Earthworm-induced shifts in microbial diversity in soils with rare versus established invasive earthworm populations

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Abstract:

European earthworms have colonised many parts of Australia, although their impact on soil microbial communities remains largely uncharacterised. An experiment was conducted to contrast the responses to *Aporrectodea trapezoides* introduction between soils from sites with established (Talmo, 64 *A. trapezoides* m^{-2}) and rare (Glenrock, 0.6 *A. trapezoides* m^{-2}) *A. trapezoides* populations. Our hypothesis was that earthworm introduction would lead to similar changes in bacterial communities in both soils. The effects of earthworm introduction (earthworm activity and cadaver decomposition) did not lead to a convergence of bacterial community composition between the two soils. However, in both soils the Firmicutes decreased in abundance and a common set of bacteria responded positively to earthworms. The increase in the abundance of *Flavobacterium,* Chitinophagaceae, Rhodocyclaceae and Sphingobacteriales were consistent with previous studies. Evidence for possible soil resistance to earthworms was observed, with lower earthworm survival in Glenrock microcosms coinciding with *A. trapezoides* rarity in this site, lower soil organic matter and clay content, and differences in the diversity and abundance of potential earthworm mutualist bacteria. These results suggest that while the impacts of earthworms vary between different soils, the consistent response of some bacteria may aid in predicting the impacts of earthworms on soil ecosystems.

1. Introduction:

Earthworms are ecosystem engineers, driving soil structure and nutrient dynamics (Jones *et al.*, 1994, Lavelle *et al.*, 1997) and their importance in soil ecosystems has long been recognised. By feeding on litter and soil, burrowing and releasing casts, earthworms change soil porosity, bulk density, water infiltration, nutrient mineralisation, gas emissions, organic carbon stabilisation and plant productivity (Blouin *et al.*, 2013). However, the specific consequences of earthworm activity for soil processes can vary substantially depending on earthworm species, soil type, rainfall and plant cover (Blouin *et al.*, 2013).

Earthworms can be divided into three broad functional groups: epigeic earthworms live and feed in the surface litter layer; anecic earthworms live in permanent vertical burrows, feeding at the soil surface on litter and other organic materials and depositing their casts at the burrow entrance; endogeic earthworms feed on mineral soil and partially decomposed material as they burrow horizontally through soil (Bouché, 1977). The ecological group to which an earthworm species belongs can have a substantial effect on the way its activity affects soil ecosystems (Thakuria *et al.*, 2010). For example, Greiner *et al*. (2012) observed that two different earthworm species, the epi-endogeic *Amynthas hilgendorf* and the epigeic *Lumbricus rubellus*, both of which are invasive in North America, had different impacts on litter decomposition, nutrient mineralization and soil aggregate size.

The earthworm gut and its associated microbial community produce a variety of digestive enzymes such as polysacharidases, glycosidases and peroxidases, and earthworm activity is therefore important in mediating organic matter decomposition in terrestrial habitats (Hartenstein, 1982, Zhang *et al.*, 1993, Hong *et al.*, 2011, Shan *et al.*, 2013). Earthworm activity has been shown to increase mineralisation of bacterial and fungal cells and their constitutive parts such as peptidoglycan, protein and chitin, whilst organic C in earthworm casts may be protected from further degradation by its encapsulation within micro-aggregates and complexation with soil minerals (Shan *et al.*, 2013). Furthermore, *Lumbricus rubellus* and the anecic *Lumbricus terrestris* feeding on detritus were associated with increased cellobiohydrolasae activity in organic and surface mineral soil layers, which

was attributed to their effect on separating lignin from cellulose in plant litter (Dempsey *et al.*, 2013). Whilst earthworms consume microbial biomass present in soil and decomposing plant litter, they also select and promote the growth of other bacterial groups that aid in the decomposition of organic matter and influence nutrient cycling in soil (Aira *et al.*, 2006, Hong *et al.*, 2011). For example, the reduced oxygen levels and rich microbial population makes the earthworm gut a favourable environment for denitrification (Drake & Horn, 2007). Earthworms are therefore usually implicated in increasing emissions of nitrous oxide (N_2O), an important greenhouse gas, from soil (Costello & Lamberti, 2009). However, Nebert *et al*. (2011) showed that whereas *Lumbricus rubellus* increased N_2O emissions and the abundance of the denitrifier gene *nosZ* upon litter amendment, the endogeic *Aporrectodea caliginosa* caused only a transient increase in N_2O emissions and no effect on denitrification genes. Similarly, Bradley *et al.* (2012) showed that interactions between soil land use history and the epigeic *Eisenia Andrei* can lead to opposing effects on the gross rate of methane production.

The existing studies detailing the effects of earthworms on soil microbial community composition using culture-independent methods are often not directly comparable owing to the differences in experimental design, earthworm functional type, and treatments applied (Bernard *et al.*, 2012, Koubova *et al.*, 2012, Dempsey *et al.*, 2013, Frisli *et al.*, 2013, Koubova *et al.*, 2015, Braga *et al.*, 2016, Delgado-Balbuena *et al.*, 2016). The available information suggests that earthworms boost the growth of fast growing bacteria owing to the production of labile carbon substrates (Braga *et al.*, 2016). In accordance to the variability of their functional effects, the consequences of earthworm activity on microbial community composition has been shown to vary depending on soil conditions. For example, Koubova *et al*. (2015) observed that the effect of earthworm on soil microbial community was greater on less nutrient rich soils, while Koubova *et al*. (2012) demonstrated that soil history led to contrasting responses of methanogens to the epigeic *Eisenia andrei*. As earthworms can have diverse effects on soil properties and microbial community diversity, the spread of invasive earthworms into new environments can influence soil ecosystem function in whole landscapes, with potentially important consequences for soil biodiversity and ecological services (Greiner *et al.*, 2012).

