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Consequences of anecic earthworm removal over 18 months for earthworm assemblages and nutrient cycling in a grassland

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Highlights:

- Targeted removal of *Lumbricus terrestris* and *Aporrectodea longa* in field plots affected earthworm community
- *Lumbricus festivus* and *Satchellius mammalis* more abundant with anecics removed
- *Aporrectodea caliginosa* assimilated less ^{15}N from surface litter with anecics removed
- Low-level reduction of anecic earthworm populations has ecological consequences

Abstract

Earthworms are recognised widely for playing important roles in soil functioning, but few studies have attempted to assess the effects of separate functional groups under natural field conditions. We investigated the effects of selective removal of large anecic earthworms (primarily *Lumbricus terrestris*) over 18 months on earthworm assemblages, earthworm trophic ecology, and plant nutrient uptake in a temperate grassland. We used unenclosed field plots to simulate selective predation of large anecic individuals by alien flatworms and isotopically enriched plant material (^{13}C and ^{15}N) to trace nutrients. Though surface addition of plant material to plots increased the abundance and biomass of total and anecic earthworms, compared to control plots, earthworm composition was different and more variable where anecics had been removed. Most notably, in treatments receiving litter, abundance and biomass of the litter-feeding epi-anecic *Lumbricus festivus* and epigeic *Satchellius mammalis* were significantly greater where anecics had been removed. Addition of labelled plant material enriched individuals from all species in ^{13}C and ^{15}N , especially in litter-feeding epigeics. Similar abundances but altered isotopic compositions suggest that the removal of anecics influenced the feeding activities of other earthworm species. In particular, the soil-feeding endogeic *Aporrectodea caliginosa* was less enriched where anecics had been removed, suggesting that this species benefits from anecic surface foraging activity. Individual *L. terrestris* tended to be less enriched isotopically in the removal treatment, probably reflecting re-colonisation from outside litter addition plots. There was no effect of anecic removal on ^{15}N uptake into above-ground biomass of each of three plant functional groups, though there was a trend of greater enrichment in removal plots. Taken together, these findings provide novel evidence, from a real field setting, that low-level reduction of anecic earthworm populations (experimental removal of 4 large individuals per 1 m² plot over 18 months) can affect other earthworm species in terms of their abundance and trophic relations.

Keywords: Earthworms; exclusion experiment; grassland; litter decomposition; Lumbricidae; soil functions; stable isotopes.

1. Introduction

Earthworms impact soil processes and play important roles in mediating soil ecosystem functions that, in turn, deliver ecosystem services. In particular, earthworms are known to influence organic matter incorporation and biogeochemical cycling (Beare et al., 1995; Bohlen et al., 2004; Scullion et al., 2007; Zhang et al., 2013), soil structure and hydrology (Shipitalo & Butt, 1999; Pérès et al., 2010; Spurgeon et al., 2013; van Schaik et al., 2014), and plant productivity (Curry, 1994; Scheu 2003; van Groenigen et al., 2014). They are recognised as ecosystem engineers and functionally they are often considered the most important soil invertebrates in temperate ecosystems (Curry, 1994; Lavelle et al., 2006).

In grasslands, earthworms are particularly abundant and functionally diverse; Compared to other habitats in temperate ecosystems, they generally contain greater abundances (typically 300–600 individuals m⁻²) and species richness per sampling unit (~4–7 species) (Rutgers et al., 2009; Keith et al., 2012; Spurgeon et al., 2013). Based on their food sources, morphology and behaviour, earthworms are classified into three broad functional groups (Bouché, 1972), i.e. litter dwellers (epigeics), soil feeders (endogeics), and deep-burrowers (anecics), though many species can exhibit intermediate characteristics. Identifying particular functional groups that play key roles in soils under field conditions has been an important objective for several decades (Brussaard, 1997; Barrios, 2007).

Both *Lumbricus terrestris* and *Aporrectodea longa*, two anecic species, are widespread in agricultural ecosystems (Bouché, 1972). Anecic earthworms have been shown to affect soil ecosystem functioning by their deep-burrowing activity, thereby increasing permeability and improving soil structure, to influence biological and chemical properties in soil around burrows (Don et al., 2008; Stromberger et al., 2012; Andriuzzi et al., 2013, 2016a) and in soil more generally (Blouin et al., 2013; Fahey et al., 2013). For example, the active incorporation of dead plant material and organic residues from the surface into the soil matrix affects C and N cycling (Brown et al., 2000; Bohlen et al., 2004). The deep-burrowing anecic earthworms typically build semi-permanent, vertical burrows and collect surface litter residues and drag them into the mouths of their burrows, where decomposition is initiated by

microorganisms prior to ingestion by the earthworm (Curry and Schmidt 2007). Milcu et al., (2008) showed in the JENA grassland experiment that increased litter decomposition was related to greater anecic abundance (*L. terrestris*). The relationship between earthworms and primary producers is reciprocal, with benefits found for plant performance (reviewed in Blouin et al. 2013). However, we know less about the fate of nutrients mineralized in decomposition with and without anecic earthworm activity, for example in terms of crop uptake of N from decomposing residues, even though crop N supply is a crucial process in models of earthworm functions (Bohlen et al., 2004). It has been found in a greenhouse experiment that *L. terrestris* presence can increase the N content of grasses and legumes (Eisenhauer and Scheu 2008), but we do not know if studies on anecic effects on residue N fates conducted under laboratory conditions (see also Cortez et al., 1989; Andriuzzi et al., 2016b) can be translated to field conditions.

Given the large influence that anecic earthworms can have on the soil, disappearance of such earthworms may have cascading effects on soil ecosystem processes. Selected disappearance of certain earthworm species even from relatively 'undisturbed' grasslands is conceivable, for example caused by the exclusively earthworm-eating, invasive 'New Zealand flatworm' (*Arthurdendyus triangulatus*, Platyhelminthes: Geoplanidae), which is widespread in northern Britain and the island of Ireland (Boag and Yeates, 2001). The contention that anecic earthworms are vulnerable to predation by New Zealand flatworm was raised by Fraser and Boag (1998) and has recently been demonstrated in experimental field plots in Northern Ireland by Murchie and Gordon (2013), who found anecic earthworms to be most vulnerable to predation by *A. triangulatus*. More specifically, a negative relationship was found between densities of *A. triangulatus* and the anecics *L. terrestris* and *A. longa*, accompanied by an estimated reduction of 20% in total earthworm biomass (Murchie and Gordon, 2013). Testing the impacts of the disappearance of important components of the soil fauna, and the subsequent effects on feeding habits and interspecific interactions of the earthworm population, is an useful step to unravel links between functional biology and processes below-ground.

