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Article

Accepted Version

Milcu, A., Paul, S. and Lukac, M. ORCID:
<https://orcid.org/0000-0002-8535-6334> (2011) Belowground interactive effects of elevated CO₂, plant diversity and earthworms in grassland microcosms. *Basic and Applied Ecology*, 12 (7). pp. 600-608. ISSN 1439-1791 doi:
<https://doi.org/10.1016/j.baae.2011.08.004> Available at
<https://centaur.reading.ac.uk/21815/>

It is advisable to refer to the publisher's version if you intend to cite from the work. See [Guidance on citing](#).

To link to this article DOI: <http://dx.doi.org/10.1016/j.baae.2011.08.004>

Publisher: Elsevier

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Type of contribution: **Research article**

Number of text pages: 25

Number of tables: 1

Number of figures: 2

Number of words including references, tables and figure captions: 5348

Appendixes: 1

Belowground interactive effects of elevated CO₂, plant diversity and earthworms in model grasslands

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25 **ABSTRACT**

26 The potential interactive effects of future atmospheric CO₂ concentrations and plant diversity
27 loss on the functioning of belowground systems are still poorly understood. Using a
28 microcosm greenhouse approach with assembled grassland plant communities of different
29 diversity (1, 4 and 8 species), we explored the interactive effects between plant species
30 richness and elevated CO₂ (ambient and + 200 p.p.m.v. CO₂) on earthworms and microbial
31 biomass. We hypothesised that the beneficial effect of increasing plant species richness on
32 earthworm performance and microbial biomass will be modified by elevated CO₂ through
33 impacts on belowground organic matter inputs, soil water availability and nitrogen
34 availability. We found higher earthworm biomass in eight species mixtures under elevated
35 CO₂, and higher microbial biomass under elevated CO₂ in four and eight species mixtures if
36 earthworms were present. The results suggest that plant driven changes in belowground
37 organic matter inputs, soil water availability and nitrogen availability explain the interactive
38 effects of CO₂ and plant diversity on the belowground compartment. The interacting
39 mechanisms by which elevated CO₂ modified the impact of plant diversity on earthworms
40 and microorganisms are discussed.

41

42 **Keywords:** Climate change; Species richness; Above-belowground interactions; Water
43 availability; Soil N; *Lumbricus terrestris*

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48

49 **Introduction**

50 Understanding of the relationship between biodiversity, climate change and the ecosystem
51 services upon which we are heavily reliant emerged as a critical issue in the face of
52 increasing human-induced environmental change (Millennium Ecosystem Assessment 2005).
53 Whilst much attention has been devoted to separately studying the impacts of climate change
54 and biodiversity loss, considerably less is known about their interactive effects on ecosystems
55 and the services they provide (Balvanera, Pfisterer, Buchmann, He, Nakashizuka et al. 2006).
56 Studies which include the effects of climate change factors on the functioning of the
57 belowground compartment are even fewer and by and large highlight the considerable lack of
58 knowledge in this area (Chung, Zak, Reich & Ellsworth 2007, Kardol, Cregger, Campany &
59 Classen. 2010). Since the biological functioning and the species composition of the
60 belowground compartment is dependent on organic matter inputs from photosynthesising
61 plants, the understanding of the links between producer and decomposer subsystems is
62 essential for predicting of ecosystem-level responses to global change (Wardle 2002).

63 Grasslands constitute a widespread type of ecosystem covering ~ 27% of terrestrial
64 surface (Henwood 1998), highlighting their importance as model systems used for
65 investigating the consequences of biodiversity loss (Roscher, Temperton, Scherer-Lorenzen,
66 Schmitz, Schumacher et al. 2005, Tilman, Reich & Knops 2006) or elevated CO₂ (Niklaus,
67 Alpei, Kampichler, Kandeler, Körner et al. 2007, Reich 2009). Reich, Knops, Tilman,
68 Craine, Ellsworth et al. (2001) showed that plant diversity interacts with elevated CO₂ and
69 nitrogen availability to modify ecosystem functioning. Furthermore, elevated atmospheric
70 CO₂ concentrations usually reduce stomatal density and conductance (Woodward & Bazzaz
71 1988; Tricker, Trewin, Kull, Clarkson, Eensalu et al. 2005) which may result in increased soil
72 moisture by reducing evapotranspiratory loss of water (Morgan et al. 2001; Nelson, Morgan,
73 Lecain, Mosier, Milchunas et al. 2004) under elevated levels of CO₂. Although both CO₂ and

74 plant diversity loss have been shown to impact the decomposer functioning through changes
75 in the quality and quantity of organic matter inputs and impacts on soil moisture and nutrient
76 availability, we have a limited understanding of their combined effects on the decomposer
77 functioning (Niklaus et al. 2007) despite their important role in biogeochemical cycling, plant
78 performance and soil C storage (Bardgett 2005).