111 European earthworms are now widespread throughout southern Australia, impacting
112 terrestrial ecosystems particularly in soils used for cultivation and grazing. While the extent of
113 colonisation of invasive earthworms in native Australian ecosystems appears to be limited and poorly
114 characterised (Hendrix *et al.*, 2006), their spread in agricultural land has been associated with benefits
115 to plant yield and quality, increased nutrient availability, soil structure (Curry & Baker, 1998) among
116 other benefits. However, invasive earthworm colonisation in Australia is patchy, and the
117 environmental variables that limit or promote their spread are poorly understood (Baker *et al.*, 2006).
118 Here we examined whether one of the most common invasive earthworm species in Australia,
119 *Aporrectodea trapezoides* (Duges) (Lumbricidae) (Baker *et al.*, 2006) can cause consistent ecological
120 changes in soils representing a single ecosystem type: sheep-grazed pasture in south eastern Australia.
121 More specifically, we compared two fertilized pasture soils in close proximity (approximately 15 km
122 apart), which, although under similar climate and management practices, were particularly
123 distinguished by the presence (Talmo) or absence (Glenrock) of established populations of invasive
124 European earthworms, especially *A. trapezoides*. We used microcosms with soil from both sites which
125 were amended with *A. trapezoides*, while plant litter was added as a food source and to determine the
126 impact of the earthworms on the diversity of putative bacterial saprotrophic groups. We measured soil
127 nitrogen pools (NH_4^+-N, NO_3^--N, free amino acid N [FAA-N], dissolved organic nitrogen [DON] and
128 microbial biomass nitrogen [MBN]) and determined bacterial community diversity by high-
129 throughput sequencing of 16S rRNA gene amplicons. Our objective was to determine whether
130 inoculation of pasture soil with *A. trapezoides* would lead to consistent changes in soil nitrogen pools
131 and microbial community structure in soils with and without previous populations of this earthworm
132 species. We hypothesized that 1) earthworm utilization of added plant litter would change available
133 carbon sources for the prevailing microbial community and consequently change the bacterial
134 decomposer community; 2) earthworm activity would lead to a convergence of Glenrock and Talmo
135 soil microbial community composition, and 3) *A. trapezoides* status as an established population in
136 Talmo and their rarity in Glenrock is due to their dispersal patterns, site history and management, and
137 both soils would be equally suitable for these earthworms. Our findings improve understanding of the

impacts invasive earthworms in Australian agricultural soils and offer clues of the factors that can limit their spread into new territories.

2. Methods

2.1. Earthworm collection

Earthworms (*A. trapezoides*) were extracted manually from Talmo pasture (sampling depth was 5-15 cm, in October 2013), and incubated in Talmo soil at 15°C in the dark. The earthworms were all kept in Talmo soil within a single container for approximately one month prior to microcosm set up. *A. trapezoides* was identified using keys in Sims & Gerard (1985) and Baker & Barrett (1994). Recently, evidence has been obtained for the presence of cryptic *A. trapezoides* diversity in Australia (Martinsson *et al.*, 2015), and it is possible that the individual earthworms used in this study represented different cryptic species. While possible, it is unlikely that different cryptic variants of *A. trapezoides* were introduced non-randomly amongst the treatments used in this experiment, avoiding therefore a treatment-specific bias.

2.2. Soil collection and microcosm set up

Soils were collected from the Talmo pasture (this site is colonised with *A. trapezoides*), and Glenrock pasture (where these earthworms are very rare, see Fig. S1) sites in November 2013 by digging the top 0-20 cm of the soil in an area of approximately 2 x 2 m^2. Both pastures are used for sheep grazing and consist of a mixture of mostly non-native annual and perennial grasses, in addition to *Trifolium subterraneum* (subterranean clover). A previous survey of soil properties showed that Talmo pasture has higher moisture, total C, organic P, microbial biomass C and N and clay content, whereas Glenrock had higher C/N ratio and inorganic P (de Menezes et al., 2015, Prendergast-Miller et al., 2015). The soils were sieved through 5 mm mesh and used to make up 2.5 kg microcosms built from 20 x 15 cm PVC pipes. A total of 30 microcosms were set up, 15 for each soil. For each soil, there were five replicate microcosms with no litter or earthworms added as a control; 10 microcosms were supplemented with 5 g of roughly chopped plant litter leaves (*Medicago littoralis* var. Harbinger), known to be food source to earthworms (Gallagher & Wollenhaupt, 1997). The *Medicago* plants were grown in calcareous dune sand under controlled conditions (Ladd et al., 1981), and the leaf litter

content was 40% C, 4.5% N. All microcosms were watered to excess and left to drain for two days. The initial soil moisture content was 28% and 32% for Glenrock and Talmo, respectively. Soil moisture was monitored throughout the experiment by regular weighing and moisture addition. Meshed netting (1 mm), was placed in the microcosm openings to prevent earthworms from escaping. Twelve *A. trapezoides* adult individuals were introduced to five of the 10 microcosms containing litter in each soil. The microcosms were incubated at 15°C in the dark and their position in the incubator rotated weekly. After 17 weeks the microcosms were destructively sampled, the number of surviving earthworms counted, and soils were sampled for DNA extraction and sequencing of the 16S rRNA gene as well as for characterisation of soil nitrogen pools. Earthworm casts were also collected from the microcosm surfaces for molecular analysis.

