

# *Belowground interactive effects of elevated CO<sub>2</sub>, plant diversity and earthworms in grassland microcosms*

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**Belowground interactive effects of elevated CO<sub>2</sub>, plant diversity and earthworms in model grasslands**

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25 **ABSTRACT**

26 The potential interactive effects of future atmospheric CO<sub>2</sub> 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 CO<sub>2</sub> (ambient and + 200 p.p.m.v. CO<sub>2</sub>) 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 CO<sub>2</sub> 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 CO<sub>2</sub>, and higher microbial biomass under elevated CO<sub>2</sub> 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 CO<sub>2</sub> and plant diversity on the belowground compartment. The interacting  
39 mechanisms by which elevated CO<sub>2</sub> 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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## 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 CO<sub>2</sub> (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 CO<sub>2</sub> and  
69 nitrogen availability to modify ecosystem functioning. Furthermore, elevated atmospheric  
70 CO<sub>2</sub> 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 CO<sub>2</sub>. Although both CO<sub>2</sub> 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 CO<sub>2</sub> and  
82 plant diversity loss on their performance. The few studies which report effects of elevated  
83 CO<sub>2</sub> on earthworms predominantly measure their response in terms of activity, indicating  
84 increased activity with increasing levels of CO<sub>2</sub> (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 CO<sub>2</sub>. 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 CO<sub>2</sub> on  
93 microorganisms are still unclear due to insufficient data to predict how microbial biomass  
94 and functioning change as atmospheric CO<sub>2</sub> 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 CO<sub>2</sub> 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 CO<sub>2</sub> 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 CO<sub>2</sub>, 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 0.5mm<sup>2</sup> 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 CO<sub>2</sub> 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 ( $4.9774 \pm 0.067$ 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 CO<sub>2</sub> levels  $\times$  4 replicates per CO<sub>2</sub> 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 CO<sub>2</sub> was maintained at 600ppm for 8 hours during the photosynthetically active part of the  
142 day. Air humidity, air temperature and CO<sub>2</sub> 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 CO<sub>2</sub> 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 O<sub>2</sub> 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 ( $C_{\text{mic}}$   
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 CO<sub>2</sub> (CO<sub>2</sub>), 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 ~ CO<sub>2</sub>\*EW\*SR, random = ~1 | chamber / microcosmID),  
181 where y indicates the response variable, chamber represents the eight growth chambers at  
182 which the CO<sub>2</sub> 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 CO<sub>2</sub>  
194 nor plant species richness treatments had a significant effect on earthworm biomass (Table 1).  
195 They did, however, have a significant interactive effect (CO<sub>2</sub> x SR; Table 2) with earthworms  
196 exhibiting lower biomass in the eight species mixtures at ambient (-37.8%) than at elevated  
197 CO<sub>2</sub> (-29.5%) (Fig. 1a).

198 *Soil N.* Soil nitrogen content at the end of the experiment was not affected by the CO<sub>2</sub>  
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 CO<sub>2</sub> and earthworms  
204 (CO<sub>2</sub> x EW) with the lowest C/N ratio in the presence of legumes under ambient CO<sub>2</sub>  
205 conditions.

206 *Soil moisture content.* Elevated CO<sub>2</sub> increased the soil moisture measured at the end of the  
207 experiment from 25.3% in the ambient CO<sub>2</sub> 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 CO<sub>2</sub> concentration and the presence of earthworms (CO<sub>2</sub> 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 CO<sub>2</sub> in the presence of  
213 earthworms (Tukey HSD <0.05).

