Seabird guano and phosphorus fractionation in a rhizosphere with earthworms

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ABSTRACT

Soil phosphorus (P) is a critical nutrient for plant growth and is an important determinant of vegetation development and long-term ecosystem sustainability. We investigated the effects of rhizosphere-earthworm-guano interactions on soil P dynamics in a mesocosm involving two species of native New Zealand earthworms (*Megascolecidae* Sp.1 and *Maoridrilus transalpinus*) and introduced *Eisenia fetida*, in the context of inputs from seabird guano and the ecological restoration of a unique coastal sandplain forest. A fully factorial experimental design included a tall fibrous liliaceous perennial plant (New Zealand flax) growing in a low P forest soil, a guano-P amendment (with and without guano-P), and earthworm inoculation (with and without species of epigeics, endogeics and anecics). Soil dehydrogenase activity, CaCl$_2$-P, citrate-P and HCl-P were significantly modified by earthworm-guano interactions, altering the P status of the original forest soil. Furthermore, interactions between the rhizosphere and earthworms stimulated transformation of soil P and guano P; the proportional importance of soil microbial biomass P, organic-P and more soluble P fractions were substantially modified. These findings show that rhizosphere-earthworm interactions are likely to mediate the supply, chemical forms and plant-availability of P, and are likely to have an important role in successional processes and the trajectory of ecological restoration in coastal forests of New Zealand.

1. Introduction

After nitrogen (N), phosphorus (P) is the second most important nutrient for plant growth but differs to N by being one of the least available plant-essential nutrients in soil. Soil P exists in various chemical forms, either inorganic (typically 35–70% of total P) or organic (20–80% of total P); the proportional importance varies between soil types and with land management regime (McLaren and Cameron, 1996). Phosphorus is taken up by plants from soil solution in the form of orthophosphate, mainly as $H_2PO_4^-$ in acid soil and as HPO_4^{2-} in alkaline soil (Shen et al., 2011). It is the transformation and dynamics of chemical forms of P that determine its bioavailability in soils, itself driven by multiple physico-chemical and biological factors (Brady and Weil, 2008; Condron and Newman, 2011). Bioavailability of inorganic P is particularly limited in highly weathered soils of old ecosystems (Peltzer et al., 2010), such as in the primeval coastal forests of New Zealand in the present study (Turner et al., 2014).

Bird guano contains variable concentrations of P, typically ranging from 0.12 to 16% DW (Otero et al., 2015). Large coastal populations of seabirds in New Zealand were decimated by relatively recent human settlement, but may have previously provided a significant source of this element to coastal forest soils (Hawke and Newman, 2004; Mulder and Keall, 2001; Roberts et al., 2007). The fate of guano-P deposited on soil is known to change the ratio of organic and inorganic P fractions (Ziółek and Melke, 2014). Soil bicarbonate-extractable soil P (potential plant available P) appears to be replenished by the depletion of non-labile inorganic P forms potentially sourced from deposited and later transformed guano-P (Hawke and Condron, 2014). With regard to P mobility and bioavailability in soil, biological processes generally play a significant role in determining its chemical form and mobility; for example, through earthworm feeding, digestion, excretion, burrowing and casting which can substantially modify the physical, chemical and biological properties of soil (Bertrand et al., 2015; van Groenigen et al., 2014). However, earlier studies of relevance to the current work have mostly focused on P availability to plants (e.g. Chapuis-Lardy et al., 2011; Le Bayon and Milleret, 2009), rather than on soil P fractionation and dynamics in earthworm-inhabited rhizospheres with external P inputs.

The experimental work reported in this paper was carried out to investigate P dynamics in a simulated ecological restoration on a sandplain forest soil at Punakaiki on the West Coast of South Island, New Zealand. The inception of a broader ecological restoration project was partly due to its location being on the flight path of the only nesting site of the rare and threatened Westland Petrel (Smith et al., 2016). We considered this situation may be typical of the large colonies of seabirds that formerly inhabited the coastal forest of New Zealand and probably transferred significant amounts of bioavailable phosphorus from the ocean to deficient soils on shore. Interactions of earthworms with guano-derived P in the rhizosphere appear not to have been previously studied. This paper describes a mesocosm investigation of the interactions between New Zealand Flax, native and exotic earthworms, and inputs of seabird guano introduced into a low-P soil.

2. Materials and methods

2.1. Soil collection

Surface soil (0–20 cm) was collected using a spade from a mature Coastal Sandplain Forest Reserve at Punakaiki (42°8′38.39″S, 171°19′50.36″E). This soil is described as a Sandy Brown Dystrudept Mahinapua soil (Hewitt, 2010; Soil Survey Staff, 2014). It has a relatively low total P concentration of 400 mg kg^{-1} (Table 1), compared to soil beneath adjacent stands of New Zealand Flax which has P concentration of approximately 900 mg kg^{-1}. The mature forest soil also provides the habitat of an unnamed native endogeic earthworm (Megascolecidae Sp.1) that could be conveniently collected with large numbers of adults. Based on site observations, this mature forest plot contains all three functional groups of earthworms although there is a paucity of epigeics in the relatively depauperate O-horizon of the forest soil. After removing any surface litter, soil was sieved using a 6 mm steel sieve and stored under dark and outdoor temperature (average 22.5 °C) for 3 weeks prior to use in the mesocosm experiment.

2.2. Plants and earthworms

New Zealand Flax (*Phormium tenax* J.R. et G.Forst., Xanthorrhoeaceae) is a tall (up to 5 m), fleshy and fibrous liliaceous perennial (Moore and Edgar, 1976). This species is indigenous to New Zealand, widespread and is commonly planted in ecological restoration projects although it is largely absent from later successional stages of mature forests. The plant frequently dominates large stands of vegetation, as it occurs at the present site close to the shoreline and adjacent to the Punakaiki sandplain forest.

Three earthworm species, (*Eisenia fetida*, Lumbricidae), (*Megascolecidae* Sp.1, Megascolecidae), and (*Maoridrilus transalpinus*,

Table 1
Selected soil chemical and physical properties of the forest soil used in the mesocosm experiment (Zhong, 2017). Values are means (n = 5) with standard errors in parentheses. MBC: microbial biomass carbon; MBP: microbial biomass phosphorus.