2.3. Soil analyses

Soils from the microcosms were collected and individually homogenised. Soil subsamples were extracted with 1M KCl (1:4 w/w). Extracts were analysed for N pools: ammonium (NH_4^+-N) and nitrate (NO_3^--N) using a microplate reader (SynergyMX, BioTek; Winooski, VT) method adapted from Mulvaney *et al*. (1996) and Miranda *et al*. (2001) respectively; concentration of free amino acid nitrogen (FAA-N) was determined using the fluorimetric o-phthalaldehyde-β-mercaptoethanol (OPAME) method (Jones *et al.*, 2002) on the same microplate reader; total dissolved N (TDN) was measured using a Total Organic C analyser (Shimadzu TOC-VCSH/CSN +TNM-1; Kyoto, Japan), and dissolved organic N (DON) was calculated by subtracting the sum of NH_4^+-N and NO_3^--N from TDN. Microbial biomass N (MBN) was determined after chloroform fumigation of additional soil subsamples and extracted with 1M KCl (1:4 w/v), the values obtained were corrected using a factor of 0.54. Soil nitrogen pools are expressed on a soil dry weight basis. Soil pH was measured using a 1:5 w/v in water and soil moisture was determined gravimetrically after drying at 105 ^oC overnight. Further details of the properties of soils at their site of origin, including total, organic and inorganic phosphorus, mid-infrared [MIR] spectrometry-predicted clay, MIR-predicted particulate, humus and recalcitrant organic carbon, free amino-acid N, microbial biomass carbon and nitrogen, C/N and fungi:bacteria ratios is found in de Menezes *et al*. (2015).

192 **2.4. Sequencing**

193 For DNA sequencing, all earthworm microcosm samples were used, as well as the earthworm casts
194 and 3 soil samples from each of the original field sites taken at the same time as the microcosm soils
195 were sampled. DNA was extracted from 0.25 g of soil from a total of 46 samples (30 microcosms plus
196 10 earthworm cast samples and 6 field samples) using the MO-BIO PowerSoil® kit, following the
197 manufacturer's protocol using the Qiagen TissueLizer (Venlo, Netherlands) to lyze microbial cells
198 (full speed for 2 minutes). The DNA quality and quantity was checked using NanoDrop™ and
199 Quanti-iT™ Picogreen (Life Technologies™, Mulgrave, Australia) and sent for sequencing using the
200 Illumina MiSeq platform. Following quantification using Qubit™ (Life Technologies™, Mulgrave,
201 Australia), the V1-V3 variable regions of the bacterial 16S rRNA gene was amplified using the 27f
202 and 519r bacterial 16s rRNA primers (Winsley *et al.*, 2012), which were adapted to contain barcodes
203 and the Illumina linker sequence, and equimolar amounts of DNA were added to one MiSeq flow cell.
204 The Illumina MiSeq 500 cycle V2 kit was used for paired end sequencing. FastQC
205 (http://www.bioinformatics.babraham.ac.uk/projects/fastqc/) was used to check for sequence quality,
206 and low quality regions were trimmed and merged using FLASH (Magoc & Salzberg, 2011) with a
207 minimum overlap of 20 bp. Sequences < 400 bp and with homopolymers > 8 bp and ambiguities were
208 removed in mothur (Schloss *et al.*, 2009), resulting in a total of 20,616,999 sequences and average
209 length of 468 bp. Sequence clustering at 97% identity threshold and chimera removal was performed
210 using USEARCH/UCHIME (Edgar *et al.*, 2011). The resulting OTU sequences were classified in
211 mothur using the Greengenes reference files (DeSantis *et al.*, 2006), with a confidence threshold of
212 60%, and eukaryotic, archaeal, mitochondrial or plastid sequences were removed, in addition to those
213 sequences not classified to the domain level. The final dataset had 11,329,277 sequences, 5,123
214 OTUs, and minimum, maximum and average number of sequences was 174,028, 393,344 and
215 246,288, respectively. For beta-diversity analyses, OTUs with less than 5 copies in at least 9 of the 46
216 soil DNA sequence samples were removed, and the abundance data was log(x+1) transformed using R
217 (R Core Development Team, 2014) and the Phyloseq package (McMurdie & Holmes, 2013) as
218 described in the bioconductor workflow for microbiome data (Callahan *et al.*, 2016). In the

differential abundance analysis using DESeq2, non-rarefied OTU abundance data was used as recommended by McMurdie and Holmes (2014). Bacterial richness (number of observed OTUs and Chao1 index) were calculated in Phyloseq (McMurdie & Holmes, 2013) based on the OTU table prior to filtering of rare OTUs and log(x+1) transformation. The 16S rRNA gene sequence data has been submitted to the NCBI Sequence Read Archive (accession number SUB2851342).

2.5. Data analysis

A weighted UniFrac distance matrix (Lozupone & Knight, 2005) was calculated in Phyloseq based on the log(x+1) transformed OTU abundance data and the matrix was imported into PRIMER-E package for ecological statistical analysis (Clarke & Gorley, 2006). ANOSIM analysis was carried in PRIMER separately for Talmo and Glenrock microcosm soils, with treatment as factor and control, litter, litter+earthworm and cast as levels. ANOSIM analyses produce an R statistic which can vary from -1 to 1, and which can be interpreted as an absolute measure of the strength of the differences between groups (Clarke & Gorley, 2006). Differences in bacterial communities were visualised using principal coordinates analysis (PCoA) in R. Individual OTUs that were significantly enriched in each treatment were identified using the DESeq2 (Love *et al.*, 2014) extension of the Phyloseq package (McMurdie & Holmes, 2014). DESeq2 was run using the Wald test, with automatic filtering of low abundance OTUs, automatic calculation of adjusted *p*-values and an alpha of 0.01, and the enriched OTUs were visualised using the ggplot2 package in R (Wickham, 2009). Soil NO_3^--N data as well as the number of observed OTUs, Chao1 index and the relative abundance of specific bacterial taxa of potential functional importance were log-transformed before analysis to improve the homogeneity of variance.