79 Both earthworms and microorganisms are key decomposer groups sensitive to
80 changes in the aboveground inputs, as well as soil water and nutrient regimes. Currently we
81 have only a limited mechanistic understanding of the interactive effects of elevated CO₂ and
82 plant diversity loss on their performance. The few studies which report effects of elevated
83 CO₂ on earthworms predominantly measure their response in terms of activity, indicating
84 increased activity with increasing levels of CO₂ (Yeates, Tate & Newton 1997, Arnone &
85 Zaller 1997; Zaller & Arnone 1999c). However, an observation of community biomass and
86 composition by Zaller & Arnone (1999c) found no effects of elevated CO₂. Available data
87 also show that increasing plant species diversity (Zaller & Arnone 1999b; Speh, Josh,
88 Schmid, Alphei & Körner 2000; Niklaus et al. 2007) has a positive effect on earthworm
89 biomass and activity, but depends on species-specific relationships with earthworms (Zaller
90 & Arnone 1999c; Milcu, Partsch, Langel & Scheu 2006). Milcu et al. (2006) suggested that
91 changes in the quality rather than quantity of plant inputs associated with changes in species
92 diversity affect earthworm performance. The consequences of elevated CO₂ on
93 microorganisms are still unclear due to insufficient data to predict how microbial biomass
94 and functioning change as atmospheric CO₂ concentration continues to rise (Zak et al 2000,
95 Bardgett, Freeman & Ostle YEAR). The effect of diversity loss on soil microorganisms is
96 somewhat clearer with recent publications pointing out the positive relationship between
97 plant diversity and the functioning and biomass of microbial communities (Eisenhauer et al
98 2009).

99 The scope for interacting effects of diversity loss and elevated CO₂ on soil organisms
100 is clearly large. Climatic change is likely to both directly and indirectly (via plant responses)
101 affect soil biota, with associated consequences for ecosystem functioning. In this study we
102 explored the interaction between plant species richness, elevated CO₂ and the presence of
103 earthworms and its effects on the belowground compartment of model grassland, using a
104 microcosm greenhouse experiment. Our overarching hypothesis is that the frequently
105 observed beneficial effects of increasing the richness of plant species on earthworm
106 performance and microbial biomass will be altered by elevated CO₂, through changes in
107 belowground organic matter inputs and altered soil water and nitrogen availability.

108

109 **Materials and methods**

110 *Experimental set up*

111 Experimental grassland communities of varied plant species richness (1, 4 and 8 species)
112 were established in cylindrical microcosms made of PVC tubes, 10.3 cm in diameter and 25
113 cm in height. The microcosms were sealed at the bottom with 0.5mm² mesh, and a layer of
114 sand (0.25kg) was added to the bottom of the microcosms to ensure efficient drainage. The
115 sand was topped by 1.6 kg of sieved soil (1cm diameter sieve) taken from the A horizon of a
116 mesotrophic grassland (MG6 – *Lolium perene* - *Cynosurus cristatus*; Rodwell 1998) in
117 Silwood Park, Ascot, UK (N 51° 24.3 W 00° 38.5). Prior to use, the soil (bagshot sands, pH
118 5.35) was homogenized and defaunated by freezing at -22°C for two weeks (Huhta, Wright &
119 Coleman 1989). Subsequently, the soil was watered intermittently over a 12 day period with
120 deionised water to remove excess nutrients released by the perturbation.

121 The microcosms were planted out with eight seedlings (~ 5 cm height) pre-
122 germinated in John Innes compost from a species pool of 37 grassland species (Appendix 1).
123 The microcosms were assigned to eight air-conditioned chambers (100cm x 100cm x 53.5

124 cm) located in a greenhouse. An elevated atmospheric CO₂ treatment of 600 ppm simulating
125 the average prediction for 2100 (IPCC 2007) was applied to four of the chambers, with the
126 other four receiving air with ambient concentration of ~400 ppm.

127 The earthworm treatment (EW) was established by adding one *Lumbricus terrestris* L.
128 (4.9774 ± 0.067 g FW) to one set of the microcosms in each chamber. *L. terrestris* was chosen
129 as a typical representative colonising the grassland type (MG6) from which the soil was
130 extracted. As *L. terrestris* is an anecic litter feeding species, 3g DW of *Dactylis glomerata*
131 litter (cut into 3cm pieces) was placed onto the soil surface in each microcosm. No additional
132 litter entered the microcosms as any plant litter originating from the aboveground biomass
133 was removed weekly. Each growth chamber thus contained 36 microcosms: two replicates of
134 each species mixture, one containing a *L. terrestris* individual (EW) and one control (NW).
135 The resulting total of 288 microcosms (3 plant diversity levels \times 6 replicates/diversity level \times
136 2 earthworm treatments \times 2 CO₂ levels \times 4 replicates per CO₂ level) were fitted with 15cm
137 high cylindrical transparent plastic guards at the top of each microcosm to contain the
138 earthworms in the microcosms. The greenhouse was lit naturally, but also received additional
139 light for 8 hours a day via 400 Watt overhead 'POOT' lamps. Identical environmental
140 conditions were maintained in all chambers, apart from the 4 chambers where atmospheric
141 CO₂ was maintained at 600ppm for 8 hours during the photosynthetically active part of the
142 day. Air humidity, air temperature and CO₂ levels of each chamber were monitored
143 continuously. The microcosms were watered every 3 days, initially with 30ml of deionised
144 water, increasing to 60ml, 80ml and then 120ml over the course of the experiment to account
145 for increasing water use by growing plants. The positions of the microcosms within the
146 growth chambers were randomised every 14 days.