214 *Microbial biomass.* Elevated CO<sub>2</sub> 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<sup>-1</sup> soil dry weight) compared to monocultures  
217 (208.2 mg Cmic g<sup>-1</sup> soil dry weight, Tukey HSD <0.05), with the eight species mixtures  
218 being intermediate (217.5 mg Cmic g<sup>-1</sup> soil dry weight, Tukey HSD >0.05). Additionally, the  
219 CO<sub>2</sub> treatment, earthworm presence and plant species richness had an interactive effect (CO<sub>2</sub>  
220 × EW × SR; Table 2, Fig. 2) on the microbial biomass; under elevated CO<sub>2</sub> 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 CO<sub>2</sub> 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 CO<sub>2</sub>, 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 CO<sub>2</sub> 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 CO<sub>2</sub> 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 CO<sub>2</sub> × SR  
250 interaction non-significant, we contend that the increase in earthworm biomass under  
251 elevated CO<sub>2</sub> 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 CO<sub>2</sub> is known to generally enhance N  
254 rhizodeposition (Schultze & Merbach 2008) and N<sub>2</sub> 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 CO<sub>2</sub>. 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 CO<sub>2</sub> × 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 CO<sub>2</sub> 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 CO<sub>2</sub> 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 CO<sub>2</sub>. Changes in litter  
278 inputs, root biomass and production, soil moisture and rhizodeposition have been previously  
279 identified to affect microbial biomass under elevated CO<sub>2</sub> (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 CO<sub>2</sub> 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 CO<sub>2</sub> level; higher microbial  
295 biomass was found under elevated CO<sub>2</sub> 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 CO<sub>2</sub> 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 CO<sub>2</sub> treatments, but that a smaller  
327 reduction was observed under to elevated CO<sub>2</sub> (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 CO<sub>2</sub> 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 CO<sub>2</sub> 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 CO<sub>2</sub> 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 CO<sub>2</sub> 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 CO<sub>2</sub> 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 CO<sub>2</sub> 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

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375

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**Table 1.** Output table of mix-effects models for the effect of CO<sub>2</sub> treatment (CO<sub>2</sub>), 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 <sub>2</sub>	1,6	0.32	0.591	1,6	<b>11.12</b>	<b>0.015</b>	1,6	0.1	0.947	<b>1,6</b>	<b>0.48</b>	<b>0.514</b>	1,6	4.69	0.073
EW	n.a	n.a	n.a	<b>1,273</b>	<b>108.7</b>	<b>&lt;.001</b>	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	<b>2,273</b>	<b>7.9</b>	<b>&lt;.001</b>	<b>2,253</b>	<b>0.161</b>	<b>0.021</b>	<b>2,256</b>	<b>3.54</b>	<b>0.030</b>	<b>2,256</b>	<b>3.1</b>	<b>0.047</b>
CO <sub>2</sub> × EW	excl.	excl.	excl.	<b>1,273</b>	<b>9.7</b>	<b>0.002</b>	1,253	0.1	0.777	excl.	excl.	excl.	1,256	0.1	0.969
CO <sub>2</sub> × SR	<b>2,101</b>	<b>3.54</b>	<b>0.032</b>	excl.	excl.	excl.	excl.	excl.	excl.	2,256	1.59	0.206	2,256	1.77	0.172
CO <sub>2</sub> × EW × SR	excl.	excl.	excl.	excl.	excl.	excl.	excl.	excl.	excl.	excl.	excl.	excl.	<b>2,256</b>	<b>4.0</b>	<b>0.018</b>



## Figure legends

**Figure 1.** Two-way interaction effects of plant species richness (SR) and atmospheric CO<sub>2</sub> 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 CO<sub>2</sub> and earthworm presence on the carbon (glucose) induced microbial biomass (Cmic). Error bars represent  $\pm$  SE