3. Results:

3.1. Earthworm survival

Earthworm activity as determined by visual inspection of cast production on the surface was highest following their introduction into the microcosms, particularly in the Talmo microcosms. From weeks five to the end of the experiment cast production slowed and was mostly absent in the last two weeks for all microcosms. Although earthworms were active in both Talmo and Glenrock microcosms, burrowing and cast production were clearly greater in the Talmo microcosms. Out of a total of 60

246 earthworms added to each set of the earthworm+litter treatment microcosms, 4 (6%) and 22 (36%)
247 survived in the Glenrock and Talmo microcosms at the time of sampling, respectively. As a result of
248 the difference in earthworm survival between Talmo and Glenrock microcosms, we chose to analyse
249 treatment effects separately for each soil microcosm set. Furthermore, as a consequence of earthworm
250 death, the effects of earthworm introduction described and discussed here are a result of the
251 combination of earthworm activity and their cadaver decomposition.

252 **3.2. Nitrogen pools, soil pH and moisture**

253 In the Talmo microcosms, addition of litter led to a significant increase in NH_4^+-N ($p < 0.05$), and the
254 earthworm+litter treatment was associated with increased NO_3^--N ($p < 0.01$) and MBN ($p < 0.05$)
255 (Fig. 1, Table S1). Talmo earthworm+litter treatment showed a decrease in pH compared to the Talmo
256 litter-only treatment (5.7 to 5.4, $p < 0.05$), while differences in moisture level were only significant
257 when comparing control to earthworm+litter treatment (Table S1, Fig. S2). In Glenrock microcosms,
258 the earthworm+litter treatment showed increases in NH_4^+-N compared to litter-only treatment and
259 DON compared to the control and litter-only treatments ($p < 0.05$), FAA-N levels were greater in the
260 litter ($p < 0.01$) and litter+earthworm ($p < 0.05$) microcosms compared to the control (Fig. 1, and
261 Table S1), and litter addition led to a pH increase (5.5 to 5.7, $p < 0.001$) compared to the control
262 Glenrock microcosms (Fig. S2, Table S1). Moisture levels varied between 18-24% and 29-39% in
263 Glenrock and Talmo respectively, and these moisture levels are similar to the values observed in the
264 original sites during the wettest months, when the earthworms are active (unpublished data).
265 Differences in moisture values between treatments were not significantly different except when
266 comparing Talmo control to Talmo earthworm+litter microcosms (Table S1 and Fig. S2).

267 **3.3. Bacterial communities**

268 **3.3.1. Microbial richness**

269 There were no significant differences in microbial richness except that Talmo soils had a greater
270 number of observed OTUs and Chao1 index than Glenrock soils (t-test $p < 0.001$, Fig. 2,
271 supplementary Table S1).

272 **3.3.2. Community structure**

The different treatments were distributed along the first axis in the PCoA plot, which explained 37 % of the variability observed, while the two sites are separated along the second PCoA axis, which explained 28.9% of the variability observed (Fig. 3). This indicates that changes in microbial community composition between treatments were greater than differences between Talmo and Glenrock.

3.3.2.1. Talmo

Principal coordinate analysis (Fig. 3) and ANOSIM tests (Table 1) shows that bacterial community structure was significantly different between Talmo field soil (i.e. the original soil source) and the control microcosms (ANOSIM R value = 1). The changes in bacterial community structure between control and litter+earthworm and between litter and earthworm+litter treatments were smaller but significant (ANOSIM R value of 1 and 0.548 respectively) (Table 1). Supplementary Fig. S3A shows the phylum-level community composition of Talmo soils at the phylum level: Acidobacteria abundance was higher in the control microcosms compared to the field soils samples, whereas litter addition led to an increase in Proteobacteria and Firmicutes. Earthworm addition led to further increase in the abundance of the Proteobacteria and a decrease in the abundance of Firmicutes, whereas the abundance of the Acidobacteria decreased further in the Talmo earthworm casts. The Verrucomicrobia decreased in abundance in the control and litter microcosms compared to the field soils, while their abundance in casts increased compared to the earthworm+litter microcosms (Fig. S3A). Of the bacterial groups often associated with decomposition in soil, the Clostridiales (phylum Firmicutes) increased in abundance with the addition of litter, but the introduction of earthworms in addition to litter lowered their abundance in comparison to the litter-only treatment microcosms (supplementary Fig. S4). Differential abundance analysis using DESeq2 confirms the increase in Clostridiales OTUs after the addition of litter (Fig. 4). DESeq2 also showed that while almost all Firmicutes, most Proteobacteria, Actinobacteria and Bacteroidetes OTUs responded positively to litter addition, approximately half of the Acidobacteria and Verrucomicrobia OTUs declined in abundance compared to the control microcosms (Fig. 4).