Demonstrating relationships between soil biodiversity and functioning in natural, intact communities is challenging (therefore it is rarely attempted), but it is important to be able to test hypotheses under realistic field conditions (Bardgett, 2005). A number of studies manipulated entire earthworm communities in the field using electroshocking in an effort to examine their importance for ecosystem processes with minimal physical disturbance (Bohlen et al. 1995; Liu & Zou, 2002; Milcu et al., 2008; Szlavecz et al., 2013). For example, Bohlen et al. (1995) pioneered the experimental reduction (using repeated electrical extraction) of entire earthworm assemblages from agronomic field plots to study their effects on soil processes. More recently, total earthworm abundance was also manipulated along a gradient of plant diversity in the JENA grassland experiment using a combination of electroshocking methods to reduce and manual additions to increase earthworm abundance (Milcu et al., 2008; Fisher et al., 2014). However, the selective manipulation of particular functional groups without widespread disturbance to the soil ecosystem remains difficult. For instance, Decaëns et al. (1999) excavated soil monoliths in pasture and wrapped them in mesh to selectively exclude the large anecic earthworm *Martiodrilus carimaguensis* (Glossoscolecidae), while the species resided deeper in the soil during the dry season. The present field experiment used an approach that minimises general disturbance to the soil by targeting anecic earthworm burrows to remove large individuals; this approach has been exploited successfully to study small-scale effects associated with individual anecic burrows and burrow walls (Stromberger et al., 2012; Andriuzzi et al., 2016a).

This study used a selective exclusion approach in non-enclosed plots to assess the role of anecic earthworms in the incorporation of litter and their effects on other epigeic and endogeic earthworm species in a grassland. We tested the hypotheses that i) the removal of adult anecic earthworms affects the abundance and trophic relations of other earthworm species, and ii) the removal of adult anecic earthworms affects the uptake of surface-litter derived ^{15}N isotope tracer by functional plant groups.

2. Materials and methods

2.1 Site description and experimental design

The experiment was conducted in an eight-year-old set-aside grassland known as the ‘Slang’ at UCD Lyons Research Farm, Lyons Estate, Co. Kildare, Ireland (WGS84: 53.318504, -6.529152). This field is known to harbour twelve earthworm species (O. Schmidt, unpubl. data). Soil in the Slang, derived from alluvium, is moderately to poorly drained, consists of a loam to loamy sand texture, and is classified as a humic gley or regosol (Lalor, 2004). The site was sown to perennial ryegrass (*Lolium perenne*) and taken out of production as a set-aside field in 2001. To conform to set-aside criteria, management was restricted to topping twice annually and fertiliser addition was ceased.

For the experiment, white mustard (*Sinapis alba* L. var. Rivona) plant biomass was used as litter because it is used as a cover crop, it is relatively easy to grow and to label effectively, and preference tests with *L. terrestris* have shown that this young, N-rich and structurally soft biomass becomes palatable quickly to earthworms (Valckx et al., 2011). Plots (1 m × 1 m) were allocated randomly to treatments; they were separated by 2 m wide strips and no barriers or enclosures of any kind were used (See Figure S1 for plot layout). The treatments consisted of:

- i) an isotopic natural abundance control with undisturbed earthworm populations to which no labelled litter was added at all [*NATURAL*],
- ii) a control with undisturbed earthworm populations to which labelled litter was added [*LITTER*], and
- iii) a treatment in which large anecic earthworms were removed and labelled litter was added [*LITTER & REMOVAL*].

2.2 Anecic earthworm removal

On 6 June 2007 the vegetation was cut to ground level and the ground forked to remove poached areas in all plots. There was no predisposed difference in earthworm abundance between plots allocated to treatments (see Supplementary methods). The two anecic species present at the study site (*L. terrestris* and *A. longa*) were removed from *LITTER* & *REMOVAL* plots over a period of 18 months, with the first anecic earthworms removed on 30 August 2007, and repeatedly thereafter (DD/MM/YY: 30/08/07; 26/11/07; 02/12/07; 08/02/08; 15/02/08; 15/10/08; 19/11/08; 27/02/09). Large-bodied anecic earthworms were ejected by injecting approximately 50 mL of a dilute mustard oil irritant (2 mL allyl isothiocyanate (Sigma) dispersed in 40 mL isopropanol [2-propanol] (Sigma), then added to 20 L water and mixed thoroughly) into individual burrows with a syringe. The volume of extractant added to each *LITTER* & *REMOVAL* plot is unlikely to have had a significant influence on soil moisture given precipitation during the experimental period (see Table S1). Ejected worms were identified, weighed and their burrow location recorded and mapped. Earthworm burrows were located again on each removal date and ejected anecic earthworms removed. Nomenclature follows Sims & Gerard (1999).

2.3 Labelling of plant material and field application

White mustard plants (*Sinapis alba* L. var. Rivona) were grown to produce dual-labelled (^{13}C and ^{15}N) litter as described in full in Stromberger et al. (2012), following Schmidt and Scrimgeour (2001). Briefly, compost-grown mustard plants were sprayed with a dual-labelled ^{13}C - ^{15}N urea solution (prepared by dissolving 5 g of 99 atom% ^{13}C urea and 100 mg 99 atom% ^{15}N urea in 2 L distilled water) with the addition of a wetting agent (Citowett, BASF, Ludwigshafen, Germany) using an ordinary trigger powered mister ('Spraymist', Hozelock Ltd, Aylesbury, UK). Seedlings were first sprayed 21 days after sowing when true leaves emerged and at a height of 100–120 mm, and repeated 8 times over 3 weeks (see Table S1 for details).

Labelled above-ground mustard biomass was harvested on 19 March 2009, mixed thoroughly and stored unaltered at 4°C overnight to be applied to experimental plots the following day. Five randomly selected individual mustard plants were oven-dried at 65°C for 24 h to determine average moisture content and isotopic enrichments after thorough mixing. The isotopic enrichment (mean \pm SD) in the labelled mustard litter was -8.5 ± 6.0 ‰ $\delta^{13}\text{C}$ (1.096 ± 0.007 atom% ^{13}C) and 163 ± 89 ‰ $\delta^{15}\text{N}$ (0.426 ± 0.032 atom% ^{15}N).