147

148 *Plant diversity experimental design*

149 A pool of 37 South-East England mesotrophic acid grassland plant species belonging to three
150 functional groups (grasses, herbs and legumes) was used to produce a total of 18 different
151 species mixtures (see Appendix A) using independent random draws with replacement (i.e.
152 species mixtures were created by randomly selecting species from the species pool with all
153 species being available for to be drawn for any species mixture). The established gradient of
154 species diversity (1, 4 and 8) had 6 replicates per diversity level. Any sampling effects were
155 reduced by using species pool much larger than the maximum diversity level (Aarssen 1997).
156

157 *Sampling and analytical procedures*

158 The experiment has been harvested after four months of CO₂ exposure when the majority of
159 plants showed signs of senescence. Immediately prior to destructive sampling, the soil
160 moisture level of each microcosm was measured (ThetaProbe Soil Moisture Sensor - ML2x,
161 Delta-T Products). Soil C/N content was analysed using a CNS elemental analyser (Thermo
162 Scientific FlashEA 1112 series). Earthworms were hand collected during the final harvest,
163 washed, dried for 1 min on filter paper and weighed. A sub-sample of soil was sieved to
164 remove any roots (2mm dia.) and stored at 4°C. After 2 weeks the soil was used to assess the
165 microbial biomass using the substrate induced respiration (SIR) method (Anderson and
166 Domsch, 1978) using electrolytic O₂ microcompensation apparatus (Scheu 1992). The
167 microbial respiratory response to the addition of glucose was measured at 30-minute intervals
168 for 24h at 22°C. Sufficient amount of glucose in solution was added to the substrate in order
169 to saturate the catabolic activity of microorganisms (4 mg glucose per 1g of soil dry weight).
170 The maximum initial respiratory response (MIRR [$\mu\text{g O}_2 \cdot \text{h}^{-1} \cdot \text{g soil dw}^{-1}$]) was calculated
171 as the average of the lowest three readings within the first 11 h and microbial biomass (C_{mic}
172 [$\mu\text{g C}_{\text{mic}} \cdot \text{g soil dw}^{-1}$]) was calculated as $\text{MIRR} \cdot 38 \cdot 0.7$ (Anderson & Domsch 1978).

173

174 *Statistical analysis*

175 Mixed effects models, as implemented in the R Statistical package (lme function, R version
176 2.10.1), was used to analyse the fixed effects of CO₂ (CO₂), earthworm presence (EW), plant
177 species richness (SR) and their interactions on earthworm biomass, microbial biomass, soil
178 moisture and soil N and C/N ratio. The random-effects of the maximal model, fit by
179 maximum likelihood, had a microcosm within chamber random structure to account for the
180 nested design [model <-lme (y ~ CO₂*EW*SR, random = ~1 | chamber / microcosmID),
181 where y indicates the response variable, chamber represents the eight growth chambers at
182 which the CO₂ treatment has been applied and microcosmID represents the individual
183 microcosms]. Minimum adequate models were achieved by sequentially excluding non-
184 significant terms (starting with highest-order interactions) and comparing each model with its
185 predecessor using Akaike information criterion (Crawley 2007). Tukey's HSD posthoc test
186 was used to perform pairwise comparison of treatments with more than two levels. Several
187 continuous explanatory variables such as plant biomass (shoot, root, total), soil moisture and
188 soil N content were tested as covariables in the models for identifying potential mechanisms.
189 We used Statistica 8 package (StatSoft Inc) for graphical presentation of data.

190

191 **Results**

192 *Earthworm body weight.* In total, 81 % of the 144 *Lumbricus terrestris* used were retrieved at
193 the end of the experiment and on average, their biomass decreased by 32%. Neither the CO₂
194 nor plant species richness treatments had a significant effect on earthworm biomass (Table 1).
195 They did, however, have a significant interactive effect (CO₂ x SR; Table 2) with earthworms
196 exhibiting lower biomass in the eight species mixtures at ambient (-37.8%) than at elevated
197 CO₂ (-29.5%) (Fig. 1a).

