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1     **Transfer of sulfidized silver from silver nanoparticles, in sewage sludge, to plants**  
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19 **Abstract**

20 Consumer products containing silver nanoparticles (AgNPs) release silver (Ag) to the  
21 environment, particularly wastewater. Sewage sludge (SS), which contains numerous  
22 contaminants including Ag, is recycled by spreading on agricultural land. Although slight  
23 impacts and bioaccumulation of Ag sulfide (Ag<sub>2</sub>S, the main species found in SS) in terrestrial  
24 organisms have been demonstrated, possible trophic transfer into plants and subsequently  
25 animal species has not been examined. Accordingly, the present study experimentally  
26 measured the transfer of Ag from AgNPs and sulfidized Ag into plants and primary  
27 consumers and compared their bioavailability. Nine plant cultivars were grown in soil mixed  
28 with SS containing Ag, which revealed that bioaccumulation of Ag by plants is species-  
29 dependent. Ryegrass (the plant species with the greatest accumulation – up to 0.2 mg kg<sup>-1</sup>)  
30 was then cultivated on a larger scale to expose snails and locusts for several weeks. While  
31 locusts did not accumulate Ag after two weeks of exposure, snails exhibited Ag  
32 bioaccumulation after 5 weeks when soil was accessible. Sulfidized Ag derived from AgNPs  
33 were less available (bioaccumulation up to 2.5 mg kg<sup>-1</sup>) than the Ag from the original AgNPs  
34 (bioaccumulation up to 15 mg kg<sup>-1</sup>). This transfer potential of Ag could have consequences  
35 for food webs due to chronic exposure linked to SS spreading practices. This study shows that  
36 transformations of AgNPs in treatment plants attenuate but do not completely eliminate the  
37 risk of Ag to plant and animal species SS.

38

39 **Keywords**

40 Sulfidized silver, ryegrass, snails, locusts, food chain, bioaccumulation

## 41 **1. Introduction**

42 The various properties of silver nanoparticles (AgNPs) most notably biocidal action  
43 (Reidy et al., 2013), have led to their widespread use in the pharmaceutical, cosmetic, textile,  
44 electronics and optics industries, among many other applications (European commission,  
45 2012, 2014; McGillicuddy et al., 2017). Throughout their life cycles, nanofunctionalized  
46 consumer products containing AgNPs release Ag into the environment and especially  
47 wastewater (Adam et al., 2018; Donner et al., 2015). Silver (Ag) is mostly trapped in  
48 biosolids thanks to the efficiency of wastewater treatment methods (Kaegi et al., 2011; Tiede  
49 et al., 2010). However, these biosolids, which contain between 1 and 20 mg kg<sup>-1</sup> of silver (Ag)  
50 on average (depending on the source: Gottschalk et al., 2013; U.S. EPA, 2009) and maximum  
51 concentrations up to 850 mg kg<sup>-1</sup>, are often spread on agricultural fields as fertilizer  
52 (European commission, 2017; Eurostat, 2017). According to a scenario analyzed by Blaser et  
53 al. (2008) an estimated 140 tons of Ag enter agricultural soils each year due to the spreading  
54 of sewage sludge (SS) in the European Union alone.

55 In response to these practices, the toxicity and ecotoxicity of Ag have been widely  
56 studied. Most of these works have studied Ag ions or intact AgNPs but it is now known that  
57 AgNPs released in wastewater undergo transformations in sewers and during the biosolid  
58 treatment (Levard et al., 2012; Ma et al., 2014; Pradas del Real et al., 2016). Indeed, AgNPs  
59 and Ag<sup>+</sup> ions can react with sulfur to give silver sulfide (Ag<sub>2</sub>S) (Sekine et al., 2015). It is  
60 precisely Ag<sub>2</sub>S that is the predominant Ag species in SS, which has different properties than  
61 AgNPs or Ag<sup>+</sup> (Levard et al., 2013). Another previous study showed that the speciation of Ag  
62 after anaerobic digestion was Ag<sub>2</sub>S and Ag linked to thiols, that is to say two sulfidized forms  
63 of silver (Courtois et al., 2020). These Ag species have received less attention, and studies  
64 using SS containing realistic amounts of sulfidized Ag are scarce (Courtois et al., 2019).

65 Under simplified conditions, Ag can be bioaccumulated by plants (Geisler-Lee et al.,  
66 2012, 2014; Kaveh et al., 2013; Nair and Chung, 2014; Rui et al., 2017; Song et al., 2013;  
67 Stampoulis et al., 2009; Yasur and Rani, 2013) and animal species like nematodes (Luo et al.,  
68 2016) and earthworms (Baccaro et al., 2018; Diez-Ortiz et al., 2015). Studies performed under  
69 more realistic conditions have also shown an Ag accumulation in species like woodlouse  
70 (Kampe et al., 2018) or earthworms (Lahive et al., 2017; Velicogna et al., 2017). Studies  
71 under realistic conditions have also reported that the accumulation of Ag from sulfidized Ag  
72 in plants was lower than that of Ag from AgNPs but was still significant (Doolette et al.,  
73 2015; Pradas del Real et al., 2016, 2017; Stegemeier et al., 2015; Wang et al., 2015). These  
74 preliminary results are worrying and suggest that Ag could transfer up the food chain and  
75 reach a wide range of animal species.

76 For the first time, the bioavailability and the toxicity of various forms of Ag to a plant  
77 species and 2 soil invertebrates will be compared in a trophic chain experiment in order to  
78 assess the transfer of sulfidized Ag (Ag<sub>2</sub>S and Ag linked to thiols) from soil to plants and two  
79 complementary primary consumers. Small herbivorous invertebrates that are staple foods for  
80 many small carnivores and omnivores, including humans : the terrestrial snail *Cantareus*  
81 *aspersus* and the locust *Locusta migratoria* (Barker and Watts, 2002; Poma et al., 2017).  
82 These two species are complementary: one is a soft body invertebrate in close contact with  
83 soil, whereas the other has a cuticle and spends most of its life on plant cover. During this  
84 experiment, the Ag content in soil, plants and animals was quantified and vital parameters  
85 such as biomass and survival were monitored.

86

## 87 **2. Materials and methods**

### 88 ***2.1 Soil***

89 Soil from organic agriculture was collected from the Haut de France region (GPS  
90 coordinates: 50°35'58.1"N 3°09'01.5"E) in January 2019 for the preliminary experiment and  
91 in September 2019 for the main experiment. The soil is a slightly leached to calcareous  
92 (presence of chalk granules) brown soil developed on wind-blown silts on chalky substrate,  
93 shallow to deep (soil map by DRAAF, 2013) classified as Luvisol (LV) according to WRB  
94 (World Reference Base) (Food and Agriculture Organization of the United Nations, 2015).  
95 For both experiments, the first 20 cm of soil was collected a few days before the beginning of  
96 the experiment. The soil was not dried in order to preserve the natural microbiota. To remove  
97 stones and roots and to homogenize and aerate the soil, the soil was sieved wet with a 5 mm  
98 mesh. The pH was and approximately  $6.76 \pm 0.01$  (measured in KCl 1M according to ISO  
99 10390:2005) and the total carbon and the dissolved organic carbon were  $15.44 \pm 1.34$  mg g<sup>-1</sup>  
100 and  $27.31 \pm 1.34$  mg L<sup>-1</sup>. The characterized metal content of the dry soil was:  $0.53 \pm 0.34$  mg  
101 kg<sup>-1</sup> Ag,  $0.32 \pm 0.06$  mg kg<sup>-1</sup> cadmium (Cd),  $18.68 \pm 0.36$  mg kg<sup>-1</sup> copper (Cu),  $336.18 \pm 9.83$   
102 mg kg<sup>-1</sup> manganese (Mn),  $11.45 \pm 0.17$  mg kg<sup>-1</sup> nickel (Ni),  $52.73 \pm 1.61$  mg kg<sup>-1</sup> lead (Pb),  
103  $49.78 \pm 0.40$  mg kg<sup>-1</sup> zinc (Zn) (method described in 2.7.2).

104

## 105 ***2.2 Sewage sludge***

106 Four wastewater treatment plants (WWTPs) situated in southern Poland were pre-  
107 selected in order to monitor the content of Ag during a two-year period. Among the four  
108 facilities, the facility that produced SS with the lowest level of contamination in metals  
109 (silver, arsenic, cadmium, copper, manganese, nickel, lead) and a preferable C:N ratio was  
110 selected. According to a previous study, SS from the selected WWTP is a good source of  
111 nutrients for earthworms without inducing stress related to the presence of contaminants  
112 (Suleiman et al., 2017). The selected WWTP (Poland, GPS coordinates: 50°55'22.81  
113 19°07'10.41) is a small-sized plant that uses activated sludge technology to support an

114 agricultural area (flow: 1,000 m<sup>3</sup>/d, population equivalents: 20,000). The metal content of the  
115 dry SS was as follows: 11.53 ± 1.43 mg kg<sup>-1</sup> Ag, 0.95 ± 0.22 mg kg<sup>-1</sup> arsenic (As), 1.10 ± 0.24  
116 mg kg<sup>-1</sup> Cd, 140.62 ± 22.55 mg kg<sup>-1</sup> chromium (Cr), 145.43 ± 37.38 mg kg<sup>-1</sup> Cu, 186.78 ±  
117 59.13 mg kg<sup>-1</sup> Mn, 19.11 ± 9.02 mg kg<sup>-1</sup> Ni, 32.21 ± 5.86 mg kg<sup>-1</sup> Pb, and 2510.36 ± 615.99  
118 mg kg<sup>-1</sup> Zn (average based on measurements between March and December 2016, according  
119 to the method described in 2.7.2.). Before agricultural reuse, anaerobic stabilization of the SS  
120 was performed at laboratory scale.

121

### 122 *2.3 Silver species*

123 The AgNPs used for this study were the standard reference materials Ag NM300K  
124 from the European Commission Joint Research Centre (JRC), which have been fully  
125 characterized (Klein et al., 2011). These nanoparticles are spherical and are formulated as a  
126 colloidal dispersion with a nominal Ag content of 10.2% by weight, dispersed in 4% w/w  
127 each of polyoxyethylene glycerol trioleate and polyoxyethylene (20) sorbitan mono-laurate  
128 (Tween 20). The Tween 20, the dispersant solution of AgNPs, will be called “Dispersant” or  
129 “Dis” throughout the rest of the manuscript. The nominal size of 99% of the particles is  
130 approximately 15 nm, without coating. Transmission electron microscopy indicated a size of  
131 17 ± 8 nm. Smaller nanoparticles of approximately 5 nm are also present (Mendes et al.,  
132 2015). The size distribution determinations by using zeta-sizer analysis resulted in about 100  
133 nm (Klein et al., 2011). The NM300K nanoparticles were kindly provided by the Fraunhofer  
134 Institute for Molecular Biology and Applied Ecology IME (Schmallenberg, Germany). Each  
135 bottle (lot) contained approximately 2 g of NM300K diluted in dispersant corresponding to a  
136 volume of 2 mL. This solution contained 10% (w/w) Ag (0.1 g of Ag per 1 mL).



137 A solution of AgNO<sub>3</sub> was prepared by dissolving AgNO<sub>3</sub> powder in milliQ water,  
138 taking into account the molar mass of each atom so that the concentration obtained  
139 corresponds well to a concentration of Ag and not of AgNO<sub>3</sub>.

140 These two solutions of nanoparticles and silver ions were diluted with milliQ water to  
141 obtain Ag concentrations of approximately 2 mg ml<sup>-1</sup>.

142

## 143 **2.4 Plant species**

### 144 *2.4.1 Preliminary experiment*

145 Nine plant cultivars (of which two cultivars were the same species) were used in this  
146 study: turnip, cabbage, hemp, lettuce, ryegrass cultivar 1 (calao variety), ryegrass cultivar 2  
147 (bocage variety), radish, Dutch clover and wheat. These species were chosen because they are  
148 consumed by humans or livestock, and can either be cultivated directly after sewage sludge  
149 spreading, or after a rotation of land use where the spreading of sewage sludge has taken place  
150 previous years, either are conventionally studied in the laboratory. *Brassica napus* (Emerald  
151 variety, reference 9273), *Brassica oleracea* (Proteor variety, ref. 4890), *Lactuca sativa* (Pierre  
152 Benite variety, ref. 5775) and *Raphanus sativus* (“18 days” variety, ref. 7190) seeds were  
153 obtained from Baumaux Seeds Company (<https://www.graines-baumaux.fr/>). These seeds  
154 were untreated for conventional cultivation. *Cannabis sativa* (Futura 75 variety) seeds were  
155 obtained from HEMP it (<https://hemp-it.coop/>). *Lolium perenne* variety *calao* seeds were  
156 from Semences de France (<https://www.semencesdefrance.com/>). *Lolium perenne* variety  
157 *bocage* and *Trifolium repens* seeds were from Carneau (<http://carneau.fr/>). *Triticum aestivum*  
158 *L.* (L., KWS Mistral variety) seeds were from Florimond Desprez ([https://www.florimond-](https://www.florimond-desprez.com/)  
159 [desprez.com/](https://www.florimond-desprez.com/)).

160

### 161 *2.4.2 Main experiment*

162 For this experiment, ryegrass cultivar 2 (*Lolium perenne*, bocage variety) was selected  
163 as the plant species, as explained in the results. The seeds used in the main experiment were  
164 from the same batch used for the preliminary experiment.

165

## 166 ***2.5 Invertebrate species for the main experiment***

167 Two species at the bottom of many food chains and widely consumed by humans in  
168 several regions of the world were studied (Barker and Watts, 2002; Poma et al., 2017). The  
169 first tested species was *Cantareus aspersus* (ex *Helix aspersa*), a phytophagous and  
170 detritivorous pulmonate gastropod mollusk, known to accumulate some metallic trace  
171 elements and is even used as an indicator of habitat contamination (Baroudi et al., 2020;  
172 Beeby, 1985; Carbone and Faggio, 2019; De Vaufleury, 2015; Hopkin, 1989). This species is  
173 native to the peri-Mediterranean area and occupies a wide range of habitats worldwide (ISO  
174 15952:2018). The snails used in this study were from the farm of laboratory Chono-  
175 Environment (University of Franche-Comté, Besançon, France) and were raised as described  
176 by Gomot de Vaufleury (2000). Juvenile individuals weighing  $995.7 \pm 214.6$  mg ( $n = 369$ )  
177 were used. The second tested species was *Locusta migratoria*, a phytophagous orthopteric  
178 arthropod found almost everywhere in the world (Jarwar et al., 2019; Zhang et al., 2019),  
179 which has been less studied in this context. Few studies have shown the capacity of this  
180 member of Orthoptera to accumulate Cd and Pb (Devkota and Schmidt, 2000; Zhang et al.,  
181 2012). Sub-adult locusts were purchased online from La ferme aux insectes (Gng distribution  
182 sarl, Crevecoeur le Grand, France) (<https://www.lafermeauxinsectes.com/fr/>). Upon delivery,  
183 individuals were housed under observation and depuration for 12 h before installation in the  
184 mesocosms, that is, 21 days after the addition of snails in the mesocosms, and 7 weeks after  
185 plant seeding. The average weight of the locusts was  $646.6 \pm 84.0$  mg ( $n = 194$ ). These two

186 species are complementary: one is a soft body invertebrate in close contact with soil, whereas  
 187 the other has a cuticle and spends most of its life on plant cover.

