 (0.6)^{b}	7.6 (0.5)^{c}	2.6 (0.2)^{ab}
Rhizobia	No earthworm	54.4 (4.1)^{b}	11.0 (1.9)^{a}	8.2 (1.4)^{a}	2.8 (0.5)^{a}
	Eisenia fetida	57.6 (1.5)^{ab}	9.6 (0.4)^{a}	6.8 (0.3)^{a}	2.8 (0.3)^{a}
	Maoridrilus transalpinus	57.6 (4.0)^{ab}	11.7 (1.4)^{a}	8.3 (1.1)^{a}	3.3 (0.3)^{a}
	Maoridrilus sp.2	60.4 (3.2)^{ab}	10.0 (0.7)^{a}	7.6 (0.5)^{a}	2.4 (0.2)^{a}
	O. cyaneum	65.4 (1.5)^{a}	9.9 (0.8)^{a}	7.2 (0.6)^{a}	2.7 (0.2)^{a}
Two-way ANOVA (p value)	Species	0^{***}	0.057^{ns}	0.036^{*}	0.152^{ns}
	Rhizobia	0.042^{*}	0.021^{*}	0.006^{**}	0.421^{ns}
	Interaction	0.002^{**}	0.181^{ns}	0.126^{ns}	0.453^{ns}

[†]Yeast mannitol broth (YMB) addition without rhizobial inoculant.

native earthworms (*Maoridrilus* spp.) significantly enhanced shoot length more than other treatments. There were no marked differences in shoot length and dry weight between earthworm species in the earthworm-rhizobia inoculation treatment. In the rhizobia-inoculation treatment, only *O. cyaneum* significantly increased shoot length more than in pots without earthworms.