198 *Soil N.* Soil nitrogen content at the end of the experiment was not affected by the CO₂
199 treatment alone or by the presence of earthworms (Table 1). Increasing plant species richness
200 led to significantly lower soil N (Table 1), with the lowest N concentration in the eight
201 species mixtures (0.13% N) compared to one and four species mixtures (0.14% N).

202 *Soil C/N ratio.* Increasing plant diversity led to significantly higher C/N ratio (Fig. 1b).
203 Furthermore, the C/N ratio was affected by the interaction between CO₂ and earthworms
204 (CO₂ x EW) with the lowest C/N ratio in the presence of legumes under ambient CO₂
205 conditions.

206 *Soil moisture content.* Elevated CO₂ increased the soil moisture measured at the end of the
207 experiment from 25.3% in the ambient CO₂ to 29.3%. Increasing plant species diversity led
208 to significantly lower soil moisture in the four (26.1%) and eight (25.9%) species richness
209 compared to monocultures (30.1%, Table 1). Soil moisture was also affected by the
210 interaction between CO₂ concentration and the presence of earthworms (CO₂ x EW; Table 1
211 and Figure 1c), with significantly less available soil water in the earthworm treatment and
212 with the lowest amount of available soil water content at ambient CO₂ in the presence of
213 earthworms (Tukey HSD <0.05).

214 *Microbial biomass.* Elevated CO₂ marginally increased microbial biomass (+6.2%) (Table 1).
215 Plant species richness also affected the microbial biomass, with significantly higher biomass
216 in the four species richness (226.0 mg Cmic g⁻¹ soil dry weight) compared to monocultures
217 (208.2 mg Cmic g⁻¹ soil dry weight, Tukey HSD <0.05), with the eight species mixtures
218 being intermediate (217.5 mg Cmic g⁻¹ soil dry weight, Tukey HSD >0.05). Additionally, the
219 CO₂ treatment, earthworm presence and plant species richness had an interactive effect (CO₂
220 × EW × SR; Table 2, Fig. 2) on the microbial biomass; under elevated CO₂ only four and
221 eight species mixtures increased microbial biomass (Tukeys HSD <0.05) compared to

222 monocultures if earthworms were present, while in the absence of earthworms microbial
223 biomass was higher in the monocultures (Fig. 2).

224

225 **Discussion**

226 Despite their small scale and somewhat artificial nature, microcosm experiments have a good
227 track record of providing an indication of processes and mechanisms directing ecosystem
228 functioning at much larger scales (Benton, Solan, Travis & Sait 2007). Given that all
229 microcosms were constructed by using homogenised soil with standardised plant litter and no
230 additional litter entered the soil, we contend that the observed effects of elevated CO₂ were
231 occurring through the rhizosphere. Although the experiment discussed in this paper lasted
232 only for the equivalent of one vegetative season, the observations show several interactive
233 effects of elevated CO₂, earthworm presence and plant diversity on the functioning of the
234 belowground compartment of a model temperate grassland.

235

236 *Earthworms*

237 It is widely accepted that the main drivers through which elevated CO₂ will affect soil
238 organisms are soil moisture (Niklaus et al. 2007) and plant derived organic matter inputs such
239 as litter and rhizodeposition (O'Neill 1994, Meehan et al. 2010). Changes in both quantity
240 and quality of soil organic matter inputs have been shown to change with increasing plant
241 diversity, due to alterations in the amount and chemical composition of organic matter
242 entering the soil. This may potentially have beneficial effects on the earthworm community
243 (Edwards 1996; Milcu, Partsch, Scherber, Weisser & Scheu 2008) though the relationship
244 between plant species diversity and earthworm performance is unlikely to be straightforward
245 with idiosyncratic effects often reported (Wardle, Bonner, Barker, Yeates, Nicholson et al.
246 1999; Eisenhower, Milcu, Sabais, Bessler, Weigelt et al. 2009). We observed higher

247 earthworm biomass in microcosms with higher plant diversity (8 species), but only under
248 elevated CO₂ treatment. However, since soil moisture, plant shoot, root and total biomass,
249 when used as potential covariables in the statistical model, did not render the CO₂ × SR
250 interaction non-significant, we contend that the increase in earthworm biomass under
251 elevated CO₂ is not a result of changes in soil moisture or plant biomass. As no aboveground
252 inputs entered the system, the greater earthworm biomass could only have resulted from
253 additional belowground inputs. Elevated CO₂ is known to generally enhance N
254 rhizodeposition (Schultze & Merbach 2008) and N₂ fixation in legumes (Stephanie,
255 Fischinger, Hristozkova, Mainassara & Schultze 2009) leading to increased total
256 belowground N transport. This likely explains the higher biomass of *L. terrestris*, an anecic
257 litter feeding species which has been previously shown to be closely linked to N availability
258 (Milcu et al. 2008). Interestingly, the lowest biomass of earthworms was observed at the
259 highest plant diversity level under ambient CO₂. This is in contradiction with existing
260 literature which documents positive or idiosyncratic (Hedlund, Santa Regina, Van Der
261 Putten, Lepš, Diaz et al. 2003; Eisenhauer et al. 2009), but no detrimental effects of
262 increasing plant diversity on earthworm performance. The observed decrease in earthworm
263 biomass was likely caused by the high competition for N between plants and earthworms as
264 documented by the lower soil N and higher C/N ratio in the eight species diversity mixtures
265 in an already low-N soil (0.13% N) (Fig. 1b). Indeed fitting the C/N ratio in the model
266 renders the CO₂ × SR interaction non-significant ($P > 0.05$) and increases the fitness of the
267 model as estimated by the AIC.

