

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

3 Alexandre B. de Menezes<sup>1,2\*</sup>, Miranda T. Prendergast-Miller<sup>3,4</sup>, Lynne M. Macdonald<sup>3</sup>, Peter Toscas<sup>5</sup>,  
4 Geoff Baker<sup>1</sup>, Mark Farrell<sup>3</sup>, Tim Wark<sup>6</sup>, Alan E. Richardson<sup>1</sup> and Peter H. Thrall<sup>1</sup>

5

6 <sup>1</sup>CSIRO Agriculture & Food, PO Box 1700, Canberra, ACT 2601, Australia

7 <sup>2</sup>Microbiology Department, National University of Ireland, Galway, University Road, Galway, Ireland

8 (present address)

9 <sup>3</sup>CSIRO Agriculture & Food, Locked bag 2, Glen Osmond, SA 5064, Australia

10 <sup>4</sup>Environment Department, University of York, Heslington, York, YO10 5NG, UK (present address)

11 <sup>5</sup>Data61, Private Bag 10, Clayton South, VIC 3169, Australia

12 <sup>6</sup>Data61, QCAT, Pullenvale, QLD 4069, Australia

13

14

15

16 *\*corresponding author:* Alexandre B. de Menezes

17 Microbiology Department, School of Natural Science, National University of Ireland, Galway,

18 University Road, Galway, Ireland

19 Email: ademenez@gmail.com

20

21

22

23

24

25

26

27

28

29 **Abstract:**

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

45  
46  
47  
48  
49  
50  
51  
52  
53  
54  
55  
56

57 **1. Introduction:**

58 Earthworms are ecosystem engineers, driving soil structure and nutrient dynamics (Jones *et al.*, 1994,  
59 Lavelle *et al.*, 1997) and their importance in soil ecosystems has long been recognised. By feeding on  
60 litter and soil, burrowing and releasing casts, earthworms change soil porosity, bulk density, water  
61 infiltration, nutrient mineralisation, gas emissions, organic carbon stabilisation and plant productivity  
62 (Blouin *et al.*, 2013). However, the specific consequences of earthworm activity for soil processes can  
63 vary substantially depending on earthworm species, soil type, rainfall and plant cover (Blouin *et al.*,  
64 2013).

65 Earthworms can be divided into three broad functional groups: epigeic earthworms live and  
66 feed in the surface litter layer; anecic earthworms live in permanent vertical burrows, feeding at the  
67 soil surface on litter and other organic materials and depositing their casts at the burrow entrance;  
68 endogeic earthworms feed on mineral soil and partially decomposed material as they burrow  
69 horizontally through soil (Bouché, 1977). The ecological group to which an earthworm species  
70 belongs can have a substantial effect on the way its activity affects soil ecosystems (Thakuria *et al.*,  
71 2010). For example, Greiner *et al.* (2012) observed that two different earthworm species, the epi-  
72 endogeic *Amyntas hilgendorf* and the epigeic *Lumbricus rubellus*, both of which are invasive in  
73 North America, had different impacts on litter decomposition, nutrient mineralization and soil  
74 aggregate size.

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

84 was attributed to their effect on separating lignin from cellulose in plant litter (Dempsey *et al.*, 2013).  
85 Whilst earthworms consume microbial biomass present in soil and decomposing plant litter, they also  
86 select and promote the growth of other bacterial groups that aid in the decomposition of organic  
87 matter and influence nutrient cycling in soil (Aira *et al.*, 2006, Hong *et al.*, 2011). For example, the  
88 reduced oxygen levels and rich microbial population makes the earthworm gut a favourable  
89 environment for denitrification (Drake & Horn, 2007). Earthworms are therefore usually implicated in  
90 increasing emissions of nitrous oxide (N<sub>2</sub>O), an important greenhouse gas, from soil (Costello &  
91 Lamberti, 2009). However, Nebert *et al.* (2011) showed that whereas *Lumbricus rubellus* increased  
92 N<sub>2</sub>O emissions and the abundance of the denitrifier gene *nosZ* upon litter amendment, the endogeic  
93 *Aporrectodea caliginosa* caused only a transient increase in N<sub>2</sub>O emissions and no effect on  
94 denitrification genes. Similarly, Bradley *et al.* (2012) showed that interactions between soil land use  
95 history and the epigeic *Eisenia Andrei* can lead to opposing effects on the gross rate of methane  
96 production.