Texture	Silt loam
pH (1:5 DI water)	4.8 (0.2)
EC (ds m^{-1})	0.12 (0.01)
Total C (%)[a]	3.58
Total N (%)[a]	0.23
C:N ratio[a]	15.9
NH$_4$-N (mg kg^{-1})	2.6 (1.0)
NO$_3$-N (mg kg^{-1})	0.8 (0.2)
MBC (mg kg^{-1})	671 (186)
MBP (mg kg^{-1})	5.8 (1.7)
Total P (mg kg^{-1})	400 (78)
Organic P (mg kg^{-1})	247 (71)

[a] Modified from Smith et al. (2016).

Megascolecidae) were selected for this experiment, representing epigeic, endogeic and anecic functional groups respectively (Buckley et al., 2015; Kim et al., 2015). Two of the species are native earthworms, but the common exotic tiger worm, *E. fetida*, was obtained from local compost heaps in the absence of viable source of native epigeics. Although a native epigeic species, *Deinodrilus gorgon*, (Boyer et al., 2011) was found in the litter layer of the forest soil, its scarcity in the field and low survivorship under laboratory conditions made it unsuitable for the glasshouse pot trial. The two native species were selected mainly because of the abundance of adults during field sampling and their relatively high survivorship under laboratory conditions. One of these species is currently undescribed, as is often the case for earthworms in New Zealand, but details of its identification are provided elsewhere (Kim et al., 2015, 2017). The three earthworm species were kept in a laboratory incubator separately for up to three weeks and checked routinely to pick out dead or injured individuals, so that healthy earthworms in sufficient numbers were available for the experimental work.

2.3. Guano

Substantial efforts were made to collect guano from around the Western Petrel (*Procellaria westlandica*, Procellariidae) nesting sites, but its scarcity, consistency and solubility during frequent rainfall events meant that less than 5 g of dry guano were gathered in two sampling trips. As an alternative, guano of the Spotted Shag (*Stictocarbo punctatus*, Phalacrocoracidae) was collected from a roosting site at King Billy Island (43°38′5.39″S, 172°41′8.14″E) in Lyttelton Harbour on the opposite eastern coast of South Island. Guano was air-dried, ground, then sieved through 0.5 mm brass sieve, and stored in clean screw-top polyethylene containers. Only the shag guano was used in the experiment, but the elemental composition of both guanos was determined using ICP-OES (Varian 720 ES, Australia) following microwave digestion (Microwave digester, CEM MARS Xpress, USA) in 5 M HNO$_3$ (International Soil Analysis Exchange, WEPAL Soil Material). Total C and N contents were determined using the Dumas combustion method on a CNS Elemental Analyser (LECO Elemental Analyser, NSW, Australia) (Blakemore et al., 1987). Organic phosphorus concentrations were estimated by subtracting the 0.5 M H$_2$SO$_4$-extracted phosphorus after and before ignition (Saunders and Williams, 1955). Results are expressed on an oven-dry (105 °C) basis.

2.4. Experimental design

The mesocosm experiment was conducted in a glasshouse at Lincoln University. One-year-old flax (*Phormium tenax*) seedlings were obtained from the NZ Department of Conservation Motukarara Nursery. The plant plugs contained potting mix, which was a barked-based medium amended with lime and nutrients. This was gently washed off the roots when transplanting plants into 45 plastic pots (5 L) each filled with forest soil (approx. 5 kg fresh weight each pot). To prevent earthworms escaping and to maintain uniform conditions, the underside of each plant pot was sealed with fine mesh, which was also secured to the upper surface of all of the pots and around the stem bases of the plants. Plants were acclimatised to glasshouse conditions for one week prior to inoculation with earthworms. Treatments consisted of: 3 species of earthworms [×3], each with (+) and without (−) guano additions [×2], with 5 replicates per treatment [×5]. Two further sets of 5 pots were allocated either single- (+) or double-amounts (++) of added guano without earthworms [+10], and 5 pots were planted without guano and earthworms additions [+5]. Five additional pots were provided with a single-amount (+) guano without flax and earthworms [+5]. This provided a total of 50 mesocosms.

Four earthworms of a single species were weighed and then added to each of the earthworm-treatment pots. Earthworm mortality at the surface of the pots was checked on a daily basis; in the first 5 days, all of

the endemic earthworms died in two of the pots, and they were removed and replaced with the same species of earthworm. Subsequently, any dead earthworms on the soil surface were removed without being replaced. After 5 days, 1.5 g of guano (equivalent to 26 kg P ha^{-1}) was added to the surface of the soil in relevant pots, and then twice more at fortnightly intervals. Applications of double amounts of guano were made in appropriate treatments at the same time.

Mesocosms were arranged in a complete randomized block design and maintained in a glasshouse with average day and night temperatures of 30/25 °C throughout the trial. Soil moisture content was maintained by adding the same amount of water to each pot on a daily basis, as subjectively judged to be optimal.

2.5. Analytical

This pot trial was harvested 9 weeks after earthworm inoculation. Following the harvest of plants, roots were gently washed and surface dried on absorbent paper. Shoots and roots were separated, stored in paper bags, dried for 48 h at 60 °C, then weighed. Earthworms were carefully removed from each treated pot and weighed. During separation, moist soil was carefully sampled from the rhizosphere, represented as soil detached from roots gently crushing and shaking. Soils were crumbled and sieved (< 2 mm) to remove fine roots, and stored in clean zip-lock polyethylene bags at 4 °C for less than 1 week. Soil sub-samples were air-dried (25 °C, 1 wk), crushed using a mortar and pestle, sieved (500 μm brass sieve) and stored in zip-lock polyethylene bags at room temperature.