268

269 *Microbial biomass*

270 Like earthworms, soil microorganisms are often found to respond to elevated

271 CO₂ by changing their community composition, overall biomass and activity (De Graaff,
272 Mavan Groenigen, Six, Hungate & Van Kessel 2006; Blagodatskaya, Blagodatsky,
273 Dorodnikov & Kuzyakov 2010). The magnitude and the sign of the effect of elevated CO₂ on
274 microorganisms is not very clear, with no effects (Holmes et al 2006), detrimental (Hungate
275 et al. 1996) or idiosyncratic responses having been documented (Zak, Pregitzer, King &
276 Holmes 2000; Kanerva, Palojarvi, Rämö & Manninen. 2008). Here we only found a
277 marginally increased microbial carbon biomass (C_{mic}) in the elevated CO₂. Changes in litter
278 inputs, root biomass and production, soil moisture and rhizodeposition have been previously
279 identified to affect microbial biomass under elevated CO₂ (Zak 2000). However, whilst we
280 could not identify the exact mechanism, it is worth noting that this increase was mediated by
281 belowground effects of elevated CO₂ as no aboveground plant litter entered the soil.
282 Moreover, we found significant changes in response to plant diversity. Higher C_{mic} at higher
283 plant diversity levels have recently been reported in a 7 year experiment (Eisenhauer et al.
284 2009). In our microcosms, we found the eight and four plant species mixtures to have higher
285 C_{mic} than the monocultures. However, contrary to our expectations, the highest biomass was
286 found in the four and not in the eight plant species mixtures. Higher C_{mic} at intermediate plant
287 diversity levels have been previously found in early stages of experiments manipulating
288 diversity as the effects of plants on the C_{mic} increased with time (Eisenhauer et al. 2009).

289 The presence of animal ecosystem engineers such as anecic earthworms also affects
290 the functioning of the belowground systems and microbial biomass has been noted to
291 increase due to accelerated incorporation of nutrient rich litter into the soil. Sheehan, Kirwan,
292 Connolly & Bolger (2008) suggested that the positive effect of anecic species on microbial
293 biomass was greatest with increased food supply. We found that microbial biomass varied
294 with the presence of earthworms, plant species richness and CO₂ level; higher microbial
295 biomass was found under elevated CO₂ at higher plant diversity levels when earthworms

296 were present, but also at the lower diversity level in the absence of earthworms. Given the
297 inconclusiveness in observed effects, we could not identify the exact mechanism behind this
298 interaction as none of the measured covariates (root biomass, total plant biomass, soil
299 moisture or soil N content) affected the significance level of the interaction. This highlights
300 the complexity of belowground C fluxes, it is possible that a longer-term experiment might
301 be able to uncover such a mechanism by allowing the systems to stabilise and transient
302 effects to lose their influence.

303

304 *Soil moisture*

305 Water availability frequently limits the functioning of the detritivore system (Swift, Heal &
306 Anderson 1979; Austin 2002) with many soil decomposers migrating into deeper soil layers
307 or to entering a dormant stage under water stress, with consequences for the functioning of
308 the ecosystems. Water availability is essential for normal earthworm physiology due to their
309 cutaneous mode of respiration and for maintaining their coelomic hydrostatic pressure at
310 levels that allow locomotion (Lavelle 1988). Although highly dependent on soil water,
311 earthworm activities (e.g. burrowing, casting, midden formation) can affect the soil water
312 dynamics in a feedback loop through effects on soil physical properties. The mechanisms
313 include changes in soil porosity and stability of organomineral aggregates. By burrowing and
314 by the creation of organomineral casts, earthworms are generally thought to exert a beneficial
315 influence on soil water availability through increased water infiltration and by improving soil
316 water holding capacity (Edwards 2004). However, negative effects of earthworm activity on
317 soil water retention have also been reported (Blouin, Lavelle & Laffray 2007) and there is
318 increasing evidence that elevated CO₂ affects belowground communities also through
319 improved plant and soil water status due to reduced evapotranspiration via reduced leaf
320 conductance (Niklas et al 2007). This could play an important role in the functioning of the