2.4 Sampling and isotope ratio analysis

On 20 March 2009, 2.1 kg fresh labelled mustard litter (equivalent to 200 g dry mass m^{-2}) were applied to the 90 cm \times 90 cm core area of each plot of *LITTER* and *LITTER & REMOVAL* treatments (see Figure S1), and covered by coarse, transparent plastic mesh (20 mm mesh size) to prevent litter loss by wind. Sampling took place on 28 and 29 April 2009 (38–39 days after application of mustard litter). Anecic earthworm burrows (located from burrow maps) and evidence of fresh burrows were marked with flagged stakes, for sampling of burrow wall material reported in a separate paper (Stromberger et al., 2012). Any mustard litter remaining on the surface was collected, bagged, oven-dried (80°C for 12 h) and recorded as dry mass. Living, above-ground plant biomass was harvested in the 50 cm \times 50 cm core area centred within each plot, manually sorted and separated into grasses, herbs and legumes, and weighed after oven-drying. A 50 cm \times 50 cm frame (10 cm height) was pressed lightly into the ground over this area and dilute mustard oil solution (prepared as above) was applied using a watering can within the frame. All earthworms expelled to the surface over a 20 minute period were collected using plastic forceps and rinsed in water. All extracted earthworms were transported back to the laboratory, where they were identified, counted and weighed the following day, thus allowing quantitative assessment of treatment effects on species populations and community composition. We acknowledge that Bouché's (1972) ecological group names are endpoints and that most species are found on a continuum between groups. However, to be able to analyse ecological group responses, we assigned species to discrete

groups. In particular, we assigned *L. festivus* (classified as intermediate between epigeic and anecic by Bouché) to epigeics for the purpose of group analysis because in our experience its population dynamic resembles that of typical epigeics much more than that of anecics (Schmidt et al., 2001).

To measure stable isotope composition, oven-dried (65°C for 24 h) vegetation samples (separately for grasses, herbs and legumes) were powdered using a steel ball mill (Retsch, Haan, Germany) and ~3 mg dry material were weighed into tin capsules (Elemental Microanalysis Ltd., Okehampton, UK). Individual earthworms were allowed to void their guts for 24 hours on moist tissue paper, rinsed and sedated by freezing while keeping them separated throughout processing. Tissue of 2–5 individuals per species per treatment was freeze-dried, powdered in a steel ball mill (as above), and ~0.5 mg dry weight transferred into tin capsules. Stable isotope ratios ($^{13}\text{C}/^{12}\text{C}$ and $^{15}\text{N}/^{14}\text{N}$) were measured by Elemental Analysis-Isotope Ratio Mass Spectrometry (EA-IRMS) at Iso-Analytical Ltd. (Cheshire, UK), see Schmidt and Scrimgeour (2001) and Stromberger et al. (2012) for details.

2.5 Statistical analysis

Statistical data analyses were conducted using R v3.2.3 (R Development Core Team, 2015). Normality and homogeneity of variances were calculated for all univariate earthworm and plant response variables by assessing normality and residuals plots, and using Shapiro-Wilk and Levene's tests. Most data did not conform to assumptions for parametric ANOVA, therefore differences between all treatments were tested by non-parametric one-way ANOVA (Kruskal-Wallis rank sum tests). Differences in means were explored *post-hoc* with Tukey contrasts using *nparcomp* v2.6 package (Konietschke et al., 2015). Differences between labelled treatments in the composition of ^{13}C and ^{15}N in earthworm species were tested by permutational multivariate analyses of variance (PerMANOVA) using the *adonis* function in the *vegan* package v2.3-3 (Oksanen et al., 2016).

Treatment effects on count data of separate earthworm species were assessed using a generalised linear model (*glm*) with the count data assumed to conform to a *quasi*-Poisson

distribution with a log-link function. Associated P-values are based on χ^2 test from analyses of deviance (ANODEV) and significance was assumed to be at $P < 0.05$. Multivariate responses of earthworm assemblages were also tested by PerMANOVA using the *adonis* function (Oksanen et al., 2016). For this, Bray-Curtis dissimilarity matrices were computed between samples using raw earthworm abundance data, both including and excluding anecic species (*A. longa* and *L. terrestris*). Homogeneity of multivariate dispersion (using *betadist* in *vegan*) with deviation from centroids was calculated in conjunction with each PerMANOVA as an indication of beta-diversity. All multivariate P-values are computed based on 9999 permutations. Earthworm assemblage dissimilarities were visualised using 2D non-metric Multidimensional scaling (monoMDS engine through *vegan*) on raw abundance data. Each nMDS was calculated using a maximum of 250 trials, until the best fit was reached (least 2D stress).

3. Results

3.1 Anecic earthworm removal

Earthworm removal from *LITTER & REMOVAL* plots resulted in a total of 24 ejected individuals (including adults, sub-adults and large juveniles), with *L. terrestris* being the most abundant (Supplementary Table S3). Between 3 and 7 individuals (median = 4) were removed per plot and the biomass (live-weight) sum of these ranged between 6.1–19.1 g (median = 11.4 g) per plot (1 m × 1 m). Several burrows were re-occupied by an earthworm during the 18-months removal period, with *L. terrestris* found reoccupying two previously evacuated burrows.

3.2 Effects of anecic removal on earthworm assemblages

The abundance of earthworms was significantly affected by the treatments (Table 1), with more earthworms recovered from the *LITTER* and *LITTER & REMOVAL* plots compared to the untreated *NATURAL* plots. Mirroring this, fresh-weight biomass in the *NATURAL* plots was significantly less than in treatments with litter added regardless of the removal of anecics

(Table 1). There were significantly more anecic earthworms in the treated plots compared to the untreated ones, but the removal of anecics did not have a significant effect on the final abundance of anecics, nor total abundance (Table 1). There was, however, a greater abundance of epigeic earthworms in *LITTER & REMOVAL* compared to the *LITTER* plots (Table 1). Species richness (SR) and the Shannon-Wiener index (H') were not significantly affected by any of the treatments (Table 1).