Soil microbial biomass carbon (MBC) was measured by CHCl$_3$ fumigation and 0.5 M K$_2$SO$_4$ extraction on moist soils (Vance et al., 1987), using a conversion factor (Kc) of 0.45 (Wu et al., 1990). Soil microbial biomass phosphorus (MBP) was measured following CHCl$_3$ fumigation and 0.5 M NaHCO$_3$ extraction of moist soils, applying a conversion factor (Kp) of 0.4 (Brookes et al., 1982). To measure Soil Dehydrogenase Activity (DHA), 2 g moist soil was incubated with 2 ml of 1% TTC buffer (pH = 7.6) at 25 °C for 24 h in 25 ml falcon tubes in dark incubator (Gong, 1997). After extraction with 10 ml methanol, the supernatant was centrifuged (3000 rpm, 10 min) and absorbance (485 nm) determined using a spectrophotometer (UV 160A, Shimadzu, Japan).

Soil pH and electrical conductivity (EC) were measured on air-dried soil (1:5 soil to DI water) using pH and EC probes (calibrated with pH 4 and 7 buffer solutions) (Mettler Toledo, Australia). Soil total organic matter (% SOM) was measured as loss on ignition in a muffle furnace at 500 °C. Determination of mineral nitrogen (NH$_4$-N and NO$_3$-N), following extraction of fresh soil in 2 M KCl, used a FIAstar 5000 triple channel analyser with SoFIA software version 1.30 (Foss Tector AB, Sweden) (Clough et al., 2001).

The Soil P fractionation scheme employed in the present study was designed to extract biologically-based phosphorus fractions, aiming to simulate mechanisms of plant and microbial phosphorus acquisition (DeLuca et al., 2015). Sequentially, the CaCl$_2$-P fraction has high bioavailability, followed by medium bioavailability of citrate-P fraction, and then the low bioavailability of the HCl-P fraction. Each of these three P fractions was extracted by shaking a 1 g of air-dried soil with 20 ml of each extractant for 3 h. Extracts were then centrifuged at 2280g for 30 min, and filtered through Whatman No. 42 filter papers. Filtrates were stored in fridge prior to analysis. The first extraction, *0.01 M CaCl$_2$ extractable P* (*CaCl$_2$-P*), represents soluble and weakly adsorbed inorganic P, simulating P acquired by direct root interception. The second extraction, *0.01 M citrate extractable P* (*citrate-P*), represents active inorganic P pool adsorbed to clay particles or weakly bound in inorganic precipitates, simulating the P pool that could potentially be released by organic acids produced from plant root and microorganisms. The third extraction, *1 M HCl extractable P* (*HCl-P*), simulates more recalcitrant P pool that could potentially be solubilized via proton excretion (inorganic acids) promoted by plant root and microbial

processes. Additionally, the *soil organic P concentration* (*organic-P*) was estimated by subtracting the 0.5 M H$_2$SO$_4$-extracted phosphorus after and before ignition (Saunders and Williams, 1955). *Soil total P* concentration was estimated by NaOH fusion in a nickel crucible (Smith and Bain, 1982). *Soil inorganic phosphorus* was calculated as the difference between total P and organic P.

2.6. Statistical analysis

Data were analysed using Minitab (Minitab Inc., State College, Pennsylvania, USA), with one-way ANOVA and Fisher's least-significance-difference post-hoc test, and two-way ANOVA (guano and earthworm interactions) of selected soil chemical properties and P fractions, and the main effect analysis of an independent variable (guano or earthworm) at each level of the other independent variable (guano or earthworm) on selected soil chemical properties and P fractions.

3. Results

3.1. Bird guano, earthworms and plant growth

Shag guano had several times higher P and Ca concentrations than petrel guano, but less than 5% of the Cd concentration of petrel guano (Table 2). Shag guano used in the mesocosm experiment had a C:N ratio of 3.5.

On completion of the glasshouse pot trial, all of the native earthworms had died whilst all *E. fetida* survived. The biomass of this species, with and without guano inputs, increased by 106% and 23.5% respectively (data not shown). Despite the death of native earthworms, there was clear visual evidence of substantial burrowing activity in all inoculated pots (Fig. 1). The lower input rates of guano increased plant growth in the absence of earthworms by a similar amount that could be attributed to the presence of the two native earthworms without guano addition (Fig. 2). Combinations of native earthworms and guano did not have an additive effect on plant growth. There were no significant differences in plant root/shoot ratios between treatments, which ranged from 0.4 to 0.6% (data not shown).

3.2. Soil chemistry

Soils were marginally acidified when guano was applied in the absence of plants and earthworms (Table 3 cf. Table 1), but changed little in the presence of rhizospheres and earthworms. Without guano additions, soil organic matter was reduced in the presence of a plant rhizosphere and earthworms. Guano additions substantially increased

Table 2
Chemistry of Spotted Shag and Westland Petrel guanos. Values in Shag guano column are means (n = 3) with standard errors in parenthesis. Petrel guano column lacks error values due to limited guano availability.