97         The existing studies detailing the effects of earthworms on soil microbial community  
98 composition using culture-independent methods are often not directly comparable owing to the  
99 differences in experimental design, earthworm functional type, and treatments applied (Bernard *et al.*,  
100 2012, Koubova *et al.*, 2012, Dempsey *et al.*, 2013, Frisli *et al.*, 2013, Koubova *et al.*, 2015, Braga *et*  
101 *al.*, 2016, Delgado-Balbuena *et al.*, 2016). The available information suggests that earthworms boost  
102 the growth of fast growing bacteria owing to the production of labile carbon substrates (Braga *et al.*,  
103 2016). In accordance to the variability of their functional effects, the consequences of earthworm  
104 activity on microbial community composition has been shown to vary depending on soil conditions.  
105 For example, Koubova *et al.* (2015) observed that the effect of earthworm on soil microbial  
106 community was greater on less nutrient rich soils, while Koubova *et al.* (2012) demonstrated that soil  
107 history led to contrasting responses of methanogens to the epigeic *Eisenia andrei*. As earthworms can  
108 have diverse effects on soil properties and microbial community diversity, the spread of invasive  
109 earthworms into new environments can influence soil ecosystem function in whole landscapes, with  
110 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 ( $\text{NH}_4^+$ -N,  $\text{NO}_3^-$ -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

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

## 140 **2. Methods**

### 141 **2.1. Earthworm collection**

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

### 151 **2.2. Soil collection and microcosm set up**

152 Soils were collected from the Talmo pasture (this site is colonised with *A. trapezoides*), and Glenrock  
153 pasture (where these earthworms are very rare, see Fig. S1) sites in November 2013 by digging the  
154 top 0-20 cm of the soil in an area of approximately 2 x 2 m<sup>2</sup>. Both pastures are used for sheep grazing  
155 and consist of a mixture of mostly non-native annual and perennial grasses, in addition to *Trifolium*  
156 *subterraneum* (subterranean clover). A previous survey of soil properties showed that Talmo pasture  
157 has higher moisture, total C, organic P, microbial biomass C and N and clay content, whereas  
158 Glenrock had higher C/N ratio and inorganic P (de Menezes *et al.*, 2015, Prendergast-Miller *et al.*,  
159 2015). The soils were sieved through 5 mm mesh and used to make up 2.5 kg microcosms built from  
160 20 x 15 cm PVC pipes. A total of 30 microcosms were set up, 15 for each soil. For each soil, there  
161 were five replicate microcosms with no litter or earthworms added as a control; 10 microcosms were  
162 supplemented with 5 g of roughly chopped plant litter leaves (*Medicago littoralis* var. Harbinger),  
163 known to be food source to earthworms (Gallagher & Wollenhaupt, 1997). The *Medicago* plants were  
164 grown in calcareous dune sand under controlled conditions (Ladd *et al.*, 1981), and the leaf litter

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

### 175 **2.3. Soil analyses**

176 Soils from the microcosms were collected and individually homogenised. Soil subsamples were  
177 extracted with 1M KCl (1:4 w/w). Extracts were analysed for N pools: ammonium ( $\text{NH}_4^+$ -N) and  
178 nitrate ( $\text{NO}_3^-$ -N) using a microplate reader (SynergyMX, BioTek; Winooski, VT) method adapted  
179 from Mulvaney *et al.* (1996) and Miranda *et al.* (2001) respectively; concentration of free amino acid  
180 nitrogen (FAA-N) was determined using the fluorimetric o-phthalaldehyde- $\beta$ -mercaptoethanol  
181 (OPAME) method (Jones *et al.*, 2002) on the same microplate reader; total dissolved N (TDN) was  
182 measured using a Total Organic C analyser (Shimadzu TOC-VCSH/CSN +TNM-1; Kyoto, Japan),  
183 and dissolved organic N (DON) was calculated by subtracting the sum of  $\text{NH}_4^+$ -N and  $\text{NO}_3^-$ -N from  
184 TDN. Microbial biomass N (MBN) was determined after chloroform fumigation of additional soil  
185 subsamples and extracted with 1M KCl (1:4 w/v), the values obtained were corrected using a factor of  
186 0.54. Soil nitrogen pools are expressed on a soil dry weight basis. Soil pH was measured using a 1:5  
187 w/v in water and soil moisture was determined gravimetrically after drying at 105 °C overnight.  
188 Further details of the properties of soils at their site of origin, including total, organic and inorganic  
189 phosphorus, mid-infrared [MIR] spectrometry-predicted clay, MIR-predicted particulate, humus and  
190 recalcitrant organic carbon, free amino-acid N, microbial biomass carbon and nitrogen, C/N and  
191 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 lyse 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



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

## 224 **2.5. Data analysis**

225 A weighted UniFrac distance matrix (Lozupone & Knight, 2005) was calculated in Phyloseq based on  
226 the  $\log(x+1)$  transformed OTU abundance data and the matrix was imported into PRIMER-E package  
227 for ecological statistical analysis (Clarke & Gorley, 2006). ANOSIM analysis was carried in PRIMER  
228 separately for Talmo and Glenrock microcosm soils, with treatment as factor and control, litter,  
229 litter+earthworm and cast as levels. ANOSIM analyses produce an R statistic which can vary from -1  
230 to 1, and which can be interpreted as an absolute measure of the strength of the differences between  
231 groups (Clarke & Gorley, 2006). Differences in bacterial communities were visualised using principal  
232 coordinates analysis (PCoA) in R. Individual OTUs that were significantly enriched in each treatment  
233 were identified using the DESeq2 (Love *et al.*, 2014) extension of the Phyloseq package (McMurdie  
234 & Holmes, 2014). DESeq2 was run using the Wald test, with automatic filtering of low abundance  
235 OTUs, automatic calculation of adjusted *p*-values and an alpha of 0.01, and the enriched OTUs were  
236 visualised using the ggplot2 package in R (Wickham, 2009). Soil  $\text{NO}_3^-$ -N data as well as the number  
237 of observed OTUs, Chao1 index and the relative abundance of specific bacterial taxa of potential  
238 functional importance were log-transformed before analysis to improve the homogeneity of variance.