321 ecosystems particularly in conditions of water shortage when burrows increases water
322 drainage and evaporative water loss. Our results are in line with the findings of Blouin et al.
323 (2007), showing a significant decrease in soil moisture content in the presence of *L. terrestris*
324 via preferential water flow through the vertical burrows which can account to up to 10% of
325 the water flow (Edwards, Shipitalo, Owens & Norton 1989). Given that in our experiment the
326 soil water content was reduced by earthworms in both CO₂ treatments, but that a smaller
327 reduction was observed under to elevated CO₂ (Fig. 1c), this suggests that both reduced soil
328 water retention and increased water use efficiency (Wullschleger, Tschaplinski & Norby
329 2002) contribute to the observed effects. However, it might not be possible to extrapolate our
330 this finding to field conditions due to differences in drainage and water movement between
331 microcosms and undisturbed soil profile. Our observations indicate that deep vertical
332 macropores created by anecic earthworms may modify the effect of elevated CO₂ on soil
333 water availability, the mechanism behind this interaction remains to be verified in the field.

334

335 *Soil nitrogen*

336 In an extensive meta-analysis considering the effects of elevated CO₂ on nutrient
337 cycling De Graaff et al. (2006) found no effects on N mineralisation rates, but
338 documented increased soil C inputs leading to increased soil C/N ratio. Although
339 we found no significant effect of CO₂ on the soil N or C/N ratio, we observed a decrease of
340 soil N and an increase in the soil C/N ratio with increasing plant diversity. These kind of
341 effects have previously been reported (e.g. Oelmann, Wilcke, Temperton, Buchmann, Roscher
342 et al 2007 and Roscher, Thein, Schmid & Scherer Lorenzen 2008) and they are attributed to a
343 better usage of the total resources, whether they be nitrogen or water (i.e. increased
344 complementarity). This mechanism contributes to the positive relationship between species
345 richness and above-ground biomass production and is often paralleled by a decrease in

346 belowground available N due to increased efficiency in exploiting the available resource pool
347 but facilitating an increased accumulation of plant biomass as compared to low-diversity
348 communities (Roscher et al 2008). In support of this we found an increase of total plant
349 biomass with increasing diversity ($F_{2,270} = 6.6294$, $p = 0.002$).

350 Earthworm presence is known to increase N cycling and availability (Scheu 2003;
351 Edwards 2004), however, in this study the presence of earthworms did not have any effect on
352 the availability of soil N. In our experiment this is presumably related to the low resource
353 (litter) inputs in our system, but we acknowledge that by measuring only changes in total soil
354 N we could have missed potential changes in available N.

355

356 In conclusion, the findings of this experiment emphasize several interacting
357 mechanisms by which elevated CO₂ can modify the established relationship between plant
358 diversity, earthworms and microorganisms. The results confirm our over-arching hypothesis
359 that changes in belowground resource allocation, soil water and N availability mediated by
360 elevated CO₂ alter the relationship between plant diversity and the functioning of the
361 belowground compartment. Some of the observed interactions might be transient or
362 magnified by the nature of this microcosm experiment. However, these results confirm the
363 existence of several mechanisms which could determine the response and adaptation of
364 essential soil functions such as decomposition, nutrient cycling, carbon storage, water
365 filtration and primary productivity to elevated CO₂ at the ecosystem level. Such mechanisms
366 and interactions need further investigation in order to understand and predict the interactive
367 effects of climate change and diversity loss on ecosystem functioning and stability.

368

369 **Acknowledgements**

370 We thank NERC for funding the placement student. We gratefully acknowledge Prof. Stefan
371 Scheu for allowing performing the microbial biomass analyses in his lab. We also thank Prof.
372 Mick Crawley for comments on the R statistical analysis, Dennis Wildman and Tom Sloan
373 for their invaluable technical support and Nico Eisenhauer and Alessandra Lagomarsino for
374 their comments on the manuscript.

375

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Table 1. Output table of mix-effects models for the effect of CO₂ treatment (CO₂), earthworm presence (EW), plant species richness (SR) and their interactions on the belowground response variables. Excluded effects refer to the treatment factors which have been removed from the model in order to achieve minimum adequate models (Crawley 2007).

	Belowground measurements														
	Earthworm biomass			Soil moisture			Soil N			Soil C to N ratio			Microbial biomass		
	df	<i>F</i>	<i>P</i>	df	<i>F</i>	<i>P</i>	df	<i>F</i>	<i>P</i>	df	<i>F</i>	<i>P</i>	df	<i>F</i>	<i>P</i>
CO ₂	1,6	0.32	0.591	1,6	11.12	0.015	1,6	0.1	0.947	1,6	0.48	0.514	1,6	4.69	0.073
EW	n.a	n.a	n.a	1,273	108.7	<.001	1,253	1.1	0.284	1,256	2.75	0.098	1,256	0.9	0.342
SR	2,101	1.61	0.205	2,273	7.9	<.001	2,253	0.161	0.021	2,256	3.54	0.030	2,256	3.1	0.047
CO ₂ × EW	excl.	excl.	excl.	1,273	9.7	0.002	1,253	0.1	0.777	excl.	excl.	excl.	1,256	0.1	0.969
CO ₂ × SR	2,101	3.54	0.032	excl.	excl.	excl.	excl.	excl.	excl.	2,256	1.59	0.206	2,256	1.77	0.172
CO ₂ × EW × SR	excl.	excl.	excl.	excl.	excl.	excl.	excl.	excl.	excl.	excl.	excl.	excl.	2,256	4.0	0.018