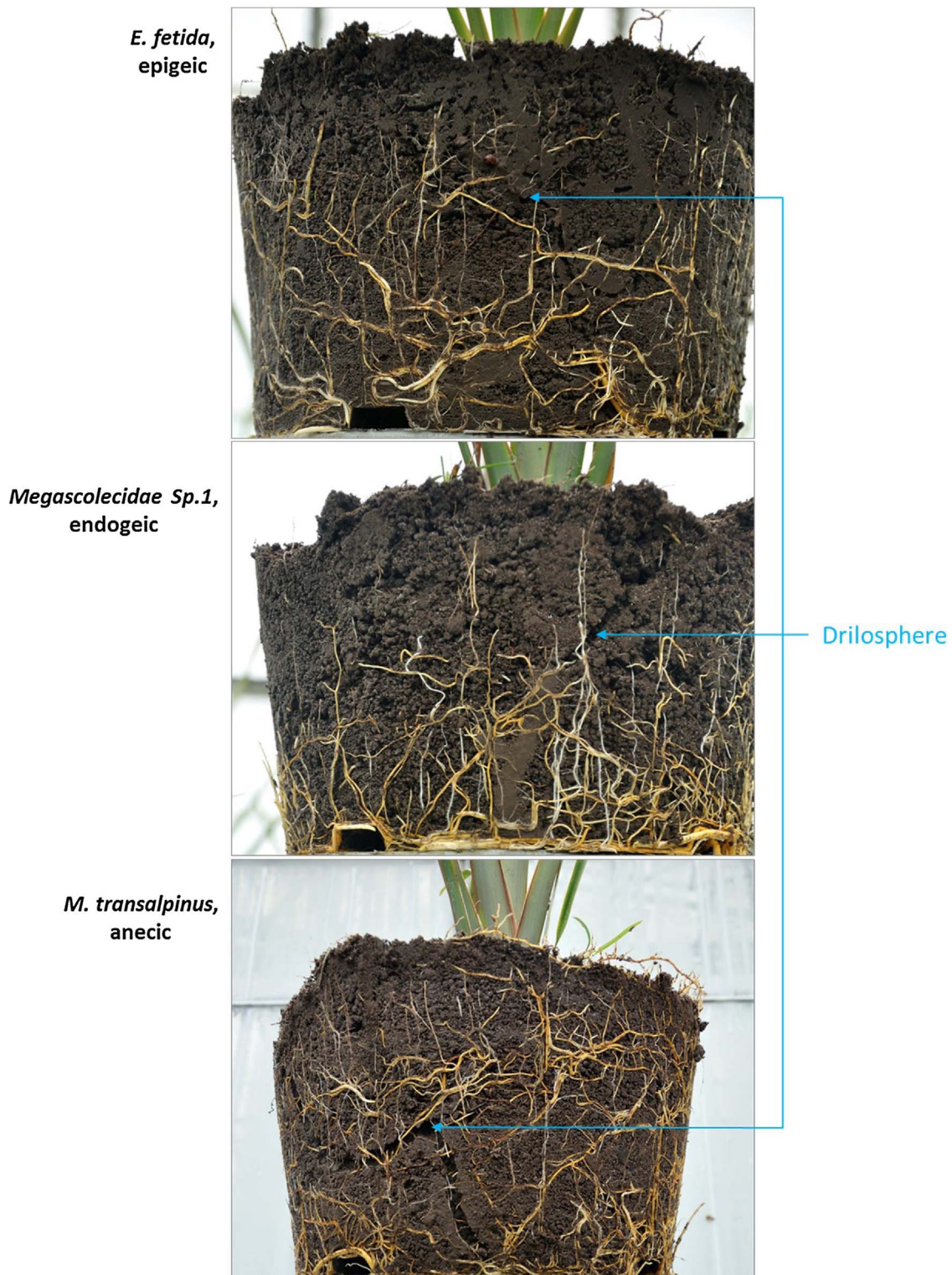|	Shag guano	Petrel guano
Total C%	11.4 (0.3)	–
Total N%	3.5 (0.5)	–
Total P%	12.1 (0.2)	1.4
Organic P%	0.08 (0.01)	–
Ca%	23.0 (0.4)	2.4
K%	0.7 (< 0.01)	0.5
S%	0.7 (0.03)	0.2
Mg%	0.6 (0.1)	0.3
Na%	0.4 (0.03)	0.1
Al%	0.3 (0.04)	0.2
Fe%	0.2 (0.03)	0.5
Zn%	0.05 (< 0.01)	0.02
Mn%	0.02 (< 0.01)	0.02
Cd (mg kg^{-1})	0.2 (< 0.01)	4.2
mg Cd/kg P	1.7 (0.3)	305

Fig. 1. Evidence of earthworm casting and burrowing activities, 9 weeks after inoculation. (For interpretation of colour in this figure, the reader is referred to the web version of this article.)

both NH_4-N and NO_3-N concentrations in soil, but these mobile forms of N were apparently sorbed in the presence of a rhizosphere, except with the higher application rate of guano which provided a surge of NH_4-N. There were no treatment effects on MBC, but MBP was much higher with larger inputs of guano and was enhanced in the presence of *E. fetida*. DHA was enhanced in the presence of a rhizosphere, except at the high level of guano input.

As would be expected, Total-P, Organic-P and $CaCl_2$-P were increased by guano inputs, but all declined substantially during the experiment in the presence of plants and earthworms compared to no-flax/no-earthworm pots, both singly and together (Fig. 3). Total-P was reduced more by earthworms than plants. The three inorganic fractions of P supplied by guano appeared to be reduced less by *E. fetida* than by other earthworms or by plants alone.
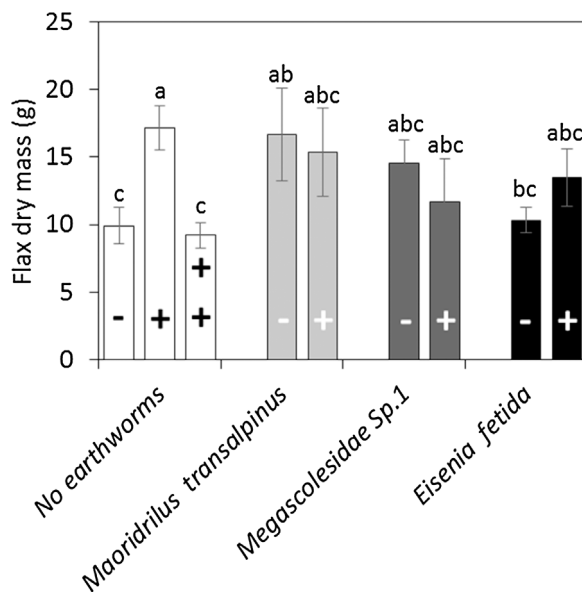
Fig. 2. Dry mass of flax after 9 weeks. Values are means ± standard errors (n = 5). The same letters indicate no significant difference (p < 0.05). '−' and "+" indicate without and with guano addition, '+ +' indicates double amount of guano addition.

Two-way ANOVA showed a significant interaction between the effects of guano and earthworm additions on soil DHA, CaCl$_2$-P, citrate-P, and HCl-P (Table 4). The main effects analysis showed that earthworm species had significantly more effects on soil DHA and CaCl$_2$-P, while guano additions had significantly more effects on citrate-P and HCl-P.