## 239 **3. Results:**

### 240 **3.1. Earthworm survival**

241 Earthworm activity as determined by visual inspection of cast production on the surface was highest  
242 following their introduction into the microcosms, particularly in the Talmo microcosms. From weeks  
243 five to the end of the experiment cast production slowed and was mostly absent in the last two weeks  
244 for all microcosms. Although earthworms were active in both Talmo and Glenrock microcosms,  
245 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  $\text{NH}_4^+\text{-N}$  ( $p < 0.05$ ), and the  
254 earthworm+litter treatment was associated with increased  $\text{NO}_3^-\text{-N}$  ( $p < 0.01$ ) 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  $\text{NH}_4^+\text{-N}$  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**

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

#### 278 **3.3.2.1. Talmo**

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

#### 299 **3.3.2.2. Glenrock**

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

### 317 **3.3.3. Bacterial taxa responsive to earthworm introduction**

#### 318 **3.3.3.1. Talmo**

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

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

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

#### 344 3.3.3.2. Glenrock

345 Fig. 5 shows that there was a greater number of OTUs which were significantly more abundant in the  
346 earthworm+litter treatment in the Glenrock microcosms (the site where originally *A. trapezoides* was  
347 very rare) compared to Talmo. As seen in Talmo, the OTUs that responded positively to the  
348 earthworm+litter treatment were mainly classified to the Verrucomicrobia, Bacteroidetes and  
349 Proteobacteria. There were 13 OTUs that were enriched both at Glenrock and Talmo in the  
350 earthworm+litter treatment microcosms, and these belonged to the *Flavobacterium* (seven OTUs),  
351 *Comamonas*, *Pedobacter* and *Pelomonas* (one OTU each) genera as well as unclassified OTUs  
352 belonging to families Cerasicoccaceae, Methylophilaceae and auto67\_4W (Verrucomicrobia,  
353 Pedosphaerales) (one OTU each). As observed in the Talmo microcosms, *Flavobacterium* OTUs had

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

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

#### 369 3.3.4. Nitrogen cycling bacteria

370 Supplementary Fig. S6 shows the combined abundance of all OTUs classified to the genera  
371 *Nitrosovibrio* and *Nitrospira*. *Nitrosovibrio* is a member of the Nitrosomonadales, which is mostly  
372 associated with  $\text{NH}_3^+$  oxidation to  $\text{NO}_2^-$ , while the *Nitrospira* are associated with the oxidation of  $\text{NO}_2^-$   
373 to  $\text{NO}_3^-$ . The abundance of the *Nitrosovibrio* increased substantially in a stepwise fashion from the  
374 field soils to the control, litter, litter+earthworm treatments and casts in both Talmo and Glenrock  
375 microcosms. The *Nitrosovibrio* were particularly abundant in the earthworm casts, reaching ca. 2% of  
376 the bacterial 16s rRNA genes in casts from Glenrock soil microcosms. The genus *Nitrospira* showed  
377 the opposite trend compared to *Nitrosovibrio* in the Talmo microcosms, with highest abundance  
378 observed in the Talmo field soils (0.07% of sequences), declining in a stepwise fashion in the control,  
379 litter and earthworm+litter treatments. While *Nitrospira* comprised 0.01% of the sequences in the

380 Talmo earthworm+litter microcosms, in Glenrock this genus was entirely absent in the same  
381 treatment. Other typical  $\text{NO}_2^-$ -N oxidisers (i.e. *Nitrobacter* spp.) were not detected in this study.

### 382 **3.3.5. Differences in OTU abundance between Talmo and Glenrock**

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

## 392 **4. Discussion**

### 393 **4.1. Changes in bacterial community structure**

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

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

407 Talmo and Glenrock soils were consistently different and did not converge under the different  
408 treatments. The two soils are different in several physical and chemical properties (de Menezes *et al.*,  
409 2015), and are likely to differ in further, unquantified variables, such as soil texture and bulk density.  
410 The data presented here suggests that the litter and litter+earthworm treatments were unable to  
411 challenge the ecological stability of either soil, however, the treatments applied did lead to consistent  
412 changes similar in both soils. The increase in Acidobacteria abundance in the control microcosms may  
413 be related to the lack of any C inputs in this system, while the additional plant litter led to a decrease  
414 in Acidobacteria abundance and the flourishing of saprotrophic Firmicutes (particularly members of  
415 the Clostridiales) in Glenrock and in Talmo microcosms to a lesser extent. The Proteobacteria, and in  
416 particular the Betaproteobacteria, benefited from the addition of litter and earthworm introduction in  
417 both sets of microcosms, likely due to the fact that the Betaproteobacteria includes many fast growing  
418 bacteria that benefit from the organic C levels inputs from litter and earthworm activity and the  
419 decomposition of earthworm biomass (Fierer *et al.*, 2007). Similarly, Acidobacteria abundance was  
420 even lower in the earthworm casts, which would be consistent with the greater expected available  
421 nutrients derived from earthworm mucus and excreta.