188 The metal contents of both species were measured at the beginning of the mesocosm  
 189 experiment (Table 1).

190

191 *Table 1: Metals contents in animals (whole locusts and snails without shell) at the initial time*  
 192 *point (after fasting) in mg kg<sup>-1</sup> (dry matter). <LOQ means under limit of quantification i.e.*  
 193 *<0.4 μg L<sup>-1</sup> for Ag, <1,9 μg L<sup>-1</sup> for Pb and < 6,7 μg L<sup>-1</sup> for Sb.*

	Ag	As	Cd	Co	Cr	Cu	Hg	Mn	Mo	Ni	Pb	Sb	Zn
<i>C. aspersus</i>	<LO	0.828	0.760	1.100	1.828	152.8	0.058	92.20	0.640	1.924	<LO	0.013	310.9
	Q	±	±	±	±	36 ±	±	8 ±	±	±	Q	±	78 ±
		0.129	0.015	0.084	0.136	42.23	0.100	14.06	0.022	0.042		0.022	37.82
						1		6					8
<i>L. migratoria</i>	<LO	0.825	0.045	0.043	1.622	44.00	0.024	6.688	1.357	1.116	0.128	<LO	130.0
	Q	±	±	±	±	1 ±	±	±	±	±	±	Q	64 ±
		0.122	0.041	0.038	1.540	2.573	0.042	1.600	0.865	0.865	0.201		10.12
													7

194

## 195 **2.6. Experimental scheme of the two-step mesocosm experiment**

### 196 **2.6.1. Anaerobic digestion of sewage sludge**

197 A batch anaerobic digestion (AD) of SS was performed in four continuous stirred-tank  
 198 bioreactors at the beginning of 2017. These bioreactors were glass vats filled with 6 L of SS  
 199 maintained under mesophilic conditions at a temperature of 37 °C with constant mixing (180  
 200 rpm) using a mechanical stirrer. Details of the equipment as well as the methods of analysis of  
 201 pH, volatile fatty acids, volatile solids, total solids and ammonium nitrogen were described  
 202 previously (Grosser, 2017).

203 In one bioreactor, 6 L of SS was digested without any addition. In a second bioreactor, the SS  
 204 was spiked with 240 mg of AgNPs NM300K. In a third bioreactor, the SS was spiked with the  
 205 corresponding quantity of dispersant used to suspend the AgNPs, and in a fourth bioreactor,  
 206 the SS was spiked with 240 mg of AgNO<sub>3</sub>. The chosen Ag concentration (40 mg L<sup>-1</sup>) in the  
 207 bioreactors is justified in Supplemental information 1.

208 After 31 days of AD, the process had stabilized, the bioreactors were stopped, and the 4  
209 digestates were collected and centrifuged at 12100 rcf for 15 minutes. The SS was frozen until  
210 use and was thawed slowly at 8 °C for 3 days before use.

211 Tiede et al. (2010) and Kaegi et al. (2011) showed that SS treatment traps approximately 90%  
212 of Ag in biosolids. In our study, approximately 216 mg of Ag (of the 240 mg initially added)  
213 should have remained in the SS. After AD, each bioreactor contained approximately 950 g of  
214 fresh SS. The Ag concentration in the fresh SS was therefore estimated to be approximately  
215 227 mg kg<sup>-1</sup> (calculation: [(0.9\*240)\*1000]/950 ).

216

### 217 2.6.2. *Experimental design of preliminary exposure*

218 The preliminary experiment consisted of Ag exposure of several plant species in order  
219 to observe the accumulation behaviors in each plant.

220 The 9 plant cultivars (of which two cultivars were the same species) were grown in a mixture of  
221 the agricultural soil and SS digested with AgNO<sub>3</sub>. The mixture contained 19.9 kg of  
222 agricultural fresh soil (water content: 18.3 %) and 1.27 kg of thawed SS (water content 76.7  
223 %) (i.e. 60 g of SS per kg of final mixture, justification in Sup. Inf. 2). The mixture was  
224 stirred frequently each day for 7 days and sifted immediately before distribution into boxes to  
225 ensure a relatively powdery soil facilitating seed germination. AgNO<sub>3</sub>, like AgNPs, can react  
226 with sulfur and give Ag<sub>2</sub>S and Ag linked to thiols molecules. In a previous experiment, we  
227 were able to verify that the Ag<sup>+</sup> ions added to sewage sludge did indeed transform into Ag<sub>2</sub>S  
228 and Ag linked to thiols molecules during anaerobic fermentation (Courtois et al., 2020).

229 Each plant cultivar was sown in 3 boxes to establish triplicates. Each box (17 cm x 13 cm x 5  
230 cm) contained 740 g of fresh mixture. An identical number of seeds was sown in each of the 3  
231 replicate boxes, but this number varied according to the characteristics of the species,  
232 specifically, plant size and acceptable plant density (Sup. Inf 3).

233 The plants were grown for 49 days in greenhouses (temperature maintained at 20 °C, with 16  
234 h of light and 8 h of darkness). Watering varied according to the needs of the boxes but the  
235 triplicates of one plant cultivar received the same quantity of water.

236

### 237 2.6.3. Experimental design of main exposure

238 *Lolium perenne* cv. *bocage* seeds were sown in 6 different treatments (Figure 1):

239 (a) 3 without SS: soil only (mesocosms named “Control”), soil soaked with dispersant  
240 (“Dis”), and soil soaked with AgNPs dispersed in dispersant (“AgNPs”), and

241 (b) 3 with digested SS: SS only (“AD-control”), SS with dispersant (“AD-dis”), and SS with  
242 AgNPs (“AD-AgNPs”).

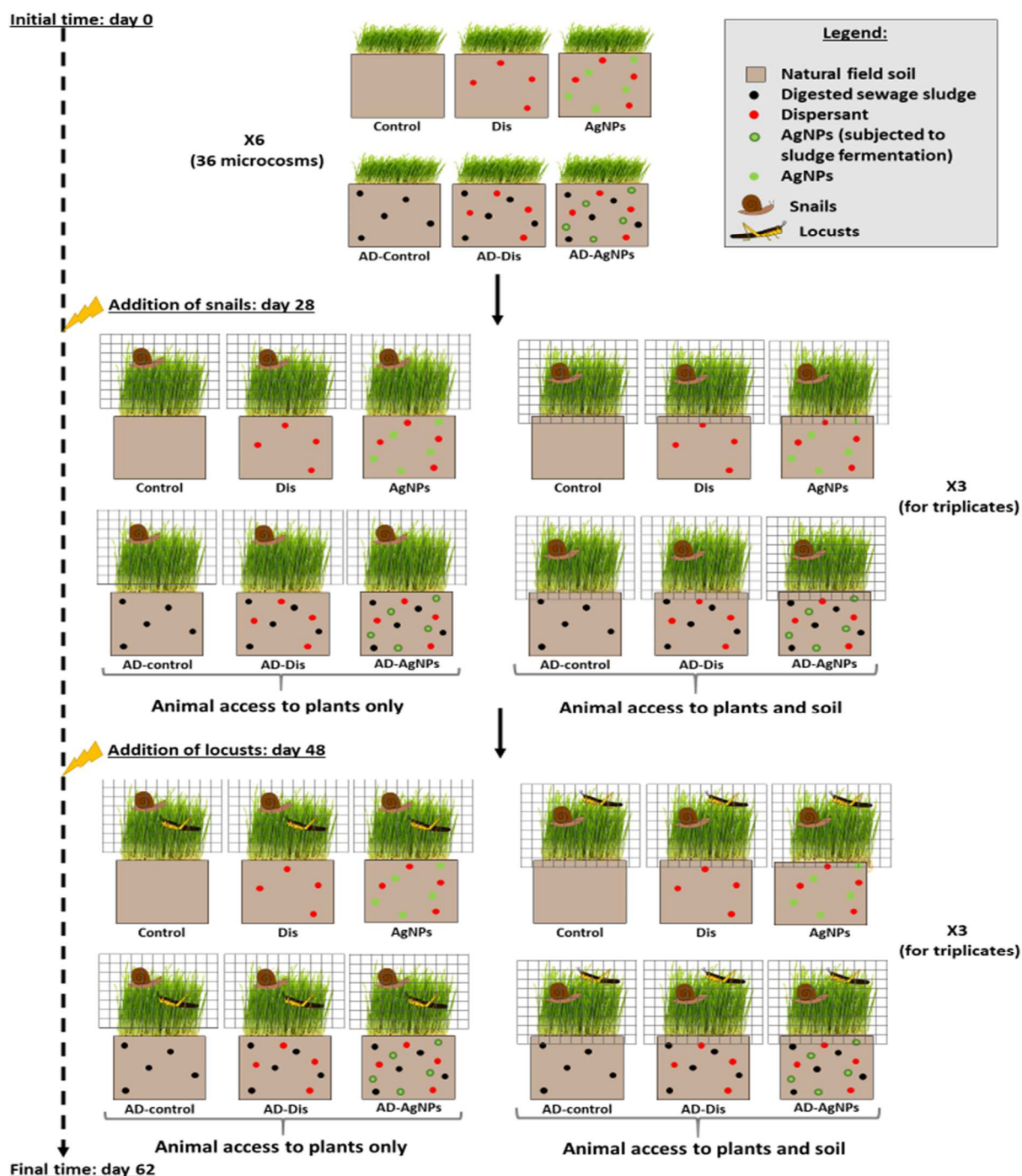
243 The proportions of SS and soil were identical to the preliminary exposure (60 g of fresh SS  
244 for 1 kg of mixture). The mixtures with SS contained 12.847 kg of agricultural wet soil and  
245 820 g of thawed SS. The mixtures without SS contained 13.572 kg of agricultural wet soil and  
246 95 mL of solution (AgNPs solution, dispersant solution or milli Q water). Thus, the amount of  
247 dispersant or AgNPs was the same in the conditions with and without SS.

248 The mixtures were stirred frequently each day for 7 days and then sifted immediately before  
249 distribution in boxes to ensure a relatively powdery soil facilitating seed germination. These 6  
250 types of mixtures were used to prepare 6 mesocosms containing exactly 2.000 kg of fresh  
251 mixture (rectangle plastic boxes of 29 cm x 14 cm x 10 cm). The use of larger boxes in a  
252 much sunnier period (for natural light) was intended to avoid these two potential sources of  
253 stress observed during the preliminary experiment (see the results 3.1.).

254 In half of the replicates, cages allowed the animals access to soil and plants. In the other half  
255 of the replicates, a net positioned 3-4 cm above the soil limited the animals’ access to plants  
256 only.

257 An identical mass of seeds was placed in each box ( $1.500 \pm 0.001$  g of seeds per box). The  
258 plants were grown for 28 days in greenhouses (temperature maintained at 20 °C with 16 h of  
259 light and 8 h of darkness and approximately 50% air humidity). Daily watering with rainwater  
260 was identical for all boxes.

261 After these 28 days of growth, 10 snails were added to each mesocosm. After 48 days of plant  
262 growth (20 days after adding snails), 5 locusts were added to each mesocosm. The addition of  
263 locusts to the mesocosms was purposely shifted in time to ensure that the snails were exposed  
264 for a sufficient time: we expected that the locusts, which are known to be voracious, would  
265 quickly consume the vegetation. The experiment was stopped when the quantity of plants  
266 became too small to continue. In total, the snails were exposed to these conditions for 34 days  
267 and cohabitated with the locusts for the last 14 of these 34 days. In each mesocosm, a small  
268 opaque dark plastic flowerpot cut in half served as a shelter for the animals.



270

271 *Figure 1: Scheme of the main experimental design. The “Control”, “Dis” and “AgNPs”*  
 272 *conditions correspond to soil only, soil mixed with dispersant solution or soil mixed with*  
 273 *AgNPs NM300K solution, respectively. The “AD-control” condition corresponds to soil*  
 274 *mixed with sewage sludge digested without any added Ag. The “AD-dis” and “AD-AgNPs”*  
 275 *conditions correspond to soil mixed with sewage sludge supplemented with either a*  
 276 *dispersant solution or a solution of AgNPs NM300K before anaerobic digestion.*

277

278 **2.7. Analyses**

279 *2.7.1 Life traits of plants and animals*

280 The appearance and size of the plant leaves were analyzed only visually, throughout  
281 the two experiments.

282 For animals, the following life traits were measured in the main experiment:

283 - The survival of the snails and locusts was observed by counting.

284 - Groups of 10 snails or 5 locusts were weighed before and after exposure, just after initial or  
285 final fasting. Before and after exposure, the animals were fasted 24 h in empty clean  
286 containers without food to empty the gut. Evolution of biomass (measured just after fasting)  
287 was evaluated by calculating the percentage of weight lost/gained by the group in one  
288 mesocosm.

289 - During the final fasting, feces of snails and locusts were collected to provide an estimate of  
290 food consumption. Feces were dried for 48 h in an oven at 60 °C and weighed. The quantity  
291 of feces produced during fasting was divided by the number of animals present in the box at  
292 the end of the experiment (which varied depending on mortality in the mesocosms). This  
293 parameter reflects the consumption (plant and/or soil) of the animals a few hours/days before  
294 the end of exposure.

295

296 *2.7.2 Metal content in sewage sludge, soil, plants and invertebrates.*

297 For both experiments (preliminary and main), soil samples were collected at the  
298 beginning of exposure and dried at 80 °C until total dehydration (stabilization of the weight).

299 The dried samples were ground with a mortar and pestle.

300 Leaves of plants were also analyzed in both experiments. For the preliminary experiment, 3  
301 samples of leaves of each species were collected at three time points: day 21 (T1), day 35  
302 (T2) and day 49 (T3). For the main experiment, leaves were collected at 28 days (initial time



303 for snails) and at 62 days (final time) of exposure. The leaves were rinsed several times with  
304 milli Q water, wiped, dried in an oven at 40 °C until total dehydration and crushed.  
305 Invertebrates were analyzed only in the main experiment. At the beginning of the experiment,  
306 several animals were frozen at -80 °C just after the initial fasting. At the final time point,  
307 when the number of survivors allowed, 6 snails and 3 locusts per mesocosm were frozen at -  
308 80 °C (just after final fasting). The snails were removed from their shell, and then whole  
309 locusts and snails (without shells) were dried separately in an oven at 60 °C until reaching a  
310 constant weight and ground to a powder using a mortar and pestle. In addition, for 1-3 snails  
311 of each Ag condition (number depending on mortality), the shell, foot and visceral mass were  
312 separated after thawing according to the method of Gomot de Vaufleury and Pihan (2002).  
313 Some studies have shown that the digestive gland is the main site for storing metals in snails  
314 (Hopkin, 1989; Pauget et al., 2015). The viscera and foot were separately dried and ground to  
315 powder in the same way as the intact bodies. No replicates could be made for this last  
316 measurement. As little data is available in snails for the bioaccumulation and distribution of  
317 Ag between the foot and the visceral mass, these two parts were analyzed separately. It was a  
318 first attempt to see if Ag uptake and subsequent distribution in snail tissues varied depending  
319 on the sources of exposure (plant or soil and plant). Due to the small size of the NPs, it was  
320 predicted that for snails exposed to soil, dermal transfer of Ag could occur.

321 For the preliminary experiment, soil mineralization was performed by digesting 500  
322 mg of soil in aqua regia (HNO<sub>3</sub>:HCl 2:5, v:v ultratracés, Optima®, Fisher Scientific, Illkirch,  
323 France). The resulting solutions were analyzed by ICP-MS (inductively coupled plasma mass  
324 spectrometry, Thermo Scientific X Series II, Courtaboeuf, France) to quantify As, Cd, Co, Cr,  
325 Cu, Mn, Ni, Pb and Zn; Ag was quantified by ICP-AES (inductively coupled plasma atomic  
326 emission spectroscopy, ICAP 6500 Radial de Thermo Scientific, Courtaboeuf, France). A  
327 certified material (loamy clay 1, CRM052, LGC Standards, Molsheim, France) was employed

328 the same day with both instruments for verification and gave recovery rates of 85 % to 120%  
329 for all metallic elements. For the main experiment, 300 mg of sample was digested in 7 mL of  
330 concentrated HNO<sub>3</sub> using a Berghof microwave digestion system (speed wave MWS-2-  
331 Microwave pressure digestion, Berghof, Eningen, Germany), and same metals were analyzed  
332 by ICP-OES (inductively coupled plasma optical emission spectrometry, Thermo Scientific,  
333 Courtaboeuf, France). The protocol used to analyze the metal contents in the sewage sludge  
334 monthly during the year 2016 was exactly the same than the one for the main experience.  
335 For mineralization of plants and animals, digestion was performed in acid medium (using  
336 HNO<sub>3</sub>, H<sub>2</sub>SO<sub>4</sub> and HCl<sub>4</sub>) at high temperature as described by Bernard et al. (2010). Lanier et  
337 al. (2019) showed that this method was effective for the reference organisms. The obtained  
338 solution was then used to quantify As, Cd, Co, Cr, Cu, Mn, Ni, Pb, Zn and Ag by ICP-OES  
339 (Agilent Technologies 5110, USA, for the preliminary experiment and Varian 720-ES, USA,  
340 for the main one). Some additional metals were quantified only in the main experiment: Sb,  
341 Mo and Hg. These metals are conventionally analyzed in foodstuffs intended for humans and  
342 animals (Directive 2002/32 of European Commission, 2002; Regulation n° 1881/2006 of  
343 European Commission, 2006) or in the context of ecotoxicological analyses. The  
344 effectiveness of the mineralization was verified and validated by mineralizing reference  
345 materials (IC-INCT-OBTL-5 from LabMix24 Germany for plants and TORT-3 and DOLT-5  
346 from National Research Council Canada for animals).