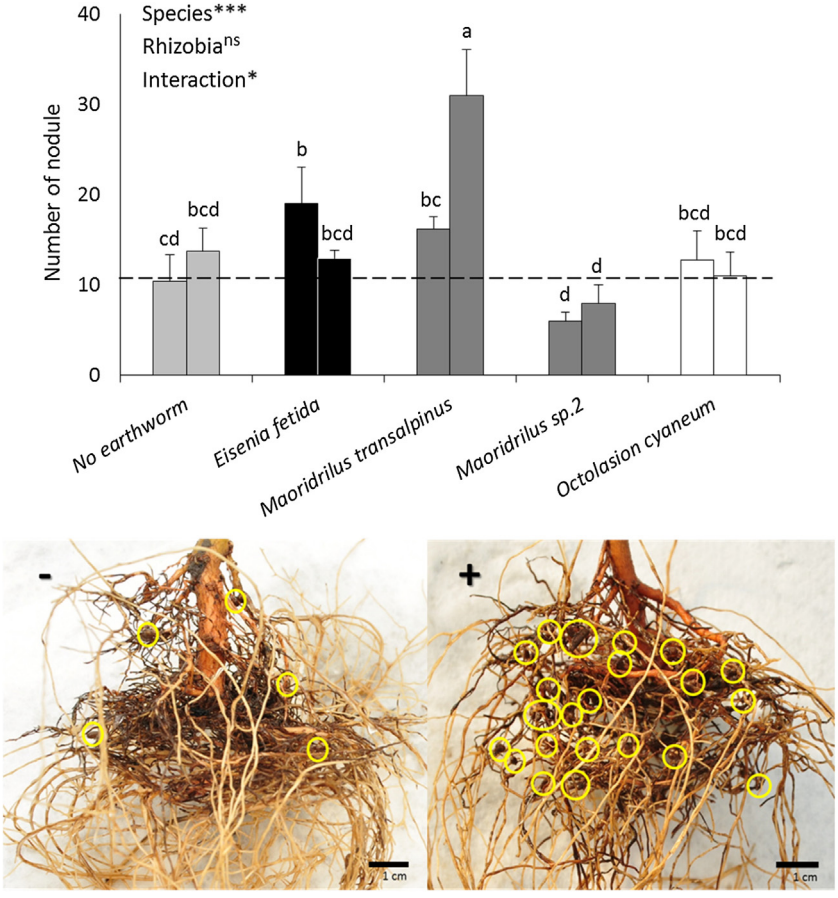

Fig. 3. Root nodulation of *S. microphylla* after 8 weeks. Asterisks indicate Two-Way ANOVA analysis showing the effect of earthworm species, rhizobial inoculation, and interaction ($^{*}p < 0.05$, $^{***}p < 0.001$, ns = not significant). Symbols refer to absence (−) or presence (+) of rhizobial inoculation. Bars are means ± standard error (n = 5). Same letters indicate no significant difference (LSD, $p < 0.05$). Shading indicates different ecological groups of earthworms: epigeic (■), anecic (▣), endogeic (□) and control without earthworms (▣). Photographs show the effects of *M. transalpinus* activity on nodulation, marked with yellow circles. Dashed line indicates YMB addition without rhizobial inoculant. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

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Table 2
Effects of earthworms and rhizobia on soil properties after 8 weeks. Values in brackets represent standard error of the mean (n = 5). Same letters indicate no significant difference (LSD, $p < 0.05$). Results of two-way ANOVA analysis to determine the effect of earthworm species, rhizobia addition, and their interaction are indicated with superscripts (ns for not significant, $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$).

Treatment		pH	EC	Mobile N		Olsen P	Moisture content	Total C	Total N	Ratio of C/N
		(1:5W)	($dS \cdot m^{-1}$)	NH_4-N ($mg \cdot kg^{-1}$)	NO_3-N ($mg \cdot kg^{-1}$)	($mg \cdot kg^{-1}$)	(%)	(%)	(%)	
Control	No inoculant[†]	5.4 (0.1)	0.06 (0.01)	0.3 (0.1)	19 (7)	29 (2)	26.3 (0.4)	2.41 (0.08)	0.22 (0.01)	10.8 (0.2)
No Rhizobia	No earthworm	5.3 (<0.1)^{a}	0.08 (0.00)^{b}	0.5 (0.2)^{a}	29 (3)^{b}	30 (<1)^{b}	27.6 (0.4)^{a}	2.50 (0.05)^{a}	0.24 (0.01)^{ab}	10.5 (0.1)^{b}
	Eisenia fetida	5.4 (<0.1)^{a}	0.07 (0.01)^{b}	0.3 (0.1)^{a}	18 (5)^{b}	30 (1)^{b}	25.6 (0.5)^{b}	2.35 (0.06)^{a}	0.22 (0.01)^{bc}	10.9 (0.1)^{ab}
	Maoridrilus transalpinus	5.1 (<0.1)^{b}	0.12 (0.01)^{a}	1.1 (0.6)^{a}	57 (3)^{a}	33 (1)^{a}	24.9 (0.3)^{bc}	2.52 (0.06)^{a}	0.24 (0.01)^{a}	10.5 (0.2)^{b}
	Maoridrilus sp.2	5.4 (<0.1)^{a}	0.08 (0.01)^{b}	0.5 (0.1)^{a}	26 (4)^{ab}	32 (1)^{ab}	23.6 (0.8)^{c}	2.48 (0.07)^{a}	0.21 (0.01)^{c}	11.7 (0.5)^{a}
	Octolasion cyaneum	5.4 (<0.1)^{a}	0.08 (0.00)^{b}	0.4 (0.1)^{a}	30 (3)^{ab}	33 (1)^{a}	25.8 (0.4)^{b}	2.41 (0.06)^{a}	0.22 (0.00)^{abc}	10.9 (0.1)^{ab}
Rhizobia	No earthworm	5.5 (<0.1)^{a}	0.06 (0.01)^{b}	0.2 (<0.1)^{b}	14 (2)^{b}	30 (1)^{a}	26.2 (0.2)^{a}	2.42 (0.08)^{a}	0.22 (0.01)^{a}	10.7 (0.1)^{b}
	Eisenia fetida	5.4 (<0.1)^{ab}	0.07 (0.01)^{b}	0.4 (0.1)^{c}	19 (4)^{b}	29 (1)^{a}	23.9 (0.5)^{b}	2.25 (0.03)^{ab}	0.21 (0.00)^{a}	10.8 (0.1)^{ab}
	Maoridrilus transalpinus	5.3 (<0.1)^{b}	0.09 (0.01)^{a}	0.8 (0.3)^{a}	30 (6)^{a}	30 (1)^{a}	24.9 (0.4)^{b}	2.33 (0.08)^{ab}	0.22 (0.01)^{a}	10.8 (0.2)^{ab}
	Maoridrilus sp.2	5.4 (<0.1)^{a}	0.07 (0.01)^{b}	0.5 (<0.1)^{bc}	17 (3)^{b}	30 (1)^{a}	24.6 (0.5)^{b}	2.39 (0.06)^{ab}	0.20 (0.02)^{a}	12.2 (1.0)^{ab}
	Octolasion cyaneum	5.4 (<0.1)^{a}	0.06 (0.00)^{b}	0.6 (0.1)^{b}	16 (2)^{b}	20 (1)^{a}	23.9 (0.5)^{b}	2.24 (0.02)^{b}	0.20 (0.00)^{a}	11.0 (0.1)^{ab}
Two-way ANOVA (p value)	Species	0***	0***	0.133^{ns}	0***	0.123^{ns}	0***	0.045*	0.017*	0.01**
	Rhizobia	0.002**	0***	0.493^{ns}	0***	0.002**	0.008**	0.002**	0.006**	0.379^{ns}
	Interaction	0.132^{ns}	0.274^{ns}	0.678^{ns}	0.013*	0.272^{ns}	0.009**	0.873^{ns}	0.875^{ns}	0.930^{ns}