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



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

#### 450 **4.2. Bacterial richness**

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

461 bacterial richness in Talmo soils is related to the greater earthworm survival or increased  $\text{NO}_3^-$ -N in  
462 the earthworm+litter treatment is uncertain.

### 463 **4.3. Nitrogen pools and N-cycling bacteria**

464 The different moisture levels and earthworm survival rates between Glenrock and Talmo microcosms  
465 hinders comparisons of the effect of earthworm activity on changes in soil N pools between soils.  
466 While litter addition led to an increase in  $\text{NH}_4^+$ -N levels in Talmo microcosms, earthworm+litter  
467 treatment showed greater  $\text{NO}_3^-$ -N levels compared to the litter-only treatment, as seen in previous  
468 studies (Araujo *et al.*, 2004, Nebert *et al.*, 2011, Xu *et al.*, 2013). The increase in  $\text{NO}_3^-$ -N in the Talmo  
469 earthworm+litter microcosms was accompanied by a decline in  $\text{NH}_4^+$ -N levels, which indicate that the  
470 presence of the earthworms changed N cycling in these soils. Only two well-known nitrifying  
471 bacterial groups were detected in Talmo microcosm soils. The *Nitrosovibrio* (order Nitrosomonadales,  
472 a group associated with  $\text{NH}_4^+$  oxidation) increased in relative abundance from negligible in the field  
473 soils to 0.3-2% of the community 16S rRNA gene sequences in the earthworm casts. Members of the  
474 Nitrosomonadales catalyze the first step of nitrification, converting  $\text{NH}_4^+$  to  $\text{NO}_2^-$ , however, they are  
475 not capable of oxidizing  $\text{NO}_2^-$  to  $\text{NO}_3^-$  (Kowalchuk & Stephen, 2001). The only well-known  
476 autotrophic bacterial  $\text{NO}_2^-$  oxidizer detected in this study was from the order Nitrospirales, including  
477 the genus *Nitrospira*. Despite not being positively affected by the presence of earthworms, in the  
478 absence of any other known nitrite oxidisers, the Nitrospirales may have been key to the increased  
479  $\text{NO}_3^-$ -N accumulation in Talmo earthworm+litter microcosms.

480 In the Glenrock microcosms, the introduction of litter led to an increase in  $\text{NH}_4^+$ -N levels as  
481 seen in the Talmo microcosms, however earthworm+litter microcosms showed a further increase in  
482  $\text{NH}_4^+$ -N and a small decrease in  $\text{NO}_3^-$ -N levels. The increase in  $\text{NH}_4^+$ -N in the Glenrock litter and  
483 litter+earthworm treatments agree with the co-occurrent increase in the abundance of *Nitrosovibrio*,  
484 while death and decomposition of earthworms also likely contributed to the increase of this N pool. In  
485 addition, the low  $\text{NO}_3^-$ -N levels in Glenrock microcosms in all treatments is consistent with the near  
486 absence of known  $\text{NO}_2^-$  oxidisers in these samples.

### 487 **4.4. Earthworm survival**

488 Earthworm survival and activity were higher in the Talmo microcosms. This was unexpected as both  
489 microcosm sets received the same amount of plant litter and supported earthworm populations  
490 (invasive or native) in the field, while other soil properties measured (i.e. pH and moisture) were not  
491 considered unsuitable to earthworms in the Glenrock microcosms (Baker, 2007). Some soils are  
492 considered unfavourable to earthworms, particularly those low in organic carbon, low clay, high C/N  
493 ratio, sandy soils with low pH (Mathieu *et al.*, 2010). In Australia, soil pH, moisture, and the length of  
494 time the soils stay moist have been shown to influence survival and growth of *Aporrectodea longa*  
495 (Baker & Whitby, 2003). Likewise, previous studies have shown that earthworms tend to select areas  
496 with existing populations or previous presence of earthworms (Mathieu *et al.*, 2010, McTavish *et al.*,  
497 2013), and evidence has been obtained which suggests that earthworm activity may condition the soil  
498 for their own benefit (Simmons *et al.*, 2015). Talmo pasture soil had higher total C (28.4 vs. 25.1 mg  
499 C g<sup>-1</sup> soil for Talmo and Glenrock respectively), total N (2.2 vs. 1.7 mg g<sup>-1</sup> soil), organic C (sum of  
500 MIR-predicted organic C fractions of 24.7 vs. 20.7 mg g<sup>-1</sup> soil), higher clay (325.5 vs. 247.5 mg g<sup>-1</sup>  
501 soil) and lower C/N ratio (13.0 vs. 15.1) compared to Glenrock pasture soils (de Menezes *et al.*,  
502 2015). The different quantity and quality of soil C between Glenrock and Talmo microcosms may  
503 have led to differences in earthworm feeding habits, as earthworms show plasticity in their food  
504 preferences depending on food quality and environmental conditions (Neilson *et al.*, 2000, Amador *et*  
505 *al.*, 2013). The greater levels of soil C in Talmo soils may have represented additional food source to  
506 the earthworms, allowing greater survival than at the Glenrock microcosms where the earthworms  
507 would have been more reliant on the added plant litter. However, Glenrock soils are relatively similar  
508 to soils considered suitable to earthworms (Mathieu *et al.*, 2010), and although abiotic factors likely  
509 contributed to lower earthworm survival, biotic resistance or biological conditioning remains a  
510 possible contributing factor for the lower earthworm survival in Glenrock.