Ten different earthworm species (including “morphospecies”) were identified from all plots: *Allolobophora chlorotica* green morph, *A. chlorotica* pink morph, *Aporrectodea caliginosa*, *A. rosea*, *A. longa*, *Lumbricus castaneus*, *L. festivus*, *L. terrestris*, *L. rubellus*, and *Satchellius mammalis* (see Figure 1 for assigned ecological groups). The most abundant species collected from the experimental field was *S. mammalis* (Figure 1a). The abundance and biomass of the *L. festivus* and *S. mammalis* were both significantly greater in the *LITTER & REMOVAL* plots compared to the *NATURAL* and *LITTER* plots (Figure 1a and b). The most abundant endogeic earthworm species was the green morph of *A. chlorotica*, but they were not significantly affected by the removal of the anecics. Two anecics, *L. terrestris* and *A. longa*, were recovered from removal (*LITTER & REMOVAL*) plots, with significantly more individuals of *A. longa* in the plots that had received litter. There was, however, no significant effect of the removal on the abundance of *A. longa* or *L. terrestris*.

Given that earthworm community structure was purposely manipulated in one treatment by removing anecics, separate analyses of earthworm community structure were undertaken for (i) all species recovered (Figure 2a) and (ii) endogeic and epigeic species only (Figure 2b). Beta-diversity of the whole community was significantly different between the treatments, with assemblages in the *LITTER & REMOVAL* treatment significantly more variable than either the *NATURAL* and *LITTER* treatments. This was also the case for assemblages analysed with the anecic earthworms excluded (Figure 2b). Earthworm assemblage structure was significantly different between treatments (Figure 2a), with those in *NATURAL* being significantly different from the *LITTER* and *LITTER & REMOVAL* treatments (multivariate pairwise tests: $F_{1,8} = 2.72$, $P = 0.041$ and $F_{1,8} = 2.91$, $P = 0.024$, respectively). When excluding

the anecics from the analysis, however, earthworm assemblages were not significantly different between treatments (Figure 2b).

3.3 Effects of anecic removal on isotopic compositions of different earthworm species

Earthworms extracted from the *NATURAL* plots (except *L. castaneus* whose specimens died during the gut voiding process and were lost) had natural abundance stable isotope values ranging from -27.83 to -26.89‰ for $\delta^{13}\text{C}$ and 2.60 to 6.27‰ for $\delta^{15}\text{N}$, with no significant difference in ^{13}C between species ($\chi^2 = 9.44$, $df = 7$, $P = 0.221$). The natural abundance values of ^{15}N were significantly different between species ($\chi^2 = 21.3$, $df = 7$, $P = 0.003$), with endogeic species having larger ^{15}N values than the epigeic and anecic species.

The addition of labelled litter had a significant effect on the stable isotope values of earthworm tissue, with significantly enriched ^{13}C and ^{15}N values of individuals of all earthworm species (Figure 3 and 4). There was a positive relation between assimilated ^{15}N and ^{13}C , with the incorporation of the isotope tracers being least in soil-feeding species (Figure 3), and most in litter-feeding species (Figure 4). This was especially the case for the epi-anecic *L. festivus* (Figure 4b) and epigeic *L. rubellus* (Figure 4c). In the *NATURAL* and *LITTER & REMOVAL* plots, tissues of the endogeic earthworm species (*A. caliginosa*, *A. rosea* and *A. chlorotica*) had slightly elevated ^{13}C and ^{15}N values, but without a consistent effect of anecic earthworm removal (Figure 3). *A. caliginosa* individuals in *LITTER* plots, however, appeared to be more generally enriched than those in *LITTER & REMOVAL* plots (Figure 3a); this was statistically significant when one unlabelled outlier was removed (Table 2).

By contrast to the endogeics, the tissues of both the epigeic and anecic litter-feeding species (*S. mammalis*, *L. rubellus*, *L. festivus*, *A. longa* and *L. terrestris*) included a large proportion of specimens with highly enriched ^{15}N values in the litter treatments (Figure 4), reflecting substantial assimilation of ^{15}N from the surface residues. Nevertheless, there were no consistent effect of anecic removal on the ^{15}N enrichment of other anecics and endogeics (Table 2), and recovered individuals of *L. terrestris* were less enriched overall compared to other litter feeders in the *LITTER & REMOVAL* and had variable enrichments in the two litter

treatments (Figure 4d). There was a tendency for *L. festivus* to have assimilated less ^{13}C and ^{15}N when anecics had been removed compared to the *NATURAL* treatment where *L. terrestris* was not manipulated (Figure 4b); there was an opposite tendency for *L. rubellus* (Figure 4c).

3.4 Anecic removal, plant biomass and nutrient uptake

The applied litter largely disappeared during the experimental period (~9–14% mass remained after 39 days), but there was no significant effect of the removal of anecic earthworms on remaining mustard litter (Table 3). Legumes and herbs accounted for a small proportion only (~5%) of the harvested above-ground plant biomass and their mean dry weight yield was not significantly different between treatments (Table 3). By contrast, grasses accounted for 95% of the harvested total above-ground plant biomass and there was more grass in the plots with litter applied compared to the untreated *NATURAL* plots, regardless of the removal of anecics. Total harvested above-ground plant biomass from treatments with mustard residue (median = 144.0 g m⁻² in *LITTER*, median = 134.4 g m⁻² in *LITTER & REMOVAL*) was significantly greater than that harvested from the non-mustard control (*NATURAL*: median = 89.2 g m⁻²; $\chi^2 = 8.07$, df = 2, P = 0.018).

Of the different plant functional groups, legumes acquired the least ^{15}N tracer from the applied mustard litter, while herbs and grasses took up substantial amounts (Table 3). The isotopic composition of above-ground grass, herb and legume biomass was significantly affected by the litter treatment, but the removal of anecics did not measurably affect the isotopic composition of the above-ground vegetation (Table 3).

4. Discussion

4.1 Litter addition attracted earthworms

The addition of mustard litter had a significant, positive impact on the number of earthworms recorded in unenclosed plots, with greater abundance and biomass of total and anecic earthworms. It is likely that the mustard litter, as an N-rich, easily decomposable food source,

acted as a strong attractant in this unfertilised, nutrient limited system (Curry and Schmidt, 2007) and that many earthworms moved into the litter addition plots during the 39 days following litter application, including large-bodied species, as reflected by a significantly higher earthworm biomass. We accept the likelihood that such effects of litter addition to unenclosed plots may have altered, tempered or masked effects of anecic removal. Nevertheless, a number of findings following targeted anecic removal suggests that reductions in their populations can affect other earthworm species in terms of abundance and trophic activity.