[†]Yeast mannitol broth (YMB) addition without rhizobial inoculant.

3.3. Root nodulation

Surprisingly rhizobial inoculation had no effect on root nodulation, although it varied with species of earthworm and there was apparent interaction between these treatments (Fig. 3). The number of nodules increased in the presence of earthworms, particularly with *E. fetida*. With addition of rhizobia inocula, *M. transalpinus* almost doubled the number of root nodules. With other species of earthworms, the addition of the rhizobial inoculum made lesser or no difference. *Maoridrilus* sp.2 appeared to deplete nodule numbers compared to the control.

3.4. Soil properties

M. transalpinus had the most significant effect on soil properties, marginally lowering soil pH, enhancing EC, and substantially increasing soil concentrations of mobile forms of nitrogen (Table 2). There was significant interaction between these two treatments ($p < 0.05$), in terms of increased nitrate concentrations. Effects of the treatments on Total C and N, and mobile P were negligible.

3.5. Microbial activity

In the absence of rhizobia-inoculation, earthworms activated production of more dehydrogenase enzyme (DHA) in soil (Fig. 4). The presence of the native species, *M. transalpinus* and *Maoridrilus* sp.2, increased DHA by 65% and 51%, respectively. However, the rhizobial inoculant consistently reduced this effect; DHA decreased by at least 20%. Compared with exotic earthworms, both native *Maoridrilus* spp. significantly enhanced microbial activity.

3.6. N_2O emission

When combined with rhizobial inoculation, none of the earthworm species enhanced the release of nitrous oxide from soil (Fig. 5).

4. Discussion

4.1. Survivorship and behaviour of earthworms

M. transalpinus had higher mortality rates than the other species of earthworms after 8 weeks in the mesocosms. Several freshly-dead individuals of this species were found towards the end of the experiment, and soil in all the pots containing these earthworms showed clear evidence of having been well worked through burrowing. This indicated survivorship was much higher for a large part of the experimental period. Other more recent studies we have carried out (Kim, 2016) have shown that high levels of survivorship in this species in similar pot experiments are seldom maintained for more than 6 weeks, unless the earthworms are provided with an abundant food source.