#### 511 **4.5. Soil resistance and earthworm conditioning**

512 The lower earthworm survival in the Glenrock microcosms raises the question of whether the  
513 previous existence of *A. trapezoides* populations in the Talmo site may have made these soils more  
514 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

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

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

#### 557 **Funding**

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

#### 561 **Acknowledgements**

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

#### 567 **Conflicts of Interest**

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

569

570

571 **References**

- 572 Aira M, Monroy F & Dominguez J. *Eisenia fetida* (Oligochaeta, Lumbricidae) activates fungal  
573 growth, triggering cellulose decomposition during vermicomposting. *Microb Ecol* 2006; **52**: 738-747.
- 574 Amador JA, Winiarski K & Sotomayor-Ramirez D. Earthworm communities along a forest-coffee  
575 agroecosystem gradient: preliminary evidence supporting the habitat-dependent feeding hypothesis.  
576 *Trop Ecol* 2013; **54**: 365-374.
- 577 Araujo Y, Luizao FJ & Barros E. Effect of earthworm addition on soil nitrogen availability, microbial  
578 biomass and litter decomposition in mesocosms. *Biol Fert Soils* 2004; **39**: 146-152.
- 579 Arthur E, Schjonning P, Moldrup P, *et al.* Soil resistance and resilience to mechanical stresses for  
580 three differently managed sandy loam soils. *Geoderma* 2012; **173**: 50-60.
- 581 Baker G. Differences in nitrogen release from surface and incorporated plant residues by two  
582 endogeic species of earthworms (Lumbricidae) in a red-brown earth soil in southern Australia. *Eur J*  
583 *Soil Biol* 2007; **43**: S165-S170.
- 584 Baker GH & Barrett VJ. *Earthworm Identifier*. Canberra: CSIRO, 1994.
- 585 Baker GH & Whitby WA. Soil pH preferences and the influences of soil type and temperature on the  
586 survival and growth of *Aporrectodea longa* (Lumbricidae). *Pedobiologia* 2003; **47**: 745-753.
- 587 Baker GH, Brown G, Butt K, *et al.* Introduced earthworms in agricultural and reclaimed land: their  
588 ecology and influences on soil properties, plant production and other soil biota. *Biol Invasions* 2006;  
589 **8**: 1301-1316.
- 590 Bernard L, Chapuis-Lardy L, Razafimbelo T, *et al.* Endogeic earthworms shape bacterial functional  
591 communities and affect organic matter mineralization in a tropical soil. *ISME J* 2012; **6**: 213-222.
- 592 Blouin M, Hodson ME, Delgado EA, *et al.* A review of earthworm impact on soil function and  
593 ecosystem services. *Eur J Soil Sci* 2013; **64**: 161-182.
- 594 Bohlen PJ, Scheu S, Hale CM, *et al.* Non-native invasive earthworms as agents of change in northern  
595 temperate forests. *Front Ecol Environ* 2004; **2**: 427-435.

596 Bouché MB. Strategies lombriciennes. In: Lohm U & Persson T (eds.). *Soil organisms as components*  
597 *of ecosystems*. Stockholm, Sweden: Ecological Bulletin, 1977, 122-132.

598 Bradley RL, Chronakova A, Elhottova D, *et al.* Interactions between land-use history and earthworms  
599 control gross rates of soil methane production in an overwintering pasture. *Soil Biol Biochem* 2012;  
600 **53**: 64-71.

601 Braga LPP, Yoshiura CA, Borges CD, *et al.* Disentangling the influence of earthworms in sugarcane  
602 rhizosphere. *Sci Rep* 2016; **6**: 38923.

603 Callahan B, Sankaran K, Fukuyama J, *et al.* Bioconductor workflow for microbiome data analysis:  
604 from raw reads to community analyses [version 2; referees: 3 approved]. *FI000Research* 2016; **5**:  
605 1492.

606 Chung EJ, Park TS, Jeon CO, *et al.* *Chitinophaga oryziterrae* sp nov., isolated from the rhizosphere  
607 soil of rice (*Oryza sativa* L.). *Int J Syst Evol Micr* 2012; **62**: 3030-3035.