3.3.2.2. Glenrock

Microbial community structure in microcosm soils was substantially more different to field soils for Glenrock compared to Talmo soils (weighted UniFrac distance between field soils and control microcosms of 0.09 and 0.05 for Glenrock and Talmo, respectively, data not shown). Compared to Talmo, Glenrock field soils had four-fold higher relative abundance of the Firmicutes, while the Chloroflexi, Actinobacteria and Planctomycete phyla were also more abundant in this site (Fig. S3B). The abundance of the Acidobacteria was approximately two-thirds of the value for Talmo, and Verrucomicrobia was also less abundant in Glenrock field soils (Fig. S3B). Furthermore, microbial community structure was consistently different between Talmo and Glenrock soils in all treatments. ANOSIM showed that treatments had comparable effects on the overall bacterial community structure as seen in Talmo (Table 1). Increases in the Acidobacteria were observed when comparing control with field soils, while the abundance of the Proteobacteria and the Firmicutes increased in the litter treatment when compared to the control. Likewise, as observed in Talmo microcosms, earthworm+litter treatment led to an increase in the abundance of the Proteobacteria and a decrease in the abundance of the Firmicutes compared to the litter-only treatment (Fig S3B). DESeq2 (Fig. 4) showed that while the number of individual OTUs that changed in abundance following litter addition was greater in Glenrock than Talmo, there were 12 orders containing OTUs that responded positively to litter amendment in both soil sets.

3.3.3. Bacterial taxa responsive to earthworm introduction

3.3.3.1. Talmo

Fig. 5 shows that when comparing litter+earthworm to litter-only treatments the number of OTUs that responded positively to the earthworm+litter was smaller than the number of OTUs that responded to litter-only treatment in the litter-only vs. control comparison (Fig. 4). Overall, compared to the litter-only treatment the earthworm+litter treatment microcosms showed an increase in the abundance of OTUs classified to Verrucomicrobia, Bacteroidetes and Proteobacteria, while the OTUs classified to the Firmicutes decreased in abundance. The *Flavobacterium* genus seems to be particularly favoured by the presence of earthworms, as 7 OTUs responded positively in the earthworm+litter treatment. In addition, of the OTUs that responded positively to the earthworm+litter treatment, those classified to

the genus *Flavobacterium* were the most abundant, with their total abundance increasing from 0.4% in the litter-only microcosms to 1.7 and 18% of total 16S rRNA sequences in the earthworm+litter microcosms and earthworm casts, respectively (Fig. 6). Fig. 5 also shows that two OTUs classified to bacterial families or genera associated with earthworm nephridia (Davidson *et al.*, 2013) were significantly more abundant in the earthworm+litter treatment in Talmo microcosms (*Achromobacter*, *Pedobacter*). When analysing the relative abundance of genera that were detected specifically in the nephridia of Lumbricidae earthworms (Davidson *et al.*, 2013), the genera *Mesorhizobium* (family Phyllobacteriaceae), *Ochrobactrum* (Brucellaceae) and particularly *Pedobacter* (Sphingobacteriaceae) were found to respond positively to the presence of *A. trapezoides* (supplementary Fig. S5).

In the Talmo earthworm+litter treatment microcosms, evidence was obtained of an increase in the abundance of bacterial groups which are potentially aerobic or micro-aerophilic saprotrophs: Sphingobacteriales (Stursova *et al.*, 2012, Salka *et al.*, 2014), *Flavobacterium*, (Ulrich *et al.*, 2008, Hrynkiewicz *et al.*, 2010), *Pedobacter* (Margesin *et al.*, 2003) (Talmo and Glenrock); *Burkholderia* (Ulrich *et al.*, 2008), Xanthomonadaceae (Eichorst & Kuske, 2012) (Talmo). In contrast, several OTUs from the Firmicutes phylum (particularly the Clostridiales), which are well known efficient anaerobic cellulose degraders (Leschine & Canaleparola, 1983, Leschine, 1995) declined in abundance in the earthworm+litter treatment microcosms (Fig. 5).

3.3.3.2. Glenrock

Fig. 5 shows that there was a greater number of OTUs which were significantly more abundant in the earthworm+litter treatment in the Glenrock microcosms (the site where originally *A. trapezoides* was very rare) compared to Talmo. As seen in Talmo, the OTUs that responded positively to the earthworm+litter treatment were mainly classified to the Verrucomicrobia, Bacteroidetes and Proteobacteria. There were 13 OTUs that were enriched both at Glenrock and Talmo in the earthworm+litter treatment microcosms, and these belonged to the *Flavobacterium* (seven OTUs), *Comamonas*, *Pedobacter* and *Pelomonas* (one OTU each) genera as well as unclassified OTUs belonging to families Cerasicoccaceae, Methylophilaceae and auto67_4W (Verrucomcrobia, Pedosphaerales) (one OTU each). As observed in the Talmo microcosms, *Flavobacterium* OTUs had

the highest combined abundance of those taxa that responded positively to the earthworm+litter treatment, increasing from 0.04% in the litter microcosms to 0.8 and 9% in the earthworm+litter microcosms and casts, respectively (Fig. 6). Furthermore, of the Lumbricidae nephridia-associated taxa, 2 *Pedobacter* OTUs were significantly more abundant in Glenrock earthworm+litter treatments compared to the litter-only treatment (Fig. 5), while the same taxa that showed a generally positive response to earthworm+litter in Talmo also responded positively at Glenrock microcosms (Fig. S5).

As seen in Talmo microcosms, the earthworm+litter microcosms showed increased abundance of OTUs classified to taxa associated with aerobic or micro-aerophilic, potentially saprotrophic bacteria. In addition to Sphingobacteriales, *Flavobacterium, Pedobacter* OTUs which also increased in abundance at Talmo earthworm+litter microcosms, OTUs classified to Chitinophagaceae (Chung *et al.*, 2012), Myxococcales (Eichorst & Kuske, 2012), Actinomycetales (McCarthy, 1987) also showed increases in Glenrock earthworm+litter microcosms. However, despite the overall abundance of the phylum Firmicutes clearly decreasing in Glenrock earthworm+litter microcosms in comparison to the litter-only treatment (Fig. S3B), only one Firmicute OTU showed decreased abundance in this comparison when analyzed by differential abundance analysis (Fig. 5).