Figure legends

Figure 1. Two-way interaction effects of plant species richness (SR) and atmospheric CO₂ treatment on earthworm mass loss (A), effects of SR on soil C/N ratio (B) and two-way interaction effects of SR and earthworm presence on soil moisture (C). Error bars represent \pm SE.

Figure 2. Three-way interaction effects between plant species richness (SR), atmospheric CO₂ and earthworm presence on the carbon (glucose) induced microbial biomass (Cmic). Error bars represent \pm SE

Figure 1

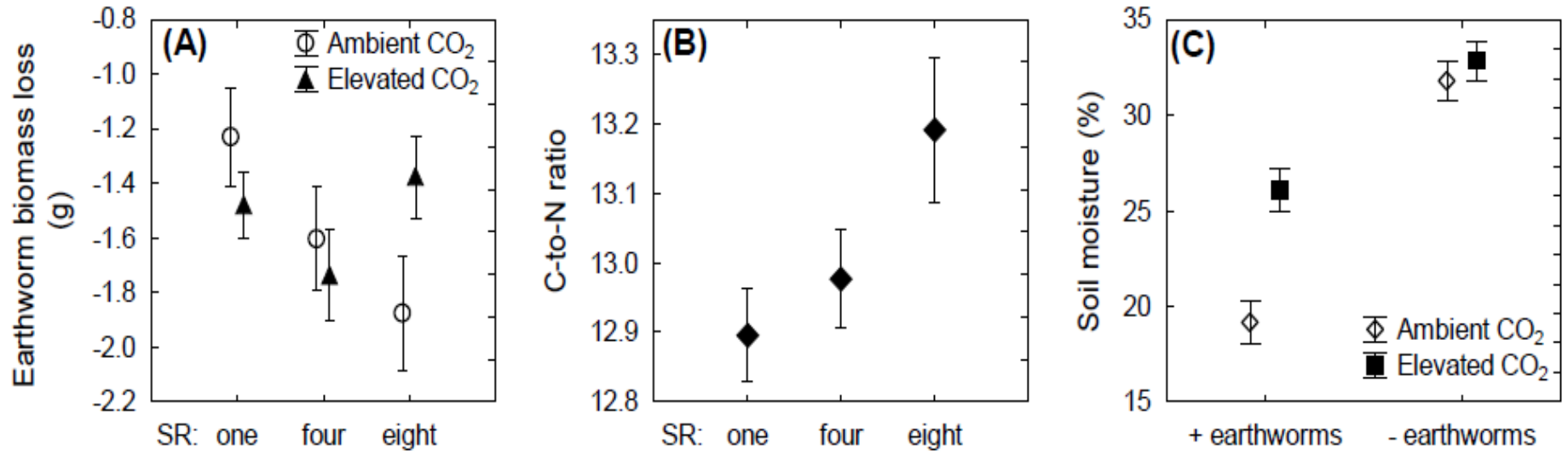
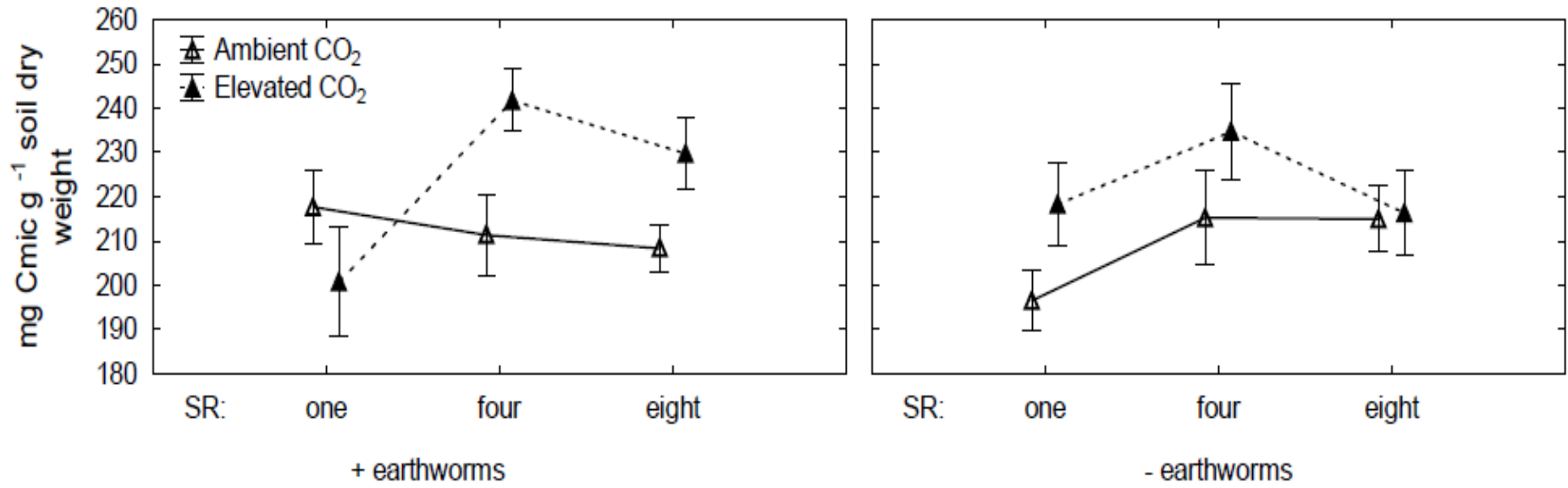