4.2 Anecic removal affected other earthworm functional groups

Anecic earthworm species have the potential to influence the activity and trophic behaviour of other earthworm populations (Uvarov, 2009), but very few studies were set in realistic field conditions (e.g. Decaëns et al., 1999). The current study differs from other removal studies that extracted the entire earthworm community first and then reintroduced ambient or elevated communities (for example Bohlen et al., 1995; reviewed by Brown and Doube, 2004). Using targeted extraction, we showed that removal of anecic earthworms in a field setting affected populations of other earthworm species and the transfer of C and N from labelled surface litter to other earthworms.

Interspecific competition may be expected between anecic and epigeic earthworms since they both use litter as a resource. For example, in a meta-study of mostly laboratory-derived data, Uvarov (2009) found that interactions between *L. terrestris* (anecic) and *L. rubellus* (epigeic) were predominantly negative. Lowe and Butt (1999) showed that such interspecific interactions between earthworm species can have longer term impacts on growth and reproductive output. Our findings provide novel field-based support for the interactive portraits between functional groups generated by Uvarov (2009), with the abundance and biomass of the litter-feeding *L. festivus* (epi-anecic) and *S. mammalis* (epigeic) being greater in the treatment receiving litter where anecics had been removed. Though not significantly so, *L. rubellus* abundance tended to be greater where anecics had been removed, but there may

also have been inter-specific interactions between epigeic species which complicate the observed outcome.

The removal of anecic earthworms, organisms considered as ecosystem engineers, may leave an ecological vacuum, filled by a subsequent increase in the epigeic population. Such effects of anecic removal on the abundance of these epigeic taxa are largely responsible for the finding in this study of earthworm composition being different and more variable where anecics had been removed compared to the other treatments. Such potential impacts on earthworm assemblages are representative of the current problem of predation by the invasive New Zealand flatworm; in particular, it has been demonstrated in a Northern Ireland experiment that *L. terrestris* was reduced to the greatest extent in existing grassland populations (Murchie and Gordon, 2013). These changes in the functional composition of earthworms may have, as yet unknown, consequences for soil processes.

Natural abundance isotope measurements of the earthworm taxa studied here clearly reflected typical endogeic (soil feeding) and anecic/epigeic (litter feeding) feeding behaviours (Hendriksen, 1990; Schmidt et al., 2004). The addition of labelled litter enriched individuals from all species in both ^{13}C and ^{15}N , but it was not consistently different between treatments within species. Though there were few significant impacts of anecic removal on isotopic values of epigeic taxa, biplots of $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ suggested that taxa may respond differently; for instance, *L. rubellus* tended to be much more enriched in removal plots, whereas *L. festivus* was less enriched. The combination of population and isotopic responses provide opportunity to speculate on mechanisms of inter-specific interactions; the tendency for greater enrichment and increased abundance in *L. rubellus*, with removal of *L. terrestris*, supports competition for litter resources between these species.

The finding that *L. terrestris* extracted from the anecic removal plots had less isotopic enrichment compared to other litter feeders suggests that these *L. terrestris* individuals had colonised the plots only a short time before sampling. It is likely that vacant burrows are exploited by wandering conspecifics, because burrows represent a valuable asset for this species' niche (Odling-Smee et al., 1996). The semi-permanent burrows of anecic earthworms

are known to be re-used once vacated (Nuutinen, 2011; Grigoropoulou and Butt, 2015).

While the plots in this study were non-enclosed to allow natural dynamics, new field studies should permanently exclude this functional group in enclosed plots in order to clarify the impacts of a complete loss of anecic earthworms in grassland ecosystems.

The abundance and biomass of individual soil-feeding species were unaffected by treatment. However, similar abundances but altered isotopic composition suggest that the removal of anecics (primarily *L. terrestris*) influenced the feeding activities of other endogeic earthworm species. Specifically, the soil feeder *A. caliginosa* was less enriched with ^{13}C and ^{15}N where anecics had been removed, suggesting strongly that *A. caliginosa* benefits from the activity of anecics, albeit it remains unclear exactly how. The meta-study by Uvarov (2009) found both positive and negative effects of *L. terrestris* on *A. caliginosa* but overall the presence of anecic taxa resulted in a greater proportion of positive effects on other species. Similarly, the abundance of endogeic Ocnerodrilid earthworms was reduced following exclusion of the anecic *M. carimaguensis* from pastures in Colombia (Decaens et al., 1999).

The anecic *L. terrestris* (but also the epigeic *L. rubellus*) were shown to promote the mineralisation of crop residues applied in a mesocosm study (Postma-Blaauw et al., 2006). Anecic earthworms may therefore be seen to provide higher quality resource for soil-feeding endogeic taxa. It was suggested that *A. caliginosa* benefits through better access to mineralised nutrients (Postma-Blaauw et al., 2006) and by preferential use of anecic burrows and feeding on burrow linings (Jégou et al., 2001). It has been shown recently that the drilosphere (i.e. the zone of influence surrounding the burrows) of anecic earthworms extends further than previously expected (Andriuzzi et al., 2013). Their influence on microbial communities and soil properties also appears to differ compared to other earthworm functional groups (Dempsey et al., 2013; Fahey et al., 2013; Andriuzzi et al., 2016a). Related research at the level of individual *L. terrestris* burrows suggests that residue-derived C is incorporated rapidly into the drilosphere and that microbial communities of the drilosphere are different from that in bulk soil (Stromberger et al., 2012). The effects of anecic removal

on the isotopic composition of the endogeic *A. caliginosa* in this study therefore corroborate these earlier findings.

4.3 Plant uptake of nutrients unaffected by anecic removal

Our second hypothesis was that the removal of large anecic earthworms would reduce the uptake of surface-litter derived ^{15}N by functional plant groups, since these earthworms are known to incorporate litter and promote its decomposition. Earlier studies showed that greater anecic abundance (*L. terrestris*) is related to increased litter decomposition, particularly for legume litter (Milcu et al., 2008). In the present study, while there was clear uptake of litter-derived N, there was no significant effect of anecic removal on ^{15}N uptake into the above-ground biomass of any plant functional group, although there was a trend of greater enrichment in removal plots.

