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

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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⁻²) and rare (Glenrock, 0.6 *A. trapezoides* m⁻²) *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.

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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 (N₂O), an important greenhouse gas, from soil (Costello &
91 Lamberti, 2009). However, Nebert *et al.* (2011) showed that whereas *Lumbricus rubellus* increased
92 N₂O emissions and the abundance of the denitrifier gene *nosZ* upon litter amendment, the endogeic
93 *Aporrectodea caliginosa* caused only a transient increase in N₂O 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 (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

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 m². 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 (NH_4^+ -N) and
178 nitrate (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- β -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 NH_4^+ -N and 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 NO₃⁻-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 NH_3^+ oxidation to NO_2^- , while the *Nitrospira* are associated with the oxidation of NO_2^-
373 to 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 NO₂⁻-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 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 NH_4^+ -N levels in Talmo microcosms, earthworm+litter
467 treatment showed greater 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 NO_3^- -N in the Talmo
469 earthworm+litter microcosms was accompanied by a decline in 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 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 NH_4^+ to NO_2^- , however, they are
475 not capable of oxidizing NO_2^- to NO_3^- (Kowalchuk & Stephen, 2001). The only well-known
476 autotrophic bacterial 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 NO_3^- -N accumulation in Talmo earthworm+litter microcosms.

480 In the Glenrock microcosms, the introduction of litter led to an increase in NH_4^+ -N levels as
481 seen in the Talmo microcosms, however earthworm+litter microcosms showed a further increase in
482 NH_4^+ -N and a small decrease in NO_3^- -N levels. The increase in 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 NO_3^- -N levels in Glenrock microcosms in all treatments is consistent with the near
486 absence of known 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⁻¹ soil for Talmo and Glenrock respectively), total N (2.2 vs. 1.7 mg g⁻¹ soil), organic C (sum of
500 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
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.

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567 **Conflicts of Interest**

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

569

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