347

### 348 *2.7.3 Statistical analyses*

349 The majority of the data did not follow a normal distribution, and the variances were  
350 not homogeneous (Shapiro, Lilliefors and Bartlett tests). Thus, Scheirer-Ray-Hare  
351 nonparametric tests and post-hoc tests based on ranks were used. For data following a normal

352 distribution with homogeneous variances, ANOVA and Tukey's post-hoc tests were used.  
353 Tests were realized in R software (R Core Team, 2008).

354

### 355 **3. Results**

#### 356 ***3.1. Preliminary experiment***

##### 357 *Metal content in soil*

358 The Ag contents were slightly lower than expected. The metal contents in soil mixed  
359 with sewage sludge containing Ag sulfide were:  $9.649 \pm 0.303$  mg kg<sup>-1</sup> (dry matter) Ag,  $8.666$   
360  $\pm 0.268$  mg kg<sup>-1</sup> As,  $0.633 \pm 0.096$  mg kg<sup>-1</sup> Cd,  $5.439 \pm 0.168$  mg kg<sup>-1</sup> Co,  $22.228 \pm 0.572$  mg  
361 kg<sup>-1</sup> Cr,  $23.112 \pm 0.685$  mg kg<sup>-1</sup> Cu,  $358.650 \pm 15.556$  mg kg<sup>-1</sup> Mn,  $11.613 \pm 0.381$  mg kg<sup>-1</sup>  
362 Ni,  $74.376 \pm 3.264$  mg kg<sup>-1</sup> Pb, and  $88.105 \pm 3.549$  mg kg<sup>-1</sup> Zn. These metal contents will be  
363 compared with the values in the main experiment (Table 2 in section 3.2.).

364

##### 365 *Life traits of plants*

366 All plant cultivars grew normally on the medium except clover. For all species, some  
367 leaves turned yellow in the last week of exposure.

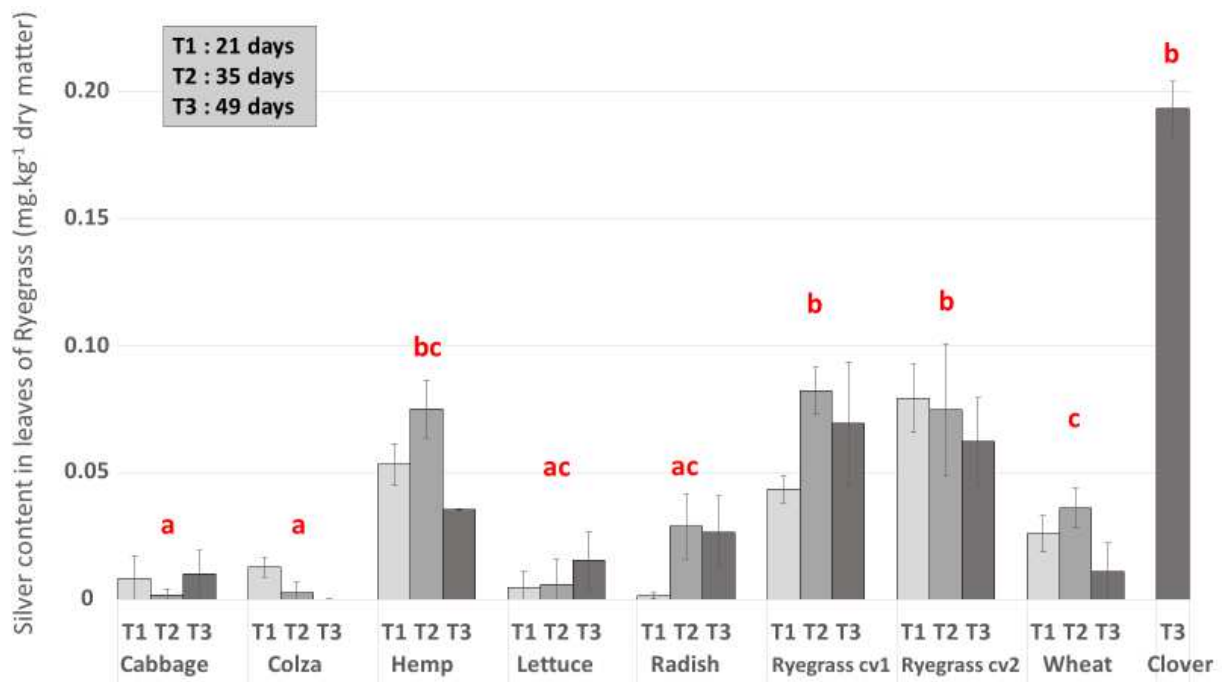
368 The yellowing of the leaves observed at the end of the preliminary experiment suggested  
369 harmful effects on the plants. Such effects might alter the Ag bioaccumulation capacity of the  
370 plants, but only at time T3, since the plants had a healthy appearance for the first 6 weeks.  
371 Although it is possible that the yellowing was caused by Ag, it is more likely due to a  
372 deficiency related to the limited amount of substrate and/or the successive drying and  
373 watering, as the intensity of the artificial lights in the greenhouse dried the soil surface very  
374 quickly.

375

##### 376 *Metal accumulation in plants*

377 Clover could not be sampled at T1 and T2 due to a lack of biomass. Among the other  
 378 plant cultivars, hemp and the two ryegrass cultivars accumulated the most Ag in their leaves  
 379 (mean of 0.06 mg kg<sup>-1</sup> of dry leaves for these species) (Figure 2). The accumulation of Ag  
 380 was rapid and was maintained over time because the amount of bioaccumulated Ag was  
 381 statistically similar among T1, T2 and T3 for all species.

382



383

384 *Figure 2: Silver concentration in leaves of plants in mg kg<sup>-1</sup>(dry matter). Significant*  
 385 *differences are indicated by different letters (comparison between species only, data from all*  
 386 *time points pooled since there were no significant differences among the 3 time points for*  
 387 *each species). T1, T2 and T3 correspond to the dates of sample collection, i.e. 21, 35 and 49*  
 388 *days, respectively.*

389

390 Ryegrass cultivar 2 was selected for the main experiment because it had the lowest variation  
 391 of Ag content over time and the highest concentration of Ag.

392

### 393 **3.2. Main experiment**

#### 394 *Metal contents in mesocosm mixtures*

395 In general, there was little difference in metal concentrations among the 6 mesocosm  
 396 conditions (Table 2). Zn content was higher in the conditions with SS than in those without.

397 Ag content was significantly higher in the conditions with AgNPs or AD-AgNPs than in  
 398 Control and Disor AD-control and AD-dis, respectively. However, the Ag content was  
 399 significantly lower in AD-AgNPs than in the AgNPs mesocosms (almost two times less). It is  
 400 therefore important to carefully interpret the comparisons of the AgNPs and AD-AgNPs  
 401 conditions.

402 In the preliminary experiment, the Ag concentration ( $9.65 \pm 0.30 \text{ mg kg}^{-1}$  of dry  
 403 matter) was intermediate between the two Ag concentrations in the main experiment.

404  
 405 *Table 2: Metal concentration in the soil at the beginning of the experiment in the 6 mesocosm*  
 406 *conditions (in  $\text{mg kg}^{-1}$ , in dry matter). Standard errors were indicated. Statistically significant*  
 407 *results are indicated by stars. Red stars indicate that the silver concentrations were higher in*  
 408 *the AgNPs and AD-AgNPs conditions than the others, although the Ag concentrations were*  
 409 *significantly different between the AgNPs and AD-AgNPs conditions. Green stars indicate*  
 410 *that the Zn concentrations were higher in the conditions with sludge than in the sludge-free*  
 411 *conditions.*

	Control	Dis	AgNPs	AD-control	AD-dis	AD-AgNPs
<b>Ag</b>	$0.529 \pm 0.340$	$0.576 \pm 0.258$	* $11.150 \pm 0.420$	$2.124 \pm 0.252$	$1.903 \pm 0.810$	* $6.993 \pm 0.223$
<b>As</b>	$4.131 \pm 0.128$	$4.168 \pm 0.141$	$3.950 \pm 0.059$	$4.374 \pm 0.256$	$4.301 \pm 0.157$	$3.888 \pm 0.212$
<b>Cd</b>	$0.320 \pm 0.061$	$0.308 \pm 0.061$	$0.310 \pm 0.027$	$0.296 \pm 0.037$	$0.323 \pm 0.029$	$0.304 \pm 0.026$
<b>Co</b>	$4.173 \pm 0.092$	$3.879 \pm 0.042$	$3.789 \pm 0.066$	$4.105 \pm 0.117$	$4.102 \pm 0.105$	$3.854 \pm 0.188$
<b>Cr</b>	$25.675 \pm 1.656$	$24.775 \pm 0.834$	$24.300 \pm 1.036$	$28.300 \pm 1.089$	$26.550 \pm 0.580$	$26.275 \pm 1.150$
<b>Cu</b>	$18.675 \pm 0.359$	$18.425 \pm 0.050$	$17.475 \pm 0.222$	$19.900 \pm 0.141$	$19.775 \pm 0.150$	$18.500 \pm 0.424$
<b>Mn</b>	$336.175 \pm 9.831$	$326.425 \pm 22.948$	$307.775 \pm 4.844$	$336.725 \pm 0.974$	$337.250 \pm 17.989$	$307.100 \pm 15.112$
<b>Ni</b>	$11.450 \pm 0.173$	$11.225 \pm 0.050$	$10.950 \pm 0.191$	$11.875 \pm 0.096$	$11.900 \pm 0.245$	$11.175 \pm 0.275$
<b>Pb</b>	$52.725 \pm 1.611$	$51.875 \pm 0.957$	$51.150 \pm 3.527$	$52.900 \pm 0.173$	$52.333 \pm 0.896$	$50.375 \pm 2.251$
<b>Zn</b>	$49.775 \pm 0.403$	$49.025 \pm 0.450$	$47.200 \pm 0.424$	* $76.350 \pm 3.753$	* $72.125 \pm 2.114$	* $69.100 \pm 1.633$

412  
 413 *Plant life traits: leaf growth*  
 414 Ryegrass growth was observed in all mesocosms. From the second week, it appeared visually  
 415 that the *Lolium perenne* grew much faster in the mesocosms with SS than those without SS  
 416 (Sup. Inf. 4). This visual difference in biomass was maintained until the end of the  
 417 experiment. Visually, there was therefore no effect of Ag on the length and the biomass of  
 418 ryegrass; only the effect of SS was visible.

419

420 *Animal life traits: survival and biomass*

421 Snail and locust survival were not correlated with condition or soil access according to the  
 422 statistical tests (non-parametric ANOVA) (Table 3).

423 Locust biomass did not vary significantly between the different treatments and the two types  
 424 of access. However, for snails, there was on average a gain in mass or a very small mass loss  
 425 for snails in contact with the soil. By comparison, a greater mass loss was observed for  
 426 individuals who did not have access to the soil, although the differences between access to  
 427 soil and plants and access to plants only were significant only in 2 of 6 conditions (AD-  
 428 control and AD-AgNPs). No effect of Ag (regardless of the initial chemical form) or  
 429 dispersant was observed.

430

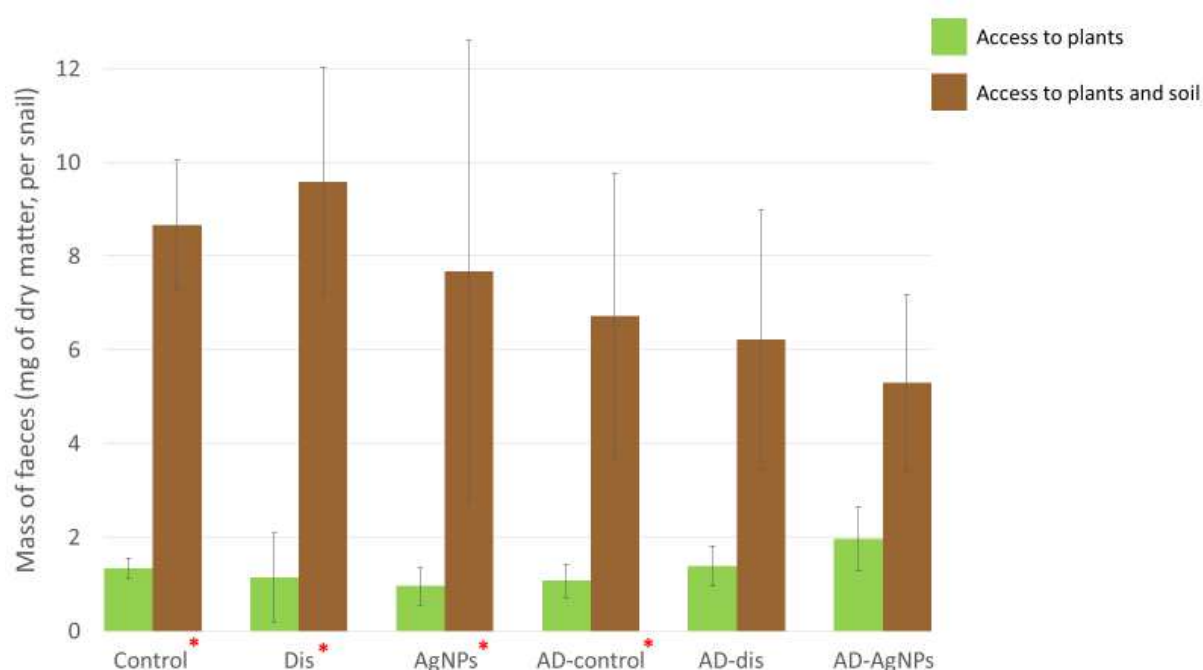
431 *Table 3: Survival and biomass gained or lost between the beginning and end of the*  
 432 *experiment. P means ‘access to plants only’, and P+S means ‘access to plants and soil’.*  
 433 *Standard error were indicated. Statistical tests showed no correlation between the survival or*  
 434 *the biomass and the Ag presence.*

<u>Condition</u>	<u>Access</u>	<u>Snails</u>		<u>Locusts</u>	
		<u>Survival (%)</u>	<u>Gain/Loss of weight (%)</u>	<u>Survival (%)</u>	<u>Gain/Loss of weight (%)</u>
Control	P	76.67 ± 25.17	- 0.15 ± 0.12	86.67 ± 23.09	+ 0.06 ± 0.04
	P+S	73.33 ± 11.55	+ 0.04 ± 0.07	60.00 ± 20.00	+ 0.05 ± 0.20
AD-control	P	93.33 ± 11.55	- 0.17 ± 0.08	53.33 ± 23.09	+ 0.07 ± 0.09
	P+S	83.33 ± 15.28	+ 0.29 ± 0.04	53.33 ± 23.09	+ 0.16 ± 0.08
Dis	P	93.33 ± 5.77	- 0.11 ± 0.03	86.67 ± 23.09	0.00 ± 0.09
	P+S	63.33 ± 11.55	- 0.01 ± 0.23	46.67 ± 23.09	- 0.14 ± 0.08
AD-dis	P	83.33 ± 15.28	- 0.26 ± 0.27	60.00 ± 34.64	- 0.04 ± 0.05
	P+S	86.67 ± 15.28	+ 0.10 ± 0.02	60.00 ± 20.00	+ 0.06 ± 0.08
AgNPs	P	83.33 ± 15.28	- 0.15 ± 0.04	53.33 ± 41.63	+ 0.01 ± 0.14
	P+S	83.33 ± 15.28	+ 0.04 ± 0.07	66.67 ± 23.09	+ 0.09 ± 0.09
AD-AgNPs	P	66.67 ± 23.09	- 0.16 ± 0.16	46.67 ± 23.09	+ 0.12 ± 0.21
	P+S	76.67 ± 15.28	+ 0.32 ± 0.14	60.00 ± 20.00	+ 0.03 ± 0.17
<i>Average</i>		<i>80.28 ± 15.94</i>	<i>- 0.02 ± 0.21</i>	<i>61.11 ± 24.82</i>	<i>+ 0.04 ± 0.13</i>

435

436 Feces production by locusts and snails was the same under all conditions (control, dispersant,  
 437 AgNPs, AD-control, AD-dis, AD-AgNPs), indicating similar levels of consumption in the  
 438 days preceding the end of the experiment. Feces production was greater for snails in contact  
 439 with the soil than those not in contact with the soil (Figure 3), consistent with the biomass  
 440 results. For locusts, feces production was not significantly linked to the soil access, form of  
 441 Ag or SS presence/absence.