3.3.4. **Nitrogen cycling bacteria**

Supplementary Fig. S6 shows the combined abundance of all OTUs classified to the genera *Nitrosovibrio* and *Nitrospira*. *Nitrosovibrio* is a member of the Nitrosomonadales, which is mostly associated with NH_3^+ oxidation to NO_2^-, while the *Nitrospira* are associated with the oxidation of NO_2^- to NO_3^-. The abundance of the *Nitrosovibrio* increased substantially in a stepwise fashion from the field soils to the control, litter, litter+earthworm treatments and casts in both Talmo and Glenrock microcosms. The *Nitrosovibrio* were particularly abundant in the earthworm casts, reaching ca. 2% of the bacterial 16s rRNA genes in casts from Glenrock soil microcosms. The genus *Nitrospira* showed the opposite trend compared to *Nitrosovibrio* in the Talmo microcosms, with highest abundance observed in the Talmo field soils (0.07% of sequences), declining in a stepwise fashion in the control, litter and earthworm+litter treatments. While *Nitrospira* comprised 0.01% of the sequences in the

Talmo earthworm+litter microcosms, in Glenrock this genus was entirely absent in the same treatment. Other typical NO_2^--N oxidisers (i.e. *Nitrobacter* spp.) were not detected in this study.

3.3.5. Differences in OTU abundance between Talmo and Glenrock

Using differential abundance analysis to perform pairwise comparisons of OTU abundance between sites at each treatment (Fig. SA7-C), Glenrock showed a greater number of differentially abundant Firmicute OTUs, particularly the anaerobic and often saprotrophic Clostridiales in the litter and earthworm+litter treatments (number of Clostridiales OTUs more abundant in Glenrock vs. Talmo: 55 and 14 [control], 85 and 8 [litter treatment], 86 and 3 [earthworm+litter]) (Fig. S7A-C). Of relevance to N cycling and in agreement with Fig. S6, there were 12, 7 and 6 Nitrospirales OTUs which were more abundant in Talmo control, litter and earthworm+litter treatments respectively when compared to Glenrock microcosms of the same treatment, and none which were more abundant in Glenrock microcosms.

4. Discussion

4.1. Changes in bacterial community structure

Glenrock soils microbial community structure went through considerably greater change during microcosm set up compared to Talmo soils, suggesting that the soil microbial community at Talmo is more resistant to physical disturbance. While the drivers of soil microbial community structure resistance are complex, variable and not fully understood (Griffiths & Philippot, 2013), the greater soil organic matter and clay content may have conferred greater structural resistance to Talmo soils, potentially providing greater protection to the microbial community compared to Glenrock soil (Kuan *et al.*, 2007, Arthur *et al.*, 2012, Corstanje *et al.*, 2015).

In contrast to the effect of physical manipulation of the soil for microcosm set up, the subsequent experimental treatments affected the soil community composition to a similar extent for both sites. The bacterial community data showed that compared to Talmo, the Glenrock field soils taken at the time of sampling had a greater abundance of the Firmicutes and the Bacteroidetes, whereas Talmo field soils had substantially greater abundance of Acidobacteria, often considered "oligotrophic" organisms (Jones *et al.*, 2009). Importantly, microbial community composition of

Talmo and Glenrock soils were consistently different and did not converge under the different treatments. The two soils are different in several physical and chemical properties (de Menezes *et al.*, 2015), and are likely to differ in further, unquantified variables, such as soil texture and bulk density. The data presented here suggests that the litter and litter+earthworm treatments were unable to challenge the ecological stability of either soil, however, the treatments applied did lead to consistent changes similar in both soils. The increase in Acidobacteria abundance in the control microcosms may be related to the lack of any C inputs in this system, while the additional plant litter led to a decrease in Acidobacteria abundance and the flourishing of saprotrophic Firmicutes (particularly members of the Clostridiales) in Glenrock and in Talmo microcosms to a lesser extent. The Proteobacteria, and in particular the Betaproteobacteria, benefited from the addition of litter and earthworm introduction in both sets of microcosms, likely due to the fact that the Betaproteobacteria includes many fast growing bacteria that benefit from the organic C levels inputs from litter and earthworm activity and the decomposition of earthworm biomass (Fierer *et al.*, 2007). Similarly, Acidobacteria abundance was even lower in the earthworm casts, which would be consistent with the greater expected available nutrients derived from earthworm mucus and excreta.

Earthworm+litter treatment showed a changed community of saprotrophs in both soils compared to the litter-only treatment, with decreases in the abundance of Firmicute bacteria and increases in proteobacterial decomposers. Taken together these results indicate that the presence of earthworms improved aeration of the soil and affected the bacterial decomposer community, favoring aerobic groups (Schellenberger *et al.*, 2011). Alterations in the decomposer community were also likely to be due to changes in litter quality caused by litter passage through the earthworm gut, which is known to secrete polysaccharidases and to harbour plant polysaccharide-degrading microorganisms (Hartenstein, 1982, Zhang *et al.*, 1993, Hong *et al.*, 2011). Gut passage is also thought to increase microbial access to the cellulose imbedded within the plant-cell wall matrix (Dempsey *et al.*, 2013). In addition, earthworm mucus may have also contributed to the observed changes in bacterial community structure. For example, Bernard *et al*. (2012) concluded that the increase in the abundance of the Flavobacteriaceae following the introduction of the endogeic earthworm *Pontoscolex corethrurus* in combination with straw amendment was due to the increased nitrogen from earthworm