608 Clarke K & Gorley R. *PRIMER v6: User Manual/Tutorial*. Plymouth: 2006.

609 Corstanje R, Deeks LR, Whitmore AP, *et al.* Probing the basis of soil resilience. *Soil Use Manage*  
610 2015; **31**: 72-81.

611 Costello DM & Lamberti GA. Biological and physical effects of non-native earthworms on nitrogen  
612 cycling in riparian soils. *Soil Biol Biochem* 2009; **41**: 2230-2235.

613 Curry JP & Baker GH. Cast production and soil turnover by earthworms in soil cores from South  
614 Australian pastures. *Pedobiologia* 1998; **42**: 283-287.

615 Dallinger A & Horn MA. Agricultural soil and drilosphere as reservoirs of new and unusual  
616 assimilators of 2,4-dichlorophenol carbon. *Environ Microbiol* 2014; **16**: 84-100.

617 Davidson SK, Powell R & James S. A global survey of the bacteria within earthworm nephridia. *Mol*  
618 *Phylogenet Evol* 2013; **67**: 188-200.

619 de Menezes AB, Prendergast-Miller MT, Richardson AE, *et al.* Network analysis reveals that bacteria  
620 and fungi form modules that correlate independently with soil parameters. *Environ Microbiol* 2015;  
621 **17**: 2677-2689.

622 Delgado-Balbuena L, Bello-Lopez JM, Navarro-Noya YE, *et al.* Changes in the bacterial community  
623 structure of remediated anthracene-contaminated soils. *PLoS One* 2016; **11**: e0160991.

624 Dempsey MA, Fisk MC, Yavitt JB, *et al.* Exotic earthworms alter soil microbial community  
625 composition and function. *Soil Biol Biochem* 2013; **67**: 263-270.

626 DeSantis TZ, Hugenholtz P, Larsen N, *et al.* Greengenes, a chimera-checked 16S rRNA gene  
627 database and workbench compatible with ARB. *Appl Environ Microb* 2006; **72**: 5069-5072.

628 Drake HL & Horn MA. As the worm turns: The earthworm gut as a transient habitat for soil microbial  
629 biomes. *Annu Rev Microbiol* 2007; **61**: 169-189.

630 Edgar RC, Haas BJ, Clemente JC, *et al.* UCHIME improves sensitivity and speed of chimera  
631 detection. *Bioinformatics* 2011; **27**: 2194-2200.

632 Eichorst SA & Kuske CR. Identification of cellulose-responsive bacterial and fungal communities in  
633 geographically and edaphically different soils by using stable isotope probing. *Appl Environ Microb*  
634 2012; **78**: 2316-2327.

635 Fierer N, Bradford MA & Jackson RB. Toward an ecological classification of soil bacteria. *Ecology*  
636 2007; **88**: 1354-1364.

637 Frisli T, Haverkamp THA, Jakobsen KS, *et al.* Estimation of metagenome size and structure in an  
638 experimental soil microbiota from low coverage next-generation sequence data. *J Appl Microbiol*  
639 2013; **114**: 141-151.

640 Gallagher AV & Wollenhaupt NC. Surface alfalfa residue removal by earthworms *Lumbricus*  
641 *terrestris* L in a no-till agroecosystem. *Soil Biol Biochem* 1997; **29**: 477-479.

642 Greiner HG, Kashian DR & Tiegs SD. Impacts of invasive Asian (*Amyntas hilgendorfi*) and  
643 European (*Lumbricus rubellus*) earthworms in a North American temperate deciduous forest. *Biol*  
644 *Invasions* 2012; **14**: 2017-2027.

645 Griffiths BS & Philippot L. Insights into the resistance and resilience of the soil microbial community.  
646 *FEMS Microbiol Rev* 2013; **37**: 112-129.

647 Hartenstein R. Soil macroinvertebrates, aldehyde oxidase, catalase, cellulase and peroxidase. *Soil Biol*  
648 *Biochem* 1982; **14**: 387-391.

649 Heijnen CE & Marinissen JCY. Survival of bacteria introduced into soil by means of transport by  
650 *Lumbricus rubellus*. *Biol Fert Soils* 1995; **20**: 63-69.



651 Hendrix PF, Baker GH, Callaham MA, *et al.* Invasion of exotic earthworms into ecosystems inhabited  
652 by native earthworms. *Biol Invasions* 2006; **8**: 1287-1300.

653 Hong SW, Lee JS & Chung KS. Effect of enzyme producing microorganisms on the biomass of  
654 epigeic earthworms (*Eisenia fetida*) in vermicompost. *Bioresource Technol* 2011; **102**: 6344-6347.

655 Hrynkiewicz K, Baum C & Leinweber P. Density, metabolic activity, and identity of cultivable  
656 rhizosphere bacteria on *Salix viminalis* in disturbed arable and landfill soils. *J Plant Nutr Soil Sc*  
657 2010; **173**: 747-756.