The anecic removal treatment did not reduce the disappearance of surface-applied mustard residue, probably because the young mustard material was easily decomposable and accessible to other earthworms. This was reflected in the significant increase in grass biomass in mustard addition plots and the uptake of substantial amounts of mustard-derived N by plants, especially herbs and grasses. N uptake by legumes was lower because they also use fixed atmospheric N_2 , which dilutes soil or litter derived N. The low natural abundance $\delta^{15}\text{N}$ levels in legumes suggest that N_2 fixation was active and important in this set-aside system (Handley and Scrimgeour, 1997). Indirect earthworm effects on plants, such as via soil structural functions (Scullion et al., 2007), are only measurable over longer time periods.

5. Conclusions

The impacts measured in this field experiment of anecic removal on the abundance and trophic ecology of other earthworm species are especially remarkable because so few anecic earthworms were removed experimentally, namely 4 (median) large individuals per 1 m^2 plot over 18 months. These findings underline just how functionally active and

ecologically important this functional group of earthworms is. They also suggest that even a partial removal of these species – for example through selective predation by alien flatworms – is likely to have ecological consequences for soil communities and soil functions.

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Table 1. Treatment effects on earthworm community measures, including abundance and biomass (m^{-2}) for the different functional groups (as in Figure 1), and diversity indices with species richness (SR) and Shannon-Wiener index (H'). Data are median values of $n = 5$. P-values of the Kruskal-Wallis rank sum tests (KW-test) are on 2 df, superscript letters indicate significant differences between treatments based on non-parametric multiple comparisons at $P < 0.05$.

| Community measure | Treatment | | | KW-test | |
|---|-------------------|-------------------|-----------------------------|----------|--------------|
| | <i>NATURAL</i> | <i>LITTER</i> | <i>LITTER & REMOVAL</i> | χ^2 | P-value |
| ABUNDANCE (m^{-2}) | | | | | |
| Anecic | 12 ^a | 20 ^b | 24 ^b | 6.51 | 0.038 |
| Endogeic | 32 | 40 | 32 | 0.46 | 0.794 |
| Epigeic | 24 ^a | 48 ^b | 80 ^c | 7.49 | 0.024 |
| Juveniles | 40 | 72 | 64 | 2.95 | 0.229 |
| Total | 100 ^a | 176 ^b | 192 ^b | 6.42 | 0.040 |
| BIOMASS (g m^{-2}) | | | | | |
| Anecic | 24.5 ^a | 50.2 ^b | 50.1 ^b | 7.94 | 0.019 |
| Endogeic | 7.3 | 8.8 | 6.7 | 0.96 | 0.619 |
| Epigeic | 6.4 ^a | 5.3 ^a | 24.2 ^b | 8.18 | 0.017 |
| Juveniles | 10.4 | 23.8 | 18.7 | 3.26 | 0.196 |
| Total | 45.1 ^a | 86.3 ^b | 106.6 ^b | 11.2 | 0.003 |
| DIVERSITY | | | | | |
| SR all spp. | 7 | 8 | 7 | 0.52 | 0.771 |
| SR epi. & endo* | 5 | 6 | 5 | 0.30 | 0.863 |
| H' all spp. | 1.71 | 1.83 | 1.40 | 0.78 | 0.677 |
| H' epi. & endo* | 1.32 | 1.54 | 1.03 | 1.86 | 0.395 |

* For comparison of non-manipulated species, epigeics and endogeics were also analysed separately.

Table 2. Multivariate tests of effect of anecic removal (*LITTER* vs *LITTER & REMOVAL* treatments) on $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ for each earthworm species. DF = Degrees of freedom; values in parentheses represent the test following removal of one outlier for *A. caliginosa*.

| Species | PERMANOVA | | | |
|---------------------------------|----------------|----------------|--------------|------------------|
| | F | R-squared | DF | P-value |
| <i>Aporrectodea caliginosa</i> | 1.87 (4.46) | 0.19 (0.39) | 1,9 (1,8) | 0.218 (0.028) |
| <i>Aporrectodea rosea</i> | 2.46 | 0.29 | 1,7 | 0.160 |
| <i>Allolobophora chlorotica</i> | 0.87 | 0.10 | 1,9 | 0.373 |
| <i>Satchellius mammalis</i> | 0.28 | 0.03 | 1,9 | 0.641 |
| <i>Lumbricus festivus</i> | 1.35 | 0.16 | 1,8 | 0.283 |
| <i>Lumbricus rubellus</i> | 1.24 | 0.20 | 1,6 | 0.293 |
| <i>Aporrectodea longa</i> | 0.23 | 0.03 | 1,9 | 0.681 |
| <i>Lumbricus terrestris</i> | 1.31 | 0.16 | 1,8 | 0.355 |

Table 3. Treatment effects on mustard litter remaining (g dry mass m⁻²), above-ground plant biomass (g dry mass m⁻²) and isotopic composition ($\delta^{13}\text{C}$ and $\delta^{15}\text{N}$, ‰) of plant functional groups (Grass, Herb and Legumes), at time of earthworm sampling. Values represent median values of n = 5. P-values of the Kruskal-Wallis rank sum tests (KW-test) are on 2 df, superscript letters indicate significant differences between treatment based on non-parametric multiple comparisons at P < 0.05.

| | Treatment | | | KW-test | |
|-----------------------|---------------------|---------------------|-----------------------------|----------|-------|
| | <i>NATURAL</i> | <i>LITTER</i> | <i>LITTER & REMOVAL</i> | χ^2 | P |
| MUSTARD | | | | | |
| Biomass | Not added | 13.6 ^a | 21.2 ^a | 0.88 | 0.347 |
| GRASS | | | | | |
| Biomass | 80.0 ^a | 134.8 ^b | 130.0 ^b | 7.98 | 0.019 |
| $\delta^{13}\text{C}$ | -30.33 ^a | -30.78 ^b | -30.77 ^b | 9.38 | 0.009 |
| $\delta^{15}\text{N}$ | 1.85 ^a | 29.25 ^b | 30.91 ^b | 9.62 | 0.008 |
| HERB | | | | | |
| Biomass | 1.2 | 2.8 | 3.2 | 0.98 | 0.613 |
| $\delta^{13}\text{C}$ | -29.14 | -29.32 | -28.69 | 3.38 | 0.185 |
| $\delta^{15}\text{N}$ | 3.02 ^a | 18.86 ^b | 26.25 ^b | 9.98 | 0.007 |
| LEGUME | | | | | |
| Biomass | 1.2 | 1.2 | 1.6 | 1.50 | 0.471 |
| $\delta^{13}\text{C}$ | -29.07 ^a | -30.18 ^b | -29.88 ^b | 9.62 | 0.008 |
| $\delta^{15}\text{N}$ | -1.28 ^a | 7.48 ^b | 8.76 ^b | 10.22 | 0.006 |