mucus, which induced these bacteria to mine for phosphorus in recalcitrant soil organic carbon. While the increase in *Flavobacterium* abundance in the earthworm+litter treatments may partly be due to a return of the microbial community towards the soil's field state, this increase only occurred when the earthworms were present in the microcosms, and is consistent with the presence of *A. trapezoides* in the Talmo original site. It would appear therefore that the earthworms played a role in *Flavobacterium* abundance increase in this experiment, perhaps due to a similar mechanism as described by Bernard *et al*. (2012). There were further similarities between the bacterial groups that responded positively to earthworm presence in this study and that of Bernard *et al*. (2012), such as the Chitinophagaceae, Rhodocyclaceae and Sphingobacteriales, all of which had OTUs that were more abundant after earthworm addition in this study. The increased abundance of the Chitinophagaceae, Rhodocyclaceae and the Sphingobacteria following earthworm introduction was attributed to their potential ability to degrade insoluble polysaccharides such as cellulose, hemicelluloses and chitin (Bernard *et al.*, 2012). Therefore, the data presented here suggests that as earthworms can boost specific microbial groups in contrasting soils, promoting subtle changes in microbial community composition despite the overall stability of the local microbial communities.

4.2. Bacterial richness

The treatments applied had mostly minor, non-significant effect on bacterial richness. Talmo microcosms, in general, had greater microbial richness than Glenrock, and this was the case in all treatments. Higher biodiversity levels have been implicated in greater ecological stability of communities of higher organisms, while the relationship between species richness and stability in microbial communities is less clear (Shade *et al.*, 2012, Shade, 2017). Talmo soil microbial community structure changed less between the original field soils and the microcosms compared to Glenrock, and this coincided with the higher alpha-diversity of the Talmo soil microbiome. Whether the greater microbial community stability observed for Talmo soils is a result of the greater bacterial diversity, as described by van Elsas (2012), or due to the differences in soil properties between Talmo and Glenrock discussed above, cannot be ascertained in this study. Similarly, whether the greater

bacterial richness in Talmo soils is related to the greater earthworm survival or increased NO_3^--N in the earthworm+litter treatment is uncertain.

4.3. Nitrogen pools and N-cycling bacteria

The different moisture levels and earthworm survival rates between Glenrock and Talmo microcosms hinders comparisons of the effect of earthworm activity on changes in soil N pools between soils. While litter addition led to an increase in NH_4^+-N levels in Talmo microcosms, earthworm+litter treatment showed greater NO_3^--N levels compared to the litter-only treatment, as seen in previous studies (Araujo *et al.*, 2004, Nebert *et al.*, 2011, Xu *et al.*, 2013). The increase in NO_3^--N in the Talmo earthworm+litter microcosms was accompanied by a decline in NH_4^+-N levels, which indicate that the presence of the earthworms changed N cycling in these soils. Only two well-known nitrifying bacterial groups were detected in Talmo microcosm soils. The *Nitrosovibrio* (order Nitrosomonadales, a group associated with NH_4^+ oxidation) increased in relative abundance from negligible in the field soils to 0.3-2% of the community 16S rRNA gene sequences in the earthworm casts. Members of the Nitrosomonadales catalyze the first step of nitrification, converting NH_4^+ to NO_2^-, however, they are not capable of oxidizing NO_2^- to NO_3^- (Kowalchuk & Stephen, 2001). The only well-known autotrophic bacterial NO_2^-oxidizer detected in this study was from the order Nitrospirales, including the genus *Nitrospira*. Despite not being positively affected by the presence of earthworms, in the absence of any other known nitrite oxidisers, the Nitrospirales may have been key to the increased NO_3^--N accumulation in Talmo earthworm+litter microcosms.

In the Glenrock microcosms, the introduction of litter led to an increase in NH_4^+-N levels as seen in the Talmo microcosms, however earthworm+litter microcosms showed a further increase in NH_4^+-N and a small decrease in NO_3^--N levels. The increase in NH_4^+-N in the Glenrock litter and litter+earthworm treatments agree with the co-occurrent increase in the abundance of *Nitrosovibrio*, while death and decomposition of earthworms also likely contributed to the increase of this N pool. In addition, the low NO_3^--N levels in Glenrock microcosms in all treatments is consistent with the near absence of known NO_2^- oxidisers in these samples.

4.4. Earthworm survival

Earthworm survival and activity were higher in the Talmo microcosms. This was unexpected as both microcosm sets received the same amount of plant litter and supported earthworm populations (invasive or native) in the field, while other soil properties measured (i.e. pH and moisture) were not considered unsuitable to earthworms in the Glenrock microcosms (Baker, 2007). Some soils are considered unfavourable to earthworms, particularly those low in organic carbon, low clay, high C/N ratio, sandy soils with low pH (Mathieu *et al.*, 2010). In Australia, soil pH, moisture, and the length of time the soils stay moist have been shown to influence survival and growth of *Aporrectodea longa* (Baker & Whitby, 2003). Likewise, previous studies have shown that earthworms tend to select areas with existing populations or previous presence of earthworms (Mathieu *et al.*, 2010, McTavish *et al.*, 2013), and evidence has been obtained which suggests that earthworm activity may condition the soil for their own benefit (Simmons *et al.*, 2015). Talmo pasture soil had higher total C (28.4 vs. 25.1 mg C g^{-1} soil for Talmo and Glenrock respectively), total N (2.2 vs. 1.7 mg g^{-1} soil), organic C (sum of MIR-predicted organic C fractions of 24.7 vs. 20.7 mg g^{-1} soil), higher clay (325.5 vs. 247.5 mg g^{-1} soil) and lower C/N ratio (13.0 vs. 15.1) compared to Glenrock pasture soils (de Menezes *et al.*, 2015). The different quantity and quality of soil C between Glenrock and Talmo microcosms may have led to differences in earthworm feeding habits, as earthworms show plasticity in their food preferences depending on food quality and environmental conditions (Neilson *et al.*, 2000, Amador *et al.*, 2013). The greater levels of soil C in Talmo soils may have represented additional food source to the earthworms, allowing greater survival than at the Glenrock microcosms where the earthworms would have been more reliant on the added plant litter. However, Glenrock soils are relatively similar to soils considered suitable to earthworms (Mathieu *et al.*, 2010), and although abiotic factors likely contributed to lower earthworm survival, biotic resistance or biological conditioning remains a possible contributing factor for the lower earthworm survival in Glenrock.

