1 **Title:** Response of temperate anecic earthworm individual biomass to species interactions

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14

Abstract

16   Earthworms contribute to a wide range of ecosystem services provided by the soil.

17 Nevertheless, synecology of these organisms is still not properly elucidated especially in terms of

18 species interactions. The aim of the present study was to determine the effects of anecic earthworm

19 species interactions on their individual biomass. These effects were measured using three epi-

20 anecic species, *Lumbricus rubellus rubellus* (Hoffmeister, 1843), *Lumbricus centralis* (Bouché,

21 1972), *Lumbricus terrestris* (Linné 1758), and three strict-anecic species, *Aporrectodea caliginosa*

22 *meridionalis* (Bouché 1972), *Aporrectodea nocturna* (Evans, 1946), *Aporrectodea giardi*

23   (Savigny, 1826). Twenty-one pairs of individuals were established following five assemblages:

24   monospecific pairwise assemblages of epi- and strict-anecic earthworms (2×3 treatments),

25   bispecific pairwise assemblages within epi- and within strict-anecic earthworms (2×3 treatments)

26   and bispecific pairwise assemblages with one epi- and one strict-anecic earthworm (3×3

27   treatments). Treatments were maintained in mesocosms for 30 days under controlled conditions

28   (food provided at the soil surface at the beginning of the experiment) and changes in the earthworm

29   individual biomass were measured. Strict-anecic earthworms in monospecific or bispecific

30   assemblages maintained their initial biomass. In contrast, epi-anecic earthworms exhibited an

31   increase of 12.4% and 23.7% of their biomass in monospecific and bispecific assemblages,

32   respectively. In bispecific assemblages combining one epi- and one strict-anecic earthworm, epi-

33   anecic earthworms solely gained biomass leading to a total increase of a 6.9%. Surprisingly, the

34   biomass' changes were not homogenous within the two sub-categories as the six earthworm species

35   exhibited species-specific responses. The greatest increases in individual biomass were recorded

36   for epi-anecic earthworms in the bispecific assemblages. This study provides further evidence for

37   the distinction between the two anecic sub-categories, as it indicates that species interactions is

38   positive only for epi-anecic earthworm biomass.

39

40   **1. Introduction**

41   Earthworms are widespread organisms and constitute the most important animal biomass

42   in European soil under temperate climates (Hole, 1981; Curry, 1994; Bar-On et al., 2018).

43   According to their physiology, morphology and behaviour, earthworms are classified into three

44   ecological categories: epigeic, endogeic and anecic species (Bouché, 1972, 1977). Nevertheless,

several studies highlighted that within anecic earthworms two ecological sub-categories can be identified based mainly on their feeding (Ferlian et al., 2014; Andriuzzi et al., 2016; Larsen et al., 2016) and burrowing (Bastardie et al., 2003) behaviours. Briefly, epi-anecic earthworms (*Lumbricus* sp.) feed preferentially on surface organic matter, mainly composed of leaf litter, which they can bury into their main permanent burrows. In contrast, strict-anecic earthworms (*Aporrectodea* sp.) feed on humified organic matter already incorporated into the soil with a slight proportion of leaf litter and they establish a denser burrow network than epi-anecic earthworms. Consequently, ecosystem services provided by earthworms belonging to these two anecic sub-categories are expected to be different.

In this context, improving our understanding of the assembly rules of anecic earthworm communities is important since (i) from one to six different anecic earthworm species can coexist in European soils under temperate climates (Poier and Richter, 1992; Murchie et al., 2015) and (ii) inoculations of anecic earthworms are frequently realised in order to enhance soil functions (Butt, 2008; Forey et al., 2018). Previous studies reported that interactions within anecic earthworms could either delay or increase their growth rate depending on the degree of niche overlap between species (Butt, 1998; Lowe and Butt, 1999, 2002; Uvarov, 2009). However, our knowledge about these interactions within and between epi- and strict-anecic earthworms is still limited.

The present study aimed to determine the effects of anecic earthworm interactions on individual biomass as a specific proxy of earthworm fitness (Butt, 1991; Butt et al., 1994). We measured changes in earthworm individual biomass after 30 days of experimentation with leaf litter placed at the soil surface to mimic natural leaf litter deposition. First, we hypothesized a loss of biomass in mono- and bispecific mixtures within each ecological sub-category due to resource

67 competition. Second, we hypothesized no change in individual biomass when combining one epi-

68 and one strict-anecic species due to their different feeding behavior (no niche overlap expected).