442



443  
 444 *Figure 3: Quantity of faeces produced by snails during the final fasting depuration in mg of*  
 445 *dry matter. Red stars indicate significant differences between 'access to plants' and 'access to*  
 446 *plants and soil' within the same condition.*

447

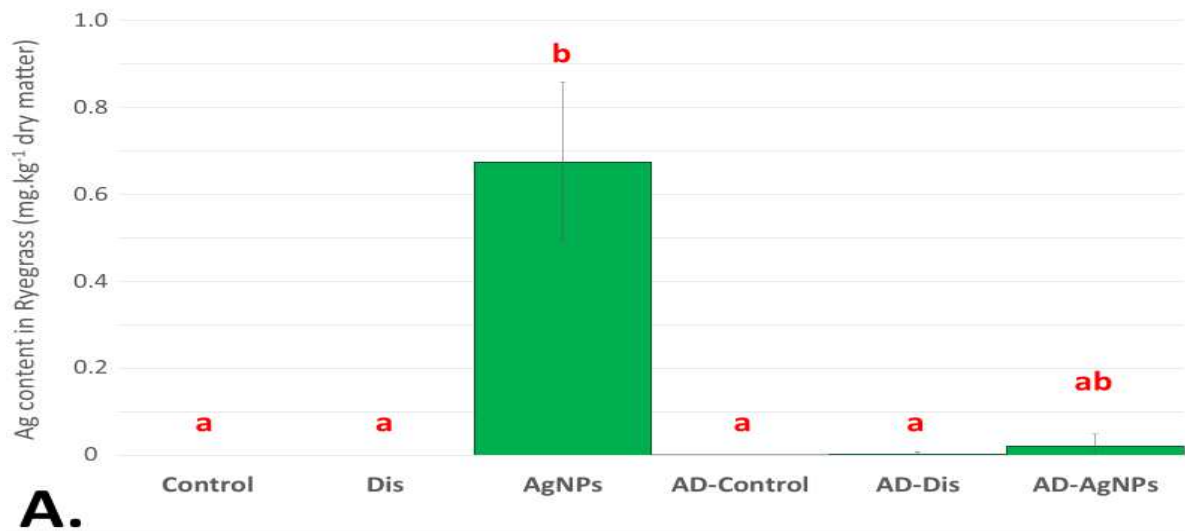
#### 448 *Metal accumulation in plants*

449 Consistent with the results of the preliminary experiment, ryegrass accumulated Ag when  
 450 cultivated in soil enriched with Ag (Figure 4 A). In the condition without SS (AgNPs), the Ag  
 451 content in the aerial parts of ryegrass was very significant (mean of 0.68 mg kg<sup>-1</sup> of Ag in dry  
 452 matter). By comparison, in the AD-AgNPs condition, Ag was detectable in ryegrass, but the  
 453 content was lower (0.02 mg kg<sup>-1</sup> in mean) and did not differ significantly from that in the  
 454 mesocosm without Ag. These results indicated that the sulfidized Ag contained in the SS was

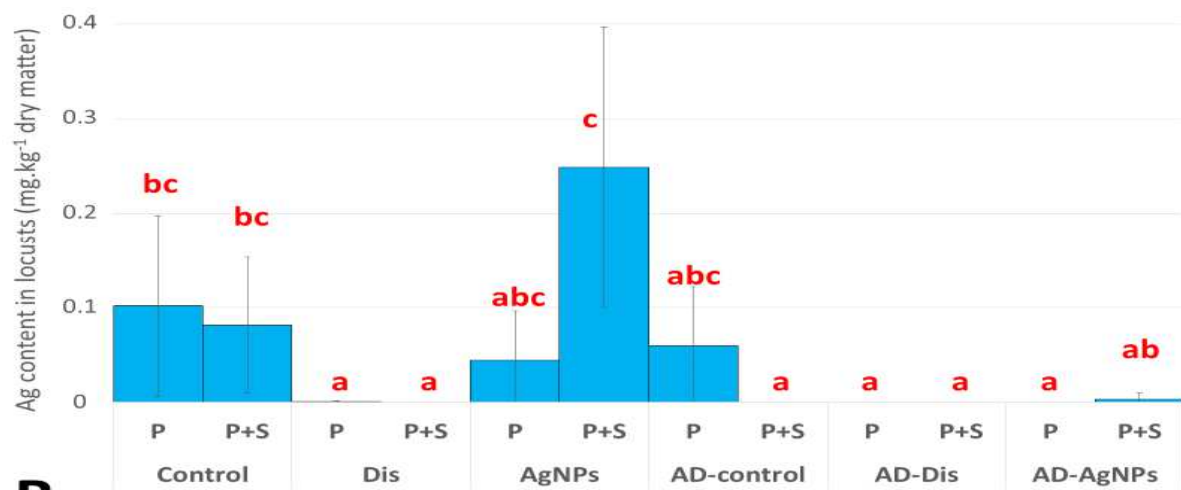
455 less bioavailable to ryegrass than AgNPs. This conclusion was confirmed by the calculation  
456 of the bioaccumulation factor (BAF) (Sup. Inf. 8): the BAF for ryegrass was 0.061 in the  
457 AgNPs mesocosms but 0.003 in the AD-AgNPs mesocosms, a difference of 20-fold.

458

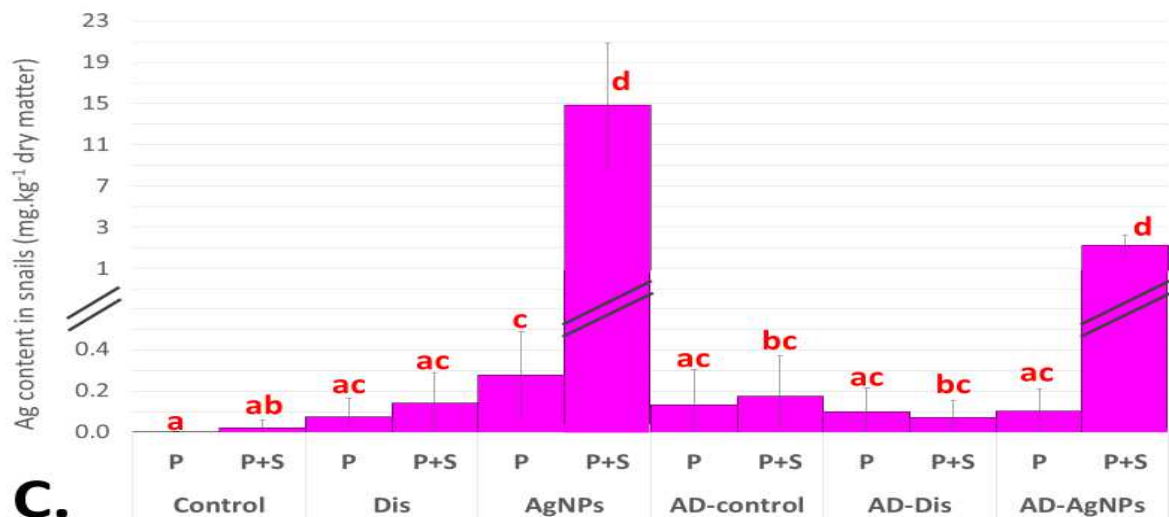




**A.**



**B.**



**C.**

459

460 Figure 4: Silver concentration in organisms at the end of experiment. A. Silver content in the  
 461 aerial part of ryegrass in mg kg<sup>-1</sup> (dry matter). Significant differences are indicated by  
 462 different letters. B. Silver content in whole locusts in mg kg<sup>-1</sup>(dry matter). Significant  
 463 differences are indicated by different letters. C. Silver content in snails (without shell) in mg  
 464 kg<sup>-1</sup>(dry matter). Significant differences are indicated by different letters.

465

466 At the final time point, the accumulation of As, Cd, Co, Cr, Cu, Hg, Pb, Sb and Zn in  
467 the aerial parts of ryegrass was similar under all conditions. For Mn, Mo and Ni, the  
468 accumulation was lower when the plants were cultivated under SS conditions compared to  
469 conditions without SS, regardless of the Ag concentration in the soil (Sup. Inf. 5). In general,  
470 ryegrass rapidly accumulated metals, and between 4 and 9 weeks, the content of these metals  
471 in the plants remained relatively unchanged.

472

#### 473 *Metal accumulation in locusts.*

474 The Ag content in whole locusts was very low (Figure 4 B), and there was no  
475 significant difference in Ag content between locusts exposed to Ag conditions and those  
476 exposed to conditions without Ag.

477

478 The accumulation of Cd, Cu, Co, Sb, As, Mo, Hg, Ni, Pb and Zn was quite similar under all  
479 conditions (Sup. Inf. 6). The few significant differences in Cr or Mn content, like the  
480 differences in the BAF for Cr (Sup. Inf. 8), cannot be attributed to Ag, SS or type of access.  
481 The BAF for Zn was lower in all mesocosms with SS (2.20 on average) than in the  
482 mesocosms without SS (3.27 on average) (Sup. Inf. 8).

483

#### 484 *Metal accumulation in snails.*

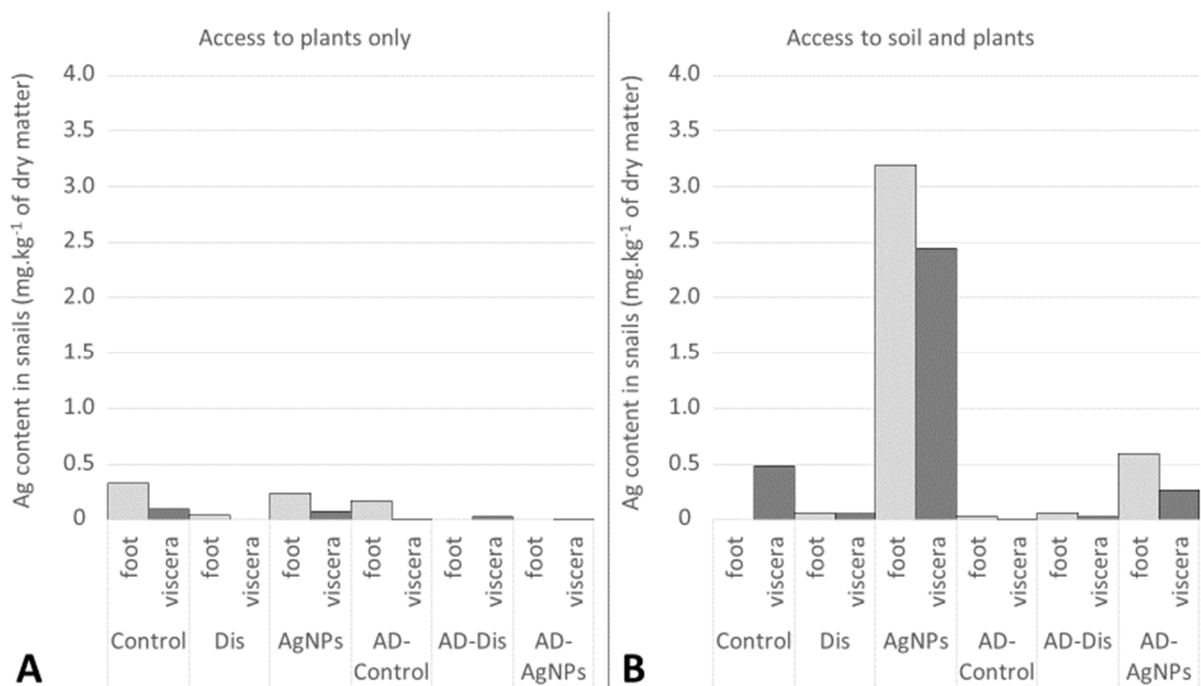
485 In contrast to the results observed in locusts, snails significantly bioaccumulated Ag  
486 under two conditions: AgNPs with soil and plant access, which yielded the highest  
487 concentrations ( $15.25 \pm 5.73$  mg kg<sup>-1</sup> of dry matter), and AD-AgNPs with soil and plant  
488 access (Figure 4 C). The BAF for AD-AgNPs with soil and plant access was 4 times lower  
489 than that for AgNPs with soil and plant access (Sup. Inf. 8). In the other conditions with Ag

490 without access to soil, the Ag content in snails was not different from that of snails that were  
491 not exposed to Ag.

492

493 Moreover, Ag accumulation was higher in the feet than in the viscera of snails exposed to Ag  
494 conditions (Figure 5). This result should be considered preliminary because the mortality  
495 observed in the mesocosms did not allow replicas to be made.

496



497

498 *Figure 5: Silver concentration (in mg kg<sup>-1</sup> of dry matter) in snail foot and viscera exposed to*  
499 *(A) to plants only and (B) to soil and plants. The absence of error bars and statistical*  
500 *comparisons is due to the impossibility of making replicates.*

501

502 For snails, the accumulation of Cu, Sb, As, Cr, Mn, Mo, Hg, Ni and Zn was similar in all  
503 conditions. However, the accumulated amount of Cd was significantly greater in snails that  
504 were in contact with soil than in snails without access to soil (Sup. Inf. 7). The average BAF  
505 for Cd was 5.99 for snails with plant+soil access but 3.33 for snails with plant access only  
506 (Sup. Inf. 8). Similar effects were observed for Co and Pb, but the differences between the  
507 two types of access were not statistically significant.

508

#### 509 **4. Discussion and conclusion**

##### 510 *Transfer and effects of Ag on plants*

511           The results of the preliminary experiment showed that the 9 plant cultivars studied  
512 accumulated a small amount of Ag in their aerial parts when Ag was added to the soil in  
513 sulfide form via SS. These low concentrations are consistent with the results of Pradas del  
514 Real et al. (2016), who found that Ag levels in plants were not detectable with a similar  
515 experimental design and Ag soil concentration. Ag accumulated quickly and reached a  
516 threshold value (aft 3 weeks). The level of accumulation varied and ranged from 0.005 to  
517 0.200 mg kg<sup>-1</sup> (dry matter) depending on the plant species, implying that the magnitude of the  
518 effects of spreading of SS rich in Ag could vary by crop type. Additional analyses of the main  
519 crops for which spreading of SS is a common practice in Europe or even globally would  
520 enable informed decisions on the choice of plots to amend with SS based on plant species to  
521 be cultivated. SS very rich in Ag could even be recycled in a manner other than as a biosolid  
522 on agricultural plots, for example, in the remediation of highly polluted soil, in order to  
523 "dilute" the contaminants already present on site ; the risks of this method are still being  
524 assessed (Jaskulak et al., 2020; Kubátová et al., 2016).

525

526           The beneficial effects of SS on plant growth and biomass have been known for several  
527 years (Kumar et al., 2017) and were confirmed in the main experiment on ryegrass. Ag did  
528 not visually affect the appearance and aerial biomass of this species, consistent with the  
529 observations of Pradas del Real et al. (2016). In their study, sulfidized Ag affected the root  
530 growth of rape and wheat even with Ag concentrations around 1 mg kg<sup>-1</sup>. Moreover, our  
531 results confirmed the lower bioavailability of sulfidized Ag compared with AgNPs previously  
532 reported in the literature (Doolette et al., 2015; Pradas del Real et al., 2017, 2016; Stegemeier

533 et al., 2015; Wang et al., 2015), since the bioaccumulation of sulfidized Ag by ryegrass was  
534 approximately 20 times lower than that of AgNPs according to the BAF calculations. Indeed,  
535 Ag<sub>2</sub>S is very poorly soluble compared with AgNPs (Levard et al., 2013; Pradas del Real et al.,  
536 2017), which reduces the release of Ag<sup>+</sup> ions that can easily penetrate plant cells, particularly  
537 since the Ag added to SS before anaerobic digestion persists for several weeks in sulfidized  
538 form in soil (according to a study using the same soil, SS from the same treatment plant, and  
539 similar anaerobic digestion with identical Ag concentrations; Courtois et al., 2020).