During the separation of plant roots from soil, it was observed that the two *Maoridrilus* species were invariably intertwined within the root system and there was noticeably more evidence of burrowing and mixing of the soil, compared to the other species. *Octolasion* tended to dwell beneath the roots towards the bottom of the pot, whilst *E. fetida* inhabited soil close to the soil surface. Enhanced plant growth appeared to be related to the different ecological grouping of earthworms. There are substantial differences in terms of burrowing behaviour between these ecological groups of earthworm, the consequences of which are almost certainly masked by the uniformly mixed soils without natural vertical horizons in this mesocosm experiment.

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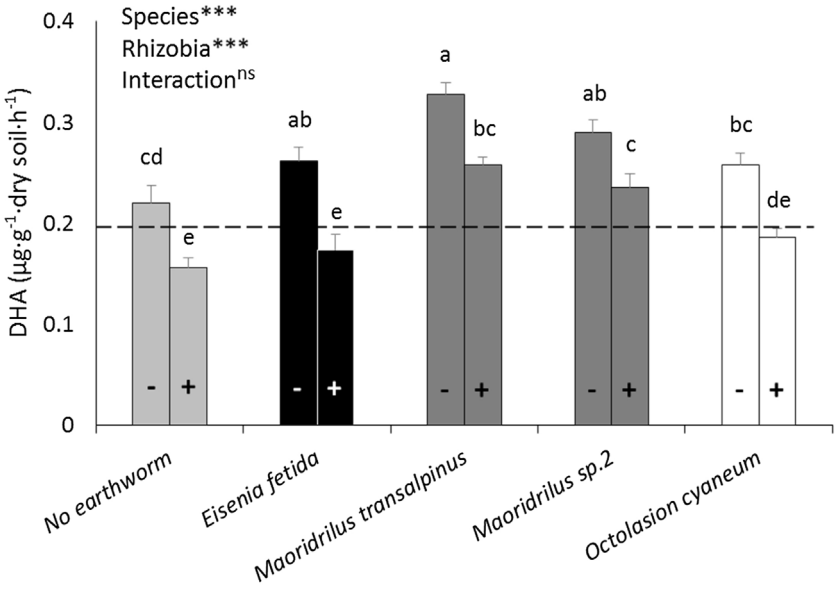

Fig. 4. Dehydrogenase enzyme activity (DHA) in soil after 8 weeks. Asterisks indicate Two-Way ANOVA analysis showing the effect of earthworm species, rhizobial inoculation, and interaction (***$p < 0.001$, ns = not significant). Symbols refer to absence (−) or presence (+) of rhizobial inoculation. Symbols refer to rhizobia strain present (+) or absent (−). Bars are means ± standard error (n = 5). Same letters indicate no significant difference (LSD, $p < 0.05$). Shading indicates different ecological groups of earthworms: epigeic (■), anecic (▩), endogeic (□) and control without earthworms (▩). Dashed line indicates YMB addition without rhizobial inoculant.

Poor survivorship of *M. transalpinus* was considered to be an acceptable limitation of this experiment; previous studies have shown that decaying earthworm bodies do not significantly contribute to the amount of N otherwise released into soil by earthworms (Whalen et al., 1999; van Groenigen et al., 2014). There were no reference pots without plants in the present study, although better growth of Lumbricid earthworms has been previously recorded in the presence of legumes (Milcu et al., 2008).

4.2. Effect on plant growth and nodulation

Whalen et al. (1999) found that, despite the relatively small amount of nitrogen, 70% of that released by decaying earthworms