69

70 **2. Materials and methods**

71   We studied three strict-anecic earthworm species, *Aporrectodea caliginosa meridionalis*

72 (Bouché, 1972), *Aporrectodea nocturna* (Evans, 1946), *Aporrectodea giardi* (Savigny, 1826) and

73 three epi-anecic earthworm species, *Lumbricus rubellus rubellus* (Hoffmeister, 1843), *Lumbricus*

74 *centralis* (Bouché, 1972), *Lumbricus terrestris,* (Linné, 1758). We collected the soil (5-20 cm

75 depth), fresh leaf litter of *Lolium perenne* (Linné, 1753; a typical grassland species), and the six

76 anecic earthworm species from temporary grasslands in the Long Term Ecological Research

77 (LTER) site ''Zone Atelier Armorique'' (48°50' N, -1°58' W), Brittany, France. We determined

78 soil and leaf litter characteristics according to the protocols described in Hoeffner et al. (2018). We

79 identified the soil as a brown soil with 48.2% sand, 37.5% silt and 14.3% clay, characterized by

80 2.9% organic matter, a C:N ratio of 9.7 and a pH of 6.4. The leaf litter was characterized by a C:N

81 ratio of 13.9, a phenolic concentration of 3.4%, a water holding capacity of 392.6% and a specific

82 leaf area of 330.2 $cm^2.g^{-1}$.

83   Two weeks before the beginning of the experiment, we selected adult earthworms from the

84 six species, we grouped them in monospecific boxes containing the soil previously hand-sieved at

85 4-mm and fed them with air-dried leaves of *L. perenne*. Thirty-six hours before the start of the

86 experiment, we placed each earthworm on a moist sponge in a plastic box to void its gut content

87 (depuration).

4

88   We filled mesocosms (PVC cylinder, 30 cm high, 10 cm diameter) with 4.9 kg of fresh

89   sieved soil, placed 3.5 g of air-dried leaves of *L. perenne* at the soil surface to mimic the natural

90   leaf litter deposition and re-humidified them with deionized water. Then, we determined the

91   individual biomass of adult earthworms (i.e. presence of a turgid clitellum, to ensure taxonomic

92   identity) and placed them in the corresponding mesocosm. The six earthworm species presented an

93   initial mean biomass gradient varying from 0.62 g for *L. rubellus* to 4.72 g for *L. terrestris*

94   (Supplementary Fig. S1). We placed the mesocosms in a climatic chamber at 12 °C, with a relative

95   humidity of 85% and a 12 h: 12 h light: dark regime. From the six anecic earthworm species, we

96   established 21 pairs of individuals in five replicates following five assemblages (Supplementary

97   Fig. S2): monospecific pairwise assemblages of strict- and epi-anecic earthworms (2 sub-ecological

98   categories × 3 earthworm species treatments), bispecific pairwise assemblages within strict- and

99   within epi-anecic earthworms (2 sub-ecological categories × 3 earthworm species treatments) and

100   bispecific pairwise assemblages with one strict- and one epi-anecic earthworm (3 epi-anecic

101   species × 3 strict anecic species treatments). We maintained soil moisture by spraying deionized

102   water on the soil surface twice a week. Given the large number of mesocosms, we established

103   replicates of each treatment one day apart leading to five blocks of 21 mesocosms.

104   After 30 days, leaf litter was visually still available at the soil surface and we collected

105   earthworm individuals by destroying the mesocosms. We determined each individual earthworm

106   biomass after depuration, as previously described. We calculated the percentage of change in

107   earthworm individuals' biomass following the formula: ((final fresh biomass − initial fresh

108   biomass) / initial fresh biomass) × 100%. For monospecific assemblages, we assigned the biomass

109 of the lightest earthworm at the end of the experiment to the lightest one at the beginning of the

110 experiment.

111   We performed statistical analyses with the R software 3.2.3 (R. Core Team, 2017). We

112 evaluated significance in all cases at $P < 0.05$. Data met the conditions of normality and

113 homoscedasticity. We excluded four soil mesocosms that contained dead earthworms (from

114 random species and random assemblages) from the data analysis. First, we used a one-way

115 ANOVA, followed by a Tukey HSD test for post hoc pairwise comparisons, to assess differences

116 in initial biomass between the six earthworm species. Second, within the two ecological categories,

117 we used two-way ANOVAs to test for the effects of species assemblage and block on earthworm

118 biomass change. Third, we used two-way ANOVAs to test for the effects of species combinations

119 and block on the biomass change of the 6 studied species. Finally, we used separated one-sample

120 t-tests with adjusted P-values due to multiple comparisons to test whether (i) earthworm species

121 and (ii) earthworm assemblages significantly lost or gained biomass during the experiment.

122

123 **3. Results**

124   During the experiment, four earthworms over the 210 died. Whatever the treatment, the

125 surviving earthworms remained adults (with a turgid clitellum) until the end of the experiment.