540

#### 541 *Transfer and effects of Ag on soil invertebrates*

542 Similar to the results in plants, sulfidized Ag did not appear to have a strong impact on  
543 the life traits of animals. At the internal concentration measured in the present study, Ag did  
544 not affect the life traits (biomass and survival) of locusts and snails. Studies of woodlice and  
545 earthworms have yielded similar results (Kampe et al., 2018; Lahive et al., 2017; Velicogna et  
546 al., 2017; Courtois et al., 2020), although these studies used a different mode of contamination  
547 (direct exposure to soil and not via contaminated food).

548 While locusts did not significantly accumulate Ag, their period of exposure was  
549 shorter than that of snails. Studies have shown that for snails, 2 weeks is sufficient to observe  
550 significant bioaccumulation of Cd (Coourdassier et al., 2002; Gimbert et al., 2006), whereas 4  
551 weeks is required for Ag, Cu, Pb and Zn (De Vaufleury, 2015; Mariet et al., 2017). We can  
552 therefore conclude that the duration of exposure of snails in the present study (almost 5  
553 weeks) was sufficient to allow the bioaccumulation of Ag and other metals, whereas 2 weeks  
554 of exposure of locusts may not have been sufficient to observe significant bioaccumulation of  
555 metals like Ag. Comparable data are not available for locusts because most studies recover  
556 animals from the field and therefore the duration of exposure is unknown (Devkota and  
557 Schmidt, 2000; Soliman and El-Shazly, 2017; Zhang et al., 2012). This first reassuring result

558 should therefore be confirmed by longer exposure of locusts. However, in addition to the  
559 short exposure time, the Ag levels in locusts may not be detectable due to the low Ag levels in  
560 plants.

561         When snails could get to the soil, they ate more and gained more weight. The snails  
562 were likely more comfortable in this type of mesocosm configuration, as access to the soil  
563 probably offers higher local humidity and more hiding places, resulting in higher soil  
564 consumption and contact. When Ag was present, the snails bioaccumulated more Ag when  
565 access to soil was permitted. It is possible that Ag was transferred to the snails via the pedal  
566 sole due to direct contact or was only adsorbed on the foot via mucus. The snails may also  
567 ingest more Ag by consuming soil particles to supplement their nutritious intake, as described  
568 previously by Gomot et al. (1989) and Pauget et al. (2012). Both channels of Ag accumulation  
569 are likely since Ag was found not only in the foot but also the viscera. However, studies have  
570 shown that the locations of metal accumulation do not necessarily indicate the route of  
571 contamination. Studies of *H. aspersa* (Coourdassier et al., 2002) and *Arion ater* (Ireland,  
572 1982) showed that regardless of the mode of Cd exposure, the bioaccumulated metal was  
573 distributed in a similar way, indicating redistribution of the metal in the organism. A similar  
574 phenomenon can be envisaged for Ag contamination. Moreover, although the production of  
575 mucus during movements when fasting must have allowed the majority of the particles  
576 adsorbed on the foot to detach from the animal, a small part of the Ag content in snails might  
577 have only been adsorbed on the foot, limiting the potential negative effect on the measured  
578 endpoints (survival, mass). Indeed, it has been shown that intake of AgNPs with food  
579 significantly affects other parameters, e.g. lipid peroxidation levels, catalase and glutathione-  
580 S-transferase activities, cell death and immunological parameters, even when the  
581 concentration of Ag in snail tissues is not detectable (Radwan et al., 2019). However, when a  
582 predator consumes the snail, this small amount of Ag still transferred via the food chain. A

583 comparative tomographic analysis of snails in contact with plants only and those previously in  
584 contact with soil might reveal whether part of the Ag is adsorbed on the foot or if all of it is  
585 passed into the tissues of the snail. Such an analysis would also identify the main sites of  
586 bioaccumulation of Ag in this snail species.

587 In the most realistic condition (AD-AgNPs with access to soil), the snails accumulated  
588 approximately  $2.3 \text{ mg kg}^{-1}$  of Ag, compared with  $15 \text{ mg kg}^{-1}$  in the same condition with  
589 AgNPs. These contents are clearly higher than those found in a study by Mariet et al. (2017)  
590 that examined the bioaccumulation of Ag in different areas of an old lead-silver mine. In  
591 Mariet et al. (2017), the Ag concentration reached  $1.14 \text{ mg kg}^{-1}$  in the digestive gland (a part  
592 of the viscera) of *Cantareus asperses* snails exposed to soil containing  $14.5 \text{ mg kg}^{-1}$  of Ag  
593 (BAF: 0.054). Several factors may explain the lower transfer observed in this previous field  
594 study, for instance, soil characteristics, metal speciation in soil, reduced contact and eating of  
595 soil due to the humus cover and the possibility of eating various plants exhibiting different  
596 capacities of Ag accumulation. Regardless, our results are reassuring in the context of land  
597 spreading of SS and are consistent with previous studies of ryegrass and earthworms  
598 (Courtois et al., 2020 ; Velicogna et al., 2017): the Ag found in SS is less bioavailable than  
599 AgNPs due to its speciation. The higher organic matter content in soil supplemented with SS  
600 may have increased this bioaccumulation gap (between snails in AgNPs P+S and in AD-  
601 AgNPs P+S). In fact, the snails under AD-AgNPs conditions probably consumed fewer soil  
602 particles to meet their nutritional needs than those under AgNPs conditions, and therefore the  
603 latter also consumed more metals. In addition, it would be interesting to compare the  
604 speciation of Ag in plants grown under AgNPs conditions and AD-AgNPs conditions. Wang  
605 et al. (2015) and Pradas del Real et al. (2017) showed that the speciation of bioaccumulated  
606 Ag varied according to plant treatment. In these studies,  $\text{Ag}_2\text{S}$ -NPs treatment resulted in  
607 bioaccumulation of Ag mainly in  $\text{Ag}_2\text{S}$  form, AgNPs treatment resulted in bioaccumulation of

608 Ag mainly in metallic form and linked to a thiol molecule, and AgNO<sub>3</sub> treatment resulted in  
609 bioaccumulation of Ag mainly linked to a thiol molecule. If Ag<sub>2</sub>S could enter plants via the  
610 roots, the bioaccumulated Ag<sub>2</sub>S might be less bioavailable, resulting in less bioaccumulation  
611 in animals.

612 In the present study, snails in contact with soil contaminated by AgNPs bioaccumulated high  
613 levels of Ag (with a mean BAF equal to 1.367). AgNPs are sometimes directly applied to  
614 crops for their nanopesticide or nanofertilizer properties (Chhipa, 2019; Khan and Rizvi,  
615 2017). Such practices could increase the content of Ag entering the trophic network, although  
616 the absorption of sulfidized Ag by soybean roots has been shown to be greater than the  
617 absorption of AgNPs by its leaves (Dang et al., 2019). In the latter study, soybean was grown  
618 hydroponically, so it is possible that sulfidized Ag linked to organic matter in soil and / or SS  
619 would be less bioavailable.

620

621 In conclusion, the addition of Ag to agricultural soil via the spreading of contaminated  
622 SS resulted in transfer of this metal to plants, in a species-dependent manner, especially  
623 ryegrass which reached Ag concentration up to 0.2 mg kg<sup>-1</sup>. Snails and locusts exposed only  
624 to ryegrass did not notably bioaccumulate the Ag unlike snails exposed also to the soil as  
625 occurs in the field. The transfer of Ag in snails was higher when AgNPs were directly applied  
626 to the soil (up to 15 mg kg<sup>-1</sup>) but still reached 2.5 mg kg<sup>-1</sup> when Ag was brought by SS  
627 showing a reduced bioavailability to snails of sulfidized Ag compared to AgNPs, both  
628 indirectly due to a lower transfer to plant and directly from the soil. It is the first-ever  
629 demonstration that sulfidized Ag and to a lower extent AgNPs in soil is bioavailable to snails  
630 whereas this is not the case for locust. The experimental design used provide information of  
631 great significance either for the SS recycling and the potential risk of the release of Ag NP in  
632 the environment whether regarding the possible human exposure by eating cultivated Ag-



633 contaminated plants or exposure of various consumers via food web transfer of Ag.  
634 Transformations of AgNPs in treatment plants attenuate but do not completely prevent the  
635 accumulation of Ag in plant and animal species. SS spreading is a common practice, and it  
636 may be possible to make informed decisions on the choice of plots to amend with SS  
637 according to the plant species planned for cultivation. Thus, it seems important to add Ag to  
638 the list of metals controlled in SS (European directive 86/278/CEE, 1986) in order to ensure  
639 that SS containing Ag is spread primarily on plots that will receive cultivated species that  
640 accumulate little Ag. When SS is too rich in Ag, other strategies for SS recycling, could be  
641 employed, such as remediation of polluted soil.

642

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655

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657 **References**

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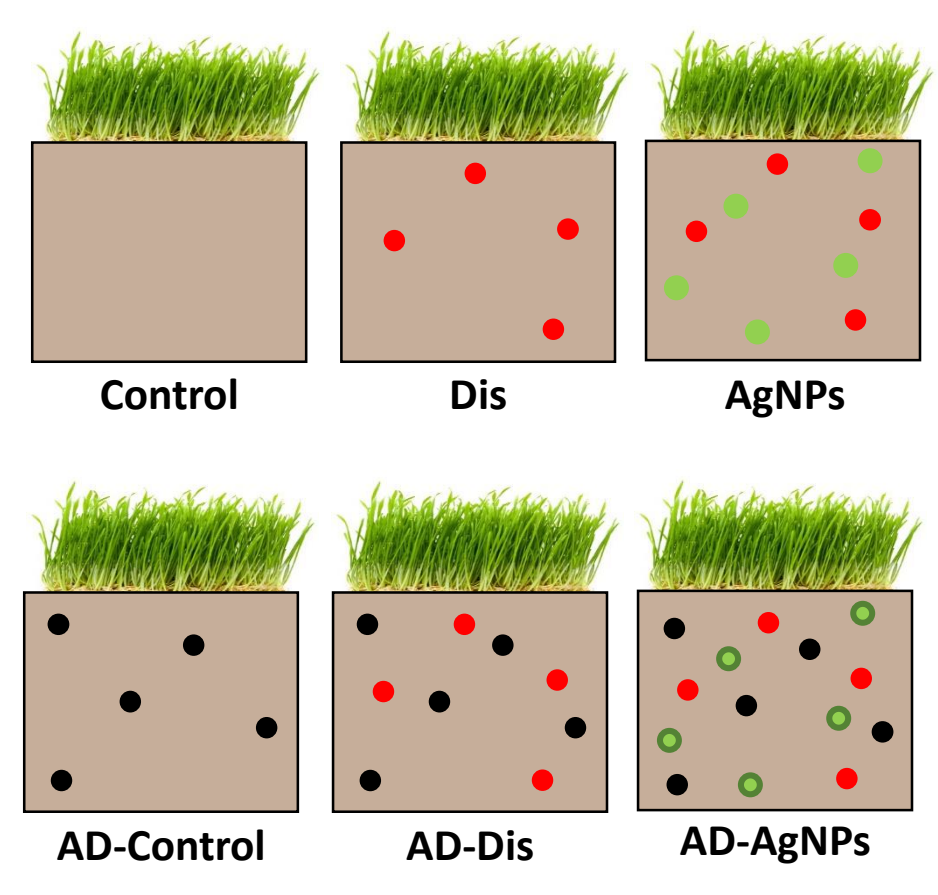
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926

Initial time: day 0

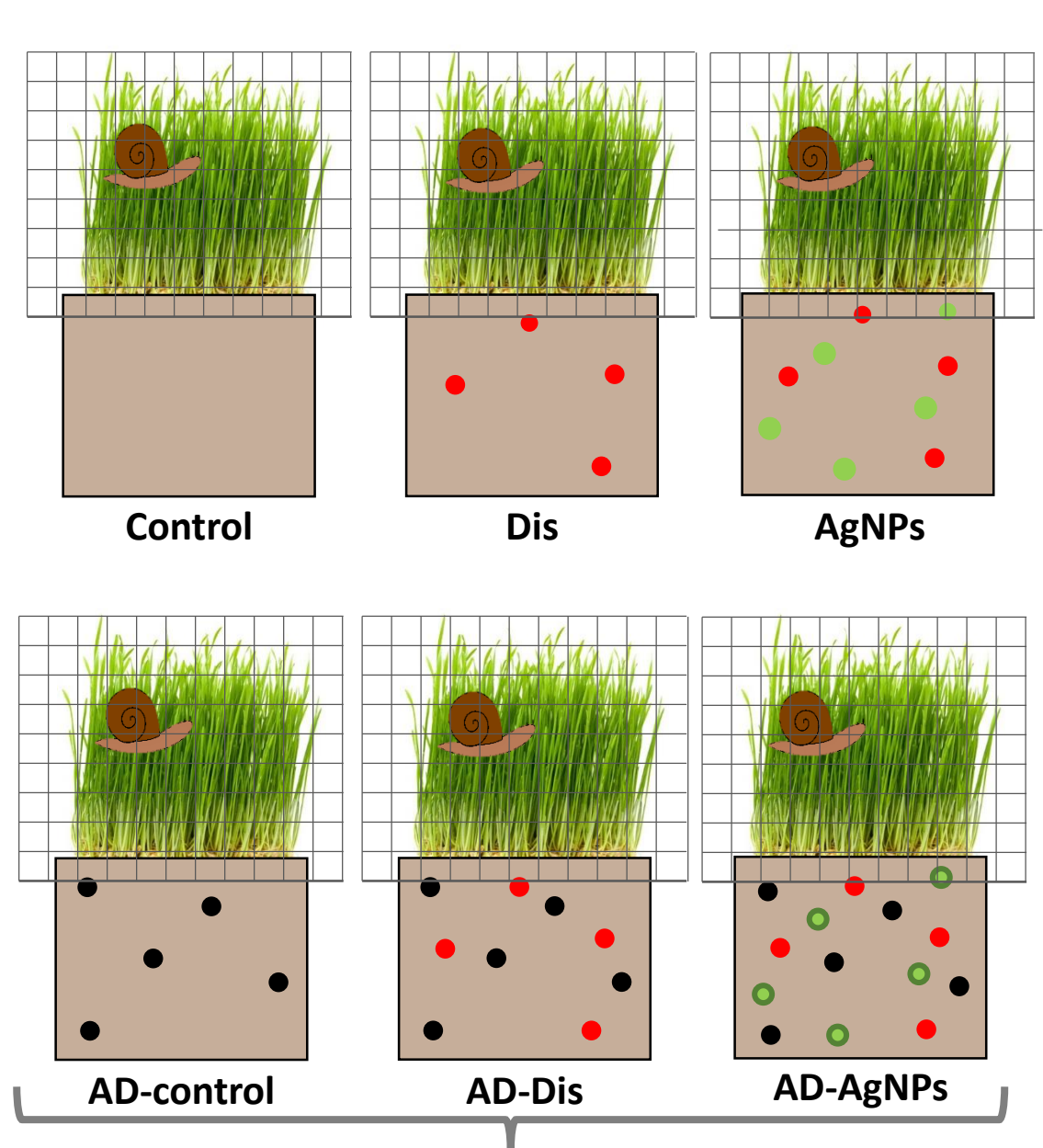
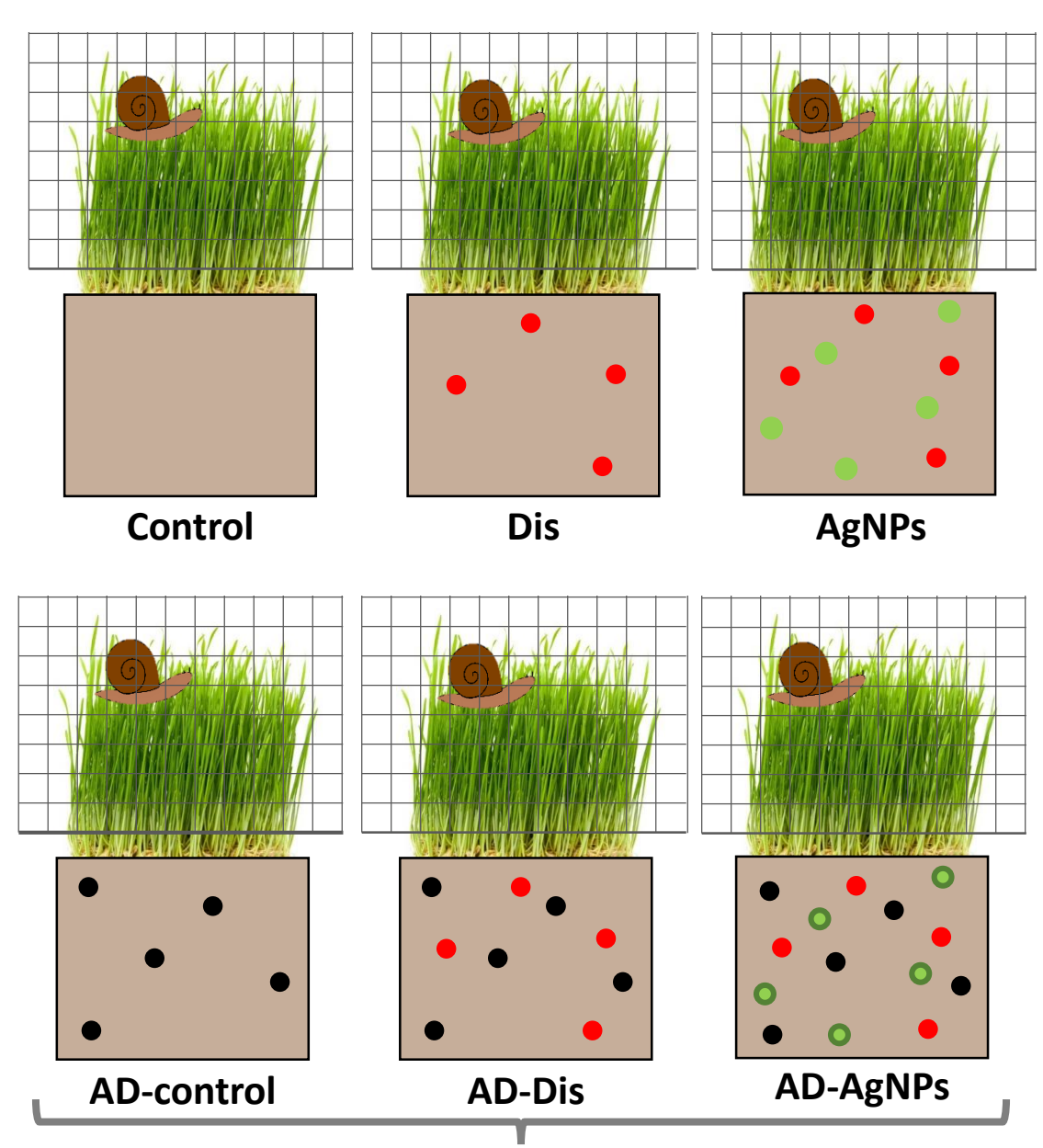
X6  
(36 microcosms)