Figure captions

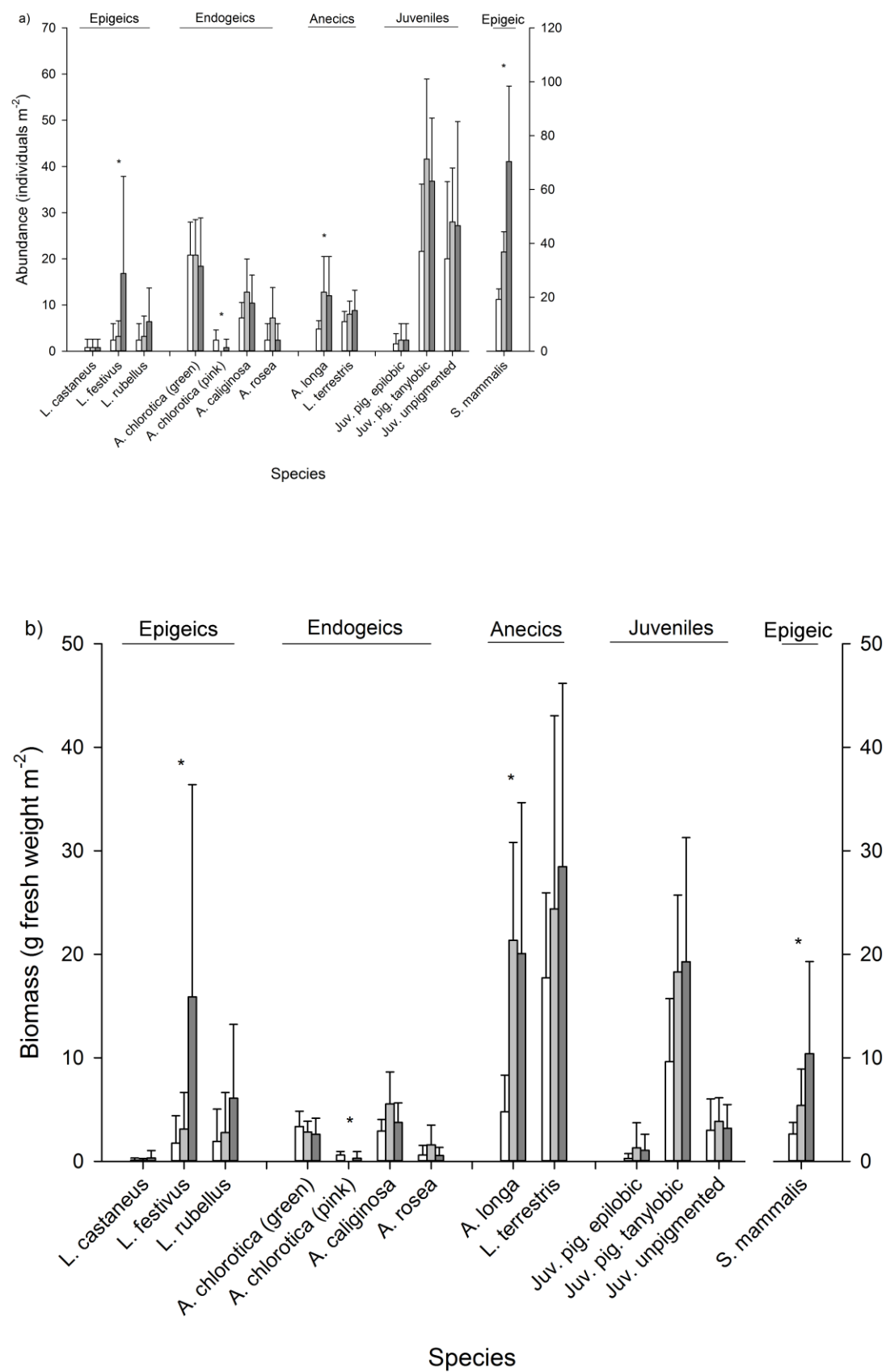
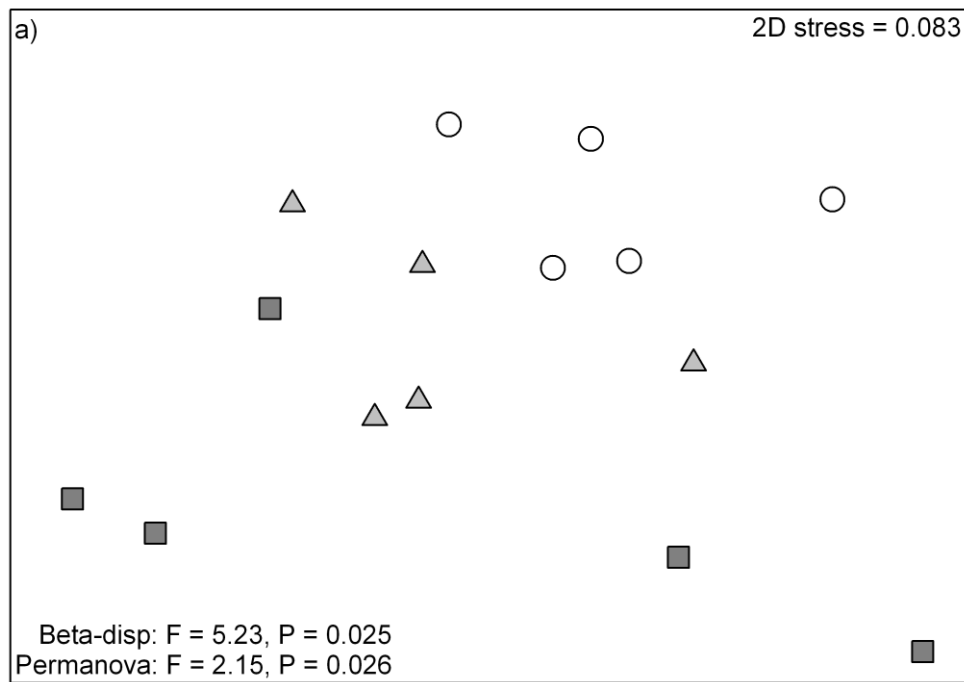


Figure 1. (a) Abundance and **(b)** live biomass of identified earthworm species and unidentified juvenile groups in *NATURAL* (open), *LITTER* (light grey), and *LITTER & REMOVAL* (dark grey) treatments. Values represent means \pm 1 SD. Asterisks indicate a significant difference between treatments at $P = 0.05$ assuming a quasi-Poisson distribution. Juv. = juvenile, pig. = pigmented. Please note different axis scale for *Satchellius mammalis* density on the right.