Figure 1

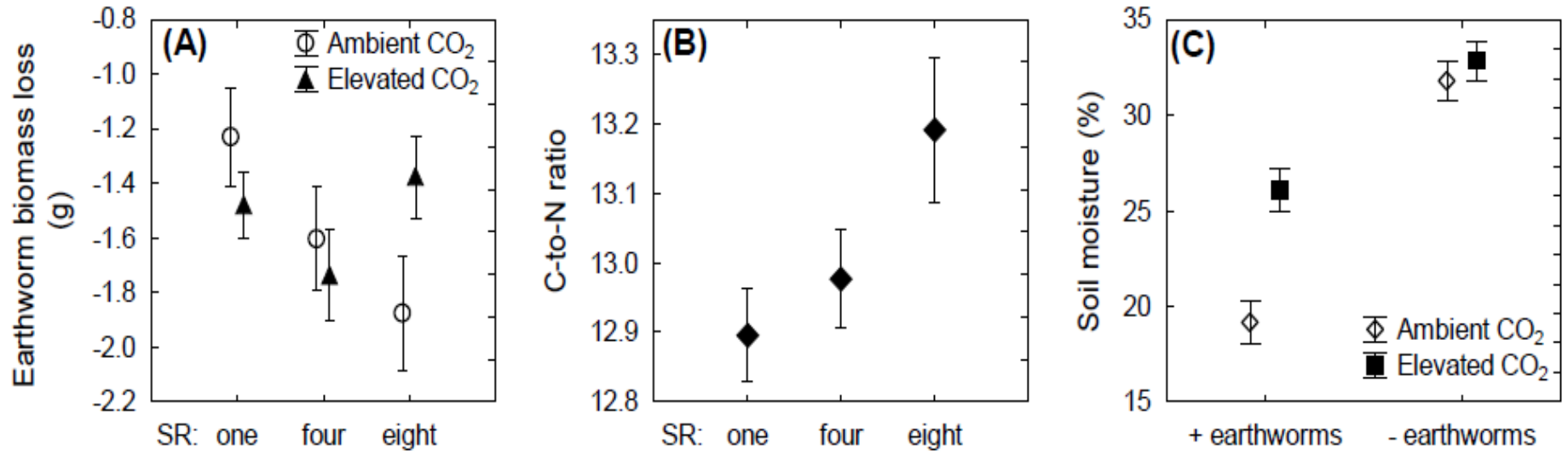
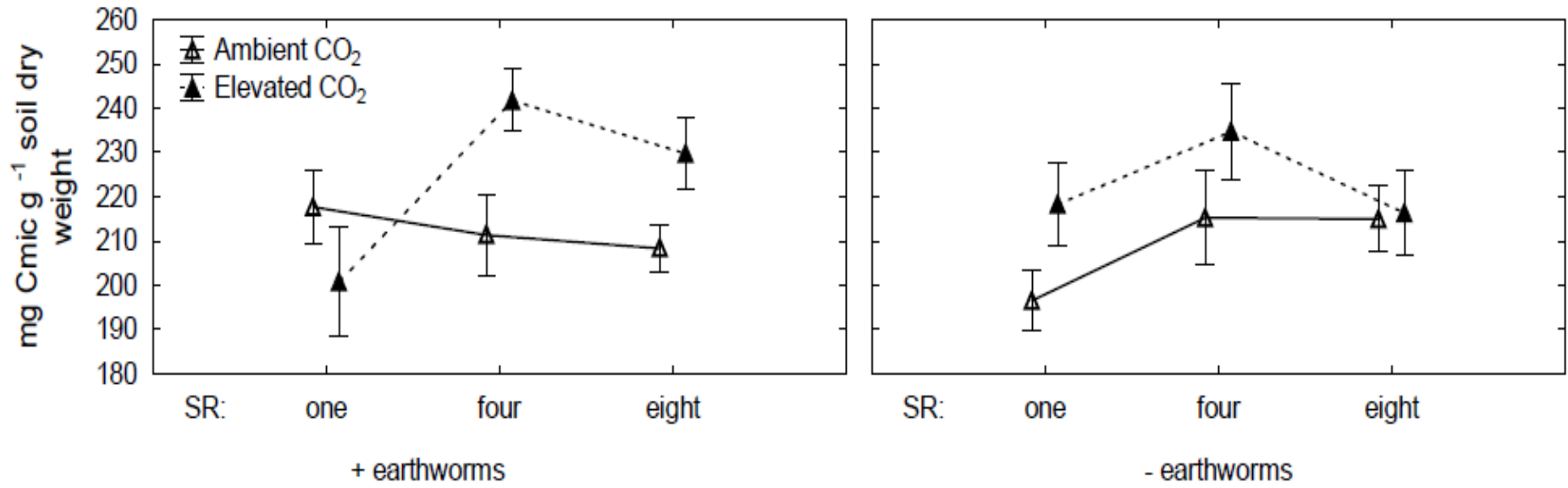


Figure 2



**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 perene</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 perene</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