658 Jones CG, Lawton JH & Shachak M. Organisms as ecosystem engineers. *Oikos* 1994; **69**: 373-386.

659 Jones DL, Owen AG & Farrar JF. Simple method to enable the high resolution determination of total  
660 free amino acids in soil solutions and soil extracts. *Soil Biol Biochem* 2002; **34**: 1893-1902.

661 Jones RT, Robeson MS, Lauber CL, *et al.* A comprehensive survey of soil acidobacterial diversity  
662 using pyrosequencing and clone library analyses. *ISME J* 2009; **3**: 442-453.

663 Koubova A, Chronakova A, Pizl V, *et al.* The effects of earthworms *Eisenia* spp. on microbial  
664 community are habitat dependent. *Eur J Soil Biol* 2015; **68**: 42-55.

665 Koubova A, Goberna M, Simek M, *et al.* Effects of the earthworm *Eisenia andrei* on methanogens in  
666 a cattle-impacted soil: A microcosm study. *Eur J Soil Biol* 2012; **48**: 32-40.

667 Kowalchuk GA & Stephen JR. Ammonia-oxidizing bacteria: a model for molecular microbial  
668 ecology. *Annu Rev Microbiol* 2001; **55**: 485-529.

669 Kuan HL, Hallett PD, Griffiths BS, *et al.* The biological and physical stability and resilience of a  
670 selection of Scottish soils to stresses. *Eur J Soil Sci* 2007; **58**: 811-821.

671 Ladd JN, Oades JM & Amato M. Distribution and recovery of nitrogen from legume residues  
672 decomposing in soils sown to wheat in the field. *Soil Biol Biochem* 1981; **13**: 251-256.

673 Lavelle P, Bignell D, Lepage M, *et al.* Soil function in a changing world: the role of invertebrate  
674 ecosystem engineers. *Eur J Soil Biol* 1997; **33**: 159-193.

675 Leschine SB. Cellulose degradation in anaerobic environments. *Annu Rev Microbiol* 1995; **49**: 399-  
676 426.

677 Leschine SB & Canaleparola E. Mesophilic cellulolytic clostridia from freshwater environments. *Appl*  
678 *Environ Microb* 1983; **46**: 728-737.

679 Love MI, Huber W & Anders S. Moderated estimation of fold change and dispersion for RNA-seq  
680 data with DESeq2. *Genome Biol* 2014; **15**: 550.

681 Lozupone C & Knight R. UniFrac: a new phylogenetic method for comparing microbial communities.  
682 *Appl Environ Microb* 2005; **71**: 8228-8235.

683 McCarthy AJ. Lignocellulose-degrading actinomycetes. *FEMS Microbiol Rev* 1987; **46**: 145-163.

684 Magoc T & Salzberg SL. FLASH: fast length adjustment of short reads to improve genome  
685 assemblies. *Bioinformatics* 2011; **27**: 2957-2963.

686 Margesin R, Sproer C, Schumann P, *et al.* *Pedobacter cryoconitis* sp nov., a facultative psychrophile  
687 from alpine glacier cryoconite. *Int J Syst Evol Micr* 2003; **53**: 1291-1296.

688 Martinsson S, Cui YD, Martin PJ, *et al.* DNA-barcoding of invasive European earthworms (Clitellata:  
689 Lumbricidae) in south-western Australia. *Biol Invasions* 2015; **17**: 2527-2532.

690 Mathieu J, Barot S, Blouin M, *et al.* Habitat quality, conspecific density, and habitat pre-use affect the  
691 dispersal behaviour of two earthworm species, *Aporrectodea icterica* and *Dendrobaena veneta*, in a  
692 mesocosm experiment. *Soil Biol Biochem* 2010; **42**: 203-209.

693 McMurdie PJ & Holmes S. phyloseq: an R package for reproducible interactive analysis and graphics  
694 of microbiome census data. *PLoS One* 2013; **8**: e61217.

695 McMurdie PJ & Holmes S. Waste not, want not: why rarefying microbiome data is inadmissible. *PLoS*  
696 *Comput Biol* 2014; **10**: e1003531.

697 McTavish MJ, Basiliko N & Sackett TE. Environmental factors influencing immigration behaviour of  
698 the invasive earthworm *Lumbricus terrestris*. *Can J Zool* 2013; **91**: 859-865.

699 Miranda KM, Espey MG & Wink DA. A rapid, simple spectrophotometric method for simultaneous  
700 detection of nitrate and nitrite. *Nitric Oxide-Biol Ch* 2001; **5**: 62-71.

701 Mulvaney RL. Nitrogen – inorganic forms. In: Sparks DL, Page AL, Helmke PA & Loeppert RH  
702 (eds.). *Methods of Soil Analysis Part 3 Chemical Properties*. Madison, WI, USA: Soil Science Society  
703 of America and American Society of Agronomy, 1996, 1123-1184.