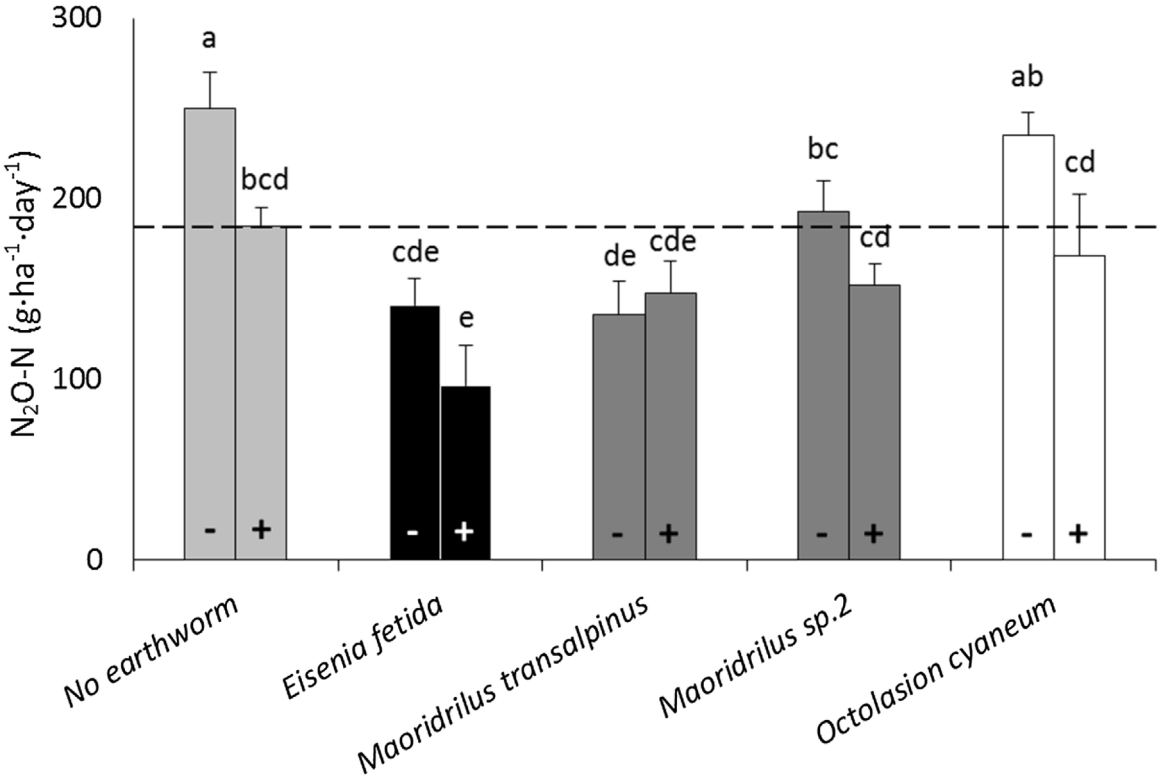

Fig. 5. Release of nitrous oxide (N_2O) after 8 weeks. Symbols refer to rhizobia strain present (+) or absent (−). Bars are means ± standard error (n = 5). Same letters indicate no significant difference (LSD, $p < 0.05$). Shading indicates different ecological groups of earthworms: epigeic (■), anecic (▩), endogeic (□) and control without earthworms (▩). Dashed line indicates YMB addition without rhizobial inoculant.

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could be incorporated into plant shoots within 16 days. In the present study, it would seem highly likely that 75% mortality may explain better plant growth in the *M. transalpinus* treatment without rhizobial inoculation. However, this simple explanation is contradicted by the absence of a plant growth response with rhizobial inoculation and 60% earthworm mortality. Rhizobial inoculation led to lower rates of shoot growth, but neither earthworms nor rhizobia significantly modified root biomass. The growth period was quite short in the present study, but a recent meta-analysis study showed an average 23% increase in above-ground plant biomass due to the presence of earthworms, largely through release of N from organic matter (van Groenigen et al., 2014).

Rates of root nodulation were only enhanced in the presence of *M. transalpinus*, where the number of nodules per plant was 3× higher than the control. In pots without rhizobial inoculation, there was also a significant amount of root nodulation, suggesting that the soil or plants already contained inocula. The source of this soil inoculant may have been small amounts of clover in the dairy farm ryegrass sward, or plants may have acquired inocula from the nursery. However, Tan et al. (2015) found that closely-related *Mesorhizobium* type strains from other genera of legumes were unable to nodulate *Sophora microphylla*, which required its own specific type strains for nodulation. Genotypic data on rhizobia suggest co-evolution of rhizobial symbionts with *Sophora* in isolation from major areas of legume evolution has provided unique identities and novel characteristics (Tan et al., 2012). It is clear that there is significant interaction between earthworm species, plant growth and nodulation, but the complexity of these relationships has not been revealed in the present study.