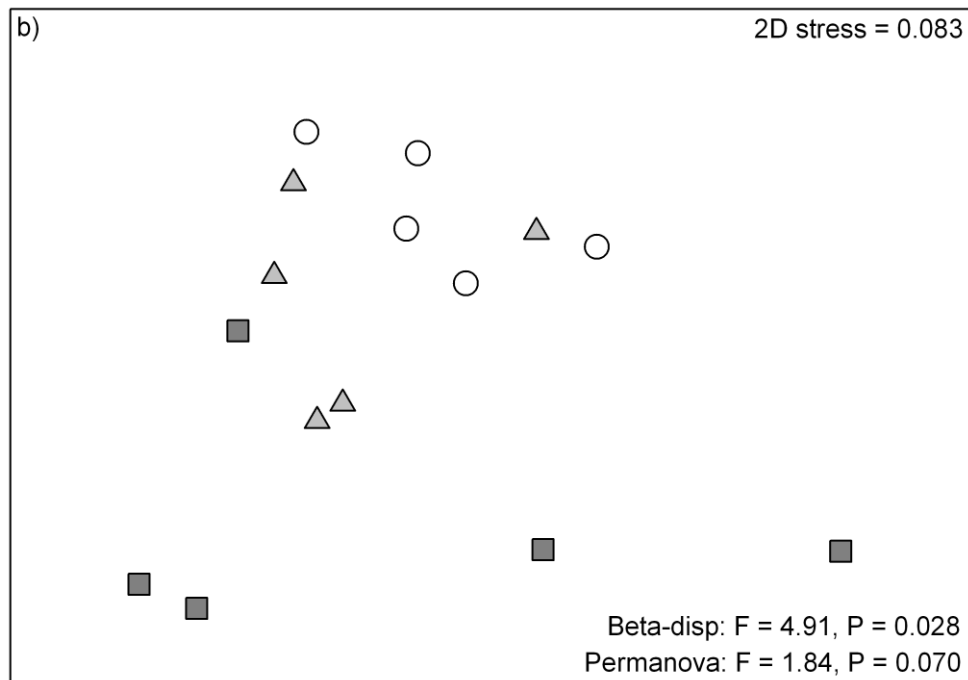


Figure 2. Non-metric multidimensional scaling plots of earthworm assemblages with (a) all earthworms present and (b) assemblages without anecics (*Aporrectodea longa* and *Lumbricus terrestris*), in NATURAL (open circles), LITTER (light grey triangles), and LITTER & REMOVAL (dark grey squares) treatments. Included are 2D stress values as a “goodness of fit”, beta-dispersion analyses among centroids and permanova with F statistics on $df_1 = 2$, $df_2 = 12$ and P values based on 9999 permutations.

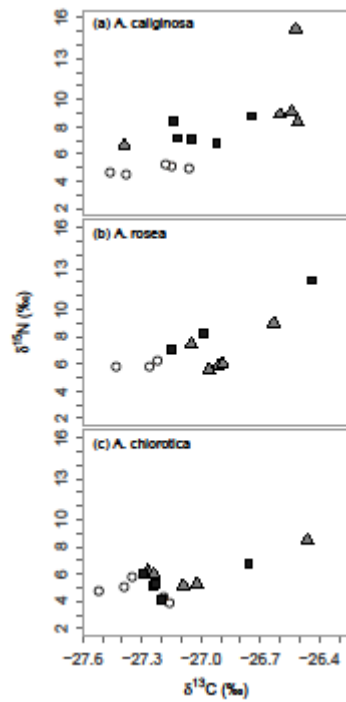


Figure 3. Isotope composition (biplots of $\delta^{13}\text{C}$ ‰ and $\delta^{15}\text{N}$ ‰) of earthworm tissue from the soil feeding species **(a)** *Aporrectodea caliginosa*, **(b)** *Aporrectodea rosea*, and **(c)** *Allolobophora chlorotica* in NATURAL (open circles), LITTER (light grey triangles), and LITTER & REMOVAL (dark grey squares) treatments.

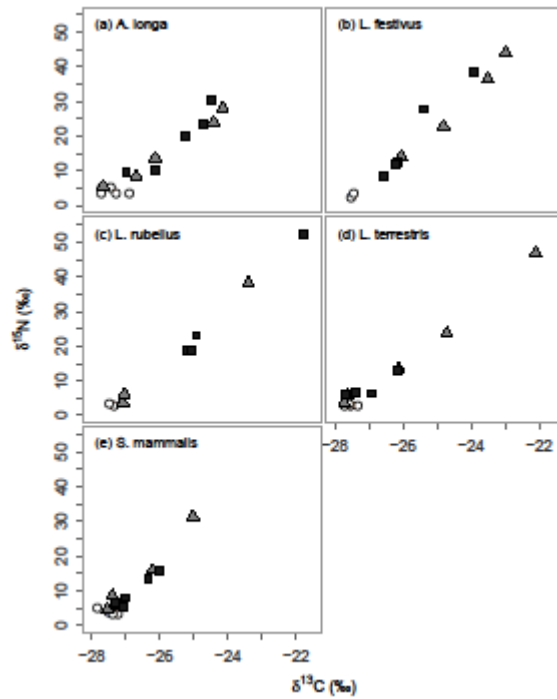


Figure 4. Isotope composition (biplots of $\delta^{13}\text{C}$ ‰ and $\delta^{15}\text{N}$ ‰) of earthworm tissue from the litter feeding species (a) *Aporrectodea longa*, (b) *Lumbricus festivus*, (c) *L. rubellus*, (d) *L. terrestris*, and (e) *Satchellius mammalis* in NATURAL (open circles), LITTER (light grey triangles), and LITTER & REMOVAL (dark grey squares).