**Legend:**

- Natural field soil
- Digested sewage sludge
- Dispersant
- AgNPs (subjected to sludge fermentation)
- AgNPs
- Snails
- Locusts

⚡ Addition of snails: day 28

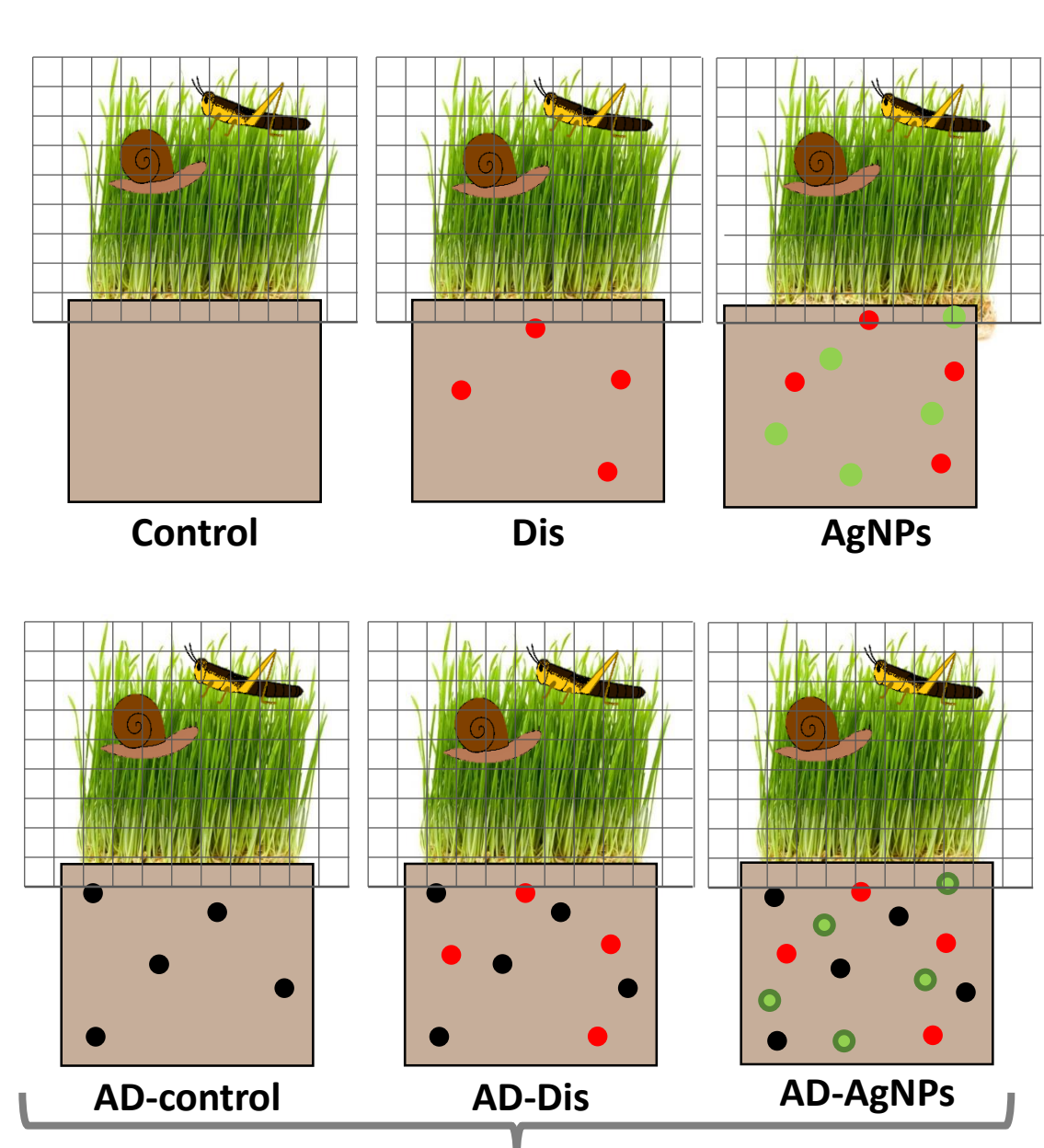
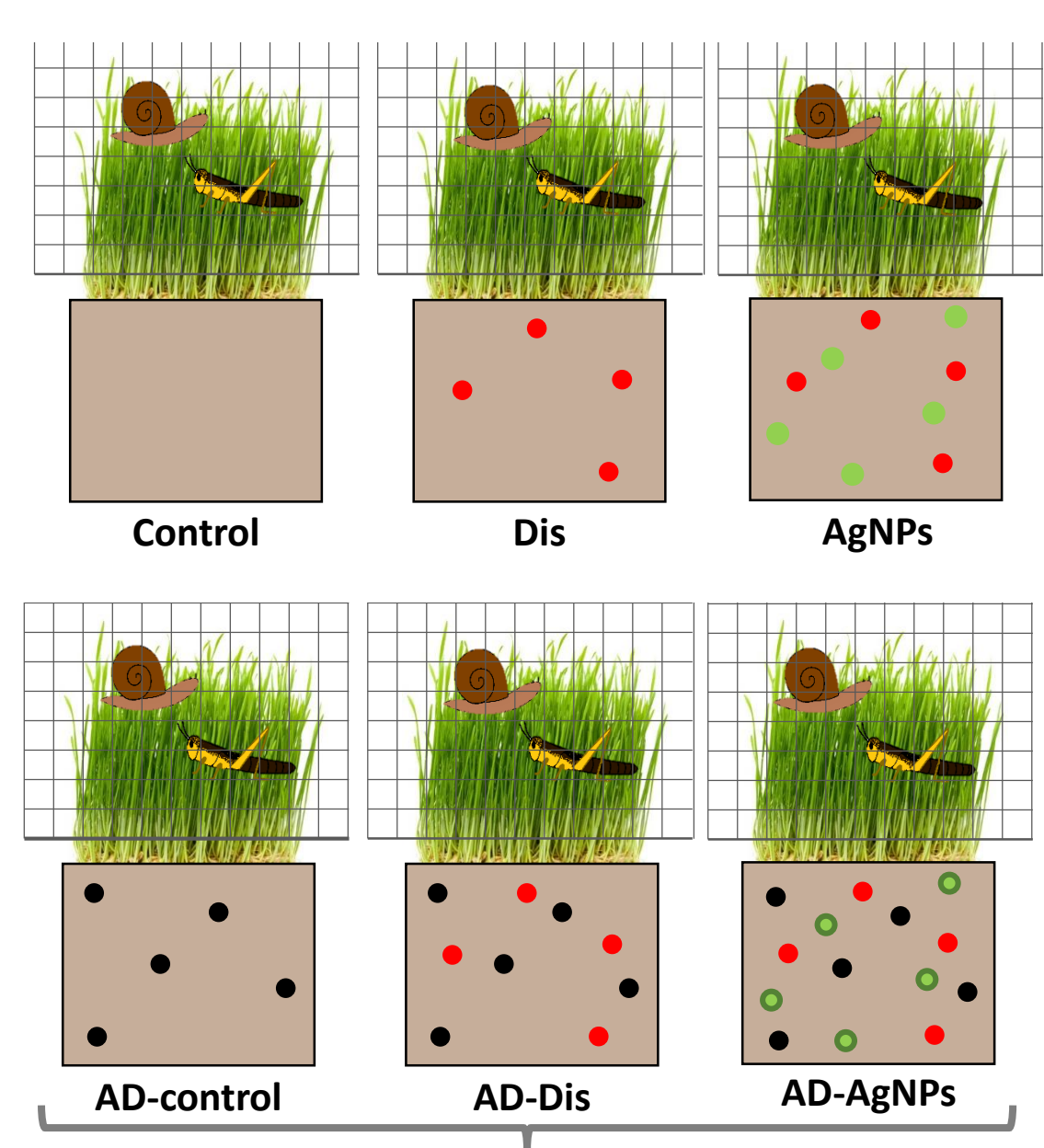


X3  
(for triplicates)

Animal access to plants only

Animal access to plants and soil

⚡ Addition of locusts: day 48



X3  
(for triplicates)

Animal access to plants only

Animal access to plants and soil

Final time: day 62

Silver content in leaves of Ryegrass (mg.kg<sup>-1</sup> dry matter)

T1 : 21 days  
T2 : 35 days  
T3 : 49 days

0.20  
0.15  
0.10  
0.05  
0

T1 T2 T3 T1 T2 T3 T1 T2 T3 T1 T2 T3 T1 T2 T3 T1 T2 T3 T1 T2 T3 T3  
Cabbage Colza Hemp Lettuce Radish Ryegrass cv1 Ryegrass cv2 Wheat Clover