126 Overall, strict anecic earthworms in mono- and bispecific pairwise assemblages within their sub-

127 category maintained their initial biomass during the experiment (Fig. 1A). In contrast, the biomass

128 of epi-anecic earthworms in mono- and bispecific pairwise assemblages within their sub-category

129 increased (Fig. 1B), and this increase was twice higher in bispecific compared to monospecific

130 assemblages (+23.7% *vs.* +12.4%, respectively, Fig. 1B). The biomass of earthworms in bispecific

131   assemblages combining one epi- and one strict-anecic was 6.9% higher than at the beginning of

132   the experiment, but this increase was solely due to the biomass gained by epi-anecic earthworms

133   (+17.0%, Fig. 1B).

134   The biomass' changes were not homogenous within the two anecic sub-categories as the

135   six earthworm species exhibited species-specific responses (Fig. 2). Contrary to the two other

136   strict-anecic species, *A. giardi* lost biomass in monospecific assemblage (-9.2%) as well as in

137   bispecific assemblages with *A. nocturna* (-7.3%), *L. rubellus* (-7.5%) and *L. terrestris* (-13.6%,

138   Fig. 2). While the biomass of *A. caliginosa meridionalis* and *A. nocturna* did not vary significantly

139   in both mono- and bispecific assemblages with an epi-anecic species, these two strict-anecic

140   species gained biomass in presence of *A. giardi* (+6.8% and +9.0% for *A. caliginosa meridionalis*

141   and *A. nocturna*, respectively, Fig. 2). Within the epi-anecic species, the biomass of *L. centralis*

142   only increased in presence of *A. caliginosa meridionalis* (Fig. 2), while *L. terrestris* gained biomass

143   whatever the pairwise assemblage considered (Fig. 2). Finally, the biomass of *L. rubellus* increased

144   in monospecific assemblage (+12.0%) as well as in bispecific assemblages with *L. centralis*

145   (+29.8), *A. nocturna* (+21.2%) and *A. caliginosa meridionalis* (+12.3%, Fig. 2).

146

147   **4. Discussion**

148   In contrast to our first hypothesis of a negative effect of species interaction on biomass

149   change in pairwise assemblages within each anecic ecological sub-category (Butt, 1998; Lowe and

150   Butt, 1999, 2002; Frazão et al., 2018), we did not observe any change in the biomass of strict-

151   anecic earthworms but a gain of biomass of epi-anecic earthworms. However, the organic resources

152   and the experimental time used in previous studies differed to those applied in the present study

153   (i.e. *Lolium perenne* and 30 days). For example, Lowe and Butt (1999) used separated cattle solids

154   during 84 days and Frazão et al. (2018) a mixture of *Triticum aestivum* and *Raphanus sativus*

155   during 61 days. Postma-Blaauw et al. (2006) observed that the assemblage of *L. rubellus* with *L.*

156   *terrestris* fed with *Solanum tuberosum* enhanced the bacterial biomass in their burrows. In addition,

157   Hoeffner et al. (2018) observed specific fungal communities within *L. rubellus*, *L. centralis* and *L.*

158   *terrestris* burrows fed with *L. perenne*. These previous observations of positive effects of epi-

159   anecic species on microbial communities could lead to enhanced leaf litter decomposition and then

160   to higher organic matter assimilation by epi-anecic earthworms (Hoeffner et al., 2018). To confirm

161   that these observed effects are due to the ecological category of earthworms and not the genus they

162   belong to, it would be interesting to study other genus within anecic earthworms such as

163   *Scherotheca*, *Octodrilus* or *Fitzingeria*.

164   Overall, the effect of the interaction between one strict- and one epi-anecic earthworm was

165   mainly additive confirming our second hypothesis of reduced niche overlap. Nevertheless, we

166   observed some gain or loss of biomass depending on the earthworm assemblages. It may be

167   assumed that other mechanisms, such as facilitation or competition for resource access and gallery

168   formation, could explain these differences and some additional experiments are required.

169   Within strict-anecic earthworms, *A. caliginosa meridionalis* and *A. nocturna* in

170   monospecific assemblages maintained their initial biomass while *A. giardi* lost biomass. These

171   findings are in agreement with previous studies that also reported no change in *A. caliginosa*

172   *meridionalis* biomass after 31 days when fed with *Castanea sativa* (Cortez and Bouché, 2001) and

173   a 38% loss of biomass of *A. giardi* after 31 days when fed with *Triticum aestivum* (Cortez et al.,

174   1989). Overall, epi-anecic earthworms in monospecific assemblages gain biomass (Shipitalo et al.,

175   1988; Binet and Trehen, 1992; Hoeffner et al., 2018). For example, Shipitalo et al. (1988) reported

176   a 100% and a 35% increase in biomass of *L. rubellus* and *L. terrestris* when fed with *Medicago*

177   *sativa* litter after 32 and 36 days of experimentation, respectively. Thus, the present study confirms

178   the two distinct behaviours to process the leaf litter of strict- and epi-anecic earthworms and

179   provides support to this sub-category distinction. In fact, feeding on leaf litter at the surface by epi-

180   anecic earthworms (Larsen et al., 2016; Hoeffner et al., 2018, 2019) allowed them to increase their

181   biomass with our experimental conditions. In contrast, strict-anecic earthworms by feeding mainly

182   on soil organic matter had restricted access to organic matter (2.9% of soil content). Additionally,

183   we also cannot exclude that soil organic matter consumption by strict-anecic earthworms was

184   restricted due to the low soil volume of 1571 cm^{-3} in the experimental mesocosms.