The proportional importance of P fractions was modified by the flax-earthworm-guano treatments (Table 5). The effect of *E. fetida* and high guano inputs on MBP described above, was reflected in its proportion of soil organic-P. Compared to the original forest soil, the proportional concentration of Organic P to Total P increased during the pot trial, irrespective of treatment. CaCl$_2$-P accounted for more of the inorganic P in pots without plants or earthworms at the end of the experiment. The citrate-P and HCl-P fractions increased with high guano inputs and in the presence of *E. fetida*.

4. Discussion

4.1. Guano chemistry

Distinct differences in chemical composition between Spotted Shag and Westland Petrel guano may be attributed to dietary and feeding differences. Spotted Shag is abundant along the coast and feeds close to the shore (Lalas, 1983). Westland petrels travel much further out to sea, although they have been found to opportunistically rely on scavenging fishery waste from fishing boats during the chick-rearing period which is the only time they spend on land (Freeman, 1998). A large proportion of fish bone regurgitant was visible in the shag guano which accounts for the high concentrations of Ca and P. Nitrogen concentrations in the shag guano (3.5%) were low compared to concentration reported for other seabirds (Irick et al., 2015), but this concentration together with a C:N ratio of 3.5 indicate a readily available source of this nutrient for plants. Some of the guano may have been quite old when it was collected, and some N might have already been lost through NH$_3$ emissions. More than 30% of N excreted from seabirds has been found to be volatilized as NH$_3$-N (Blackall et al., 2008), as was evident in the stench beneath the shag colony.

Cadmium inputs to soil associated phosphate fertilisers are a major environmental concern in New Zealand's agricultural soils. Concentrations of this toxic metal in petrel guano is within the range of 27–641 mg kg^{-1} that is typical of sedimentary rock phosphate (McLaughlin and Hamon, 2001), and shag guano was at the lower end of the range. These differences would result in different Cd loadings from guano of shags and petrels of 1.7 and 305 mg Cd kg^{-1} of applied P. Applications rates of fertiliser P to agricultural pasture are typically in the order of 50 kg P ha^{-1}, which would add 0.85–15.25 g Cd ha^{-1} if guano of either of these species were used as a fertiliser. Whilst these amounts are small, soil Cd accumulation rates could differ substantially in the longer term with repeated application, according to the source of guano.

4.2. Earthworms, guano and plant growth

A high proportion of organic matter (11% C) in the forest soil provided good food resource for *E. fetida*, as reflected in increased biomass, and it was unsurprising that all individuals of this species survived;

Table 3
Selected soil properties, nine weeks after earthworm inoculation, with and without earthworms and rhizospheres, and with and without guano inputs. Values in columns are means (n = 5) with standard errors in parenthesis. The same letters in each column indicate no significant difference (p < 0.05). SOM: soil organic matter; MBC: microbial biomass carbon; MBP: microbial biomass phosphorus; DHA: dehydrogenase activity; '−' and "+": without and with guano additions; '+ +': double amount of guano addition.

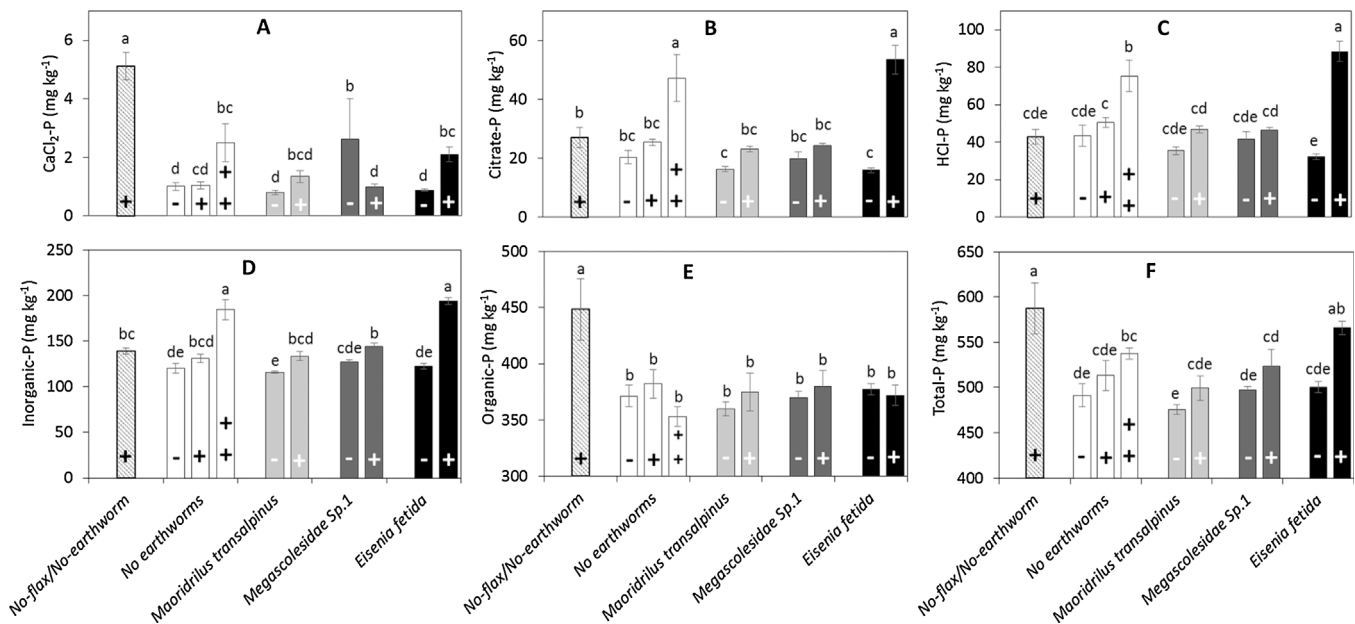| Treatments		pH	EC	% SOM	NH$_4$-N	NO$_3$-N	MBC	MBP	DHA
Earthworms	Guano	(1:5 H$_2$O)	(ds m^{-1})		(mg kg^{-1})	(mg kg^{-1})	(mg kg^{-1})	(mg kg^{-1})	(mg kg^{-1} dry soil h^{-1})
No-flax/No-earthworm	+	4.41 (0.04)[e]	0.08 (< 0.01)[a]	2.9 (0.3)[a]	13.3 (1.1)[ab]	33.4 (3.5)[a]	611 (56)[a]	5.7 (2.8)[b]	1.65 (0.05)[c]
No-earthworms	−	4.77 (0.08)[abc]	0.06 (0.02)[cd]	2.2 (0.2)[bcd]	3.5 (0.2)[d]	3.8 (0.6)[bc]	531 (30)[a]	2.0 (0.7)[b]	1.9 (0.08)[b]
	+	4.66 (0.05)[cd]	0.07 (< 0.01)[b]	2.3 (0.1)[bcd]	5.5 (1.2)[cd]	3.7 (0.9)[bc]	556 (32)[a]	3.8 (0.3)[b]	1.91 (0.07)[b]
	+ +	4.63 (0.01)[d]	0.10 (0.03)[a]	2.6 (0.1)[abc]	19.4 (3.6)[a]	7.6 (2.4)[b]	490 (35)[a]	15.2 (4.5)[a]	1.54 (0.02)[c]
Maoridrilus transalpinus	−	4.67 (0.02)[bcd]	0.07 (< 0.01)[b]	2.0 (0.1)[d]	5.3 (1.2)[cd]	3.1 (0.2)[c]	529 (38)[a]	3.1 (0.7)[b]	2.01 (0.09)[ab]
	+	4.73 (0.03)[abcd]	0.07 (< 0.01)[bc]	2.1 (0.1)[cd]	8.6 (2.7)[bcd]	3.3 (0.3)[c]	616 (46)[a]	3.6 (0.5)[b]	1.96 (0.09)[b]
Megascolesidae Sp.1	−	4.76 (0.01)[abc]	0.05 (< 0.01)[d]	2.0 (0.2)[cd]	4.0 (0.8)[d]	2.3 (0.3)[c]	599 (41)[a]	4.2 (1.6)[b]	2.00 (0.05)[ab]
	+	4.70 (0.05)[abcd]	0.07 (< 0.01)[b]	2.8 (0.4)[ab]	10.8 (3.1)[bc]	3.2 (0.5)[c]	495 (54)[a]	3.8 (1.4)[b]	1.85 (0.02)[b]
Eisenia fetida	−	4.78 (0.02)[ab]	0.05 (< 0.01)[d]	1.9 (0.1)[d]	2.9 (0.1)[d]	2.9 (0.2)[c]	582 (49)[a]	4.2 (1.0)[b]	1.91 (0.04)[b]
	+	4.80 (0.04)[a]	0.07 (0.01)[bc]	2.8 (0.2)[ab]	8.3 (3.5)[bcd]	2.6 (0.2)[c]	550 (78)[a]	16.6 (8.1)[a]	2.16 (0.08)[a]