4.3. Effects on soil biochemistry

Previous field studies elsewhere have shown that earthworms enhance N mineralization by reducing microbial immobilization, which may increase leaching losses of NO_3 (Blair et al., 1997; Domínguez et al., 2004). In the present study, *M. transalpinus* had the largest influence on soil physicochemistry including soil pH, EC, and solubility of N. Over the 8 week period of this study little change could be expected in Total C and N concentrations in soil, but changes were detectable in these labile fractions. However, there was little apparent effect of the earthworms on mobile P in soil, even though this element is known to play a critical role in N fixation (Valentine et al., 2011; Vardien et al., 2016).

Dehydrogenase enzyme activity in soil was consistently lower with rhizobial inoculation but was enhanced by earthworms, particularly by *Maoridrilus* spp. Dehydrogenases provide a measure of overall soil microbial activity and play a significant role in the biological oxidation of soil organic matter, but most of this enzyme is produced by anaerobic microorganisms and would be expected to be lower under aerobic conditions (Wolińska and Stępniewska, 2012). In the presence of native anecic species the bulk soil in the pots was certainly aerated through burrowing and disturbance (Brown et al., 2004; Lemtiri et al., 2014), probably providing better conditions for rhizobial development and nodulation. At the same time, when soil was removed from the pots, our observations indicated higher moisture in soil fractions that were obviously from drilosphere walls (Horn et al., 2006; Lemtiri et al., 2014); this may have provided locations of increase anaerobic conditions that are required for DHA production. This may indicate that aeration of soil by earthworms provides improved conditions for nodulation in the rhizosphere, but also localised anaerobic conditions that favour anaerobic microroganisms and intracellular DHA production. These changed soil conditions may begin to explain why rhizobial inoculation in combination with earthworms decreased microbial activity.

4.4. Effects on GHGs emission

Changed moisture conditions and aeration of soil may also be responsible for differences in N_2O production, which was generally reduced in the presence of earthworms (Fig. 5). Nitrification by aerobic, ammonia-oxidizing bacteria requires well-drained and aerated soils to produce nitrate from ammonium, as well as some nitrous oxide (Wrage et al., 2004; Barnard et al., 2005). However, denitrification of nitrate produces more nitrous oxide, but requires anaerobic conditions. Differences in N_2O production, with and without rhizobial inoculation, reflect the same patterns as DHA