**a**

**a**

**bc**

**ac**

**ac**

**b**

**b**

**c**

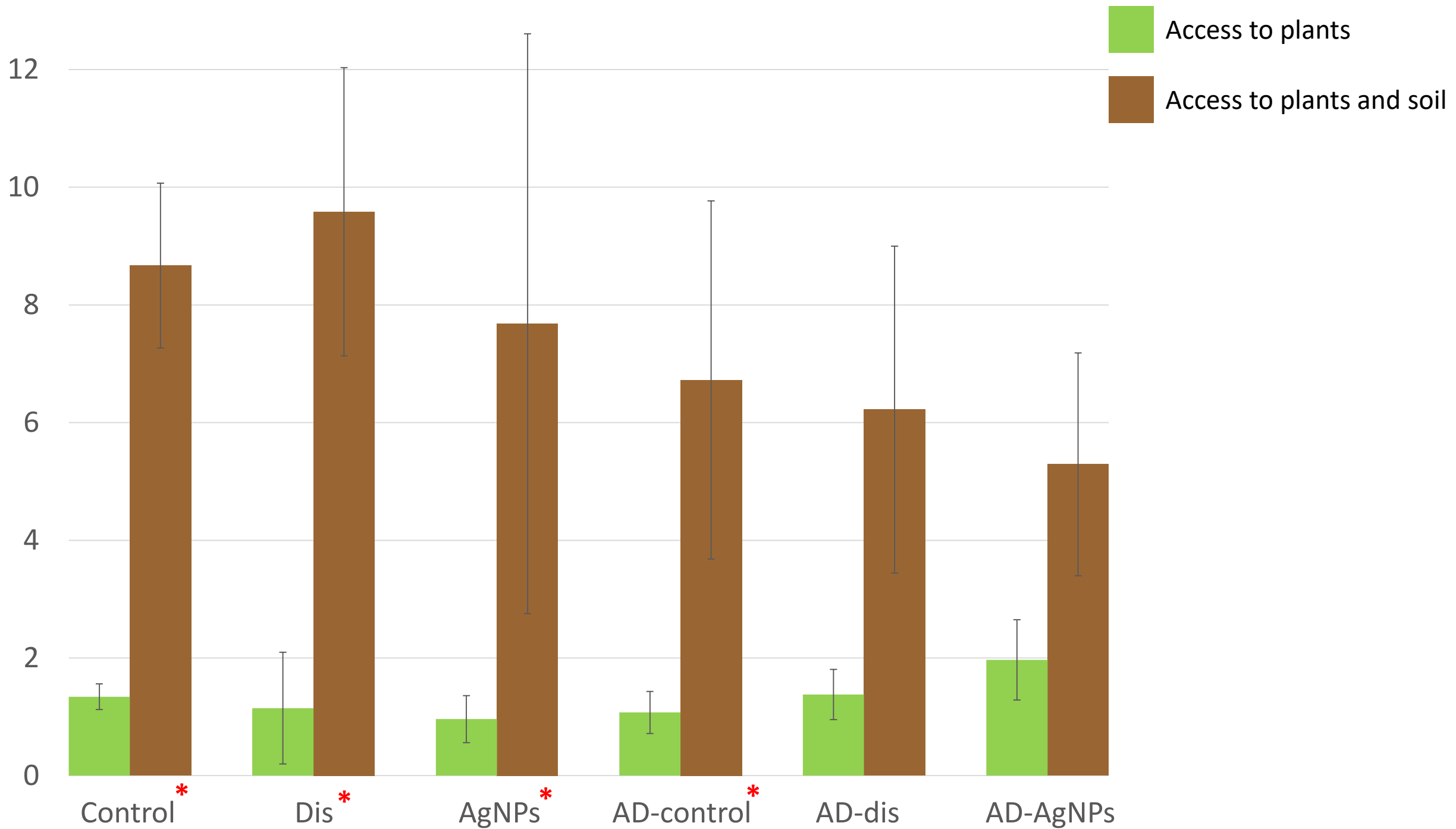
**b**

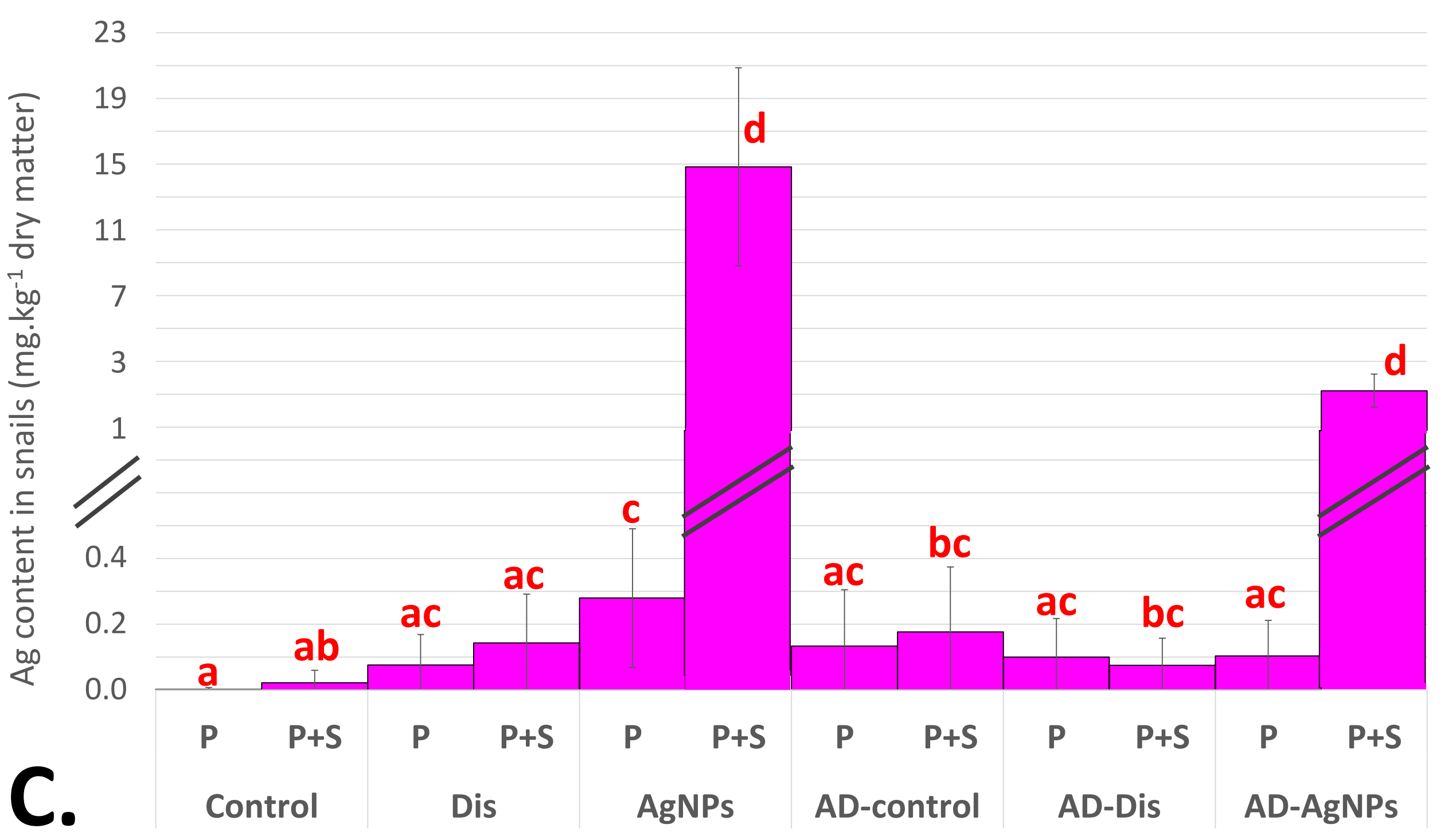
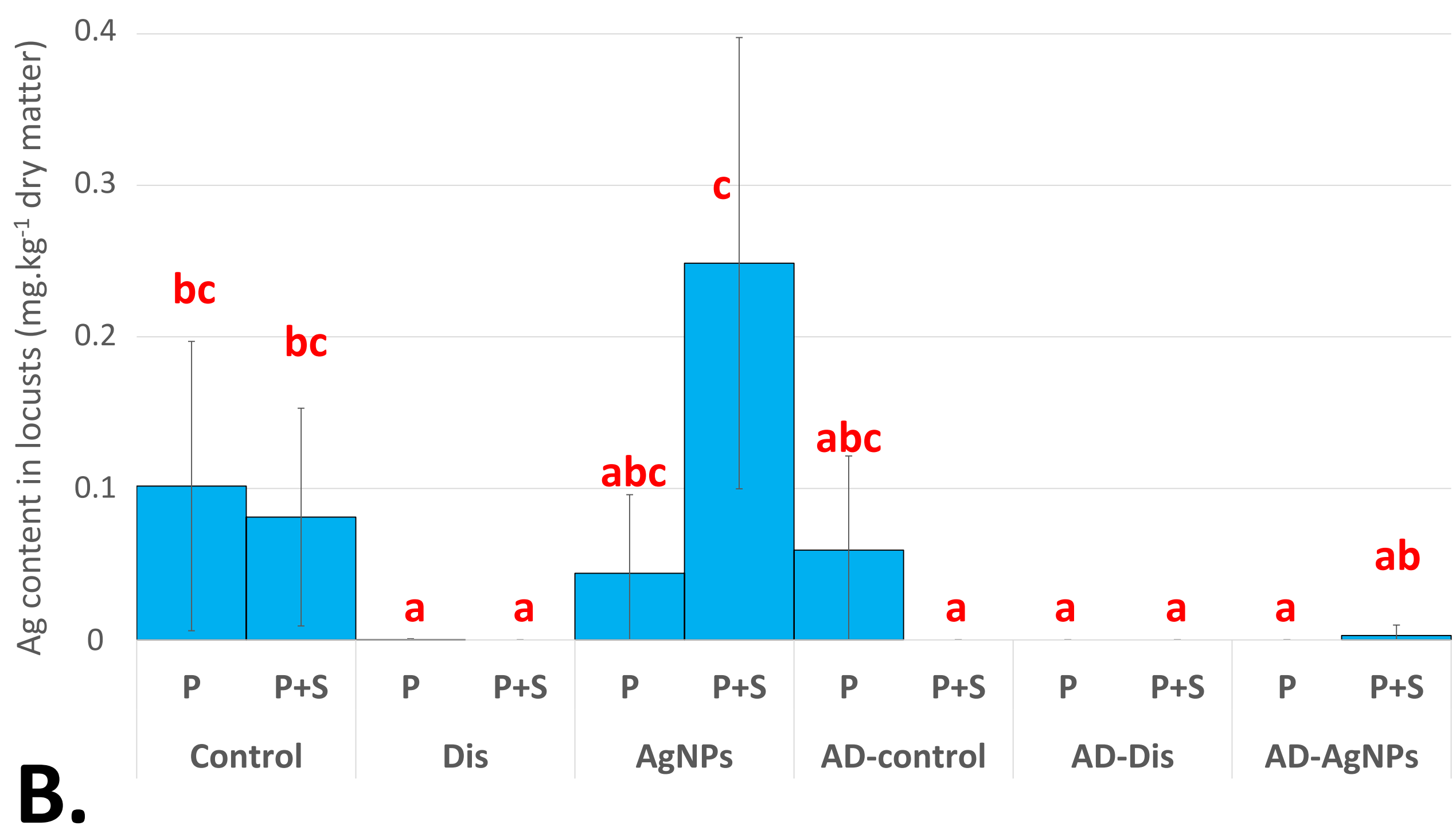
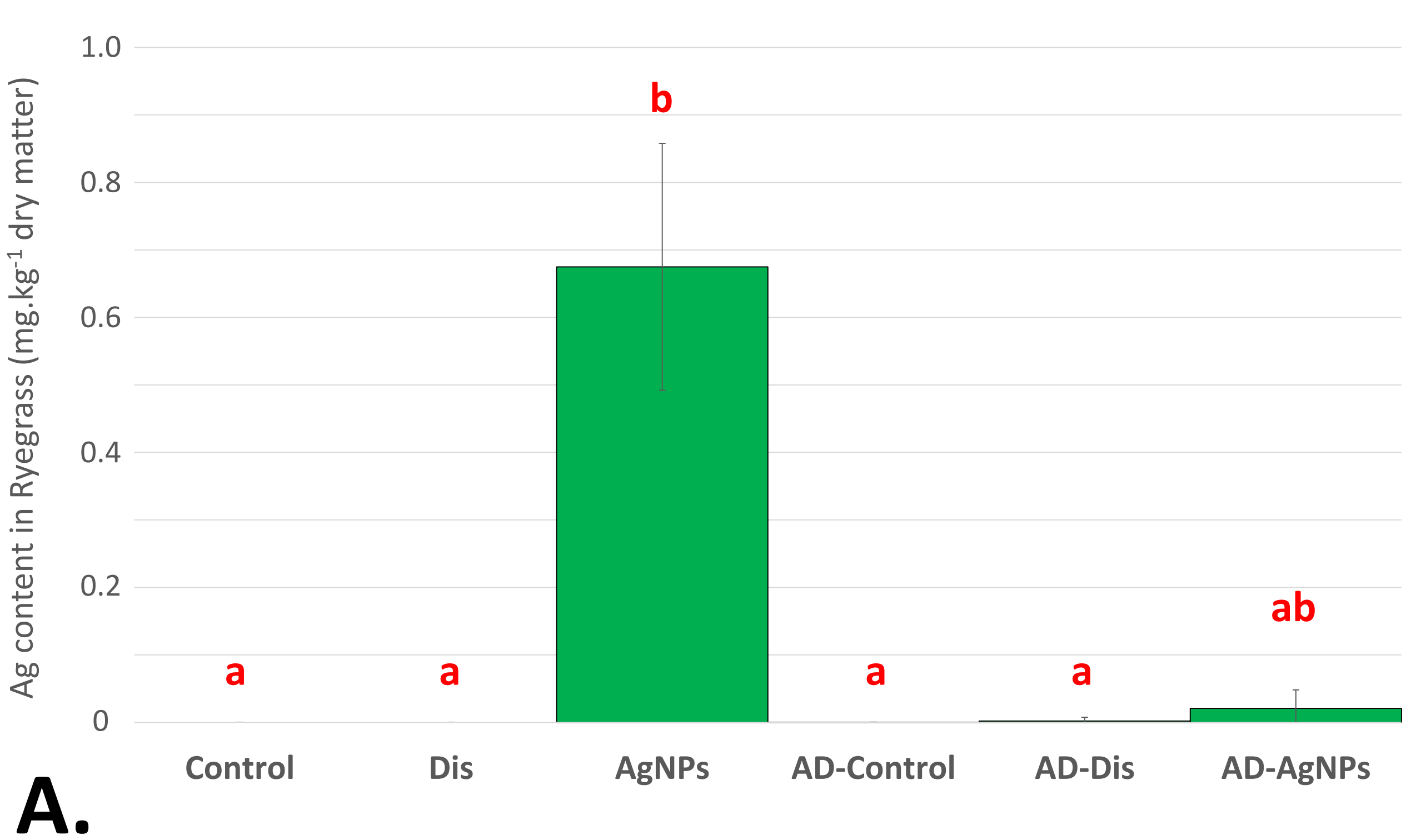


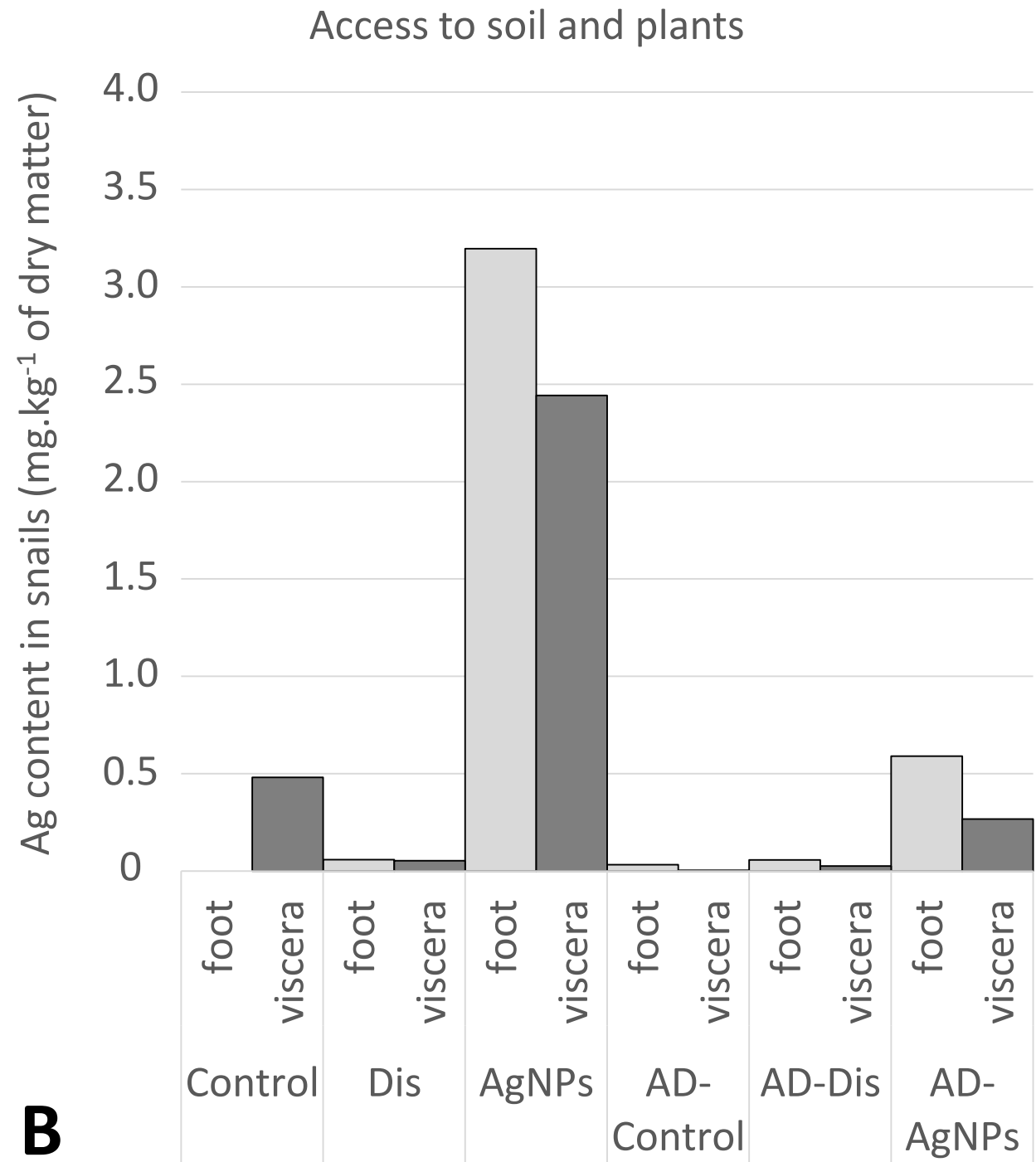
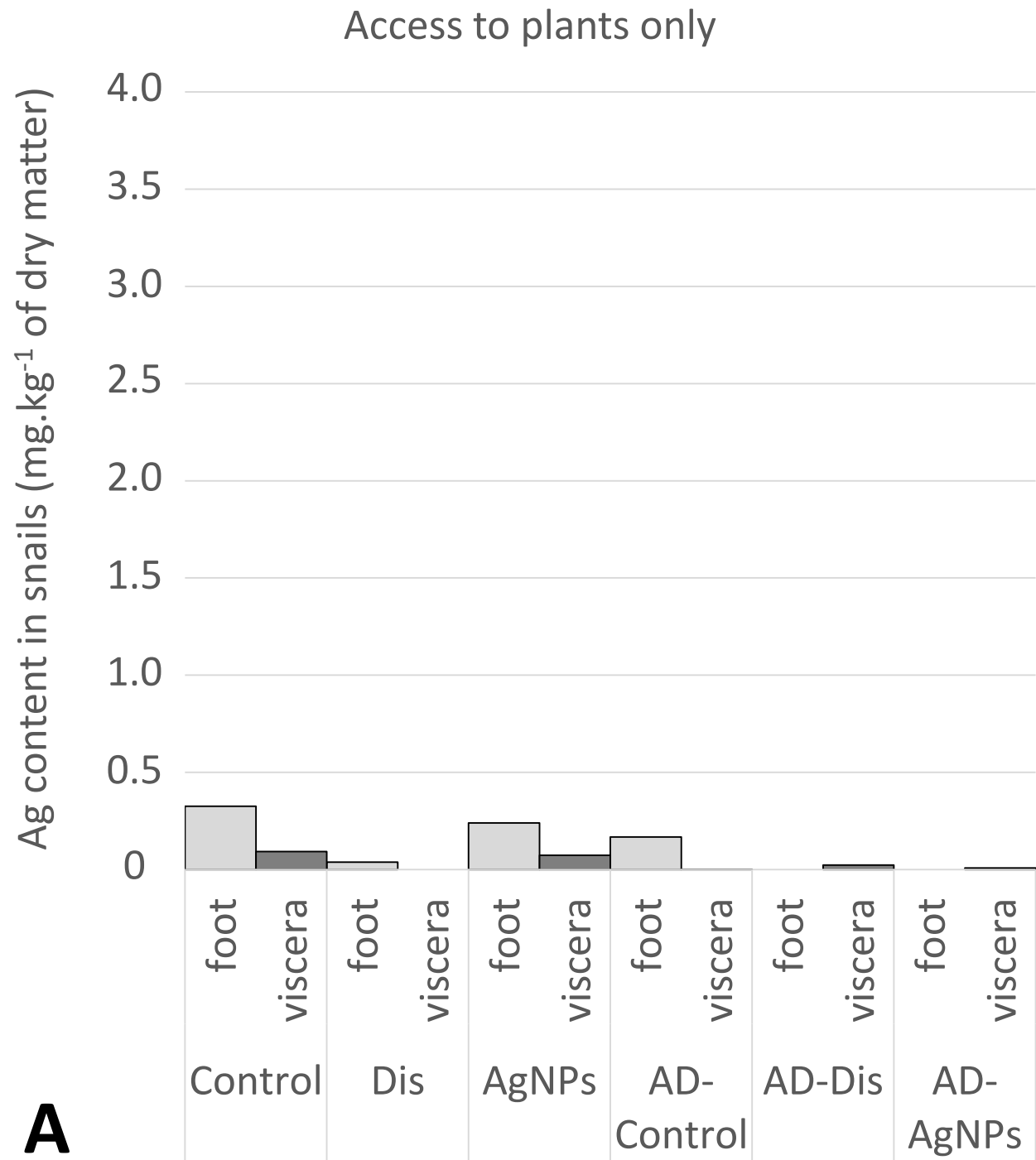
Plant	T1 (21 days)	T2 (35 days)	T3 (49 days)	Significance
Cabbage	~0.01	~0.00	~0.01	a
Colza	~0.015	~0.00	~0.00	a
Hemp	~0.055	~0.075	~0.035	bc
Lettuce	~0.005	~0.005	~0.015	ac
Radish	~0.00	~0.03	~0.025	ac
Ryegrass cv1	~0.045	~0.085	~0.07	b
Ryegrass cv2	~0.08	~0.075	~0.065	b
Wheat	~0.025	~0.035	~0.01	c
Clover	-	-	~0.195	b