704 Nebert LD, Bloem J, Lubbers IM, *et al.* Association of earthworm-denitrifier interactions with  
705 increased emission of nitrous oxide from soil mesocosms amended with crop residue. *Appl Environ*  
706 *Microb* 2011; **77**: 4097-4104.

707 Neilson R, Boag B & Smith M. Earthworm delta  $\delta^{13}\text{C}$  and delta  $\delta^{15}\text{N}$  analyses suggest that putative  
708 functional classifications of earthworms are site-specific and may also indicate habitat diversity. *Soil*  
709 *Biol Biochem* 2000; **32**: 1053-1061.

710 Prendergast-Miller MT, de Menezes AB, Farrell M, *et al.* Soil nitrogen pools and turnover in native  
711 woodland and managed pasture soils. *Soil Biol Biochem* 2015; **85**: 63-71.

712 Salka I, Srivastava A, Allgaier M, *et al.* The draft genome sequence of *Sphingomonas* sp. strain  
713 FukuSWIS1, obtained from acidic lake grosse fuchskuhle, indicates photoheterotrophy and a potential  
714 for humic matter degradation. *Genome Announc* 2014; **2**: e01183-14.

715 Schellenberger S, Drake HL & Kolb S. Functionally redundant cellobiose-degrading soil bacteria  
716 respond differentially to oxygen. *Appl Environ Microb* 2011; **77**: 6043-6048.

717 Schloss PD, Westcott SL, Ryabin T, *et al.* Introducing mothur: open-source, platform-independent,  
718 community-supported software for describing and comparing microbial communities. *Appl Environ*  
719 *Microb* 2009; **75**: 7537-7541.

720 Schonholzer F, Hahn D, Zarda B, *et al.* Automated image analysis and *in situ* hybridization as tools to  
721 study bacterial populations in food resources, gut and cast of *Lumbricus terrestris* L. *J Microbiol*  
722 *Meth* 2002; **48**: 53-68.

723 Shade A. Diversity is the question, not the answer. *ISME J* 2017; **11**: 1-6.

724 Shade A, Peter H, Allison SD, *et al.* Fundamentals of microbial community resistance and resilience.  
725 *Front Microbiol* 2012; **3**: 417.

726 Shan J, Liu J, Wang Y, *et al.* Digestion and residue stabilization of bacterial and fungal cells, protein,  
727 peptidoglycan, and chitin by the geophagous earthworm *Metaphire guillelmi*. *Soil Biol Biochem* 2013;  
728 **64**: 9-17.

729 Simmons W, Dávalos A & Blossey B. Forest successional history and earthworm legacy affect  
730 earthworm survival and performance. *Pedobiologia* 2015; **58**: 153-164.

731 Sims RW & Gerard BM. Earthworms. In: Barnes RSK & Crothers JH (eds.). *Synopsis of the British*  
732 *Fauna*. London: Linnaean Society London, 1985, 1-169.

733 Stursova M, Zifcakova L, Leigh MB, *et al.* Cellulose utilization in forest litter and soil: identification  
734 of bacterial and fungal decomposers. *FEMS Microbiol Lett* 2012; **80**: 735-746.

735 R Core Development Team. R: A Language and Environment for Statistical Computing. 2014, Viena,  
736 Austria.

737 Thakuria D, Schmidt O, Finan D, *et al.* Gut wall bacteria of earthworms: a natural selection process.  
738 *ISME J* 2010; **4**: 357-366.

739 Ulrich A, Klimke G & Wirth S. Diversity and activity of cellulose-decomposing bacteria, isolated  
740 from a sandy and a loamy soil after long-term manure application. *Microb Ecol* 2008; **55**: 512-522.

741 van Elsas JD, Chiurazzi M, Mallon CA, *et al.* Microbial diversity determines the invasion of soil by a  
742 bacterial pathogen. *P Natl Acad Sci USA* 2012; **109**: 1159-1164.

743 Wickham H. *ggplot2: elegant graphics for data analysis*. Springer Publishing Company, 2009.

744 Winsley T, van Dorst JM, Brown MV, *et al.* Capturing greater 16S rRNA gene sequence diversity  
745 within the domain Bacteria. *Appl Environ Microb* 2012; **78**: 5938-5941.

746 Wuest PK, Horn MA & Drake HL. Clostridiaceae and Enterobacteriaceae as active fermenters in  
747 earthworm gut content. *ISME J* 2011; **5**: 92-106.

748 Xu D, Li Y, Howard A, *et al.* Effect of earthworm *Eisenia fetida* and wetland plants on nitrification  
749 and denitrification potentials in vertical flow constructed wetland. *Chemosphere* 2013; **92**: 201-206.

750 Zhang BG, Rouland C, Lattaud C, *et al.* Activity and origin of digestive enzymes in gut of the tropical  
751 earthworm *Pontoscolex corethrurus*. *Eur J Soil Biol* 1993; **29**: 7-11.

752 Zhang WX, Hendrix PF, Snyder BA, *et al.* Dietary flexibility aids Asian earthworm invasion in North  
753 American forests. *Ecology* 2010; **91**: 2070-2079.

754