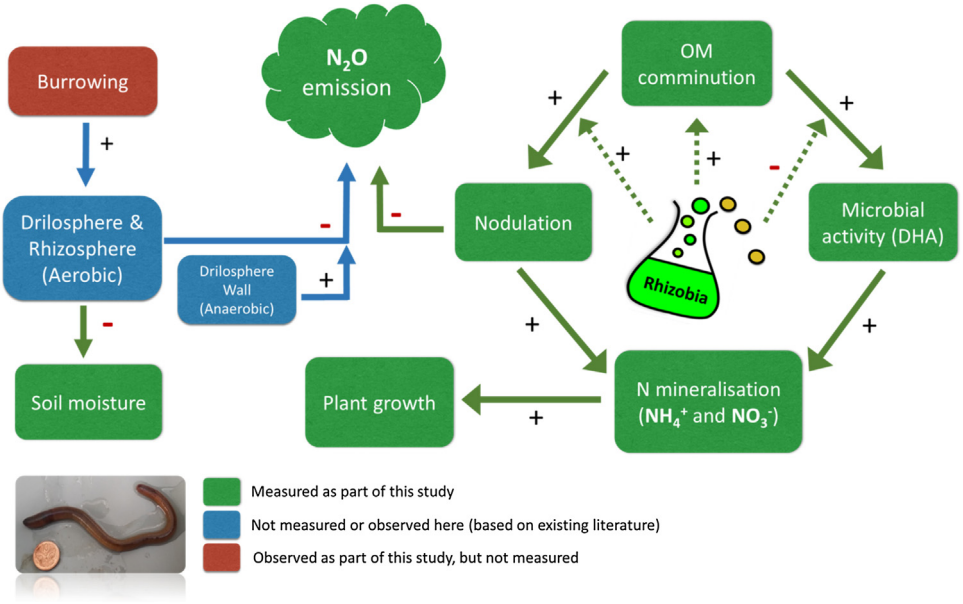

Fig. 6. Tentative interpretation of the results of the mesocosm experiment, based on data for *Maoridrilus transalpinus*. Positive (+) and negative (−) effects of the earthworm and rhizobial-inoculant are indicated as solid and dashed lines with arrows, respectively.

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except for pots with *M. transalpinus*. This species clearly had the most substantial influence on the results of the current experiment. It appears that, when there are more rhizobia, in more aerobic conditions, there is consequently less N_2O production. A higher level of burrowing activity appeared to provide aerobic conditions for higher nodulation that outweighed anaerobic conditions within drilosphere walls. This species consumed more organic matter, but increased aeration that provided more nitrate from its decomposition, but less conversion of NO_3 to N_2O. This differs to some previous studies that have shown increased N_2O production in the presence of earthworms in both laboratory and field conditions (Lubbers et al., 2013a,b).

4.5. Integration of findings

We found previously that individual species of earthworm could be separated on the basis of their modification of soil biogeochemistry, with differences particularly evident in terms of organic matter consumption, nitrogen and phosphorus mineralisation, soil microbial biomass and solubility of soil nutrients (Kim et al., 2015; Kim, 2016). Obviously this becomes more complex in the presence of plant roots. It has been shown previously that plant roots improve macroaggregate stability, which is decreased by earthworms (Milleret et al., 2009). The drilosphere provides numerous benefits for plants, including pathways for root elongation, increased aeration and enhanced supply of plant-available nutrients (Brown et al., 2000; Kautz et al., 2013).

The results of the present study showed significant interactions between native earthworms, *Sophora* and N dynamics. *M. transalpinus* produced considerable amounts of drilosphere soils in the pots, enhancing microbial activity, N solubility and nodulation. It appears that this species effectively dispersed rhizobia, increased aeration and facilitated more nodulation, in turn supressing N_2O emissions.

Invasive exotic legumes are known to be capable of fixing up to 200 kg N ha^{-1} $annum^{-1}$ in New Zealand (Magesan et al., 2012); an amount equivalent to current standard fertiliser applications in intensive agricultural land. Nitrate leaching does not appear to be higher under leguminous plants (Pattinson and Pattinson, 1985), but little is known of the N-fixing capacity of native legumes. Stands of invasive exotic gorse (*Ulex europaeus*) have been proven to have a role in restoration, providing nurse environments for native New Zealand plants (Burrows et al., 2015). However, this species has largely established on former agricultural land from which native earthworms have probably disappeared. The challenge remains to understand the role of both native legumes and native earthworms in the restoration trajectory, and their influence on both NO_3 and N_2O transfer to the wider environment.

The most desirable outcome would be to restore both native legumes and native earthworms into agricultural landscapes, whilst mitigating environmental concerns related to nitrogen. This would also have beneficial conservation outcomes. Results of the present study have demonstrated the interdependence between earthworms, root nodulation of *Sophora* and soil physicochemistry. Some combination of these factors mediates nitrogen cycling and influences the release of NO_3 and N_2O to the wider environment (Fig. 6). This work is a first step towards a better integrated understanding of the effects of plant growth, earthworm and microbial communities on N-cycling.

Acknowledgements

The authors thank the Punakaiki Coastal Restoration Project (PCRP) and Stuart Rhodes and Closure & Legacy Management, Rio Tinto Services Ltd. for their sponsorship of this work. The assistance of Hongtao Zhong and Shanshan Li for help with experimental work and Heng Wee Tan for providing rhizobial cultures is gratefully acknowledged.

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