Fig. 3. Soil concentrations of different P fractions after 9 weeks. Values are means ± standard errors (n = 5). The same letters indicate no significant difference (p < 0.05). '−' and "+" indicate without and with guano additions, '+ +' indicates a double amount of guano addition.

Table 4
Two-way ANOVA (*p* values) of guano-earthworm interaction of selected soil chemical properties and P factions, and the main effects analysis of independent variable (guano or earthworm) on the soil DHA, $CaCl_2$-P, citrate-P and HCl-P. SOM: soil organic matter; MBC: microbial biomass carbon; MBP: microbial biomass phosphorus; and DHA: dehydrogenase activity; n.a. indicates no main effect analysis applicable since the earthworm-guano interaction is not significant.

Factor	pH	EC	% SOM	NH_4-N	NO_3-N	MBC	MBP	DHA	$CaCl_2$-P	Citrate-P	HCl-P	Organic-P
Guano	0.335	0.143	0.001	0.002	0.333	0.657	0.041	0.372	0.559	< 0.001	< 0.001	0.823
Earthworm	0.137	0.072	0.239	0.821	0.101	0.721	0.184	0.052	0.606	0.004	0.002	0.584
Interaction	0.088	0.708	0.140	0.725	0.671	0.248	0.229	0.014	0.050	0.001	< 0.001	0.836
Main effect	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	Earthworm	Earthworm	Guano	Guano	n.a.

higher biomass in guano-treated pots indicated either that guano or guano-enriched organic matter provided better nutrition. This is a resilient earthworm species that can survive in extreme environments (e.g. Contreras-Ramos et al., 2005; Edwards and Bater, 1992; Kinney et al., 2012), unlike the two native species of which much less is known (Kim, 2016). Whilst different burrowing groups of earthworms were used in the experiments, our observations suggested that behavioural differences between the burrowing groups are not adequately separated in small-scale experiments of this type in which soils are uniformly mixed, shallow and contained. This also may have been a factor

influencing the lack of survivorship of native species, but there was clear visual evidence that all species had burrowed extensively through the soil (Fig. 1). The most likely distinction between species was solely the adaptation of *E. fetida* to the warmer environment of compost heaps, allowing it to proliferate in the higher temperatures of the mesocosm soils. It would be unreasonable to suggest that our experiment would distinguish any other functional differences between species, and we consider that this justifies grouping the functional effects of the earthworms together. It is worth noting that there were no obviously visible dead earthworm bodies or decaying tissues from the two native

Table 5
Proportional importance of different chemical fractions of phosphorus in comparison with the original forest soil. Values in columns are means (n = 5) with standard errors in parenthesis. The same letters indicate no significant difference (p < 0.05). '−' and "+" indicate without and with guano addition, '+ +' indicates a drouble amount of guano additions. Where MBP: microbial biomass phosphorus; n.d. means no data available.