Figure 2



Appendix A. Experimental design layout of the 18 different species mixtures. Grasses are shown in yellow, herbs in green and legumes in dark green.

Nr	Diversity level	Plant Species in Microcosm							
1	Monoculture	<i>Bromus hordeaceus</i>							
2	Monoculture	<i>Cynosurus cristatus</i>							
3	Monoculture	<i>Achillea millefolium</i>							
4	Monoculture	<i>Dipsacus fullonum</i>							
5	Monoculture	<i>Trifolium repens</i>							
6	Monoculture	<i>Vicia cracca</i>							
7	4-species-mixture	<i>Holcus lanatus</i>	<i>Lolium perenne</i>	<i>Vulpia bromoides</i>	<i>Festuca pratensis</i>				
8	4-species-mixture	<i>Taraxacum officinale</i>	<i>Rumex acetosella</i>	<i>Dipsacus fullonum</i>	<i>Daucus carota</i>				
9	4-species-mixture	<i>Trifolium dubium</i>	<i>Trifolium pratense</i>	<i>Medicago lupulina</i>	<i>Vicia sativa</i>				
10	4-species-mixture	<i>Bromus hordeaceus</i>	<i>Lolium perenne</i>	<i>Leontodon hispidus</i>	<i>Vicia sativa</i>				
11	4-species-mixture	<i>Bromus sterilis</i>	<i>Plantago lanceolata</i>	<i>Taraxacum officinale</i>	<i>Lotus corniculatus</i>				
12	4-species-mixture	<i>Alopecurus pratensis</i>	<i>Myosotis arvensis</i>	<i>Trifolium pratense</i>	<i>Lathyrus tuberosus</i>				
13	8-species-mixture	<i>Holcus lanatus</i>	<i>Anthoxanthum odoratum</i>	<i>Festuca pratensis</i>	<i>Alopecurus pratensis</i>	<i>Agrostis capillaris</i>	<i>Festuca rubra</i>	<i>Bromus sterilis</i>	<i>Poa annua</i>
14	8-species-mixture	<i>Galium mollugo</i>	<i>Plantago lanceolata</i>	<i>Matricaria recutita</i>	<i>Daucus carota</i>	<i>Prunella vulgaris</i>	<i>Leontodon hispidus</i>	<i>Myosotis arvensis</i>	<i>Rumex acetosella</i>
15	8-species-mixture	<i>Trifolium dubium</i>	<i>Vicia sativa</i>	<i>Lathyrus pratensis</i>	<i>Vicia cracca</i>	<i>Lotus corniculatus</i>	<i>Medicago sativa</i>	<i>Trifolium medium</i>	<i>Medicago lupulina</i>
16	8-species-mixture	<i>Elytrigia repens</i>	<i>Anthoxanthum odoratum</i>	<i>Festuca rubra</i>	<i>Plantago lanceolata</i>	<i>Prunella vulgaris</i>	<i>Dipsacus fullonum</i>	<i>Medicago lupulina</i>	<i>Lotus corniculatus</i>
17	8-species-mixture	<i>Lolium perenne</i>	<i>Bromus sterilis</i>	<i>Achillea millefolium</i>	<i>Matricaria recutita</i>	<i>Leucanthemum vulgare</i>	<i>Vicia sativa</i>	<i>Trifolium medium</i>	<i>Medicago sativa</i>
18	8-species-mixture	<i>Dactylis glomerata</i>	<i>Holcus lanatus</i>	<i>Festuca pratensis</i>	<i>Leucanthemum vulgare</i>	<i>Achillea millefolium</i>	<i>Trifolium dubium</i>	<i>Vicia cracca</i>	<i>Lathyrus tuberosus</i>

Grass
Herb
Legume