4.5. Soil resistance and earthworm conditioning

The lower earthworm survival in the Glenrock microcosms raises the question of whether the previous existence of *A. trapezoides* populations in the Talmo site may have made these soils more suitable for this earthworm species or whether the Glenrock soil was of inherently lower quality for

515 their survival. The possibility that some soils offer biotic or abiotic resistance to earthworm
516 colonisation has been explored previously, particularly in North America where invasive earthworms
517 are having a substantial impact on forest ecology (Bohlen *et al.*, 2004).

518 Firmicute bacteria may have influenced earthworm survival in Glenrock soils, especially
519 members of the order Clostridiales, which was ca. 4-fold more abundant in Glenrock litter and
520 earthworm+litter microcosms compared to Talmo microcosms. Although some Clostridiales are
521 thought to aid in earthworm nutrition by contributing to litter decomposition in the earthworm gut
522 (Wuest *et al.*, 2011), the litter decomposition carried out by the Clostridiales in the bulk soil may have
523 lowered the quality of the added plant litter, leading to lower earthworm survival.

524 The Lumbricidae earthworms such as *A. trapezoides* show the presence of several groups of
525 bacteria in their nephridia (Davidson *et al.*, 2013) and of these the genera *Mesorhizobium*,
526 *Ochrobactrum* and particularly *Pedobacter* were found to respond positively to the presence of *A.*
527 *trapezoides* in this study. Therefore, the presence of the earthworms in the microcosms led to a
528 detectable increase in the abundance of potential earthworm symbiotic bacteria in soil. Differences in
529 the presence and abundance of potential earthworm mutualist bacteria were also found between
530 Talmo and Glenrock soil. In particular, the genus *Flavobacterium*, which was the most abundant of
531 the taxa that increased in abundance in the earthworm+litter treatment microcosms in both soils, was
532 2-14-fold more abundant in Talmo soils compared to Glenrock. While *Flavobacterium* spp. is not
533 listed as an earthworm symbiont, the genus has nevertheless been associated with earthworm presence
534 and activity in several previous studies (Heijnen & Marinissen, 1995, Schonholzer *et al.*, 2002,
535 Bernard *et al.*, 2012, Dallinger & Horn, 2014), making the genus a possible earthworm-beneficial
536 group. Therefore, the possibility that *A. trapezoides* presence in the original site boosted earthworm-
537 beneficial bacteria that increased their subsequent survival in Talmo soil microcosms merits further
538 attention, as it is thought that earthworms can improve soil quality by boosting their microbial
539 mutualists as seen in plant-soil feedbacks (Simmons *et al.*, 2015). Interestingly, as the Glenrock
540 pasture site had an existing population of native Australian earthworms, any beneficial conditioning
541 effect in this experiment would be specific to *A. trapezoides*. The specificity of soil beneficial
542 conditioning by earthworms would be consistent with the study of Zhang *et al*. (2010) which

attributed antagonism between two species of invasive earthworms in North America to the conditioning of soil microbial communities instead of direct resource competition.

In conclusion, this study has shown that the activity of earthworms and earthworm cadaver decomposition led to a change in the soil decomposer community away from anaerobic Firmicutes to aerobic or facultative-aerobic saprotrophic Bacteroidetes and Proteobacteria. Despite the differences in soil properties and moisture, a set of bacterial OTUs responded positively to earthworm presence in both soils, consistent with previous studies (Bernard *et al.*, 2012). This suggests that there may be a discrete set of widespread endogeic earthworm-responsive bacterial taxa. The differences in earthworm survival in the two soils may be connected to a combination of abiotic and biotic soil properties, while evidence for biotic conditioning of soils by earthworms deserves further investigation. In order to better predict the spread of invasive earthworms and its consequences, future field-based studies examining the long-term impacts of invasive earthworm activity are needed to establish whether these ecosystem engineers can overcome any soil resistance and promote consistent ecological changes in varied soil ecosystems.

Funding

This work was supported by the CSIRO Transformational Biology Capability Platform, the CSIRO Sensors and Sensor Network Capability Platform and the CSIRO Agriculture Flagship as part of the 'Sensors and Sequences for Soil Biological Function' project.

Acknowledgements

We would like to acknowledge the enthusiastic support from the property owners, Tony Armour (Glenrock) and Chris Shannon (Talmo) who allowed us prompt access to their farms for soil sampling and experimentation. Shamsul Hoque (CSIRO Agriculture Flagship) helped to collect earthworms and provided outstanding technical support in the lab, while Andrew Bissett (CSIRO Oceans and Atmosphere) provided advice on the 16S rRNA gene sequencing approaches.

Conflicts of Interest

We declare that there are no conflicts of interest in the production of this manuscript.

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