185   Finally, the positive species interaction between epi-anecic species highlighted in our lab

186   experiment could partly explain field species assemblages reported in previous studies (Decaëns et

187   al., 2008; Murchie et al., 2015). For example, Murchie et al., (2015) observed three epi-anecic

188   species and only one strict-anecic species in a same temperate grassland suggesting facilitation

189   within epi-anecic species. Furthermore, Decaëns et al. (2008) observed a higher co-occurrence rate

190   between epi-anecic species than between strict anecic species in twenty temperate grasslands.

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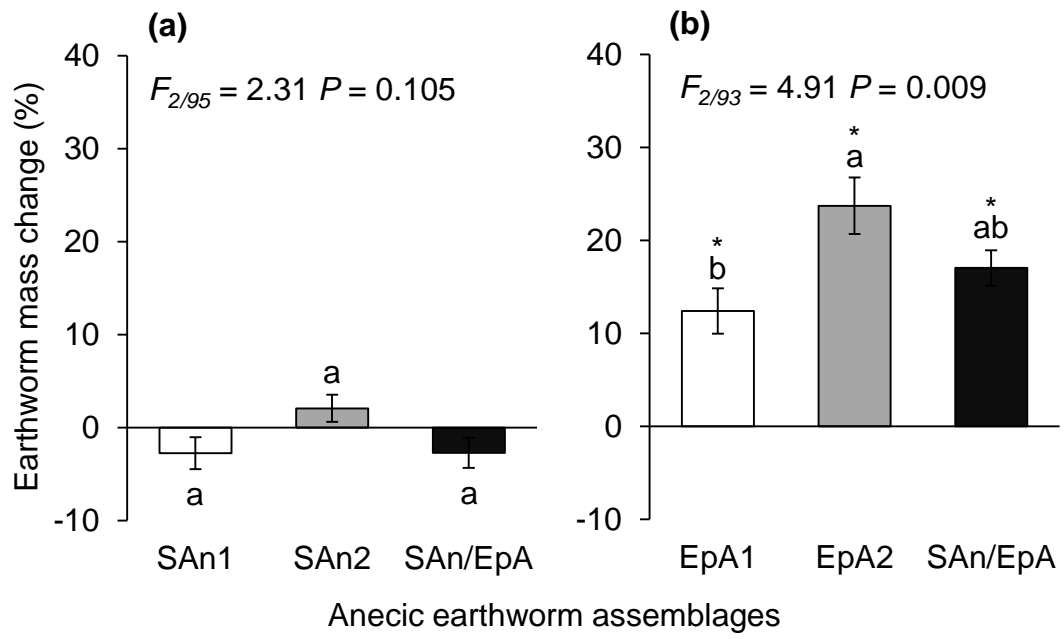
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270 **Figure legends**

271 **Fig. 1.** Earthworm biomass changes according to anecic earthworm assemblages of (a) strict- and

272 (b) epi-anecic earthworms. Values are means ± SD. Different letters denote significant differences

273 among earthworm assemblages with a > b (post hoc Tukey test results). Biomass changes

274 significantly different from 0 are indicated with a star. SAn1 = Monospecific assemblages of strict-

275 anecic, n= 28; SAn2 = Bispecific assemblages of strict-anecic, n= 30; SAn/EpA = Bispecific

276 assemblages with one strict- and one epi-anecic, n= 44; EpA1 = Monospecific assemblages of epi-

277 anecic, n= 30; and EpA2 = Bispecific assemblages of epi-anecic, n= 26.

278

279

280 **Fig. 2.** Biomass changes of earthworms in mono- and bispecific pairs of AM, AN, AG, LR, LC

281 and LT. Values are means ± SD, n = 4 to 10. Biomass changes significantly different from 0 are

282 indicated with a star. AM = *A. caliginosa meridionalis*; AN = *A. nocturna*; AG = *A. giardi*; LR =

283 *L. rubellus rubellus*; LC = *L. centralis*; LT = *L. terrestris*.

284   **Figure 1**

(a) $F_{2/95} = 2.31$ $P = 0.105$

(b) $F_{2/93} = 4.91$ $P = 0.009$

Earthworm mass change (%)

Anecic earthworm assemblages

SAn1   SAn2   SAn/EpA

EpA1   EpA2   SAn/EpA

285

Figure 2