Treatment		MBP/organic-P	organic-P/total P	$CaCl_2$-P/inorganic-P	citrate-P/inorganic P	HCl-P/inorganic P	inorganic-P/total P
Earthworms	Guano	(%)	(%)	(%)	(%)	(%)	(%)
No-flax/No-earthworm	+	1.3 (0.6)[b]	76.1 (1.2)[a]	0.037 (0.003)[a]	19.4 (2.2)[b]	30.6 (2.5)[cd]	23.9 (1.2)[d]
No-earthworms	−	0.5 (0.2)[b]	75.6 (0.8)[ab]	0.008 (0.001)[c]	16.7 (1.2)[bcd]	35.5 (3.1)[bc]	24.4 (0.8)[cd]
	+	1.0 (0.1)[b]	74.4 (0.3) [abc]	0.008 (0.001)[c]	19.4 (0.4)[b]	38.4 (1.1)[b]	25.6 (0.3)[bcd]
	+ +	4.3 (1.2)[a]	65.7 (1.8)[d]	0.013 (0.013)[bc]	24.9 (2.9)[a]	40.3 (2.1)[ab]	34.3 (1.8)[a]
Maoridrilus transalpinus	−	0.9 (0.2)[b]	75.6 (0.5)[ab]	0.007 (0.001)[c]	14.1 (0.6)[cd]	30.6 (1.7)[cd]	24.4 (0.5)[cd]
	+	1.0 (0.1)[b]	73.2 (0.4)[bc]	0.010 (0.002)[bc]	17.4 (0.9) [bc]	34.9 (1.0)[bc]	26.8 (0.4)[bc]
Megascolesidae Sp.1	−	1.1 (0.5)[b]	74.5 (0.6)[abc]	0.021 (0.011)[b]	15.4 (1.7)[bcd]	32.6 (2.4)[c]	25.5 (0.6)[bcd]
	+	1.1 (0.4)[b]	72.4 (0.4)[c]	0.007 (0.001)[c]	16.9 (0.6)[bcd]	32.2 (1.0)[c]	27.6 (0.4)[b]
Eisenia fetida	−	1.1 (0.3)[b]	75.4 (0.5)[ab]	0.007 (< 0.001)[c]	12.9 (0.5)[d]	26.2 (0.9)[d]	24.6 (0.5)[cd]
	+	4.6 (2.3)[a]	65.7 (0.9)[d]	0.011 (0.01)[bc]	27.4 (2.0)[a]	45.4 (2.0)[a]	34.3 (0.9)[a]
Forest soil	−	2.7	58.9	0.011[†]	n.d.	18.8	41.1

[†]This percentage is based on NH_4Cl-extractable P values representing soluble or loosely bound P fraction, which is considered to be equivalent to $CaCl_2$-P (Zhong, 2017).

earthworm treated pots at the end of the experiment, indicating they died a significant time before the end of the experiment.

It would seem possible that nutrients released from decaying earthworm tissues of the native species promoted flax growth, as found by Whalen and Parmelee (1999). Higher flax biomass in *M. transalpinus* pots than in *Megascolesidae* Sp.1 pots could perhaps also be explained by different biomass of the species (8.8 and 4.8 g pot^{-1} respectively). The equivalent value for *E. fetida* was 1.1 g pot^{-1}. However, we consider that earthworm death and decay does not explain the results; our mass balance calculations (data not shown) indicated that only a very small proportion of total mesocosm nutrients was contained within earthworm biomass, ranging between 0.01–0.07% of total mesocosm P. Death and decay of the native earthworms would have contributed only a negligible amount of < 10 mg P pot^{-1} (equivalent to < 4.5 kg P ha^{-1}) to any of the soil fractions of this element, based on the same relative abundance of earthworms. Instead it is likely that enhanced flax growth was attributable to either earthworm functionality, nutrients from guano, or some combination of both. Guano and earthworms had a similar effect of increasing plant growth, but these effects apparently were neither additive nor synergistic. Guano amendments probably provided readily-available nutrients to the plants; as highest flax biomass was in no-earthworm (+) pots. The double-guano treatments may have inhibited flax growth through ammonium or phosphate toxicity. P-deficient soils are typical of the west coast (McLaren and Cameron, 1996). Other recent work in our group showed that flax growth is enhanced by N additions, but not by P (Franklin et al., 2015). Studies elsewhere have shown that earthworms increase plant growth (Scheu, 2003). In a recent meta-analysis, van Groenigen et al. (2014) showed that earthworms promote aboveground plant growth primarily through improved soil structure and increased nutrient bioavailability, the latter due to excretion and increased microbial activity which promote plant growth by providing phytohormones such as cytokinins (Zhang et al., 2014). The enhancement of plant growth by earthworms is mainly due to increased soil N mineralization and availability, although it is also known that earthworms also increase P availability (Vos et al., 2014). Overall amounts of P in the mesocosm, calculated in terms of mass balance at the end of the experiment, showed that $\leq 0.5\%$ was in the plants (data not shown).

4.3. Soil biogeochemistry

Guano additions marginally acidified the soil but this effect disappeared in the presence of rhizospheres and earthworms. Production of $CaCO_3$-rich excretions by earthworms is likely to have marginally buffered soil pH, as found by Lambkin et al. (2011), and high concentration of Ca and other base cations in guano could have also contributed both to higher soil pH and EC. Reference treatment soils with guano additions, but without plants or earthworms, had similar OM concentrations to the original forest soils. Otherwise OM concentrations declined, indicating enhanced soil respiration. Added guano was substantially mineralized to ammonium-N and further nitrified to nitrate-N, which is consistent with higher soil MBC. The effects of earthworms in promoting organic N mineralization was most evident in *E. fetida* treatment pots, and this is consistent with previous studies (e.g. Kim et al., 2015; Sheehan et al., 2006).

Without guano inputs, soil organic matter was reduced, but MBC did not appear to be affected by the addition of earthworms and rhizospheres. The main effects of earthworms with guano inputs were to increase DHA and MBP. This is consistent with an earlier suggestion that selective feeding by anecic and edogeic earthworms on soil microorganisms initially reduces soil microbial biomass, but later increases the activity of soil microbial communities (Scheu et al., 2002; Zhang et al., 2000). In the field situation, this may be different in the case of epigeic earthworms which mainly feed on surface litter and organic detritus. In the present study, decomposition of earthworms of the two native species may have refuelled microbial communities.