Mass of faeces (mg of dry matter, per snail)







**A**

**B**

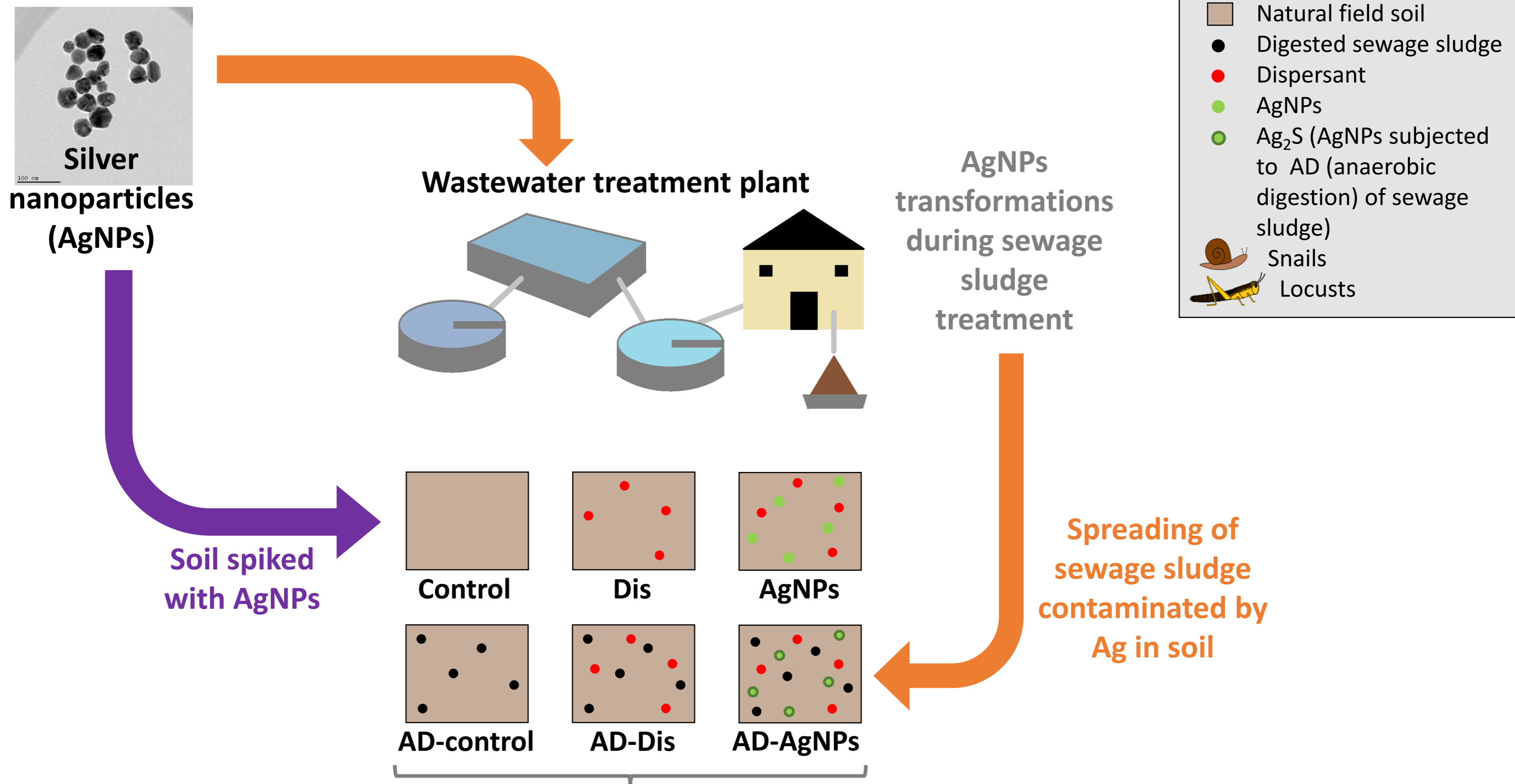


*Table 2: Metal concentration in the soil at the beginning of the experiment in the 6 mesocosm conditions (in mg kg<sup>-1</sup>, in dry matter). Standard errors were indicated. Statistically significant results are indicated by stars. Red stars indicate that the silver concentrations were higher in the AgNPs and AD-AgNPs conditions than the others, although the Ag concentrations were significantly different between the AgNPs and AD-AgNPs conditions. Green stars indicate that the Zn concentrations were higher in the conditions with sludge than in the sludge-free conditions.*

	Control	Dis	AgNPs	AD-control	AD-dis	AD-AgNPs
<b>Ag</b>	0.529 ± 0.340	0.576 ± 0.258	*11.150 ± 0.420	2.124 ± 0.252	1.903 ± 0.810	*6.993 ± 0.223
<b>As</b>	4.131 ± 0.128	4.168 ± 0.141	3.950 ± 0.059	4.374 ± 0.256	4.301 ± 0.157	3.888 ± 0.212
<b>Cd</b>	0.320 ± 0.061	0.308 ± 0.061	0.310 ± 0.027	0.296 ± 0.037	0.323 ± 0.029	0.304 ± 0.026
<b>Co</b>	4.173 ± 0.092	3.879 ± 0.042	3.789 ± 0.066	4.105 ± 0.117	4.102 ± 0.105	3.854 ± 0.188
<b>Cr</b>	25.675 ± 1.656	24.775 ± 0.834	24.300 ± 1.036	28.300 ± 1.089	26.550 ± 0.580	26.275 ± 1.150
<b>Cu</b>	18.675 ± 0.359	18.425 ± 0.050	17.475 ± 0.222	19.900 ± 0.141	19.775 ± 0.150	18.500 ± 0.424
<b>Mn</b>	336.175 ± 9.831	326.425 ± 22.948	307.775 ± 4.844	336.725 ± 0.974	337.250 ± 17.989	307.100 ± 15.112
<b>Ni</b>	11.450 ± 0.173	11.225 ± 0.050	10.950 ± 0.191	11.875 ± 0.096	11.900 ± 0.245	11.175 ± 0.275
<b>Pb</b>	52.725 ± 1.611	51.875 ± 0.957	51.150 ± 3.527	52.900 ± 0.173	52.333 ± 0.896	50.375 ± 2.251
<b>Zn</b>	49.775 ± 0.403	49.025 ± 0.450	47.200 ± 0.424	*76.350 ± 3.753	*72.125 ± 2.114	*69.100 ± 1.633

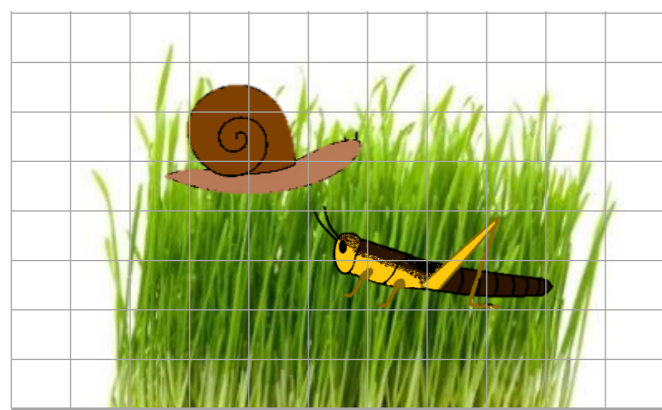
*Table 3: Survival and biomass gained or lost between the beginning and end of the experiment. P means ‘access to plants only’, and P+S means ‘access to plants and soil’. Standard error were indicated. Statistical tests showed no correlation between the survival or the biomass and the Ag presence.*

<u>Condition</u>	<u>Access</u>	<u>Snails</u>		<u>Locusts</u>	
		<u>Survival (%)</u>	<u>Gain/Loss of weight (%)</u>	<u>Survival (%)</u>	<u>Gain/Loss of weight (%)</u>
Control	P	76.67 ± 25.17	- 0.15 ± 0.12	86.67 ± 23.09	+ 0.06 ± 0.04
	P+S	73.33 ± 11.55	+ 0.04 ± 0.07	60.00 ± 20.00	+ 0.05 ± 0.20
AD-control	P	93.33 ± 11.55	- 0.17 ± 0.08	53.33 ± 23.09	+ 0.07 ± 0.09
	P+S	83.33 ± 15.28	+ 0.29 ± 0.04	53.33 ± 23.09	+ 0.16 ± 0.08
Dis	P	93.33 ± 5.77	- 0.11 ± 0.03	86.67 ± 23.09	0.00 ± 0.09
	P+S	63.33 ± 11.55	- 0.01 ± 0.23	46.67 ± 23.09	- 0.14 ± 0.08
AD-dis	P	83.33 ± 15.28	- 0.26 ± 0.27	60.00 ± 34.64	- 0.04 ± 0.05
	P+S	86.67 ± 15.28	+ 0.10 ± 0.02	60.00 ± 20.00	+ 0.06 ± 0.08
AgNPs	P	83.33 ± 15.28	- 0.15 ± 0.04	53.33 ± 41.63	+ 0.01 ± 0.14
	P+S	83.33 ± 15.28	+ 0.04 ± 0.07	66.67 ± 23.09	+ 0.09 ± 0.09
AD-AgNPs	P	66.67 ± 23.09	- 0.16 ± 0.16	46.67 ± 23.09	+ 0.12 ± 0.21
	P+S	76.67 ± 15.28	+ 0.32 ± 0.14	60.00 ± 20.00	+ 0.03 ± 0.17
<i>Average</i>		<i>80.28 ± 15.94</i>	<i>- 0.02 ± 0.21</i>	<i>61.11 ± 24.82</i>	<i>+ 0.04 ± 0.13</i>



**RESULTS OF TRANSFER**

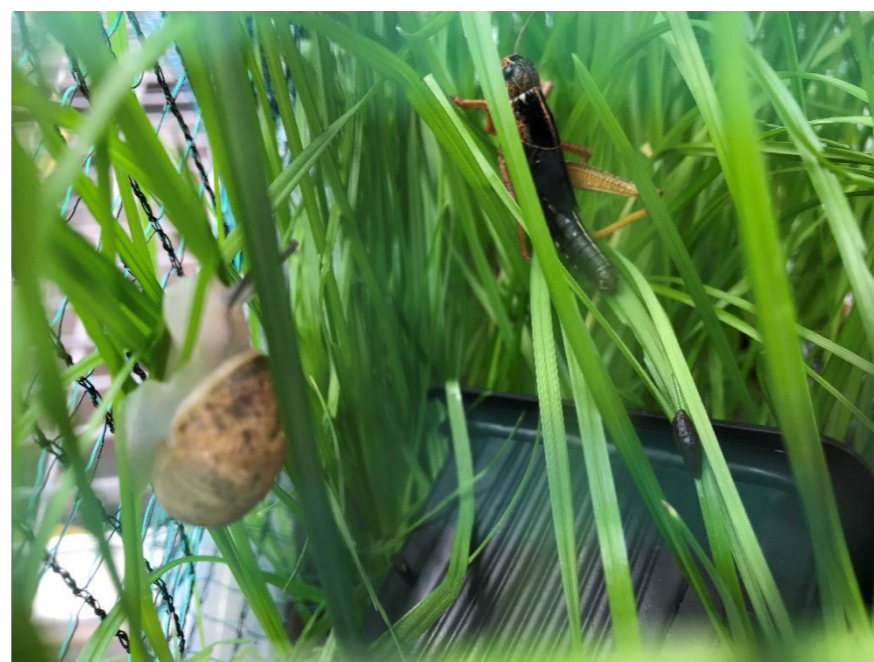
**Ryegrass: significant bioaccumulation**



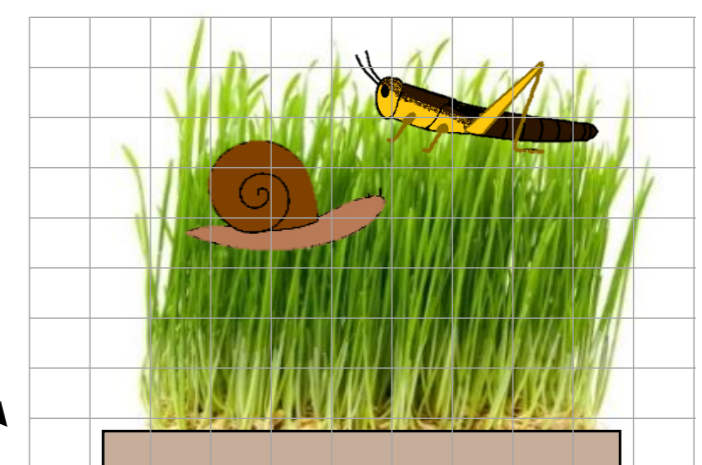
**Exposure to plants only**

**Locust: no Ag**

**Sulfidized Ag is transferred to the food chain but at lower levels than AgNPs**



**Ryegrass: weak but detectable bioaccumulation**



**Exposure to plants and soil**

**Locust: no Ag**

**Snail: Ag bioaccumulation ++ IF access to the soil**

**Snail: Ag bioaccumulation + IF access to the soil**