Guano P is also likely to have provided a good source of quickly-available P to either soil microbes or earthworms or both. In guano-treated (+) pots, *E. fetida* significantly promoted MBP to similar levels as in no-earthworm (++) pots.

Guano may have also increased soil DHA which consists mainly of oxidoreductase enzymes that play a critical role in the initial stages of oxidizing soil organic matter (Gu et al., 2009). In a long-term fertilization experiment, Chu et al. (2007) found soil DHA was greatly increased. In the preent experiment, it is possible that *E. fetida* created localized anaerobic conditions through burrowing, soil consumption and mucus secretion (Edwards, 2004; Lubbers et al., 2013), thereby favouring DHA by anaerobic microorganisms (most DHA is produced by anaerobic microorganisms (Włodarczyk et al., 2002)). Clearly, further research is required to elucidate this.

4.4. Phosphorus fractionation

It is clear that the soil P pool had been biotically modified both by earthworms and the rhizosphere, and also by their interactions with each other and with guano additions. Most previous studies have simply measured available-P or P speciation in earthworm casts, compared with bulk or uningested soil (e.g. Chapuis-Lardy et al., 2009; Kim et al., 2015; Kuczak et al., 2006; Vos et al., 2014). These studies have invariably reported higher available-P concentrations (NaOH-, NaHCO$_3$- or water extractable-P) in earthworm casts. Causal factors have been considered to include facilitated mechanical breakdown of organic matter, raised soil pH (6.0–6.8), earthworm mucus, mobilization of clay particles, and increased microbial and enzymatic activities during gut transition. In the present study, Total P, Organic-P and CaCl$_2$-P in soil were lower in the presence of a rhizosphere and earthworms (Fig. 3). The proportion of Total-P in soil that was represented by Organic-P over the duration of the experiment was higher than the original forest soil, but CaCl$_2$-P formed a much lower proportion of inorganic P (Table 5).

The higher input of guano combined with *E. fetida* had a marked effect on raising soil concentrations of MBP, Citrate-P and HCl-P (Table 3, Fig. 3). At the same time HCl-P came to represent a smaller proportion of inorganic-P (Table 5). The contribution of decaying earthworm tissues to soil soluble or weakly adsorbed P pool in the present experiment would have been slight, because of the relatively low P phosphorus content in earthworm biomass and the competition for this from soil microbes and flax roots. Decaying earthworm tissues from two native species are likely to have contributed to soil MBP when comparing pots between no-earthworms ($-$) and native earthworms ($-$) treatments only (Table 3). It has been previously suggested that earthworm residues decompose rapidly and become available for microbial assimilation first, but later plants capture more released nutrients (Hodge et al., 2000).

Comparing the effects of guano additions with and without flax, uptake of soluble forms of P by flax plants may have reduced the supply available to soil microbes. Soil pH of 6–7 provides optimal P availability in soil (Brady and Weil, 2008), and lowered soil pH (4.4–4.8) would be expected to limit the pool of soluble or weakly adsorbed (CaCl$_2$-extractable) P. High soluble-P with large error bar in *Megascolecidae* Sp.1 ($-$) pots could be explained if this species survived closer to end of experiment, since other P fractions were unchanged.

Soils with *E. fetida* maintained higher concentrations of the more soluble P fractions (CaCl$_2$-P, citrate-P, and HCl-P), particularly in comparison with pots without earthworms. Aira and Domínguez (2014) recorded higher acetic acid-extractable P in casts, which is reasonably comparable to citrate-P in the present study. Kuczak et al. (2006) found HCl-extractable inorganic P concentrations to be higher in earthworm casts, and suggested earthworms indirectly enhance the binding of P to Fe/Al clay particles. Guano contains mostly inorganic P, and the present work shows that flax-earthworm interactions substantially modify the impact of guano-P on soil P dynamics. Soil microbial activities significantly promoted the build-up of organic P and moderately bound

HCl-P fractions, at the same time maintaining a reasonable level of soluble or weakly adsorbed P fractions. The exclusive contribution of soil microorganisms to the transformation of guano-P into different P fractions was reflected in no-flax/no-earthworm (+) pots, compared to original forest soil (Table 5). Flax-earthworm interactions then further stimulated the transformation of different P fractions. Compared to original forest soil, organic P rather than inorganic P became more significant, representing 60% compared to 40% of total P respectively. The proportional importance of acid-extractable P to inorganic P was also higher than original forest soil, accounting for approximately 19% of inorganic P. MBP accounted for was proportionally less of the organic P (approximately 3%) than in the original forest soil, except in the case of *E. fetida* (+) and no-earthworm (++) pots.

5. Conclusions

Diet differences and the constituents of guano provide substantial differences in guano chemistry, particularly of the content of phosphorus, calcium and cadmium. Guano provided a readily-available source of nutrients for uptake by flax, but flax growth was primarily stimulated by the supply of mineral N, but was also modified by the interaction between guano and earthworms.

Burrowing differences between ecological groups of earthworms were not differentiated by the limitations of the pot experiment. Low soil pH of the forest soil probably had a limiting effect on the pool of more mobile forms of P.

Soil microbial activities played a significant role transforming guano P, promoting the build-up of organic P and moderately bound HCl-P fractions, at the same time maintaining a supply of soluble or weakly adsorbed fractions of P. Earthworms interacted with guano by modifying soil DHA and $CaCl_2$-P. Guano had a more pronounced effect on Citrate-P and HCl-P fractions. The interaction of earthworms and flax rhizospheres had a further substantial and interactive effect on the transformation of soil P and guano P into different P fractions, decreasing Total P, Inorganic-P and $CaCl_2$-P, but increasing Organic P. Soils inoculated with *E. fetida* maintained higher concentrations of these biologically-based P fractions of citrate-P and HCl-P.

Patterns of P mobility have emerged from the experiment that show it would be fallacious to describe the chemistry of this element in the coastal forest soil of New Zealand without understanding the impacts of seabird guano and interactive roles of microbial communities, rhizospheres and earthworms in mediating its mobility. The complexity of these interactions requires further elucidation.

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